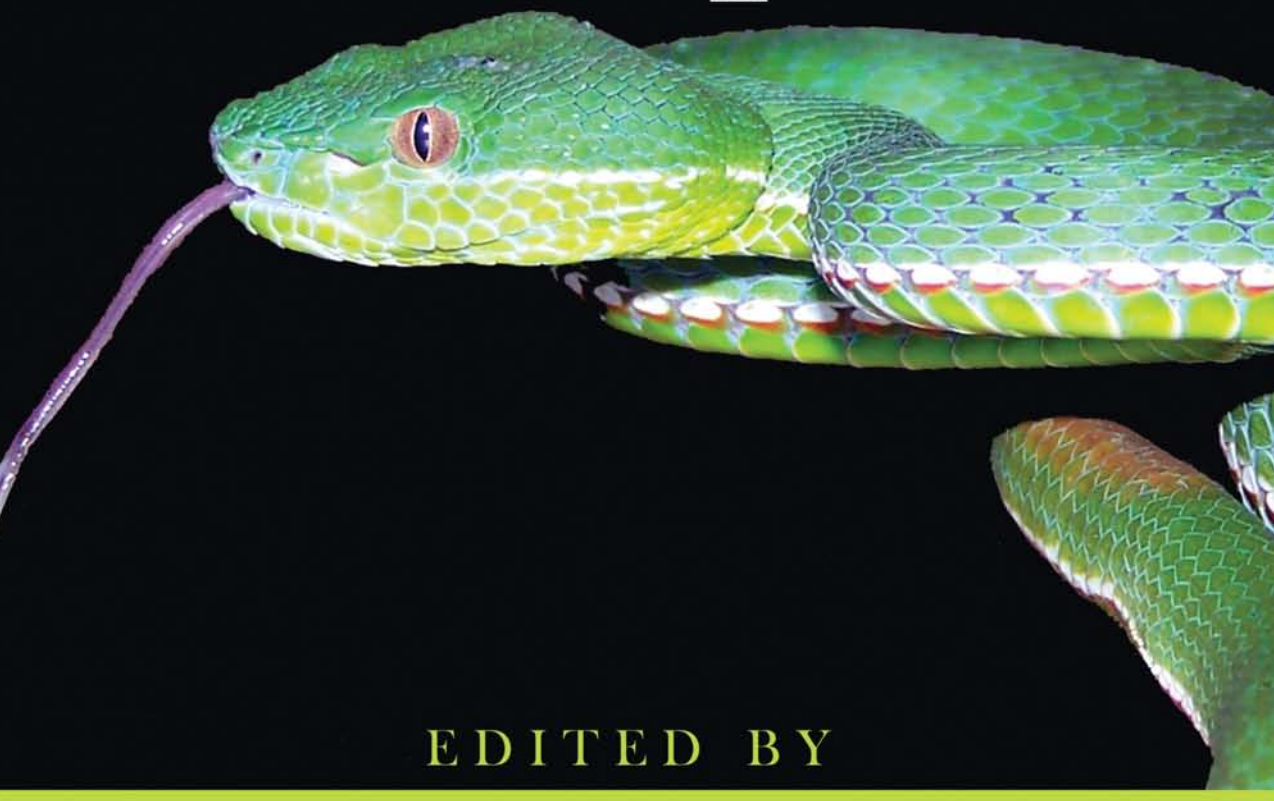
HANDBOOK OF

Venoms and Toxins of Reptiles



EDITED BY

Stephen P. Mackessy

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SECTION IV Envenomation: Occurrence, Prevention, Treatment

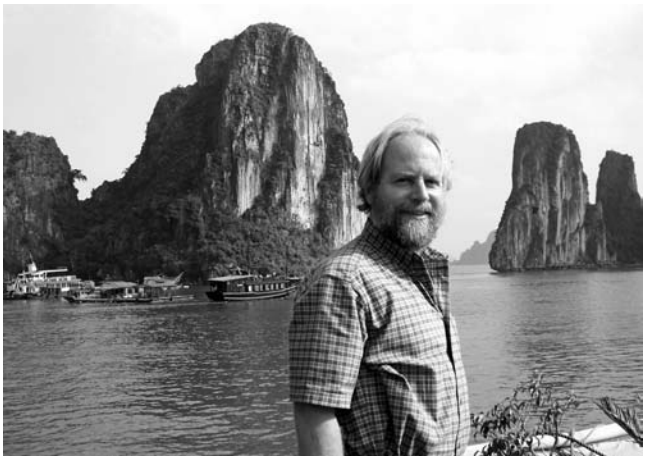
Preface

One of the fascinating aspects of venomous animals and their venoms is the simple observation that minute amounts of this specialized toxic material, venom, when injected into the body of prey or other animals, can cause intense pain, profound physiological changes, or death, often in a very short time. For many millennia and throughout most cultures, humans, from Cleopatra to Steve Irwin, have exploited the public awe of the creatures producing venoms, particularly the snakes, for a wide variety of purposes. We are simultaneously captivated and terrified by venomous snakes, in part for good reason. Envenomation by snakes was likely a significant daily concern for our primordial ancestors, and it remains a substantial health issue in many parts of the world; worldwide, it is estimated that nearly 3 million envenomations and 125,000 deaths occur annually. Morbidity and loss of functions, particularly following viper bites, add to this annual toll, making snakebite an important (though often overlooked) source of human suffering. But venomous animals are much more than just a source of danger to humans. These animals have a long evolutionary history, and their venoms have evolved as a means of assisting them to obtain sustenance, a basic requirement of all life. Venomous reptiles have likely existed for well over 120 million years, and in that time, myriad toxins have evolved that allow them to incapacitate, paralyze, kill, and digest their prey with a high degree of efficiency. The biological potency and specificity of some of these venom toxins is truly astounding, and therein lies much of the attraction for toxinologists.

Reptile venoms and toxins have a potential for tremendous contribution to treatment of human diseases, and some of this potential has been realized in the production of drugs based on or modeled from venom toxins. These nonhuman combinatorial chemists have (teleologically speaking) usurped many regulatory compounds from various physiological processes, turning them against their prey at concentrations orders of magnitude greater than normal. It is therefore not surprising that reptile venoms contain toxins that can be directed against human cancers, hemostatic disorders, and even diabetes. Further, because many toxins interact with receptors/ligands with a high degree of specificity, they are also an excellent source of novel drug leads and design.

In the following twenty-four chapters, produced by leading toxinologists, biologists, biochemists, and physicians from twelve countries worldwide, there is a wealth of new and reviewed information concerning many aspects of reptile venoms. The first section provides a context for understanding the diversity of activities present in the venoms, while the second and third sections present detailed information on many of the enzymes and toxins found in these venoms. The final section brings into focus the worldwide extent of the occurrence and complexity of human envenomations by reptiles. It is hoped that the content presented here will help to stimulate new and continued interest in venoms and the animals that produce them. Many unanswered questions remain in the field of reptile toxinology, and collaborations between specialists from very different fields can produce unique and interesting results. I thank all of the authors for their fine contributions and their patience with the process necessary to bring this book to fruition. The assistance of Patricia Roberson, Gail Renard, and John Sulzycki at Taylor and Francis/CRC Press is also greatly appreciated. Finally, I thank my wife, Jennifer, and daughter, Elizabeth, for their patience and understanding of the many idiosyncrasies inherent in one studying venomous animals and venoms, and I dedicate this book to them.

About the Editor



Stephen P. Mackessy is currently professor of biology in the School of Biological Sciences at the University of Northern Colorado (UNC). His research broadly encompasses the biology of venomous snakes and the biochemistry of snake venoms, and he has published over one hundred scientific papers, book chapters, and natural history notes. His research has included many graduate and undergraduate students, as well as collaborations with colleagues from Singapore, Spain, Mexico, Argentina, Brazil, France, and various other universities in the United States. Several ongoing projects are centered on understanding the evolution of venom systems in snakes and the biological significance of venom compositional variation, with a particular interest in the interface of snake ecology/evolution and venom biochemistry/pharmacology. To this end, broad sampling of venoms from many species of rattlesnakes (*Crotalus*, *Sistrurus*) and numerous species of rear-fanged snakes has resulted in extensive fieldwork in the southwestern United States, Mexico, Guam, and Southeast Asia. Recent projects have focused on the effects of venoms and toxins on metastatic cell proliferation and the investigation of novel toxins for new drug leads. His research program has been supported by numerous local, state, and national funding agencies.

Dr. Mackessy also teaches numerous graduate and undergraduate courses in biomedicine (Toxinology, Current Topics in Biomedical Research, Parasitology) and vertebrate biology (Herpetology, Comparative Anatomy, Mammalogy) at UNC, where he has received awards in recognition of outstanding research and teaching. He earned a BA and an MA in biology (ecology and evolution section) at the University of California at Santa Barbara, Department of Biology (with Dr. S. S. Sweet), and his PhD (with a minor in biochemistry) was received from Washington State University, Department of Zoology (with Dr. K. V. Kardong). He was a postdoctoral research associate at Colorado State University, Department of Biochemistry and Molecular Biology (with Dr. A. T. Tu) before joining the Department of Biological Sciences at UNC. He was the managing editor of the *Journal of Natural Toxins* for seven years. Personal interests include fieldwork with venomous snakes, travel and motorcycles, as well as traveling and camping with his family.

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Section I

Reptile Toxinology, Systematics, and Venom Gland Structure

1 The Field of Reptile Toxinology
Snakes, Lizards, and Their Venoms

Stephen P. Mackessy

CONTENTS

Reptile venoms are typically complex mixtures of primarily peptides and proteins, and the myriad biological effects these molecules produce in envenomated prey and humans are similarly complex and potent. In this book, the many authors discuss the venom apparatus of reptiles, consider the current status of phylogenetic relations of venomous reptiles, explore specific families of venom components, and provide current approaches to the treatment of human envenomations worldwide. In this introduction to the book, variation in venom composition and the factors leading to this variation are discussed. Major patterns of venom compositional trends are identified for the main clades of venomous reptiles, and the identification of novel toxins and interesting structural variants, as well as elucidation of their biological activities and significance, will remain fertile areas of research for many years to come.

I. INTRODUCTION

The production of toxic materials by animals, plants, and microorganisms has fascinated humanity for millennia, for reasons practical, nefarious, and inquisitive. However, only much more recently has the study of these compounds, toxinology, become a formalized discipline. Like many areas of the sciences, toxinology began as a primarily descriptive venture, and technical limitations restrained understanding of the many toxic compounds produced by life-forms. There is still a considerable need for basic descriptive work on venoms and toxins, as the venoms of many species are wholly unknown, and many high-throughput techniques are not yet sufficient at detecting subtle aspects of structure-function differences in many molecules that share a common structural fold but have very different pharmacologies. But as toxinology has moved beyond descriptive work, it has become clear how critical toxins are used as tools for understanding normal homeostatic mechanisms of humans and other animals. Further, study of toxins has contributed greatly to rational drug

design efforts, and many compounds first isolated from natural sources are now used as highly effective drugs for treating human ailments (Opie and Kowolik, 1995; Smith and Vane, 2003; Lewis and Garcia, 2003; Fox and Serrano, 2007). In the last 20 years, particularly with the tremendous advances in genomics and proteomics, we have seen a great increase in the discovery, description, and utilization of purified toxins, and the field of toxinology now includes aspects of virtually all areas of modern life sciences. The use of toxins as "molecular tweezers" has allowed dissection and clarification of numerous important physiological processes, including many aspects of neurotransmission, apoptosis, hemostasis, and signal transduction.

Reptiles include the largest of the venomous vertebrates, and many species produce very large quantities of potent venoms. Envenomations worldwide remain a significant source of morbidity and mortality for humans and their domestic animals in many countries. Species producing venoms are found in several different clades of squamate reptiles, including the snake families Atractaspididae, Elapidae, Viperidae, and the polyphyletic "Colubridae," as well as the lizard family Helodermatidae (Figure 1.1). Within this fascinating and ancient group of animals, there are many interesting and unanswered biological questions, ranging from species diversity and distribution to the ecology and evolution of these (often) highly specialized reptiles. As snakes evolved from a mechanical means of overpowering prey (constriction) to a chemical means (venom injection; Kardong et al.,

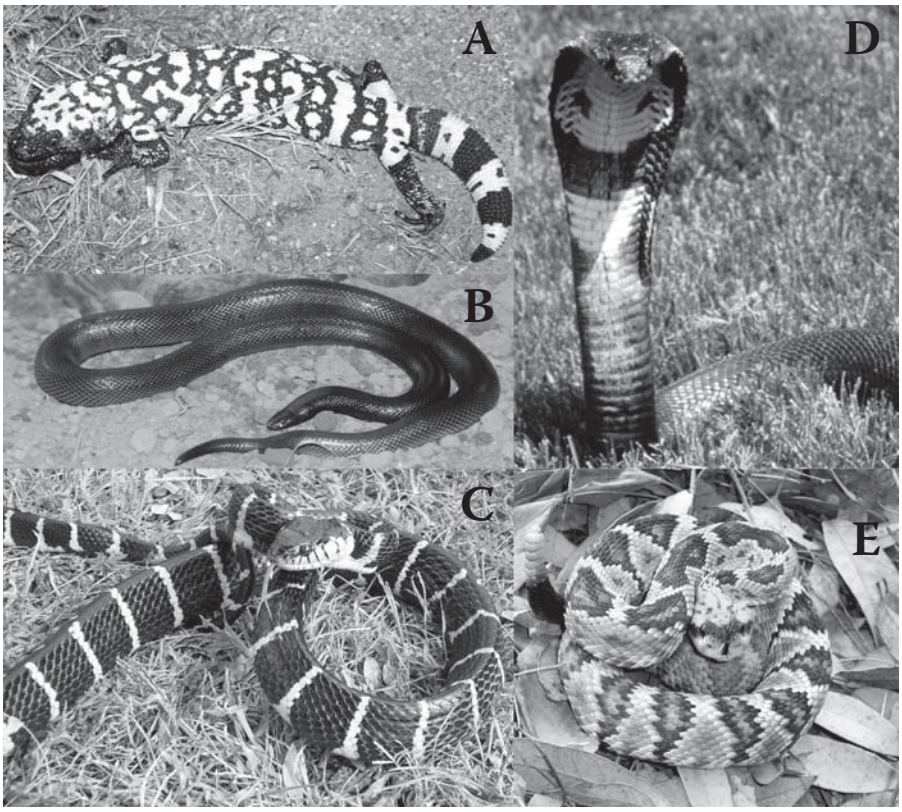


FIGURE 1.1 ***(A color version of this figure follows page 240.)*** Representative examples of venomous squamate reptiles. (A) Gila monster (*Heloderma suspectum*), a member of the family Helodermatidae. (B) Small-scaled burrowing asp (*Atractaspis microlepidota*), family Atractaspididae. (Photograph by Kristen Wiley, courtesy Kentucky Reptile Zoo.) (C) Mangrove catsnake (*Boiga dendrophila*), family Colubridae. (D) Monacled cobra (*Naja kaouthia*), family Elapidae. (E) Northern blacktail rattlesnake (*Crotalus molossus molossus*), family Viperidae.

1997), natural selection has favored fine-tuning of predator-prey interactions, and in the process a wide array of compounds with almost unbelievable specificities have evolved. Although many invertebrates, and a few fish, are also important sources of human envenomations, only venoms and envenomations produced by reptiles will be considered here. Thus, for biologists (in the broadest sense of the word) there is truly something for everyone among venomous reptiles and their venoms.

Included in this book are twenty-four chapters produced by some of the finest researchers in the field. Many chapters focus on specific components of venoms, while others consider the structure and function of the highly specialized venom systems of squamate reptiles, as well as the evolutionary relationships of these animals. The first section of this book places venoms and their study in a broader biological context and will provide source information for toxinologists that is typically omitted from a classical treatment of the field. Specifically, an overview of relationships among venomous animals (systematics) is presented, as well as a summary of the main structural features of the glands producing venoms. Though a structural chemist working in toxinology may have little direct need for such information, it provides a more detailed glimpse into biologically relevant structure-function relationships at levels of organization above the molecular level.

Many different toxins and several new classes of proteins (such as helveprins/cysteine-rich secretory proteins [CRiSPs]) have been described since the last edition of the *Handbook of Natural Toxins* (Tu, 1991), and the increase in the level of sophisticated techniques to reevaluate known toxins and venoms has been impressive. A major section of the book will include a thorough treatment of many enzymatic components found in venoms. Though there are several classes of toxins that have enzymatic activities as well as specific sites of ligand-mediated actions, I believe that it is useful to group those compounds that have catalytic activity (classically, enzymes) vs. ligand-binding mediated activities (classically, toxins). There are many different activities included here, and much new information is presented.

Another major section of the book will include the nonenzymatic proteins and peptides found in venoms. This section will summarize many of the major classes of such toxins. Numerous primary structure and gene sequences for a variety of different toxins are now available via public databases, and these data have greatly increased understanding of gene structure, structure-function relationships among proteins, evolution of toxin families, and generalized patterns of venom protein expression. Some additional venom components that are neither enzymes nor toxins include nonpeptide organic constituents (such as nucleosides), small peptide components of venoms (of both intrinsic and extrinsic action), and inorganic and metal ion constituents of venoms, and these also contribute to the biological potency of venoms.

The last section logically follows the preceding sections and includes chapters on envenomation by reptiles in several different areas of the world, summarizing the significant advances in treatment of the often confusing sequelae of envenomation and identifying problems unique to (and common among) each area. Though antivenins still remain the main course of treatment for envenomations, advances in production and manufacture have increased efficacy and decreased side reactions. Access to health care is still a major concern in many parts of the world, and a contrast between ideal treatment and what is possible in many regions will be apparent. This section has contributions from clinicians/physicians familiar with envenomations as well as from individuals involved in the research, development, and production of antivenoms.

The intent behind this broader treatment of topics within the general field of reptile toxinology is to provide a better context for understanding the complexity of these venoms, which have been shaped by evolutionary and ecological forces. Envenomation is a complex syndrome involving dysregulation of many homeostatic mechanisms simultaneously, and it is hoped that by having a broader understanding of the many factors shaping venoms and envenomation, more effective treatments can be developed. Further, venoms are important natural sources of compounds useful as drugs and

probes of many physiological processes (Fox and Serrano, 2007), and by understanding the context in which venoms evolved, one may be able to exploit novel compound sources more effectively.

II. VENOMS AND TOXINS DEFINED

The definition of venoms has been somewhat contentious, but a venom is here considered to be a simple to complex secretion produced in a specialized gland that is typically delivered via specialized envenomation systems, including a secretory gland, often (but not always) specialized teeth (Vonk et al., 2008), and a suite of specific behaviors allowing delivery of the venom. Further, venoms must be introduced (commonly injected) into recipient tissues in order for deleterious effects to occur, while poisons are typically ingested (Mackessy, 2002a). Thus, reptiles representing an envenomation risk to humans and prey animals are referred to as venomous, not poisonous. Only one species, *Rhabdophis tigrinus*, is known to be both venomous and poisonous, because it possesses a Duvernoy's gland that produces venom (e.g., Sawai et al., 2002) *and* a saccular nuchal gland that sequesters toad toxins, poisonous to potential predators (Akizawa et al., 1985; Hutchinson et al., 2007; Mori and Burghardt, 2008).

In snakes, the venom apparatus consists of bilaterally paired specialized glands (a venom gland or Duvernoy's gland, which are homologous structures) located medial to the upper labial scales, posterior to the nostrils, and behind/below the eyes. In the front-fanged snakes (families Atractaspididae, Elapidae, and Viperidae), this apparatus consists of a large venom gland with a (typically) large basal lumen, allowing for storage of secreted venom for immediate deployment (Mackessy, 1991; Mackessy and Baxter, 2006). There is often a primary duct leading to an accessory gland, and a secondary duct connects the glands to the base of a hollow (and often long) hypodermic fang. Contraction of a specialized compressor muscle pressurizes the gland and delivers a bolus of venom under moderate pressure into recipient tissues. Rear-fanged snakes (the polyphyletic family "Colubridae"; see Chapter 2 for an updated phylogeny) have a somewhat different apparatus. A homologous gland, the Duvernoy's gland, lies in a position similar to that of the front-fanged snakes' venom gland, but it lacks the compressor muscle and a large basal lumen. Instead, the gland is held in place by connective tissue attached to the upper labial scales and a posterior ligament that runs to the rictus of the jaws (Figure 1.2, top); when envenomating prey, jaw adductor muscles pull the ligament posteriorly and labial scales tight, compressing the gland and delivering venom to the base of posterior maxillary teeth with varied morphologies (simple, enlarged, single, multiple, shallowly or deeply grooved, etc.; Figure 1.2, bottom). Venom, which initially was largely stored intracellularly, is then exocytosed and travels through a duct to the rear teeth, where it is introduced into prey tissues. Whereas front-fanged snakes deliver venom rapidly via a pair of enlarged hollow fangs, rear-fanged snakes may introduce venom more slowly (Kardong and Lavín-Murcio, 1993) but at multiple sites via numerous puncture wounds produced as the snake chews on prey. For example, the green vine snake (*Oxybelis fulgidus*), a nonconstricting rear-fanged snake, grasps and holds prey (mouse or lizard) until it becomes quiescent; during this period, obvious adductor muscle contractions without concomitant movement of prey are observed, which could assist venom delivery (unpublished observation). Brown treesnakes (*Boiga irregularis*) use both constriction and venom when subduing prey (Mackessy et al., 2006; personal observation); lizards are held in the jaws without constriction until quiescent, while mice are immediately constricted. Differential behavioral strategies utilized when feeding/biting (e.g., Deufel and Cundall, 2006), as well as differences in venom apparatus architecture and biochemical composition of the venom, can greatly influence the outcome of human envenomations by colubrid snakes, some of which may be quite serious. Whereas a front-fanged snake such as a rattlesnake can initiate and complete a strike in less than 0.5 s (Kardong and Bels, 1998), most colubrid snakes cannot deliver a large bolus of venom rapidly, and contact (bite) time appears to be a significant determinant of severity of envenomation by colubrid snakes (Mackessy, 2002a).

A specialized venom apparatus, found among lizards only in members of the family Helodermatidae (Figure 1.1A), is both unusual and enigmatic (reviewed in Beck, 2005). Modified

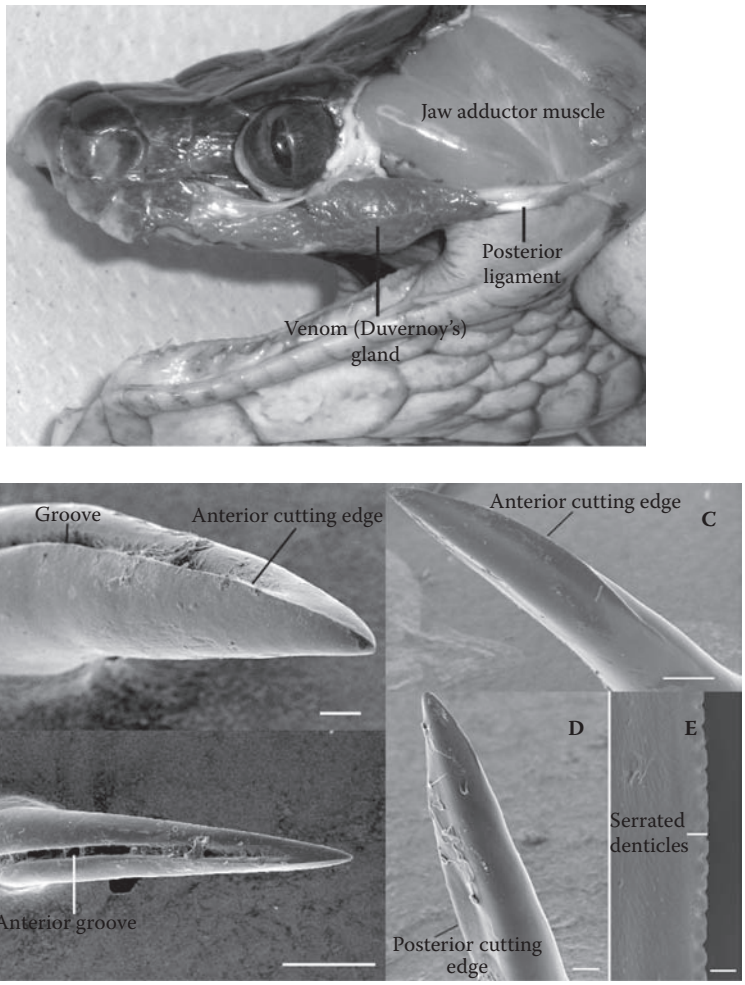


FIGURE 1.2 Reptile glands and teeth. Top: Venom gland of the brown treesnake (*Boiga irregularis*); the skin has been removed from the lateral surface of the head. Muscle fibers do not insert directly on or around the venom gland. (Photograph by C. Rex.) Bottom: Scanning electron micrographs of representative teeth of several squamate reptiles. (A and B) Rear maxillary fang of mangrove catsnake (*Boiga dendrophila*)—note the deep groove, characteristic of many *Boiga* sp. (C) Rear fang of false water cobra (*Hydrodynastes gigas*). (D) Rear fang of night snake (*Hypsiglena torquata*)—note that the cutting edge may be either anterior or posterior in colubrids. (E) Anterior edge of mandibular tooth, crocodile monitor (*Varanus salvadorii*), a large nonvenomous varanoid lizard—note serrated cutting edge, characteristic of most varanids. Scale bars: A, D, and E, 100 μm; B and C, 500 μm.

submandibular glands on the lower jaw produce a complex venom that is released via ducts leading to the base of grooved mandibular teeth. Venom is also primarily stored intracellularly, and as for rear-fanged snakes, delivery of significant volumes of venom requires much longer contact time than is needed by front-fanged snakes. Venoms from helodermatid lizards also contain peptide toxins known as exendins, of which one, Exenatide, has become the "poster child" for development of novel drugs from reptile venom components (e.g., Heine et al., 2005). In order to approach novel venom investigations rationally and effectively, it is important to understand the basics of how venomous organisms use their venoms in a natural predator-prey context. Chapter 3 provides greater detail on comparative aspects of venom apparatus morphology.

III. SOURCES OF VARIATION IN VENOM COMPOSITION

Many reports in the literature have documented different levels of variation in composition of venoms, among major and minor taxonomic groups, between different parts of the same population of one species, during different ages of the animal, and several other factors (see Chippaux et al., 1991, for a review). Venoms can be quite different, at both macro- and microvariation levels, but they also share many compounds across broad taxonomic levels. As venomous reptiles co-opted various regulatory molecules from numerous metabolic pathways and conscripted them as venom constituents (e.g., Fry, 2005), the "evolutionary selection" seems to have been somewhat limited, and venom proteins belong to a relatively small number of protein families (Calvete et al., 2007). However, once conscripted, this limited diversity of proteins has undergone rapid evolution *in situ*, resulting in the production of myriad activities within a single conserved molecular fold. This common motif is seen repeatedly among venom constituents, particularly among the three-finger toxins (3FTXs) (e.g., Kini, 2002; Pawlak et al., 2006, 2009), the phospholipases A_2 (Nakashima et al., 1995; Kini, 1997; Chuman et al., 2000), many serine proteases affecting hemostasis (Deshimaru et al., 1996; Serrano and Maroun, 2005), venom CRISPs (Yamazaki and Morita, 2004), and disintegrins (Juárez et al., 2008).

Venoms can and do vary tremendously in composition, but the absolute mechanisms controlling and producing this variation are poorly understood (but see Earl et al., 2006). Because venoms are trophic adaptations that facilitate handling of prey, their effects on different organisms (including humans) are quite variable, dependent not only on dose but also on the variant molecules contained in a given venom. On the one hand, venoms of some sea snakes (family Elapidae) can be exceedingly simple in composition, containing only two major venom protein families, three-finger α-neurotoxins and phospholipases A_2 (see *Laticauda*, Figure 1.3A). On the other hand, venoms of many front-fanged snakes, such as mambas (family Elapidae) and rattlesnakes (family Viperidae), may contain fifty to one hundred protein and peptide components representing ten to twenty venom protein families (Perkins et al., 1993; Perkins and Tomer, 1995; Sanz et al., 2006). Among the approximately one thousand species of advanced snakes (Caenophidia) that produce venoms, a wide variety of members of these protein families are expressed in the venoms, and many factors interact to determine specific venom composition.

A. Phylogeny and Taxonomic Relationships as a Source of Variation

Though venom composition varies, often significantly, in composition between species (e.g., Tu, 1982, 1991; Ménez, 2002), more closely related species of reptiles generally tend to have venoms that are more similar in composition than do more distantly related venoms. However, the phylogenetic component of venom variation has only been incompletely explored, and a comprehensive analysis of venom composition and phylogeny, using species representative of the major diverse groups, would be very informative on just how important phylogenetic effects actually are. In general, however, dominance of the major protein families found in venoms follows broad phylogenetic trends; for example, at the family level, elapid venoms share more similarities within the family relative to composition in viperid snake venoms. In elapid venoms, smaller toxins predominate, particularly 3FTXs and phospholipases A_2, whereas in viperid venoms, higher-mass enzymatic toxins are prevalent (Figure 1.3). Venoms of the polyphyletic family "Colubridae" are more variable; some, like several species of *Boiga*, produce venoms rich in 3FTXs, while in other species, such as *Alsophis* and *Lioheterodon*, 3FTXs are apparently absent from the venom (Figure 1.3A). Most "colubrid" venoms assayed contain some enzymatic components, commonly metalloproteases and acetylcholinesterases (Hill and Mackessy, 2000; Mackessy, 2002b); phospholipases A_2 do not appear to be broadly distributed among colubrid venoms (but see Huang and Mackessy, 2004).

Viperid venoms are qualitatively and quantitatively very different than most elapid venoms (Figure 1.3B). The prominence of higher molecular weight components, primarily hydrolytic

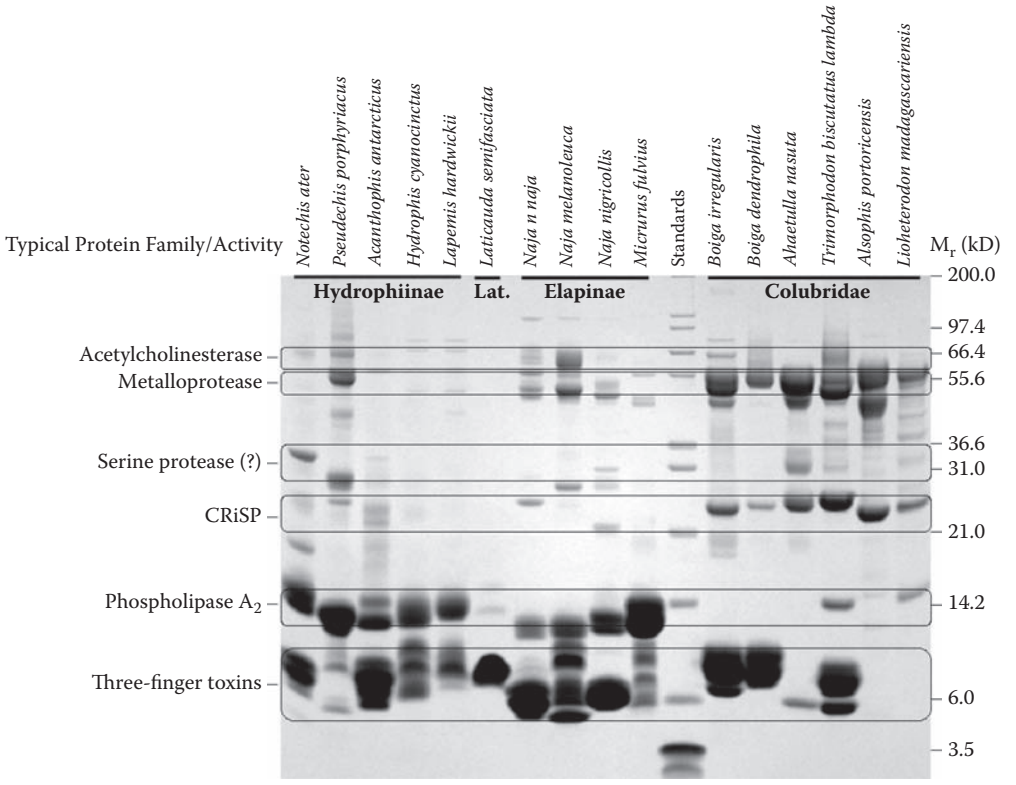
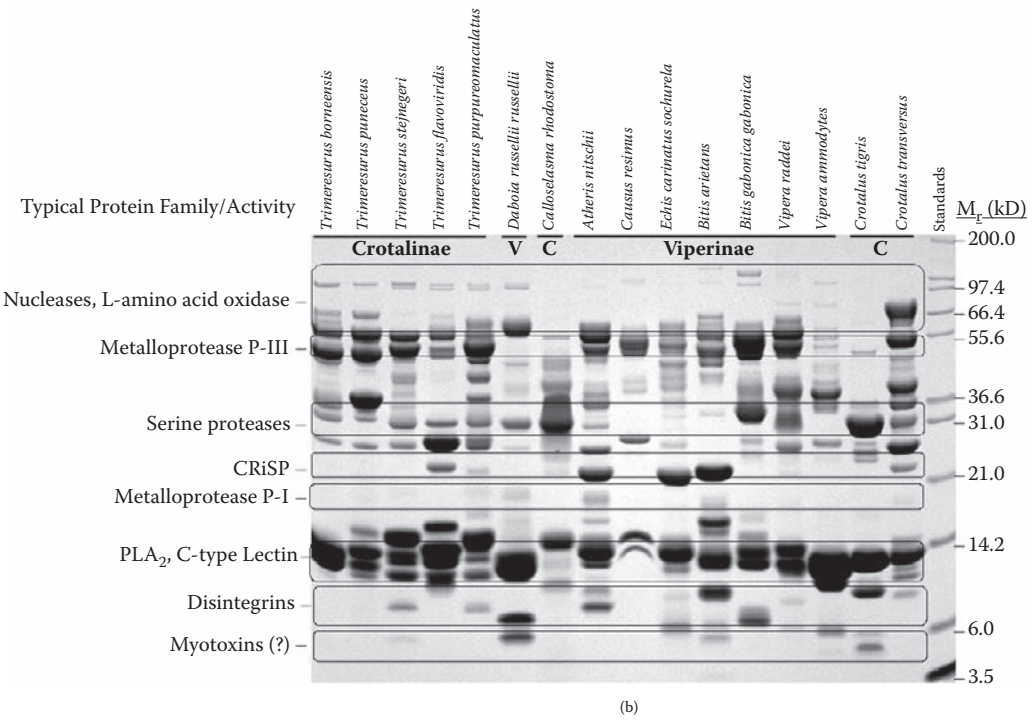


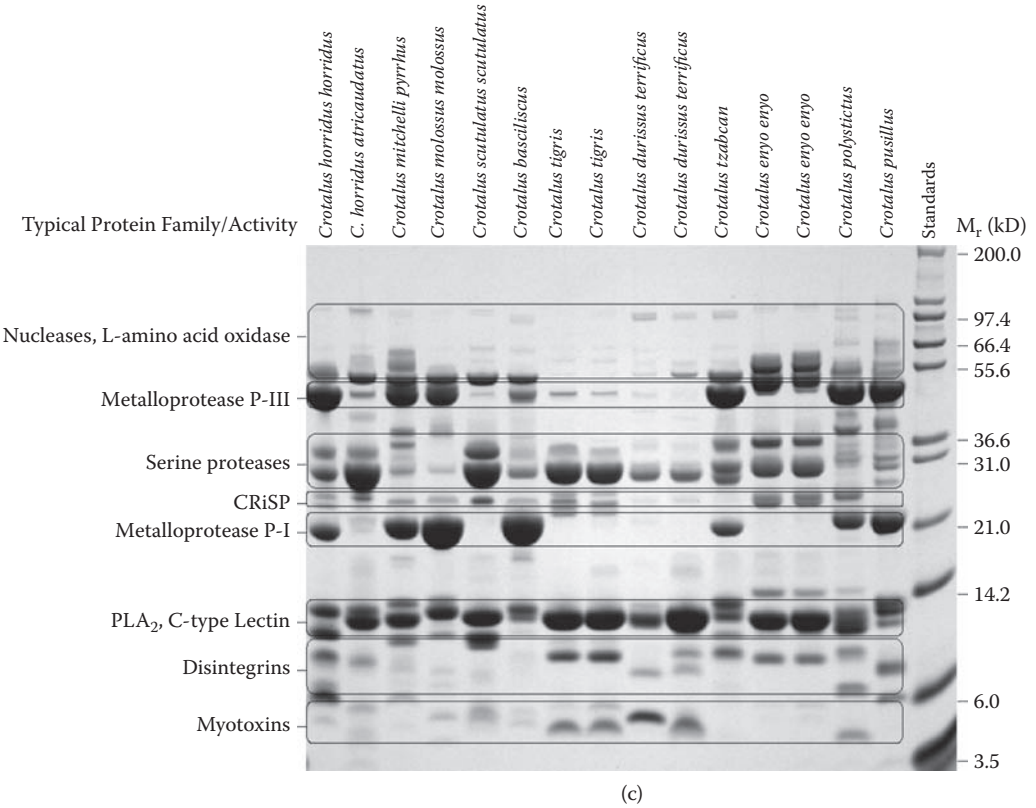
FIGURE 1.3 SDS-PAGE comparison of major venom components in the main clades of venomous snakes. (a) Representatives of the families Elapidae, subfamilies Elapinae, Laticaudinae (Lat.), and Hydrophiinae, and the "Colubridae." (b) Family Viperidae, subfamilies Crotalinae (C) and Viperinae (V). (c) Family Viperidae, subfamily Crotalinae—rattlesnakes. Each lane contains 24 µg venom; 12% acrylamide NuPage gels and MES (2-(*N*-morpholino)ethanesulfonic acid) running buffer (Invitrogen) were used. Major protein families are given on the left, and relative molecular masses (M_r) are on the right of each gel. Ovals enclose bands that are typical of protein families indicated, based on published masses; however, not all bands within a given oval are representatives of indicated families, some protein families are not indicated, and not all bands are identified. Gel C is from Mackessy (2008). See text for discussion of differences.

enzymes, is apparent, and serine proteases (thrombin-like, kallikrein-like, arginine esterase, etc.) dominate the mid-mass ranges (~28–36 kDa), which are typically missing from elapid and colubrid venoms. In general, what one notices is that the pattern of mass distributions within a family is more similar than between families. This predominance of enzymatic components in viperid venoms is strongly supported by many proteomic studies as well (e.g., Nawarak et al., 2003; Li et al., 2004; Serrano et al., 2005; Sanz et al., 2006, 2008; Angulo et al., 2008). A comparison of the families of proteins present in the major taxa of venomous reptiles highlights these trends noted above (Tables 1.1–1.4), and it is apparent that though distinct differences occur between species and families, there are many venom components that are broadly shared, indicating that evolution of venoms among reptile lineages has not been completely random or unrelated.

But phylogenetic consistency is only part of the overall pattern. Venom composition within a well-defined evolutionary lineage, the rattlesnakes (family Viperidae, *Crotalus* and *Sistrurus*), does *not* strictly follow phylogeny but instead appears to follow one of two specific trends, which may be mutually incompatible, independent of close phylogenetic relatedness (Mackessy, 2008). Type I venoms showed high levels of P-I and P-III metalloproteases and were less toxic than Type II venoms,



(b)



(c)

FIGURE 1.3 (continued).

TABLE 1.1
Some Common Components of *Heloderma* Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity
		Enzymes	
Hyaluronidase	73	Hydrolysis of interstitial hyaluronan	Decreased interstitial viscosity
Serine proteases	28–63	Kallikrein-like	
Gilatoxin/horridum toxin	31–33	Kallikrein-like; releases bradykinin	Induces rapid hypotension
Phospholipase A_2 enzymes (Group III)	13–15	Ca^{2+}-dependent hydrolysis of 2-acyl groups in 3-sn-phosphoglycerides	Myotoxicity, myonecrosis, lipid membrane damage
		Nonenzymatic Proteins/Peptides	
CRISP—helothermine	25	May induce hypothermia	Lethargy, paralysis; role in prey capture (?)
Nerve growth factor		Stimulates neuron growth	Unknown
Exendins 1–4	3.5–4.0	Bind to VIP receptors, GLP-1 receptors; stimulate amylase/insulin release, hypotension, etc.	Envenomation role unclear—relation to periodic fasting (?)
Gilatide (Exendin 4 fragment)		Binds to GLP-1 receptor; improves memory	Role in predator avoidance conditioning (?)
		Smaller Organic Compounds	
Serotonin		Neurotransmitter	Mediates inflammation, vasodilation, etc.

Note: Mass in kilodaltons (kDa). Note that this list is not all-inclusive, and that masses, functions, and activities do not apply to all compounds isolated from all venoms. Specific venoms may not contain all components. (?) indicates hypothetical function or activity.

Source: Based on Beck (2005).

which were the most toxic rattlesnake venoms and which had low to no metalloprotease activity. These gross differences in venom composition can be seen following one-dimensional SDS-PAGE (Figure 1.3C): the highly hemorrhagic and tissue-damaging venoms of *C. atrox*, *C. molossus*, and *C. ruber* (Type I) show prominent P-I and P-III bands, and the highly toxic venoms of *C. tigris* and *C. durissus terrificus* (Type II) lack these bands. To an extent, this Type I/Type II dichotomy also occurs globally, as elapid venoms typically are quite toxic and rich in smaller toxins but poor in metalloproteases (and other larger enzymatic components), while the converse is generally true for viperid venoms. What these broad patterns of venom composition variation indicate is that there are other factors that may be more important determinants of absolute venom composition and specific venom gland gene expression than phylogeny. In fact, the presence of genes encoding 3FTXs in venom gland transcriptomes from viperids (Junqueira-de-Azevedo et al., 2006; Pahari et al., 2007), but not the translated toxin in the proteome of the same species (Sanz et al., 2006), suggests that there is a potential for much greater genetic identity of the venom gland genome among venomous species than has been previously acknowledged. That the proteome of venomous reptiles can vary so significantly indicates that many other factors determine which venom genes are translated into the final product utilized by the snake or lizard.

B. Age as a Source of Variation

Age affects several parameters of venom, most obviously overall yield. Volume and total dry weight of venom produced increase exponentially with age/size in several species (Klauber, 1956;

TABLE 1.2
Some Common Components of Colubrid Snake Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity	References
		Enzymes		
Phosphodiesterase (low activity)	94–140	Hydrolysis of nucleic acids and nucleotides	Depletion of cyclic, di- and trinucleotides; hypotension/shock (?)	Mackessy, 1998, 2002; Aird, 2002
Acetylcholinesterase	55–60	Hydrolysis of acetylcholine	Depletion of neurotransmitter; tetanic paralysis (?)	Broaders and Ryan, 1997; Hill and Mackessy, 2000
Snake venom metalloproteinases: M12 reprolysins		Hydrolysis of many structural proteins, including basal lamina components	Hemorrhage, myonecrosis, prey predigestion	Hill and Mackessy, 2000; Kamiguti et al., 2000; Komori et al., 2006; Peichoto et al., 2007
P-III	48–55			
P-II (?)	38			
Serine proteases	36	Hydrolysis of fibrinogen (α and β subunits)	Hemostasis disruption (?)	Assakura et al., 1994
Phospholipase A_2 enzymes (Group I)	13–15	Ca^{2+}-dependent hydrolysis of 2-acyl groups in 3-sn-phosphoglycerides	Myotoxicity, myonecrosis, lipid membrane damage	Hill and Mackessy, 2000; Huang and Mackessy, 2004
		Nonenzymatic Proteins/Peptides		
Cysteine-rich secretory proteins (CRiSPs)/helveprins	21–29	Possibly block cNTP-gated channels	Induced hypothermia; prey immobilization (?)	Yamazaki and Morita, 2004
Dimeric three-finger toxins	17	Potent inhibitor of neuromuscular transmission; show taxon-specific effects	Rapid immobilization of prey, paralysis, death	Pawlak et al., 2009
Three-finger toxins, α-neurotoxins	6–9	Potent inhibitors of neuromuscular transmission; may show taxon-specific effects	Rapid immobilization of prey, paralysis, death	Fry et al., 2003; Lumsden et al., 2005; Kini, 2002; Pawlak et al., 2006

Note: Mass in kilodaltons (kDa). Note that this list is not all-inclusive, and that masses, functions, and activities do not apply to all compounds isolated from all colubrid venoms. Specific venoms may not contain all components. (?) indicates hypothetical function or activity.

Mackessy, 1985, 1988; Mirtschin et al., 2002; Mackessy et al., 2003, 2006). Because head size (and gland volume) increase with age, this general trend is expected for essentially all venomous reptiles, and yields of adult snakes may be one to two orders of magnitude greater that those of neonates. Protein concentration may also vary with age, and lyophilized *Boiga irregularis* venom from neonate snakes had approximately one-half the protein content (w/w) of venoms from adult snakes (Mackessy et al., 2006). However, in addition to allometric increases in overall venom quantity, venom may also vary ontogenetically in composition. For many rattlesnakes (Mackessy, 1985, 1988, 1993, 1996, 2008; Gutiérrez et al., 1991; Mackessy et al., 2003, 2006) and Latin American pit vipers (e.g., *Bothrops atrox*: Guércio et al., 2006), this results in venoms with very different biochemical composition and pharmacology at different times in the life history of an individual snake.

Venom ontogeny has been noted for several species, with lower protease activity noted in venoms from neonate/juvenile snakes, and age-related differences in composition are apparently more

TABLE 1.3
Some Common Components of Elapid Snake Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity	References
		Enzymes		
Phosphodiesterase	94–140	Hydrolysis of nucleic acids and nucleotides	Depletion of cyclic, di- and trinucleotides; hypotension/shock (?)	Mackessy, 1998; Aird, 2002
5′-nucleotidase	53–82	Hydrolysis of 5′-nucleotides	Nucleoside liberation	Rael, 1998; Aird, 2002
Alkaline phosphomonoesterase	90–110	Hydrolysis of phosphomonoester bonds	Uncertain	Rael, 1998
Acetylcholinesterase	55–60		Anderson and Dufton, 1998	
Hyaluronidase	73	Hydrolysis of interstitial hyaluronan	Decreased interstitial viscosity—diffusion of venom components	Tu and Kudo, 2001
L-amino acid oxidase (homodimer)	85–150	Oxidative deamination of L-amino acids	Induction of apoptosis, cell damage	Tan, 1998
Prothrombin activators				
Group C	>250	Activate factor VII or factor X	Induce DIC, highly toxic	Rosing and Tans, 1991, 1992
Group D	45–58	Activate factor X		
(Group A)	~45	Activates factor X		Gao et al., 2002
Snake venom metalloproteinases: M12 reprolysins		Hydrolysis of many structural proteins, including basal lamina components	Hemorrhage, myonecrosis, prey predigestion	Fox and Serrano, 2005
P-III	43–60			
Phospholipase A_2 enzymes (Group I)	13–15	Ca^{2+}-dependent hydrolysis of 2-acyl groups in 3-sn-phosphoglycerides, fibrinogen, etc.	Myotoxicity, myonecrosis, lipid membrane damage	Kini, 1997, 2003
		Nonenzymatic Proteins/Peptides		
Cysteine-rich secretory proteins (CRiSPs)/helveprins	21–29	Possibly block cNTP-gated channels	Induced hypothermia; prey immobilization (?)	Yamazaki and Morita, 2004
Nerve growth factors	14–32.5	Promote nerve fiber growth	Unknown; apoptosis (?)	Hogue-Angeletti et al., 1976; Siigur et al., 1987; Koh et al., 2004
PLA_2-based presynaptic neurotoxins (monomeric to tetrameric)	13.5–80	Blocks release of acetylcholine from axon terminus	Potent neurotoxicity; prey immobilization	Bon, 1997

(continued on next page)

TABLE 1.3 (continued)
Some Common Components of Elapid Snake Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity	References
Three-finger toxins, α-neurotoxins, cardiotoxins, fasciculins, etc.	6–9	Potent inhibitors of neuromuscular transmission, cardiac function, acetylcholinesterase, etc.	Rapid immobilization of prey, paralysis, death	Nirthanan and Gwee, 2004; Kini, 2002; Doley et al., 2008
		Smaller Organic Compounds		
Purines and pyrimidines	AMP = 0.347, hypoxanthine, inosine	Broad effects on multiple cell types (?)	Hypotension, paralysis, apoptosis, necrosis (?); prey immobilization	Aird, 2002, 2005

Note: Mass in kilodaltons (kDa). Note that this list is not all-inclusive, and that masses, functions, and activities do not apply to all compounds isolated from all elapid venoms. Specific venoms may not contain all components. (?) indicates hypothetical function or activity.

pronounced among viperids than among elapids. Two prominent changes that occur involve overall toxicity of venom to prey and total metalloproteinase content of neonate vs. adult venoms, with neonate venoms being more toxic but showing much lower levels of metalloproteinase activity. It was proposed that these biochemical differences were related to changes in prey (both taxonomic differences and physical parameters, such as bulkiness), with venoms acting optimally on prey utilized preferentially by a specific age class (Mackessy, 1988). It was also noted some time ago that among northern Pacific rattlesnakes (*Crotalus oreganus oreganus*), this shift in composition included a change from production of higher-mass metalloproteinases (P-III/P-IV) by neonate snakes to a predominance of lower molecular mass metalloproteinases (P-III, P-II, and P-I) in venoms from adult snakes (Mackessy, 1993). This same shift in composition has recently been confirmed in *Bothrops atrox* and *B. asper* by several proteomic studies (Guércio et al., 2006; Alape-Girón et al., 2008), and so it appears that this ontogenetic shift in composition may occur broadly among viperid snakes.

However, not all rattlesnakes or Latin American vipers show this same pattern of ontogenetic variation in metalloproteinase content, and this age-related shift is associated with the production of Type I venoms (see above) but not with Type II venoms. Examples of this lack of gross change in metalloproteinase production have been noted in *C. o. concolor* (Mackessy et al., 2003) and *C. durissus terrificus* (Gutiérrez et al., 1991). In both species, both juvenile and adult snakes produce very toxic venoms. In *C. o. concolor*, this constraint on venom composition may limit prey selection and breadth of foraging activity.

C. Geography as a Source of Variation

Venoms may also vary in composition as a function of geographic location. The biological significance of these differences is not clear, but they may result from the occurrence of one of two (or more) mutually exclusive evolutionary "strategies" similar to the Type I/II dichotomy noted above. Clinically, these geographic differences can have profound impacts, as the venom of the same subspecies of snake (such as *C. s. scutulatus*) from different localities may be very different in toxicity and metalloproteinase activity (Glenn and Straight, 1978; Glenn et al., 1983), resulting in very different patient presentations following envenomation. A similar difference in composition with locality

TABLE 1.4
Some Common Components of Viperid Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity	References
		Enzymes		
Phosphodiesterase	94–140	Hydrolysis of nucleic acids and nucleotides	Depletion of cyclic, di- and trinucleotides; hypotension/shock (?)	Mackessy, 1998; Aird, 2002
5′-nucleotidase	53–82	Hydrolysis of 5′-nucleotides	Nucleoside liberation	Rael, 1998; Aird, 2002
Alkaline phosphomonoesterase	90–110	Hydrolysis of phosphomonoester bonds	Uncertain	Rael, 1998
Hyaluronidase	73	Hydrolysis of interstitial hyaluronan	Decreased interstitial viscosity—diffusion of venom components	Tu and Kudo, 2001
L-amino acid oxidase (homodimer)	85–150	Oxidative deamination of L-amino acids	Induction of apoptosis, cell damage	Tan, 1998
Snake venom metalloproteinases: M12 reprolysins		Hydrolysis of many structural proteins, including basal lamina components, fibrinogen, etc.; some are prothrombin activators (groups A and B)	Hemorrhage, myonecrosis, prey predigestion	Fox and Serrano, 2005, 2008
P-III	43–85			
P-II	25–30			
P-I	20–24			
Serine proteases				
Thrombin-like	31–36	Catalysis of fibrinogen hydrolysis	Rapid depletion of fibrinogen; hemostasis disruption	Markland, 1998; Swenson and Markland, 2005
Kallikrein-like	27–34	Release of bradykinin from HMW kininogen; hydrolysis of angiotensin	Induces rapid fall in blood pressure; prey immobilization	Nikai and Komori, 1998
“Arginine esterase”	25–36	Peptidase and esterase activity	Uncertain; predigestion of prey (?)	Schwartz and Bieber, 1985
Phospholipase A_2 enzymes (Group II)	13–15	Ca^{2+}-dependent hydrolysis of 2-acyl groups in 3-sn-phosphoglycerides	Myotoxicity, myonecrosis, lipid membrane damage	Kini, 1997, 2003
		Nonenzymatic Proteins/Peptides		
Cysteine-rich secretory proteins (CRiSPs)/helveprins	21–29	Possibly block cNTP-gated channels	Induced hypothermia; prey immobilization (?)	Yamazaki and Morita, 2004
Nerve growth factors	14–32.5	Promote nerve fiber growth	Unknown; apoptosis (?)	Siigur et al., 1987; Koh et al., 2004

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TABLE 1.4 (continued)
Some Common Components of Viperid Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity	References
PLA_2-based presynaptic neurotoxins (2 subunits, acidic and basic)	24	Blocks release of acetylcholine from axon terminus	Potent neurotoxicity; prey immobilization	Aird and Kaiser, 1985; Ducancel et al., 1988; Faure et al., 1994
C-type lectins	27–29	Binds to platelet and collagen receptor	Anticoagulant, platelet modulator	Leduc and Bon, 1998
Disintegrins	5.2–15	Inhibit binding of integrins to receptors	Platelet inhibition; promotes hemorrhage	Calvete et al., 2005
Myotoxins—non-PLA_2	4–5.3	Modifies voltage-sensitive Na channels; interacts with lipid membranes	Myonecrosis, analgesia; prey immobilization	Fox et al., 1979; Laure, 1975; Bieber and Nedelhov, 1997
		Smaller Peptides		
Bradykinin-potentiating peptides	1.0–1.5	Increases potency of bradykinin	Pain, hypotension; prey immobilization	Wermelinger et al., 2005
Tripeptide inhibitors	0.43–0.45	Inhibit venom metalloproteases and other enzymes	Stabilization of venom components	Francis and Kaiser, 1993; Munekiyo and Mackessy, 2005
		Smaller Organic Compounds		
Purines and pyrimidines	AMP = 0.347, hypoxanthine, inosine	Broad effects on multiple cell types (?)	Hypotension, paralysis, apoptosis, necrosis (?); prey immobilization	Aird, 2002, 2005
Citrate	0.192	Inhibition of venom enzymes	Stabilization of venom	Freitas et al., 1992; Francis et al., 1992

Note: Mass in kilodaltons (kDa). Note that this list is not all-inclusive, and that masses, functions, and activities do not apply to all compounds isolated from all rattlesnake venoms. Specific rattlesnake venoms may not contain all components. (?) indicates hypothetical function or activity.

has been observed for the southern Pacific rattlesnake (*C. o. helleri*), and for both *C. s. scutulatus* and *C. o. helleri*, the high toxicity of venoms from some snakes is due to the expression of Mojave toxin genes and a concomitant high level of Mojave toxin and homologs in the venom (Wooldridge et al., 2001; French et al., 2004). Variation in composition in Latin American viperids has also been long noted (e.g., Jiminez-Porras, 1964), and J. M. Gutiérrez and colleagues have since greatly extended these studies (i.e., Saravia et al., 2002; Alape-Girón et al., 2008; Angulo et al., 2008).

Regional variation in phospholipase A_2 and peptide myotoxin components has also been noted for several viperids. Creer et al. (2003) noted significant variation in phospholipase A_2 (PLA_2) isoforms in the venoms of *Trimeresurus stejnegeri* from Taiwan and nearby islands, and these geographic differences were ascribed to differences in prey taken. While this geographic difference in PLA_2 isoform content may in fact be prey driven, one shortcoming of this and most studies is that there are no data indicating whether specific isoforms might have greater effects against particular prey types (see below). Geographic variation in α-neurotoxin isoform type and overall content was recorded for *Naja atra* and *Naja kaouthia* from China, Thailand, and Taiwan (Wei et al., 2003). This variation did not appear to be phylogenetically or clinally based, and the authors suggested

that variation may be associated with differences in prey or habitat. Similar to the study with venom PLA_2 isoform variation, the biological significance of this variation is unclear.

D. Diet and Venom Compositional Variation

As trophic adaptations, it is expected that venom composition would be related to some aspects of diets, because different species of prey animals are differentially sensitive to various types of toxins. Further, if venomous reptiles are significant predators in a given ecosystem, one might expect some type of coevolutionary adjustments between predator and prey species, perhaps leading to an "arms race" among the interacting species. Venoms of reptiles should be subject to selective forces shaping effectiveness toward particular prey, and the result of selection may manifest as taxon-specific toxicity of venoms or venom components.

Correlations between age-related changes in diet and venom composition have been inferred for many species (i.e., Daltry et al., 1996a), but the causal link between these features that support such claims is for the most part weak. The correlation in Pacific rattlesnakes (*C. o. helleri* and *C. o. oreganus*) was considerably strengthened by the demonstration of greater toxicity of the neonate venoms toward preferred prey, in this case lizards (Mackessy, 1988), which accompanied concomitant changes in venom composition. A similar relationship between venom toxicity and preferred prey was observed for numerous species of South American coral snakes (*Micrurus*: Jorge da Silva and Aird, 2001) and *Micrurus nigrocinctus* from Costa Rica (Urdaneta et al., 2004). In both studies, venoms were most effective against the preferred (ectothermic) prey.

However, some species of rattlesnakes, such as *C. o. concolor* (Mackessy et al., 2003) and *C. d. terrificus* (Gutiérrez et al., 1991), do not show an age-related difference in toxicity and metalloproteinase activity level as do related conspecifics, even though diet changes with age. For *C. o. concolor*, which occurs in a rather harsh temperate climate (southern Wyoming), the lack of significant metalloproteinase activity in venoms may limit the size of prey taken, a hypothesis supported by diet data (Mackessy et al., 2003). Interestingly, differences in myotoxin-a homolog levels do vary ontogenetically, and neonate snakes, which feed nearly exclusively on lizards, produce very low levels of myotoxins in their venoms. It is unknown whether peptide myotoxins are more effective on specific prey taxa.

The most striking examples of taxon-specific differences in susceptibility to venoms and venom toxins, which are almost certainly tied to diet, occur among colubrid snakes. The brown treesnake (*Boiga irregularis*) is an arboreal snake that largely feeds on birds and lizards, although mammals are taken opportunistically as well. However, as noted above, the way prey is handled varies by taxon, and mammals are typically killed via constriction. Analysis of the crude venom showed that toxicity (IP LD_{50}) to birds and lizards was very different than for mammals, and venom from adult snakes was ~15 times more toxic to birds and lizards than to mice (Mackessy et al., 2006). The venom of *B. irregularis* contains a plethora of low-mass (7–10 kDa) proteins, which were suspected to be neurotoxins (or other 3FTXs), and monomeric 3FTXs occur in the venom of the related *B. dendrophila* (Lumsden et al., 2005; Pawlak et al., 2009). In a very recent study, irditoxin (*B. irregularis* dimeric toxin), the first described member of a covalently linked dimer subfamily of 3FTXs, was shown to explain this taxon specificity (Pawlak et al., 2009). Irditoxin, which accounts for ~10% of the total venom protein content (w/w), was rapidly lethal to birds (0.22 μg/g IP) and lizards (0.55 μg/g IP) but was nontoxic to mice at doses up to 25 μg/g (highest dose tested). Venom yields from large snakes commonly exceeded 20 mg, and based on the action of irditoxin alone, this amount of crude venom could kill 9 kg-equivalents of bird (domestic chicken). It should be clear that during a predatory strike on a native bird, in which much less than 20 mg venom is expected to be expended, the potency of this venom is more than sufficient to immobilize or kill prey rapidly. It is likely that several other species of *Boiga*, as well as other rear-fanged colubrids, produce venoms with homologous dimeric 3FTXs. Further, because of the lower complexity of the venom proteome of most colubrids relative to viperids and elapids, as well as the dependency of many species on

ectothermic vertebrates or invertebrates as prey, colubrid venoms should serve as convenient models for assessing the relationship of venom composition to diet.

E. Other Possible Sources of Variation

1. Seasonal Variation

Although seasonal variation has been suggested to occur in composition of venom from several species, the evidence suggesting this supposition is lacking. In fact, one study using isoelectric focusing of venoms, which should be sensitive enough to detect minor differences, suggested just the opposite. For three species of rattlesnakes (*Crotalus atrox*, *C. molossus*, and *C. oreganus* (formerly *viridis*) *helleri*), no differences were seen in protein banding patterns of samples collected from the same snake over a period of 20 months (Gregory-Dwyer et al., 1986). This seasonal constancy in venom composition is consistent with observations on venoms from *C. viridis viridis* from Weld Co., Colorado, as well as observations on composition of venoms taken from a single adult individual (many different species) in captivity over several years (Mackessy, unpublished observation). Though there is a general belief that venoms do vary seasonally, the available evidence is scant. An earlier report suggested that venoms from *Vipera ammodytes* showed differences between summer- and winter-obtained venoms, with summer venoms containing two additional bands (lethal proteins) that were missing from samples collected from captive snakes in winter (Gubenšek et al., 1974). Seasonality as a source of compositional variation is a factor that requires further study.

2. Sex-Based Variation

Results of earlier studies have suggested that little to no differences in venom composition occur between the sexes of the same species (see Chippaux et al., 1991, for references). An isoelectric focusing study of venoms from a large number of *Calloselasma rhodostoma* noted that one band was present in venoms from females but absent from male venoms (Daltry et al., 1996b), but this band was not identified. However, recent studies using a proteomics approach (two-dimensional electrophoresis, mass spectrometry) indicate that at least subtle differences in venom composition exist between male and female *Bothrops jararaca* (Menezes et al., 2006; Pimenta et al., 2007). Using SDS-PAGE, sex-specific bands were noted, with male snakes only producing venoms with a 100 kDa protein, and female snakes' venom contained a gelatin-degrading component (likely a metalloproteinase) of ~25 kDa that was absent from male snake venoms. Following two-dimensional electrophoresis, significant differences between male and female venoms in spot intensities were also noted for several different protein groups (not identified), with female venoms generally showing more intense spots. Differences in crude venom activities toward several protein and peptide substrates were somewhat variable, but male venoms were less active toward casein and more active toward D-Val-Leu-Lys-pNA, while female venoms showed the opposite trend (Menezes et al., 2006). A MALDI-TOF-MS study of bradykinin-potentiating peptides (BPPs) identified significant individual variation in numbers and levels of this peptide, and four peptides were found only in female snake venoms (Pimenta et al., 2007). These four novel peptides were found to be cleaved BPPs that lacked the C-terminal portion (Gln-Iso-Pro-Pro), and they are apparently inactive BPPs. The biological significance (if any) of these sex-based differences in venom composition are unclear, but it is apparent that at least some sex-based differences may be expected in venoms from other species of reptiles.

IV. CONCLUSIONS

Toxinology as a field of study has grown tremendously over the last 10 to 20 years, in large part driven by the technical advances in genomics and proteomics. As these tools are utilized to probe venoms from more species in ever-increasing detail, it is important to keep in mind that these venoms and the toxins comprising them have evolved in a specific biological context, largely dominated by numerous trophic and predator-prey interactions. It is now feasible to expect full proteome and

venom gland genome catalogs to be produced for many species of venomous reptiles within the next 10 years, and these complete descriptions of venom compositional diversity will contribute greatly to our understanding of the mechanisms favoring the evolution of specific venom profiles among specific taxa. The evaluation of the biological activities of the many isoforms, presently known and yet to be described, remains a daunting task, but this information is necessary to identify the biological roles of specific components and to place venom compositional diversity into a more meaningful biological context. One of the wonderful aspects of toxinology is that there is no limit to the number of interesting questions concerning venomous reptiles and their venoms. Our job is to pose and pursue those questions, and it is hoped that this book will contribute to that pursuit in some small way.

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2 Recent Advances in Venomous Snake Systematics

Adrian Quijada-Mascareñas and Wolfgang Wüster

CONTENTS

The advanced snakes (Caenophidia) constitute the most diverse group of living snakes. They include the medically important venomous snakes and have therefore received considerable research attention. A large body of recent phylogenetic work has resulted in a consensus that caenophidians as a group are more phylogenetically complex than portrayed by previous family-level classifications. The traditional family Colubridae is nonmonophyletic and composed of multiple deep clades that deserve taxonomic recognition at the family level. Moreover, all Caenophidia (and probably all snakes and many lizards) are descended from a single venomous ancestor, making the venom apparatus a homologous feature of all snakes. Molecular markers and further exploration in several continents are revealing considerable hitherto unsuspected diversity of venomous snakes, including the discovery of new species and the reassessment of existing species, which often turn out to be more heterogeneous than previously suspected. Here we review the most recent taxonomic changes and the new discoveries involving venomous snakes worldwide.

I. INTRODUCTION: THE IMPORTANCE OF SYSTEMATICS FOR TOXINOLOGY AND THE TREATMENT OF ENVENOMATION

An understanding of the systematics and the phylogenetic relationships among venomous snakes is a vital part of any investigation into the venoms of these organisms. A robust taxonomic underpinning is essential to any toxinological research, to help ensure the replicability of research results, and also for the production of appropriate and effective antivenoms, and thus the treatment of snakebite patients. In addition, any investigation into the origin of venom delivery systems and the evolution of venom composition requires a phylogenetic framework that takes into account the evolutionary interrelationships among snakes and other reptiles.

A complicating factor is that, despite their medical importance, the taxonomy of many groups of venomous snakes is still inadequately understood. New species are being discovered regularly, and populations believed to be part of a single species often turn out to constitute different species. This process of revision and discovery owes much to concerted efforts to explore the biodiversity of previously understudied regions and the general availability of molecular genetic data. The resulting state of flux in the classification and nomenclature of many species is a common cause of confusion and even frustration among nontaxonomists, but at the same time, these discoveries have important ramifications for toxinology and the treatment of snakebite patients: taxonomic affinities may help predict patterns of variation in venom composition, which may in turn affect the treatment of patients (Wüster et al., 1997; Fry et al., 2003a). Unfortunately, toxinology and medical science have a long history of paying little attention to the systematics and taxonomy of venomous snakes. As a result, many studies cannot be related to our current and developing understanding of the systematics of the snakes concerned (Wüster and McCarthy, 1996), leading to difficulties in replicating experimental results, and even unnecessary mortality of snakebite patients (Warrell and Arnett, 1976; Warrell, 2008; Visser et al., 2008). One of the aims of this chapter is to increase the awareness of the importance not only of understanding the current state of the systematics of many groups, but also of keeping abreast of future developments. Clinicians, toxinologists, venom producers, and antivenom manufacturers have an absolute responsibility to be aware of the current understanding of the taxonomy of their chosen animals and to follow future developments as they occur. Collaboration with herpetologists working on their specific groups of snakes is one way of accomplishing this.

The purpose of this chapter is to summarize recent developments in snake systematics, particularly in those species with a well-developed venom delivery system, as well as others that have featured in the toxinological or clinical literature. Here we focus on the advances of the last 10 to 12 years, particularly in light of the advances in molecular systematics and recent explorations in species-rich regions of the planet that have revealed hitherto unsuspected diversity in many groups of snakes.

II. HIGHER-LEVEL TAXONOMY AND EVOLUTIONARY RELATIONSHIPS IN THE CAENOPHIDIA

Snakes are divided into two main groups. The fossorial scolecophidians (blindsnakes and threadsnakes, ca. 340 species) are small snakes that feed mainly on ants and termites. All other snakes, the alethinophidians (ca. 2,640 species), are ecologically diverse, feeding primarily on vertebrates (Cundall and Greene, 2000; Vidal and Hedges, 2002). Among Alethinophidia, the caenophidians or advanced snakes (ca. 2,470 species) widely use venom or constriction to subdue their prey, while the remaining alethinophidian snakes (ca. 170 species) use constriction only (Vidal and Hedges, 2002; Vidal and David, 2004).

The advent of molecular systematics has brought considerable insight to the understanding of caenophidian relationships. Recent molecular phylogenetic studies by Slowinski and Lawson (2002),

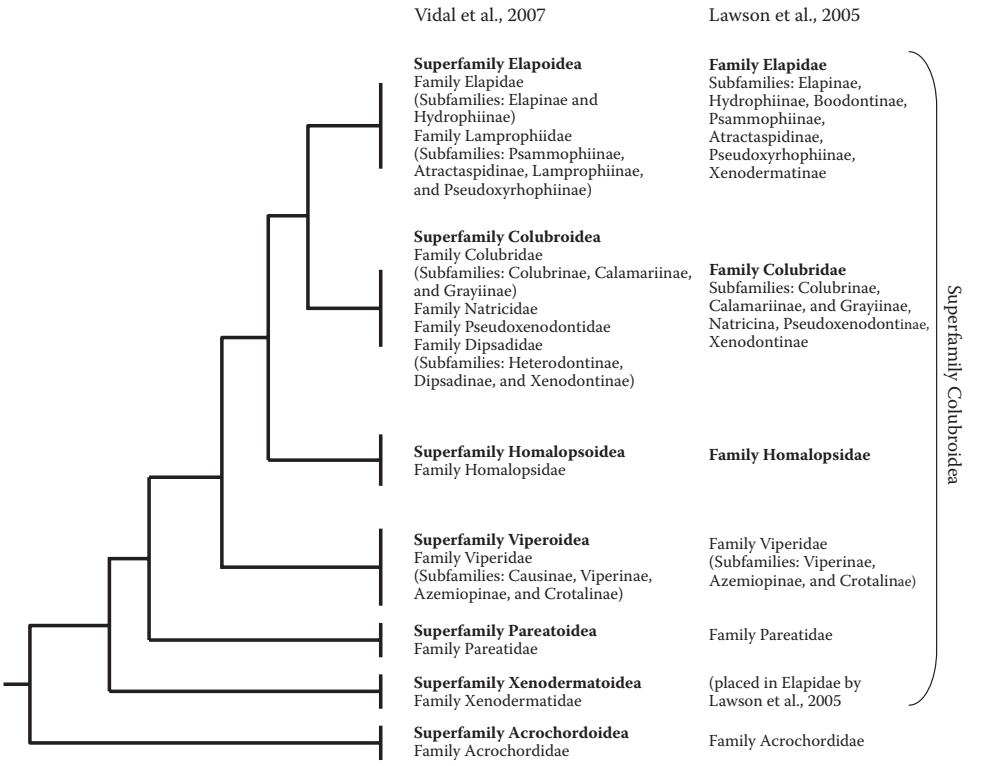

FIGURE 2.1 Simplified taxonomy and evolutionary higher-level relationships in the Caenophidia based on molecular phylogenetic studies (Lawson et al., 2005; Vidal et al., 2007).

Vidal and Hedges (2002), Lawson et al. (2005), and Vidal et al. (2007) agree that caenophidians evolved from a single common ancestor and that the family Colubridae is not a single monophyletic group as previously supposed, but represents many lineages. On the other hand, the front-fanged and medically important families Elapidae, Viperidae, and Atractaspididae do not form a clade, but each represent an independent, monophyletic lineage within the Caenophidia. These findings have allowed studies mapping venom delivery system characters and the phylogeny of the toxin-encoding genes themselves onto these phylogenies, in an effort to reconstruct the origin of the venom and its delivery systems. Several studies have mapped venom chemistry and morphological data onto the new phylogenies (e.g., Vidal, 2002; Fry et al., 2003b, 2006; Jackson, 2003; Fry and Wüster, 2004). All have reached the conclusion that the ancestor of all Caenophidia possessed at least some components of a venom delivery system, suggesting that all nonvenomous caenophidians are descended from a venomous ancestor. Moreover, Fry et al. (2006) also provided evidence that venom evolved much earlier in squamate history, and that many lizards hitherto considered nonvenomous do in fact secrete toxins (or toxin-like proteins), and that the venom apparatus of all squamates (i.e., snakes, *Heloderma*, varanid, anguid, and iguanid lizards) is homologous. In view of the homology of the venom gland and many of the toxin families secreted by them, Fry et al. (2003c) also suggested that the distinction between Duvernoy's gland and the venom glands of elapids, viperids, and many atractaspidids should be abandoned.

A near consensus of the evolutionary relationships in the Caenophidia has emerged recently, mainly based on molecular phylogenies (e.g., Kelly et al., 2003; Lee et al., 2004; Lawson et al., 2005; Vidal et al., 2007) (Figure 2.1). However, the translation of these phylogenetic data into a stable family-level taxonomy has not been reached yet (cf. Lawson et al., 2005; Vidal et al., 2007).

A key finding of recent phylogenetic work is that the old family Colubridae, a huge assemblage that traditionally included all caenophidians devoid of a front-fanged venom system, is nonmonophyletic, as the front-fanged Viperidae, Elapidae, and Atractaspididae are all nested within this group. Consequently, the modern treatment, which is reaching a general consensus, is elevating most of those subfamilies to a family rank, and restricting the name Colubridae to a single monophyletic group (Lawson et al., 2005; Vidal et al., 2007) that includes a number of species such as whip snakes, rat snakes, and king snakes, as well as many well-known rear-fanged genera of toxinological interest such as *Dispholidus*, *Thelotornis*, and *Boiga*. Table 2.1 presents the current state of family-level classification of medically or toxinologically important snakes.

Following the evolutionary relationships derived from a nuclear DNA phylogeny, Vidal et al. (2007) suggest that caenophidians have an Asian origin, due the Asian distribution of the basal lineages, the Acrochordidae, Xenodermatidae, Pareatidae, Viperidae (partly Asian and most likely of Asian origin—Wüster et al., 2008), and Homalopsidae (Vidal and Hedges, 2002). The remaining majority of caenophidian species fall into the remaining clade, in turn divided into two subclades, the superfamilies Colubroidea and Elapoidea (Vidal et al., 2007; see Figure 2.1). Note that this use of the term *Colubroidea* is much more restrictive than previous usage, where the term included all Caenophidia except the Acrochordidae (e.g., Lawson et al., 2005). The Colubroidea include the cosmopolitan and highly diverse Colubridae, Natricidae, and the American Dipsadidae and Asian Pseudoxenodontidae, whereas the Elapoidea include the cosmopolitan, front-fanged Elapidae, as well as a number of primarily African and Malagasy taxa grouped into the family Lamprophiidae, including the partly front-fanged Atractaspididae and the Psammophiinae.

A. Phylogeny of the Elapidae

Slowinski and Keogh (2000) reconstructed the phylogeny of the Elapidae based on mitochondrial DNA (mtDNA) sequences and found that a basal split separates the African, Asian, and American elapids from the Australasian and marine radiation, and thus supported the classification proposed previously by Slowinski et al. (1997), whereby the African, Asian, and American elapids form the subfamily Elapinae, whereas the Australasian and marine radiation (including *Laticauda*) forms the subfamily Hydrophiinae. The latter, long considered to contain solely the marine elapids, also now includes all terrestrial Australo-Papuan elapids (e.g., *Acanthophis*, *Notechis*, *Oxyuranus*) and the genus *Laticauda*. Castoe et al. (2007) confirmed this result with additional sequence data and also confirmed the basal split within the Elapinae as being between coral snakes (*Micrurus*, *Micruroides*, *Calliophis*, *Sinomicrurus*) and other Old World genera, including *Hemibungarus*. Sanders and Lee (2008) and Sanders et al. (2008) reconstructed the phylogeny of the Australasian and marine elapids and found evidence that this clade is the result of a recent and very rapid adaptive radiation in Australia. Relationships among the major clades have been difficult to reconstruct in the past, most likely as a result of the rapidity of the radiation of this clade.

B. Phylogeny of the Viperidae

The phylogeny of the Viperidae has been the subject of a number of recent studies. However, most of these have focused primarily on either the Viperinae or the Crotalinae, with a relative lack of studies of the entire family. The phylogeny of the Viperinae was reconstructed from mitochondrial DNA sequences by Lenk et al. (2001), who found that most major genera were monophyletic (but see below) but was unable to resolve the basal nodes of the tree. Several molecular studies of the phylogeny of the Crotalinae revealed that *Azemiops* appears to be the sister group to all pit vipers (Cadle, 1992; Knight and Mindell, 1993), and that the New World pit vipers form a monophyletic group that originated from a single invasion of the New World from Asia (Kraus et al., 1996; Parkinson, 1999; Parkinson et al., 2002; Malhotra and Thorpe, 2004a; Castoe and Parkinson, 2006).

TABLE 2.1
Recent Advances in Venomous Snake Systematics (1998–2008)

Family / Subfamily	Genus	New Taxa	Taxonomic Combinations: Previous Name	Taxonomic Combinations: New Name	Reference
Viperidae					
Viperinae	*Atheris*	*A. acuminata*			Broadley (1998)
			A. nitschei rungweensis	*A. rungweensis*	
			A. anisolepis	*A. squamigera*	Lawson and Ustach (2000)
		A. broadleyi			Lawson (1999)
			Adenorhinos barbouri	*A. barbouri*	Lenk et al. (2001)
		A. subocularis (Res)			Lawson et al. (2001)
		A. hirsuta			Ernst and Rödel (2002)
	Daboia		*D. russelii russelii*	*D. russelii*	Thorpe et al. (2007)
			D. russelii siamensis	*D. siamensis*	
			Macrovipera mauritanica	*D. mauritanica*	Lenk et al. (2001), Wüster et al. (2008)
			Macrovipera deserti	*D. deserti*	
			Vipera palaestinae	*D. palaestinae*	
	Bitis		*B. gabonica rhinoceros*	*B. rhinoceros*	Lenk et al. (1999)
			B. cornuta-inornata complex	*B. cornuta*	Branch (1999)
				B. inornata	
				B. rubida	
				B. armata	
				B. albanica	
	Cerastes	*C. cerastes hoofieni*			Werner et al. (1999)
		C. gasperettii mendelssohni			
	Echis	*E. coloratus terraesanctae*			Babocsay (2003)
		E. omanensis			Babocsay (2004)
	Montivipera		*Vipera xanthina*	*M. xanthina*	Nilson et al. (1999)
			Vipera albizona	*M. albizona*	
			Vipera albicornuta	*M. albizona*	
			Vipera bornmuelleri	*M. bornmuelleri*	

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TABLE 2.1 (continued)
Recent Advances in Venomous Snake Systematics (1998–2008)

Family			Taxonomic Combinations		
Subfamily	**Genus**	**New Taxa**	**Previous Name**	**New Name**	**Reference**
			Vipera latifii	*M. latifii*	
			Vipera bulgardaghica	*M. bulgardaghica*	
			Vipera wagneri	*M. wagneri*	
			Vipera raddei	*M. raddei*	
	Pseudocerastes	*P. urarachnoides*			Bostanchi et al. (2006)
	Vipera		*Vipera pontica*	*Vipera barani*	Baran et al. (2001)
			Vipera ursinii complex	*Vipera u. ursinii*	Nilson and Andrén (2001)
				Vipera u. macrops	
				Vipera u. graeca	
				Vipera u. rakosiensis	
				Vipera u. moldavica	
				Vipera renardi renardi	
				Vipera r. parursinii	
				Vipera r. tienshanica	
				Vipera anatolica	
				Vipera eriwanensis	
				Vipera ebneri	
				Vipera lotievi	
		V. orlovi			Tuniyev and Ostrovskikh (2001)
		V. magnifica			
Crotalinae					
	Agkistrodon		*A. bilineatus taylori*	*A. taylori*	Parkinson et al. (2000)
		A. b. lemosespinali			Smith and Chiszar (2001)
	Bothriopsis		*B. peruviana*	*B. oligolepis*	Harvey et al. (2005)
				B. chloromelas	
	Trimeresurus		*T. sumatranus malcolmi*	*Popeia malcolmi*	Stuebing and Inger (1998), Malhotra and Thorpe (2004a)

	T. albolabris	*Cryptelytrops albolabris*	Malhotra and Thorpe (2004a, 2004b, 2004c)
	T. albolabris insularis	*Cryptelytrops insularis*	Giannasi et al. (2001), Malhotra and Thorpe (2004a)
	T. albolabris septentrionalis	*Cryptelytrops septentrionalis*	
T. vogeli		*Viridovipera vogeli*	David et al. (2001)
T. gumprechti		*Viridovipera gumprechti*	David et al. (2002)
	T. tibetanus	*Himalayophis tibetanus*	Malhotra and Thorpe (2004a, 2004b, 2004c)
	T. karanshahi	*Himalayophis tibetanus*	Tillack et al. (2003), Malhotra and Thorpe (2004a, 2004b, 2004c)
T. truongsonensis		*Viridovipera truongsonensis*	Orlov et al. (2004), Malhotra and Thorpe (2004a, 2004b, 2004c)
	T. popeiorum sabahi	*Popeia sabahi*	Vogel et al. (2004), Malhotra and Thorpe (2004a, 2004b, 2004c)
	T. popeiorum barati	*Popeia barati*	
T. fucatus		*Popeia fucata*	
T. nebularis		*Popeia nebularis*	
	T. puniceus–T. borneensis complex	*T. puniceus*	David et al. (2006)
T. andalasensis			
		T. borneensis	
		T. wiroti	
		T. brongersmai	
	T. flavomaculatus	*Parias flavomaculatus*	Malhotra and Thorpe (2004a, 2004b, 2004c)
	T. hageni	*Parias hageni*	
	T. mcgregori	*Parias mcgregori*	
	T. schultzei	*Parias schultzei*	
	T. andersonii	*Cryptelytrops andersonii*	
	T. cantori	*Cryptelytrops cantori*	
	T. erythrurus	*Cryptelytrops erythrurus*	
	T. fasciatus	*Cryptelytrops fasciatus*	

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TABLE 2.1 (continued)
Recent Advances in Venomous Snake Systematics (1998–2008)

Family / Subfamily	Genus	New Taxa	Taxonomic Combinations: Previous Name	Taxonomic Combinations: New Name	Reference
			T. kanburiensis	*Cryptelytrops kanburiensis*	
			T. labialis	*Cryptelytrops labialis*	
			T. macrops	*Cryptelytrops macrops*	
			T. purpureomaculatus	*Cryptelytrops purpureomaculatus*	
			T. venustus	*Cryptelytrops venustus*	
			T. macrolepis	*Peltopelor macrolepis*	
			T. medoensis	*Viridovipera medoensis*	
			T. stejnegeri	*Viridovipera stejnegeri*	
	Popeia	*P. buniana*			Grismer et al. (2006)
	Cryptelytrops	*C. honsonensis*			Grismer et al. (2008)
	Tropidolaemus	*T. laticinctus*			Kuch et al. (2007)
			T. wagleri complex	*T. wagleri*	Vogel et al. (2007)
				T. subannulatus	
				T. philippensis	
	Protobothrops		*Trimeresurus cornutus*	*P. cornutus*	Herrmann et al. (2004)
			Ceratrimeresurus shenlii	*P. cornutus*	David et al. (2008)
	Atropoides	*A. indomitus*			Smith and Ferrari-Castro (2008)
	Bothriechis	*B. supraciliaris* (resurrected)			Solórzano et al. (1998)
		B. thalassinus			Campbell and Smith (2000)
	Ophryacus		*Porthidium melanurum*	*Ophryacus melanurus*	Gutberlet (1998)
	Bothrocophias	*B. myersi*			Gutberlet and Campbell (2001)
			Porthidium hyoprora	*B. hyoprora*	
			Bothrops microphthalmus	*B. microphthalmus*	
			Bothrops campbelli	*B. campbelli*	
	Bothrops	*B. muriciensis*			Ferrarezzi and Freire (2001)
			B. osbornei	*B. punctatus osbornei*	McDiarmid et al. (1999)
			B. pradoi	*B. leucurus*	Puorto et al. (2001)

	B. alcatraz			Marques et al. (2002)
		B. neuwiedi neuwiedi	*B. neuwiedi*	Da Silva (2004)
		B. n. urutu		
		B. n. meridionalis		
		B. n. goyazensis		
		B. n. paranaensis		
		B. n. diporus	*B. diporus*	
		B. iglesiasi	*B. lutzi*	
		B. n. lutzi		
		B. n. piauhyensis		
		B. n. mattogrossensis	*B. mattogrossensis*	
		B. n. bolivianus		
		B. n. pauloensis	*B. pauloensis*	
		B. n. pubescens	*B. pubescens*	
	Bothrops marmoratus			Da Silva and Rodrigues (2008)
Cerrophidion	*C. petlalcalensis*			López-Luna et al. (1999)
Porthidium	*P. porrasi*			Lamar and Sasa (2003)
		P. lansbergii arcosae	*P. arcosae*	Campbell and Lamar (2004)
Crotalus		*C. exsul*	*C. ruber*	Smith et al. (1998)
		C. tortugensis	*C. atrox*	Castoe et al. (2006)
		C. viridis nuntius	*C. viridis viridis*	Douglas et al. (2002)
		C. v. abyssus	*C. abyssus*	
		C. v. cerberus	*C. cerberus*	
		C. v. concolor	*C. concolor*	
		C. v. helleri	*C. helleri*	
		C. v. lutosus	*C. lutosus*	
		C. v. oreganus	*C. oreganus*	
	C. tancitarensis			Alvarado-Díaz and Campbell (2004)
		C. durissus ssp. (South America)	*C. durissus*	Campbell and Lamar (2004)
		C. durissus dryinas	*C. durissus durissus*	

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TABLE 2.1 (continued)
Recent Advances in Venomous Snake Systematics (1998–2008)

Family			Taxonomic Combinations		
Subfamily	**Genus**	**New Taxa**	**Previous Name**	**New Name**	**Reference**
			C. durissus durissus (Central America)	*C. simus*	
			C. durissus totonacus	*C. totonacus*	
			C. durissus culminatus	*C. culminatus*	Wüster et al. (2005a)
			C. durissus tzabcan	*C. tzabcan*	
			C. durissus cascavella	*C. durissus terrificus*	
			C. durissus collilineatus	*C. durissus terrificus*	
			C. mitchellii stephensi	*C. stephensi*	Douglas et al. (2007)
		C. ericsmithi			Campbell and Flores-Villela (2008)
	Lachesis		*L. m. muta*	*Lachesis muta*	Zamudio and Greene (1997)
			L. m. melanocephala	*Lachesis melanocephala*	
			L. m. stenophrys	*Lachesis stenophrys*	
		L. acrochorda (revalidated)			Campbell and Lamar (2004)
	Gloydius		*Agkistrodon blomhoffi blomhoffi*	*Glodyus blomhoffii blomhoffii*	
			A. b. brevicaudus	*G. b. brevicaudus*	
			A. b. dubitatus	*G. b. dubitatus*	
			A. b. siniticus	*G. b. siniticus*	
			A. halys halys	*G. halys halys*	
			A. h. caraganus	*G. h. caraganus*	
			A. h. cognatus	*G. h. cognatus*	
			A. intermedius	*G. intermedius*	
			A. i. caucasicus	*G. i. caucasicus*	
			A. i. stejnegeri	*G. i. stejnegeri*	
			A. saxatilis	*G. saxatilis*	
			A. ussuriensis	*G. ussuriensis*	McDiarmid et al. (1999)

	Protobothrops	*Triceratolepidophis sieversorum*		*Protobothrops sieversorum*	Ziegler et al. (2000), Guo et al. (2007)
			Trimeresurus mangshanensis, Ermia mangshanensis, Zhaoermia mangshanensis	*Protobothrops mangshanensis*	Gumprecht and Tillack (2004), Guo et al. (2007)
	Ovophis/Garthius		*Ovophis chaseni*	*Garthius chaseni*	Malhotra and Thorpe (2004a)
Homalopsidae					
	Enhydris	*E. gyii*			Murphy et al. (2005)
		E. chanardi			Murphy and Voris (2005)
Natricidae	*Rhabdophis*	*R. t. formosanus*			Ota et al. (1999)
	Amphiesma		*A. pryeri*	*A. pryeri*	
				A. concelarum	
				A. ishigakiense	Ota and Iwanaga (1997)
		A. kerinciense			David and Das (2003)
		A. andreae			Ziegler and Quyet (2006)
		A. leucomystax			David et al. (2007)
Colubridae					
Colubrinae	*Thelotornis*	*T. usambaricus*			Broadley (2001)
			T. capensis mossambicanus	*T. mossambicanus*	
	Xyelodontophis	*X. ulugurensis*			Broadley and Wallach (2002)
	Boiga	*B. tanahjampeana*			Orlov and Ryabov (2002)
		B. bengkuluensis			Orlov et al. (2003)
		B. ranawanei			Samarawickrama et al. (2005)
			B. ocellata	*B. siamensis*	Pauwels et al. (2006)
	Ahaetulla	*A. prasina medioxima*			Lazell (2002)
	Trimorphodon		*T. biscutatus vilkinsonii*	*T. vilkinsonii*	LaDuc and Johnson (2003)
			T. biscutatus complex	*T. tau*	DeVitt et al. (2008)
				T. quadruplex	
				T. biscutatus	
				T. lyrophanes	
				T. paucimaculatus	
				T. lambda	

(continued on next page)

TABLE 2.1 (continued)
Recent Advances in Venomous Snake Systematics (1998–2008)

Family			Taxonomic Combinations		
Subfamily	**Genus**	**New Taxa**	**Previous Name**	**New Name**	**Reference**
Dipsadidae	*Clelia*	*C. hussami*			Morato et al. (2003)
	Hydrodynastes	*H. melanogigas*			Franco et al. (2007)
	Hypsiglena			*H. torquata*	Mulcahy (2008)
				H. affinis	
			Eridiphas slevini	*H. slevini*	
				H. tanzeri	
				H. sp. *nov.*	
				H. jani	
				H. chlorophacea	
				H. ochrorhyncha	
	Pseudoboa	*P. martinsi*			Zaher et al. (2008a)
	Liophis		*L. miliaris intermedius*	*L. reginae*	Dixon and Tipton (2003)
			L. m. semiaureus	*L. semiaureus*	Giraudo et al. (2006)
	Philodryas		*P. oligolepis, P. affinis*	*P. laticeps*	Zaher et al. (2008b)
			P. pallidus	*Liopholidophis varius*	Thomas and Di-Bernardo (2001)
Lamprophiidae					
Atractaspidinae	*Atractaspis*		*A. microlepidota complex*	*A. microlepidota*	Trape et al. (2006)
				A. micropholis	
				A. watsoni	
	Psammophis	*P. zambiensis*			Hughes and Wade (2002)
			P. leightoni trinasalis	*P. trinasalis*	Broadley (2002)
			P. leightoni namibensis	*P. namibensis*	
			P. subtaeniatus orientalis	*P. orientalis*	
			P. sibilans brevirostris	*P. brevirostris*	
			P. sibilans leopardinus	*P. leopardinus*	

			Dipsina multimaculata	*P. multimaculata*	Kelly et al. (2008)
			Dromophis lineatus	*P. lineatus*	
			Dromophis praeornatus	*P. praeornatus*	
	Psammophylax		*Rhamphiophis acutus*	*Psammophylax acutus*	
	Malpolon		*Malpolon monspessulanus*	*M. monspessulanus*	Carranza et al. (2006)
			M. m. insignitus	*M. insignitus insignitus*	
			M. m. fuscus	*M. insignitus fuscus*	
	Madagascarophis		*M. citrinus*	*M. colubrinus*	Nagy et al. (2007)
Elapidae					
Elapinae					
	Elapsoidea		*E. semiannulata boulengeri*	*E. boulengeri*	Broadley (1998)
		E. trapei			Mane (1999)
	Micrurus		*M. frontalis balicoryphus*	*M. baliocoryphus*	Jorge da Silva and Sites (1999)
			M. f. brasiliensis	*M. brasiliensis*	
			M. f. frontalis	*M. frontalis*	
			M. f. multicinctus	*M. altirostris*	
			M. f. diana	*M. diana*	
			M. f. pyrrhocryptus	*M. pyrrhocryptus*	
			M. pyrrhocryptus tricolor	*M. tricolor*	
		M. pachecogili			Campbell (2000)
		M. pacaraimae			Morato de Carvalho (2002)
		M. tamaulipensis			Lavvin-Murcio and Dixon (2004)
		M. silviae			Di-Bernardo et al. (2007)
			M. surinamensis nattereri	*M. nattereri*	Passos and Fernandes (2005)
	Naja	*N. mandalayensis*			Slowinski and Wüster (2000)
		N. nubiae			Wüster and Broadley (2003)
			N. annulifera anchietae	*N. anchietae*	Broadley and Wüster (2004)

(continued on next page)

TABLE 2.1 (continued)
Recent Advances in Venomous Snake Systematics (1998–2008)

Family			Taxonomic Combinations		
Subfamily	**Genus**	**New Taxa**	**Previous Name**	**New Name**	**Reference**
		N. ashei			Wüster and Broadley (2007)
			N. nigricollis nigricincta	*N. nigricincta nigricincta*	Wüster et al. (2007)
			N. nigricollis woodi	*N. nigricincta woodi*	
			Boulengerina annulata	*N. annulata*	
			Boulengerina christyi	*N. christyi* (by implication)	
			Paranaja multifasciata	*N. multifasciata*	
	Walterinnesia		*Naja morgani* = eastern populations of *W. aegyptia*	*Walterinnesia morgani*	Nilson and Rastegar-Pouyani (2007)
	Calliophis			*C. beddomei*	Slowinski et al. (2001)
				C. bibroni	
				C. gracilis	
				C. maculiceps	
				C. melanurus	
				C. nigrescens	
			Maticora intestinalis	*C. intestinalis*	
			Maticora bivirgata	*C. bivirgatus*	
		C. haematoetron			Smith et al. (2008)
	Hemibungarus		*Calliophis calligaster*	*H. calligaster*	Slowinski et al. (2001)
	Sinomicrurus		*Hemibungarus hatori*, *Calliophis hatori*	*S. hatori*	Slowinski et al. (2001)
			Calliophis japonicus, *Hemibungarus japonicus*	*S. japonicus*	
			Calliophis kelloggi, *Hemibungarus kelloggi*	*S. kelloggi*	
			Calliophis macclellandi, *Hemibungarus macclellandi*	*S. macclellandi*	
			Calliophis sauteri, *Hemibungarus sauteri*	*S. sauteri*	

	Bungarus	*B. slowinskii*			Kuch et al. (2005)
			B. javanicus	*B. candidus*	Kuch and Mebs (2007)
	Aspidelaps		*Aspidelaps lubricus infuscatus*	*A. l. cowlesi*	Broadley and Baldwin (2006)
Hydrophiinae	*Oxyuranus*	*O. temporalis*			Doughty et al. (2007)
	Demansia			*D. papuensis*	Shea (1998)
			D. atra	*D. vestigiata*	
	Hydrophis	*H. laboutei*			Rasmussen and Ineich (2000)
		H. sibauensis			Rasmussen et al. 2001
	Laticauda	*L. guineai*			Heatwole et al. (2005)
		L. saintgironsi			Cogger and Heatwole (2006)
		L. frontalis (revalidated)			
	Simoselaps	*S. morrisi*			Horner (1998)
	Elapognathus		*Drysdalia coronata*	*Elapognathus coronatus*	Keogh et al. (2000)
	Notechis		*N. ater, N. scutatus*	*Notechis scutatus*	Keogh et al. (2005)
	Paroplocephalus		*Echiopsis atriceps*	*Paroplocephalus atriceps*	Keogh et al. (2000)
	Pseudechis		*Pailsus*	*Pseudechis*	Wüster et al. (2005b)
			Pailsus rossignolii	Pseudechis rossignolii	Williams and Wüster (2005)

Wüster et al. (2008) analyzed all Viperidae together and, like Nagy et al. (2005), found that *Causus* is nested within the Viperinae and does not warrant recognition of a separate subfamily Causinae; their study confirmed many of the results mentioned above, including the status of *Azemiops* as the sister taxon of the Crotalinae.

III. ACCOUNTS OF RECENT DEVELOPMENTS IN VENOMOUS SNAKE SYSTEMATICS AT THE GENUS AND SPECIES LEVEL

Although advances in our understanding of the high-level phylogeny of snakes are essential to understand the origin and evolution of venom and the distribution of venom among the advanced snakes, they are not otherwise of particular importance for the identification of venoms and the interpretation of toxinological research data. In contrast, correct species-level taxonomy is of fundamental importance for the interpretation and replication of toxinological and medical research. Misidentified or unidentifiable venoms lead to wasted research effort (e.g., by researchers failing to find compounds of interest because they have been misled by sloppy taxonomy in another paper) and potentially to ineffective antivenoms and unnecessary snakebite fatalities (Warrell, 2008). Since our understanding of venomous snake diversity has advanced rapidly in the last decade, it is important for toxinologists to be aware of developments in the field. Here we present a synopsis of recent taxonomic changes and new taxa of venomous caenophidians discovered. An overview of taxonomic changes is provided (see Tables 2.1 and 2.2). We follow the higher-level Caenophidia taxonomy proposed by Vidal et al. (2007) to provide a framework in which to discuss genus- and species-level changes in detail.

A. Superfamily Colubroidea

1. Natricidae

Rhabdophis—*Asiatic Keelbacks*

Rhabdophis is a genus of snakes, generally called keelback snakes, found primarily in Southeast and Eastern Asia. The species *Rhabdophis tigrinus* is widespread in China, Korea, Japan, and Taiwan. Morphological and karyotype differences between these and other populations suggest that the Taiwanese populations should be recognized as a separate subspecies, *R. t. formosanus* (Ota et al., 1999).

Amphiesma—*Asiatic Keelbacks*

Ota and Iwanaga (1997) reviewed the systematics of *Amphiesma pryeri* and its subspecies in the Ryukyu Archipelago, Japan. Based on hemipenial morphology, karyotype, and reproductive mode, they consider *A. pryeri*, *A. concelarum*, and *A. ishigakiense* as valid species. In addition, several species have been described recently: *A. kerinciense* is described from the slopes of Gunung Kerinci, western Sumatra, Indonesia (David and Das, 2003), *A. andreae* from the Truong Son (Annamite mountain range) of Quang Binh Province in central Vietnam (Ziegler and Quyet, 2006), and *A. leucomystax* from central Vietnam and possibly Thailand (David et al., 2007).

2. Colubridae

Coluber—*Racers*

Coluber was a generic name for a large number of New and Old World species of racers and whipsnakes. Nagy et al. (2004) studied the phylogenetic relationships among Old and New World representatives using mitochondrial and nuclear genes. Based on their phylogeny, they restricted the usage of the name *Coluber* to the New World taxa and discuss the synonymy with *Masticophis*. Among the Old World racers, *Platyceps*, *Hemorrhois*, *Spalerosophis*, and *Hierophis* are validated. *Hierophis* seems to be paraphyletic with *Eirenis* nested within it. The authors recommend a subdivision of *Hierophis*

TABLE 2.2
Systematics of Gloydius, Illustrating the Different and Often Contradictory Interpretations of the Systematics and Nomenclature of This Genus

Golay et al. (1993)	Bour (1993)	Zhao and Adler (1993)	Gloyd and Conant (1990)	Harding and Welch (1980)	David and Ineich (1999)	McDiarmid et al. (1999)	Orlov and Barabanov (1999)	Gumprecht et al. (2004)	Vogel (2006)
A. blomhoffi blomhoffi			*A. blomhoffii blomhoffii, A. affinis*	*A. blomhoffii blomhoffii*	*G. blomhoffii blomhoffii*	*G. blomhoffii blomhoffii*	*A. blomhoffii blomhoffii*	*G. blomhoffii blomhoffii*	*G. blomhoffii blomhoffii*
A. blomhoffi brevicaudus		*A. blomhoffii brevicaudus*	*A. blomhoffii brevicaudus*	*A. blomhoffii brevicaudus*	*G. blomhoffii brevicaudus*	*G. blomhoffii brevicaudus*	*A. brevicaudus*	*G. brevicaudus*	*G. brevicaudus*
A. blomhoffi dubitatus		*A. blomhoffii brevicaudus*	*A. blomhoffii dubitatus*		*G. blomhoffii brevicaudus*	*G. blomhoffii dubitatus*			
A. blomhoffi siniticus		*A. blomhoffii brevicaudus*	*A. blomhoffii siniticus*	*A. blomhoffii brevicaudus*	*G. blomhoffii brevicaudus*	*G. blomhoffii siniticus*	*A. blomhoffii siniticus*	*G. blomhoffii siniticus*	*G. blomhoffii siniticus*
A. halys halys	*A. halys mogoi*	*A. halys halys*	*A. halys halys*	*A. halys halys*	*G. halys mogoi*	*G. halys halys, G. halys mogoi (?)*	*A. halys halys*	*G. halys halys*	*G. halys mogoi*
A. halys caraganus	*A. halys halys*		*A. halys caraganus*	*A. halys caraganus*	*G. halys halys*	*G. halys caraganus*	*A. halys caraganus*	*G. halys caraganus*	*G. halys halys*
A. halys cognatus	*A. halys cognatus*	*A. intermedius*	*A. halys cognatus*		*G. intermedius*	*G. halys cognatus*	*A. halys cognatus*	*G. halys cognatus*	
A. intermedius intermedius		*A. intermedius*	*A. intermedius intermedius*	*A. halys intermedius*	*G. intermedius*	*G. intermedius*	*A. halys halys*		
A. intermedius caucasicus			*A. intermedius caucasicus*	*A. halys caucasicus*	*G. halys caucasicus*	*G. intermedius caucasicus*	*A. halys caucasicus*	*G. halys caucasicus*	
A. intermedius stejnegeri		*A. intermedius*	*A. intermedius stejnegeri*		*G. intermedius*	*G. intermedius stejnegeri*	*A. halys stejnegeri*	*G. halys stejnegeri*	
A. saxatilis		*A. saxatilis*	*A. intermedius saxatilis*	*A. saxatilis*	*G. saxatilis*	*G. saxatilis*	*A. intermedius*	*G. intermedius*	*G. intermedius*
A. ussuriensis		*A. ussuriensis*	*A. caliginosus, A. blomhoffii ussuriensis*	*A. caliginosus, A. halys ussuriensis*	*G. ussuriensis*	*G. ussuriensis*	*A. ussuriensis*	*G. ussuriensis*	*G. ussuriensis*

Note: In the absence of a thorough study of the systematics, we present these different schemes for comparison. Toxinologists need to ensure that their venoms will remain identifiable in the light of future studies. Some older references listed here used the name *Agkistrodon*. We have followed the original nomenclature here, but *Gloydius* is now universally accepted.

into three monophyletic genera. The name *Hierophis* is restricted to the European group containing the taxa *H. viridiflavus* and *H. gemonensis*. *Eirenis* is retained with the inclusion of *H. spinalis*. A third group composed of *H. jugularis*, *H. caspius*, *H. schmidti*, *H. gyarosensis*, and *H. cypriensis* is moved to *Dolichophis*. The Afrotropical racers remain as *Coluber* in the broad sense.

Thelotornis—*African Twig Snakes*

Studies on the systematics of the genus *Thelotornis* in eastern Africa indicate a very diverse group (Broadley, 2001). *T. usambaricus* was described from Usambara Mountains of Tanzania. *T. capensis mossambicanus* from eastern Africa (from central Mozambique northward) was raised to the status of a full species, *T. mossambicanus*. In addition, the following taxa are recognized: *T. kirtlandii* from the forests of central and western Africa, *T. capensis capensis* from northeastern South Africa and adjoining areas, and *T. c. oatesi* from Zambia, Zimbabwe, Mozambique, southern Congo, Botswana, southern Angola, and Namibia (Broadley, 2001).

Xyelodontophis—*Dagger-Toothed Vine Snake*

Based on two specimens, a new genus and species, *Xyelodontophis uluguruensis*, was described from the Uluguru Mountains of Tanzania (Broadley and Wallach, 2002). Externally, these snakes are most similar to *Thelotornis*, but lack a horizontal pupil and differ in several internal characters. In particular, the posterior maxillary teeth are greatly enlarged, ungrooved, and flat and curved—hence the suggested common name of dagger-toothed vine snake. The Duvernoy's gland is intermediate in size between *Dispholidus* and *Thelotornis*, so that this species should be regarded as potentially dangerous.

Ahaetulla—*Asian Vine Snake*

Ahaetulla include approximately eight species found predominantly from India through to China and much of Southeast Asia, including many Pacific islands. Lazell (2002) described a new subspecies, *A. prasina medioxima*, from the island of Shek Kwu Chau, Hong Kong, and four subspecies are now recognized in *A. prasina*.

Boiga—*Mangrove/Cat Snakes*

Boiga is a large genus (ca. thirty-five species) of rear-fanged colubrid snakes found throughout Southeast Asia, India, and Australasia. Several species had been described recently: *Boiga tanahjampeana* from the island of Tanahjampea, south of Sulawesi, Indonesia (Orlov and Ryabov, 2002), *B. bengkuluensis* from Bengkulu Province, SW Sumatra (Orlov et al., 2003), and *B. ranawanei* from Kandy District, Sri Lanka (Samarawickrama et al., 2005). Distinctiveness of these forms is based mainly on scale morphometrics and body color patterns.

Trimorphodon—*American Lyre Snakes*

Lyre snakes range throughout the southwestern United States, from Texas to California as well as most of Mexico and down into Central America. The systematics of the species complex has been revised by LaDuc and Johnson (2003) and Devitt et al. (2008). Most subspecies have been elevated to species based on phylogenetic and morphometric analysis (Table 2.2). Therefore, the complex is composed of seven recognizable species.

3. Dipsadidae

Clelia hussami—*Mussuranas*

Mussuranas (or muçuranas) are large snakes of mainly ophiophagous habits distributed from Guatemala to Brazil. A new species, *Clelia hussami*, was described from a narrow area of the Araucaria forest in southern central Paraná and northern central Santa Catarina states, Brazil (Morato et al., 2003). This form can be distinguished from other members of the genus by a combination of

nineteen dorsal scale rows, fifty-six or fewer subcaudals, a dark mid-dorsal line that is no more than three scale rows wide, and immaculate supralabials and ventrals.

Leptodeirini—*Neotropical Cat-Eyed Snakes*

The group Leptodeirini was previously considered monophyletic and consists of the genera *Leptodeira*, *Imantodes*, *Eridiphas*, *Hypsiglena*, *Pseudoleptodeira*, and *Cryophis*. Mulcahy (2007) analyzed the phylogenetic relationships of the group using mtDNA. Monophyly of Leptodeirini is not supported. Instead, clades containing *Imantodes* and *Leptodeira*, another containing *Hypsiglena* and *Eridiphas*, with the latter placed closer to *Cryophis* and other dipsadine genera (*Sibon*, *Dipsas*, and *Atractus*), were supported.

Hypsiglena—*North American Night Snakes*

Mulcahy (2007) used phylogeographic analysis to examine species boundaries of *Hypsiglena torquata*. He recognized six species: one is a previously undescribed species, and two were previously recognized as subspecies (*H. torquata*, *H. affinis*, *H. tanzeri*). The remaining three are widespread, polymorphic lineages, composed of multiple subspecies: *H. jani*, *H. chlorophaea*, and *H. ochrorhyncha*. The Baja California night snake, *Eridiphas slevini*, was placed back in the genus *Hypsiglena*.

Hydrodynastes melanogigas

A new species of the semiaquatic genus *Hydrodynastes* has been described from the state of Tocantins, central Brazil (Franco et al., 2007). The new species differs from *H. gigas* and *H. bicinctus* primarily through its melanism, the lack of a postocular stripe and ventral coloration.

Liophis

The genus *Liophis* ranges south of Central America (Costa Rica and Panama) and the Caribbean to Argentina. Recent taxonomic advances in the genus include the recognition of *L. miliaris intermedius* as a synonym of *L. reginae* (Dixon and Tipton, 2003). Based on morphological and color pattern analysis, Giraudo et al. (2006) elevated *L. m. semiaureus* to species level (*L. semiaureus*).

Pseudoboa

Zaher et al. (2008a) described a new species, *P. martinsi*, from the Amazon Basin of Brazil, with records from the states of Pará, Amazonas, Roraima, and Rondônia. The new species is distinguished from the other five species of the genus by a combination of scalation and coloration characters. Individuals of the new species were found in both primary and disturbed forested areas.

Philodryas

Twenty-two species of this genus were recognized until recently, but this number has been reduced by recent research. The holotype of *Philodryas pallidus* was mistaken as a member of the Neotropical group. The actual identity corresponds to *Liopholidophis varius* from Madagascar (Thomas and Di-Bernardo, 2001). *Philodryas laticeps* was previously known only from the holotype. Based on morphological data, Zaher et al. (2008b) concluded that the similar species *P. oligolepis* and *P. affinis* are junior synonyms of *P. laticeps*.

B. Superfamily Elapoidea

1. Lamprophiidae

Atractaspis—*Stiletto Snakes*

Members of the genus *Atractaspis* occur mostly in sub-Saharan Africa, with a limited distribution in Israel and the Arabian Peninsula. The taxonomy of the genus remains in a state of disarray due to the lack of comprehensive revisions and the paucity of material of most species. Trape

et al. (2006) examined patterns of morphological variation in four described taxa of the *Atractaspis microlepidota* complex. Their findings suggest that *A. microlepidota* is restricted to westernmost Africa (Senegal, Gambia, and Mauritania) and confirm the status of *A. micropholis* as a distinct species. A related form, *A. watsoni*, which has long been considered a synonym of *A. microlepidota*, is found to be a valid species with a distribution extending from Mauritania to Sudan, through which it is sympatric with the similar *A. micropholis*, which is found from Senegal to Nigeria. The status of eastern African and Arabian populations previously assigned to *A. microlepidotus* remains to be clarified. Dobiey and Vogel (2007) referred to the eastern African populations as *Atractaspis fallax*, and suggested that the taxa *A. phillipsii* and *A. magrettii* may be part of the same species.

Psammophis *and Allies—Sand Snakes*

Psammophis is a genus of mainly African rear-fanged snakes with massive venom glands and complex venom. Broadley (2002) elevated to species level several forms previously included in other taxa: *Psammophis trinasalis*, *P. namibensis*, *P. orientalis*, *P. brevirostris*, and *P. leopardinus.* On the other hand, *Psammophis zambiensis*, a member of the former *P. sibilans* complex, is described from northern and eastern Zambia (Hughes and Wade, 2002). The phylogeography of the widespread species *P. schokari* was studied by Rato et al. (2007), and the validity of *P. aegyptius* is corroborated. Kelly et al. (2008) studied the phylogeny and species delimitation in *Psammophis* and allied genera. Psammophiinae is considered at the family level by these authors. The monotypic genus *Dipsina* was transferred to *Psammophis*. *Dromophis* is deeply nested within *Psammophis*, and was therefore synonymized with that genus. The *Psammophis sibilans* species complex was found to consist of two monophyletic entities: the *phillipsii* and *subtaeniatus* complexes. *P. p. phillipsii* and *P. mossambicus* are not distinct. On the other hand, *P. cf. phillipsii occidentalis* is elevated to species status. Finally, *Rhamphiophis acutus* was transferred to the genus *Psammophylax*.

Malpolon—*Montpellier Snake*

Carranza et al. (2006) studied the phylogenetic and biogeographic affinities of *Malpolon monspessulanus* in most of its distribution. The western and eastern forms of *M. monspessulanus* have different dorsal color patterns, differences in skull structure, and exhibit species-level genetic divergence in mtDNA. Therefore, Carranza et al. recommended that they should be treated as separate species: *M. monspessulanus* (sensu stricto) and *M. insignitus*, the latter including the subspecies *M. i. fuscus*.

Madagascarophis—*Malagasy Cat-Eyed Snakes*

Malagasy cat-eyed snakes are found in Madagascar, from montane regions to rain forest. Nagy et al. (2007) studied mitochondrial and nuclear divergence in *Madagascarophis* species except *M. ocellatus*. They identified six major clades, which only partly agreed with previously proposed classifications. Three clades are considered as distinct species: *M. colubrinus*, *M. meridionalis*, and an undescribed species; *M. citrinus* is a synonym of *M. colubrinus*.

2. Elapidae

Acanthophis—*Death Adders*

Acanthophis is one of the more complex and poorly understood genera of elapids. Considerable morphological diversity produced a confusing taxonomy in the past, and there are no comprehensive revisions of the genus. A recent phylogeographic study (Wüster et al., 2005a) revealed previously unsuspected patterns of genetic diversity that disagreed profoundly with conventionally accepted species limits. The New Guinea populations, previously shoehorned into either *A. antarcticus* or *A. praelongus*, comprise two lineages, the *A. laevis* complex and *A. rugosus*. The *A. laevis* complex is widespread in New Guinea and the Moluccas, and it may contain more than one species.

Acanthophis praelongus is confined to northern Queensland and is most closely related to *A. antarcticus*. Death adder populations from the top end of Australia, previously considered part of *A. praelongus*, are related to *A. rugosus*, and appear to comprise two major lineages: one comprises populations from southern New Guinea and hilly parts of the Northern Territory, Western Australia, and western Queensland, and is probably best considered as *A. rugosus*; the other contains populations from floodplains in the Northern Territory and western Queensland, and probably represents a separate species, *A. hawkei*. However, this group may include several additional species-level lineages and requires more work (Wüster et al., 2005a).

Demansia—*Australian Whip Snakes*

The Australian whip snakes include a number of fast-moving, diurnal elapids from Australia and New Guinea. They are generally regarded as relatively innocuous. Shea (1998) studied the patterns of geographic variation in the *Demansia papuensis/vestigiatus* complex in northern Australia and Papua New Guinea. The status of some populations, particularly those of New Guinea, was unclear until recently. Two species are recognized: *Demansia papuensis*, found in northern Australia from the Kimberleys to central eastern Queensland, and *D. vestigiata*, found from extreme northeastern Western Australia to extreme southeastern Queensland, and also in southern Papua New Guinea. The name *Demansia atra*, widely used in the literature until now, is a junior synonym of *D. vestigiata*.

Simoselaps—*Australian Shovel-Nosed Snakes*

Horner (1998) described a new species of *Simoselaps*, *S. morrisi*, from the northern Arnhem Land, Northern Territory, Australia. These small, secretive snakes are normally regarded as inoffensive to humans, but their venoms remain largely unstudied.

Elapsoidea—*African Garter Snakes*

Despite the common name, African garter snakes are unrelated to the harmless North American garter snake species. Broadley (1998a) reviewed the taxonomy of the *Elapsoidea semiannulata* complex and the taxon *E. s. boulengeri* was elevated to species. Previously in southeastern Senegal *Elapsoidea trapei* was described (Mané, 1999). Therefore, ten species are recognized: *E. boulengeri* (southern parts of Africa), *E. broadleyi* (Somalia), *E. chelazziorum* (Somalia), *E. guentheri* (south-central Africa), *E. laticincta* (Central Africa), *E. loveridgei* (Central and East Africa), *E. nigra* (Tanzania), *E. semiannulata* (widespread south of Sahara), *E. sunderwallii* (southern Africa), and *E. trapei* (Senegal).

Micrurus—*New World Tropical Coral Snakes*

During the past two decades, a large number of new species in the genus *Micrurus* appeared in the literature: *M. pachecogili* from the highlands of southern Puebla, México (Campbell, 2000); *M. pacaraimae* from the Brazilian-Venezuelan border area in Roraima (Morato de Carvalho, 2002); *M. camilae* from Córdoba Province, Colombia (Renjifo and Lundberg, 2003); *M. tamaulipensis* from the Sierra de Tamaulipas, Tamaulipas, Mexico (Lavin-Murcio and Dixon, 2004); and *M. silviae* from Rio Grande do Sul in southern Brazil (Di-Bernardo et al., 2007).

In addition, several revisions of species complexes were published. The *Micrurus frontalis* complex was revised (Jorge da Silva and Sites, 1999); the complex now includes seven species: *M. frontalis*, from central Brazil and eastern Paraguay; *M. altirostris*, from southern Brazil, eastern Paraguay, Uruguay, and northwestern Argentina; *M. baliocoryphus*, from northeastern Argentina and southwestern Paraguay; *M. brasiliensis*, from northern central Brazil; *M. diana*, from Santa Cruz Province, Bolivia; *M. pyrrhocryptus*, from Argentina, northwestern Paraguay, and southern Bolivia; and *M. tricolor*, from Brazil and eastern Bolivia. More recently, *M. surinamensis nattereri*, from the Orinoco and Rio Negro drainages of Venezuela, Colombia, and Brazil, was elevated to species level as *M. nattereri* (Passos and Fernandes, 2005).

Drysdalia/Elapognathus—*Crowned and Short-Nosed Snakes*

Crowned snakes (*Drysdalia* spp.) possess relatively mild venom and are native to parts of southern and eastern Australia. The genus *Elapognathus* (short-nosed snake) was previously considered monotypic, containing only *Elapognathus minor.* Keogh et al. (2000) investigated the phylogenetic affinities of *E. minor* and *D. coronata*. The results show *D. coronata* to be more closely related to *E. minor* than to other species of *Drysdalia*. As a result, *D. coronata* is recognized as *E. coronatus.*

Paroplocephalus/Echiopsis—*Lake Cronin Snake*

The Lake Cronin snake is only found in the region of Lake Cronin, Western Australia. Keogh et al. (2000) examined the phylogenetic position of the Lake Cronin snake, most often assigned to *Echiopsis atriceps*. The species appears to be most closely related to the broad-headed snakes, *Hoplocephalus.* Since the Lake Cronin snake is nonetheless highly distinct from *Hoplocephalus*, Keogh et al. assigned it to a new genus, *Paroplocephalus*, of which it is the only species.

Hydrophis—*Sea Snakes*

The genus *Hydrophis* occurs mostly the in sea waters of Indo-Australia and Southeast Asia. Rasmussen et al. (2000) described *Hydrophis laboutei* from New Caledonia, and *Hydrophis sibauensis* was described from an affluent of the River Kapuas, West Kalimantan, Indonesia (Rasmussen et al., 2001). The latter species is unique, as it occurs a long distance from the sea. The only other strictly freshwater sea snakes live in lakes close to the coast.

Calliophis, Maticora*, and* Sinomicrurus—*Oriental Coral Snake*

Until recently, the oriental coral snakes constituted a poorly understood group, and several species have been moved back and forth between different genera. Slowinski et al. (2001) analyzed the phylogeny of the group, including also the New World coral snakes. Based on morphology and mitochondrial DNA sequences, they identified three monophyletic groups, which were considered distinct genera: *Calliophis*, a tropical Asian genus including the species *C. beddomei*, *C. bibroni*, *C. gracilis*, *C. maculiceps*, *C. melanurus*, *C. nigrescens*, *C. intestinalis*, and *C. bivirgatus*; *Hemibungarus*, containing the single Philippine species *H. calligaster*; and a new genus, *Sinomicrurus*, which contains *S. hatori*, *S. japonicus*, *S. kelloggi*, *S. macclellandi*, and *S. sauteri*. Oriental coral snake appears to be the sister group to the New World coral snakes (Slowinski et al., 2001). Smith et al. (2008) described a new species, *Calliophis haematoetron*, from central Sri Lanka. This is the second species of coral snake known from the island country (after *C. melanurus*).

Naja, Paranaja, Boulengerina—*Cobras*

The cobras have been heavily revised at both species and genus level in recent years. Nagy et al. (2005) analyzed the phylogeny of African caenophidian snakes using mitochondrial and nuclear genes, and synonymized the genus *Boulengerina* with *Naja*, an approach followed by Branch (2005). Wüster et al. (2007) confirmed this and also synonymized *Paranaja* with *Naja*.

Several species of spitting cobras have been described recently: *Naja mandalayensis*, from the area around the city of Mandalay, central Burma (Slowinski and Wüster, 2000); *N. nubiae*, the Nubian spitting cobra from northeastern Africa (Wüster and Broadley, 2003), previously regarded as a variety of *N. pallida*; and *N. ashei*, the giant spitting cobra, from eastern and northern Kenya, southern Ethiopia, southern Somalia, and eastern Uganda, previously considered a regional variant of *N. nigricollis*, the black-necked spitting cobra (Wüster and Broadley, 2007). Based on mtDNA analysis, Wüster et al. (2007) showed that *N. nigricincta*, the zebra spitting cobra, clearly represents a separate species from *N. nigricollis.* Broadley and Wüster (2004) analyzed morphological variation and mtDNA in *N. annulifera*. Both analyses show that the subspecies *N. a. annulifera* and *N. a. anchietae* represent clearly distinct species.

Notechis—*Australian Tiger Snakes*

Tiger snakes are found in southern regions of Australia, including its coastal islands and Tasmania. These snakes display great geographic variation in color and size. Keogh et al. (2005) analyzed the phylogeography of the genus *Notechis* using sequences from mitochondrial genes. They found extremely low levels of genetic divergence across the genus. The main subdivision was between populations from southwestern and southeastern Australia. Within southeastern Australia, divergences were very small despite the presence of great variation in body size and pattern. Therefore, all previously recognized species (*N. scutatus* and *N. ater*) and their subspecies represent a single geographically variable species, *Notechis scutatus*.

Pseudechis—*Australian Black Snakes*

Australian black snakes are potentially lethal and found in every Australian state with the exception of Tasmania, and two species are found in New Guinea. Kuch et al. (2005a) and Wüster et al. (2005b) analyzed mitochondrial DNA sequences of all species and a number of populations. The genus *Pailsus*, described in an amateur publication, was found to be a synonym to *Pseudechis*; the taxa previously known as *Pailsus pailsi* and *Pailsus rossignolii* probably represent valid species of the genus *Pseudechis* (Williams and Wüster, 2005). Kuch et al. (2005a) analyzed the phylogeography of *P. australis* using sequences of two mitochondrial genes. Their results, coupled with morphological differences, suggest the existence of five distinct species in the complex, although the authors refrain from discussing their nomenclature due to existing confusion in this regard.

Laticauda—*Sea Kraits*

Cogger and Heatwole (2006) analyzed morphological variation in *Laticauda colubrina*, with special emphasis on populations in New Caledonia and Vanuatu. They recognize two additional species previously confounded with *L. colubrina*: *L. frontalis* is restricted to Vanuatu and the Loyalty Islands of New Caledonia; *L. saintgironsi* is restricted to the coastal waters of the island of New Caledonia and some of the Loyalty Islands. Heatwole et al. (2005) had described another new species of the *L. colubrina* complex, *L. guineai*, from the southern coast of Papua New Guinea.

Aspidelaps—*African Shield Cobras*

Broadley and Baldwin (2006) surveyed morphological and pattern variation in *Aspidelaps* in southern African. They consider *A. l. infuscatus* as a synonym of *A. l. cowlesi*. Additionally, the authors also identified a differentiated form of *A. l. lubricus* from northwestern South Africa and southwestern Namibia, which may represent an undescribed taxon.

Oxyuranus temporalis—*Central Ranges Taipan*

Doughty et al. (2007) describe a new species of taipan from the central ranges of Western Australia, near the state line with the Northern Territory: *Oxyuranus temporalis*. Phylogenetic analysis of mtDNA sequences showed it to be the sister species of the two previously known taipans. The new species is known from a single specimen, so very little is known of its natural history, and nothing of its venom.

Bungarus—*Kraits*

Kraits are found in the Indian subcontinent, including Sri Lanka and eastern Pakistan, and Southeast Asia, including Indonesia and Borneo. Kuch et al. (2005b) describe a new species of krait from Lao Cai and Yen Bai Provinces, northern Vietnam: *Bungarus slowinskii*. Kuch and Mebs (2007) examine variation in morphology, mitochondrial DNA, and alpha-bungarotoxin gene sequence in *Bungarus* spp. in Java. Their findings demonstrate that the uniformly black kraits described as a separate species, *B. javanicus*, are in fact conspecific with the widespread species *B. candidus*.

Walterinnesia—*Desert Black Snake*

Desert black snakes are native to dry habitats of the Middle East and can be found in the countries of Egypt, Israel, Lebanon, Syria, Jordan, Iraq, Iran, Kuwait, and Saudi Arabia. Nilson and Rastegar-Pouyani (2007) examined morphological variation in the genus across its distribution, from Egypt to Iran. The eastern populations (from Turkey and Saudi Arabia to Iran) were found to differ in morphological characters from farther west (Egypt, Israel, Jordan). Therefore, the eastern form is recognized as a distinct species, *Walterinnesia morgani*.

C. Superfamily Viperoidea

1. Viperidae—Viperinae

Atheris—*African Bush Vipers*

African bush vipers, genus *Atheris*, are found in forest habitats in tropical sub-Saharan Africa, excluding southern Africa. They show many ecomorphological similarities to the arboreal pit vipers of Asia and South America. Lenk et al. (2001) found *Atheris ceratophorus* to be more closely related to *Adenorhinos barbouri* than to other species of *Atheris*. The authors therefore suggest placing the species *barbouri* into the genus *Atheris* to avoid paraphyly of the latter. In addition, several species of the genus were described recently: *A. acuminata* was described from western Uganda (Broadley, 1998b); *A. broadleyi* was described from southeastern Cameroon and western Central African Republic (Lawson, 1999); and *A. hirsuta* was described from the Taï National Park in Ivory Coast, West Africa (Ernst and Rödel, 2002). *Atheris subocularis* was rediscovered and revalidated (Lawson et al., 2001). Lawson and Ustach (2000) examined the distinction between *A. squamigera* and *A. anisolepis*, concluding that they are synonyms.

Bitis—*African Vipers*

Lenk et al. (1999) studied the phylogenetic relationships among species of *Bitis*. In addition to proposing four subgenera of the genus *Bitis*, they noted a considerable differentiation between two recognized subspecies of *B. gabonica*: *B. g. gabonica* and *B. g. rhinoceros*. Both are as different from each other as each is from *B. nasicornis*, and *B. g. rhinoceros* is elevated to species, *B. rhinoceros*.

Branch (1999) reviewed the *B. cornuta-inornata* complex, recognizing five species: *B. cornuta* is from the Atlantic coastal regions of South Africa and southwestern Namibia; *B. rubida* is found in the Western Cape Province; *B. armata* is found in the southwestern corner of the Western Cape; *B. inornata* is restricted to the Sneeuberg region of the Eastern Cape; and *B. albanica* is restricted to coastal areas in the Eastern Cape, between Port Elisabeth and Grahamstown.

Cerastes—*Horned Vipers*

Werner et al. (1999) analyzed the population systematics of *Cerastes cerastes* and *C. gasperettii* from the Arabian Peninsula and the Arava Valley. Populations of *C. cerastes* from the southwestern Arabian Peninsula are described as a new subspecies, *C. cerastes hoofieni*. The population of *C. gasperettii* from the Arava Valley is described as a new subspecies, *C. g. mendelssohni*.

Vipera/Macrovipera/Montivipera/Daboia

Lenk et al. (2001) carried out the first wide-ranging study of the phylogeny of the Viperinae. The large Eurasian vipers were found to be monophyletic, but clustered into a number of clades that do not correspond to current generic classification. Unlike in previous studies (Herrmann et al., 1992), the present study places the species *Macrovipera lebetina* and *M. schweizeri* as sister group in the *V. xanthina-raddei* complex, whereas the North African *Macrovipera mauritanica* and *M. deserti* appear to be more closely related to *Daboia russelii* and *Vipera palaestinae*. Lenk et al. therefore suggested placing these four species in the genus *Daboia*.

The data presented by Lenk et al. also support the recognition of the subgenus *Montivipera*, described by Nilson et al. (1999) for the *xanthina-raddei* group, as a full genus. Nilson et al. (1999) reviewed the systematic status of the *Vipera xanthina* group. Based on the evidence suggesting that this group constitutes a distinct lineage within *Vipera*, the authors described a new subgenus, *Montivipera*, and included nine taxa. These results were echoed by Lenk et al. (2001) and Wüster et al. (2008). Based on these results, Joger (2005) recognized *Montivipera* as a full genus. However, the status of some of the species included in *Montivipera* is controversial (Schätti et al., 1991; Nilson and Andrén, 1992).

Tuniyev and Ostrovskikh (2001) described two new species of small viper from the Caucasus, *Vipera orlovi* and *V. magnifica*. Nilson and Andrén (2001) revised the systematics of the *V. ursinii* complex based on the analysis of morphological variation as well as protein electrophoretic and immunological distance data, recognizing twelve taxa (Table 2.2).

Echis—*Burton's Carpet and Oman Saw-Scaled Vipers*

Babocsay (2003) used multivariate morphometrics to investigate patterns of geographic variation in *Echis coloratus* in the Near East, describing a new subspecies, *E. c. terraesanctae*. Using multivariate morphometrics in the *Echis coloratus* complex, Babocsay (2004) defined and described the populations from northern Oman and the United Arab Emirates as a new species, *Echis omanensis*.

Pseudocerastes urarachnoides

Bostanchi et al. (2006) described *Pseudocerastes urarachnoides* from the Zagros Mountains of western Iran. Its geographic distribution lies between that of the two other species of the genus, *P. persicus* and *P. fieldi*. This species is characterized by the possession of a number of long, bristle-like scales along its tail tip, giving the appearance of having an arachnid attached to the tail. This bottlebrush-shaped tail tip appears to be used in caudal luring displays.

Daboia russelii—*Russell's Viper*

Thorpe et al. (2007) used mitochondrial DNA sequences and morphological data to analyze the systematics of the Russell's viper (*Daboia russelii*) complex in Asia. Two main monophyletic groups were revealed and two species proposed: *Daboia russelii* being the western (India, Sri Lanka, Pakistan, Nepal) form and *Daboia siamensis* the eastern (Myanmar, Thailand, Cambodia, China, Taiwan, Indonesia) form. Thorpe et al. note that variation in clinical symptoms of bites does not reflect the phylogenetic affinities of the populations concerned.

2. Viperidae—Crotalinae

Bothriechis—*Palm Pit Vipers*

Solórzano et al. (1998) investigated geographic variation in *Bothriechis schlegelii* in Costa Rica and revalidated the species *Bothriechis supraciliaris*, from southwestern Valle del General and parts of Puntarenas Province, Costa Rica. Campbell and Smith (2000) described a new species of Palm Pit Viper from the Atlantic versant of Guatemala and Honduras: *Bothriechis thalassinus*. Its distribution extends along a series of mountains along the Guatemala-Honduras border.

Porthidium melanurum/Ophryacus melanurus—*Mexican Black-Tailed Pit Viper*

In a phylogenetic analysis of various Central American pit vipers, Gutberlet (1998) found that the Mexican black-tailed pit viper (previously known as *Porthidium melanurum*) is more closely related to *Ophryacus undulatus*, the Mexican horned pit viper, than to the hognosed pit vipers of the genus *Porthidium*. Consequently, this species was transferred to the genus *Ophryacus*, becoming *Ophryacus melanurus*.

Cerrophidion—*Central American Montane Pit Vipers*

López-Luna et al. (1999) described a new species of Montane pit viper from the highlands of southwestern Veracruz State, México: *Cerrophidion petlalcalensis*. So far, the species has only been recorded from Cerro Petlalcala, near the city of Orizaba. Anecdotal details of a bite inflicted on one of the authors by the holotype are given.

Gloydius—*Mamushis*

Orlov and Barabanov (1999) revised the classification of *Gloydius*, under the old generic name *Agkistrodon*. Reexamination of the holotype of *Gloydius intermedius* led to the conclusion that this belongs to the species *G. saxatilis*. The name *intermedius* was published before the name *saxatilis*; consequently, the correct name for the rock mamushi of the Russian Far East is *G. intermedius*. However, a rigorous revision of the genus *Gloydius* has not yet been overtaken, and species limits within the genus remain inadequately understood (see Table 2.3). Toxinologists should ensure that they source their venoms from populations of known geographic origin so that their identity can be related to future systematic revisions. If possible, samples for DNA analysis should be taken from specimens providing venoms and should be deposited in suitable natural history collections after their death. Note that the genus name *Gloydius* is now universally accepted, as multiple phylogenetic studies of pit viper phylogeny have shown that the Asian taxa formerly classified in the genus *Agkistrodon* are unrelated to North American *Agkistrodon* (Parkinson, 1999; Parkinson et al., 2002; Malhotra and Thorpe, 2004a; Castoe and Parkinson, 2006).

Agkistrodon bilineatus *and* A. taylori—*Cantils*

Parkinson et al. (2000) used mitochondrial DNA to analyze the phylogeny of the genus *Agkistrodon*, and in particular the affinities of the different subspecies of *A. bilineatus*. The northeastern subspecies, *A. b. taylori*, represents a highly distinct lineage. In addition to genetic differences, consistent differences in pattern and the presence of strong sexual dimorphism in pattern indicate that this form is a distinct species, *Agkistrodon taylori*. Smith and Chiszar (2001) described a new subspecies of *Agkistrodon bilineatus* on the basis of a single specimen from near Palma Sola, Veracruz, Mexico: *A. b. lemosespinali*. However, the validity of this taxon has been questioned based on distribution and morphological characters (Bryson and Mendoza-Quijano, 2007).

Bothrocophias

Gutberlet and Campbell (2001) described a new genus of pit viper, *Bothrocophias*. This includes several species of pit viper of problematic generic affinities: *B. hyoprora* was generally regarded as part of *Porthidium* (e.g., Campbell and Lamar, 1989) but transferred to *Bothrops* on the basis of several phylogenetic analyses (Kraus et al., 1996; Parkinson, 1999), whereas the species *B. microphthalmus* and *B. campbelli* had almost invariably been regarded as part of *Bothrops*. In addition, Gutberlet and Campbell noted that specimens from the lowlands of southwestern Colombia previously assigned to *B. campbelli* belong to a different species that they described as *B. myersi*, whereas the name *B. campbelli* was restricted to the highland species from the Ecuadorian Andes.

Bothrops—*Lanceheads*

Several taxonomic discoveries and changes relevant to the genus *Bothrops* appeared in recent literature. Wüster et al. (1999) analyzed mitochondrial DNA variation in the *Bothrops atrox* complex. Previous taxonomy does not correspond to the mtDNA haplotype lineages identified, and neither do patterns of morphological variation, and the status of most of the recognized species is questioned. Puorto et al. (2001) analyzed mitochondrial DNA and morphological variation in the *B. atrox* complex along the Atlantic coast of Brazil, concluding that *B. leucurus* and *B. pradoi* are part of one single species, *B. leucurus*. New species descriptions include *Bothrops muriciensis*, described from the state of Alagoas, northeastern Brazil (Ferrarezzi and Freire, 2001), and *B. alcatraz*, a form

TABLE 2.3
Family-Level Classification of Snake Genera of Documented or Potential Medical or Toxinological Interest

Viperidae		Homalopsidae	Colubridae	Dipsadidae	Natricidae	Lamprophiidae	Elapidae		
Azemiopinae	**Crotalinae**		*Ahaetulla*	*Alsophis*	*Amphiesma*	**Atractaspidinae**	***Elapinae***	**Hydrophiinae**	*Kolpophis*
Azemiops	*Agkistrodon*	*Cerberus*	*Boiga*	*Apostolepis*	*Balanophis*	*Atractaspis*	*Aspidelaps*	*Acalyptophis*	*Lapemis*
	Atropoides	*Enhydris*	*Coelognathus*	*Boiruna*	*Macropisthodon*	*Homoroselaps*	*Bungarus*	*Acanthophis*	*Laticauda*
Viperinae	*Bothriechis*	*Homalopsis*	*Coluber*	*Clelia*	*Rhabdophis*	*Macrelaps*	*Calliophis*	*Aipysurus*	*Loveridgelaps*
Atheris	*Bothriopsis*		*Crotaphopeltis*	*Coniophanes*	*Thamnophis*		*Dendroaspis*	*Aspidomorphus*	*Micropechis*
Bitis	*Bothrocophias*		*Dispholidus*	*Conophis*		**Psammophiinae**	*Elapsoidea*	*Astrotia*	*Notechis*
Causus	*Bothrops*		*Gonyosoma*	*Diadophis*		*Malpolon*	*Hemachatus*	*Austrelaps*	*Ogmodon*
Cerastes	*Calloselasma*		*Hemorrhois*	*Elapomorphus*		*Psammophis*	*Hemibungarus*	*Cacophis*	*Oxyuranus*
Daboia	*Cerrophidion*		*Leptophis*	*Erythrolamprus*		*Psammophylax*	*Leptomicrurus*	*Demansia*	*Parahydrophis*
Echis	*Crotalus*		*Oxybelis*	*Heterodon*		*Rhamphiophis*	*Micruroides*	*Denisonia*	*Parapistocalamus*
Eristicophis	*Cryptelytrops*		*Platyceps*	*Hydrodynastes*			*Micrurus*	*Drysdalia*	*Paroplocephalus*
Macrovipera	*Deinagkistrodon*		*Rhamnophis*	*Hypsiglena*		**Pseudoxyrhophiinae**	*Naja*	*Echiopsis*	*Pelamis*
Montatheris	*Garthius*		*Spalerosophis*	*Leptodeira*		*Langaha*	*Ophiophagus*	*Elapognathus*	*Praescutata*
Montivipera	*Gloydius*		*Telescopus*	*Liophis*		*Leioheterodon*	*Pseudohaje*	*Emydocephalus*	*Pseudechis*
Proatheris	*Himalayophis*		*Thelotornis*	*Phalotris*		*Madagascarophis*	*Sinomicrurus*	*Enhydrina*	*Pseudonaja*
Pseudocerastes	*Hypnale*		*Thrasops*	*Philodryas*			*Walterinnesia*	*Ephalophis*	*Rhinoplocephalus*
Vipera	*Lachesis*		*Trimorphodon*	*Tachymenis*				*Furina*	*Salomonelaps*
	Ophryacus		*Xyelodontophis*	*Thamnodynastes*				*Glyphodon*	*Simoselaps*
	Ovophis			*Xenodon*				*Hemiaspis*	*Suta*
	Parias							*Hoplocephalus*	*Thalassophis*
	Peltopelor							*Hydrelaps*	*Toxicocalamus*
	Popeia							*Hydrophis*	*Tropidechis*
	Porthidium							*Kerilia*	*Vermicella*
	Protobothrops								
	Sistrurus								
	Trimeresurus								
	Tropidolaemus								
	Viridovipera								

Source: According to Vidal et al., 2007.

closely related to *B. jararaca* and *B. insularis*, from Ilha dos Alcatrazes, a small island off the coast of São Paulo State, southeastern Brazil (Marques et al., 2002).

The *Bothrops neuwiedi* complex was regarded as a single species, *B. neuwiedi*, with twelve highly variable subspecies. Da Silva (2000, in Campbell and Lamar, 2004) analyzed multiple morphological characters, and *B. neuwiedi* was found to consist of seven species. Six species were already named by da Silva (2000, in Campbell and Lamar, 2004): *B. neuwiedi* (Brazil: Bahia, Goiás, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, and Santa Catarina), *B. diporus* (northern Argentina, Paraguay, Brazil: Mato Grosso do Sul, São Paulo, Paraná, Santa Catarina, Rio Grande do Sul), *B. lutzi* (Brazil: Piauí, Bahia, Pernambuco, Goiás, northern Minas Gerais), *B. mattogrossensis* (Paraguay, Bolivia, southeastern Peru, Brazil: southwestern Amazonas, Rondônia, Mato Grosso, Mato Grosso do Sul, Tocantins, Goiás, São Paulo), *B. pauloensis* (Brazil: Minas Gerais, Goiás, Mato Grosso, Mato Grosso do Sul, São Paulo; possibly Bolivia), and *B. pubescens* (Uruguay, Brazil: Rio Grande do Sul). The seventh species, from the Brazilian states of Goiás, Tocantins, and western Minas Gerais, was described by da Silva and Rodrigues (2008) as *Bothrops marmoratus*.

Bothriopsis/Bothrops oligolepis, peruviana, *and* chloromelas

Harvey et al. (2005), in a review of Bolivian pit vipers, revised the status of a group of rarely seen pit vipers from the Andes of Bolivia and Peru: *Bothriopsis oligolepis* and *Bothriopsis peruviana*. They examined the holotype of *B. peruviana* (Boulenger, 1903), and found that this form is a synonym of *B. oligolepis*. Consequently, the available name for the relatively noncontrasting species from southern Peru and Bolivia is *Bothriopsis oligolepis*. Moreover, Harvey et al. (2005) revalidated *Bothriopsis chloromelas* for the brightly patterned species found in the central Andes of Peru. The validity of the genus *Bothriopsis* is contentious: several phylogenetic studies (Salomão et al., 1997; Wüster et al., 2002; Castoe and Parkinson, 2006) have shown that it is nested within *Bothrops*. Some authors have advocated synonymizing *Bothriopsis* with *Bothrops* (Salomão et al., 1997; Wüster et al., 2002), whereas others have retained usage of *Bothriopsis* despite the resulting paraphyly of *Bothrops* (Campbell and Lamar, 2004; Castoe and Parkinson, 2006). Wüster et al. (2002) showed that the species often referred to as *Bothriopsis punctata* is more closely related to species such as *Bothrops atrox* than to other species classified as *Bothriopsis*. Irrespective of other considerations on the validity of the genus *Bothriopsis* (see above), this species should be assigned to the genus *Bothrops*.

Crotalus—*Rattlesnakes*

Recent advances in the systematics of *Crotalus* are characterized by multiple splits of previously recognized, widespread species, and subspecies elevated to species level. The *C. viridis* complex was found to be composed of multiple species-level lineages, contrary to the previous view, and several subspecies were elevated to species (Pook et al., 2000; Ashton and de Queiroz, 2001; Douglas et al., 2002), although there is as yet no clear consensus as to how many species should be recognized in the complex (but see Crother et al., 2003).

The Neotropical *C. durissus* complex, long a taxonomic minefield, was split into three species, *C. simus* (Central America), *C. totonacus* (northeastern Mexico), and *C. durissus* (South America), by Campbell and Lamar (2004) and Savage et al. (2005). However, *C. simus* appears to be polyphyletic and to consist of multiple species-level lineages (Wüster et al., 2005; Quijada-Mascareñas and Wüster, 2006), leading Wüster et al. (2005) to recognize *C. tzabcan* and *C. culminatus* as separate species. The same authors also relegated the Brazilian subspecies *C. durissus cascavella* and *C. durissus collilineatus* to the synonymy of *C. d. terrificus*.

The panamint rattlesnake, *C. m. stephensi*, was found to be the sister taxon of the mainland taxa *C. m. mitchellii* and *C. m. pyrrhus*, and to differ consistently by a single nuclear polymorphism in the nuclear sequences. The congruence between mitochondrial and nuclear DNA led Douglas et al. (2007) to elevate the panamint rattlesnake to species status, *C. stephensi*.

Castoe and Parkinson (2006) studied the phylogeography of *C. atrox*, including allied insular forms of the Gulf of California. *C. atrox* has a relatively limited phylogeographic structure, and the Tortuga Island rattlesnake (*C. tortugensis*) and the form from Santa Cruz Island are placed in the synonymy of *C. atrox*.

Finally, two morphologically defined species were described recently: *C. tancitarensis* from Cerro Tancítaro, Michoacán, México (Alvarado-Díaz and Campbell, 2004) and *C. ericsmithi* from the mountains of Guerrero, Mexico (Campbell and Flores-Villela, 2008).

Trimeresurus *Complex—Asiatic Arboreal Pit Vipers*

The taxonomy of the Asiatic arboreal pit vipers *Trimeresurus* has been highly impacted by further exploration and the use of molecular markers. Malhotra and Thorpe (2004a) used phylogenetic analysis of mtDNA, hemipenial morphology, and scalation and identified a number of well-defined species groups, particularly within *Trimeresurus* sensu lato. On this basis, the genus *Trimeresurus* was split into seven genera.

Multiple species have been described recently, mostly by distinguishing known populations from other species with which they had previously been confused: *T. vogeli* from southeastern Thailand (David et al., 2001), *T. gumprechti* from northeastern Thailand (David et al., 2002), and *T. truongsonensis* from Phong Nha-Ke Bang National Park in Quang Binh Province, central Vietnam (Orlov et al., 2004) are part of the *stejnegeri* complex (genus *Viridovipera*—Malhotra and Thorpe, 2004a; Dawson et al., 2008).

Within Malhotra and Thorpe's genus *Popeia*, Vogel et al. (2004) described *Popeia fucata* (as *Trimeresurus fucatus*) from southern Thailand, southern Myanmar (Burma), and much of Peninsular Malaysia, and *P. nebularis* (as *T. nebularis*) from the Cameron Highlands, Pahang, Malaysia. The latter was independently described as *Popeia inornata* by Sanders et al. (2004), but since this description appeared after that of *T. nebularis*, *Popeia inornata* becomes a synonym of *Popeia nebularis*. In addition, Vogel et al. (2004) considered the subspecies *T. p. sabahi* from Borneo and *T. p. barati* from Sumatra to constitute separate species. Also within *Popeia*, Grismer et al. (2006) described the pit viper *Popeia buniana*, for which they suggest the common name "fairy pit viper," from the island of Pulau Tioman, Malayan Peninsula. The Tioman population was previously classified as *Trimeresurus fucatus* by Vogel et al. (2004) and as *Popeia sabahi* by Sanders et al. (2006), on both molecular phylogenetic and morphological grounds.

Within *Cryptelytrops*, Giannasi et al. (2001) used amplified fragment length polymorphisms (AFLPs) to test systematic relationships previously inferred from mtDNA sequence evidence (Malhotra and Thorpe, 1997, 2000). In view of the congruent results of mtDNA sequences and the AFLP data, Giannasi et al. regard the Lesser Sunda and East Java populations as a separate species, *T. insularis*, and the Nepalese population as a full species, *T. septentrionalis* (now *Cryptelytrops insularis* and *Cryptelytrops septentrionalis*, respectively). Grismer et al. (2008) described *Cryptelytrops honsonensis* sp. nov. from Hon Son Island in Rach Gia Bay, southern Vietnam. The new species is morphologically closest to *C. venustus* from southern Thailand.

Within what is now *Protobothrops*, Stuebing and Inger (1998) analyzed variation in *Trimeresurus sumatranus* on the island of Borneo. They came to the conclusion that the high-elevation populations from Mt. Kinabalu should be treated as a separate species, *T. malcolmi*.

Herrmann et al. (2004) provide a redescription, review, and phylogenetic assessment of *T. cornutus*, indicating that this species is the sister species of *Protobothrops jerdonii*. Consequently, the authors support its reclassification as *P. cornutus*.

David et al. (2006) revised the taxonomy of the *T. puniceus* group of pit vipers, using multivariate analysis of morphological characters and mtDNA, leading to the recognition of five distinct species within the complex. *Trimeresurus puniceus* is confined to Java and southern Sumatra. A possibly distinct species from western Sumatra is flagged but not named by the authors. *T. borneensis* is confined to the island of Borneo, whereas populations previously assigned to this species from the

Malayan Peninsula are assigned to *T. wiroti*, a species with a history of controversy and confusion with *T. puniceus* and *T. borneensis*. *T. brongersmai* is recognized from the islands of Siberut and Simeulue, off western Sumatra. Finally, a new species, *T. andalasensis*, is described from northern Sumatra. This species was previously regarded as being conspecific with *T. borneensis*.

Atropoides—*Jumping Vipers*

Castoe et al. (2003) used phylogenetic analysis of two mitochondrial gene sequences to reconstruct the phylogeny of the genus *Atropoides*. The monophyly of the genus was neither supported nor contradicted by their data; *A. nummifer* was found to be genetically diverse, and *A. olmec* was found to be nested among the *A. nummifer* lineages. Moreover, *A. olmec* haplotypes were recovered not just from the type locality of that species in Veracruz, but also from northeast Oaxaca, Mexico, and Baja Verapaz, Guatemala, suggesting that the species may have a wider distribution than previously anticipated. The various subspecies of *A. nummifer* probably deserve species status. Smith and Ferrari-Castro (2008) described *Atropoides indomitus* from central Honduras. The new species differs from most other species of the genus by having higher ventral scale counts, and from *A. picadoi* in pattern. The population concerned had previously been flagged as distinct by Castoe et al. (2003) based on mtDNA sequence analysis.

Porthidium—*Hognosed Pit Vipers*

Lamar and Sasa (2003) described a new species of hognosed pit viper, *Porthidium porrasi*, from the Peninsula de Osa and the mainland on the opposite side of the Golfo Dulce, on the Pacific coast of Costa Rica. The population was previously regarded as a differentiated population of *Porthidium nasutum*. It differs from *P. nasutum* in retaining a white tail tip into adulthood, in having 25 to 27 rather than 23 dorsal scale rows at mid-body, and in having a more banded pattern compared to the blotched pattern of *P. nasutum*. Mitochondrial DNA sequence analysis confirmed the divergence between the new species and *P. nasutum*, and its status as the sister species of the latter. Campbell and Lamar (2004) recognize the Manabi hognosed pit viper, previously described as *Porthidium lansbergii arcosae*, as a separate species, *Porthidium arcosae*. The species is restricted to dry forests on the western coast of Ecuador. Castoe et al. (2005) demonstrated the monophyly of *Porthidium* with *Cerrophidium* and *Atropoides* as the sister taxa. Further analysis of the group found an arid-adapted clade formed by *P. hespere*, *P. dunni*, and *P. ophryomegas* (Bryson et al., 2008).

Ermia, Zhaoermia, Triceratolepidophis, *and* Protobothrops

Occasionally, major taxonomic discoveries are made in the most unexpected places. Ziegler et al. (2000) described a new genus and species of pit viper from Vietnam: *Triceratolepidophis sieversorum*. The only known specimen at the time was found preserved in a bottle of rice liquor in the house of a local medicine man. It had been caught in a local chicken coop.

Gumprecht and Tillack (2004) note that the generic name *Ermia*, erected by Zhang (1993) for the species previously known as *Trimeresurus mangshanensis*, is preoccupied by a genus of locusts, and therefore not available for the Mangshan pit viper. They therefore proposed the new generic name *Zhaoermia* as a replacement name for *Ermia*. Like the original genus name, it honors the eminent Chinese herpetologist Zhao Ermi. However, Guo et al. (2007) provided evidence that the genus *Protobothrops* is paraphyletic if *Zhaoermia* and *Triceratolepidophis* are excluded, and therefore synonymized these two genera with *Protobothrops*. The correct names for the species concerned are therefore *Protobothrops mangshanensis* and *Protobothrops sieversorum*.

Garthius/Ovophis

During their phylogenetic studies of Asian pit vipers, Malhotra and Thorpe (2000, 2004a) found that the genus *Ovophis* is polyphyletic: the *Ovophis monitcola* group (*Ovophis* sensu stricto) clustered as the sister group of the *Protobothrops* group, whereas *O. okinavensis* clustered with *Gloydius*, and *O. chaseni* occupies an isolated, basal position among the pit vipers. A new genus, *Garthius*, was

described by Malhotra and Thorpe (2004a) to accommodate the latter, the correct name for which is now *Garthius chaseni*. The separate position of *Ovophis okinavensis* (together with the closely related and equally misplaced *Trimeresurus gracilis*) remains pending.

Lachesis—*Bushmasters*

Bushmasters are the longest vipers in the western hemisphere and the only ones that lay eggs. Three species of bushmasters have been recognized in recent years (Zamudio and Greene, 1996): *Lachesis muta*, from the Amazon Basin, the Guianas, and the Brazilian Atlantic forest; *Lachesis melanocephala*, from the southern Pacific versant of Costa Rica; and *Lachesis stenophrys*, from the Atlantic versant of Costa Rica, western Panama, and southern Nicaragua. Campbell and Lamar (2004) also identified and recognized the bushmasters from Panamá and northwestern South America as a distinct species, *Lachesis acrochorda*.

Tropidolaemus

Kuch et al. (2007) revised the genus *Tropidolaemus* and described a new species from Sulawesi, *T. laticincta*, with a diagnostic strongly ornate head and body pattern. Likewise, the authors refer to *T. wagleri* populations from eastern Indonesia and the Philippines as separate species from *T. wagleri*, and refer to them as the *T. subannulatus* complex. Vogel et al. (2007) revised the systematics of the *T. wagleri* complex using multivariate morphometric analyses. Their data reveal the presence of three distinct taxa within the complex regarded as different species: *T. wagleri* from Sumatra, the Malayan Peninsula, and Bangka Island; *T. subannulatus* complex from Borneo, Sulawesi, and most of the Philippines; and *T. philippensis* from southern and western Mindanao Island, Philippines.

D. Homolapsidae

Cerberus—*Bockadam Snake*

Formerly, the genus *Cerberus* was considered monophyletic and composed of three species: *C. australis* (from Australia), *C. microlepis* (known only from Lake Buhi in the Philippines), and the widely distributed *C. rynchops* (India to Wallacea). Recently, the monophyly of the group has been questioned based on mtDNA sequences (Karns et al., 2000; Alfaro et al., 2004). The species *C. australis* is highly divergent from all other lineages. The geographically widespread *C. rynchops* is composed by four clades (Indian and Myanmar, Philippines, Greater Sunda Islands and Sulawesi, and the Thai-Malay Peninsula and the Gulf of Thailand). The authors of these studies made no taxonomic recommendations, arguing that more sampling in other areas is needed.

Enhydris—*Rainbow Water Snakes*

The genus *Enhydris* is the most complex and species rich of the oriental-Australian rear-fanged Homalopsidae. Voris et al. (2002) used mtDNA to demonstrate that the genus *Enhydris* is paraphyletic. *Enhydris bocourti* was shown to be part of a clade containing *Cerberus rynchops*, *Erpeton tentaculatum*, and *Homolopsis buccata*; while *Enhydris punctata* was the sister species to the Australian mangrove-dwelling snake *Myron richardsonii*. No taxonomic recommendations were made by these authors. On the other hand, two species were described recently: *E. gyii* from the Kapuas river system, west Kalimantan, Indonesia (Murphy et al., 2005), and *E. chanardi* from Bangkok, Thailand (Murphy and Voris, 2005).

IV. CONCLUSIONS

The above summary of recent taxonomic discoveries among venomous snakes demonstrates the rapid pace of knowledge acquisition in this field. Moreover, there is no reason to expect the pace

of research and new developments to slow down: there is ample evidence that many undiscovered species still exist, and that assemblages of populations long thought to constitute single, widespread species may in fact consist of multiple, hitherto unsuspected but highly distinct species.

For toxinologists, physicians, and antivenom producers, this pace of discovery poses a number of challenges. The first is to remain abreast of systematic developments in the taxa of interest. The second major challenge is to ensure the value and interpretability of their work against the possibility of as yet unknown future taxonomic changes, in particular against the possibility that the species they are working on may be found to be a composite of multiple species. While this is not necessarily easy, a number of safeguards can help (Wüster and McCarthy, 1996):

1. Toxinological work needs to be put into a clear taxonomic context: scientific names change their meanings—for instance, whereas the species name *Naja naja* once referred to all Asian cobras, it now refers only to the spectacled cobra of India, Pakistan, Sri Lanka, Nepal, and Bangladesh. A clearly stated taxonomic framework is essential to ensure the interpretability of names.
2. Locality information on experimental venoms or animals used to supply venom is crucial for several reasons. From the point of view of taxonomy, it not only helps confirm the stated identity of a venom, but also greatly increases the likelihood that a venom will remain attributable to a specific species if future taxonomic revisions result in a new understanding of the systematics of a group. Providing detailed locality information is one of the easiest ways of "future-proofing" toxinological work. Moreover, in any case, locality information is essential for any toxinological work, since variation in venom composition is common even within otherwise homogenous species and may not reflect the taxonomy of the group concerned (Daltry et al., 1996; Thorpe et al., 2007). Toxinologists should refuse to buy or use venom from suppliers unable to provide locality information.
3. Where possible, the specimens involved in accidents reported in the medical literature or used to supply experimental venoms should be preserved and vouchered in suitable natural history collections upon their deaths. This will allow a reassessment of the identity of the specimens in the light of future work. Tissue samples or blood samples can be obtained for DNA analysis during the specimens' lifetime and used to confirm identity. This should be done as a matter of course in complex or controversial groups of snakes, in collaboration with interested herpetological systematists.
4. Venoms of particular interest but unclear taxonomic provenance can be attributed to their species by means of DNA barcoding (Pook and McEwing, 2005).
5. Perhaps most importantly, toxinologists, antivenom manufacturers, and physicians should collaborate with systematists to ensure the value of their work. This could simply involve confirmation of the identity of individual snakes and explanation of the systematic background of the snakes under study. However, increased collaboration among evolutionary biologists, systematists, and toxinologists should ideally consist of two-way communication. This has the potential to lead to the exploration of new directions in research on the evolution of venoms.

In order to ensure the replicability of research results, avoid waste of effort, and improve snakebite patient outcomes, it is of great importance that those working in toxinology or the medical aspects of snakebite consider keeping abreast of systematic developments as a fundamental part of their professional development. We hope that the recommendations and information provided in this chapter will help increase taxonomic awareness among toxinologists, lead to more efficient and useful toxinological research, and lead to new collaborative partnerships in our quest to understand the nature and evolution of venoms and the animals that produce them.

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3 Reptile Venom Glands
Form, Function, and Future

Scott A. Weinstein, Tamara L. Smith, and Kenneth V. Kardong

CONTENTS

True venom systems evolved at least twice in extant reptiles—once early in helodermatid lizards and second much later in advanced snakes (colubrids, viperids, elapids, and atractaspidids). In helodermatids, the venom gland lies along the lower jaw and empties near grooved, multiple teeth within the mouth. As these slow-moving lizards feed largely on eggs and nestlings, this venom system is probably part of a defensive strategy. Within venomous snakes, the venom gland lies in the temporal region. In viperids and elapids it consists of a main venom gland, pressurized during the strike by directly attached striated muscles, and an accessory gland with connecting ducts eventually emptying into a hollow fang. Atractaspidids possess only a main venom gland, although it too is pressurized by striated muscles. These venom systems are closed, producing a sudden, high-pressure discharge of the venom bolus drawn from a reservoir within the gland. In contrast, many colubrid snakes possess a relatively lower-pressure system based on a Duvernoy's gland lacking a large reservoir, which releases secretion ("venom") more slowly into oral epithelia adjacent to teeth that are sometimes deeply grooved but never hollow. Consequently, predatory systems based on a Duvernoy's system may employ an adaptive strategy different from that of front-fanged venomous snakes. In viperids, elapids, and atractaspidids, the venom system discharges a bolus of venom quickly, dispatching the prey (or thwarting a predator). Such differences in deployment of these oral glands in an adaptive context account for variation in gland structure and in the composition of their secretions. Although early research has focused on the toxic properties of these oral secretions, it is

now clear that venom components, including those of Duvernoy's glands, perform multiple biological functions. However, biological roles must be based on experimental evidence, not conjecture, where it is shown that the oral secretions in fact are injected at levels capable of producing favorable prey capture results. Elucidating these neglected adaptive roles of reptile oral secretions will significantly improve our understanding of the evolution of the complexity of composition and function of these secretions.

I. INTRODUCTION

True venom delivery systems have evolved in several living groups of reptiles: advanced venomous snakes (e.g., colubrids, atractaspidids, vipers, pit vipers, cobras, and allies) and helodermatid lizards (Gila monster, *Heloderma suspectum*, and beaded lizard, *Heloderma horridum*) (Kochva, 1978; Minton and Minton, 1980; Zug, 1993). These squamate groups, as well as other reptiles, possess an extraordinary variety of oral glands (Gabe and Saint-Girons, 1969) and accompanying secretions with an incompletely characterized variety of functions. Some snakes have independently evolved an oral system capable of producing medically significant bites; others are completely harmless to humans. Understandably, investigation of these systems has focused on medically relevant effects of the oral secretions. Consequently, the vast majority of research (approximately 95%) on reptile oral secretions has emphasized the medical and pharmacological effects of these complex mixtures (Kardong, 2002a). This is largely due to practical considerations, as snakebite is a serious public health problem in many regions, especially in underdeveloped countries (White, 1995; see also Section IV, this volume). Estimates of worldwide snakebite incidence range up to 2.5 million bites/annum (Chippaux and Goyffon, 1998).

Many studies of squamate oral secretions have determined lethal potency and experimentally assessed additional deleterious biological effects. Unfortunately, as relatively little attention has been given to the functional and evolutionary roles (sensu, Bock, 1980) of these substances, some aspects of the basic biological significance of these oral secretions remain speculative (Weinstein and Kardong, 1994; Kardong, 1996b; Aird, 2002). Resolving the adaptive significance of venom components requires experimental investigation of the role of specific squamate oral secretions in survival strategies. Presumptive assignment of biological significance without such verification (e.g., Fry et al., 2006) only confounds the study of adaptive processes (Leroi et al., 1994).

Here, we first consider the comparative structure of oral glands. With this anatomical grounding in hand, we will then examine the diversity of secretory products, the functional and evolutionary significance, and a proposal for a richer and more promising research paradigm.

II. STRUCTURE

A. Phylogeny

The sister group to the squamates (lizards and snakes) is Sphenodontida, which dates to at least the Late Triassic, about 230 million years ago (mya). The oldest lizard dates to the Late Jurassic (160 mya), and oldest snakes to the Middle Creataceous (100 mya), although these groups are now extinct. The most ancient group of extant lizards is the Gekkota (Middle Cretaceous), while that of extant snakes is the Aniliidae (Late Cretaceous). Helodermatid-like lizards extend back 98 mya, at least to the Late Cretaceous and perhaps earlier (Gilmore, 1928; Gao and Hou, 1996), but these earliest groups may lack grooved teeth, as are present in later helodermatids (Nydam, 2000). Fossil evidence of boids also dates to the Late Cretaceous. All venomous snakes belong to the advanced snakes, the Caenophidia (Colubroidea), which includes most extant snakes (Figure 3.1). The Caenophidia include three separate lineages, the Atractaspididae, Elapidae, and Viperidae, which have been recognized as dangerously venomous snakes because of their clinical significance and capacity to produce human morbidity and mortality (Warrell, 2004; Kuch et al., 2006). This

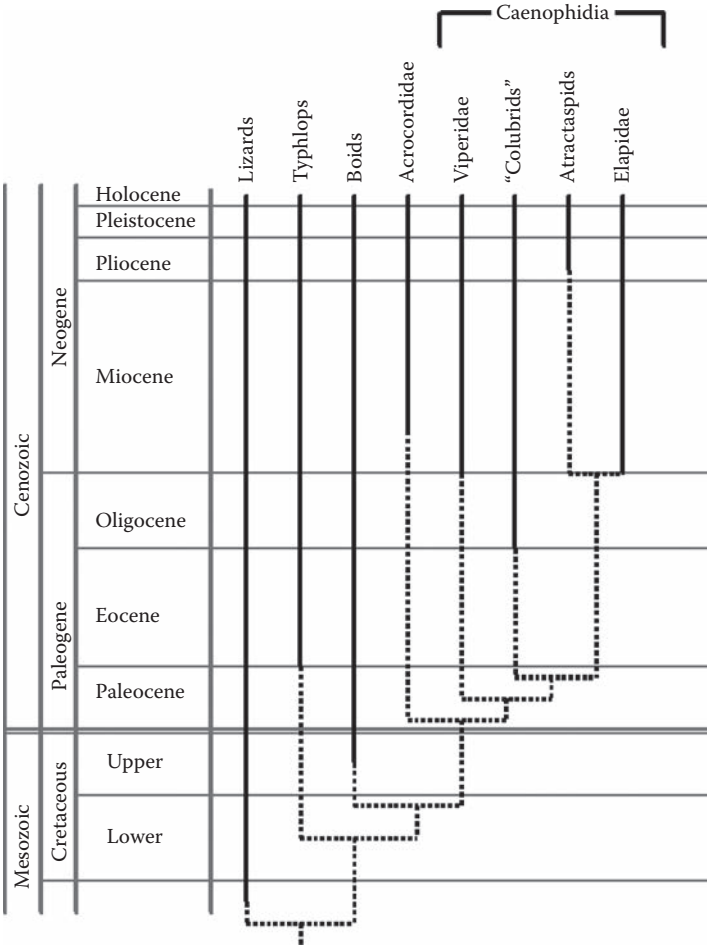

FIGURE 3.1 Stratogram. Stratographic occurrence and phylogenetic relationships of selected squamate groups. Lizards and basal snakes (Typhlopidae, Boidae) appear early, followed by aquatic species (Acrochordidae), and then in the Cenozoic by the advanced snakes. An asterisk (*) denotes clades with a front-fanged venom system, tubular fangs, and specialized venom apparatus. "Colubrids," in parentheses to recognize their paraphyletic feature, have an earlier stratographic debut than the front-fanged venomous snakes. Note that front-fanged venom systems evolved once in viperids and again in atractaspidids and elapids. (Phylogeny based on Benton, 1997; Kuch et al., 2006; Vidal et al., 2007.)

recognition is based also on biological function, as their venom apparatus is designed to bring about rapid prey death (Kardong, 2002a). Other lineages within the Caenophidia are currently incompletely resolved (but see Vidal et al., 2007; Chapter 2, this volume) and their taxonomy unsettled, but for convenience are referred to as colubrids (i.e., members of the unresolved family Colubridae). The colubrids are a paraphyletic group that includes several independent clades. A few species may cause severe human envenomations and even fatalities (FitzSimons and Smith, 1958; Mittleman and Goris, 1978; McKinstry, 1983; Ogawa and Sawai, 1986; Minton, 1990; Kuch and Mebs, 2002), but most colubrids do not represent a significant risk to humans (Kardong, 2002a).

Living families of advanced snakes all debut in the fossil record in the Cenozoic, beginning with the colubrids (Oligocene, 34 mya), followed by elapids (cobras and allies) and viperids (vipers and pit vipers), both at about the start of the Miocene (23 mya). Currently, viperids are thought to derive early within the radiation of advanced snakes, and elapids more directly from colubrids (Figure 3.1).

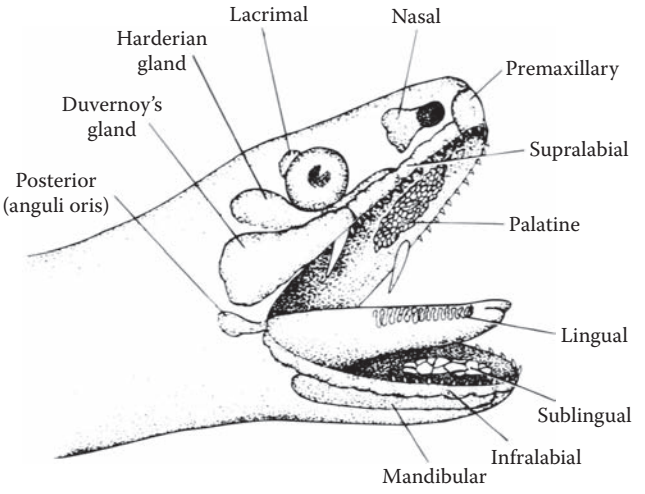

FIGURE 3.2 Oral glands of reptiles. Not all oral glands shown are present in all squamates. The venom gland of advanced snakes is a phylogenetic derivative of the Duvernoy's gland, located in the temporal region behind the eye. (From Kardong, 2002b, after Kochva, 1978. With permission.)

Thus, within the snake radiation, modern venomous snakes appear late, derived from the more basal and nonvenomous snakes, and they in turn from the even earlier lizards.

B. Anatomy of Reptilian Oral Glands

In reptiles, a great variety of glands are present in and around the oral cavity (reviewed by Kochva, 1978). Some are in the tongue, along the upper and lower lips, near the nasal cavity, or near the eye; others are specialized to contribute selective secretions to the mouth (Figure 3.2). Those associated with the nasal cavity and eye bathe these structures, keep them moist, and perhaps perform related functions yet undiscovered. Those that release products immediately into the oral cavity similarly lubricate the oral cavity, but also lubricate food to ease its passage during swallowing.

1. Lizards

a. Helodermatid Lizards

The reptilian oral glands that have received the most attention are those of the venomous helodermatid lizards and venomous snakes. In the helodermatids, the venom apparatus apparently serves a defensive function, as these lizards are slow moving, with the lowest metabolism of any lizard studied to date (Beck, 2005), and feed largely upon prey (e.g., bird eggs, fledglings, juvenile mammals, reptile eggs) swallowed with little resistance (Herrel et al., 1997). Alternative or additional roles for the venom system have not been sufficiently considered. For example, the specialized diet of helodermatids suggests that food is available for a limited part of the year, thereby placing a premium on efficient digestion of gathered prey. Their venom may contribute to heightened digestive processing of prey during this brief period, similar to that proposed in some populations of North American rattlesnakes, which often face a similar brief abundance of prey availability in the early spring (Thomas and Pough, 1979; Kardong, 1986b; Beck, 2005).

Venom secretion in helodermatids likely evolved independently from that in snakes. Unlike venomous snakes, the venom gland, a specialized mandibular gland, lies along the lower jaw, opening into multiple ducts (*Heloderma suspectum*) (Stahnke et al., 1970) or a single duct (*H. horridum*) (Kochva, 1978) that conduct venom to the mandibular tooth row (Figure 3.2). Mandibular and

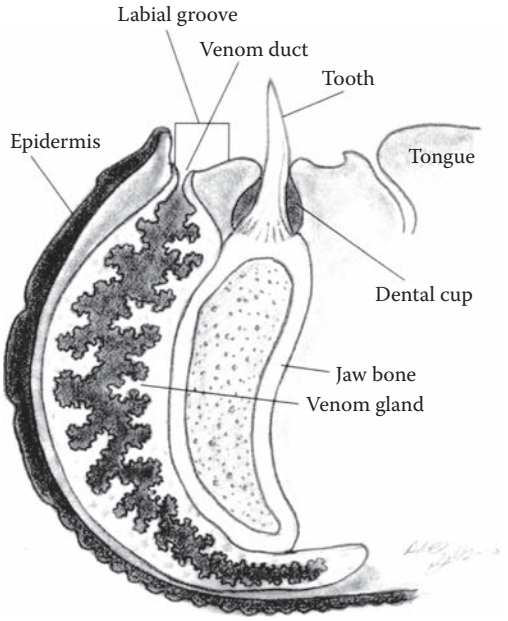

FIGURE 3.3 Venom gland of a helodermatid lizard. Cross section of one of the sacs emptying near a dentary tooth. (After Beck, 2005. With permission.)

maxillary teeth are grooved, but not tubular, perhaps aiding flow or distribution of oral secretion. The venom gland encapsulates multiple lobules emptying into a slightly expanded central lumen (Figure 3.3), but there is no evidence of storage of large volumes of venom in reservoirs, as in many venomous snakes (Bogert and Campo, 1956).

Thus, grossly, the structure of the helodermatid venom gland is readily distinguishable from venom systems in snakes. The complexity of helodermatid venoms is comparable to many snake venoms, and similarly, helodermatid venom biochemistry is reasonably well known. Numerous toxins and other biologically active polypeptides have been isolated from *Heloderma* venoms. These include hemorrhagins, gilatoxin (a kallikrein-like component; Utaisinchaeroen et al., 1993), vasomotor-active peptides (helodermins; Uddman et al., 1999), cell-specific ion channel toxins (helothermine; Nobile et al., 1996), and numerous enzymes and biogenic amines (Mebs and Raudonat, 1967; Hendon and Tu, 1981). The glycoprotein gilatoxin exhibits a murine i.v. lethal potency similar to that of the crude venom (2.7 mg/kg; Hendon and Tu, 1981). Several glucagon-like peptides (i.e., exendin-4) have been isolated from *Heloderma* venoms, and a derivative of these components, Byetta® (exenatide), has been added to the pharmaceutical armamentarium for management of type II diabetes mellitus. These venoms are antigenically distinct from snake venoms. *Heloderma suspectum* and *H. horridum* venoms showed no reactivity in immunodiffusion against twenty-four different monovalent and polyvalent antivenins against snake venoms (Minton, 1974). Interestingly, *Heloderma* venoms exhibit marked thermostability, retaining toxicity after autoclaving at 100°C for 20 minutes (Mebs, 1972). A snake venom with similar documented thermostability is that of Wagler's pit viper, *Tropidolaemus wagleri* (Weinstein, 1991). Although the literature pertaining to *Heloderma* venoms has been comprehensively reviewed (Russell, 1980; Tu, 1991; Mebs, 2002; Campbell and Lamar, 2004; Beck, 2005), the biological role of helodermatid venom has received little attention (Beck, 2005).

Envenomations inflicted by helodermatids produce recognizable clinical poisoning characterized by severe pain, hypotension (and hypotensive shock), nausea/vomiting, diaphoresis, and local edema (Hooker et al., 1994; Roller, 1977; Strimple et al., 1997; Cantrell, 2003; see also Chapter 23).

Myocardial infarction and consumptive coagulopathy following *Heloderma suspectum* envenomation have been reported (Bou-Abboud and Kardassakis, 1988; Preston, 1989), indicating that these envenomations can be life threatening.

b. Other Lizards

Although there is a report of toxic components and transcripts encoding several classes of toxin-like proteins in oral secretions of non-helodermatid lizards (Fry et al., 2006), such as iguanids, agamids, and varanids, there is no current evidence that these proteins are introduced into prey in the wild at levels significant enough to produce rapid subjugation or immobility. Instead, complications of these bites are more likely the result of secondary bacterial infection. Isolated reports of patients bitten by varanids (particularly the desert monitor, *Varanus griseus*, Sopiev et al., 1987) and presenting with clinically significant envenomations or "toxic effects," such as dysphagia, dyspnea, chest discomfort, and other signs/symptoms (Ballard and Antonio, 2001), have been published. However, these and similar cases require careful evidence-based and physician-based evaluation. This is particularly important because there are enormous numbers of varanid, agamid, and iguanid lizards in captivity, and bites from some of these are probably common. However, there are no noteworthy recent reports from medical facilities documenting the clinical evolution of such episodes. Instead, well-documented clinical sequelae of varanid and iguanid bites feature mechanical trauma (severity may be related to the involved anatomical region) and infectious complications. Presentations may include severe lacerations, extensive soft tissue injury, type I hypersensitivity, and cellulitis (Kelsey et al., 1997; Hsieh and Babel, 1999; Merin and Bush, 2000; Bibbs et al., 2001; Levine et al., 2003). Typically, larger specimens inflict correspondingly more serious wounds.

Selection in lizards favors increased relative bite performance associated with increasing cranial size as well as ontogenetically related growth of jaw adductors (Herrel and O'Reilly, 2006). Over one dozen bites inflicted by large varanids (*V. niloticus*, *V. bengalensis*, *V. salvator*, *V. varius*) either personally experienced, medically managed, or observed firsthand by one of the authors (SAW), presented as purely lacerations with reactive erythema and edema. In these cases, increased size of the varanid was associated with increased severity of the resulting injury. Broad-spectrum antibiotic coverage (amoxicillin/clavulanate, 875 mg, b.i.d.) was prescribed in one of three cases managed by SAW. None of these three cases, or the bites experienced personally, had any clinically significant sequelae.

Some investigators have noted the regional beliefs that have anecdotally assigned toxicity to varanids (Smith, 1935). Rarely observed clinical effects of bites inflicted by the Komodo monitor (*Varanus komodoensis*) have been ascribed to pathogenic serotypes of *Staphylococcus* sp. or various Enterobacteriaceae. *Escherichia coli* was the most common bacteria isolated from saliva of wild *V. komodoensis*, while *Staphylococcus capitis* and *S. caseolyticus* were most common in saliva from captive specimens (Montgomery et al., 2002). These investigators identified over fifty taxa of pathogenic organisms in *V. komodoensis* saliva. Interestingly, *Pasteurella multocida* was isolated from the blood of mice succumbing to injections of saliva from wild specimens. The wild *V. komodoensis* studied also had plasma antibody against *P. multocida*. The wounds inflicted by *V. komodoensis* are likely associated with sepsis (Montgomery et al., 2002). In addressing the potential infectious sequelae of *V. komodoensis* bites, Auffenberg (1981) weighed his own extensive experience with anecdotal reports collected in the Flores Islands. He reported two uncomplicated aseptic bites inflicted by 1.0–1.2 m specimens. Reports from islanders described variously severe outcomes from bites inflicted on humans, including rare fatalities. Some included reported predatory behavior. Culture of oral secretions collected from wild lizards yielded *Staphylococcus* sp. and several taxa of Enterobacteriaceae. Persistence of specific populations of oral bacterial flora may depend on re-inoculation from carrion (Auffenberg, 1981).

Many lizards possess a mandibular gland parallel with the infralabial gland along the lower jaw (Figure 3.2). However, outside of helodermatids, the mandibular gland exhibits no distinctive, large

lumen or specializations for venom production and storage. In varanids, teeth are not grooved (or tubular) but are typically serrated. In the absence of any scientific confirmation and clinical verification to the contrary, medical manifestations following bites are most parsimoniously attributed to bacterial infection (Gillespie et al., 2002).

2. Front-Fanged Venomous Snakes

a. Elapids and Viperids

In contrast to helodermatid lizards, venom of elapid, viperid, and atractaspid snakes is produced in and delivered by a specialized venom apparatus along the upper jaw that includes specializations of glands, muscles, teeth, venom, and behavior (Kochva, 1978; Kardong, 1979, 1980, 1982; Jackson, 2003). The venom glands of elapid (including sea snakes and allies) and viperid snakes exhibit some variability in morphology and size, but all share a similar basic design in that there is a main venom gland and an accessory gland. In viperids, the main venom gland empties via a single primary duct into the accessory gland, and from here via a secondary duct into the base of the tubular fang (Figure 3.4). In most elapids, the accessory gland is next to the main venom gland and surrounds the primary venom duct emptying the main venom gland (Figure 3.5) (Rosenberg, 1967). In some sea snakes, the main and accessory glands do not abut one another but instead are separated, connected by the primary venom duct (Gopalakrishnakone and Kochva, 1990, 1993). The main venom glands of both viperids and elapids consist of clumped tubular cisternae lined with secretory cells (Kochva and Gans, 1966), although elapid venom

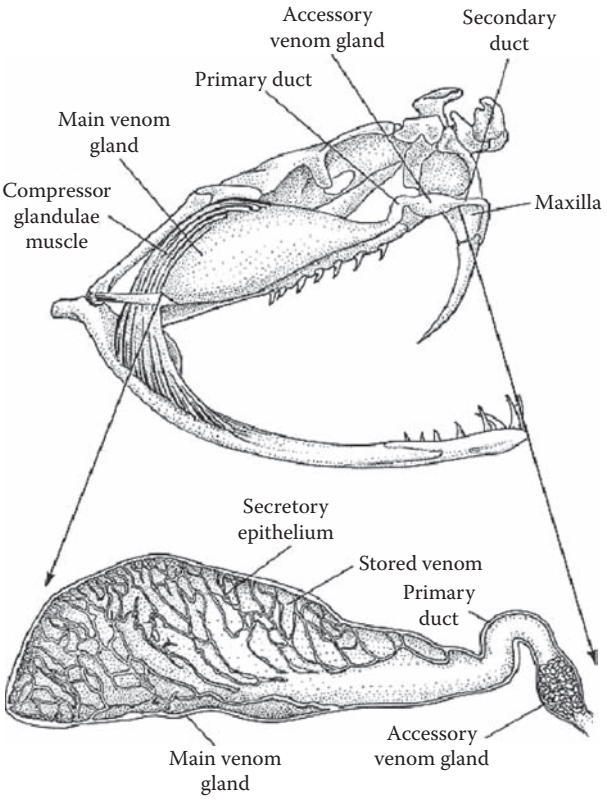

FIGURE 3.4 Viperid venom gland. The secretory epithelium releases venom stored in the collective lumen of the gland where large quantities accumulate, ready for an envenomating strike. During the strike, contraction of the compressor glandulae muscles pressurize the gland, forcing a bolus of venom through the ducts and into the prey. (From Kardong, 2002b, after Mackessy, 1991. With permission.)

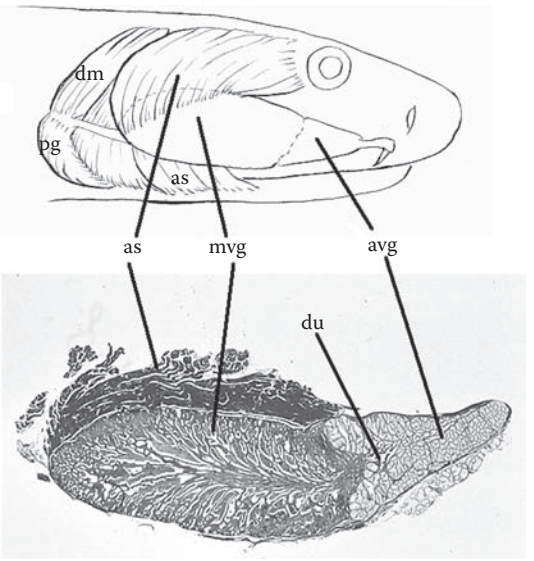

FIGURE 3.5 Elapid venom gland. Head of a representative elapid snake. Note presence of adductor superficialis (as) muscle, which inserts directly on the venom gland (mvg), pressurizing it during the strike. Accessory venom gland (avg), venom duct (du), depressor mandibulae (dm), pterygoidus (pg). Histomicrograph is of *Walterinnesia aegyptii* (kindly supplied by E. Kochva).

glands tend to have longer secretory tubules than those observed in viperid glands (Rosenberg, 1967). Slowly cycling columnar cells with apical granular secretory activity and mucous secretion contribute to venom formation. A diverse cellular population contributes to a wide array of venom components. Recent data support previous studies and hypotheses regarding the origin of venom components as derived molecular species encoded as a consequence of conserved physiological functions (Kochva, 1987; Ho et al., 1997; Cousin et al., 1998; Fry, 2005). The viperid and elapid gland compressors are, respectively, the compressor glandulae muscle, derived from the adductor externus profundus, and the superficialis muscle, derived from the adductor externus superficialis (Jackson, 2003). Further subdivision of the crotaline compressor glandulae into fascicular columns may endow finer control over the volume of expressed venom (Young et al., 2000).

Venom glands reside next to the upper jaw behind the eye, not along the mandible, as in helodermatid lizards. In viperid snakes, venom is produced in a specialized gland and stored extracellularly in a large basal lumen (Figure 3.4) (Mackessy, 1991). Venomous snakes hold stored venom during extended periods of fasting, but it remains ready when feeding resumes after hibernation or in defense; there is no reported turnover of the stored venom protein (Mackessy and Baxter, 2006). If manually depleted (extracted, or "milked"), the secretory epithelium of the main venom gland exhibits rapid protein synthesis (Kochva et al., 1980; Carneiro et al., 1991; Mackessy, 1991) with subsequent exocytosis replenishing venom stores in the ductules and large lumen. This process is completed in about 16 days (Kochva, 1987). However, when expending venom during natural strikes, venom is replenished more rapidly, or less total venom is expended initially, as judged by the rapid recovery of lethal envenomation of prey (Kardong, 1986b).

The action of metalloproteases can produce autolysis of the venom constituents. Stabilization of venom components appears to be accomplished by regulation of pH levels. This is accomplished by mitochondria-rich cells of the main venom gland that acidify the mixture, and by endogenous inhibitors that inhibit enzymatic activity of venom during storage. When injected, activation is spontaneous (Mackessy and Baxter, 2006). These mitochondria-rich cells are morphologically similar to parietal cells of the gastric pit in the mammalian stomach. In the stomach, acidification activates

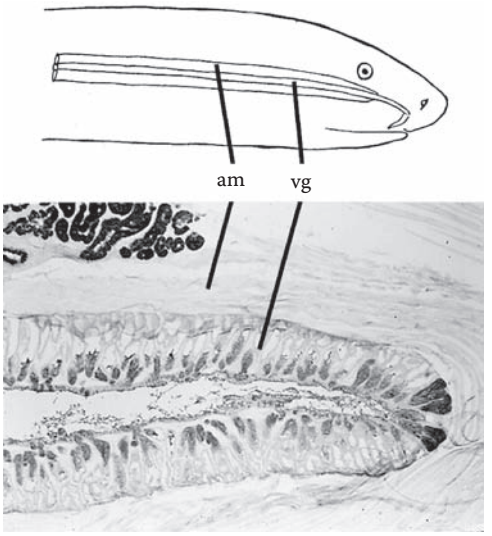

FIGURE 3.6 Atractaspidid venom gland. (a) This venom gland (vg) is elongate, typical of some atractaspidids. (b) Longitudinal section of venom gland. The specialized adductor externus medialis (am) muscle runs parallel with and inserts on the venom gland, presumably pressurizing it during a bite. Note lumen (lu) into which the radially arranged secretory tubules empty. An accessory gland is absent. Histomicrograph is of *Atractaspis engaddensis* (kindly supplied by E. Kochva).

digestive enzymes, but in the venom gland acidification inhibits venom enzymes (Mackessy and Baxter, 2006).

b. Atractaspidids

Atractaspidids have a different venom gland arrangement (Kochva et al., 1967). The centrally located lumen is elongated and surrounded by spoke-like tubules. In some species the gland may be located in the temporal region, but in other species it extends posteriorly out of the region and along the sides of the body (Figure 3.6). It is accompanied by striated compressor muscles involved directly in emptying the gland. In this variation of venom gland topography, it is similar to *Causus* and *Maticora*. Unlike elapids and viperids, the venom gland of atractaspidids lacks a discrete accessory gland and possesses a different histochemical profile (Kochva, 1978). The gland compressor muscle, also unlike viperids and elapids, is derived instead from the adductor externus medialis (Jackson, 2003).

The Duvernoy's gland (see below), a common oral gland in colubrids, is homologous with the true venom gland (Gygax, 1971; Kochva, 1965, 1978; Kochva and Wollberg, 1970). In some atractaspid species, in addition to a venom gland, a Duvernoy's gland is claimed to be present, diagnosed by its macroscopic appearance (coarsely lobulated) and position (dorsolaterally, at the corner of the mouth) (Haas, 1931; McDowell, 1986; Greene, 1997). However, such an interpretation is problematic (Wollberg et al., 1998; Underwood, 2002), and its hypothesized presence may actually be a misinterpretation of the rictal gland. If it is present, the simultaneous presence of a venom gland and a Duvernoy's gland in some atractaspidids has unknown significance. Possibly, the specialized venom gland now adds the role of producing a venom, and other oral gland functions are retained by the persistent Duvernoy's gland (McDowell, 1986).

c. Accessory Glands

The accessory gland, smaller than the main venom gland, consists of two parts recognized by histochemical (Kochva and Gans, 1965; Mackessy and Baxter, 2006) and ultrastructural (Hattingh et al., 1984; Mackessy, 1991) profile. An extract of *Agkistrodon piscivorus* accessory gland injected

intraperitoneally in mice is essentially nontoxic, with doses of up to 100 mg/kg resulting in no ill effects (Gennaro et al., 1963). Its function may be to condition or activate venom passing through during injection (Gans and Elliott, 1968). The presence of serous cells caudally followed rostrally by mucus-secreting epithelium (Hattingh et al., 1984; Mackessy, 1991) implies that lytic venom components passing through are activated by the caudal portion (Mackessy and Baxter, 2006). The accessory gland, especially the rostral part, may contribute substances to the venom during injection. However, electrophoresis and RP-HPLC analysis find no peptide or protein components added to the venom bolus exiting the intact apparatus, compared with main venom gland alone (Mackessy and Baxter, 2006).

As mentioned above, the accessory gland in viperids is separate from but connected via a primary duct to the main venom gland, encircles the venom duct in elapids, and is absent in atractaspidids. The relative size of the accessory gland may vary considerably, especially in specialized species (Gopalakrishnakone and Kochva, 1990).

3. Colubrid Snakes

The structure of venom glands in viperid and elapid snakes is considerably different than the jaw and gland apparatus of colubrids (Figure 3.7), and many species even lack its homologous counterpart, the Duvernoy's gland (Taub, 1966). About 17% of colubrid snakes lack evidence of a Duvernoy's gland, although in some groups as many as 90% of those examined were without a Duvernoy's gland (Taub, 1967). Those colubrids with a Duvernoy's gland exhibit a gland with structure significantly different from the venom gland of front-fanged snakes (Zalisko and Kardong, 1992). Although Duvernoy's glands may show variation, especially in size, they typically do not have any significant storage reservoir, possess a duct system readily distinguishable from that of venom glands of front-fanged

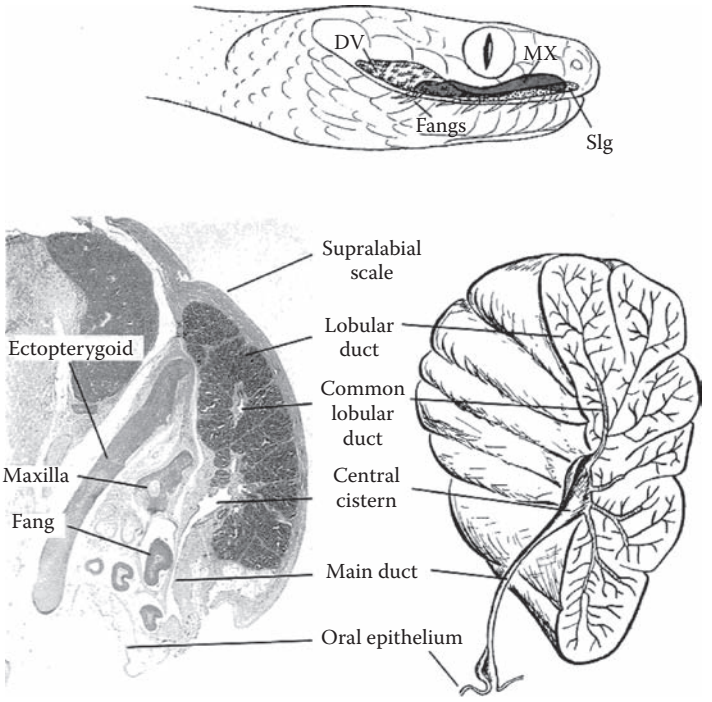

FIGURE 3.7 Duvernoy's gland, *Boiga irregularis*. Top: Duvernoy's gland (DV) lies within the temporal region posterior to the maxilla (MX) and is distinct from the supralabial gland (slg). Lower left: Cross section of right upper labial region to show internal structure of the Duvernoy's gland (lobular duct, common lobular duct, central cistern, main duct) and relationship to adjacent structures. Lower right: Schematic illustration of Duvernoy's gland and its duct system. (After Zalisko and Kardong, 1992.)

snakes, and usually have no direct striated muscle insertion to pressurize the gland (Taub, 1967). The gland, composed primarily of serous cells, is encased in a capsule of connective tissue (Taub, 1967). Teeth associated with Duvernoy's gland are never tubular (hollow) but instead are solid, often enlarged, and sometimes deeply grooved (Weinstein and Kardong, 1994; Young and Kardong, 1996). Rather than pressure discharge of a bolus by mechanical action of striated muscles, release of secretion appears to be primarily via autonomic stimulation (Rosenberg, 1992). The gland is tightly adhered to the overlying skin, and a ligament runs from the posterior end of the gland and inserts on the distal end of the quadrate bone. Contraction of the jaw adductor muscles may therefore contribute to gland pressurization. Released secretion is conveyed by a duct into a loose cuff near or around rear, often enlarged, maxillary teeth (Zalisko and Kardong, 1992). Alternatively, several ducts may carry secretion to the vicinity of various maxillary teeth (Fry et al., 2007).

These basic structural and functional features of Duvernoy's gland are also present in some colubrid species that are known to cause severe bites in humans (e.g., *Dispholidus* and *Thelotornis*; Fitzsimmons and Smith, 1958; Pope, 1958). The Duvernoy's gland is enlarged, but the departing duct serves a grooved maxillary tooth, not a hollow fang (Kardong, 1979; Young and Kardong, 1996). This means that in these venomous colubrids, as in all others with a Duvernoy's gland, the delivery system is necessarily low pressure. The venom system of these colubrids is built on a different morphology than the venom systems of viperid, elapid, and atractaspid snakes. Various caenophidian snakes exhibit atypical or specialized gland morphologies (e.g., *Causus*, *Aipysurus*; Fry et al., 2007), including some colubrids (e.g., *Dasypeltis*; Gans, 1974), some with derived specialized functions (e.g., *Dispholidus*; duToit, 1980). Recognizing these differences in morphology (Duvernoy's vs. front-fanged venom gland) and delivery (low vs. high pressure; McDowell, 1986, 1987; Greene, 1997) may help clarify differing biological roles and evolutionary strategies within caenophidians possessing different venom systems.

III. FUNCTIONS OF THE VENOM APPARATUS

As mentioned above, the functions of oral secretions in reptiles have often been interpreted in their roles in production of clinically significant morbidity and mortality (Meier, 1990), and the pharmacology of these secretions referenced almost exclusively to their supposed significance as a venom system (e.g., Fry et al., 2006). Unfortunately, this has had the effect of underestimating the variety of complex roles played by snake oral secretions in the biology of reptiles, produced a very narrow view of oral secretions, and resulted in misinterpretation of reptilian evolution. In fact, reptilian oral secretions contribute to many biological roles other than to quickly dispatching prey.

A. Delivery of Oral Secretions

Secretions released into the buccal cavity help condition dental structures (Gans, 1978) and certainly coat captured prey with mucus to aid its passage during swallowing (Greene, 1997). Contributions to the mucus are secretions released from supralabial and infralabial glands (Figure 3.2) under autonomic nervous system stimulation, as well as from the mucous lining of the buccal cavity. Depending upon the species, other oral glands may also contribute. These secretions collect relatively slowly as the jaws are walked with reciprocating displacement over the prey (e.g., Kardong, 1986a).

The venom glands of viperids (Kardong and Lavín-Murcio, 1993), elapids (Rosenberg, 1967), and atractaspidids (Kochva, 2002) are part of high-pressure delivery systems. The venom bolus is quickly expelled; rattlesnakes can deliver venom in less than half a second (Kardong and Bels, 1998). Although the specific gland compressor is different in each family (Jackson, 2003), all of these venom systems exhibit notably direct striated muscle insertion. When the gland compressor muscle contracts, the main venom gland is pressurized, producing expulsion of a presynthesized, stored, venom bolus. From venom gland to exit orifice at the tip of the tubular fang, this system is closed when activated, not open to ambient pressures, and therefore can develop, under striated muscle

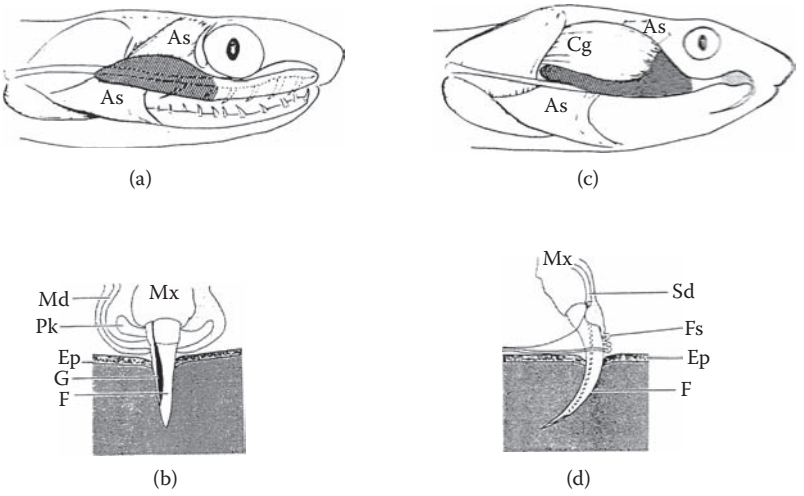

FIGURE 3.8 Duvernoy's gland versus viperid venom gland. (a) The Duvernoy's gland (shaded area), when present in colubrids, is located in the temporal region. The adductor superficialis muscle passes medially to the gland but typically does not insert on the gland, leaving the gland with no direct striated muscle action to pressurize it. (b) The released venom (Duvernoy's secretion) passes into a loose cuff around the posterior maxillary tooth. (c) In the viperid apparatus, the compressor glandulae muscle inserts on the venom gland (shaded) and pressurizes it during the strike. (d) The venom is released under significant pressure and flows through a relatively closed system, enters the erect fang, passes through the fang lumen, and then enters the prey (shaded). As, adductor superficialis; Cg, compressor glandulae; Ep, epidermis of prey; F, grooved maxillary tooth (in b), fang (in d); Fs, fang sheath; G, open groove; Md, main duct; Mx, maxilla; Pk, secretory pocket; Sd, secondary duct. (After Weinstein and Kardong, 1994. With permission.)

action, a sustained high-pressure head until venom enters the prey or predator (cf. Rosenberg, 1967). Penetration of the integument, of prey or predator, by the hollow fang lifts the fang sheath, which remains on the surface of the integument, and thereby opens the route of venom flow, allowing rapid discharge of a bolus of venom (Young et al., 2001, 2003, 2004; Young and O'Shea, 2005).

In comparison with that of a front-fanged venom system, the Duvernoy's gland is necessarily a low-pressure system due to its fundamental anatomical differences and more limited envenomation abilities (Kardong and Lavín-Murcio, 1993). The release of Duvernoy's secretion into a loose cuff of oral epithelium followed by access to a solid or grooved tooth means that this colubrid jaw apparatus is an open, low-pressure system, unable to produce or sustain a high-pressure head (Figure 3.8). In an extensive survey of squamate jaw muscles, Haas (1973) reported that no striated muscles insert directly on the Duvernoy's gland, but as Hass and others (Kochva and Wollberg, 1970) observe, in the colubrid snake *Dispholidis typus* (boomslang) some fibers of the adductor externus superficialis may actually insert on the gland, forming a modest compressor glandulae. Even if not directly attached, the adductor externus superficialis common to colubrids (and all snakes) runs medial to Duvernoy's gland such that when it contracts and bulges, it could theoretically exert a small mechanical lateral force on the nearby gland, further encouraging release of secretion (Jansen and Foehring, 1983).

The special case of *Dispholidus* is an exception among colubrids, and the structure, mechanism of secretion release, and contribution to prey handling distinguish the Duvernoy's gland from the venom gland of front-fanged venomous snakes (Kardong and Lavín-Murcio, 1993). Therefore, interpretation of how such a Duvernoy's system is deployed during prey capture, swallowing, and defense would benefit by recognizing its distinctive structure. Some have been tempted to view the Duvernoy's system as presumably an inefficient venom system (Jackson, 2007). This is unfortunate, but understandable, because its secretions have typically been interpreted in a medical context rather than in a biological one (Kardong, 2002a; but see Mackessy et al., 2006; Pawlak et al., 2006,

2008). Instead, we should consider that its primary biological roles may be those other than producing rapid prey death, and hence interpret its distinctive structural and functional features as serving other survival roles (Kardong, 1996b).

B. Biological Roles of Duvernoy's Secretion (Venom)

The secretions produced by Duvernoy's glands are a highly variable cocktail of chemical entities (primarily proteins), each with individual and synergistic roles. Many of these components exhibit toxicity. Certainly, viperid and elapid venoms provide the biological role of killing prey rapidly, and do so because of toxic components. But the reverse is not necessarily true. If an oral secretion, such as Duvernoy's secretion, is toxic, then we cannot automatically conclude that the secretion is a venom without evidence on how it is utilized during predation. A biological role cannot be determined on the basis of a chemical property alone, but only by directly documenting the role in an organism's survival. Because of the preoccupation with toxicity, alternative functions of Duvernoy's secretion have not been extensively examined and remain largely ignored. However, there are some possibilities, not necessarily mutually exclusive (reviewed in Greene, 1997; Kardong, 2002a; Mackessy, 2002).

During prey capture, the snake must subjugate the prey to prevent its escape and eventually turn it into a meal. The snake also faces the danger of retaliation by the prey, inflicting injury that might injure the snake. Snakes have evolved a variety of mechanisms to deal with these difficulties. Certainly oral secretions function to *kill prey rapidly.* Even some colubrids possess an oral gland system capable of producing secretions with high toxicity (see above) and occasionally human deaths (FitzSimons and Smith, 1958; Mittleman and Goris, 1978; Sawai et al., 1985; Ogawa and Sawai, 1986; Minton, 1990). Certainly this suggests that a few specialized Duvernoy's systems can kill prey rapidly. However, synthesis of such a toxic venom is metabolically costly (McCue, 2006). Other Duvernoy's systems may not rapidly kill but rather immobilize/tranquilize prey (Rodríguez-Robles, 1992; Rodríguez-Robles and Thomas, 1992; Thomas and Leal, 1993). This may reduce prey struggle, but leaves open the possibility of retaliation, escape, and continued metabolic expense. The colubrid snake *Diadophis punctatus* was reported to produce immobility or protracted time to death of squamate prey. This suggests that Duvernoy's secretions are used during prey capture (Gehlbach, 1974; Anton, 1994; Hill and Mackessy, 2000). If oral secretions from the ringneck snake (*D. p. occidentalis*) are injected in high doses intra-abdominally into a natural prey such as the garter snake *Thamnophis ordinoides*, 100% mortality may occur after 3 h (O'Donnell et al., 2007). Unfortunately, such results do not answer the question of whether the ringneck snake in nature actually can or does deliver oral secretions at levels similar to the dose levels used in these laboratory experiments. Without reference to actual prey handling techniques, such toxic effects demonstrated in the laboratory may have no relevance to the actual biological functions. For example, when preying on the black-fronted nunbird (*Monasa nigrifrons*), a green vine snake (*Oxybelis fulgidus*) was observed grasping the bird by the head without constriction. The bird was allowed to hang until immobile. This arboreal snake then swallowed the prey without any sign of struggle (Endo et al., 2007). This could suggest that the properties of Duvernoy's secretion relevant to the snake's survival are the immobilizing properties that incapacitate the bird, not the toxic properties that may immediately kill it.

Besides chemical means, there are mechanical means of prey capture. Constriction offers one mechanism whereby coils of the snake's body encircle and compress the prey, preventing its escape, ultimately leading to death by asphyxiation/thoracic trauma and subsequently facilitating ingestion (Greene and Burghardt, 1978). Large snakes simply overpower prey, using strong jaws to do so. After physically subduing prey, by whatever means, they swallow it. Snakes swallow prey whole, without significant mastication. Swallowing whole prey, especially if covered in fur or feathers, presents significant friction, reduced if lubricating oral secretions coat the prey surface (Gans, 1961). If injected deep into prey during capture or swallowing motions, oral secretions may contribute to

the chemical breakdown of tissues (Thomas and Pough, 1979; Kardong, 1986b; Mackessy, 1988; Hayes et al., 1993) and hence aid digestion. Even if deposited only in tooth punctures in the skin, oral secretion components (enzymatic and nonenzymatic) may contribute to opening such breeches in the integument, thereby facilitating entry of digestive enzymes as the prey passes through the gastrointestinal tract (Hayes et al., 1993).

C. Multifunctionality of Venoms

Snake venoms contain numerous components that serve a wide variety of functions (see Sections II and III). For example, many venoms contain antimicrobial components (Stiles et al., 1991; Lu et al., 2002; Gomes et al., 2005; Nair et al., 2007). Some of these, such as L-amino acid oxidase (LAO), exhibit potent catalytic activity as well as notable bacteriocidal potency, and some of the organisms sensitive to the effects of venom LAOs are common pathogens (*Aeromonas hydrophilia*) of reptiles and amphibians (Stiles et al., 1991). Such components likely are multifunctional and may have potent antimicrobial activity as a coincident consequence of the primary action (production of bacteriocidal oxygen radicals, H_2O_2, as a reaction product of the oxidative deamination of L-amino acids to form α-ketoacids and ammonia) of the enzyme. Such secondary effects may contribute to the conservation, genetic diversification, and duplication of venom components that offer multifunctional utilities for survival.

The use of venom for prey capture and defense, which has been the focus of our discussion, represents a complex strategy that involves multiple functions of venom components and specialized predatory behaviors. For example, the rattlesnake predatory strike may target and deliver a venom bolus to a highly vascularized part of the prey, the thorax holding the lungs and heart (Kardong, 1986b). Typically a rattlesnake, once injecting venom, quickly releases its prey (Klauber, 1956), often within less than half a second (Kardong and Bels, 1998). This strike and quick release behavior is attributed to the advantages of removing the rattlesnake's vulnerable head from biting retaliation by the prey (Lee et al., 1988; Furry et al., 1991). But the cost of this behavior from the snake's standpoint is that the envenomated and released prey must be located again, usually by following chemosensory cues (Chiszar, 1978; Chiszar et al., 1992b,c, 1999). Failure to relocate the struck prey means failure to secure a meal, loss of nutritional support to meet the snake's metabolic needs, and a decrease in fitness.

1. Locomotor Inhibition

The chance to relocate the envenomated prey can be improved by reducing the distance the prey travels after being struck by the rattlesnake. A rapid lethal effect is one way to do this. Another is to disrupt the prey's locomotor system immediately, before death occurs. Within a predatory context it has been noted that well before toxic components bring about death, the envenomated rodent exhibits paralysis of its locomotor system, producing "knockdown" and significantly reducing the distance it travels after being struck and envenomated (Minton, 1969). Crotamine or its close homolog in venom has been shown to produce such effects (e.g., Gonçalves, 1956), and hindlimb paralysis has been used for some time as a bioassay for crotamine (Schenberg, 1959). Crotamine, purified from the venom of the rattlesnake *Crotalus durissus terrificus*, is composed of forty-two amino acid residues, three disulfide bridges (Nicastro et al., 2003), and belongs to the highly conserved myotoxin protein family, designated small basic polypeptide myotoxins (SMPMs) (Ownby, 1998). The homolog myotoxin-α is generally present in the venoms of rattlesnakes (*Crotalus* and *Sistrurus*, Bober et al., 1988). Crotamine has moderate toxicity (i.p., LD_{50} = 6.0 mg/kg; Boni-Mitake et al., 2001), produces myonecrosis, and may have analgesic activity. The hindlimb paresis has been attributed to inhibition of voltage-sensitive Na^+ channels (Nicastro et al., 2003; Oguiura et al., 2005). However, some recent data suggest that preferential antagonism of fast-twitch muscles involving an unknown mechanism may account for the observed paralysis (Rizzi et al., 2007). A crotamine homolog is present in the venom of the northern Pacific rattlesnake, *Crotalus oreganus oreganus* (Bober et al., 1988; Ownby,

1998), and we have observed, following an envenomating strike, the rapid onset of this characteristic spastic hindlimb paresis (Kardong, 1986b).

The spastic paretic effect of crotamine was used by Hampe and Belló (1997) as a sensitive bioassay to determine the concentration of crotamine in a solution. By first injecting mice with a series of purified and known crotamine concentrations and then scoring the time onset of hyperextension paralysis in the hindlimbs, they were able to produce a dose-response (time) curve that could detect doses as low as 0.32 mg/kg (Hampe and Belló, 1997). A regression line of this curve produced the equation:

$$\log \mathbf{t} = 3.20 - 0.80 \log \mathbf{D}$$

where the relationship between the log of the time (**t**) to onset of hyperextension and the log of amount of crotamine (mg/kg) injected (**D**) is determined. We used this equation to calculate the amount of crotamine injected by snakes. To do so, we scored the time from strike to first appearance of hindlimb hyperextensive paralysis in mice naturally struck by *Crotalus oreganus*, northern Pacific rattlesnake. Our results indicate **t** = 14.5 sec (1–56 s). This translates into an average concentration of myotoxin injected to **D** = 0.0028 mg/kg, a level well below the LD_{50} (6.0 mg/kg; Boni-Mitake et al., 2001) and certainly well below the ALD_{100} (absolute lethal dose) upon which the snake in the wild depends to consistently kill its prey.

In natural prey such as deer mice (*Peromyscus maniculatus*), the total time to death, strike to last muscular twitch (Kardong, 1986b), may average just under 2 minutes (117.8 s) (Kuhn et al., 1991). Assuming that prey traveled at 3 cm/s poststrike, this could result in the envenomated prey traveling about 3.5 m before toxic effects alone stopped its displacement (based on Kuhn et al., 1991). However, the quicker paralysis of the locomotor system by myotoxin (here 14.5 s average) means that essentially the mouse is stopped, on average, about 43.5 cm from the snake, reducing the poststrike travel distance by about 88%, and leaving it closer to the snake. This increases the chances of poststrike relocation of the prey and reduces the time the trailing snake itself is exposed to its own community of predators. We hypothesize that its primary biological role, rather than lethality, is more likely to be in reducing the escape distance of envenomated and released prey.

We are well aware that this hypothesis is speculative, as it is built on several separate studies. We present it here to illustrate an example of the biological functions that may be addressed more frequently by pharmacological studies. Restricting experimental focus on the toxic effects of venoms tends to limit our understanding of the totality of venom functions. Certainly crotamine may, when injected, have a concentrated effect in critical organs (Boni-Mitake et al., 2006) or play a synergistic role in quickly dispatching prey. Our point is that broadening the pharmacological analysis of venom components would be welcome, including a test of this hypothesis. Such nonlethal functions may be more important than our first estimates suggest. For example, our estimates of poststrike travel distance may be underestimates, as field studies by others have shown considerable travel of prey after being envenomated (Clark, 2004). This would make the inducement of locomotor disruption all the more important as a survival strategy for the rattlesnake. Thus, based on evidence currently available, myoxins and their homologs seem not to play a significant adaptive role in quickly killing prey. Rather, their most obvious effect is in producing spastic paralysis where they play the primary biological role of reducing prey travel postenvenomation.

2. Precipitous Hypotension and Prey Subjugation

The diversity of biologically active components present in venoms affords direct and synergistic mechanisms of prey subjugation/immobilization. Induction of precipitous hypotension provides a means of rapid disruption of prey locomotion, thereby preventing escape. There is a voluminous literature regarding the hypotensive effects of some snake venoms and envenomation-induced hypotension (with a strong experimental bias toward crotaline venoms). The pharmaceutical exploitation of bradykinin-potentiating peptides from *B. jararaca* venom led to the discovery of one of the most commonly used classes of antihypertensive medications, the angiotensin-converting enzyme inhibitors.

Earlier reports (Russell et al., 1962) demonstrated that an intravenous bolus of *C. adamanteus*, *C. atrox*, *C. ruber*, or *C. oreganus* (formerly *viridis*) *helleri* venom caused immediate hypotension and shock. Several studies have provided evidence of species-specific susceptibility to the hypotensive effects of crotaline venoms (Vick et al., 1967; Schaeffer et al., 1973, 1984; Russell, 1980), perhaps due to the vascular dynamics of venous sequestration in the splanchnic-hepatic circulation (Vick et al., 1967; Russell, 1980). The rapid appearance of radiolabeled crotaline venoms in the lungs and the development of shock, independent from changes in cardiac output, suggested a strong pulmonary role in postenvenomation shock (Gennaro and Ramsey, 1959; Bonta et al., 1970; Russell, 1980). This is especially interesting when considering observations that suggest the specific targeting of predatory strikes to the thoracic cavity (see previous section).

Undoubtedly, the immediate hypotensive effects of many venoms are due to multiple venom components acting both individually and in concert. Components such as bradykinin-potentiating peptides (Ondetti, 1971; Greene et al., 1972; Murayama et al., 2000), rhexic hemorrhagins (Ownby, 1982), and other serine proteases and metalloproteases (Hung and Chiou, 2001; Weinberg et al., 2004) have been implicated in venom-induced hypotensive effects. In addition, some studies have suggested a mechanism related to the loss of central nervous system autoregulation after intravenous administration of *Naja nivea* venom (DiMattio et al., 1985). Other contributing mechanisms may include purinergic receptor activation (Aird, 2002; see also Chapter 20, this volume). This mechanism could function on several levels, including stimulating release of vasoactive peptides and autocoids and inhibiting quantal release from presynaptic terminals and central excitatory neurons, as well as interaction with the effects of other venom constituents (Aird, 2002). These proposed mechanisms merit further investigation. It is noteworthy that some clinical studies have considered the role of elevated purines in hypotensive events concomitant with cellular ischemia (Woolliscroft and Fox, 1986).

Therefore, the hypotensive effects that may occur following envenomation likely result from the complex action of a combination of venom components. These effects probably play an integral role in the rapid immobilization of envenomated prey, both reducing the distance traveled after the strike and reducing danger of prey retaliation. Effective delivery of toxins strongly influences the likelihood of successful preimmobilization. For instance, the biological role of hypotensive effects induced by Duvernoy's secretion (venom) from *Rhamphiophis oxyrhynchus* in anesthetized rats (Lumsden et al., 2005) must be considered in relation to the associated secretory delivery system. Successfully dispatching prey is more complicated than just rapidly killing it. From the snake's standpoint, reducing escape distance and retaliation are also adaptive features of prey capture based on primary functions of venom components. Future research investigating the mechanisms of hypotension induced by ophidian venoms (particularly when conducted in prey species correlated with a specific venom of interest) will advance our understanding of the biological functions of these complex substances.

D. Clinical Implications of Colubrid Venoms: Comparable to Elapids and Viperids?

The detection of neurotoxins in Duvernoy's secretions of colubrid snakes requires careful interpretation and reference to similar toxins in other venomous snakes. For example, it is incorrect to compare the toxic potential of elapids such as *Acanthophis* spp., *Naja* sp., etc., with those of colubrids such as *Boiga dendrophila* to humans directly, without specifying the animal model used. Superficial comparison of murine lethal potencies may suggest a similar level of toxicity between secretions of some colubrids and the venoms of some crotaline or elapid snakes. Unfortunately, for the layperson and nonexpert, this implies a similar level of medical importance and equivalent potential human danger that in fact is not present. It is similarly inaccurate to relate the magnitude of antagonism observed from *in vitro* nerve-muscle preparation assays to potential lethal potency *in vivo*. While such observations can reflect the medical importance of highly potent venoms (such as those from the aforementioned elapids) due to the high proportion of toxins and efficiency of venom delivery

systems, it is misleading to compare these with colubrid toxins. For example, the specificity and ontogenetic nature of the acetylcholine receptor (AchR) subunit composition at the murine motor end plate dictate the action of waglerin 1 from venom of the crotaline viperid, *Tropidolaemus wagleri* (Aiken et al., 1992). This peptide exhibits potent activity in the murine nerve-muscle assay; however, the venom has modal lethal potency in mice, and the purified peptide shows no AchR-binding activity when tested in assays using human or avian tissues (Weinstein et al., 1991; McArdle et al., 1999). Human envenomations by *T. wagleri* typically feature mild to moderate local edema and pain without manifestations of neurotoxicity (S. Minton, personal communication, 1984; Cox, 1991). Most colubrid secretions assayed to date exhibit modal or low potencies in the murine model (see Weinstein and Kardong, 1994, for comparison of lethal potencies), but in avian and lizard models, high toxicity and potency have been observed (Mackessy et al., 2006; Pawlak et al., 2006, 2008).

Having a toxin within the oral gland is not the same thing as delivering it, or delivering it at medically significant levels. Therefore, statements insinuating that one colubrid secretion is as potent as a given elapid venom are overreaching and may be incorrect, likely to produce misplaced concerns regarding medical importance. Such statements do not factor in the venom apparatus, mode of delivery, and possible prey specificity of secreted toxins present in venoms of front-fanged venomous snakes and oral secretions of colubrids. Comments clearly comparing magnitude of *in vitro* assay activity could be accurate in conveyance of observations made regarding the similarity of activity of composite neurotoxins in each venom or secretion. However, such comments will likely be misunderstood, unless succinctly qualified. These considerations assume greater importance due to the explosion of herpetofauna popularity in the pet industry. Incorrect information in the popular press only complicates the need to balance caution with reason in considering potential risks to the reptile hobbyist. On the other hand, it is important that medical professionals obtain an increased awareness of the potential importance of colubrid taxa termed "mildly venomous," or of those with unknown toxicity. The toxicity of oral secretions in the vast majority of colubrid snakes remains unknown, but there are likely taxa of several subfamilies that secrete venoms of clinical importance. Some large adult colubrids with modal or low lethal potency may also pose a risk to pediatric or geriatric patients and to those with chronic illness.

All biological toxins introduced into prey or humans exhibit variability in bioavailability and metabolism. This is particularly relevant as a number of *Boiga* sp. oral secretions exhibit markedly variable protein content (Weinstein and Smith, 1993). This may reflect a broad range of toxin content intraspecifically, as is observed in other venomous caenophidians (Bonilla et al., 1971; Minton and Weinstein, 1986; Chippaux et al., 1991). The lack of a significant volume of stored Duvernoy's secretion contributes further to the differences between the dynamics of colubrid oral secretions and delivery, and those of proteroglyphous and solenoglyphous snakes. Also, as mentioned previously, the unpredictable delivery of colubrid toxins due to the low-pressure delivery systems of these taxa (Kardong and Lavín-Murcio, 1993) and probable species-specific toxin susceptibility may figure prominently when considering colubrid secretion potency. Hypotheses regarding species specificity of colubrid toxins (Weinstein and Smith, 1993; Weinstein and Kardong, 1994; Mackessy, 2002) are supported by data demonstrating saurian- or avian-specific toxins present in some colubrid venoms (Mackessy et al., 2006; Pawlak et al., 2006, 2008).

Undoubtedly, there are unstudied colubrid toxins that are medically important. However, claims of medically significant manifestations of a colubrid bite require careful clinical assessments (Warrell, 2004). As mentioned previously, the majority of serious human envenomations resulting from colubrid bites present as consumptive coagulopathies (disseminated intravascular coagulopathy resulting in hemorrhagic diathesis). To date, clinical evidence indicates that life-threatening colubrid envenomings are due to bites inflicted by the Asian natricine colubrids, *Rhabdophis subminiatus* and *R. tigrinus*, as well as the African dispholidines, *Dispholidus typus*, *Thelotornis kirtlandii*, and *T. capensis* (Visser and Chapman, 1978; Atkinson et al., 1980; Aitchison, 1990; Smeets et al., 1991; Minton, 1990; Li et al., 2001; Seow et al., 2000). Possible

neurotoxic colubrid envenomings have few supporting data and may be misinterpretations of symptoms. Unlike the voluminous documentation of neurotoxic envenomings inflicted by many elapid species and a lesser number of viperids, which can include bulbar and extrabulbar manifestations, there are very limited data regarding neurotoxicity as a consequence of colubrid envenomations. Gonzales (1979) reported neurotoxic effects (ptosis, dysphagia, and respiratory distress) of *Malpolon monspessulanus* envenomation. This single report is supported by the recent case documented by Pommier and de Haro (2007), who report ptosis, blurred vision, and oculomotor palsy in a patient envenomated by an adult *M. monspessulanus* in France. The clinical assessment in this case provides a good evidence base, as the patient was evaluated by an ophthalmologist. Reports of ptosis, respiratory failure, and spasticity among a series ($n = 11$) of pediatric patients (all <4 years of age) bitten by *Boiga irregularis* on Guam (Fritts et al., 1994) could represent evidence of neurotoxic envenoming. In this series, all of the patients with the aforementioned symptoms were less than 1 year old (average = 2.9 months of age). However, the predatory behavior of this species and its ontogenetic variation in venom properties complicate interpretation of these limited documented cases.

Studies of primarily captive *B. irregularis* suggest that small prey are swallowed directly while large prey are constricted (Chiszar et al., 1992a; Hayes et al., 1993). Rodents envenomated by captive specimens were found to accumulate a large proportion (46%) of venom in the integument (Hayes et al., 1993). The murine i.p. lethal potency of adult *B. irregularis* secretion (venom) is 10.3 mg/kg (Weinstein et al., 1991). Interestingly, *B. irregularis* venom exhibits an ontogenetically related decrease in postsynaptic neurotoxin content (Weinstein et al., 1993) and concomitantly increased lethal potency in mice. *Boiga irregularis* implicated in serious bites on Guam were large specimens; mean body length was approximately 1.17 m (Fritts and McCoid, 1999). Further, although increasing *B. irregularis* body size correlates with larger secretion yields, small specimens are capable of substantial yields (Chiszar et al., 1992b). Therefore, numerous variables associated with opisthoglyphous colubrids in general, and with *B. irregularis* biology specifically, contribute to the inconsistent clinical presentations resulting from envenomations by this species (Kardong, 1999). But this presents a paradox. Bites inflicted on human neonates and infants by large specimens resulted in the most concerning clinical presentations. Yet, these large specimens produce secretions with lower murine toxicity. The smaller snakes have oral secretions with significant neurotoxin content, a low toxicity, and are not implicated with serious human envenomations. Thus, *Boiga irregularis* presents a risk to neonates and infants; however, the source of the medical sequelae remains unclear and unconfirmed. Fritts and McCoid (1999) considered the possibility that *Boiga* spp. envenomations may be misidentified as bites inflicted by sympatric elapid species such as *Bungarus* spp. in some locations. This accentuates the need for careful documentation, whenever possible, of colubrid envenomations, including information detailing the verified identity (ideally, with deposition of the voucher specimen in a recognized institution), size, weight, and provenance of offending snakes, and presenting history, lab data, investigations, and clinical observations.

IV. DISCUSSION AND CONCLUSIONS

A. Multiple Functions and Biological Roles in the Wild

Oral secretions of squamates are chemical cocktails with a richness and diversity of functions and biological roles. Even a toxic peptide such as crotamine in the venom of some rattlesnakes fulfills a primary role not of initially killing prey. Rather, its probable primary function is to disable the locomotor system of the released prey, preventing its escape beyond a recovery range before death occurs. Some components of venomous snakes may similarly be toxic, but play more primary roles in spreading venoms, disrupting blood supply, or promoting rapid circulatory spread of the venom (Minton and Minton, 1980; Russell, 1980; Mebs, 2002). Certainly squamate oral secretions may be defined as true venoms and promote rapid prey death, but many have additional roles,

such as producing quiescence/immobilization of prey (Rodríguez-Robles, 1992; Rodríguez-Robles and Leal, 1993), lubrication, digestion, poststrike trailing, defense, and others (Kardong, 2002a). Although crotamine and other myotoxins are widely perceived in the biomedical literature as toxins contributing directly to prey death, their primary biological role likely is to reduce prey escape distance traveled by envenomated prey.

In truly venomous snakes, the venom components that cause rapid prey death are pharmacologically toxic. But the opposite is not necessarily true—simply because a given biological substance is toxic should not alone infer that the animal producing it is necessarily venomous. "Venomous" has been interpreted to imply a verified biological role (Kardong, 1996b). Toxicity is a property, like the color yellow, while venomous implies a biological role, how it is used (Bock, 1980; Kardong, 1996b). In fact, such pharmacological data alone can actually be misleading when making inferences about biological role. For example, human saliva contains a complex array of bioactive substances and bacteria. The chemical constituents may include histatins (cationic, histidine-rich, antifungal peptides; Situ and Bobek, 2000, platelet-activating factor [PAF] and PAF inhibitor [Smal and Baldo, 1991], lysozyme, α-amylase, the α-7 acetylcholine receptor antagonist kynurenic acid [Kuc et al., 2006]), as well as numerous mucins, proteases, and protease inhibitors. A recent investigation of the human salivary proteome catalogued 309 proteins from whole human saliva (Hu et al., 2005), some of which are toxic (Bonilla et al., 1971). However, there is no objective or useful sense in which humans can be described as venomous animals. The murine toxicity of human saliva is an epiphenomenon, a secondary characteristic with no adaptive advantage, but an accidental by-product of its biochemistry. While our saliva may be toxic (i.e., a property), humans are not venomous (biological role).

Squamate secretions injected into laboratory animals may show pharmacological effects of toxicity or deleterious physiological responses. Such results may interest toxinologists, but without further examination, they shed little light on how the lizard or snake actually uses, or does not use, these features of its oral secretion. For example, the colubrid snake *Boiga irregularis* possesses grooved rear teeth (Young and Kardong, 1996) and delivers a pharmacologically toxic (Weinstein et al., 1991) oral secretion (Duvernoy's secretion) (Zalisko and Kardong, 1992) to its prey (Hayes et al., 1993). But if delivery of this secretion is experimentally blocked, there is no significant effect on its prey capture abilities or defense (using mice), suggesting that this "toxic" secretion plays no significant biological role in prey capture or defense (in captive scenarios) in the life of the snake (Rochelle and Kardong, 1993). However, the observation that venom from this species is much more toxic to lizard prey than to mice, coupled with the behavioral differences of the snakes toward these prey (lizards are held until quiescence, while mice are constricted), strongly suggests that snakes utilize different predatory modes, envenomation or constriction, toward different prey (Mackessy et al., 2006). By comparison, if the glandular secretion (venom) of a viperid snake (which does not also constrict) is blocked, prey capture ability may be severely disrupted (Kardong, 1996a). A snake must possess the specialized venom equipment that is sufficient to deliver, in a timely manner, large enough quantities to give the toxin biological significance. Otherwise, the pharmacological properties may be epiphenomena and may mislead interpretation of actual biological roles. Stated another way, an oral secretion may be pharmacologically toxic, but biologically inconsequential if the snake lacks the venom system to inject it at levels sufficient to contribute to prey capture.

B. "Protovenoms": Preadapted for Later Roles

Evolutionary biologists long ago recognized that features in derived species make their debut in basal species, although often in a different biological role. This evolutionary phenomenon is exaptation (preadaptation) (Gould and Vrba, 1982). Paraphrasing Stephen J. Gould, this involves previous characters of ancestors in one biological role being co-opted to new biological roles in later descendants (Gould, 2002). Toxic oral substances, when biochemically documented in colubrid snakes and basal squamates, do not automatically qualify the reptile as venomous. Instead, these toxins may be

involved in different biological roles in basal groups, later to be co-opted into a new role in the true venom system of derived snakes. Herein, genes and their products (toxins) are exapted from earlier phylogenetic roles into new derived roles (Arthur, 2002). The only way to confirm a toxin's biological role in basal squamates is by experimental confirmation.

Taking all the varied and diverse toxic components of squamate oral secretions and collapsing them into a venom system can mask, not illuminate, the variety of specialized morphologies, functions, and biological roles (Leroi et al., 1994) present in squamate reptiles. Such an expansive use of the term *venomous*, even mapped on a robust phylogeny, simply repeats past mistakes. What some have described in basal squamates as a venom system (Fry et al., 2006) is likely not an early evolution of a venom system at all, but an example of chemical preadaptation within oral secretions of a clade of squamates. Calling all in this clade venomous implies an overall potential danger that does not exist, misleads in the assessment of medical risks, and confuses the biological assessment of squamate biochemical systems.

If used in a more restricted sense, we see that venom systems in squamates in fact evolved independently multiple times. Each includes specializations of glands, muscles, teeth, oral secretion (venom), and behavior sufficient to deploy this jaw apparatus in effective defense or in the rapid dispatch of prey. One such venom system is found in helodermatid lizards that includes enhanced activity of the mandibular gland along the lower jaw, teeth, and venom secretion. In a few colubrids, a venom system arose that is built upon the Duvernoy's gland that releases and can deliver surprisingly toxic, occasionally even medically important, secretions. Within the front-fanged advanced snakes, three high-pressure venom systems have apparently independently evolved among atractaspidids, elapids, and viperids. The different structural and functional features of these venom systems suggest that they represent different solutions to environmental challenges of prey capture or defense. The evolution of venom systems in squamates is complex, which is why we urge greater experimental attention to actual prey handling techniques in order to verify natural biological functions, rather than claims based on extrapolation or conjecture. Such an experimental approach, building a better understanding of their varied functions and biological roles, will help to clarify this complex evolution of venom systems. Finally, this overview of venom gland form and function highlights the importance of interdisciplinary research that defines contemporary toxinological investigation. It is likely that future research will provide discoveries useful to basic biomedical science, expand comprehension of the basic biology of venomous reptiles, and contribute to clinical and laboratory medicine.

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Section II

Reptile Venom Enzymes

4 Snake Venom Metalloproteinases

Jay W. Fox and Solange M. T. Serrano

CONTENTS

There have been intensive investigations performed on viperid venoms in order to understand the biochemical and physiological bases for the severe pathologies associated with envenomation by these snakes. One group of toxins in these venoms, the snake venom metalloproteinases (SVMPs), has received particular attention due to their association with several prominent symptoms, most notably local and systemic hemorrhage. In this chapter we discuss some of the basic principles of the structure and function of SVMPs and highlight recent developments, which have elucidated some of the mechanisms underlying the function of these toxins. We provide a concise discussion of recent developments leading to the current understanding of key aspects of SVMPs but will not attempt an in-depth review of these metalloproteinases (see recent review: Fox and Serrano, 2005). Instead, we concentrate on critical and significant advances in the understanding of this fascinating group of toxins.

I. SNAKE VENOM METALLOPROTEINASES: KEY MEMBERS OF THE REPROLYSIN SUBFAMILY OF METALLOPROTEINASES

The M12 subfamily of metalloproteinases is comprised of the SVMPs and ADAMs (a disintegrin and metalloproteinase) groups of proteins that share certain salient structural features, including a homologous metalloproteinase domain as well as, in certain cases, domain structures carboxy to the proteinase domain. The ADAMs family of proteinases represents a diverse structural family of proteins with a variety of functional characteristics (for a review see White, 2003). The SVMPs are considered to have evolved from the ADAMs as a result of adaptive pressure on snakes to produce venom appropriate for its needs in terms of hunting and prey capture (Jia et al., 1996; Fry et al., 2006).

A. SVMP Description

Early in the scientific characterization of snake venom, proteinases in the venom were isolated that demonstrated a relatively broad proteolytic specificity. Furthermore, the proteolytic activity could be effectively inhibited by chelating agents such as EDTA or o-phenanthroline (Bjarnason and Fox, 1995). Thus, these proteins were dubbed metalloproteinases, and subsequently, following more intensive characterization, they were shown to be dependent on zinc for their catalytic activity (Bjarnason and Fox, 1995). Furthermore, in many instances these proteins were shown to cause hemorrhage and were thus termed hemorrhagic factors or toxins; however, as we now know, there are SVMPs that display other activities besides causing hemorrhage (see Section IV).

B. Classification

The SVMPs were originally organized into structural classes based on the presence of various domain structures (Bjarnason and Fox, 1995). All SVMPs share a metalloproteinase domain characterized by the signature HEXXHXXGXXH zinc-binding motif and, as would be expected for extracellular proteins, a number of structurally stabilizing disulfide bonds. cDNA sequence analysis of SVMP structure indicated that the proteinases are synthesized in a latent or zymogen form that is processed by a Cys-switch mechanism into the active form in a manner similar to that of the matrix metalloproteinases (Hite et al., 1992; Shimokawa et al., 1996). Figure 4.1 shows the most recently modified classification scheme of the SVMPs originally developed (Fox and Serrano, 2005). SVMPs that are comprised of only a metalloproteinase domain are classified as class P-I SVMPs. In their nascent form they have pre-pro-domains, which are proteolytically processed in the mature SVMP, as well as a "spacer" domain carboxy to the metalloproteinase domain. The P-II class of SVMPs is characterized by the presence, in its nascent form, of a disintegrin domain that in most cases hosts the canonical RGD motif. The disintegrin domain is found carboxy to the spacer domain, which is proteolytically processed giving rise to a "free" disintegrin. In the P-IIb class the disintegrin domain is not processed but remains part of the structure of the proteinase. The P-IIc class represents the dimeric form of the P-IIb. The P-IId class is just a representation of a precursor form which gives rise to the homodimeric disintegrins observed in venoms, whereas the P-IIe class represents the P-II SVMP, which gives rise to a heterodimeric RGD-containing disintegrin. There are four subclassifications of the P-III SVMPs. All of these in their nascent forms have a disintegrin-like and cysteine-rich domain carboxy to the spacer domain. The disintegrin-like domain, although sharing some sequence homology with the disintegrin domains of the P-II class, are distinct in that they have a very different disulfide bond structure where the RGD integrin-binding site is found in the disintegrins. Therefore, it has been postulated that the functionality of the disintegrin-like domain, if any, in the P-III subclass is indeed very different from that of the disintegrins proper (Fox and Serrano, 2005).

In the P-IIIa subclass the disintegrin-like and cysteine-rich domains are not processed from the proteinase, whereas in the P-IIIb class the spacer, disintegrin-like, and cysteine-rich domains are proteolytically processed from the metalloproteinase domain. The mechanism or structural basis

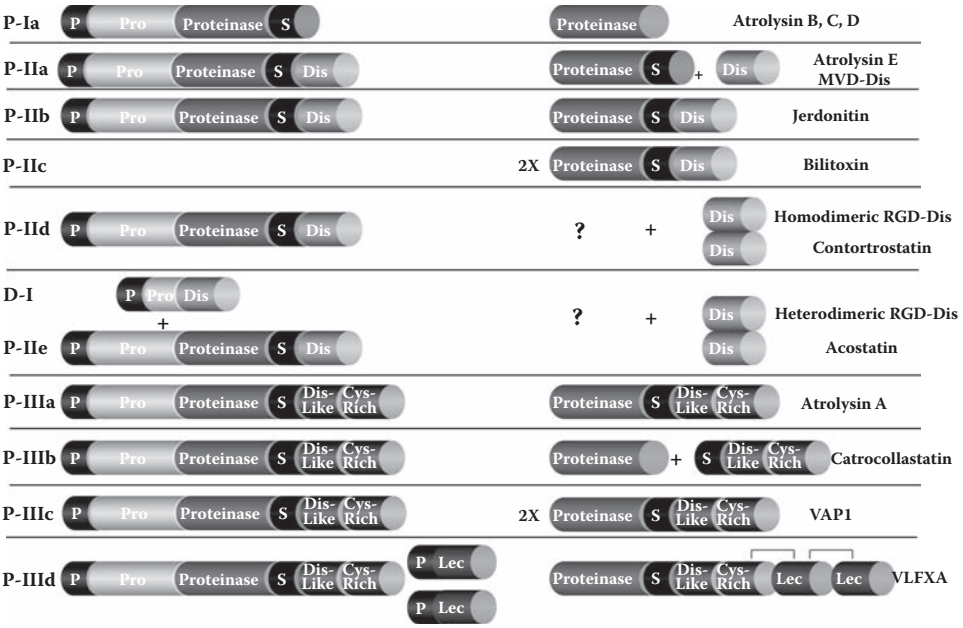

FIGURE 4.1 ***(A color version of this figure follows page 240.)*** Schematic of SVMP classes. (Reprinted from Fox and Serrano, 2008, with permission from Wiley.)

for the processing from the metalloproteinase domain of the spacer region and the disintegrin-like and cysteine-rich domains in contrast to the P-IIIa subclass, which remains intact, is uncertain. However, there are some experimental data described in the literature that suggest it may have something to do with disulfide bond arrangement or folding (Moura-da-Silva et al., 2003). The P-IIIc subclass is a dimeric form of the P-IIIa subclass, whereas the P-IIId subclass is comprised of the P-IIIa structure with the addition of two disulfide-bonded lectin-like domains posttranslationally coupled to the P-IIIa structure. It will be interesting, as additional genomic sequence information is provided on the SVMPs, to see how some of the detailed features of transcription, translation, and posttranslation contribute to the diversity of this toxin family.

II. SVMP STRUCTURE

A. Processing of SVMPs

Based on the observations of Hite and colleagues (1992) that SVMPs were synthesized as a preproenzyme form, there have been significant efforts to understand the nature of zymogen activation within the gland and maintenance of SVMP functional and structural integrity in the secreted form found in the venom lumen. Toward that end, Shimokawa and colleagues using recombinant pro-atrolysin E (a P-IIa SVMP from *Crotalus atrox*) demonstrated that *in vitro* autolytic processing occurred via an initial proteolytic cleavage to release the pro-domain followed by a second cleavage to remove the disintegrin domain (Shimokawa et al., 1996). Interestingly, unlike the matrix metalloproteinases, organomercurials could not activate the pro-form of the protein. This was somewhat surprising in that studies have shown that like the matrix metalloproteinases, SVMPs also have a functional cys-switch mechanism for maintaining enzyme latency (Grams et al., 1993).

Another interesting feature of the P-IIIb class of SVMPs is the observation of this class undergoing processing to release a disintegrin-like/cysteine-rich domain that mirrors the platelet-aggregation inhibition functionality of the P-IIIa SVMPs. Two examples of this are catrocollastatin

from *Crotalus atrox* and jararhagin from *Bothrops jararaca*, which give rise to catrocollastatin-C and jararhagin-C, respectively. What is curious is that it appears that only a portion of the population of the proteinases undergoe this processing. To address this, Moura-da-Silva and colleagues examined the primary structure of naturally occurring jarahagin-C and jararhagin-C that was produced *in vitro* from jararhagin following perturbation of structure by acetonitrile or long incubation times at room temperature (Moura-da-Silva et al., 2003). What was observed is that the amino-termini of the naturally occurring jararhagin-C and *in vitro* produced jararhagin-C were different. The authors interpreted this to suggest that there were different folding isomers of jararhagin in venom, perhaps due to different disulfide bond patterns. One possible explanation for this is that various folding isomers could be differentially susceptible to processing of the disintegrin-like/cysteine-rich domain and as such would contribute to venom complexity and function.

B. Disulfide Bonds in SVMPs

As noted earlier, all the SVMPs are characterized by having numerous disulfide bonds that serve to maintain structural and functional stability outside the venom gland cell. Typically, the P-I subclass has two to three disulfide bonds in the metalloproteinase domain, whereas P-II and P-III have three (Table 4.1). Further, in the P-II and P-III subclasses there is often an odd cysteinyl residue that in some cases may be represented as a free cysteine or in a disulfide bond with a cysteinyl residue in another domain, or involved in formation of dimeric structures with another subunit. The disulfide bond number in the disintegrin domains of the P-II subclass ranges from 5 to 7, and in the disintegrin-like domains of the P-III subclass the disulfide bond number is 8. The disulfide bond number in the cysteine-rich domain of the P-III subclass is 6. In the P-IIId subclass there is an odd cysteinyl that is involved in a disulfide bond with the lectin-like domain.

As mentioned above, the disulfide bond arrangement, as well as the presence of the so-called odd cysteinyl residues in the various domains, likely plays an important role in contributing to venom complexity due to potential for posttranslational disulfide bond formation with other subunits to produce different proteins with potentially different activities. Further, the potential for forming different disulfide bond arrangements during synthesis may result in alternative posttranslational proteolytic processing, which would also contribute to venom complexity and function. Many of the molecular mechanisms underlying these processes are yet to be determined and are under active investigation.

C. Unusual P-III SVMPs

There are two P-III SVMPs that currently do not precisely fit the classification structure shown in Figure 4.1. Kaouthiagin is an approximately 51 kDa protein found in the venom of *Naja kaouthia*, which has been shown to function as a vWF-binding and -cleaving proteinase to inhibit ristocetin-induced platelet aggregation (Hamako et al., 1998; Ito et al., 2001). Primary structural analysis of kaouthiagin revealed that in the disintegrin-like domain of the protein there was a segment missing that includes four cysteinyl residues (Figure 4.2), which would suggest that the structure of this disintegrin-like domain is likely very different from that of the typical P-III disintegrin-like domains. If and how this may impact on the functionality of kaouthiagin is unknown; however, recently jararhagin, a P-III SVMP with a typical disintegrin-like domain, has been demonstrated to bind and cleave vWF at its A1 domain (Serrano et al., 2007), suggesting that the deletion in the disintegrin-like domain of kaouthiagin has little effect on that activity.

The cDNA sequence of a very interesting metalloproteinase, BjussMP-I, from *Bothrops jararacussu* venom glands, has also been reported (Mazzi et al., 2006). Based on the cDNA sequence, the authors suggested that the protein belongs to the P-III SVMP class. Analysis of the deduced primary structure of BjussMP-I reveals a very unusual sequence in what is typically termed the

TABLE 4.1
Cysteinyl Residues in Domains of SVMPs

SVMP Class	Number of Cys Residues in Domain						Proteolytically Processed Disintegrin/Disintegrin-Like Domains
	Pre	Pro	Proteinase	Disintegrin/ Disintegrin-Like	Cysteine-Rich	Lectin	
				P-I			
Atrolysin B	1	3	4	N.A.	N.A.	N.A.	N.A.
Atrolysin C	1	3	4	N.A.	N.A.	N.A.	N.A.
ACLPREF	1	3	6	N.A.	N.A.	N.A.	N.A.
Acutolysin A	1	3	7	N.A.	N.A.	N.A.	N.A.
				P-II			
Trigramin	1	3	7	12	N.A.	N.A.	Yes—monomer
Flavoridin	1	3	7	12	N.A.	N.A.	Yes—monomer
Atrolysin E	1	3	7	10	N.A.	N.A.	Yes—monomer
Lebetase	1	3	6	12	N.A.	N.A.	N.R.
Flavostatin	1	3	6	10	N.A.	N.A.	Yes—monomer
MT-d	1	3	5	12	N.A.	N.A.	Yes
Contortrostatin	1	3	6	14	N.A.	N.A.	Yes—homodimer
Acostatin							
Alpha chain	1	0	N.A.	10	N.A.	N.A.	Yes—heterodimer
Beta chain	1	4	6	10	N.A.	N.A.	
Jerdonitin	1	3	6	14	N.A.	N.A.	No
Bilitoxin-1	N.R.	N.R.	7	15	N.A.	N.A.	No
				P-III			
Brevilysin H6	N.R.	N.R.	7	16	12	N.A.	Yes
HR1a	1	3	7	16	12	N.A.	Yes
HR1b	1	3	7	16	12	N.A.	Yes
Catrocollastatin	1	3	7	16	12	N.A.	Yes
Jararhagin	N.R.	3	7	16	12	N.A.	Yes
Bothropasin	1	3	7	16	12	N.A.	Yes
Acurhagin	1	3	7	16	12	N.A.	Yes
HF3	1	3	6	16	12	N.A.	N.R.
Berythractivase	1	3	7	16	13	N.A.	N.R.
Atrolysin A	N.R.	N.R.	7	16	12	N.A.	N.R.
Kaouthiagin	N.R.	N.R.	6	12	12	N.A.	N.R.
BjussuMP_I	N.R.	3	6	12	13	N.A.	N.R.
VAP1-homodimeric	0	3	7	16	12	N.A.	N.R.
HV1-homodimeric	1	3	7	16	12	N.A.	N.R.
RVV-X	N.R.	N.R.	8	16	13	LC1 = 7	N.R.
VLFXA	0	3	7	16	13	LC1 = 7 LC2 = 8	N.R.

N.R., not reported; N.A., not applicable.

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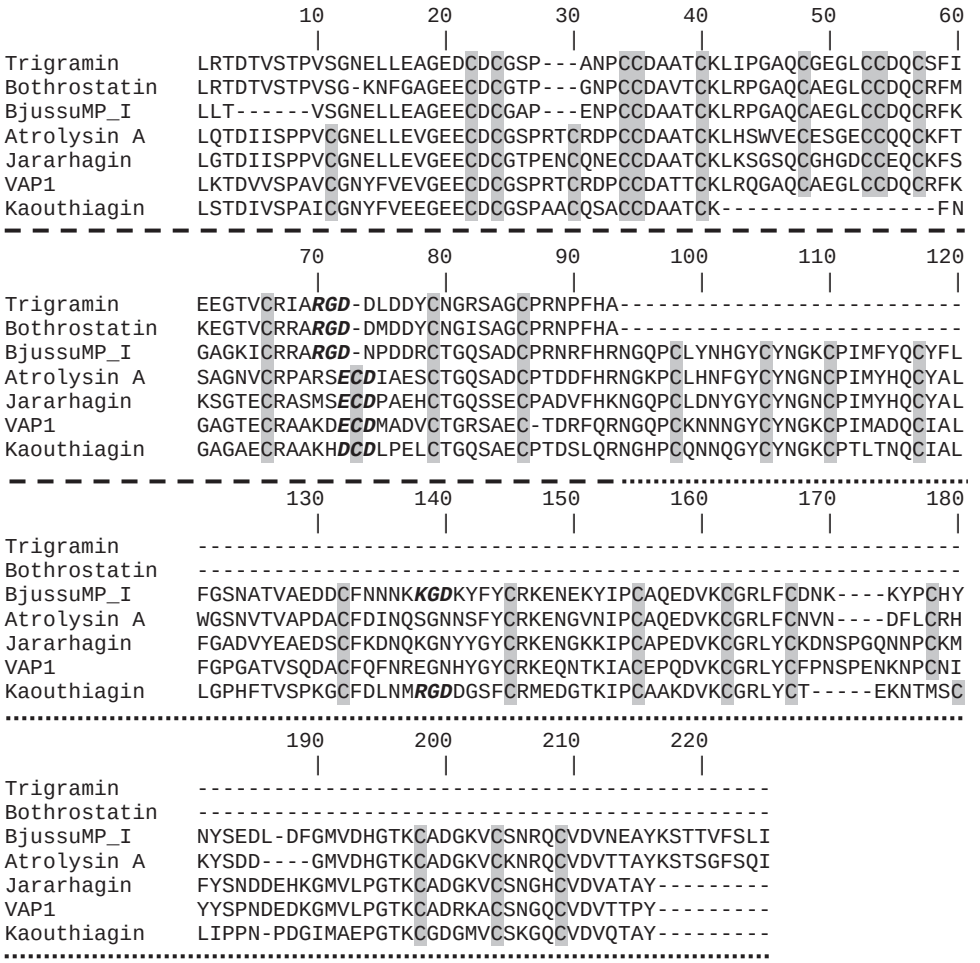

FIGURE 4.2 Sequence alignment (ClustalW) of disintegrins (trigramin (CAA35910) and bothrostatin (AAK15542)) and disintegrin-like/cysteine-rich domains of P-III SVMPs (atrolysin A (AAA03326), jararhagin (CAA48323), VAP1 (2EROA), BjussuMP_I (ABD73129), and Kaouthiagin (P82942)). (---) corresponds to the disintegrin-like domain of P-III SVMPs; (·····) corresponds to the cysteine-rich domain of P-III SVMPs, according to Takeda et al. (2006). Cys residues are highlighted in gray. Disintegrin and disintegrin-like sequences are shown in ***italic boldface***.

disintegrin-like domain (Figure 4.2). First, in lieu of the common ECD motif in the disintegrin-like domain is found the canonical RGD integrin-binding motif. In the ECD → RGD substitution, there is a key different structural factor, the lack of the cysteinyl residue associated with the ECD sequence. Furthermore, alignment of the cysteinyl residues of disintegrins and P-III disintegrin-like domains showed that BjussMP-1 displays typical disintegrin structure, not the disintegrin-like structure commonly associated with P-III SVMPs. Thus, assuming the sequence interpretation is correct, BjussMP-I represents a novel structure falling outside the current SVMP classification scheme. Since this is the only example of such a structure, we are withholding the formation of yet another P-III subclass; however, should additional homologous structures be reported, then perhaps an additional subclass would be warranted. Given this unusual structure, one could envision atypical activities to be associated with such a subclass. BjussMP-I does in some sense show a typical P-III proteolytic activity in that it can lyse fibrin clots (Mazzi et al., 2006). However, it also was

demonstrated to inhibit ADP-induced platelet aggregation, an activity typically found in the RGD disintegrins proper, whereas most P-IIIs are competent at inhibiting collagen-stimulated platelet aggregation. As such, this reinforces the concept that the typical P-III SVMPs interact with integrins via their cysteine-rich domain, whereas BjussMP-I could have integrin-binding functionalities at both its disintegrin domain and cysteine-rich domain. In addition to isolating more examples of this proteinase, further investigation will be required to determine conclusively the functionality of the disintegrin and cysteine-rich domains of this type of SVMP.

D. Three-Dimensional Structure of SVMPs

The crystal structures for a number of P-I SVMPs have been determined (Fox and Serrano, 2005), and they all share similar structural features, particularly in the region of the zinc-binding site, underscoring a common catalytic mechanism. The P-I metalloproteinase domain is observed to be comprised of two subdomains, with the amino-terminal subdomain being the larger of the two. The amino-terminal subdomain contains the canonical HEXXHXXGXXH zinc-binding motif and the carboxy-terminal subdomain contains the CIM-turn motif (Gomis-Ruth et al., 1994). The two or three disulfide bonds found in these structures are found aggregated together in the interior of the molecule and contribute to the compact, globular structure of the protein. Comparisons of P-I SVMP structure with their fellow reprolysins, ADAMs 17 and 33, have been made (Gomis-Ruth et al., 1994; Orth et al., 2004). The most notable difference in structures is that the metalloproteinase domain of the ADAM 17 is significantly longer, and thus there are two surface bulges. Furthermore, ADAM 17 lacks the typical P-I calcium-binding site but is functionally replaced by a disulfide bond to stabilize the region. The authors did note, however, that the ADAM 17 structure may in fact be unique, and that other ADAMs may more closely resemble SVMP P-I structure (Maskos et al., 1998).

Recently, the crystal structures of a dimeric and a monomeric P-III SVMP have been elucidated. Takeda and colleagues have reported on the structure of VAP-1, a nonhemorrhagic SVMP from *Crotalus atrox* venom (Takeda et al., 2006). The significance of this work is twofold: it is the first report of a P-III crystal structure, and therefore sheds light on the overall spatial organization of the domain structure of the metalloproteinase, and it allows for speculation on the structure of the ADAMs subfamily of the reprolysins, given the sequence similarities shared between the two groups. Examination of VAP1 shows an overall crescent-shaped structure, with the metalloproteinase domain opposing the disintegrin-like domain and the cysteine-rich domain tightly packed against the disintegrin-like domain (Figure 4.3). It has been postulated that the ECD region of the disintegrin-like domain is responsible for interaction with integrins (Jia et al., 1997). Takeda and colleagues, based on their structural observations, suggested that the domain structural organization of VAP1 would preclude a ligand functionality for the ECD region of the disintegrin-like domain in that it would be inaccessible for binding to integrin receptors. Furthermore, they suggested that more likely for receptor engagement was the hypervariable region at the distal arm of the cysteine-rich domain. This is in keeping with experimental evidence demonstrating functional importance of the cysteine-rich domain of the P-III SVMPs, with regard to the vWF A domain interaction (see Section IV). Crystal structures of a monomeric P-III catrocollastatin from *C. atrox* venom have also been reported (Igarashi et al., 2007). In this report the authors commented that the monomeric structure of catrocollastatin is very similar to that of the dimeric VAP1, and thus in these instances dimerization has little effect on tertiary structure (Igarashi et al., 2007). Furthermore, they noted from the various crystal structures observed of catrocollastatin that there is a certain degree of flexibility in the structure, which is likely due in part to the spacer or hinge region between the metalloproteinase and disintegrin-like domains. The authors made an interesting speculation that this spacer region in the ADAMs is somewhat variable in length, and as such it may play an important role in terms of providing structural flexibility for substrate recognition and proteolytic cleavage, as well as proteolytic processing between the metalloproteinase and disintegrin-like domains.

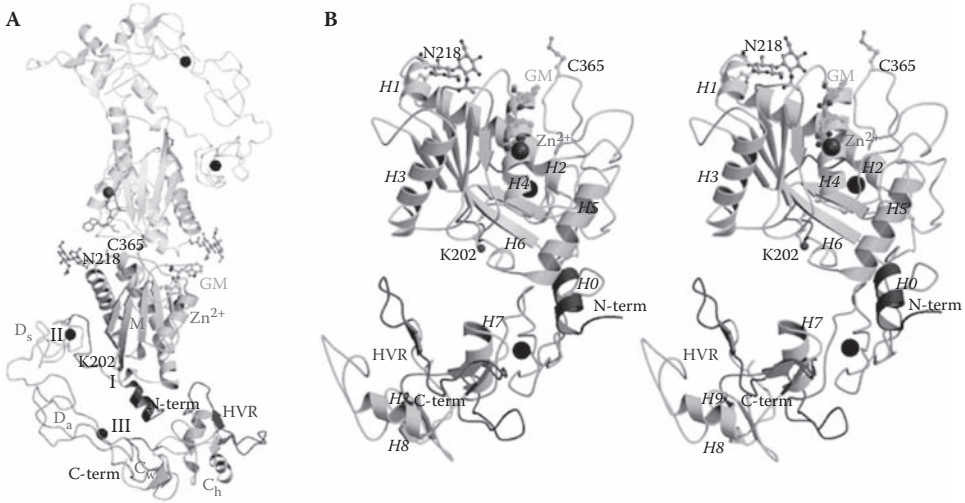

FIGURE 4.3 ***(A color version of this figure follows page 240.)*** Metalloproteinase-disintegrin-cysteine-rich (MDC) architecture. (A) VAP1 dimer viewed from the noncrystallographic twofold symmetry (NCS) axis. The N-terminal helix (H0-helix), metalloproteinase domain (M-domain), linker, "shoulder" (D_{s-}), "arm" (D_{a-}), "wrist" (C_{w-}), and "hand" (C_{h-}) domains and hypervariable regions (HVRs) belonging to the one monomer are shown in red, yellow, gray, cyan, pink, gray, green, and blue, respectively. The disulfide-linked counterpart is shown in gray. Zinc and calcium ions are represented as red and black spheres, respectively. The NAG (*N*-acetyl-glucosamine, in orange) moieties linked to Asn218, the calcium-mimetic Lys202, and the bound inhibitor GM6001 (GM, in green) are in ball-stick representations. (B) Stereo view of VAP1 monomer from the direction nearly perpendicular to (A). The helix numbers are labeled. (Reprinted from Takeda et al., 2006, with permission from Macmillan Publishers Ltd.)

Certainly, additional SVMP structures as well as structures for ADAMs will be needed before we can appropriately generate a tertiary structural classification for the reprolysins and better understand the impact of these structures on the functionality of the various domains.

III. SVMP ACTIVITIES

A. Proteolytic Activities

A wide variety of biochemical and biological activities have been ascribed to the SVMPs, and clearly from these activities one can envision their critical role in the overall toxicity of the venom. A partial listing of activities of selected members of SVMP subclasses is seen in Table 4.2. In reviewing these data, one must be aware that there could be multiple activities for a particular SVMP, but they are not shown simply because that specific activity was not assayed. For example, most of the P-I SVMPs likely display fibrinogenolytic activity, but in many cases that activity was not reported. In the P-I subclass, both hemorrhagic and nonhemorrhagic activities have been found, but in general with the hemorrhagic P-I SVMPs the minimum hemorrhagic dose is significantly higher than that observed for the P-III subclass. This characteristic is thought to do with the presence of additional domain structures in the P-IIIs that contribute to the enhanced activity/functionality of the toxins (Fox and Serrano, 2005).

The P-II subclass, in addition to having members that are hemorrhagic, also have members that inhibit platelet aggregation due to the presence of the unprocessed disintegrin domain in the proteinase. The P-III subclass has members with a variety of activities, including hemorrhagic, apoptotic, vWF cleavage, activation of prothrombin, and activation of factor X.

TABLE 4.2
Biological Activities of Selected SVMPs

SVMP	Activity	References
	P-I	
Atrolysin C	Hemorrhagic	Shannon et al., 1989; Zhang et al., 1994
Acutolysin A	Hemorrhagic	Gong et al., 1998; Liu et al., 1999
BaP1	Hemorrhagic; myonecrotic; inflammatory	Gutiérrez et al., 1995; Rucavado et al., 1995
Fibrolase	Fibrinolytic	Markland, 1996
HT-2	Hemorrhagic	Mori et al., 1987; Takeya et al., 1990
Atroxase	Fibrinolytic	Willis and Tu, 1988
LHF-II	Hemorrhagic	Sanchez et al., 1991
H2-proteinase	Proteolytic; nonhemorrhagic	Takeya et al., 1989
HR2A	Hemorrhagic	Takahashi and Ohsaka, 1970; Yamada et al., 1999
Graminelysin I	Apoptotic	Wu et al., 2001
	P-II	
Atrolysin E	Hemorrhagic	Hite et al., 1992; Shimokawa et al., 1996
MT-d	Proteolytic	Jeon and Kim, 1999
Jerdonitin	Inhibition of platelet aggregation	Chen et al., 2003
Bilitoxin-I	Hemorrhagic	Imai et al., 1989; Nikai et al., 2000
	P-III	
Atrolysin A	Hemorrhagic; inhibition of platelet aggregation	Fox and Bjarnason, 1995; Jia et al., 1997
Catrocollastatin	Inhibition of platelet aggregation	Zhou et al., 1995, 1996
Jararhagin	Hemorrhagic; inhibition of platelet aggregation	Paine et al., 1992; Kamiguti et al., 1996
HF3	Hemorrhagic; activation of macrophage phagocytosis	Assakura et al., 1986; Silva et al., 2004
HR1a	Hemorrhagic	Omori-Satoh and Sadahiro, 1979; Kishimoto and Takahashi, 2002
HR1b	Hemorrhagic	Omori-Satoh and Sadahiro, 1979; Kishimoto and Takahashi, 2002
Kaouthiagin	Cleavage of vWF; inhibition of platelet aggregation	Hamako et al., 1998; Ito et al., 2001
VAP1	Apoptotic	Masuda et al., 1998, 2000
HV1	Apoptotic	Masuda et al., 2001
Acurhagin	Hemorrhagic; inhibition of platelet aggregation	Wang and Huang, 2002
Ecarin	Activation of prothrombin	Kornalik and Blomback, 1975; Nishida et al., 1995
Berythractivase	Activation of prothrombin	Silva et al., 2003
RVV-X	Activation of factor X	Takeya et al., 1992; Gowda et al., 1994
VLFXA	Activation of factor X	Siigur et al., 2001, 2004

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As mentioned earlier, a review of the literature generally indicates that certain SVMPs do not lack particular activities, but only that the activity has or has not been assayed for a particular SVMP. Thus, one is never sure, unless it is explicitly pointed out in the literature, what activities SVMPs do or do not have beyond the assays described.

B. Nonproteolytic Activities

In general, when describing nonproteolytic activities of the SVMPs, one is referring to activities associated with the nonmetalloproteinase domains found in the P-II and P-III. As previously

mentioned, viperid venoms commonly contain disintegrins, which are small proteins that generally contain the RGD integrin-binding motif that are potent inhibitors of platelet aggregation (Calvete et al., 2005; see also Chapter 17, this volume). These disintegrins can be found as processed independent proteins in the venom or as a carboxy domain in conjunction with a metalloproteinase domain in the P-II subclass. In the context of the P-II structure one can envision the disintegrin domain playing a role for proteinase-substrate targeting or inhibiting RGD-dependent integrin function. The disintegrin-like domain of the P-III subclass in conjunction with the amino-terminal metalloproteinase domain and the carboxy-terminal cysteine-rich domain could likewise be postulated as playing a role in substrate targeting. Based on recent ligand-binding and structural studies, it now seems unlikely that it is directly involved in integrin binding (Serrano et al., 2006, 2007). As stated above, the region that has been speculated to support integrin binding, the ECD region, is not exposed in the P-III structure and thus is not accessible for receptor engagement (Takeda et al., 2006), though synthetic peptides from this region can inhibit platelet aggregation (Jia et al., 1997). Previously, Kamiguti and colleagues identified regions in the cysteine-rich domain of the P-III jararhagin to be capable of inhibiting platelet aggregation (Kamiguti et al., 2003). Pinto and colleagues (2007a), using similar peptides from the cysteine-rich domain from the P-III jararhagin, were able to show binding to vWF, and based upon the structure of VAP1, they modeled the structure for jararhagin, from which it appeared that these peptides were accessible for vWF interaction (Figure 4.4). More recently, Serrano and colleagues have demonstrated that the cysteine-rich domain of jararhagin could bind to the A1 domain found in a variety of proteins and act to target the SVMP to the protein for subsequent proteolytic cleavage (Serrano et al., 2007). Thus, there is mounting evidence that in the P-III SVMPs, the cysteine-rich domain, as well as the processed disintegrin-like/cysteine-rich domains, bind to A domains in proteins to block the function of the A domain and to target the protein for proteolytic cleavage.

In the P-IIId SVMPs, RVV-X, and VLFXA, it is the lectin-like domains that give rise to the unique substrate specificity of this subclass. Although it has not been experimentally demonstrated, one can speculate that it is the covalently bound lectin-like domains that target the proteinases to factor X for subsequent proteolytic activation.

C. Role of Nonmetalloproteinase Domains in SVMP Activities

It is apparent that the general activity of SVMPs is largely dependent on the proteolytic activity of the metalloproteinase domain. However, it is becoming increasingly clear that in the case of P-IIb and P-IIIa, c, d SVMPs, the presence of the nonproteinase domains play an important role in modulating the overall functionality of the SVMP. In most cases, this is thought to occur via targeting the SVMP to a particular region or substrate whereby local proteolysis by the metalloproteinase results in a site- or substrate-specific activity. For example, the P-IIb SVMP jerdonitin, isolated from *Trimeresurus jerdonii* venom, has been demonstrated to be an inhibitor of ADP-induced platelet aggregation, suggesting that it can block αIIbβ3 integrin-mediated aggregation (Chen et al., 2003). The role of the metalloproteinase domain in the toxicity of this SVMP has not been established, but one can speculate that proteolysis of the integrin, or proteins proximal to the integrin and targeted by jerdonitin, may be cleaved.

In the case of the P-III SVMPs there have been significant recent advances in our understanding of the function of the nonproteinase domains. Serrano and colleagues determined that the cysteine-rich domains of certain P-III SVMPS were capable of targeting the SVMPs to various extracellular proteins harboring a vWF A domain (Serrano et al., 2006). The FACIT collagens XII and XIV, matrillins 1, 2, and 3, and vWF itself were shown to bind the cysteine-rich domain of jararhagin, followed by proteolysis of the protein at sites in or adjacent to the A domain. This resulted in disruption of the extracellular matrix structure, and in the case of vWF, rendered it incapable of promoting platelet aggregation (Serrano et al., 2007).

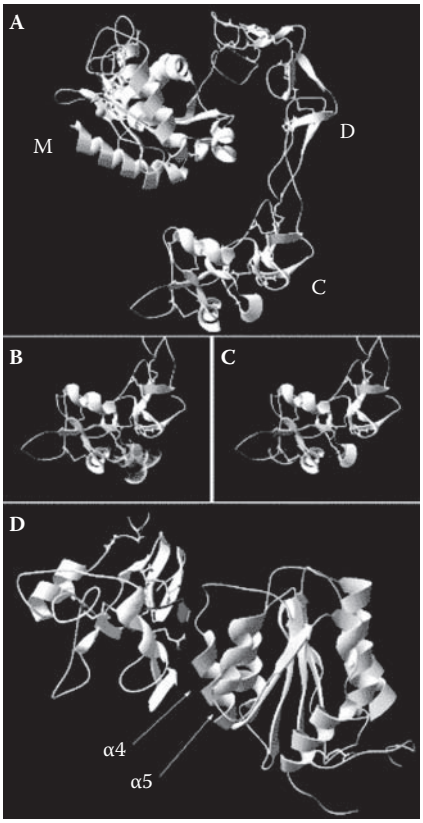

FIGURE 4.4 ***(A color version of this figure follows page 240.)*** Structural analysis of the P-III SVMP jararhagin model. (A) View of jararhagin. Metalloproteinase (M), disintegrin (D), and cysteine-rich (C) domains are indicated. Zinc ion is represented as a gray sphere. (B and C) Peptides 365PCAPEDVKCG374 (Jar6) and 372KCGRLYCK379 (Jar7) are represented in blue. Solvent accessibility of the residues in each peptide is represented in dotted spheres and colored by atoms (carbon in white, oxygen in red, and nitrogen in blue). (D) Docked structure of jararhagin cysteine-rich domain (gray) and vWF A1 domain (light blue). The peptide Jar6 region is colored in red. vWF A1 a4 and a5 helices are indicated. Disulfide bonds are represented by yellow sticks. (Reprinted from Pinto et al., 2007a, with permission from Elsevier.)

The factor X–activating SVMP russellysin (RVV-X), isolated from the venom of Russell's viper (*Daboia russellii*), has been demonstrated to inhibit ADP-induced platelet aggregation, as well as activate coagulation factor X via proteolysis (Takeya et al., 1992). Although the experimental data have not been produced to explain these activities definitively, one can speculate that the platelet aggregation inhibition activity could be modulated by the disintegrin-like domain. This is similar to that observed for the P-III SVMPs, and the C-type lectin domains may function by specifically targeting the P-IIId SVMP to factor X or some member of the coagulation pathway that is proximal to and within a distance sufficient for proteolysis by RVV-X. This again underscores how the complexity of function of the SVMPs can be modulated by the presence of specific, nonproteinase domains.

D. Direct vs. Indirect Activities of SVMPs

For venoms containing SVMPs, many of the typical biological effects associated with envenomation are thought to be due to the direct proteolytic activity on a particular substrate. One of the better-studied examples is the hemorrhagic P-III metalloproteinases, which for many years

have been thought to produce hemorrhage and subsequently tissue necrosis due to proteolytic degradation of the specialized extracellular matrix (the basement membrane) surrounding capillaries, thereby allowing extravasation into the surrounding tissue (Baramova et al., 1989; Shannon et al., 1989; Serrano et al., 2006). Although there is little question as to the importance of P-III SVMPs and extracellular matrix degradation in the direct production of hemorrhage, there could be other proteolytic activities ascribed to the SVMPs that also contribute, albeit indirectly, to the pathogenesis of the toxins. We have termed these types of effects the indirect effects of toxins. Using either crude viperid venom or an isolated P-III SVMP, we have examined effects on the ability to alter gene expression patterns in tissue culture. Using crude *Crotalus atrox* venom at concentrations that do not give rise to any macro- or microscopic changes in cell morphology or adhesion, Gallagher and colleagues (2003) investigated the change in gene expression of venom-treated human umbilical vein endothelial cells. The ontology of the upregulated genes indicated a pro-apoptotic class of genes being affected, specifically those associated with Fas ligand/TNF-α receptor apoptotic pathways. Given the presence of endothelial cell-specific SVMPs, such as VAP1 in *C. atrox* venom, this result was not particularly surprising and suggested potential mechanisms that contribute to necrosis often associated with viperid envenoming. In a better-defined experiment using human fibroblasts in culture treated with the P-III jararhagin, or mice injected with jararhagin followed by laser microdissection of the affected area, changes in gene expression were analyzed (Gallagher et al., 2005). In both experimental approaches, dramatic upregulation of genes associated with pro-inflammatory pathways, such as IL-1b, IL-6, IL-8, CXCL1, and CXCL2, was observed. Furthermore, microscopic analysis of the affected area showed a dramatic influx of inflammatory cells, likely as a result of the upregulation pro-inflammatory genes. Inhibition of the proteolytic activity of jararhagin abrogated the effects on gene expression in these experiments, thereby highlighting the role of proteolysis in this phenomenon. Although one cannot conclude that these indirect effects on host gene expression were the sole contributor to inflammation and necrosis, one can have confidence that this form of pathogenesis is important to the process.

More recently, using a proteomic approach with human fibroblasts in culture, Pinto and colleagues demonstrated the ability of the P-III atrolysin A to release annexin V from fibroblast cell surfaces (Pinto et al., 2007b). Annexin V is known to be important in coagulation pathways, and thus the ability of P-III to release the cell surface sequestered protein into a soluble form bearing potential coagulatory activities could also be an example of an indirect effect for a SVMP in the overall pathogenesis associated with viper envenoming. In addition to annexin V, some matrikines were observed to be released from the cell-secreted extracellular matrix, but the biological properties of those products are unknown, and hence we cannot say whether they play an indirect role in SVMP-induced pathogenesis.

E. Proteolytic Assays to Characterize SVMPs

Routinely, the endopeptidase activity of SVMPs can be determined by using substrates such as casein and azocasein, or fluorogenic oligopeptides. The proteolytic activity on casein is usually measured using a casein 1% solution prepared in a 50 mM Tris-HCl, pH 8–9 buffer containing 1–2 mM $CaCl_2$. After incubation at 37°C, the reaction is stopped by adding trichloroacetic acid to a final concentration of 0.5–1% and the mixture is centrifuged at 18,000 rpm for 5 min. If casein is used, hydrolysis products in the supernatant can be determined by spectrophotometric measurement of absorbance at 280 nm; if azocasein is the substrate, absorbance of the supernatant is determined at 450 nm. One unit of proteolytic activity corresponds to the amount of enzyme that induces a change in absorbance of 1.0/min.

Fluorogenic oligopeptide substrates containing a (7-methoxycoumarin-4-yl)acetyl (MCA) group can be used to determine kinetic parameters of hydrolysis of peptide bonds by SVMPs.

Intramolecularly quenched fluorogenic peptide substrates containing the groups aminobenzoyl (Abz) at the N-terminus and 2,4-dinitroanilinoethylamide (Dna) at the C-terminus have also been used to measure the activity of SVMPs. The oligopeptide sequence can be designed, for instance, according to the sequence of known cleavage sites at macromolecular substrates. Because of the sensitivity of this kind of assay, fluorogenic oligopeptides are used at much lower concentrations (0.5–1.0 μM) than macromolecular substrates such as casein. Likewise, low concentrations of SVMPs, in the range of 10–20 nM, are used in the assay. As hydrolysis of the peptide occurs, increase of fluorescence is followed on a fluorescence spectrophotometer, and specific activity and kinetic parameters such as *Km* and *Vmax* can be determined (Takeya et al., 1993).

The B-chain of oxidized insulin has been used as a standard substrate to determine the primary specificity of SVMPs. It is incubated with the proteinase, and the time course hydrolysis is followed by reversed-phase HPLC of aliquots taken from the reaction mixture. In the past, cleavage sites were determined by amino acid analysis of hydrolysis products, but in more recent years this has been done by mass spectrometric analysis of released peptides. SVMPs usually cleave the B-chain of insulin at the bonds Ala14-Leu15, Tyr16-Leu17, His10-Leu11, Leu15-Tyr16, and Phe24-Phe25 (Fox et al., 1986; Reichl et al., 1993).

SVMPs are sensitive to EDTA, EGTA, and o-phenanthroline, chelating compounds that remove zinc and calcium ions, thereby inhibiting their proteolytic activity. For complete inhibition, SVMPs are generally incubated with 5–10 mM of these chelators for around 30 min, followed by removal of excess inhibitor by dialysis before testing for the desired proteolytic activity.

F. Surface Plasmon Resonance Assays to Analyze the Interaction between SVMPs and Their Ligands

Interactions of SVMPs (full-length native proteins or recombinant domains) with adhesion proteins such as collagens and von Willebrand factor can be analyzed by surface plasmon resonance technology. It allows for a label-free interaction analysis in real time. In this kind of technique, the *ligand* is immobilized to a gold-plated sensor chip, and repeated injections of *analyte* (binding partner) are then flowed on top of the sensor chip. A variety of sensor chip surfaces and immobilization chemistries are available to fit the interactions to be analyzed. Events of analyte-ligand association and dissociation are monitored by changes in molecular mass accumulation at the solid-liquid interface on the sensor chip surface. The acquired interaction data can provide information on specificity, concentration, stoichiometry, kinetics (k_a, k_d), and affinity, expressed as $KD = k_d/k_a$ (Rich and Myszka, 2000).

Interactions between the SVMPs atrolysin A and jararhagin, and the recombinant cysteine-rich domain of atrolysin A (all considered analytes), with several ligands (full-length von Willebrand factor, collagens I, XII, and XIV, and matrilins 1, 2, and 3) were recently analyzed in the BIAcore™ system, using CM-5 (carboxylated dextran matrix) sensor chips to which the ligands were immobilized (Serrano et al., 2005a, 2006, 2007). Increasing concentrations of the analytes were injected over the sensor chip surface, and kinetic constants were calculated using the nonlinear fitting of association and dissociation curves according to a 1:1 model and proprietary software (BIAevaluation software).

IV. PROTEOMICS OF SVMPs

A. Shotgun Approach

Based on both simple examination of venoms from all families of venomous snakes, as well as in-depth proteomic and transcriptome analysis, it appears that SVMPs, to varying levels, are found in all venoms, although the amounts and population of subclasses vary among the families, genera,

species, and geographic locations (Serrano et al., 2005b; Zhang et al., 2006; Ching et al., 2006). In general, the approach to SVMP proteomics has been by either shotgun, whereby all proteins in a venom are assessed (Fox et al., 2006; Yanes et al., 2007), or specifically examining venoms for identifying characteristics of SVMPs in the venoms. The shotgun approach, when carefully performed, allows for a good description of the various SVMPs present in the venom (particularly if the transcriptome for the venom is available), as well as some degree of information of the relative amounts of the various SVMPs. For example, using this approach Calvete and colleagues determined that SVMPs comprise approximately 23% of the total venom proteins of the snake *Bitis gabonica gabonica* (Calvete et al., 2007). Of interest is that there was not particularly good concordance between the proteomic analysis and the transcriptome analysis of this snake, which was attributed to the lack in the cDNA library of a complete set of transcripts for the proteins. This highlights the value of an in-depth proteomic analysis of the venom if a more complete understanding of the venom is desired.

B. Directed Proteomic Approach

Although the information obtained is not as detailed and in-depth as that obtained from a careful shotgun approach to proteomics, the directed proteomic approach for identifying specific proteins in a venom based on their individual characteristics can be very insightful in terms of understanding the nature of the venom. Some of the specific identifiers of SVMPs include gelatinolysis (P-I to P-III), disintegrin/disintegrin-like domains (P-II to P-III), cysteine-rich domains (P-III), and C-lectin-like domains (P-IIId) (Fox and Serrano, 2005). Serrano and colleagues made use of these identifiers to probe several venoms with regard to the presence and levels of SVMPs compared to other typical venom proteins such as phospholipases and serine proteinases (Serrano et al., 2005b). Using a combination of domain-specific antibodies and zymography, the authors were able to develop a rather complete, albeit descriptive, catalog of SVMP subclasses found in several viperid venoms. Based on their observations one can envision this approach to be a reasonable complement to the shotgun, detailed approach described above.

V. SVMP EVOLUTION

The understanding of venom evolution over the past several years has been significantly enhanced, primarily due to the explosion of proteomic, transcriptomic, and genomic data generated. These data have allowed hypotheses to develop regarding venom protein evolution and ultimately led to approaches to test those hypotheses.

A. General Concepts on Venom Protein Evolution

Fry and colleagues described features of the evolution of venom systems in lizards and snakes (Fry et al., 2006). This was followed in 2007 by a more complete analysis of venom evolution in which several principles were established. First is that most of the snake toxins were recruited from established protein families; at least twenty-four such events were determined to have occurred. Further, two additional toxin derivatives, the CRISPs and kallikreins, were determined to have been derived from existing salivary proteins. In general, the recruitment for toxin evolution is thought to occur via gene duplication followed by function divergence (neofunctionalization) based in part on novel prey species (Lynch, 2007). One additional interesting observation made by Fry was that of the various toxin types, there seemed to be an advantage associated with the level of disulfide bond cross-linking in terms of the generation of functionally diverse toxin multigene families (Fry, 2005).

B. Evolution of SVMPs

The P-III SVMPs evolved from ADAM family members; most likely, the ancestors were from the ADAM 7 and ADAM 28 clades (Jia et al., 1996; Fry, 2005). ADAM 7 is a noncatalytic metalloproteinase-like protein and ADAM 28 is a proteolytic metalloproteinase with activity on extracellular matrix (White, 2003). The structural similarities of the ADAMs proteins and SVMPs have been discussed (Fox and Serrano, 2005). Mutations within the three domains of the P-III subclass are the likely basis for the evolving neofunctionalization of the SVMPs. The P-II and P-I subclass evolved from the P-III subclass via loss of carboxy domains and mutagenesis. The disintegrins evolved from the P-II subclass via sequential loss of introns and mutations to give rise to the precursors of the long, medium, and short disintegrins (Bazaa et al., 2007). As we obtain a greater understanding of the structural basis of SVMP function, further insight into SVMP evolution is likely.

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5 Snake Venom Metalloproteinases
Biological Roles and Participation in the Pathophysiology of Envenomation

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CONTENTS

Zinc-dependent metalloproteinases are abundant components in snake venoms, especially in those of species of the families Viperidae and Colubridae. Four main classes of snake venom metalloproteinases (SVMPs) have been described on the basis of their domain constitution. The large diversity of SVMPs is the consequence of an accelerated evolutionary process that occurred in their genes after an early recruitment of an ADAM-like gene before the radiation of the advanced snakes. The largest diversification of SVMPs occurred in the family Viperidae. From a biological standpoint, SVMPs have two fundamental roles, associated with prey immobilization and digestion. SVMPs play a key role in viperid snakebite envenomations, since they induce a complex series of local and systemic pathophysiological effects. At the site of injection, SVMPs induce hemorrhage, edema, myonecrosis, blistering, dermonecrosis, and a prominent inflammatory reaction. Hemorrhage induced by SVMPs is a consequence of their ability to hydrolyze key components of the basement membrane that surrounds, and gives support to, endothelial cells in capillaries. The consequent weakening in

capillary mechanical stability prompts the distention and eventual disruption of the capillary wall due to the hemodynamic biophysical forces normally operating in the microcirculation. As a consequence of their drastic effects in the microvasculature, SVMPs induce a severe impairment in the process of skeletal muscle regeneration, thus contributing to the permanent tissue loss characteristic with viperid envenomations. Moreover, SVMPs induce systemic hemorrhage and coagulopathy, thus contributing to hypovolemia and cardiovascular collapse. Owing to their primary role in the pathophysiology of snakebite envenomation, the search for novel natural and synthetic inhibitors of SVMPs is a relevant task in the improvement of snakebite envenomation therapy.

I. INTRODUCTION

Zinc-dependent metalloproteinases are common constituents of the venom proteomes and venom gland transcriptomes of viperid and colubrid species (Mackessy, 2002; Francischetti et al., 2004; Li et al., 2004; Kashima et al., 2004; Serrano et al., 2005b; Ching et al., 2006; Cidade et al., 2006; Calvete et al., 2007), and have also been described in the venoms of some species of the families Elapidae and Atractaspididae (Ovadia, 1987; Tan and Saifuddin, 1990; Ito et al., 2001; Guo et al., 2007). Snake venom metalloproteinases (SVMPs) belong to the M12 family of metalloproteinases (Fox and Serrano, 2005); based on the consensus sequence of amino acid residues at the catalytic site (i.e., HEXXHXXGXXH followed by a Met turn), SVMPs have been grouped within the metzincins, together with matrix metalloproteinases (MMPs), astacins, serralysins, and ADAMs (a disintegrin and metalloproteinase) (Bode et al., 1993). ADAMs and SVMPs, in turn, comprise the subfamily of reprolysins, since they share a similar domain constitution (Fox and Serrano, 2005).

ADAMs were initially described in mammalian reproductive tissues, but it is now recognized that they are present in many species and tissues and play highly diverse functions, including the shedding of membrane-bound protein domains (e.g., shedding of tumor necrosis factor-α [TNF-α] by TNF-α converting enzyme [TACE], or ADAM-17; Black et al., 1997), cell-cell interactions, the remodeling of extracellular matrix (ECM), and the release of biologically active peptides from ECM (White, 2003). Mature ADAMs are comprised of a metalloproteinase and disintegrin-like and cysteine-rich domains, followed by epidermal growth factor-like, transmembrane, and cytoplasmic domains (White, 2003). A group of related proteins, the ADAMTs, also present a thrombospondin-type repeat (Kuno et al., 1997). It is widely accepted that the early evolution of SVMPs included the recruitment of ADAM genes, which through processes of gene duplication and accelerated mutation generated the amazing diversity of SVMPs present in these secretions (Moura-da-Silva et al., 1996a; Fry, 2005). These enzymes play very important roles in the biology of these reptiles as well as in the pathophysiology of envenomations.

II. CLASSIFICATION OF SVMPs

SVMPs have been classified into four classes, based on their domain composition (Fox and Serrano, 2005; also see Chapter 4, this volume). A brief description of the characteristics of these classes follows in order to have a structural background for the discussion on the biological and pathophysiological roles of these enzymes.

A. Class P-I

The most simplified version of SVMPs comprises class P-I, which includes precursors containing a signal sequence followed by a pro-domain, and a metalloproteinase domain. Strictly speaking, the classification of a SVMP within the P-I class should be confirmed at the cDNA sequence level, since there are mature proteins comprising only the metalloproteinase domain that are derived from P-II SVMPs through proteolytic processing (see below). P-I SVMPs are usually not glycosylated. The pro-domain inhibits enzymatic activity by a Cys switch mechanism (Hite et al., 1994); this domain

is proteolytically cleaved to generate the mature protein, comprising only the metalloproteinase domain, which includes the zinc-binding consensus sequence (HEXXHXXGXXH) followed by a Met turn. The zinc atom is tetrahedrally coordinated by the three histidines and by a water molecule, which in turn is bound to the nearby glutamate (Gomis-Rüth et al., 1994; Watanabe et al., 2003).

In addition to the complete amino acid sequence of many P-I SVMPs, the crystal structures of seven members of this class have been elucidated (Gomis-Rüth et al., 1993, 1994; Zhang et al., 1994; Kumasaka et al., 1996; Gong et al., 1998; Huang et al., 2002; Watanabe et al., 2003; Lou et al., 2005). These studies show a similar three-dimensional structure, with two subdomains separated by the active site cleft: a major subdomain with four α-helices and a β-sheet, and a minor subdomain formed by one α-helix and several loops. There are differences among P-I SVMPs concerning their disulfide bond arrangement, since some of them contain six Cys, whereas others contain only four. In addition, there are variations in the structural features of the hydrophobic pocket located at the S1′ site, as well as differences in the loop comprising residues 153 to 176, which may have functional consequences (Watanabe et al., 2003). Such variations are likely to have implications for the proteolytic specificity and the biological activities of these enzymes.

B. Class P-II

The precursor proteins of class P-II SVMPs comprise, in addition to a pro-domain and a metalloproteinase domain, a disintegrin domain, connected to the metalloproteinase domain by a short spacer sequence (Bjarnason and Fox, 1994; Tsai et al., 2000; Fox and Serrano, 2005; Ramos and Selistre de Araujo, 2006). However, many of these proteinases undergo a posttranslational proteolytic processing that results in the release of the disintegrin domain. Disintegrins, which are polypeptides that interact with integrins in cell membranes, had been known to be present in snake venoms, although the fact that they are synthesized as part of a larger protein of the SVMP family did not become evident until cDNA sequences were determined (Hite et al., 1992; Paine et al., 1992). Therefore, it is likely that many SVMPs classified within the class P-I on the basis of their mature protein sequence may actually correspond to processed P-II SVMPs on the basis of their cDNA sequence, and there are examples of SVMPs comprising only the catalytic domain that originated from P-II precursors (Miyata et al., 1989; Siigur and Siigur, 1991; Modesto et al., 2005; Deshimaru et al., 2005).

Only a few P-II SVMPs whose mature protein includes both the metalloproteinase domain and the disintegrin domain have been characterized, such as the dimeric enzyme bilitoxin-I, from the venom of *Agkistrodon bilineatus*, and the monomeric SVMP jerdonitin, from *Trimeresurus jerdonii* (Nikai et al., 2000; Chen et al., 2003). It is likely that Cys residues at positions 220 and 241, absent in other P-II SVMPs, play a role at preventing the proteolytic separation of metalloproteinase and disintegrin domains in these SVMPs (Fox and Serrano, 2005). A subclassification of P-II SVMPs was proposed by Fox and Serrano (2005), which takes into consideration the presence or absence of the disintegrin domain in the mature protein, as well as the dimerization of some of these enzymes. Interestingly, several recent reports described cDNA sequences that code for a disintegrin alone, without the sequence of metalloproteinase domain (Okuda et al., 2002; Francischetti et al., 2004). Whether these findings reveal a novel gene structure for some disintegrins, or whether they reflect a processing of the mRNA, remains to be determined.

Disintegrins, which are peptides of 40 to 100 residues released from the proteolytic processing of P-II SVMPs precursors, constitute a versatile group of proteins with a wide pharmacological profile. From a structural standpoint, they are classified as small (41–51 residues), medium (70 residues), long (~84 residues), and dimeric (subunits of 67 residues), having a variable and peculiar disulfide bond arrangement (Calvete et al., 2005). They have in common their ability to interact with integrins, which are cell membrane proteins that bind to ECM proteins or to proteins present in the plasma membrane of neighbor cells (Calvete et al., 2005; Marcinkiewicz, 2005).

The functional role of the disintegrin domain in the P-II SVMPs that are not proteolytically processed, such as bilitoxin and jerdonitin, has not been disclosed, although it is highly likely that they target these enzymes to integrins in cell membranes. Bilitixon is a potent hemorrhagic toxin (Ownby et al., 1990; Nikai et al., 2000), and it is tempting to speculate that the presence of the disintegrin domain contributes to this effect, as will be discussed for P-III SVMPs. Deglycosylation of bilitoxin significantly reduces its hemorrhagic activity, thus suggesting a role for its carbohydrate moieties (Nikai et al., 2000). Jerdonitin, which has the typical disintegrin sequence RGD, inhibits platelet aggregation, whereas no such activity was described for bilitoxin, whose sequence at the disintegrin loop is MGD, a variation that may have changed the specificity of this protein for integrins (Nikai et al., 2000; Chen et al., 2003). Recombinant forms of a P-II SVMP from *Agkistrodon halys* venom were produced, one of which comprised the entire sequence (metalloproteinase and disintegrin domains, the latter containing the RGD sequence), whereas the other included only the enzymatic domain. The protein comprising the complete sequence was able to inhibit platelet aggregation (Jeon and Kim, 1999). Interestingly, the substrate specificity of these two recombinant SVMPs differed, thus suggesting that the disintegrin domain modulates the substrate specificity of the catalytic domain (Jeon and Kim, 1999).

C. Class P-III

Class P-III SVMPs comprise, in their precursor form, a pro-domain, a metalloproteinase domain, a disintegrin-like domain, and a cysteine-rich (Cys-rich) domain (Fox and Serrano, 2005). The majority of mature P-III SVMPs characterized are monomers having the three latter domains, although a few of them occur as dimers (Masuda et al., 1998, 2001). Some venoms contain proteins corresponding to the sequence of the disintegrin-like and Cys-rich domains, thus evidencing proteolytic processing of P-III SVMPs; such is the case of jararhagin C, alternagin C, and catrocollastatin C found in the venoms of *B. jararaca*, *B. alternatus*, and *Crotalus atrox*, respectively (Usami et al., 1994; Shimokawa et al., 1997; Cominetti et al., 2004). Interestingly, these venoms also contain the complete form of the precursor SVMPs. The coexistence of mature and processed forms in the same venom may be explained by the presence of isoforms having alternative disulfide bond pairings, and therefore different susceptibility to proteolytic processing, as evidenced in the case of jararhagin (Moura-da-Silva et al., 2003).

The disintegrin-like domain in P-III SVMPs contains, in the vast majority of sequenced proteins or cDNAs, the sequence XCD instead of the sequence RGD characteristic of P-II disintegrins (Fox and Serrano, 2005), although there are very few cases of P-III SVMPs having an RGD sequence in their disintegrin domain (Mazzi et al., 2006). These sequences in the disintegrin-like domain contribute to inhibition of collagen-induced platelet aggregation *in vitro* and of integrin-mediated cell adhesion by P-III SVMPs and their processed disintegrin-like and Cys-rich domains (Kamiguti et al., 1996a; Moura-da-Silva et al., 1999; Souza et al., 2000). On the other hand, the Cys-rich domain also plays a key role in the ability of P-III SVMPs, or their processed disintegrin-like and Cys-rich proteins, to inhibit platelet aggregation *in vitro* (Jia et al., 2000; Kamiguti et al., 2003) and to bind proteins having a von Willebrand factor (vWF) A domain, such as vWF, FACIT (fibril-associated collagens with interrupted triple helices) collagens XIV and XII, and matrilins (Serrano et al., 2005a, 2006; Pinto et al., 2007).

The analysis of the role of disintegrin-like and Cys-rich domains in the function of P-III SVMPs has been hampered by the lack of three-dimensional structures of these enzymes. However, the recent description of the structure of VAP1, a P-III SVMP from the venom of *Crotalus atrox*, constitutes a significant breakthrough in this area (Takeda et al., 2006). In this structure, the disintegrin-like domain is packed as a C-shaped arm in which the integrin-binding loop, containing the sequence ECD, is inaccessible for protein-protein interactions. In contrast, a region in the Cys-rich domain, described as a hyper-variable region, constitutes a potential site for target recognition and is stabilized by conserved disulfide bridges (Takeda et al., 2006). A sequence in the Cys-rich domain

of jararhagin was identified as playing a role in the binding of vWF A domain (Pinto et al., 2007). These observations stress the relevance of the Cys-rich domain in target recognition, and question the potential role of disintegrin-like domain of P-III SVMPs in such recognition; they suggest instead a structural scaffolding role for the disintegrin-like domain. Nevertheless, the processing of P-III SVMPs, and the release of proteins consisting of disintegrin-like and Cys-rich domains, may allow the disintegrin-like domain to participate in protein-protein interactions, implying that different pharmacological effects of the various domains of P-III SVMPs may depend on their proteolytic processing. P-III SVMPs are glycosylated proteins, and a role for carbohydrates in the biological profile, i.e., hemorrhagic activity, of these enzymes has been proposed (Nikai et al., 2000; García et al., 2004; Fox and Serrano, 2005; Ramos and Selistre-de-Araujo, 2006).

D. Class P-IV

Several SVMPs contain, besides the domains described for P-III enzymes, additional subunits constituted by C-type lectin-like domains linked to the main proteinase chain by disulfide bonds (Fox and Serrano, 2005). C-type lectin-like proteins are abundant in viperid venoms; they vary in their ability to bind several targets, and therefore display a diverse pharmacological profile (Morita, 2005). A few examples of this SVMP class have been characterized, such as factor X activators from *Daboia* (*Vipera*) *russellii* and *Vipera lebetina* (Siigur et al., 2001), and prothrombin activators from *Echis carinatus* and *E. multisquamatus* (Yamada et al., 1996; Yamada and Morita, 1997). The cDNA sequence of *V. lebetina* factor X activator comprises the pro-domain and metalloproteinase, disintegrin-like, and Cys-rich domains, whereas the C-type lectin-like chains are encoded by different cDNA, demonstrating that the formation of the mature P-IV protein involves post-translational events associated with the oligomerization of these protein chains by disulfide bond formation (Siigur et al., 2004). Whether this is also the situation in other P-IV SVMPs remains to be investigated. It has been suggested that the presence of a Cys residue at position 400 may be a determinant for the ability of the main chain to form a disulfide bond with a Cys in the lectin-like chain (Fox and Serrano, 2005). (*Note*: recent work has indicated that PIV metalloproteinases are included with the Class P-III metalloproteinases [Fox and Serrano, 2008].)

III. SVMPs FROM A BIOLOGICAL PERSPECTIVE

The presence of SVMPs in the venoms of snakes belonging to the families Viperidae, Elapidae, Colubridae, and Atractaspididae suggests that an early recruitment event of an ADAM-type gene, comprising a multidomain metalloproteinase, occurred before the radiation of advanced snakes of the superfamily Colubroidea (Moura-da-Silva et al., 1996a; Fry, 2005; Fry et al., 2006). This evolutionary scenario is supported by the observation that large molecular mass SVMPs, i.e., class P-III enzymes, have been described in venoms of viperid (Fox and Serrano, 2005), elapid (Tan and Saifuddin, 1990; Ito et al., 2001; Guo et al., 2007), colubrid (Kamiguti et al., 2000; Mackessy, 2002; Ching et al., 2006; Peichoto et al., 2007), and atractaspid (Ovadia, 1987; UniProt KB database, accession number Q9PT48) species. Therefore, the recruited ADAM gene was modified in order to generate secreted proteins in the primitive venom gland having a class P-III structure, thus losing the additional domains present in ADAMs and absent in SVMPs, i.e., EGF-like, transmembrane, and cytoplasmic domains.

These ideas agree with the evolutionary model presented by Calvete et al. (2005) and Bazaa et al. (2007) to explain the diversity of venom disintegrins. This model proposes that P-III disintegrins, i.e., those corresponding to the disintegrin-like domain of P-III SVMPs, derived from a precursor ADAM molecule and represent the earliest evolutionary stage in the evolution of venom disintegrins. Thereafter, modifications in the cysteines, and therefore in the disulfide bond arrangements, together with loss of introns, generated the medium-sized, dimeric, and short disintegrins, in a process of minimization of genes and the size of the proteins (Calvete et al., 2005; Bazaa et al., 2007). Following this scheme, it is likely that additional events occurring in the evolution of SVMP genes were as follows:

1. The loss of the region encoding the Cys-rich domain, associated with the appearance of P-II SVMPs (Moura-da-Silva et al., 1996a; Juárez et al., 2006). Transcripts obtained from the venom gland of *B. jararaca* may constitute evidence of such evolutionary transition, since they present some typical features of P-III SVMPs but lack the cysteine-rich domain (Cidade et al., 2006).
2. The occurrence of SVMP precursors with high susceptibility for proteolytic processing at the metalloproteinase-disintegrin connecting region, with the consequent separation of disintegrins and proteinases comprising the metalloproteinase domain alone.
3. The posttranslational dimerization of P-III SVMPs with a C-type lectin-like chain to generate P-IV SVMPs. These three events seem to have occurred only after the separation of the viperid lineage from the rest of modern venomous snake families, since P-I, P-II, and P-IV SVMPs, and proteins comprising the disintegrin domain alone, have been described so far only in viperid venoms. Figure 5.1 summarizes the proposed main evolutionary events occurring during the evolution of SVMPs.

From a functional standpoint, the ancestral SVMP, having a P-III structure, is likely to have had proteolytic activity and the ability to bind to cellular or ECM targets through disintegrin-like and Cys-rich domains. Gene duplication events, followed by a process of accelerated evolution, a widely accepted mechanism of diversification of venom toxins through mutations at the coding regions of the duplicated genes (Ohno et al., 1998, 2003; Ogawa et al., 2005), generated the great diversity of functions associated with SVMPs. Acquisition of toxic functions is likely to have appeared early in the course of evolution of SVMPs. Two toxic activities emerging in these early

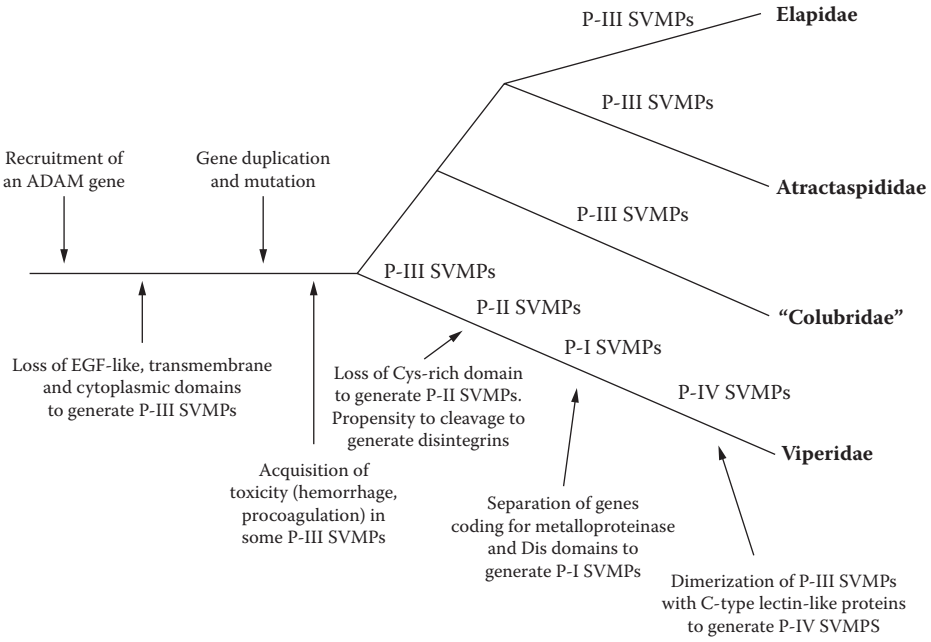

FIGURE 5.1 Hypothetical events occurring during the evolution of SVMPs in advanced venomous snake families. The original recruitment of an ADAM gene occurred before the diversification of the various advanced snake families. An ancestral gene coding for a P-III SVMP was present at an early stage, and through gene duplication and mutation, toxic SVMPs were generated, probably related to activities associated with prey immobilization, i.e., hemorrhagic and procoagulant (prothrombin activation) effects. P-III SVMPs have been described in all advanced snake families. Further evolutionary steps, associated with the appearance of P-II, P-I, and P-IV SVMPs, as well as of disintegrins, have seemed to occur only within the family Viperidae, as further adaptations to effective prey immobilization and digestion (see text for details).

stages might have been the ability to induce hemorrhage and the capacity to promote coagulation, which are displayed by P-III SVMPs described in the venoms of species of the families Viperidae (Fox and Serrano, 2005), Colubridae (Assakura et al., 1992, 1994; Kamiguti et al., 2000), Elapidae (Tan and Saifuddin, 1990), and Atractaspididae (Ovadia, 1987). The induction of bleeding and the promotion of intravascular coagulation represent highly effective ways to immobilize and kill prey. The acquisition of these effects was probably associated with changes in the enzyme structure that allowed a proper interaction with relevant targets in microvessels, to cause hemorrhage, and in clotting factors such as prothrombin, to promote coagulation. Depending on the size and type of prey, as well as the route of venom injection, coagulant SVMPs may induce rapid intravascular coagulation and death (Loría et al., 2003) or fibrinogen consumption and defibrin(ogen)ation, leading to incoagulability (Loría et al., 2003; Rucavado et al., 2005), which contributes to profuse bleeding and cardiovascular collapse.

The expression of metalloproteinase activity greatly varies between families of advanced snakes. SVMPs represent a high proportion of the content of venom proteins and venom gland cDNA transcripts in species of the family Viperidae (Francischetti et al., 2004; Serrano et al., 2005b; Cidade et al., 2006; Sanz et al., 2006; Calvete et al., 2007), and possibly the family Colubridae (Ching et al., 2006), whereas there is very little metalloproteinase content in the venoms of some elapid species (Nawarak et al., 2003; Li et al., 2004). Such divergence in the expression of SVMPs in these families might be related to the feeding behavior and type of prey, and the expression of other toxic components in the venom proteome that enable the immobilization and killing of prey by proteolytic-independent mechanisms. In the case of viperid and colubrid species, SVMPs play a key role in both prey immobilization and tissue proteolysis required for the predigestion of prey that have roughly spherical and heavy bodies, i.e., type III prey according to Cundall and Green (2000). In contrast, elapid snakes mostly feed on elongate, type II prey, which have bodies with a lower volume-to-surface ratio than type III prey (Cundall and Green, 2000). Such elongated prey do not demand a strong predigestive activity by the venom. Moreover, evolution of elapid venoms has been characterized by the diversification of genes for short postsynaptic neurotoxins and presynaptically acting neurotoxic PLA_2s, which are abundant in their proteomes (Nawarak et al., 2003; Li et al., 2004). These proteins, which were recruited in the proteomes early in snake venom evolution (Fry and Wüster, 2004; Fry, 2005), have acquired their greatest specialization in elapid venoms, with the resulting neurotoxicity becoming the predominant prey-immobilizing mechanism in these snakes. Interestingly, however, the venom of the large elapid *Ophiophagus hannah*, which feeds on relatively large ectothermic prey, contains a 63–66 kDa SVMP, probably a P-III enzyme, which constitutes 2% of the whole venom and is able to induce hemorrhage in some mammalian species (Tan and Saifuddin, 1990). Snakes of the family Atractaspididae, on the other hand, possess venoms with sarafotoxins, polypeptides that induce cardiotoxicity (Zigdon-Arad et al., 1992; see also Chapter 6, this volume), but a 50 kDa hemorrhagic SVMP has been also described (Ovadia, 1987).

The highest diversification and specialization in the evolution of SVMPs occurred within the family Viperidae, with the appearance of P-II, P-I, and P-IV classes through molecular evolutionary mechanisms discussed above. Within this evolutionary scenario, the ability to process P-II precursors and generate SVMPs comprising the metalloproteinase domain only and free disintegrins is likely to have occurred only in the viperid lineage. Disintegrins, which are highly abundant in viperid venoms, play a toxic role associated with inhibition of platelet aggregation and other integrin-dependent processes. In turn, some P-I SVMPs, albeit being generally less toxic than many P-III SVMPs, maintain their ability to induce hemorrhage (Fox and Serrano, 2005; Gutiérrez et al., 2005) and activate prothrombin (Modesto et al., 2005). Nevertheless, it is suggested that the main biological role of P-I SVMPs is digestion of prey tissues, as these enzymes are highly active proteinases. Therefore, the concomitant presence of P-III, P-II, and P-I SVMPs in viperid snake venoms allows these species to have SVMPs with predominantly prey-immobilizing roles, together with less toxic SVMPs having potent digestive action as an adaptation for feeding on type III prey. The relative proportion of the various classes of SVMP must be finely regulated within each species to

achieve an optimal balance between digestive and toxic roles. The appearance of P-IV SVMPs, which also occurred within the viperid lineage, involved the posttranslational combination of a P-III-type protein with a C-type lectin-like protein (Fox and Serrano, 2005). The latter is likely to contribute to an effective targeting of the metalloproteinase chain to relevant substrates, such as some clotting factors. A possible further step in viperid SVMPs evolution was revealed by the finding of a short disintegrin cDNA precursor coding only for the signal peptide, the pro-domain, and a dimeric disintegrin domain (Okuda et al., 2002; Francischetti et al., 2004). Although this observation could indicate processing of an mRNA precursor, analysis of genomic DNA (Bazaa et al., 2007) provided compelling evidence that short-coding regions may represent the canonical gene structure of dimeric disintegrin subunits. Thus, a genetic rearrangement in SVMP genes resulted in the separation of metalloproteinase and disintegrin genes.

IV. SVMPs IN THE CONTEXT OF THE PATHOPHYSIOLOGY OF SNAKEBITE ENVENOMATION

SVMPs play a highly significant role in the pathogenesis of local and systemic alterations induced by viperid snake venoms. One of the most serious effects induced by SVMPs is hemorrhage (Kamiguti et al., 1996b; Gutiérrez et al., 2005), but other alterations of high pathophysiological impact are also promoted by these enzymes, such as myonecrosis, blistering, coagulopathy, and effects in platelets, as well as a proinflammatory activity (Gutiérrez and Rucavado, 2000; Teixeira et al., 2005). Since most of these effects depend on the proteolytic activity of these enzymes, the role of SVMPs in envenomation can be explored by selectively inhibiting catalytic activity. This was performed with the venom of *Bothrops asper*, through the use of the synthetic hydroxamate peptidomimetic inhibitor batimastat. It was observed that when SVMP BaP1 or venom was pretreated with this inhibitor, local hemorrhage and dermonecrosis were completely abolished (Escalante et al., 2000; Rucavado et al., 2000). Regarding systemic effects, such treatment inhibited pulmonary hemorrhage and significantly reduced lethality, also inhibiting to a large extent the coagulant and defibrin(ogen)ating effects (Rucavado et al., 2004).

A. The Role of SVMPs in the Hemorrhagic Syndrome in Viperid Envenomations

Local injection of SVMPs induces a rapid hemorrhagic effect, which is typically quantified by determining the diameter of hemorrhagic lesions in the skin of experimental animals upon intradermal injections (Kondo et al., 1960; Theakston and Reid, 1983; Gutiérrez et al., 1985), or through the quantification of the amount of hemoglobin released in the tissues (Ownby et al., 1984; Gutiérrez et al., 1985). Microscopic observation of affected tissues reveals abundant extravasated erythrocytes associated with prominent damage in the microvasculature, i.e., in capillary vessels and venules (Ohsaka, 1979; Ownby et al., 1978; Lomonte et al., 1994a; Moreira et al., 1994). Similar microvascular alterations are observed in various organs when there is a systemic distribution of some SVMPs (Kamiguti et al., 1991; Escalante et al., 2003). However, not all SVMPs induce bleeding. SVMPs with the highest hemorrhagic activity belong to the class P-III, which typically are more biologically active than P-I enzymes (Gutiérrez et al., 2005), some of which lack the ability to induce hemorrhage (Bello et al., 2006). Nevertheless, this is not a general rule, as there are reports of P-III SVMPs with very low (Leonardi et al., 2007) or no (Loría et al., 2003; Silva et al., 2003) hemorrhagic activity.

Capillary vessels are defined by endothelial cells surrounded by an extracellular matrix structure known as basement membrane (BM), which is a lattice composed of type IV collagen, laminin, nidogen/entactin, and heparan sulfate proteoglycan (perlecan), together with minor components such as agrin, APARC/BM-40/osteopontin, fibulins, and other types of collagens (Timpl and Brown, 1994; Kalluri, 2003). Basement membrane plays diverse roles in microvessels: constituting

a structural scaffold, influencing endothelial cell behavior, accumulating growth factors, and representing a reserve of cryptic molecular domains (Kalluri, 2003). SVMPs induce alterations in both endothelial cells and BM. The pioneering work of Ohsaka et al. (1973) with SVMPs isolated from *Protobothrops* (*Trimeresurus*) *flavoviridis* demonstrated hydrolysis of BM preparations *in vitro*. These observations were extended in more recent works with many SVMPs, showing degradation of individual BM components upon incubation with SVMPs *in vitro* (Civello et al., 1983; Bjarnason et al., 1988; Baramova et al., 1989, 1990a, 1991; Maruyama et al., 1992; Rucavado et al., 1995, 1999; Franceschi et al., 2000). Degradation was also demonstrated *in vitro* with the BM preparation matrigel (Bjarnason et al., 1988; Escalante et al., 2006).

Recently, an immunohistochemistry study demonstrated a rapid loss in the immunostaining of laminin, nidogen, and type IV collagen upon intramuscular injection of jararhagin and BaP1 in mice, thus corroborating that BM damage also occurs *in vivo* (Escalante et al., 2006). Interestingly, however, some nonhemorrhagic SVMPs or enzymes showing extremely low hemorrhagic activity are also able to hydrolyze BM components *in vitro* (Rucavado et al., 1999; Rodrigues et al., 2000). Therefore, cleavage of BM proteins per se does not result in hemorrhage. Since BM is a structural scaffold that provides mechanical support to endothelial cells, it is likely that different cleavage patterns on BM proteins may have different effects on the structural and mechanical stability of BM. In this context, the study of the cleavage sites of BM proteins by the action of SVMPs becomes highly relevant, although there is relatively little information on this. The most detailed studies were performed with a hemorrhagic SVMP from the venom of *Crotalus atrox* (Baramova et al., 1991; Bjarnason et al., 1993; Bjarnason and Fox, 1994). Recently, the degradation patterns of laminin and nidogen in matrigel preparations were studied; a difference was observed regarding the cleavage of nidogen, since jararhagin, a potent hemorrhagic P-III enzyme, cleaved this BM component at two predominant sites. In contrast, the action of BaP1, a P-I enzyme, was restricted to the first site (Escalante et al., 2006). This difference may have functional implications, since the second cleavage site of jararhagin results in the separation of nidogen G2 domain, which interacts with type IV collagen and perlecan, from nidogen G3 domain, which interacts with laminin (Escalante et al., 2006). Since nidogen plays a critical role in the networking of basement membrane components (Fox et al., 1991), such cleavage may impair the nidogen-mediated mechanical integration of BM components, with the consequent weakening of this ECM scaffold.

When injected into experimental animals, hemorrhagic SVMPs induce rapid and prominent rupture of microvessel structure, with an explosive extravasation, clearly observed in intravital microscopic studies (Rucavado et al., 1995). Such effect is associated with a drastic disruption of endothelial cell structure, characterized by the formation of cytoplasmic projections or "blebs," a decrease in the number of pynocytotic vesicles, a reduction of endothelial cell thickness, and eventually, the rupture of cell structure, with the consequent escape of erythrocytes and other blood components to the extravascular space (Ownby et al., 1978: Ownby and Geren, 1987; Moreira et al., 1994), a process described as "hemorrhage *per rhexis*" (Ownby, 1982). These pathological events occur in capillaries within a few minutes of injection. When observations have been performed in venules, some authors have described the escape of erythrocytes through widened intercellular junctions, a process named "hemorrhage *per diapedesis*" (Ohsaka, 1979; Ownby, 1982). However, the most important impact of hemorrhagic SVMPs occurs at the capillary level by a *per rhexis* mechanism (Ownby et al., 1978; Moreira et al., 1994).

In contrast with these prominent and rapid pathological effects observed in endothelial cells *in vivo*, incubation of SVMPs with these cells in culture does not result in overt and rapid cytotoxicity (Lomonte et al., 1994b; Borkow et al., 1995; Rucavado et al., 1995; Wu et al., 2001). The only noticeable effect is a detachment of cells from the substrate, a phenomenon that depends on proteolytic activity, followed by the clustering of detached cells (Lomonte et al., 1994b; Borkow et al., 1995; Rucavado et al., 1995; Gallagher et al., 2003). However, detached cells remain viable for several hours and, when transferred to fresh medium, could be recultivated again (Borkow et al., 1995). The most evident pathological effect induced by SVMPs on endothelial cells in culture is the

induction of apoptosis, upon several hours of incubation (Masuda et al., 2000, 2001; Wu et al., 2001; You et al., 2003; Wang et al., 2003; Díaz et al., 2005; Tanjoni et al., 2005). The weight of evidence suggests that SVMP-induced endothelial cell apoptosis *in vitro* is likely to occur through anoikis, i.e., apoptosis resultant from the loss of contact of endothelial cells from their ECM (Díaz et al., 2005; Tanjoni et al., 2005). Apoptosis was described for both class P-I and class P-III SVMPs, as well as for the P-III SVMP halysase in conditions where enzymatic activity was abolished, and by a recombinant protein having the disintegrin-like and Cys-rich domains alone, thus suggesting that interference with integrin binding to ECM components may induce apoptosis (You et al., 2003).

The striking and puzzling contrast between the drastic and rapid endothelial cell damage observed *in vivo* upon hemorrhagic SVMP injection, and the lack of such effect *in vitro*, has been recently explained on the basis of the effect of hemodynamic biophysical forces operating in the microcirculation *in vivo*, which are absent in cell culture conditions (Gutiérrez et al., 2005). Blood flow and the associated hydrostatic pressure in microvessels result in transmural pressure gradients and the development of wall tension, as well as in a phenomenon known as shear stress (Ballerman et al., 1998). The ability of capillary wall to withstand such transmural pressure depends mostly on the mechanical properties of the BM. Consequently, the cleavage of key peptide bonds in BM proteins results in the weakening of this ECM structure, and the consequent distention of the wall due to the hydrostatic pressure-induced wall tension. Such distention eventually results in the rupture of endothelial cell integrity, as has been described in ultrastructural studies (Gutiérrez et al., 2005). Therefore, it has been proposed that hemorrhage occurs by a two-step mechanism: an initial proteolytic cleavage of BM components, followed by a mechanical distention of the capillary wall due to the action of hemodynamic forces, which results in the disruption of endothelial cell integrity, leading to extravasation (Figure 5.2). Experimental support for this hypothesis came from observations in which a hemorrhagic SVMP was injected into muscle tissue in conditions where blood flow was interrupted, i.e., where these biophysical forces were abrogated. In such circumstances there was no disruption of endothelial cell integrity (Gutiérrez et al., 2006).

B. Why Are P-III SVMPs More Hemorrhagic Than P-I SVMPs?

Although hemorrhagic activity has been described in SVMPs of classes P-I, P-II, and P-III, the most potent hemorrhagic toxins belong to class P-III (Bjarnason and Fox, 1994; Fox and Serrano, 2005). Given that the metalloproteinase domains of P-I and P-III enzymes are similar, the basis for this strong difference in hemorrhagic activity is likely dependent upon the disintegrin-like and Cys-rich domains of P-III SVMPs. Several possibilities have been proposed to explain this phenomenon:

1. Sequences present in disintegrin-like and Cys-rich domains may direct P-III SVMPs to relevant targets in the microvasculature, thus concentrating SVMPs where they can do the most harm (Takeya et al., 1990; Jia et al., 1997; Serrano et al., 2006). These target recognition sequences might be located at the disintegrin-like domain, thus directing the toxins to relevant integrins in endothelial cells (Souza et al., 2000; Moura-da-Silva et al., 2001). A number of recent observations, however, suggest that sequences in the Cys-rich domain are likely to play a key role in this targeting (Serrano et al., 2005a, 2006; Pinto et al., 2007). On the other hand, it has been shown that P-III SVMPs bind von Willebrand factor domain A present in various proteins, such as in FACIT collagens and matrylins (Serrano et al., 2006), through sequences present in the Cys-rich domain (Serrano et al., 2006; Pinto et al., 2007). Since FACIT collagens play a role in the stabilization of other ECM components, these findings raise the possibility that the mechanical weakening of BM results from the hydrolysis of FACIT collagens (Serrano et al., 2006). The study of *in vivo* patterns of ECM degradation by SVMPs, together with the immunohistochemical analysis of SVMP binding sites in the tissues, is a subject that needs to be investigated in order to have a more

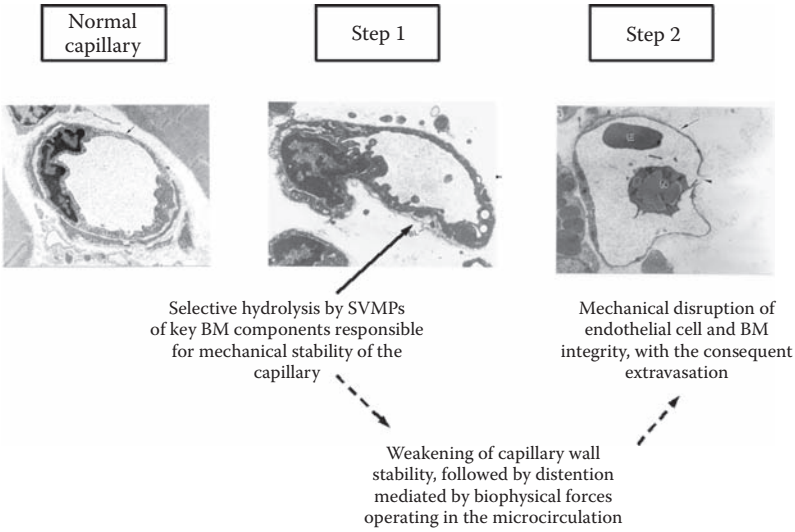

FIGURE 5.2 Proposed two-step mechanism to explain the hemorrhagic activity of SVMPs (for details, see Gutiérrez et al., 2005). Initially, there is a selective hydrolysis of key peptide bonds of BM components that play a determinant role in the mechanical stability of BM in capillary vessels (step 1). As a result of such selective cleavage, the mechanical stability of BM is jeopardized, and the hemodynamic biophysical forces that normally operate in the circulation, i.e., pressure-dependent wall tension and shear stress, provoke a distention of the capillary wall, associated with a thinning of endothelial cells, until the integrity of endothelial cells is disrupted, causing the extravasation (step 2). Left micrograph: Normal mouse capillary vessel (left). Central micrograph: A capillary 1 min after injection of SVMP BaH1 from *Bothrops asper* venom, showing early alterations, i.e., separation of endothelial cell from BM. Right micrograph: A disrupted capillary 5 min after injection of SVMP BaP1, from the venom of *B. asper.* Notice endothelial cell thinning and rupture. (Electron micrographs are reprinted from Moreira et al., 1994, and Gutiérrez et al., 2006, with permission from Elsevier Inc.)

complete understanding of the mechanisms through which these domains contribute to the hemorrhagic activity of P-III SVMPs.

2. P-III SVMPs are less susceptible to inhibition by α_2-macroglobulin, a potent plasma proteinase inhibitor, than P-I SVMPs. Incubation of P-I enzymes with human α_2-macroglobulin, or with murine murinoglobulin, results in a rapid inhibition of proteolytic and hemorrhagic activities (Baramova et al., 1990b; Estevao-Costa et al., 2000; Escalante et al., 2004). In contrast, P-III enzymes are not inactivated by macroglobulins (Kurecki and Kress, 1985; Baramova et al., 1990b; Kamiguti et al., 1994; Estevao-Costa et al., 2000; Escalante et al., 2003; Loría et al., 2003). The structural basis of this lack of inhibition is unknown at present, but it is likely that structural constraints imposed by disintegrin-like and Cys-rich domains on the binding of α_2-macroglobulin to the enzyme play a relevant role. Therefore, P-III SVMPs are able to circulate systemically without being inhibited by α_2-macroglobulin, thus becoming highly dangerous toxins owing to their ability to induce systemic bleeding (Kamiguti et al., 1991; Anderson and Ownby, 1997; Escalante et al., 2003), one of the most serious complications of viperid snakebites (Otero et al., 2002; Warrell, 2004). The finding that P-I SVMPs do not induce systemic hemorrhage likely depends on their susceptibility to inhibition by macroglobulins (Escalante et al., 2004).
3. Some hemorrhagic P-III SVMPs inhibit collagen-induced platelet aggregation *in vitro*, an effect dependent on the interaction of sequences present in disintegrin-like and Cys-rich domains with integrin $\alpha_2\beta_1$, a collagen receptor of platelets; in contrast, P-I enzymes are devoid of this activity (Kamiguti et al., 1996a, 2003; Jia et al., 1997; Moura-da-Silva et al.,

1999; Estevao-Costa et al., 2000; Escalante et al., 2004). It has been suggested that such impairment in hemostatic mechanisms may contribute to the pathogenesis of hemorrhage. However, pretreatment of mice with catalytically inhibited jararhagin, which still has the ability to inhibit collagen-induced platelet aggregation, did not potentiate the pulmonary hemorrhage induced by native jararhagin in a mouse model, thus raising doubts on the role of this platelet aggregating inhibitory activity in the pathogenesis of hemorrhage *in vivo* (Rucavado et al., 2005).

C. SVMPs Induce Blistering and Myonecrosis, and Impair Muscle Regeneration

Blistering is one of the typical local pathological alterations in viperid snakebite envenomation (Warrell, 2004). BaP1, a P-I hemorrhagic SVMP from *Bothrops asper* venom, induces blistering in a mouse model (Rucavado et al., 1998). It is likely that such an effect is due to the hydrolysis of proteins at the dermal-epidermal junction, with the consequent separation of the epidermis. In addition, experimental injections of hemorrhagic SVMPs result in acute muscle damage, i.e., myonecrosis (Ownby et al., 1978, 1990; Queiroz et al., 1985; Gutiérrez et al., 1995b; Rucavado et al., 1995). The mechanism of action of SVMPs in muscle cells is not known, but it has been proposed that necrosis is secondary to the ischemia that develops in the tissue as a consequence of microvascular damage and halting of blood perfusion (Gutiérrez et al., 1995b). In addition, SVMPs play a relevant role in the impairment of muscle regeneration in snakebite envenomation. Upon myonecrosis, muscle tissue undergoes a regenerative process that starts with the activation of myogenic satellite cells, which become myoblasts. Myoblast proliferation is followed by their fusion into myotubes and myotube maturation to become muscle fibers, a complex process orchestrated by diverse growth factors and other mediators (Chargé and Rudnicki, 2004). In order to be successful, this process requires intact blood supply, innervation, the presence of a BM around necrotic cells, and the adequate removal of necrotic detritus by phagocytic inflammatory cells (Grounds, 1991). This regenerative process is impaired in muscle injected with hemorrhagic snake venoms, very likely due to the disruption that these venoms provoke in the microvasculature (Gutiérrez et al., 1984; Santo-Neto and Marques, 2005).

Since hemorrhagic SVMPs drastically affect the capillary vessels, the blood supply to the regenerating tissue is impaired, evidenced by a reduction in the capillary:muscle cell ratio (Gutiérrez et al., 1984; Arce et al., 1991). When tissue is injected with myotoxic phospholipases A_2, which promote widespread myonecrosis but do not affect the microvasculature, there is a normal and successful regenerative process (Gutiérrez et al., 1984, 1991, 1995b). However, when mice are injected with hemorrhagic SVMPs, or with a combination of myotoxins and hemorrhagic SVMPs, regeneration is drastically impaired (Rucavado et al., 1995; Gutiérrez et al., 1995b; Salvini et al., 2001). Thus, the pathological consequences of SVMP-induced microvessel disruption go beyond the immediate hemorrhagic effect, also affecting the reparative and regenerative tissue processes that follow acute tissue damage. The inhibition of SVMPs *in situ*, by the rapid administration of natural or synthetic inhibitors, therefore represents a potentially relevant therapeutic intervention in the prevention of the prominent local tissue damage characteristic of viperid snakebite envenomations (Escalante et al., 2000; Rucavado et al., 2000; Soares et al., 2005; Gutiérrez et al., 2007).

D. SVMPs Contribute to the Hemostatic Alterations in Viperid Envenomations

Defibrin(ogen)ation is one of the most characteristic features in viperid snakebite envenomations, and results from the action of venom enzymes at different levels of the clotting cascade (Markland, 1998; White, 2005; see also Chapter 6, this volume). Potent coagulant factor X activators and prothrombin activators in snake venoms are SVMPs. Several prothrombin activators of the class P-III have been characterized from the venoms of various viperid and colubrid species (Guillin et al., 1978; Yamada and Morita, 1997; Zhang et al., 1998; Loría et al., 2003; Silva et al., 2003; Morita and Iwanaga, 1978; Senis et al., 2006). Within the classification of venom prothrombin activators,

most procoagulant SVMPs belong to group A, since they do not require any cofactor (Kini, 2005). However, few of them are group B activators, requiring Ca^{2+} for activity (Kini, 2005). Prothrombin activation by SVMPs occurs through the formation of meizothrombin (Markland, 1998). Most prothrombin activator SVMPs belong to class P-III, although P-I activators have been described in the venoms of *Bothrops insularis* and *B. cotiara* (Modesto et al., 2005; Senis et al., 2006). In addition, P-IV prothrombin activators were characterized in the venoms of *Echis carinatus* and *E. multisquamatus* (Yamada et al., 1996; Yamada and Morita, 1997). The lectin-like subunit recognizes the Ca^{2+}-bound conformation of the Gla domain in prothrombin, thus contributing to an effective targeting of the main metalloproteinase chain to prothrombin, with proteolytic cleavage and activation (Kini, 2005). When the proteolytic subunit is separated, it is able to activate prothrombin in the same way some P-III SVMPs do, i.e., without the requirement of Ca^{2+} (Yamada et al., 1996). Thus, the C-type lectin-like domain of P-IV SVMPs contributes, as has been described for the disintegrin-like and Cys-rich domains of P-III SVMPs, to the aiming of SVMPs to relevant physiological targets. Moreover, SVMPs that activate coagulation factor X have been described in the venoms of *Daboia* (*Vipera*) *russellii* (Kiesel et al., 1976) and *Bothrops atrox* (Hofman and Bon, 1987).

The most relevant pathophysiological implication of the action of these procoagulant components is the consumption of fibrinogen, which results in defibrin(ogen)ation; as a consequence, the laboratory tests for coagulation are drastically affected (White, 2005). Such clotting alterations are frequently used to monitor the severity of snakebite envenomation and the success of antivenom therapy (Warrell, 2004; White, 2005). In the case of the venom of *B. asper*, prothrombin-activating SVMPs have a predominant role in the coagulant and defibrin(ogen)ating activities (Rucavado et al., 2005), and this is likely to be the case in many other viperid venoms.

SVMPs also affect hemostasis by interfering with platelet function. In addition to the action of RGD disintegrins on platelet integrin $\alpha_{IIb}\beta_3$ (Marcinkiewicz, 2005), a number of P-III SVMPs inhibit collagen-induced platelet aggregation through the blockade of receptor $\alpha_2\beta_1$ by sequences present in the disintegrin-like and Cys-rich domains (Kamiguti et al., 1996a, 2003; Jia et al., 1997; Moura-da-Silva et al., 1999). The proteins released from some P-III precursors, comprised by the disintegrin-like and Cys-rich domains alone, also exert this inhibition (Shimokawa et al., 1997; Moura-da-Silva et al., 1999). The binding of jararhagin to integrin $\alpha_2\beta_1$ is associated not only with integrin blockade, but also with cleavage of the β_1 subunit (Kamiguti et al., 1996a). Some SVMPs are able to interact with and degrade vWF (Matsui and Hamako, 2005). Kaouthiagin, a P-III SVMP from the venom of the cobra *Naja kaouthia*, cleaves vWF; this enzyme has an HDCD sequence in the disintegrin-like domain and an unusual RGD sequence in the Cys-rich domain (Ito et al., 2001). It has been recently shown that the Cys-rich domain of P-III SVMPs interacts with vWF A domain, thus favoring hydrolysis of this protein (Serrano et al., 2005a, 2006). The *in vivo* implications of many of these *in vitro* observations are yet to be demonstrated.

The fibrino(geno)lytic activity of SVMP, especially of some nonhemorrhagic class P-I representatives, has received attention, owing to the potential role of these enzymes as fibrinolytic agents (Markland, 1998). Depending on the fibrinogen chain being hydrolyzed, fibrin(ogen)olytic proteinases are classified as α-chain fibrinogenase or β-chain fibrinogenase (Swenson and Markland, 2005). The pathophysiological role of this activity has not been clearly elucidated, but on theoretical grounds it may contribute to the reduction in fibrinogen levels, although their systemic action is likely to be inhibited by α_2-macroglobulin, since most of them are P-I SVMPs.

E. The Role of SVMPs in Venom-Induced Local Inflammation

In addition to their ability to induce direct local and systemic pathophysiological alterations, SVMPs contribute to the complex inflammatory response characteristic of viperid envenomations (Teixeira et al., 2005). SVMPs, both hemorrhagic and nonhemorrhagic, induce edema and increase vascular

permeability (Gutiérrez et al., 1995a; Rucavado et al., 1999; Rodrigues et al., 2001; Fernandes et al., 2006). This effect is likely to depend on the hydrolysis of BM components, but also on the release of inflammatory mediators from protein precursors and cells (Wei et al., 2006). SVMPs induce a prominent leukocyte inflammatory infiltrate associated with increments in circulating leukocytes (Costa et al., 2002; Fernandes et al., 2006). Leukocyte recruitment is dependent on the adhesion molecules LECAM-1, CD18, and LFA-1 (Fernandes et al., 2006). SVMP-induced complement activation is a potent fchemoattractant mechanism (Farsky et al., 2000). In addition, alternagin C, comprising disintegrin-like and Cys-rich domains, induces neutrophil migration by integrin-associated signaling pathways (Mariano-Oliveira et al., 2003). Increments in the expression of cytokines, mostly interleukin (IL)-1β and IL-6, occur in tissues injected with SVMPs (Clissa et al., 2001; Rucavado et al., 2002; Laing et al., 2003), and jararhagin is able to cleave a recombinant precursor of TNF-α (Moura-da-Silva et al., 1996b). Interestingly, the dermonecrotic activity of jararhagin is absent in knockout mice deficient in TNF receptors 1 and 2, or IL-6, thus suggesting that the ability of SVMPs to induce dermonecrosis is related to the release of these cytokines (Laing et al., 2003). At subcytotoxic concentrations, jararhagin induces the expression of genes coding for diverse inflammatory mediators in fibroblasts and macrophages (Clissa et al., 2001; Gallagher et al., 2005).

SVMPs exert diverse effects on the complement system. The P-I SVMP BaP1 activates complement *in vitro* and *in vivo* and generates chemotactic complement-derived products that contribute to leukocyte recruitment, an effect that is inhibited by pretreating serum with soluble CR1 receptor (Farsky et al., 2000). Oxiagin, a SVMP from the venom of the cobra *Naja oxiana*, inhibits the classical pathway of complement activation *in vitro* by preventing the formation of C3 convertase through a nonenzymatic mechanism (Shoibonov et al., 2005).

Interaction of SVMPs with endothelial cells can trigger diverse effects, such as apoptosis secondary to detachment (Díaz et al., 2005; Tanjoni et al., 2005), as well as increments in nitric oxide (NO) generation, prostacyclin production, and IL-8 release (Schattner et al., 2005). By cleaving ECM and other proteins, SVMPs release biologically active peptides that may induce various effects; for instance, angiostatin-like polypeptides with anti-angiogenic activity are released from plasminogen by SVMPs (Ho et al., 2002).

Injection of BaP1 induces the expression of endogenous matrix metalloproteinases (MMPs), especially proMMP-9, in muscle tissue and skin (Rucavado et al., 2002). This opens the possibility that ECM degradation in snakebite envenomations is partly due to the action of MMPs. However, zymographic analysis revealed that most of the increase corresponds to the latent forms of these enzymes, and thus it is not clear whether *in vivo* activation occurs (Rucavado et al., 2002). Crude *B. asper* venom activates proMMP-2 in a fibroblast cell culture model, a phenomenon that depends mainly on the action of serine proteinases, but in which SVMPs are also involved (Saravia-Otten et al., 2004). Activation of proMMP-2 was described by graminelysin, another SVMP (Wu and Huang, 2003). Thus, besides inducing the expression of cytokines and proMMPs, SVMPs may also activate MMPs. Therefore, the combined hydrolytic activity of SVMPs and MMPs on ECM is likely to constitute a relevant component in the complex landscape of local tissue pathology associated with viperid snakebite envenomation. Nevertheless, the contribution of P-III SVMPs to inflammation may also depend on the action of disintegrin-like and Cys-rich domains, since jararhagin C induces early inflammatory events such as leukocyte rolling and cytokine release (Clissa et al., 2006). Tables 5.1 and 5.2 summarize the most important roles played by SVMPs in the pathophysiology of viperid snakebite envenomation.

V. CONCLUDING REMARKS

SVMPs are abundant components in snake venoms, especially in those of colubrid and viperid species. A process of accelerated evolution is likely to have generated the wide diversity of SVMPs, after the recruitment of an ADAM-like gene early in the evolution of advanced snakes. The presence of disintegrin-like, Cys-rich, and C-type lectin-like domains in various classes

TABLE 5.1
Local Effects Induced by SVMPs

Target (Tissue, Cell, Structure)	Experimental Observations	Pathophysiological Consequences
Microvasculature	Disruption of capillary vessel integrity[a]	Hemorrhage, ischemia
	Reduction in capillary network density[a]	Myonecrosis, deficient skeletal muscle regeneration
Extracellular matrix	Matrix degradation[b]	Disturbance of tissue repair, regeneration and angiogenesis[c]
	Release of pharmacologically active peptides[b]	
Skin	Separation of the dermal-epidermal junction[a]	Blistering, dermonecrosis
Resident cells (macrophages, fibroblasts, endothelial cells)	Activation[a,b]	Edema
	Secretion of proinflammatory mediators (cytokines, MMPs, NO) and growth factors[a,b]	Pain
	Leukocyte recruitment[a]	Tissue damage (e.g., dermonecrosis)

[a] *In vivo* observations; for details, see the text.
[b] *In vitro* observations; for details, see the text.
[c] Possible consequences not yet demonstrated.

TABLE 5.2
Systemic Effects Induced by SVMPs

Target (Tissue, Cell, Protein)	Experimental Observations	Pathophysiological Consequences
Microvasculature	Disruption of capillary vessel integrity[a]	Hemorrhage in various organs
		Cardiovascular shock
Factor X and prothrombin	Activation; procoagulant effect[a,b]	Defibrin(ogen)ation
		Contribution to hemorrhage[c]
Platelets	Inhibition of platelet aggregation[b]	Platelet hypoaggregation
		Contribution to hemorrhage[c]
von Willebrand factor	Binding and hydrolysis[b]	Effects on hemostasis[c]
		Contribution to hemorrhage[c]

[a] *In vivo* observations; for details, see the text.
[b] *In vitro* observations; for details, see the text.
[c] Possible consequences not yet demonstrated.

of SVMPs has contributed to the acquisition of an amazing pharmacological spectrum of toxic activities that play a key role in prey immobilization and predigestion, and which generate multiple and complex pathophysiological alterations in the victims of snakebite envenomations, especially in those caused by viperid species. Thus, the search for novel SVMP inhibitors that could be applied in the therapy of these envenomations is a relevant task. Many questions remain unsolved concerning the structure, evolution, and function of this fascinating group of venom enzymes. The future offers abundant intellectual and practical challenges for those interested in SVMPs.

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6 Thrombin-Like Snake Venom Serine Proteinases

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CONTENTS

Snake venom is composed of a mixture of proteins and peptides, many of which interfere with important physiological processes in the envenomated prey. Venoms from many snakes have been shown to contain proteolytic enzymes that interact with components of the hemostatic system and catalyze a broad range of reactions involving the coagulation cascade, the kallikrein-kinin and fibrinolytic systems, the complement system, endothelial cells, and blood platelets. One group of these enzymes has been identified as thrombin-like snake venom serine proteinases (TL-SVSPs) due to their thrombin-like activity. This review details the mechanism of action of this class of enzymes and describes their biological activities, with a focus on the relationship between protein structure and function. In addition, the current state of the role of TL-SVSPs in diagnostic medicine and clinical therapy is described.

I. INTRODUCTION

Snake venom serine proteinases (SVSPs) comprise a growing group of enzymes that catalyze a broad range of reactions involving the coagulation cascade, the kallikrein-kinin, fibrinolytic, and complement systems, endothelial cells, and blood platelets. Individual SVSPs usually catalyze only one or a few of the many reactions involved in these processes. Despite a high degree of mutual sequence identity, substrate specificity among SVSPs differs considerably. A subgroup of SVSPs, thrombin-like SVSPs (TL-SVSPs), contains proteinases functionally related to thrombin. TL-SVSPs have been a subject of intense study over several decades, although sequencing and structural studies have occurred much more recently. In many ways, TL-SVSPs resemble trypsin more closely than thrombin, especially when considering the structure and primary substrate specificity of the active cleft. TL-SVSP interaction with macromolecules in some cases is thrombin-like. However, in many other instances, the proteolytic activity of the TL-SVSPs does not resemble thrombin. This chapter details what is currently known of TL-SVSPs, with a special focus on the relationship between

protein structure and function. The chapter will also describe how TL-SVSPs are currently utilized in diagnostic medicine and therapeutic applications.

II. CLASSIFICATION AND CATALYTIC MECHANISM

Peptidases are most grossly differentiated based upon which group participates in catalysis of peptide bond cleavage. Four types have been recognized: cysteine, serine, aspartic, and metalloproteinases. The SVSPs are further classified into clan PA, subclan S, family S1 (Rawlings and Barrett, 2004). These serine proteinases employ a catalytic triad of serine (Ser195), histidine (His57), and aspartate (Asp102), with residue numbering according to the chymotrypsinogen system. Serine acts as a nucleophile, and histidine as both a proton donor and an acceptor. Aspartate is thought to orient histidine properly within the catalytic cleft via hydrogen bonding (Polgar and Bender, 1969). The catalytic cleft is formed between two domains each containing a β-barrel. All enzymes within the PA clan are endopeptidases, and their catalytic domains are well conserved, from viruses to eukaryotes. Trypsin and kallikrein are prototypic examples.

Peptide bond cleavage catalyzed by SVSPs is divided into two steps, acylation and deacylation, with the second step being rate limiting (Hartley and Kilby, 1954). The first step involves nucleophilic attack of the substrate carbonyl carbon by the hydroxyl oxygen atom of Ser195. This first step is catalyzed by an imidazole nitrogen atom of His57 acting as the acceptor of the Ser195 hydroxyl proton, to which it is hydrogen bonded. The transient oxyanion of Ser195 is stabilized by hydrogen bonding with peptide backbone N-H groups of Ser195 and Gly193, the second of which is not conserved in all SVSPs. Subsequent nucleophilic attack forms the enzyme-substrate complex, a tetrahedral intermediate alongside the His57 imidazolium ion. Acid catalysis by the imidazolium ion leads to formation of the acyl-enzyme complex and release of the amine product. Deacylation is accomplished by nucleophilic attack of the acyl carbon by water. Thus, the Ser195 hydroxyl proton is lost to the amine product during each catalytic cycle and is replenished by a water-derived proton (Polgar, 1971).

Substrate specificity is dominated by interaction between the primary specificity site (S1) directly upstream of the catalytic domain and the amino acid forming the N-terminal side of the scissile bond (P1), although many aspects of the enzyme's primary, secondary, and tertiary structure are important (Sichler et al., 2002). This topic will be discussed in detail in Section IV.

III. BIOLOGICAL ACTIVITY

A summary of the biological activity, venom source, molecular mass, and inhibitors of a spectrum of TL-SVSPs is provided in Table 6.1. TL-SVSPs are defined by their ability to cleave fibrinogen (Stocker et al., 1982; Markland, 1998). Like thrombin, these enzymes release fibrinopeptides by cleaving Arg-Lys bonds on the α and β chains of fibrinogen, thus converting fibrinogen to fibrin. Unlike thrombin, which cleaves both chains, most TL-SVSPs cleave either the α or β chain, releasing fibrinopeptide A (FPA) or fibrinopeptide B (FPB), respectively. Based on this activity, TL-SVSPs have been further classified into A, B, and AB classes (Pirkle, 1998). To date, most TL-SVSPs isolated are of the class A subtype. Examples of class AB TL-SVSPs include bilineobin isolated from *Agkistrodon bilineatus* and brevinase isolated from *Agkistrodon blomhoffii brevicaudus* (Komori et al., 1993; Lee et al., 1999).

Fibrin monomers spontaneously polymerize after the release of fibrinopeptides, forming a tenuous thrombus. Covalently cross-linking fibrin polymers, catalyzed by factor XIIIa, stabilizes the thrombus. While thrombin proteolytically activates factor XIII, most TL-SVSPs do not. Thus, the thrombus formed by TL-SVSPs is quickly dissolved by plasmin. This repeated formation and subsequent dissolution of tenuous thrombi produces a consumptive coagulopathy, leading to the inability to form stable thrombi. This is in contrast to thrombin, which serves to form stable thrombi (Kumar et al., 2004). Exceptions include some SVSPs isolated from the genera *Agkistrodon*, *Bitis*,

TABLE 6.1
Properties of Thrombin-Like Snake Venom Serine Proteinases

Name	Source	Activity	Mass	Inhibitors[a]	References
Ancrod	*Agkistrodon rhodostoma*	Fibrinogenolytic (Aα)	35.4 kDa	NPGB + Agmatine + α2-macroglobulin + Antithrombin III +	Nolan et al., 1976; Burkhart et al., 1992; Au et al., 1993; Castro et al., 2004
Batroxobin	*Bothrops atrox*	Fibrinogenolytic (Aα)	41.5 kDa	Benzamidine + α2-macroglobulin + Antithrombin III – Heparin – Hirudin – Aprotinin – SBTI – ε-ACA – Tranexamic acid – Iodoacetamide –	Stocker and Barlow, 1976; Stocker et al., 1982; Sturzebecher et al., 1986; Itoh et al., 1987
Bilineobin	*Agkistrodon bilineatus*	Fibrinogenolytic (Aα and Bβ)	57 kDa	Heparin + Dithiothreitol + TLCK + Antithrombin III + Leupeptin + Argatroban – Hirudin –	Komori et al., 1993; Nikai et al., 1995
Bothrombin	*Bothrops jararaca*	Fibrinogenolytic (Aα); platelet aggregation; factor VIII activation	35 kDa	Platelet aggregation: Anti-GP IIb/IIIa + Anti-GP Ib +	Nishida et al., 1994
Brevinase	*Agkistrodon blomhoffii brevicaudus*	Fibrinogenolytic (Aα and Bβ)	2 chains: 16.5 and 17 kDa	Pefabloc + Dithiothreitol +	Lee et al., 1999
Cerastobin	*Cerastes vipera*	Fibrinogenolytic (Aα and Bβ); platelet aggregation	38 kDa	Iodoacetamide + Trasylol – SBTI –	Farid et al., 1989, 1990

(continued on next page)

TABLE 6.1 (continued)
Properties of Thrombin-Like Snake Venom Serine Proteinases

Name	Source	Activity	Mass	Inhibitors[a]	References
Cerastocytin	*Cerastes cerastes*	Fibrinogenolytic (Aα); platelet aggregation; factor X activation	38 kDa	SBTI + TLCK + TPCK + Antithrombin III – Hirudin –	Marrakchi et al., 1997a; Dekhil et al., 2003a
Cerastotin	*Cerastes cerastes*	Fibrinogenolytic (Aα); platelet aggregation	40 kDa	TPCK + TPLK + SBTI + Hirudin – Antithrombin III –	Marrakchi et al., 1997b
Contortrixobin	*Agkistrodon contortrix contortrix*	Fibrinogenolytic (Bβ); factor V activation; factor XIII activation	26 kDa	Benzamidine + DAPI + Antithrombin III –	Amiconi et al., 2000
Crotalase	*Crotalus adamanteus*	Fibrinogenolytic (Aα); kinin release	32.7 kDa	TLCK + Pro-Phe-ArgCH_2Cl + PFRCK + AFRCK + GVRCK + IPRCK + AFKCK + Tetranitromethane + 2-mercaptoethanol + Hirudin – TPCK –	Markland, 1976, 1998; Markland et al., 1981; Henschen-Edman et al., 1999
Elegaxobin II	*Trimeresurus elegans*	Fibrinogenolytic (Aα); kinin release	35 kDa	*p*-APMSF +	Oyama and Takahashi, 2003

Gyroxin	*Crotalus durissus terrificus*	Fibrinogenolytic (Aα); gyratory	32 kDa	Dithiothreitol +	Alexander et al., 1988
KN-BJ	*Bothrops jararaca*	Fibrinogenolytic (Aα); kinin release	38 kDa	Benzamine derivatives +	Serrano et al., 1998
Leucurobin	*Bothrops leucurus*	Fibrinogenolytic (Aα); gyratory	35 kDa	Benzamine + β-mercaptoethanol + SBTI – EDTA –	Magalhães et al., 2007
LM-TL	*Lachesis muta*	Fibrinogenolytic (Aα); gyratory	41-47 kDa	Agmatine + *p*-aminobenzamidine + BPTI – Ecotin – Hirugen – Bothrojaracin – Bothroatternin –	Silveira et al., 1989; Magalhães et al., 1993; Castro et al., 2001
Thrombocytin	*Bothrops atrox*	Fibrinogenolytic (Aα); factor VIII activation	36 kDa	Pro-Phe-ArgCH_2Cl + PRCK + SBTI + Antithrombin III + Heparin + FPRCK + FARCK +	Kirby et al., 1979; Castro et al., 2004; Serrano and Maroun, 2005

[a] Diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF) inhibit all tested TL-SVSPs and are included in addition to those presented in the table.

Abbreviations: +, inhibition; –, no inhibition; AFKCK, Ala-Phe-Lys chloromethyl ketone; AFRCK, Ala-Phe-Arg chloromethyl ketone; BPTI, bovine pancreatic trypsin inhibitor; DAPI, 4',6-diamidino-2-phenylindole; ε-ACA, epsilon–aminocaproic acid; FAKCK, Phe-Ala-Lys chloromethyl ketone; FARCK, Phe-Ala-Arg chloromethyl ketone; FPRCK, Phe-Pro-Arg chloromethyl ketone; GVRCK, Gly-Val-Arg chloromethyl ketone; IPRCK, Ile-Pro-Arg chloromethyl ketone; NPGB, *p*-nitro-phenyl-*p*-guanidino benzoate; PFRCK, Pro-Phe-Arg chloromethyl ketone; p-APMSF, *p*-amidinophenylmethanesulfonyl fluoride; PRCK, Pro-Arg chloromethyl ketone; SBTI, soybean trypsin inhibitor; TLCK *p*-tosyl-L-lysine chloromethyl ketone; TPCK, *p*-tosyl-L-phenylalanine chloromethyl ketone.

Bothrops, *Cerastes*, and *Trimeresurus*, which have been shown to proteolytically activate factor XIII, usually with a much reduced efficiency when compared to thrombin (Pirkle, 1998).

Thus, the term *thrombin-like* is somewhat of a misnomer. Despite the above differences, thrombin catalyzes other reactions uncommon to TL-SVSPs. Thrombin is a multifunctional enzyme that plays a central role in both hemostasis and cellular activation. Thrombin causes thrombus formation both by participating directly in the coagulation pathway at several levels and by directly causing platelet aggregation and degranulation. Besides the functions previously mentioned, thrombin cleaves protease-activated receptor 1 (PAR-1) on the cell surface of blood platelets, leading to platelet activation. Platelet activation leads to a change in platelet morphology as well as degranulation, both of which lead to platelet adhesion and aggregation. Activated platelets also provide the surface area necessary for prothrombotic factors V and VIII to function, both of which are also activated by thrombin. In this way, thrombin activation perpetuates itself in a feed-forward manner. Thrombin also acts directly upon endothelial cells and mononuclear inflammatory cells, the details of which are beyond the scope of this chapter. Lastly, thrombin can be modulated to exhibit anticoagulant activity. This occurs when it is complexed to thrombomodulin and acts via proteolytic activation of protein C (Kumar et al., 2004; Le Bonniec, 2004).

Some TL-SVSPs mimic other catalytic activities of thrombin. Contortrixobin, a type B TL-SVSP isolated from *Agkistrodon contortrix contortrix*, was shown to activate factor V with a rate 500-fold lower than thrombin. The enzyme was also found to catalyze the activation of factor XIII at a rate 250-fold lower than thrombin (Amiconi et al., 2000). Cerastocytin, a type A TL-SVSP isolated from *Cerastes cerastes*, was shown to activate both factors X and XIII (Marrakchi et al., 1995). Bothrombin, isolated from *Bothrops jararaca*, and thrombocytin, isolated from *Bothrops atrox*, are both class A TL-SVSPs that activate factor VIII, but with much lower efficacy than thrombin (Kirby et al., 1979; Niewiarowski et al., 1979; Nishida et al., 1994).

Many SVSPs induce platelet degranulation (release reaction) and aggregation. Thrombocytin was the first enzyme with this activity to be described. Although possessing less than 0.06% of the fibrinogen cleaving activity of thrombin, thrombocytin was shown to have a considerable effect on platelets, causing both serotonin release and platelet aggregation (Kirby et al., 1979; Niewiarowski et al., 1979). It has been shown that thrombocytin exerts its effects on platelets by proteolytically cleaving the N-terminal domain of PAR-1 at Arg41-Ser42 and at Arg46-Asn47. PAR-4 cleavage also appears to be involved (Santos et al., 2000). Other SVSPs induce degranulation-independent platelet aggregation. Cerastobin, a class AB TL-SVSP isolated from *Cerastes vipera*, was shown to act by hydrolyzing the platelet actin cytoskeleton directly, initiating aggregation (Farid et al., 1990). The class A TL-SVSP cerastotin (*Cerastes cerastes*) was shown to aggregate platelets in the presence of exogenous fibrinogen. It should be noted that this aggregation was not simply due to uncleaved fibrinogen bridging platelets via GpIIb-IIIa receptor binding, as aggregation was not inhibited by the addition of GpIIb-IIIa monoclonal antibodies. Rather, the platelet GpIb receptor, which binds both von Willebrand factor (vWF) and ristocetin, has been implicated in fibrinogen-dependent SVSP-induced platelet aggregation, as monoclonal antibodies to GpIb are inhibitory (Marrakchi et al., 1997b). Bothrombin is also capable of inducing platelet aggregation in the presence of fibrinogen (Nishida et al., 1994).

An interesting group of TL-SVSPs appears to produce neurologic symptoms in addition to affecting hemodynamics. These enzymes produce the gyroxin syndrome in mice, described as temporary episodes characterized by opisthotonos and rotations around the long axis of the animal. Gyroxin isolated from *Crotalus durissus terrificus* was the first of these enzymes found to cause this syndrome, but crotalase (*Crotalus adamanteus*), ancrod (*Agkistrodon rhodostoma*), leucurobin (*Bothrops leucurus*), and LM-TL (*Lachesis muta* thrombin-like enzyme) do as well (Alexander et al., 1988; Magalhães et al., 2007). It was originally hypothesized that these enzymes proteolytically release neuropeptides from endogenous precursors (Alexander et al., 1988). It has been further suggested that LM-TL is structurally homologous to an endogenous hippocampal serine protease neuropsin involved in epileptogenesis (Kishi et al., 1999; Castro et al., 2004). On the other hand, it

was shown that gyroxin does not increase radiolabeled neurotransmitter release from mouse striatal tissue, suggesting a lack of direct neurotoxic effect (Camillo et al., 2001). Clearly more work is needed to elucidate the mechanism of the gyroxin syndrome. Batroxobin is another TL-SVSP being studied for its potential effects on the central nervous system (CNS), including its roles in regulating specific CNS proteins, cerebral ischemia and reperfusion, spatial learning and memory defects, and neuronal apoptosis (Wu et al., 2000a, 2000b, 2001a, 2001b).

Some TL-SVSPs, such as crotalase, elegaxobin II from *Trimeresurus elegans*, and KN-BJ from *B. jararaca*, possess both thrombin-like and kallikrein-like functionality, but are more structurally similar to the latter (Markland, 1976; Pirkle et al., 1981; Serrano et al., 1998; Oyama and Takahashi, 2003). Likewise, some SVSPs are considered neither thrombin-like nor kallikrein-like, but do share some catalytic activity with thrombin. These SVSPs include protein C activators like ACC-C isolated from *Agkistrodon contortrix contortrix*, which function in a thrombomodulin-independent manner (Kisiel et al., 1987). Other SVSPs are totally devoid of any thrombin or kallikrein functionality and instead are functionally related to the plasminogen activators. These include TSV-PA and haly-PA (*Agkistrodon halys*), which act by cleaving the plasminogen Arg561-Val562 peptide bond (Park et al., 1998; Zhang et al., 1998).

IV. STRUCTURE-FUNCTION RELATIONSHIPS

As stated above, the secondary structure of TL-SVSPs contains two β-barrels with the catalytic cleft residing between these two barrels, also known as a β/β hydrolase fold. The secondary structure is formed and stabilized by six disulfide bridges. Five of these bridges (Cys22-Cys157, Cys42-Cys58, Cys136-Cys201, Cys168-Cys182, and Cys191-Cys220) are topographically equivalent to all S1 family serine proteinases, while the sixth (Cys91-Cys245) is unique to SVSPs (Nikai et al., 1995; Parry et al., 1998; Amiconi et al., 2000). All TL-SVSPs utilize a conserved catalytic triad composed of Ser195, Asp102, and His57. The TL-SVSP S1 site is granted its specificity by a conserved Asp189 residue. Similarly, Gly216 is the conserved residue in the S2 specificity site (Amiconi et al., 2000). Both S1 and S2 specificity site residues are conserved throughout all known TL-SVSPs, as well as trypsin and thrombin (Pirkle, 1998). S1-directed specificity ensures interaction with basic P1 residues lysine or arginine in the substrate. Studies of trypsin-substrate binding have revealed that Asp189 interacts directly with a P1 arginine residue, but uses a water molecule to mediate contact in the case of a P1 lysine residue. Perhaps this explains why trypsin has a two- to tenfold greater catalytic efficiency when the P1 residue is arginine, compared with lysine (Craik et al., 1985). Arginine is also the preferred P1 residue for thrombin (Kettner and Shaw, 1981). TL-SVSPs seem to follow this trend, as fibrinogen, PAR-1, and plasminogen are all cleaved at scissile bonds preceded by P1 arginine residues.

The catalytic cleft is a highly conserved structure among all family S1 serine peptidases. Despite this high degree of conservation, there are some important differences. Though TL-SVSPs are referred to as thrombin-like, they actually resemble trypsin more closely when considering several features of the catalytic cleft. For instance, thrombin possesses an additional three-residue loop (S1 loop) in its amino acid sequence, which allows large-substrate side chain access to the base of its catalytic cleft. Neither TL-SVSPs nor trypsin possesses this domain, a feature that could affect substrate specificity. Further, both TL-SVSPs and trypsin possess an S1 cleft proton residue (serine or threonine) at the 190 position, which acts as a proton donor and acceptor. In contrast, thrombin utilizes an alanine residue in this position, which is incapable of proton exchange (Di Cera et al., 1997; Di Cera and Cantwell, 2001; Castro et al., 2004).

Other features of the TL-SVSP catalytic domain appear to be unique. For instance, the 215 and 217 residues associated with the S2 site of thrombin have been shown to be very important in thrombin-fibrinogen interaction, with mutation in either site causing marked reduction in thrombin activity (Di Cera et al., 1997; Di Cera and Cantwell, 2001). Trp215 is conserved through most species of TL-SVSPs, whereas position 217 varies widely. Further, two additional regions (residues

82–99 and 192–193) have been shown to bear substitutions that appear to be unique to SVSPs (Amiconi et al., 2000; Wang et al., 2001). These regions are all closely associated with specificity sites. Substitutions at such positions are likely responsible not only for the differences in substrate specificity between TL-SVSPs and thrombin, but among different TL-SVSPs as well.

Another factor that may influence catalytic activity and substrate specificity is substrate access to the catalytic cleft. Substrate access has been studied mostly through comparison to TSV-PA, a plasminogen-activating SVSP for which the crystal structure has been elucidated. Even though TSV-PA is devoid of fibrinogenolytic activity, the comparison is thought to be relevant because of the high degree of sequence identity between TSV-PA and TL-SVSPs. For example, there is 64% sequence identity between TSV-PA and batroxobin. In contrast, there is only 23% identity between the sequences of TSV-PA and tissue plasminogen activator (t-PA). Several extended peptide regions were found to form loops that may affect substrate access to the active cleft, namely, the 37, 60, 70, 99, 174, 217–225, and autolysis loops. These loops frame the active cleft and vary only slightly in sequence among TL-SVSPs. Thrombin has analogous loops, with the important exception of the 60-loop (60a–h) and the autolysis loop (149a–e), which are nine and five residues longer than in TL-SVSPs, respectively (Di Cera et al., 1997; Parry et al., 1998; Di Cera and Cantwell, 2001; Wang et al., 2001; Castro et al., 2004; Serrano and Maroun, 2005). This is another example in which TL-SVSPs more closely resemble trypsin close to the catalytic cleft.

The 60 and autolysis loops play an important role in restricting access of macromolecular substrates to the catalytic cleft of thrombin. For example, basic pancreatic and soybean trypsin inhibitors are unable to alter thrombin activity. However, these compounds are able to inhibit thrombin if either the 60-loop or the autolysis loop is deleted, highlighting their importance in determining thrombin substrate specificity. SVSPs uniformly possess a C-terminal extension (245a–g) that is not present on either thrombin or trypsin (Le Bonniec et al., 1992). This extension is linked via a disulfide bridge to the 99-loop (Cys91-Cys245e) and is unique to SVSPs (Parry et al., 1998). The structure-function relationship of this extension region has not yet been established.

For thrombin, the most important areas with respect to substrate specificity outside of the active site are the anion binding exosites (ABEs). ABEs are positively charged clefts that associate with negatively charged residues on several substrates, forming salt bridges. Thrombin has two ABEs: ABE-I and ABE-II. ABE-I associates with fibrinogen and is also known as the fibrinogen-recognition exosite (FRE). ABE-I also binds PAR-1 and thrombomodulin, the latter contributing to the complex responsible for protein C activation. The thrombin ABE-I is composed of a central region containing the positively charged residues Arg73, Arg75, and Arg77, as well as a peripheral region containing Arg35, Arg67, Lys36, Lys81, Lys109, Lys110, and Lys149. ABE-II associates with heparin cofactor II, which also binds to antithrombin III, thus accelerating thrombin inhibition (Di Cera et al., 1997; Di Cera and Cantwell, 2001).

Neither ABE is conserved in the TL-SVSPs. Several studies have confirmed that SVSPs do not utilize positively charged residues structurally analogous to those in the thrombin ABE-I. Instead, it has been shown that TL-SVSPs use a series of functionally analogous arginine and lysine residues to interact with fibrinogen and PAR-1, forming an alternative FRE (Hahn et al., 1996; Wang et al., 2001; Yang et al., 2002; Maroun and Serrano, 2004). It is ultimately this alternative FRE that appears to grant most TL-SVSPs their thrombin-like substrate specificity, even though they are in many ways more similar to trypsin near the active cleft. Importantly however, ABE-dependent thrombin inhibitors such as hirudin have no effect on TL-SVSPs, suggesting that they are incapable of interacting with the TL-SVSP FRE. This further suggests that the FRE of thrombin is most likely quite different from that of the TL-SVSPs, and that the differences are probably quite complex. For example, it has been suggested that TL-SVSPs utilize hydrophobic interactions to a proportionally greater degree than thrombin, further explaining the difference in substrate specificity between the two. Indeed, it has been argued that the thrombin ABE-I is not homologously represented on TL-SVSPs at all, and rather the substrate recognition method employed by TL-SVSPs is entirely different than that used by thrombin (Castro et al., 2004; Maroun and Serrano, 2004; Serrano and

Maroun, 2005). In any case, it is likely that these differences go a long way in explaining the differences in biologic activity noted between TL-SVSPs and thrombin. ABE-II has no analog of any kind on TL-SVSPs, explaining why these molecules do not interact with heparin.

Thrombin is allosterically regulated by sodium ion (Na^+). The Na^+-thrombin complex has been deemed the fast form, as it cleaves fibrinogen at a faster rate than thrombin alone. The residue on thrombin that associates with Na^+ is Tyr225 and is located at a site structurally distinct from either exosite. SVSPs, like trypsin, do not have a tyrosine residue at position 225, but rather a proline, which does not associate with Na^+ (Wells and Di Cera, 1992; Guinto et al., 1999). Unsurprisingly, both TL-SVSPs and trypsin function in a Na^+-independent manner (Dang and Di Cera, 1996; Amiconi et al., 2000; Castro et al., 2004).

Lastly, most TL-SVSPs are glycoproteins, showing a variable number of N- or O-glycosylation sites in nonhomologous sequence positions among various TL-SVSPs. The carbohydrate content among TL-SVSPs varies widely, ranging from 0 to 62%. The function of the carbohydrate moieties present on most TL-SVSPs is still unclear, although it has been partially elucidated for some of these enzymes. Deglycosylated elegaxobin II showed a markedly reduced ability to cleave fibrinogen, as well as a 30% reduction in the kinin-releasing activity, compared to the native enzyme. On the other hand, no difference was noted in the enzyme's ability to cleave small molecules such as *p*-tosyl-L-arginine methylester (TAME) (Oyama and Takahashi, 2003). These results could suggest that the carbohydrate regions of TL-SVSPs play a role in substrate recognition. A recent study of two TL-SVSPs isolated from *Deinagkistrodon* (formerly *Agkistrodon*) *acutus* revealed that a glycosylated 37-loop is responsible for enzyme resistance to classical trypsin inhibitors (Zhu et al., 2005). More study is needed to elucidate further the role carbohydrate moieties play in the structure and function of TL-SVSPs.

V. INHIBITION

Serine protease inhibitors (SPIs) are routinely used in protein isolation protocols and other laboratory assays. Spectroscopically active SPIs are used for enzyme kinetic studies. Within the serine protease clan, SPIs can be used to differentiate between different enzymes, often highlighting important structural differences. TL-SVSPs are inhibited by diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), both of which inhibit any serine protease by irreversibly esterifying the catalytic triad serine residue. TL-SVSPs are also competitively inhibited by benzamidine and p-aminobenzamidine. Both of these molecules mimic arginine and lysine residues, which are the P1 specificity residues for SVSPs (Serrano and Maroun, 2005). Many benzamidine derivatives have been shown to inhibit TL-SVSPs as well, the most effective of which was NAPAP (Na-[(2-naphthylsulfonyl) glycyl]-4-amidinophenylalanine piperidide) (Sturzebecher et al., 1986; Serrano et al., 2000).

Inhibition studies with larger molecules allow for comparison between serine proteases. For example, the selective thrombin inhibitor D-Phe-Pro-ArgCH_2Cl has been shown to irreversibly inhibit batroxobin and thrombocytin (Kirby et al., 1979). On the other hand, the selective trypsin inhibitors antipain ([(S)-1-carboxy-2-phenyl]-carbamoyl-Arg-Val-arginal) and leupeptin (acetyl-leucylleucyl-arginal), neither able to inhibit thrombin, were shown to inhibit some of the SVSPs (Kosugi et al., 1986; Komori et al., 1993; Chang and Huang, 1995; Aguiar et al., 1996; Matsui et al., 1998). Like thrombin, most SVSPs are insensitive to bovine pancreatic trypsin inhibitor (BPTI). As previously stated, BPTI inhibition is blocked by the elongated 60 and autolysis loops in thrombin. These loops are much shorter in TL-SVSPs, akin to trypsin, and yet TL-SVSPs remain BPTI insensitive. It has been shown that this insensitivity is conferred by a glycosylated 37-loop in *D. acutus*–derived TL-SVSPs (Zhu et al., 2005). This feature is unique, however, and does not explain BPTI insensitivity in other TL-SVSPs. A docking study of BPTI with LM-TL implicated the 90-loop (Phe90-Trp100) and the C-terminal extension of TL-SVSPs as possible sites of infeasible interaction (Castro et al., 2001). Both of these sites are conserved among TL-SVSPs, and

thus may play an important role in substrate specificity. Most TL-SVSPs are also insensitive to α1-antitrypsin. Curiously, soybean trypsin inhibitor has been found to inhibit the activity of thrombocytin, calobin, and cerastocytin (Kirby et al., 1979; Marrakchi et al., 1995; Hahn et al., 1996). When taken together, these results most likely indicate that there are significant differences among TL-SVSPs at and around the active site.

The vast majority of TL-SVSPs are not affected by ABE-dependent thrombin inhibitors such as antithrombin III, hirudin, and heparin. This can be expected, as neither ABE is conserved in TL-SVSPs, and the functionally analogous FBE does not interact with these inhibitors. There are exceptions among TL-SVSPs, however, including thrombocytin (Niewiarowski et al., 1979).

VI. ISOLATION AND CHARACTERIZATION

Many aspects of TL-SVSPs can be studied in basic laboratories. Isolation of TL-SVSPs from crude venom is straightforward and usually involves gel filtration chromatography followed by ion-exchange or affinity chromatography. Purified preparations of individual TL-SVSPs may contain electrophoretically distinct isoenzymes due to slight differences in primary sequence or degrees of glycosylation. The practicality of methods to resolve these isoforms is to some extent limited by the cost and scarcity of crude snake venom.

Laboratories skilled in protein expression have circumvented the limitation of crude venom supply by expressing recombinant TL-SVSPs (rTL-SVSPs) from cDNA library sequences. Various rTL-SVSPs have been successfully expressed in bacterial, baculovirus, and yeast expression systems, with *E. coli* being the most common (Park et al., 1998; Kunes et al., 2002; Yang et al., 2002). Most rTL-SVSP expression in *E. coli* to date has resulted in the formation of insoluble bodies, which are subsequently refolded *in vitro* into the functional enzyme (Maeda et al., 1991; Zhang et al., 1997; Pan et al., 1999; Guo et al., 2001; Dekhil et al., 2003b). A notable exception is gloshedobin, which was expressed in a soluble, enzymatically active form in the presence of Mg^{2+} (Yang et al., 2003). Many protocols exist for refolding an insoluble protein. Usually, the protein is added to a large volume of a denaturing buffer (8 M urea or 6 M guanidine HCl). The denatured protein solution is then slowly added to a refolding buffer (Katoh et al., 1999). Purification of rTL-SVSPs is accomplished using the same techniques described above for TL-SVSP isolation from crude venom.

The catalytic activity of TL-SVSPs can be assessed by several different assays. In one method small spectroscopic peptide substrates are used, which contain arginine or lysine in the P1 position (Zimmerman et al., 1977). This technique can be used to study all serine peptidases. Second, TL-SVSPs can be incubated with proposed substrates, including fibrinogen, factors V, VIII, and X, protein C, fibrin, etc. No matter the level of sophistication a laboratory possesses, this latter technique is the one most often employed when studying TL-SVSP activity. The techniques used to quantify and study the products of these enzymatic reactions vary, however. A basic research laboratory may simply use a qualitative assay to show enzyme activity, such as visualization of fibrinopeptides using RP-HPLC. More advanced techniques include chromatographic isolation of reaction products and subsequent protein sequencing, as well as fluorescent resonance energy transfer (FRET) peptide studies. DNA sequencing of TL-SVSPs has allowed some laboratories to predict the secondary and tertiary structures of these enzymes through various modeling techniques. Specialized laboratories are able to study the structure of TL-SVSPs using x-ray crystallography. To date, only a few groups have been able to accomplished SVSP crystallization, but the details of these studies are beyond the scope of this chapter (Parry et al., 1998; Zhu et al., 2005).

VII. THERAPEUTIC USES

TL-SVSPs have been studied for several decades, and even the earliest papers proposed potential therapeutic uses for these enzymes. However, relatively few TL-SVSPs have been successfully

TABLE 6.2
Therapeutic Uses of Thrombin-Like Snake Venom Serine Proteinases

Name	Source	Trade Name	Indications	Clinical Trials	References
Ancrod	*Agkistrodon rhodostoma*	Viprinex™	HITT (currently only indication in United States) Acute ischemic stroke Intermittent claudication DVT Coronary artery bypass Critical ischemia	A-20-IV (completed) STAT (completed) ESTAT (completed) Undergoing phase III trials for acute ischemic stroke	Cole, 1998; Levy and Del Zoppo, 2006
Batroxobin	*Bothrops atrox*	Defibrase®	Acute cerebral infarction AMI Angina pectoris Priapism DVT Sickle cell crisis Central retinal venous thrombosis Peripheral arterial disease Perioperative anticoagulation Pulmonary embolism	rBAT undergoing phase II trials for prevention and treatment of surgical bleeding	Stocker, 1988; Bell, 1997; SinoBiomed, 2007
Reptilase	*Bothrops jararaca*	Reptilase®	Diagnostic for disfibrinogenemia	—	Stocker, 1998

Abbreviations: AMI, acute myocardial infarction; DVT, deep venous thrombosis; HITT, heparin-induced thrombocytopenia and thrombosis; rBAT, recombinant batroxobin.

transitioned into the clinical setting, and these are summarized in Table 6.2. The clinically most successful TL-SVSP is ancrod. Ancrod is currently used in the United States for the narrow indication of heparin-induced thrombocytopenia and thrombosis (HITT). This condition is caused by a type II hypersensitivity reaction with heparin acting as a hapten. Antibodies are produced against blood platelets, causing platelet activation. Activation exposes platelet cell surface components necessary for activation of coagulation cascade enzymes, ultimately leading to thrombus formation. A fibrinogenolytic TL-SVSP like ancrod is ideally suited for this disorder. Not only does the drug prevent thrombus formation acutely, but it can also be used in lieu of heparin for the patient's underlying disorder. HITT is a rare disorder, and thus ancrod is an orphan drug in the United States, treating very few patients (Cole, 1998). Ancrod has been studied extensively for use in acute ischemic stroke. Three multicenter stroke studies, A-20-IV, STAT, and ESTAT, have been conducted and examined neurological function, disability, and mortality in patients treated with ancrod versus a placebo. The first two trials showed decreased disability and mortality in ancrod-treated patients, while the third showed increased risk of intracranial hemorrhage and mortality in patients treated with ancrod (Levy and Del Zoppo, 2006). Viprinex™, a commercial preparation of ancrod, is currently undergoing phase III clinical trials for the treatment of acute ischemic stroke. In other countries, ancrod has been used for indications including intermittent claudication, prophylactic therapy for deep venous thrombosis (DVT), coronary artery bypass surgery, critical ischemia, and acute ischemic stroke (Cole, 1998). Batroxobin is another TL-SVSP that has been used clinically in other countries for acute cerebral infarction, acute myocardial infarction (AMI), angina pectoris, sickle cell crisis, DVT, central retinal vein thrombosis, peripheral arterial disease, perioperative anticoagulation in vascular surgery, pulmonary embolism (PE), and priapism. Batroxobin is commercially available as Defibrase® (Stocker, 1988; Bell, 1997). Recombinant batroxobin (rBAT) is currently in phase II clinical trials for use in the prevention and treatment of surgical bleeding (SinoBiomed, 2007).

In contrast to their limited use as therapeutic agents, TL-SVSPs have proven invaluable as diagnostic reagents. The unifunctional nature of many TL-SVSPs makes them very specific diagnostic tools, contrasted with the multifunctional nature of thrombin. Reptilase® is such a TL-SVSP used regularly in diagnostic medicine. As Reptilase acts only on fibrinogen, a prolonged Reptilase time (RT) diagnoses a disfibrinogenemia in a patient undergoing evaluation for hypercoagulability or a bleeding tendency. Unlike thrombin, TL-SVSPs are usually insensitive to fibrin degradation products (FDPs). Thus, the RT is compared with the thrombin time (TT) to identify the presence of FDPs, a sign of disseminated intravascular coagulation (DIC). A TT/RT ratio greater than 1 (TT/RT > 1) strongly supports a diagnosis of DIC. Importantly, most TL-SVSPs are not inhibited by heparin, and thus are highly useful in assaying samples taken from patients who have received heparin. Thus, RT may be used instead of TT when analyzing a heparinized sample. Other TL-SVSPs are useful in the preparation of diagnostic reagents. Since many TL-SVSPs selectively cleave either the Aα or Bβ chain of fibrinogen, they have been used to synthesize desAA and desBB fibrinogen. DesAA fibrinogen is used in a tPA functional assay (Stocker, 1998). TL-SVSPs find many uses in basic science research, as well as the diagnosis and treatment of diseases. An exhaustive list of the many uses of TL-SVSPs is beyond the scope of this chapter, but summary data describing clinical uses of TL-SVSPs are presented in Table 6.2.

VIII. CONCLUSIONS

TL-SVSPs are functionally similar to thrombin in several ways, but are also dissimilar in many ways and cannot be fully understood through the lens of this comparison alone. There are appreciable differences between thrombin and TL-SVSPs, as well as among TL-SVSPs themselves. Detailed, high-resolution studies of the structural features of TL-SVSPs, with a focus on enzyme-substrate interactions, are essential to increase the current understanding and therapeutic benefit of these enzymes. Many questions remain regarding the structure-function relationships of the TL-SVSPs, including exosites, peptide loops surrounding the active cleft, and carbohydrate side chains. Many research groups are addressing some of these questions, but there is still much work left to do.

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7 Snake Venom Nucleases, Nucleotidases, and Phosphomonoesterases

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CONTENTS

Snake venom components, acting in concert within prey, cause immobilization and initiate digestion. Additional pharmacological activities have evolved among several hydrolytic enzymes of snake venom, which interfere with numerous physiological processes of the prey and which produce these effects. However, hydrolytic enzymes such as nucleases (DNase, RNase, and phosphodiesterase), nucleotidases (5′ nucleotidase, ATPase, and ADPase), and phosphomonoesterases (acid and alkaline phosphomonoesterases) have not been extensively studied, and their pharmacological roles in venoms are not clearly defined. Also, they show overlapping substrate specificities and have other biochemical properties in common, producing uncertainty about their individual identity in venoms. For example, DNases, RNases, and phosphodiesterase share similar properties in substrate hydrolysis but differ in their pH optima and metal ion requirement for activity. Nucleotidases such as ATPases and ADPases have overlapping substrate specificities with phosphodiesterase. The differences among them are still not clear, and analyzing cDNA or amino acid sequences of the purified enzymes is necessary to resolve these differences (if any). Except for RNases, most of these enzymes are of high molecular weight, and all except DNases and RNases are known to be metalloenzymes. Of these, only 5′ nucleotidases and ADPases are known to be involved in inhibition of platelet aggregation and blood coagulation. However, the near-ubiquitous distribution of these enzymes in venoms suggests a significant role for these enzymes in envenomation. It is suggested that their major function may be in the generation of adenosine, a multitoxin. Adenosine generated

in vivo by their synergistic action on endogenous substrates is known to bring about various pharmacological effects, similar to those induced by the whole venom. Therefore, it appears that these enzymes play a central role in liberating adenosine, and through the action of adenosine, assist in prey immobilization. In addition, these enzymes could possess other pharmacological activities, which can interfere in diverse physiological processes of the prey/victim, but this has not been verified by pharmacological studies using purified enzymes. Further research is needed to characterize the biological roles of these enzymes in snake venoms and to establish clearly their role in envenomation sequelae.

I. INTRODUCTION

Snake venom is a complex mixture of biologically active components, comprising hydrolytic enzymes, nonenzymatic proteins/peptides, and small amounts of organic and inorganic molecules (Bieber, 1979; Aird, 2002, 2005). Snake venom is not primarily for self-defense, but has a more important role in prey immobilization and its subsequent digestion (Mackessy, 1988; Jorge da Silva and Aird, 2001; Aird, 2002; Urdaneta et al., 2004; Pawlak et al., 2006). Hence several digestive enzymes in venoms, in addition to their hydrolytic activity, have evolved to interfere in diverse physiological processes that help in the immobilization of prey/victim (Kochva, 1987; Aird, 2002; Fry, 2005). For example, hydrolytic enzymes such as proteases and phospholipase A_2 (PLA_2) of snake venoms are known to induce both systemic and local effects. Several PLA_2 enzymes are known to exhibit neurotoxicity (post-/presynaptic), cardiotoxicity and are pro-/anticoagulant, thereby interfering with hemostasis. Proteases and PLA_2 are also responsible for local tissue damage and cause hemorrhage, necrosis, and edema (Kini, 1997; Gutiérrez and Rucavado, 2000). Though hydrolytic enzymes such as DNase, RNase, phosphodiesterase, 5′ nucleotidase, ADPase, ATPase, and acid/alkaline phosphomonoesterases are present in almost all snake venoms, their pharmacological activities are not well characterized (Iwanaga and Suzuki, 1979; Mackessy, 1998; Rael, 1998; Aird, 2002). Since there is ambiguity about their existence due to overlapping specificities, in this chapter we discuss DNases, RNases, and phosphodiesterases under nucleases; 5′ nucleotidase, ATPase, and ADPase, all of which specifically act on nucleic acid derivatives, under nucleotidases; and the non-specifically-acting acid/alkaline phosphatases under phosphomonoesterases. The lack of interest among toxinologists in these enzymes seems to be because of the assumption that they were only involved in digestion and that they were nontoxic. However, recently there is renewed interest among toxinologists in these enzymes, as they are known to liberate purines endogenously, which act as multitoxins (Aird, 2002, 2005). The identification of free purines as endogenous constituent of venoms has further supported the role of purinergic signaling in envenomation (Lumsden et al., 2004; Aird, 2005). Purines are known to potentiate venom-induced hypotension and paralysis (Aird, 2002) via purine receptors, which are ubiquitously distributed among various organisms envenomed by snakes (Ralevic and Burnstock, 1998; Aird, 2005; Burnstock, 2006; Sawynok, 2007). In addition, some of the reports also suggest a toxic nature of these enzymes, acting either independently or synergistically with other toxins, contributing to the overall lethal effects of venoms (Boffa and Boffa, 1974; Ouyang and Huang, 1983, 1986; Aird, 2002, 2005; Dhananjaya et al., 2006). In this chapter, we have compiled the pharmacological activities associated with nucleases, nucleotidases, and phosphomonoesterases.

The distribution of these enzymes in snake venoms, their catalytic mechanisms, and assay systems to determine their activities have been described in detail in earlier reviews (Iwanaga and Suzuki, 1979; Mackessy, 1998; Rael, 1998). Only a few reviews suggest the possible pharmacological actions of these enzymes (e.g., Aird, 2002). This chapter will primarily summarize the work that has been carried out on toxic effects induced by these enzymes, emphasizing the future directions in this field of study. One of the major problems facing toxinologists is the identification and characterization of specific venom nucleases, nucleotidases, and phosphomonoesterases, because they share similar substrate specificities and biochemical properties. In this chapter, we attempt to clarify some

of the discrepancies about these enzymes, and we hope that this chapter will stimulate renewed interest among toxinologists to characterize these enzymes biologically and elucidate their role in envenomation.

II. NUCLEASES

Nucleases are enzymes that act on nucleic acids (DNA/RNA) and their derivatives. Snake venom nucleases are classified as endonucleases and exonucleases. Endonucleases include DNases, which specifically hydrolyze DNA, and RNases, which specifically hydrolyze RNA. Exonucleases include phosphodiesterases (PDEs), which hydrolyze both DNA and RNA. They are also known to exhibit endonuclease activity (Mori et al., 1987; Stoynov et al., 1997). An endonuclease activity in snake venom was first reported by Delezenne and Morel (1919). Differentiating between specific venom endonuclease activity and PDE activity is difficult since endonuclease activity is an inherent property of venom PDEs (Mori et al., 1987; Stoynov et al., 1997). Hence, most of the reported endonuclease activities may actually be due to PDE action (Sittenfeld et al., 1991; de Roodt et al., 2003). In order to differentiate PDEs from endonucleases, biochemical parameters have to be considered in addition to substrate specificities.

Even though endonucleases and PDEs hydrolyze both DNA and RNA, they exhibit distinct pH optima and metal ion requirements. A unique venom protein with an acidic pH optimum that does not require divalent cations for the hydrolysis of DNA or RNA has been considered as an endonuclease (Georgatsos and Laskowski, 1962; Mackessy, 1998), whereas all PDEs are active at basic pH and require divalent metal ion for activity (Iwanaga and Suzuki, 1979; Mackessy, 1998). The DNase activity reported by Sittenfeld et al. (1991) may be due to the action of phosphodiesterase, since the activity was measured at pH 7.0 using calf thymus DNA. A more recent study by de Roodt et al. (2003), showing DNase activity toward plasmid and calf thymus DNA in a zymogram assay, is likely to be PDE rather than DNase, since EDTA was shown to inhibit the activity. Specific endonuclease activity in the same venoms, with a pH optimum of 5.0, in addition to phosphodiesterase activity at basic pH optimum of 8.9, has been reported (Georgatsos and Laskowski, 1962; Vasilenko and Babkina, 1965; Vasilenko and Rait, 1975; Mahalakshmi and Pandit, 1987; Mahalakshmi et al., 2000). These data clearly indicate that the PDEs are distinctly different from endonucleases. However, PDE and exonuclease activity is also difficult to differentiate since there are no reports describing exclusive exonuclease activity in snake venoms. Thus, venom exonuclease activity is attributed to PDE.

A. DNases (E.C. 3.1.21.1)

Relatively few studies have been carried out with regard to specific DNases; as a result, it is difficult to say how widely they are distributed among snake venoms. A DNase activity with a pH optimum of 5.0 was purified from *Bothrops atrox* venom (Georgatsos and Laskowski, 1962). However, it was interesting to note that this preparation also showed activity toward RNA and poly-AU, in addition to DNA. During the course of preparation of PDEs from *C. adamanteus* venom, endonuclease activity was separated from exonuclease activity (Laskowski, 1980). Since the main aim of the author was to eliminate contaminating nuclease activities from exonuclease, very little is known about this isolated enzyme. This study is important, as it indicates the presence of a DNase activity in venoms, distinct from PDEs. No biological activity has been assigned to venom DNases apart from their role in digestion.

B. RNases (E.C. 3.1.21.-)

Like DNases, RNases are also not well characterized. A specific ribonuclease was isolated from the venom of *Naja oxiana*, which hydrolyzed double-stranded RNA (now called RNase V_1). The

TABLE 7.1
Properties of Purified Endonucleases from Snake Venoms

Snake Venom Source	Action on Substrate	Molecular Weight (Da)	pI	References
		DNase		
Bothrops atrox	DNA, RNA, Poly-AU	nd	5.0	Georgatsos and Laskowski, 1968
		RNase		
Naja naja oxiana	RNA	~15,900	nd	Vasilenko and Bubkina, 1965
Naja naja	Polyribocytidine, rRNA	~14,000	nd	Mahalakshmi and Pandit, 1997; Mahalakshmi et al., 2001

Note: Phosphodiesterases that also exhibit endonuclease activity have been described elsewhere.
Abbreviation: nd, not determined.

enzyme was shown to hydrolyze RNA without showing any base preference and produced oligonucleotides of two to four bases, which terminated in a 5′ phosphate (Vasilenko and Babkina, 1965; Vasilenko and Rait, 1975). More recently, an RNase with specificity for polycytidine was purified from *Naja naja* venom (Mahalakshmi and Pandit, 1987; Mahalakshmi et al., 2000). Both of these enzymes had an apparent molecular weight of ~14 to 16 kDa. Although the authors claim that the RNase preparation from *N. naja* did not show phospholipase and phosphodiesterase activity, its N-terminal sequence was identical to that of PLA_2. None of the endonucleases are reported to exhibit any pharmacological activities. The properties of endonucleases purified from various snake venoms are given in Table 7.1.

C. Phosphodiesterase (EC. 3.1.4.1)

These enzymes are known to catalyze the hydrolysis of phosphodiester bonds in a progressive fashion, beginning at the 3′ end of polynucleotides, liberating 5′ mononucleotides at basic pH. Uzawa (1932) was the first to describe phosphodiesterase (PDE) activity in snake venoms. Since then, PDE activity has been surveyed among a wide variety of taxa and found to be ubiquitously distributed in snake venoms (Iwanaga and Suzuki, 1979; Mackessy and Tu, 1993; Mackessy, 1998, 2002; Aird, 2002, and references therein). Crotalid and viperid venoms are known to contain higher PDE activity than elapid venoms (Mackessy, 1998; Aird, 2005).

PDEs act on several native substrates such as DNA, rRNA, and tRNA without showing any preference for purine or pyrimidine bases; however, it was shown that native DNA is a better substrate than denatured DNA (Iwanaga and Suzuki, 1979). They also hydrolyze oligonucleotides, including polyadenylic acid (Philipps, 1976) and cyclic nucleotides (Iwanaga and Suzuki, 1979). In addition, PDEs also hydrolyze adenosine 5′ tetraphosphate, TDP-rhamnose, UDP-glucose, GDP-mannose, poly ADP-ribose, NAD^+, $NADP^+$, and other nucleic acid derivatives (Iwanaga and Suzuki, 1979). They also hydrolyze ATP and ADP, liberating adenosine (Perron et al., 1993; Mackessy, 1989).

Venom PDEs have been isolated and characterized from numerous species of snakes. The properties of several purified venom PDEs are summarized in Table 7.2. In general, unlike RNases, PDEs are high molecular mass (>90 kDa), single polypeptide chain proteins. However, some exist as homodimers (Perron et al., 1993; Mori et al., 1987; Mackessy, 1989). They may be present in multimolecular forms or in only one form (Philipps, 1975; Mori et al., 1987; Kini and Gowda, 1984). All PDEs are metalloenzymes, as metal chelators are generally known to inhibit PDE activity (Iwanaga and Suzuki, 1979; Francis et al., 1992; Freitas et al., 1992; Mackessy, 1998, and references therein). Mori et al. (1987) showed that *Crotalus ruber ruber* PDEs contained 1.04 mol of zinc

TABLE 7.2
Properties of Purified Phosphodiesterases from Snake Venoms

Snake Venom	Action on Substrate	Molecular Weight (Da)	pI	Carbohydrate	Isoenzyme	Inhibitors	References
Bothrops atrox	Bis-pNPP, polyadenylic acid	130,000	9.2	nd	Wes (2)	EDTA	Philipps, 1976
Bothrops alternatus	Bis-pNPP	105,000	8–9.8	No	nd	EDTA	Valerio et al., 2002
Cerastes cerastes	Bis-pNPP	110,000	9.0	No	nd	EDTA, cysteine, AMP, ADP	Halim et al., 1987
Crotalus adamanteus	Bis-pNPP	115,000; 140,000	9.0	Yes	nd	nd	Philipps, 1975; Stoynov et al., 1997
Crotalus mitchelli pyrrhus	cAMP, ATP, ADP	110,000	8.5	nd	nd	EDTA	Perron et al., 1993
Crotalus rubber ruber	Native DNA/RNA, cAMP	98,000	8.5	nd	Yes	EDTA, TGA, PCMB	Mori et al., 1987
Crotalus viridis oreganus	Native DNA/RNA, cAMP	114,000	nd	nd	nd	EDTA	Mackessy, 1989
Trimeresurus flavoviridis	Bis-pNPP	nd	nd	Yes	Yes (4)	EDTA	Kini and Gowda, 1984
Trimeresurus mucrosquamatus	DNA/RNA	140,000	nd	No	nd	EDTA, PCMB	Sugihara et al., 1986

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; Bis-pNPP, bis-p-nitrophenyl phosphate; cAMP, cyclic adenosine monophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; nd, not determined; PCMB, p-chloromercuribenzoate.

TABLE 7.3
Accession Numbers of Phosphodiesterase ESTs from Various Snake Species

Snakes Species	Accession Numbers	References
Deinagkistrodon acutus	DV561486, DV563305	Oinghua et al., 2006
Lachesis muta	DY403207, DY403416	Junqueirs-de-Azevedo et al., 2006
Sistrurus catenatus edwardsii	DY587965.1	Pahari et al., 2006, 2007

per mol of enzyme. Further, zinc is also shown to be inhibitory at higher concentrations (Sugihara et al., 1986; Mori et al., 1987; Valerio et al., 2002). It is suggested that zinc is necessary for catalysis, whereas calcium and magnesium are involved in substrate binding (Dolapchiev et al., 1980). Isoforms of PDE are known to exist in *Vipera palaestinae* and *Trimeresurus flavoviridis* venoms (Levy and Bdolah, 1976; Kini and Gowda, 1984). Although, PDEs have been isolated from several venoms, there is no information on amino acid or full-length cDNA sequence. However, expressed sequence tags (ESTs) generated from cDNA libraries of *Deinagkistrodon acutus*, *Lachesis muta*, and *Sistrurus catenatus edwardsii* were found to have representatives for PDE genes (Junqueira-de-Azevedo et al., 2006; Oinghua et al., 2006; Pahari et al., 2006, 2007). The accession numbers of phosphodiesterase ESTs from various snake species are given in Table 7.3.

Although venom PDEs are widely distributed among snake taxa, only a few studies have investigated the biological activity of this near-ubiquitous venom component. An earlier study by Russell et al. (1963) showed a reduction in mean arterial pressure (MAP) and locomotor depression with partially purified PDE preparations from several snake venoms. This rapid reduction in MAP and locomotor depression can be assumed to be due to the reduction of cAMP levels. Although this preparation had contaminating proteins, this study is significant because it suggests that even in the absence of cellular disruption there is adequate substrate available for the enzyme PDE in the circulation to cause profound hypotension. Though PDEs are known to hydrolyze a wide variety of biologically important nucleotides, such as ATP, NAD^+, $NADP^+$, and GDP, this enzyme has not been investigated for other potential pharmacological activities.

III. NUCLEOTIDASES

Nucleotidases are enzymes that act upon nucleic acid derivatives and nucleic acid–related substrates, like ATP, ADP, and AMP. Since many enzymes in venoms are known to act on similar substrates, specific differentiation of nucleotidases is again difficult. It has been found that snake venoms contain both nonspecific phosphomonoesterases and 5′ nucleotidases, which specifically liberate phosphate upon hydrolysis of nucleotides. It has been observed that though 5′ nucleotidase selectively hydrolyzes 5′ nucleotides to nucleosides; these substrates are also acted upon by alkaline phosphomonoesterases (ALP) present in venoms (Sulkowski et al., 1963). Further, it has been shown that both are metal ion dependent and are active at basic pH (Rael, 1998). However, these two enzymes are differentiated based on their substrate specificity. 5′ nucleotidase is not active on 3′-AMP, ribose-5-phosphate, mononucleoside 3′, 5′ diphosphates, or higher nucleotides, but these are acted upon by ALP (Sulkowski et al., 1963; Rael, 1998). Other specific nucleotidases found in venoms are ATPases and ADPases. There is uncertainty about the existence of specific ATPase and ADPase, since venom PDE is also known to hydrolyze ATP and ADP (Mackessy, 1989; Perron et al., 1993). Further, both ATPase and PDE are metal ion dependent and active at basic pH (Kini and Gowda, 1982a, 1982b; Mackessy, 1998). Thus, the role of these enzymes is so controversial that the inhibitory effect exhibited by purified proteins on platelet aggregation is attributed to PDE by some (Mackessy, 1998) and to ADPases by others (Ouyang and Huang, 1986; Kini, 2004). Interestingly, a purified protein had exhibited both PDE activity and ADPase activity along with a

weak 5′ nucleotidase activity (Ouyang and Huang, 1986). Also, differentiation of these nucleotidases has become more complicated since *T. gramineus* 5′ nucleotidase is also known to exhibit ADPase activity (Ouyang and Huang, 1983). Pereira Lima et al. (1971) claimed that ATPase is distinct from PDE because they found disproportionate levels in different venoms; however, others have found that these two enzymes are proportionately distributed (Pfleiderer and Ortlanderl, 1963). From these studies it appears that in snake venoms either a single protein could have different domains with different activities, or truly specific nucleotidases could exist in venoms. So far there are no reports characterizing a specific 5′ nucleotidase/ATPase/ADPase and demonstrating that it is distinct from the others. Since they exhibit overlapping properties and substrate preferences, there is a possibility that different laboratories could have reported the same enzyme differently. Among nucleotidases, 5′ nucleotidase is better studied when compared with ATPases and ADPases. Although it appears logical that immobilization of prey could be achieved by depletion of ATP by the action of nucleotidases, this aspect has not been verified experimentally.

A. 5′ Nucleotidase (E.C. 3.1.3.5)

The enzyme 5′ nucleotidase preferentially catalyzes the hydrolysis of phosphate esterified at carbon 5′ of the ribose and deoxyribose of nucleotide molecules. Gulland and Jackson (1938) were the first to show the presence of 5′ nucleotidase activity in snake venoms. Since then, 5′ nucleotidase activity has been surveyed among a wide variety of taxa and found ubiquitously distributed in snake venoms (Iwanaga and Suzuki, 1979; Mackessy and Tu, 1993; Rael, 1998; Mackessy, 2002; Aird, 2002, and references therein). It has been found that viperid venoms contain more 5′ nucleotidase activity than elapid venoms (Rael, 1998; Aird, 2005).

5′ nucleotidase is known to cleave a wide variety of ribose and deoxyribose mononucleotides, including 5′-AMP, 5′-IMP, 5′-UMP, 5′-CMP, 5′-GMP, 5′-dAMP, 5′-dTMP, 5′-dCMP, 5′-dGMP, nicotinamide mononucleotide, and a number of hydroxylated, methylated, and halogenated substrates (Sulkowski et al., 1963; Rael, 1998). It has also been shown to hydrolyze ADP, thus exhibiting ADPase activity (Ouyang and Huang, 1983). However, 5′ nucleotidase prefers 5′-AMP as substrate, releasing adenosine as end product (Rael, 1998; Aird, 2002, 2005; Dhananjaya et al., 2006). It does not cleave ribose-5′-phosphate, 3′-AMP, flavin mononucleotide, or cAMP (Rael, 1998).

Only few studies have attempted to purify and characterize 5′ nucleotidase from snake venom. The properties of 5′ nucleotidase purified from various snake venoms are given in Table 7.4. 5′ nucleotidases are high molecular weight species with masses between 73 and 100 kDa (Chen and Lo, 1968; Dieckhoff et al., 1985; Ouyang and Huang, 1983, 1986). In general, venom 5′ nucleotidases are metalloenzymes, since metal chelators are known to inhibit the enzyme activity (Iwanaga and Suzuki, 1979; Ouyang and Huang, 1983; Francis et al., 1992; Freitas et al., 1992; Rael, 1998). Fini et al. (1990), using flame atomic absorption spectrometry, showed that the Zn/protein ratio was 1.85–2 mol zinc atoms per mol of protein. Further, Zn^{2+} is also known to inhibit enzymatic activity (Lin and Lin-Shiau, 1982; Ouyang and Huang, 1983). It may be that the Zn^{2+}-containing site may be the enzyme active site. Although there is no report claming the existence of isoforms, the existence of multimolecular forms in venoms was reported (Mannherz and Magener, 1979; Ouyang and Huang, 1983; Dhananjaya et al., 2006). There is no information on amino acid or full-length cDNA sequence for venom 5′ nucleotidase, but ESTs generated from cDNA libraries of *Bothrops insularis*, *L. muta*, and *D. acutus* were shown to have representatives for 5′ nucleotidase gene (Junqueira-de-Azevedo and Ho, 2002; Junqueira-de-Azevedo et al., 2006; Oinghua et al., 2006). The accession numbers of 5′ nucleotidase ESTs from the different snake species are given in Table 7.5.

Although 5′ nucleotidase is widely distributed among snake venoms, there is a lack of information about their biological activities. The *Deinagkistrodon acutus* and *T. gramineus* 5′ nucleotidases were shown to inhibit platelet aggregation (Ouyang and Huang, 1983, 1986). *Trimeresurus gramineus* 5′ nucleotidase inhibited platelet aggregation induced by ADP, collagen, sodium

TABLE 7.4
Properties of Purified Nucleotidases from Snake Venoms

Snake Venom	Action on Substrate	Molecular Weight (Da)	pI	Carbohydrate	Inhibitors	Biological Properties	References
			5′ Nucleotidases				
Deinagkistrodon (*Agkistrodon*) *acutus*	AMP	82,000	Acidic	nd	nd	Platelet aggregation inhibition	Ouyang and Hung, 1986
Trimeresurus gramineus	AMP, ADP	74,000	Basic	Yes	EDTA	Platelet aggregation inhibition	Ouyang and Hung, 1983
			ADPases				
Deinagkistrodon (*Agkistrodon*) *acutus*	ADP	94,000	Basic	No	nd	Platelet aggregation inhibition	Ouyang and Hung, 1986

Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate; nd, not determined; EDTA, ethylenediaminetetraacetic acid.

TABLE 7.5
Accession Number of Nucleotidase ESTs from Various Snake Species

Snakes Species	Accession Numbers	References
Bothrops insularis	BM401810	Junqueira-de-Azevedo and Ho, 2002
Deinagkistrodon (*Agkistrodon*) *acutus*	DV564501, DV557329, DV558168	Oinghua et al., 2006
Lachesis muta	DY403632, DY403686, DY403766	Junqueira-de-Azevedo et al., 2006

arachidonate, and the ionophore-A-23187 in platelet-rich plasma (PRP), and by thrombin in platelet-poor plasma (PPP) (Ouyang and Huang, 1983). This protein also exhibited ADPase activity. However, *D. acutus* 5′ nucleotidases inhibited ADP-induced platelet aggregation by 36%, in addition to collagen and sodium arachidonate–induced platelet aggregation, but did not possess ADPase activity or PDE activity (Ouyang and Huang, 1986). This inhibitory action of venom 5′ nucleotidases on platelet aggregation was correlated with the liberation of adenosine by its enzymatic action. Therefore, when compared with *T. gramineus* 5′ nucleotidase, the decreased inhibitory action of *D. acutus* 5′ nucleotidase on platelet aggregation could be because of the absence of associated ADPase activity. Boffa and Boffa (1974), while investigating factors from *Vipera aspis* venom affecting blood coagulation and platelet function, showed that a component displaying ADPase/5′ nucleotidase activity was the most potent inhibitor of ADP-induced platelet aggregation. It was found that the inhibitory effect was not dissociated from enzymatic activity, suggesting that the antiplatelet aggregation effect of 5′ nucleotidase may be due to the liberation of inhibitory AMP or adenosine by enzyme action on ADP released by platelets upon initiation of aggregation. Venom 5′ nucleotidase is also known to act synergistically *in vivo* with other toxins such as ADPases, phospholipases, and disintegrins to exert more pronounced anticoagulant effects (Jorge da Silva and Aird, 2001). Recently, we have shown the involvement of 5′ nucleotidase in the anticoagulant effect of *Naja naja* venom (Dhananjaya et al., 2006). *Naja naja* 5′ nucleotidase interacts directly or indirectly with factors of the intrinsic pathway to cause the observed anticoagulant effect. This study also showed that the enzyme was capable of stimulating the pharmacological action independent of catalytic activity. It is possible that during envenomation,

5′ nucleotidase acts synergistically with hemorrhagic proteases and fibrinogenases found in the venom to affect normal hemostatic functions, leading to blood loss and circulatory collapse in the prey/victim.

B. ATPases (E.C. 3.6.1.-)

These enzymes hydrolyze ATP, forming adenosine and pyrophosphate as reaction products (Johnson et al., 1953; Iwanaga and Suzuki, 1979). Zeller (1950) first showed that snake venom, upon incubation with ATP, liberated pyrophosphate. Depending upon experimental conditions, the enzyme is known to hydrolyze ATP into either AMP and pyrophosphate or ADP and phosphate (Zeller, 1950). ATPase activity has been reported from numerous snake venoms (Zeller, 1950; Johnson et al., 1953; Schiripa and Schenberg, 1964; Setoguchi et al., 1968; Pereira Lima et al., 1971; Wei et al., 1981; Kini and Gowda, 1982a, 1982b; Mukherjee et al., 2000).

Though ATPase activity is widely distributed, only a few attempts have been made to isolate and characterize it. Kini and Gowda (1982a, 1982b) partially purified toxic ATPases from *N. naja* and *Daboia russellii* venoms. They observed that ATPases of *D. russellii* (ATPase-I and -II) and *N. naja* venoms were Mg^{2+} ion dependent and basic in nature; ATPase-I was a glycoprotein, but ATPase-II did not contain any carbohydrate. However, a detailed characterization of these ATPases was not undertaken since the primary goal was to study the interaction of plant isolates with toxic venom proteins.

Because ATPase enzymes have not been purified from snake venoms, specific biological activity has not been assigned to them. Zeller (1950) termed ATPase to be toxic, as ATPase was thought to be involved in production of shock symptoms by depletion of ATP. Although it appears logical that immobilization of prey/victims could be achieved by depletion of ATP, via the action of ATPases along with other nucleotidases, this has not been verified experimentally.

C. ADPases (E.C. 3.6.1.-)

ADPases catalyze the hydrolysis of ADP to adenosine and orthophosphate (Johnson et al., 1953; Iwanaga and Suzuki, 1979). ADPase activity has been observed in several snake venoms (Schiripa and Schenberg, 1964; Setoguchi et al., 1968; Boffa and Boffa, 1974; Sekiya et al., 1975; Ouyang and Huang, 1986).

ADPase isolated from *D. acutus* venom had a molecular weight of 94 kDa, was basic in nature, and was known to inhibit platelet aggregation induced by ADP, collagen, and sodium arachidonate in platelet-rich plasma. Although it strongly inhibited ADP-induced platelet aggregation, it did not inhibit thrombin-induced aggregation in platelet-poor plasma (Ouyang and Huang, 1986). This protein was known to possess both phosphodiesterase and weak 5′ nucleotidase activities. The inhibition of platelet aggregation was assumed to be due to the generation of adenosine, which is known to inhibit platelet aggregation. *Vipera aspis* ADPase has been shown to be the most potent inhibitor of ADP-induced platelet aggregation, among others (Boffa and Boffa, 1974). The inhibitory effect was not independent of enzymatic activity. The inhibitory action was explained by the formation of inhibitory AMP or adenosine by the action of the enzyme. It is possible that a synergistic interaction of ADPases with hemorrhagic proteases and fibrinogenases (found in the same venom) occurs during envenomation, interfering with normal hemostatic mechanisms and promoting blood loss and circulatory collapse in the prey/victim. Properties of ADPases purified from various snake venoms are given in Table 7.4.

IV. PHOSPHOMONOESTERASES

These are enzymes that catalyze nonspecific hydrolysis of phosphate esters, first described in snake venoms by Uzawa (1932). The acid phosphomonoesterases (E.C. 3.1.3.1) are most active at pH 5.0, and alkaline phosphomonoesterases (E.C. 3.1.3.2) show highest activity at pH 9.5. It is apparent that

the two enzyme activities (acid/alkaline) pertain to different enzymes, because some venoms contain both the activities, while others contain only one (Tu and Chua, 1966; Iwanaga and Suzuki, 1979; Rael, 1998). Of the two phosphatases, alkaline phosphomonoesterase seems to be widely distributed and abundant in snake venoms, while acid phosphomonoesterases have more limited distribution (Uwatoko-Setoguchi, 1970; Iwanaga and Suzuki, 1979; Sifford et al., 1996; Rael, 1998). Alkaline phosphomonoesterase (ALP) activity has been surveyed among a wide variety of snake taxa and found ubiquitously distributed in snake venoms (Iwanaga and Suzuki, 1979; Mackessy and Tu, 1993; Rael, 1998; Mackessy, 2002; Aird, 2002, and references therein). It has been found that crotaline and elapid venoms contain higher ALP activity than viperine venoms (Rael, 1998; Aird, 2005).

ALP is known to hydrolyze nonspecifically ribo- and deoxyribonucleotides at different rates. Substrates include 5′-AMP, 5′-dAMP, 3′-AMP, ribose 3-phosphate, ATP, deoxy-dinucleotide phosphates, dGDP, FMN, and 5′ phosphoribose 1-pyrophosphate (Sulkowski et al., 1963).

Although ALP is known to be present commonly in snake venoms, only a few attempts have been made to purify it (Suzuki and Iwanaga, 1958a, 1958b; Sulkowski et al., 1963). In general, snake venom ALP is a high molecular weight protein (>90 kDa) (Iwanaga and Suzuki, 1979; Acosta et al., 1994; Rael, 1998). ALPs are metalloenzymes, and their activities are inhibited by metal ion chelators (Iwanaga and Suzuki, 1979; Hassan et al., 1981; Francis et al., 1992; Acosta et al., 1994; Rael, 1998).

In contrast, acid phosphomonoesterases have been partially purified only from sea snake venoms (Uwatoko-Setoguchi, 1970). These also require metal ions for activity (Uwatoko-Setoguchi, 1970; Sifford et al., 1996). The enzyme is known to be active on various substrates and differs from ALP in that glucose-1-phosphate, glucose-6-phosphate, and glycerophosphates are not hydrolyzed (Uwatoko-Setoguchi, 1970).

To our knowledge, there are no reports of the isolation and characterization of biological activity of acid or alkaline phosphomonoesterases from snake venom. However, bee venom acid phosphomonoesterase is considered an allergen and is known to be a potent releaser of histamine from sensitized human basophils (Barboni et al., 1987; Grunwald et al., 2006).

V. ADENOSINE LIBERATION DUE TO THE ACTION OF NUCLEASES/NUCLEOTIDASES/PHOSPHOMONOESTERASES

In vivo, the synergistic action of nucleases, nucleotidases, and phosphatases can result in the generation of purine and pyrimidine nucleotides (Aird, 2002). Among these nucleotides, adenosine generation is pharmacologically important, as it elicits several snake envenomation-related symptoms (Ralevic and Burnstock, 1998; Aird, 2002; Burnstock, 2006; Sawynok, 2007; also see Chapter 20, this volume).

Generation of adenosine by venom enzymes can take place by different pathways. Enzymes like nucleotidase and PDE act immediately upon envenomation on available ATP molecules to release adenosine (Figure 7.1). DNases, RNases, and PDEs liberate purine and pyrimidine nucleotides from the cell genome. The liberation of adenosine by the action of these enzymes is preceded by cell necrosis induced by venom proteases/hemorrhagins, phospholipases, myotoxins, cardiotoxins, and cytolytic peptides (Figure 7.2) (Ownby et al., 1978; Bernheimer and Rudy, 1986; Nunez et al., 2001; Ma et al., 2002). Once the cell is ruptured, the venom PDEs and DNases/RNases can hydrolyze DNA/RNA, releasing nucleotide 5′ monophosphates (NMPs). 5′ nucleotidase specifically or nonspecific phosphomonoesterases acting on these 5′-NMPs potentially liberate adenosine (Figure 7.3). There is also a possibility that the released adenosine *in vivo* is converted to inosine by the action of endogenous adenosine deaminase. However, this is also biologically important, because inosine is responsible for inducing many pharmacological actions. Some of the pharmacological actions exhibited by adenosine and inosine, as well as their relation to snake envenomations, are summarized in Table 7.6 (for more details, see Aird, 2002).

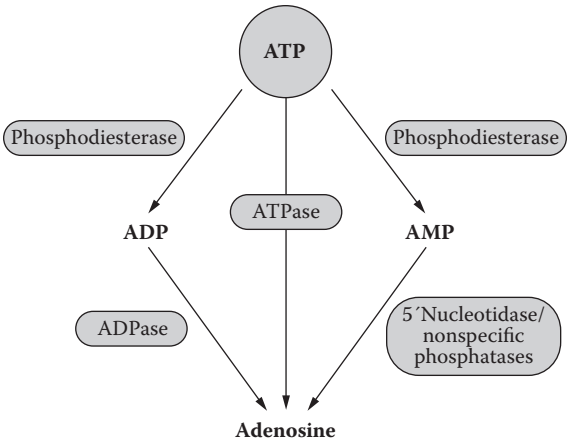

FIGURE 7.1 Schematic representation of adenosine generation from ATP hydrolysis by venom enzymes. Venom enzymes are contained in ovals, and bold letters indicate end products released upon enzyme actions.

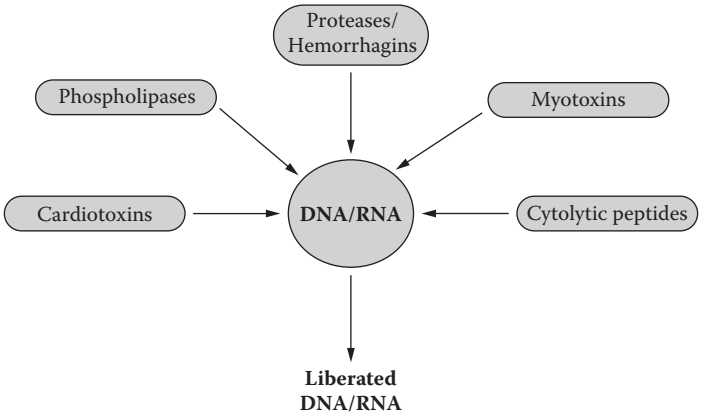

FIGURE 7.2 Cell necrosis brought about by venom enzymes. Venom enzymes are contained in ovals, and bold letters indicate end products released upon enzyme actions.

Liberated adenosine may also help in the diffusion of toxins into prey's tissues by inducing increased vascular permeability through vasodilation (Hargraves et al., 1991; Sobrevia et al., 1997) or inhibition of platelet aggregation (Seligmann et al., 1998). Along with increased vascular permeability, effects of adenosine-induced edema (Ramkumar et al., 1993) may potentiate venom-induced hypertension (Aird, 2002). In addition, adenosine is also known to cause paralysis by inhibiting neurotransmitter release at both central and peripheral nerve termini (Ralevic and Burnstock, 1998; Redman and Silinsky, 1993), thus potentiating venom-induced paralysis (Aird, 2002). Further, along with hemolytic, myolytic, and cardiolytic toxins of snake venom, adenosine may also be involved in venom-induced renal failure and cardiac arrest (Olsson and Pearson, 1990; Aird, 2002; Castrop, 2007). Other common disturbances such as nociception, locomotor alterations, and pain perceived upon envenomation may also result from increased adenosine levels (Dunwiddie and Worth, 1982; Barraco et al., 1983; Winsky and Harvey, 1986; Palmour et al., 1989; Nikodijevic et al. 1991; Jain et al., 1995; Sawynok et al., 1997; Sawynok, 1998; Aird, 2002). Therefore, it seems that adenosine could play a central role in envenomation strategies and prey immobilization (Aird, 2002, 2005). Although experimental evidence demonstrating these effects resulting from purified venom

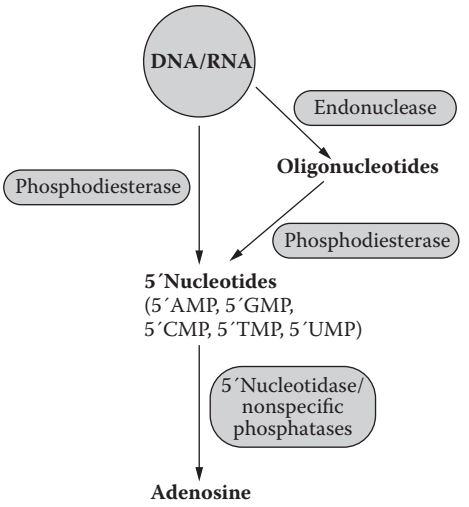

FIGURE 7.3 Schematic representation of adenosine generation by venom enzymes from DNA/RNA hydrolysis. Venom enzymes are contained in ovals, and bold letters indicate end products released upon enzyme actions.

TABLE 7.6
Pharmacological Effects of Adenosine and Inosine Related to Snake Envenomation

Pharmacological Effect	Mediated via	References
	Adenosine	
Vasodilatation	Vascular A_{2A} receptors	Hargraves et al., 1991
	Vascular A_{2B} receptors	Sobrevia et al., 1997
Cardiac block	Cardiac adenosine A_1 receptor	Olsson and Person, 1990
Vascular permeability	Mast cell A_3 receptors	Tilley et al., 2000
Inhibition of release of neurotransmitter	Adenosine A_1 receptors, central and peripheral neurons	Ralevic and Burnstock, 1998; Redman and Silinsky, 1993
Edema	Mast cell A_3 receptors	Ramkumar et al., 1993
Antiplatelet aggregation	A_1 and A_2 receptors	Seligmann et al., 1998
Renal failure	Renal adenosine A_1 receptor	Castrop, 2007
	Behavioral Effects	
Sedative effects	Central neuronal A_1 receptors	Barraco et al., 1983
Anxiolytic activity	Central neuronal A_1 receptors	Jain et al., 1995
Anticonvulsant effect	Central neuronal A_1 receptors	Dunwiddie and Worth, 1982
Aggression inhibition	Central neuronal A_1 receptors	Palmour et al., 1989
Alterations of cognitive functioning	Central neuronal A_1 receptors	Winsky and Harvey, 1986
Locomotor depression	Central A_1 and A_2 receptors	Nikodijevic et al., 1991
	Nociceptive Actions	
Analgesia	Adenosine A_1 receptor	Sawynok, 1998
Pain	Adenosine A_2 receptor	Sawynok, 1998
	Mast cell A_3 receptors	Sawynok et al., 1997
	Inosine	
Vascular permeability and inflammation	Mast cell A_3 receptors	Tilley et al., 2000

enzymes is lacking, there is evidence for direct involvement of adenosine and adenosine signaling in snake envenomation (Lumsden et al., 2004; Aird, 2005).

In addition to the liberation of adenosine, which can result in various pharmacological actions, these enzymes could interfere with many physiological processes of an organism directly. Even though they are hydrolytic enzymes, their pharmacological actions need not be based solely on their catalytic activity, and additional pharmacological activities may be inherent properties of the molecules, as venom enzymes have evolved rapidly and interfere in diverse physiological processes (Kochva, 1987; Fry, 2005). Hence, it is likely that nucleases, nucleotidases, and phosphatases also possess pharmacological activities distinct from catalytic effects, as is commonly observed among venom PLA_2s and proteases (Kini, 1997; Gutiérrez and Rucavado, 2000). This hypothesis is supported by recent work on anticoagulant effects of *N. naja* 5′ nucleotidase, and the pharmacological effect is independent of catalytic activity (Dhananjaya et al., 2006).

VI. CONCLUSIONS

Although nucleases, nucleotidases, and phosphomonoesterases are nearly ubiquitous in distribution among snake venoms, little progress has been made toward understanding these enzymes from a toxinological perspective. As discussed above, characterization of individual nucleases, nucleotidases, and phosphatases has not been clearly established, since they hydrolyze similar substrates and share similar biochemical properties. Future research on complete biophysical characterization of the purified enzymes may reveal the existence of unique venom proteins or proteins having multiple domains that contain different catalytic or biological functions. Determination of complete cDNA or amino acid sequence will also enable evaluation of the degree of homology of these enzymes from various species and families of snakes. However, a renewed interest in these enzymes in recent years is based on their involvement in generation of adenosine, a compound with multiple toxicities, but the direct involvement of these enzymes in the generation of adenosine *in vivo* has yet to be established. Nucleases, nucleotidases, and phosphomonoesterases may also possess distinct pharmacological properties that are independent of catalytic activity, a conjecture that has yet to be verified experimentally. Venom nucleotidases (ATPases, ADPases, and 5′ nucleotidase), along with other hemostatically active components, can lead to the formation of incoagulable blood, which may help in diffusion of other venom toxins to their sites of action. An analogous mechanism is found in blood-feeding organisms, where apyrases (ATP diphosphohydrolase), 5′ nucleotidases, and other enzymes provide a redundant antihemostasis barrier, which greatly limits host defenses triggered by blood feeding. Further research is needed to isolate and characterize biologically these enzymes in snake venoms, so that their biological role in venoms is clearly established.

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8 Snake Venom Phospholipase A_2 Enzymes

Robin Doley, Xingding Zhou, and R. Manjunatha Kini

CONTENTS

Phospholipase A_2 (PLA_2) enzymes are esterolytic enzymes that are found abundantly in nature. They are classified into different groups according to their three-dimensional structure, amino acid sequence, catalytic specificity, and site of expression, and this family of enzymes is rapidly expanding. Snake venoms are a particularly good source of Group I and Group II PLA_2 enzymes. Snake venom PLA_2 enzymes are similar in their primary and secondary structures to mammalian enzymes, but they induce various pharmacological effects in victims. Snake venom PLA_2 enzymes typically exist in venoms as monomers and sometimes as complexes formed between PLA_2

enzymes, as well as with other proteins, by covalent or noncovalent interactions. Snake venoms often contain many isoenzymes, and therefore care must be taken during purification to separate these, as isoenzymes may induce different pharmacological effects through their interaction with protein receptors/acceptors. This specific interaction with their target protein is mediated through specific pharmacological sites on the molecular surface. Upon binding to their target protein, they induce their effects, which may be dependent or independent of enzymatic activity. PLA_2 enzymes are known to have evolved from a nontoxic ancestral gene. Group I and mammalian pancreatic gene followed a common pathway of evolution; however, Group II enzymes evolved separately after species diversification. In this review on snake venom PLA_2 enzymes, we provide an overview of their structure, complexes, pharmacological properties, and evolution.

I. INTRODUCTION

Phospholipase A_2 (EC 3.1.1.4) enzymes are esterolytic enzymes that hydrolyze glycerophospholipids at the *sn*-2 position of the glycerol backbone, releasing lysophospholipids and free fatty acids. They occur ubiquitously in nature as $sPLA_2$ (secretory PLA_2), $cPLA_2$ (cytosolic PLA_2), $iPLA_2$ (Ca^{2+}-independent PLA_2), and PAF-AH (platelet-activating factor acetylhydrolases). $sPLA_2$ enzymes are low molecular weight, Ca^{2+}-dependent, and have an active site histidine. In contrast, $cPLA_2$, $iPLA_2$, and PAF-AH are high molecular weight, Ca^{2+}-independent intracellular enzymes, and have an active site serine residue (Six and Dennis, 2000). $sPLA_2$ enzymes are found abundantly in various biological fluids and secretions, such as inflammatory exudates, pancreatic juice, tears, body fluids, and the venoms of snakes, scorpions, bees, and lizards, whereas intracellular PLA_2 enzymes are found within all living cells (Alape-Giron et al., 1999; Valentin and Lambeau, 2000; Sugiyama et al., 2002). Mammalian PLA_2 enzymes are known to play an important role in fertilization (Fry et al., 1992), cell proliferation (Arita et al., 1991), smooth muscle contraction (Sommers et al., 1992; Nakajima et al., 1992), and hypersensitization (Vadas et al., 1993). They are also important in cellular functions such as signal transduction via biosynthesis of prostaglandins and leukotrienes, and membrane homeostasis, including the maintenance of the cellular phospholipid pools and membrane repair through deacylation/reacylation (Dennis et al., 1991; Kudo et al., 1993; Dennis, 1994). In general, mammalian enzymes are nontoxic and do not induce potent pharmacological effects. However, some of them indeed play a crucial role in numerous diseases, such as chronic inflammation, rheumatism and osteoarthritis, asthma, psoriasis, septic shocks, and adult respiratory distress syndrome (Balsinde et al., 1999; Touqui and Alaoui-El-Azher, 2001). In contrast, snake venom PLA_2 enzymes are among the major toxic proteins of the venom and play an important role in immobilization and capture of prey. In addition to their involvement in the digestion of prey, they exhibit a wide variety of pharmacological effects by interfering in normal physiological processes of prey/victims (Kini, 1997) (Table 8.1). Often, single snake venom contains a number of PLA_2 isoenzymes, and at times, different isoenzymes induce distinct pharmacological effects. However, not all PLA_2 enzymes induce all the pharmacological effects; an individual PLA_2 enzyme may exhibit one or more specific pharmacological effects. For example, β-bungarotoxin (β-Btx), a PLA_2 toxin from *Bungarus multicinctus* venom, exhibited presynaptic neurotoxicity (Strong et al., 1976) but failed to show postsynaptic neurotoxicity and anticoagulant effects (Verheij et al., 1980a). On the other hand, OHVA-PLA_2 (*Ophiophagus hannah* venom acidic PLA_2), from *Ophiophagus hannah* venom, induces myotoxicity, cardiotoxicity, and antiplatelet effects (Huang et al., 1993c, 1997; Huang and Gopalakrishnakone, 1996). In general, PLA_2 enzymes and their complexes are among the most toxic and potent pharmacologically active components of snake venoms. All known highly toxic presynaptic neurotoxins from snake venom are PLA_2 enzymes per se or contain PLA_2 as an integral part (Gubenšek et al., 1997; Bon, 1997). Similarly, PLA_2 myotoxins are more potent and faster acting than their nonenzymatic counterparts (Fletcher et al., 1997). Therefore, much effort has been put into characterizing snake venom PLA_2 enzymes, and they are one of the best-studied families of venom proteins (Kini, 1997).

TABLE 8.1
Pharmacological Effects of Venom Phospholipase A_2 Enzymes

Neurotoxicity
Presynaptic neurotoxicity
Postsynaptic neurotoxicity
Myotoxicity
Local myonecrosis
Systemic myotoxicity
Cardiotoxicity
Anticoagulant effects
Platelet aggregation initiation
Platelet aggregation inhibition
Hemolytic activity
Hemoglobinurea-inducing Activity
Internal hemorrhage
Convulsant activity
Hypotensive activity
Edema-inducing activity
Organ or tissue damage
Liver, kidney, lungs, testis, pituitary damage
Cell migration and cell proliferation
Bactericidal

The first PLA_2 enzymes were purified from the venom of *Naja naja* and *Naja tripudians* and were named as hemolysins due to their ability to hemolyze red blood cells indirectly (De, 1944). Since then, hundreds of snake venom PLA_2 enzymes have been purified and characterized (for some of the milestones in PLA_2 research, see Figure 8.1). To date, amino acid sequences of over three hundred PLA_2 enzymes have been reported from snake venom. PLA_2 enzymes share 40 to 99% identity in their amino acid sequences, and hence significant similarity in their three-dimensional folding (Scott, 1997). However, they differ greatly in their pharmacological properties (Kini, 2003). Thus, the functional differences among PLA_2 enzymes cannot be easily correlated to their structural differences, and the structure-function relationships are subtle, complicated, and challenging. The shared common ability to catalyze hydrolysis at the *sn*-2 position of phospholipids, and the lack of correlation between enzymatic activity and lethal toxicity or pharmacological potency (Rosenberg, 1997a, 1997b), make the mechanisms by which snake venom PLA_2 enzymes induce

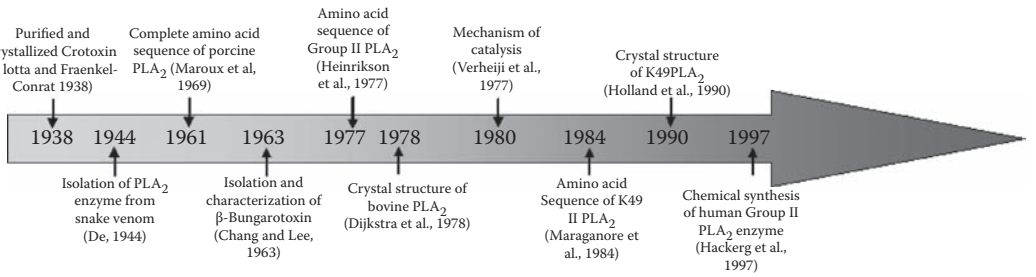


FIGURE 8.1 Milestones in PLA_2 enzyme research.

such a wide spectrum of pharmacological effects intriguing. A monograph on snake venom PLA_2 enzymes (Kini, 1997) deals with their structure, function, and mechanism, and gives an overview of snake venom phospholipase A_2 enzymes.

II. CLASSIFICATION OF PLA_2 ENZYMES

Intracellular and secretory PLA_2 enzymes have been classified into fourteen groups based on various parameters such as structure, amino acid sequence, catalysis, and expression (Schaloske and Dennis, 2006). A number of new PLA_2 enzymes are being discovered, and this superfamily of enzymes has been expanding rapidly. PLA_2 enzymes that share high sequence homology are classified under the same group. If more than one homologous PLA_2 enzyme exists within venom of the same species, then each paralog should be assigned subgrouped letters, as in the case of Group IVA, IVB, and IVC PLA_2. Those from different origins (orthologs) are not assigned separate letters and should be classified under the same subgroups. Splice variants of the same gene should be under the same group, but distinguished using Arabic numbers like VIA-1 PLA_2 and VIA-2 PLA_2 (Six and Dennis, 2000). In this classification scheme, Groups I, II, V, and X PLA_2 are closely related enzymes. They are all $sPLA_2$ enzymes and are characterized by a low molecular mass of 13–18 kDa, several disulfide bonds, a requirement of millimolar Ca^{2+} for optimal catalytic activity, and a low selectivity for phospholipids with different polar heads and fatty acids. The active site of these enzymes has a histidine residue, and they share a common mechanism for cleaving the *sn*-2 ester bond of phospholipids (Fuentes et al., 2002). Based on the amino acid sequence, three-dimensional structure, and disulfide bonding pattern, snake venom PLA_2 enzymes fall under Groups I and II (Six and Dennis, 2000).

A. Group I PLA_2 Enzymes

This group of PLA_2 enzymes is found in the mammalian pancreas and in venoms from elapid and colubrid snakes. Cobra venom PLA_2 enzymes were the first to be characterized under this group. These enzymes typically contain 115–120 amino acid residues with seven disulfide bridges, and the disulfide bond between the 11th and 77th Cys residues is unique to this group (Figure 8.2). Group I PLA_2 enzymes in snake venoms have a characteristic surface loop called the elapid loop that connects the catalytic α-helix and the β-wing. In mammalian PLA_2 enzymes, there is an additional five amino acids residue extension, which is called the pancreatic loop (residues 62–67). Thus, Group I PLA_2 enzymes can be further divided into Group IA and Group IB (Figure 8.2) based on the presence of elapid or pancreatic loop, respectively. Similar to other PLA_2 enzymes, they bind to aggregated phospholipid membrane surface (Lefkowitz et al., 1999; Gelb et al., 2000). In general, most elapid venom PLA_2 enzymes belong to Group IA, while Group IB enzymes are mainly found in mammalian pancreas. However, Group IB enzymes have also been reported in some snake venoms, such as *Oxyuranus scutellatus* (Fohlman et al., 1977), *Pseudonaja textilis* (Pearson et al., 1993), *Notechis scutatus* (Francis et al., 1995b), *Ophiophagus hannah* (Huang et al., 1997), and *Micrurus frontalis frontalis* (Francis et al., 1997). The Group IB PLA_2 enzymes in mammalian pancreas are secreted as zymogens that contains an eight amino acid residue propeptide segment that is cleaved by trypsin during maturation. These enzymes are found abundantly in the pancreatic juice, where they have an important digestive role toward dietary phospholipids. However, some of the Group IB snake venom PLA_2 enzymes retain the eight-residue propeptide segment even in the mature state (Pearson et al., 1993), while in others it is removed during maturation (Francis et al., 1997; Huang et al., 1997). More recently, trimorphin, a PLA_2 isolated from the venom of a colubrid snake, was also shown to be a member of the Group IA PLA_2 enzymes (Huang and Mackessy, 2004).

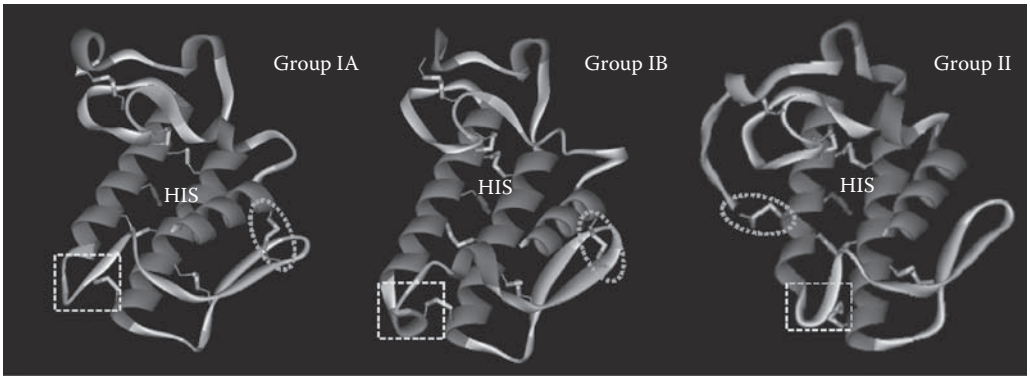


FIGURE 8.2 ***(A color version of this figure follows page 240.)*** Three-dimensional structures of snake venom PLA_2 enzymes generated and modified using ViewerLite software. PLA_2 molecules of Group IA from *Naja naja* venom (Segelke et al., 1998), Group IB PLA_2 from *Ophiophagus hannah* venom (Xu et al., 2003), and Group II PLA_2 from *Deinagkistrodon* (*Agkistrodon*) *acutus* venom (Holland et al., 1990) are shown in the figure. Structurally these PLA_2 molecules have a common scaffold comprised of two major α-helices and β-wings, though they differ in their primary amino acid sequence. The elapid loops (between the first major α-helix and β a-wing) in Groups IA and IB are shown in violet. The pancreatic loop present in Group IB is shown in green, which is absent in Group IA. The C-terminal extension in Group II is shown in green. The disulfide bridge between the cysteine residues and the active site histidine residue are also shown in the figure. The unique disulfide bridges in Groups IA and IB (Cys11–Cys77) and Group II (Cys50–Cys134) are encircled and the loops are shown in a box.

B. Group II PLA_2 Enzymes

PLA_2 enzymes from Viperidae snake venoms fall under Group II. These enzymes contain 120–125 amino acid residues and 7 disulfide bridges. They lack the pancreatic or elapid loop and differ from Group I in having an extended C-terminal tail (Figure 8.2). The 133rd cysteine residue of the C-terminal end forms a disulfide link with the 50th cysteine residue near the active site, which is unique to Group II. However, the other six disulfide bonds between the Cys residues are similar to those of Group I PLA_2 enzymes (Cys27–Cys126, Cys29–Cys45, Cys44–Cys105, Cys51–Cys98, Cys61–Cys91, and Cys84–Cys96) (Scott and Sigler, 1994). Six and Dennis (2000) have divided this group into six subgroups (IIA–IIF) on the basis of tissue specific expression:

IIA occurs in human synovial fluid, rattlesnake and viper venoms.
IIB occurs in Gaboon viper venom.
IIC occurs in rat/mouse testes.
IID occurs in human/mouse pancreas/spleen.
IIE occurs in human/mouse brain/heart/uterus.
IIF occurs in mouse testis/embryo.

Group II snake venom PLA_2 enzymes can also be divided into different subgroups on the basis of the amino acid residue in the forty-ninth position. Asp49 plays an important role in catalysis and is conserved in most snake venom PLA_2 enzymes, and hence these are identified as D49 enzymes (Scott et al., 1990). However, in some of the Group II PLA_2 enzymes this amino acid residue is replaced by lysine, serine, asparagines, or arginine, and they are identified as K49 (Maraganore et al., 1984), S49 (Polgar et al., 1996), N49 (Tsai et al., 2004), or R49 (Chijiwa et al., 2006) enzymes (Figure 8.3). Substitution of Asp in the forty-ninth position interrupts the binding of cofactor Ca^{2+}

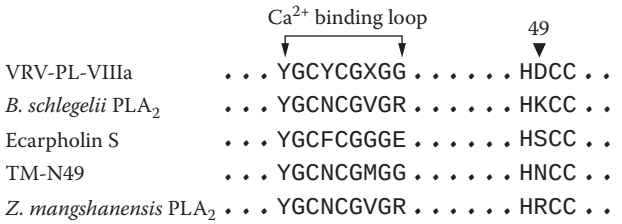


FIGURE 8.3 Alignment of calcium binding loops and forty-ninth residues in Group II PLA_2 enzymes. D49 (VRV-PL-VIIIa) from *Daboia russellii pulchella* (Gowda et al., 1994); K49 from the venom of *Bothriechis schlegelii* (Tsai et al., 2001); S49 (ecarpholin S) from the venom of *Echis carinatus* (Polgar et al., 1996); N49 (TM-N49) from the venom of *Protobothrops mucrosquamatus* (Wei et al., 2006); R49 from the venom of *Zhaoermia mangshanensis* (Mebs et al., 2006).

to the Ca^{2+} binding loop, and hence these "mutants" show low or no hydrolytic activity (Maraganore and Heinrikson, 1985). In addition, there are several substitutions in the Ca^{2+} binding loops of these mutant enzymes.

III. STRUCTURE OF PLA_2 ENZYMES

As mentioned above, snake venom PLA_2 enzymes are small proteins (~13–14 kDa) with 115–133 amino acid residues. They have fourteen conserved Cys residues that form seven disulfide bridges and stabilize the tertiary structure (Scott, 1997). The overall structures of Group I and II PLA_2 enzymes are almost similar except for the extended C-terminal end in Group II. PLA_2 enzymes consist of three major α-helices and two antiparallel β-sheets, which are held together by disulfide bridges. The conserved structures in PLA_2 enzyme are the N-terminal helix, calcium binding loop, antiparallel helix, active site, and β-wing. The N-terminal segment of PLA_2 enzymes has a highly conserved network of hydrogen bonds and stabilizes the adjacent β-sheet (Scott, 1997). Some PLA_2 enzymes that retain the N-terminal propeptide (8-mer) lack the catalytic activity, similar to the precursor of pancreatic PLA_2 enzymes. The N-terminal helix between residues 1 and 12 contributes significantly to the hydrophobic channel. The side chains of the residues in the helix form the opening of the channel, especially from the second, fourth, fifth, and ninth residues. The side chain of the fourth residue is functionally important, as it anchors the N-terminal helix to the enzyme (Scott et al., 1991).

Ca^{2+} is the most important cofactor for catalysis. During catalysis, Ca^{2+} binds to the enzyme at the conserved Ca^{2+} binding loop that lies between residues 25 and 33 with a consensus sequence (Y25-G-C-Y/F-C-G-X-G-G33). The oxygen atom from Asp49, along with three carbonyl oxygen atoms (Y/F28, G30, and G32) and two water molecules, form the pentagonal bipyramidal cage for Ca^{2+} (Banumathi et al., 2001). Two long helices (from residues 37 to 54, known as catalytic helices, and from residues 90 to 109) are oriented antiparallel and held together by disulfide bridges. The conserved side chains of these helices assist in the coordination of the primary Ca^{2+} and form the deeper contour of the hydrophobic channel (Scott, 1997). His48 is the crucial active site residue that is responsible for the catalysis and is supported by hydrogen bonds from Tyr52 to the side chain of the opposite helix (Asp99). This network, together with close coupling of Asp49 and His48, defines the active site geometry of PLA_2 enzymes. All PLA_2 enzymes have two distinct β-sheets that form the β-wing. This β-wing connects the major helices and protrudes out from the main structure into the solvent. The extended C-terminal end is the characteristic feature of Group II PLA_2 enzymes and is cross-linked to the main structure by two disulfide bridges (Heinrikson et al., 1977).

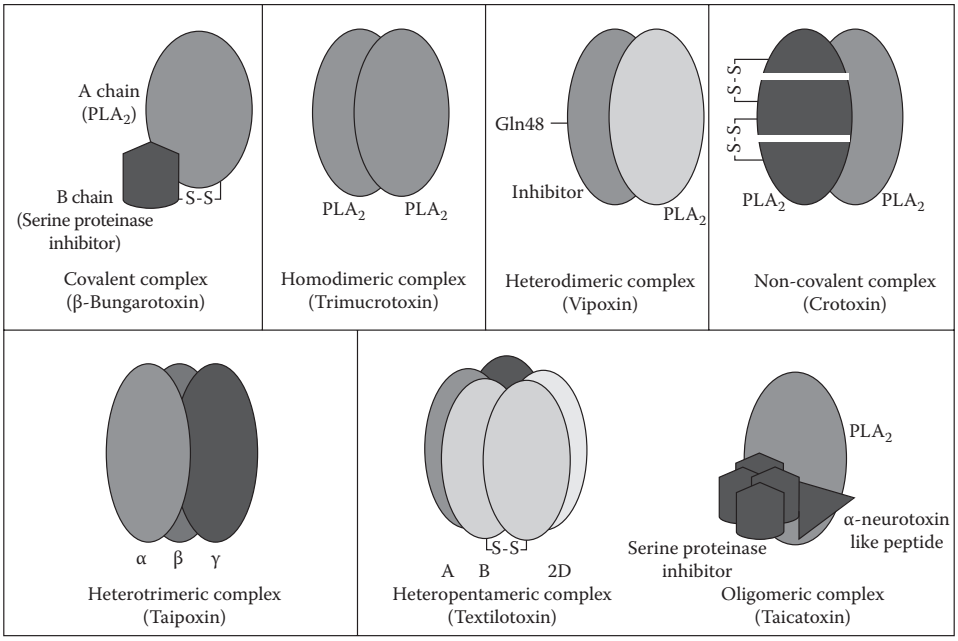


FIGURE 8.4 Schematic representation of PLA_2 complexes.

IV. PLA_2 AND ITS COMPLEXES

Although most snake venom PLA_2 enzymes exist as monomers, some of them form aggregates or complexes. These snake venom PLA_2 enzymes interact with other protein factors to form complexes (Figure 8.4). The components of these complexes are held together by either covalent or noncovalent interactions. These additional protein factors help to express their pharmacological effects to the greatest potency. Most of the PLA_2 complexes exhibit presynaptic neurotoxicity (Bon, 1997).

A. Covalent Complexes

β-bungarotoxins are among the most well-studied presynaptic neurotoxins and are isolated from the venom of *Bungarus* species. They are the only known covalent PLA_2 complexes. β-bungarotoxins consist of two dissimilar polypeptides A and B: the A chain is homologous to Group I PLA_2 enzymes, while the subunit B chain is structurally similar to the Kunitz type of serine proteinase inhibitors and dendrotoxins (Kondo et al., 1978; Bon, 1997) (Figure 8.5). The two subunits of β-bungarotoxin are held together by a single disulfide bond (Bon, 1997). So far, these types of presynaptic neurotoxins have been isolated from only a single genus, *Bungarus*. A number of isoforms of β-bungarotoxins have been isolated and characterized, formed by the association of three different forms of A chains and two different forms of B chains.

B. Noncovalent Complexes

In these PLA_2 complexes, the subunits are held together by noncovalent interactions. In most cases, PLA_2 enzymes and PLA_2-derived subunits interact with each other, forming the complex.

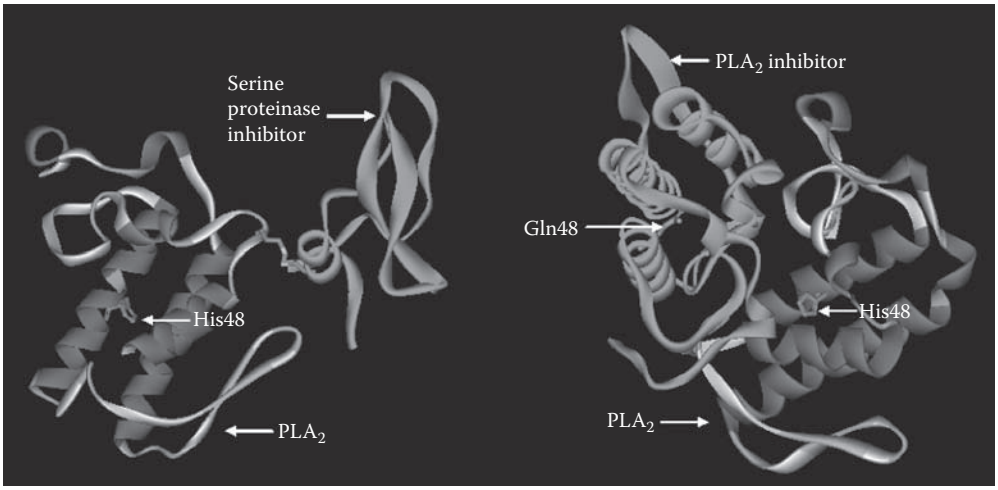


FIGURE 8.5 ***(A color version of this figure follows page 240.)*** Crystal structures of β-bungarotoxin and vipoxin complexes. In β-bungarotoxin, the serine-type proteinase inhibitor is linked to the PLA_2 molecule by a disulfide (covalent) bond between Cys15 of the PLA_2 molecule and Cys55 of the serine-type proteinase inhibitor (Kwong et al., 1995). In vipoxin, two PLA_2 molecules interact noncovalently to form the complex. In one of the molecules the His48 is replaced with Gln48, which acts as an inhibitor to the PLA_2 enzyme (Perbandt et al., 1997). The critical disulfide bond in β-bungarotoxin and active site residues of vipoxin are shown.

1. Crotoxin and Related Toxin Complexes

Crotoxin was one of the first protein complexes identified from snake venoms. This PLA_2 complex is the main neurotoxic component of South American rattlesnake *Crotalus durissus terrificus* venom (Slotta and Fraenkel-Conrat, 1938). It is a heterodimer composed of an acidic, nontoxic, and nonenzymatic subunit named crotapotin (CA) (Aird et al., 1985) and a basic, weakly toxic PLA_2 subunit (CB). Structurally, the CB subunit is a PLA_2 molecule that belongs to Group II (Aird et al., 1986). Interestingly, the CA subunit is made up three disulfide-linked polypeptide chains that are generated by proteolytic processing of a precursor Group II PLA_2 molecule (Aird et al., 1985; Bouchier et al., 1991; Faure et al., 1991). The components of crotoxin can be separated at acidic pH (<2) or in the presence of 6 M urea. The native complex can be reconstituted by mixing the components (Hendon and Fraenkel-Conrat, 1971; Rubsamen et al., 1971). Similar to native crotoxin, the CB subunit causes blockade of neuromuscular transmission (albeit with lower potency), while CA is inactive. The native crotoxin complex is at least one order of magnitude more potent than CB alone. Binding experiments show that the toxin complex dissociates upon binding to the synaptic membranes. CB binds to the membrane, while CA is released into the solution (Bon et al., 1979). If this dissociation is prevented by a covalent linkage of the subunits, the lethal potency of crotoxin is completely abolished (Hendon and Tu, 1979). CA appears to block the nonspecific binding of CB to synaptic membranes. Thus, CA acts as a chaperone of CB. Although the neurotoxicity of crotoxin has been well studied, it also exhibits a number of other biological activities, including local myotoxicity, systemic myotoxicity and myoglobinuria, inhibition of inflammatory response (Gopalakrishnakone et al., 1984; Landucci et al., 1995, 2000; Sampaio et al., 2003, 2005), initiation of platelet aggregation (Strong et al., 1976; Landucci et al., 1994), and analgesic effects (Zhang et al., 2006). Crotoxin-like neurotoxins have been purified and characterized from a number of other rattlesnake venoms: Mojave toxin from the Mojave rattlesnake *C. scutulatus scutulatus*, concolor toxin from the midget faded rattlesnake *C. viridis concolor*, vegrandis toxin from the Uracoan

rattlesnake *C. vegrandis*, and canebrake toxin from canebrake rattlesnake *C. horridus atricaudatus* (Bieber et al., 1975; Pool and Bieber, 1981; Ho and Lee, 1981; Kaiser and Aird, 1987; Straight and Glenn, 1988). In *C. durissus terrificus* venom as many as fifteen isoforms of crotoxin have been identified (Wu et al., 1983; Faure et al., 1991). These isoforms are formed by association of two subunits differing in only a few amino acid residues (Bouchier et al., 1988; Faure and Bon, 1988). CA isoforms are due to differential proteolytic processing, whereas CB isoforms are protein products of different genes (Faure et al., 1991; Faure et al., 1994).

2. Other Heterodimeric PLA_2 Complexes

Heterodimeric PLA_2 complexes have been reported primarily from viper venoms. In these complexes, one of the subunits is a PLA_2 enzyme, while the other subunit is a nonenzymatic component. Vipera toxin, isolated from the venom of *Vipera palestinae*, is a heterodimeric PLA_2 complex (Ovadia et al., 1977). It has an acidic subunit that is a PLA_2 enzyme, and the basic subunit is a nonenzymatic protein (Simon et al., 1980; Križaj et al., 1996a). Vipoxin is a heterodimeric postsynaptic neurotoxin isolated from the venom of *Vipera ammodytes meridionalis* (Freedman and Snyder, 1981). It consists of two PLA_2 subunits: a basic, highly toxic PLA_2 and an acidic, nontoxic, and catalytically inactive PLA_2 protein (Figure 8.5). In the acidic subunit, His48 is replaced by Gln, and this subunit inhibits the PLA_2 activity of the basic subunit. This is a unique example of modulation of catalytic and toxic functions of PLA_2 by an inactive, nontoxic PLA_2 (Banumathi et al., 2001). *Pseudocerastes* neurotoxin, isolated from the venom of Field's horned viper, *Pseudocerastes fieldi*, is also a heterodimeric PLA_2 complex (Batzri-Izraeli and Bdolah, 1982). It is made of two noncovalently associated subunits, a basic weakly toxic subunit (Cb II), and one of two acidic subunits (Cb Iα or Cb Iβ) (Tsai et al., 1983). All the subunits are PLA_2-like molecules. Interestingly, the acidic subunits are inactive toward phosphatidylcholine (Francis et al., 1995a), despite the fact that all the putative catalytic residues (His48, Asp49, Ca^{2+} binding loop, the hydrophobic pocket, and interfacial recognition site) are conserved. However, CbI enhances the lethal potency of the basic and weakly toxic subunit CbII (Tsai et al., 1983).

3. Homodimeric PLA_2 Complexes

Trimucrotoxin isolated from the venom of *Trimeresurus mucrosquamatus* (Taiwan habu) is a homodimeric PLA_2 complex that exhibits presynaptic neurotoxicity. The subunits dissociate into 14 kDa monomers when subjected to SDS-PAGE. The amino acid sequence of trimucrotoxin reveals that it belongs to Group II PLA_2 enzyme. It is structurally, immunologically, and pharmacologically similar to crotoxin B and agkistrodotoxin (Tsai et al., 1995).

4. Heterotrimeric PLA_2 Complexes

Taipoxin, isolated from the Australian taipan (*Oxyuranus s. scutellatus*), is a PLA_2 complex that causes neuromuscular blockade, and it is one of the most potent animal toxins known (Fohlman et al., 1976). It is a ternary complex of three subunits designated as α, β, and γ. The α subunit is the most toxic, and γ subunit is moderately toxic, while the β subunits (β-1 and β-2) are nontoxic (Fohlman et al., 1976; Lind and Eaker, 1982). Structurally, α and β subunits are PLA_2 molecules belonging to Group IA. On the other hand, γ subunit belongs to Group IB and is similar to mammalian pancreatic pro-PLA_2, with an additional eight amino acid residues at the N-terminal. This subunit is also glycosylated. Paradoxin is a presynaptic neurotoxin isolated from the venom of *Parademansia microlepidotus* (currently known as *Oxyuranus microlepidotus*) and has three subunits, α, β, and γ, and is similar to taipoxin (Fohlman, 1979; Hodgson et al., 2007). Cannitoxin is yet another toxin similar to taipoxin and was isolated and characterized from *Oxyuranus scutellatus canni* venom (Kuruppu et al., 2005).

5. Heteropentameric PLA_2 Complexes

Textilotoxin is structurally the most complex snake venom neurotoxin isolated from *Pseudonaja textilis* (Su et al., 1983). It is among the most potent neurotoxin, with an LD_{50} of 1 μg/kg (i.p.) in

mice. It consists of four subunits, A, B, C and D; all of them contain the putative PLA_2 active site and form the complex at a 1:1:1:1 ratio (Pearson et al., 1993). Subunit A shows PLA_2 activity and is lethal to mice, whereas subunit C is similar to subunit A but lacks PLA_2 activity and lethality. Subunit B is highly basic in nature and differs from subunit A in amino acid sequence in the N-terminal region and in a number of aromatic residues. Subunit B is nontoxic to mice and is similar to the β subunit of taipoxin in amino acid sequence. In contrast, subunit D consists of two identical polypeptides linked by a disulfide bond, and it contains eight extra amino acid residues at the N-terminal end, compared to the other subunits. Subunit D shows very low PLA_2 activity and is glycosylated at Asn93 (Pearson et al., 1991).

6. Other PLA_2 Complexes

The only known nonneurotoxic PLA_2 complex isolated from snake venom is taicatoxin. Isolated from the venom of Australian taipan (*Oxyuranus scutellatus scutellatus*), it is an oligomeric complex that blocks Ca^{2+} channels (Brown et al., 1987). It consists of three subunits, an α-neurotoxin-like peptide, a neurotoxic PLA_2, and a serine proteinase inhibitor; the complex is formed by noncovalent interactions at an approximate stoichiometry of 1:1:4 (Possani et al., 1992).

V. PURIFICATION OF PLA_2 ENZYMES

The quality of enzyme preparations is crucial for functional characterization and structure-function studies. Any contamination in the preparation will lead to invalid results and further complicate the structure-function studies. Snake venom is a mixture of proteins that differ in their molecular weights as well as ionic charges. Many of these proteins, particularly PLA_2 enzymes, share the same molecular weight but differ in ionic charges. Therefore, a combination of chromatographic steps should be used for their purification. The following two factors should be considered during the purification of PLA_2 enzymes from snake venom: (1) PLA_2 enzymes exist as isoenzymes, and a single snake venom may contain more than one isoenzyme. For example, *Naja naja*, *Daboia* (*Vipera russellii*), *Trimeresurus flavoviridis*, *Austrelaps superbus*, and *Pseudechis australis* venoms contain more than ten isoenzymes (Braganca and Sambray, 1967; Vishwanath et al., 1987, 1988; Takasaki et al., 1990b; Singh et al., 2000). These isoenzymes show similarities in their molecular weight, isoelectric point, and even N-terminal amino acid sequence, and therefore they might co-elute during purification. Rigorous methods should be considered in determining homogeneity of PLA_2 enzymes (Evans et al., 1980; Kini et al., 1986; Takasaki et al., 1990b; Gao et al., 2001), and sophisticated methods including capillary electrophoresis (high number of theoretical plates) and high-resolution mass spectrometry may be used in determining the homogeneity of the preparation. (2) PLA_2 enzymes interact with each other or with other proteins to form aggregates that are due to protein-protein interaction. In addition, PLA_2 enzymes interact with other venom toxins such as cardiotoxins (Condrea et al., 1970). These interactions contribute significantly to the enzymatic and pharmacological activity of PLA_2 enzymes. Therefore, a combination of different purification steps, such as gel filtration followed by ion-exchange and reverse-phase HPLC, should be employed. In some cases, the complex formation is important for the pharmacological potency and toxicity of PLA_2 toxins (discussed above). When studying such complexes, care must be taken not to disrupt the native complexes.

VI. MECHANISM OF CATALYSIS

PLA_2 enzymes are unique calcium-dependent hydrolytic enzymes that are highly water soluble and hydrolyze water-insoluble phospholipids, liberating free fatty acid and lysophospholipid (Dennis, 1983). The mechanism of catalysis by $sPLA_2$ enzyme was proposed by Verheiji and coworkers (1980b) based on structural comparison with serine proteases. The mechanism involves general base-mediated attack on the sessile bond with a conserved water molecule serving as a nucleophile.

They hydrolyze phospholipids in different phases, such as monomeric, micellar, or lipid bilayer phases. The hydrolytic activity of $sPLA_2$ enzyme has been found to increase 10,000-fold when monomeric phospholipids aggregate to form micelles at their critical micellar concentration (Verheij et al., 1981). One of the unusual properties of PLA_2 enzyme is the hydrolysis of substrate at the lipid-water interface, which is known as interfacial catalysis. The efficiency of this interfacial catalysis depends upon the absorption of the enzyme at the lipid-water interface, binding of the enzyme to the substrate, and activation of the interfacial recognition site. The interfacial recognition site is independent of the catalytic site. During interfacial catalysis, the enzyme in the interface binds to the substrate to form the enzyme-substrate complex, followed by the release of the product. Based on the differences in the interfacial binding steps, the hydrolysis of bilayer vesicles can occur in two distinct modes. In the pure hopping mode, the binding and desorption occur during each catalytic turnover cycle. In the scooting mode, the enzyme remains bound to the interface between the catalytic turnover cycles. These extreme cases can be distinguished and characterized under different experimental conditions (Jain and Berg, 1989; Ramirez and Jain, 1991). One or both modes are implicit in most models of interfacial catalysis, and the overall rate of catalytic turnover is determined by the equilibrium and kinetics of binding, catalysis, and desorption. The implications of these distinct modes for the pharmacological effects are not yet clear. The catalytic efficiency is also determined by the physical properties of the aggregate, such as packing density, phase transition temperature, liquid crystalline, and the presence of other molecules (Verheij et al., 1981; Vernon and Bell, 1992). Several studies have also proposed that the presence of defects in the bilayer structure may act as a starting point of enzyme activity (Grainger et al., 1990; Vernon and Bell, 1992). Recently, Winget and coworkers (2006) proposed a model for interfacial catalysis. According to this model, the enzyme (E) binds to the substrate (S) at the interface as the E* form. The enzyme-substrate complex (E*-S) is the interface-bound form. An additional anion activation step occurs at this stage, where an anionic phospholipid interacts at the interface of the enzyme, forming the $E^*\text{-}S^{\#}$ complex. Once activated, the PLA_2 can catalyze the formation of the enzyme-product (E*-P) complex and then release the product. After release of the product, the E* diffuse in a scooting mode to bind another substrate (S^*) for the next cycle of catalysis (Winget et al., 2006).

The mechanism by which PLA_2 enzyme hydrolyzes the phospholipid involves the highly specific interaction between the active site histidine, the Ca^{2+} cofactor, conserved water, and the glycerophospholipid substrate. His48 is conserved in $sPLA_2$ enzymes, and its role in phospholipid hydrolysis has been confirmed by chemical modification using *p*-bromophenyl bromide (*p*-BPB) (Yang and King, 1980; Verheij et al., 1980b). Other than the His48 at the active site, Asp99 and two other Tyr residues (Tyr52 and Tyr73) are also involved in the formation of the catalytic network. During catalysis, His48, assisted by Asp99, polarize the bound water molecule, which then attacks the *sn*-2 bond of the bound phospholipid to form a tetrahedral oxyanion intermediate (Scott, 1997). An alternative mechanism has also been proposed, whereby two more water molecules are involved in the formation and breakdown of the tetrahedral intermediate (Edwards et al., 2002). In both mechanisms, Ca^{2+} ion, coordinated by the oxygen atom of Asp49, serves as an electrophile during catalysis (Rogers et al., 1996; Scott, 1997; Edwards et al., 2002). Overall, catalysis by secretory PLA_2 enzymes can be summarized as follows: (1) binding of Ca^{2+} and substrate, (2) general base-mediated catalysis, (3) formation and breakdown of tetrahedral intermediate, and (4) release of the reaction product (Murakami et al., 2006). The Ca^{2+} binding loop forms one of the walls of the hydrophobic channel, and therefore it is important for enzyme-substrate interaction (Banumathi et al., 2001). In absence of Ca^{2+}, this loop is conformationally flexible. During catalysis, Ca^{2+} directs the stereospecific positioning of the substrate in the active site, and the electrophilic Ca^{2+} polarizes the sessile ester bond and stabilizes the transition state (Scott, 1997). The bound Ca^{2+} ion also supports the interactions of $sPLA_2$ enzymes with aggregated micelles, vesicles, monolayers, and membranes, since electrostatic forces play critical roles in the binding of these substrates (Scott et al., 1994). When Asp49 is substituted with other residues, the enzyme fails to bind Ca^{2+} ion, leading to the loss of catalytic activity.

VII. PHARMACOLOGICAL EFFECTS

Snake venom PLA_2 enzymes exhibit a wide variety of pharmacological effects despite their similarity in primary, secondary, and tertiary structures (Table 8.1). Some of the pharmacological effects exhibited by snake venom PLA_2 enzymes are described below.

Most of the PLA_2 enzymes are devoid of direct hemolytic activity (Fletcher et al., 1982; Jiang et al., 1989); however, in presence of exogenously added phospholipids, they exhibit potent hemolytic activity that is due to the hydrolysis products, lysophospholipid and free fatty acid, which are lytic by themselves (Condrea et al., 1964).

Some PLA_2 enzymes exhibit presynaptic or postsynaptic neurotoxicity. In presynaptic neurotoxicity, PLA_2 enzymes act on the nerve terminal, while in case of postsynaptic neurotoxicity they act on the muscle. Many snake venom PLA_2 enzymes induce myotoxicity. Some of them induce local myonecrosis, while others produce systemic myotoxicity (Gopalakrishnakone et al., 1997). In both cases, they lead to leakage of CPK (creatine phosphokinase) into the plasma of the victim (Mebs and Ownby, 1990; Mukherjee and Maity, 2002; Gutiérrez and Ownby, 2003). They induce acute muscle cell damage, causing hypercontraction and other cellular alteration, leading to the cell death (Gutiérrez and Lomonte, 1995). Studies have revealed that the catalytic activity of the enzyme does not have a significant role in the myotoxicity. For a review on the mechanism of snake venom phospholipase A_2 enzymes producing myotoxicity, see Gutiérrez and Ownby (2003).

A small number of PLA_2 enzymes exhibit cardiotoxicity (Lee et al., 1977; Fletcher et al., 1982; Chang et al., 1983; Huang et al., 1993). It has been opined that cardiotoxicity is independent of enzymatic activity, as there is no correlation between amount of phospholipid hydrolyzed and cardiotoxicity (Fletcher et al., 1982). The exact mechanism of induction of cardiotoxicity by PLA_2 enzymes has not been well established. In the case of OHV A-PLA_2, an acidic PLA_2 isolated from king cobra venom, cardiotoxicity is due to increasing levels of intracellular Ca^{2+}, as seen in isolated rat heart experiments (Huang et al., 1993).

Snake venom PLA_2 enzymes are also known to inhibit blood coagulation. These anticoagulant PLA_2 enzymes are classified into strong, weak, and nonanticoagulant enzymes, depending on the dose required to inhibit the blood coagulation (Boffa and Boffa, 1976; Verheij et al., 1980a). Strong anticoagulant PLA_2 enzymes inhibit the activation of FX to FXa by both enzymatic and nonenzymatic mechanism, and they inhibit the activation of prothrombin to thrombin by nonenzymatic mechanism (Stefansson et al., 1990; Kini and Evans, 1995; Kerns et al., 1999). On the other hand, weakly anticoagulant PLA_2 enzymes inhibit the activation of FX to FXa by an enzymatic mechanism, and they do not inhibit the activation of prothrombin to thrombin (Stefansson et al., 1990). For a review on structure, function, and mechanism of anticoagulant protein from snake venom, see Kini (2006).

Venom PLA_2 enzymes, which interfere in platelet function, can be classified into three distinct classes: A, B, and C. Class A consists of PLA_2 enzymes, which initiate platelet aggregation (Ouyang and Teng, 1979; Landucci et al., 1994). Class B PLA_2 enzymes cause only the inhibition of platelet aggregation but fail to initiate aggregation (Ouyang et al., 1983; Li et al., 1985). Inhibition of platelet aggregation by Class B PLA_2 enzymes can be either dependent on (Class B1) or independent of (Class B2) the catalytic activity (Kini and Evans, 1997). Class C show biphasic effects; at low concentration and short incubation time, they induce platelet aggregation, while at higher concentration or prolonged incubation, they inhibit platelet aggregation (Ouyang and Huang, 1984). The biphasic effects of this class of PLA_2 enzymes are either dose dependent (Class C1) or dose independent (Class C1) (Kini and Evans, 1997).

Edema-inducing PLA_2 enzymes have been reported from several snake venoms (Vishwanath et al., 1987, 1988; Angulo et al., 1997; Ali et al., 1999). The edema-inducing property of PLA_2 enzymes is due to the arachidonic acid released during the hydrolysis of phospholipids, which is converted into prostaglandins and leukotrienes. These prostaglandins and leukotrienes lead to

vascular permeability and edema formation at the site of snake bite (Angulo et al., 1997; Ali et al., 1999).

Several PLA_2 enzymes, such as mulgotoxin, *P. australis* VIII-A, and its homologs, induce myoglobinuria in experimental animals (Leonardi et al., 1979; Mebs and Samejima, 1980a, 1980b). Severe muscle damage results in the release of the myoglobin in the urine (myoglobinuria) (Takasaki et al., 1990a). In contrast, MiPLA1 from *Micropechis ikaheka* is the first known PLA_2 enzyme that induces hemoglobinuria (Gao et al., 1999). Insignificant hemolytic activity of MiPLA1 suggests that hemoglobinuria might be due to leakage in the kidney but not to lysis of RBC (Gao et al., 1999a).

Bactericidal activity of PLA_2 enzymes has been reported from several snake venoms (Forst et al., 1986, 1987; Paramo et al., 1998; Soares et al., 2000). Catalytically inactive Lys49 PLA_2 exhibits antibacterial activity, suggesting that the antibacterial activity is independent of the catalytic activity (Paramo et al., 1998).

VIII. TARGET MODEL AND PHARMACOLOGICAL SPECIFICITY

Although PLA_2 enzymes induce a variety of pharmacological effects, not all the effects are exhibited by all PLA_2 enzymes. For example, β-bungarotoxin is targeted to the presynaptic site. Similarly, *Daboia russellii* isoenzymes provide additional examples of targeting. When injected intraperitoneally, they exhibit distinct effects: one specifically induces hemorrhage in liver and kidney; the second damages the lungs, causing hemorrhage (Kasturi and Gowda, 1989); and the third kills mice through neurotoxic symptoms (T. V. Gowda, personal communication). Similar target specificity to a particular tissue was also found in *Trimeresurus flavoviridis* PLA_2 isoenzymes (Vishwanath et al., 1987). To explain the susceptibility of a tissue to a particular PLA_2 enzyme, we proposed the presence of specific target sites on the surface of target cells or tissues (Figure 8.6) (Kini and Evans, 1989b). These target sites are recognized by specific pharmacological sites on the PLA_2 molecule. These pharmacological sites are independent of, but sometimes overlapping with, the active site of the enzyme (Rosenberg, 1986). The target sites and pharmacological sites are complementary to each other in terms of charges, hydrophobicity, and van der Waal's contact surfaces (Kini and Evans, 1989b). High affinity between the target and pharmacological sites determines the specific pharmacological effects of PLA_2 enzymes. The proposed target sites could be either membrane lipids or proteins (glycoproteins). However, the ubiquitous presence of phospholipids, the susceptibility of only mature mice to β-bungarotoxin, and the species specificity argue against the membrane phospholipids as target sites (Kini and Evans, 1989b). Thus, we proposed that proteins (or glycoproteins) could act as specific target sites for PLA_2 enzymes. This can explain the observed species specificity of PLA_2 enzymes in exhibiting the pharmacological effects (Figure 8.6). The affinity toward the target sites may be altered by a simple mutation or posttranscriptional modification in the target protein. Such minor changes in the structure of the target protein among closely related species could affect the binding affinities, and thus the potency of pharmacological effects. Therefore, according to the target model, high-affinity protein-protein interaction between PLA_2 and membrane proteins in the specific target tissue determines the specific pharmacological effects of PLA_2 enzymes (Kini and Evans, 1989b). The affinity between PLA_2 and its target protein is in the low nanomolar range, whereas the binding between PLA_2 and phospholipids is in the high micromolar range. Such four to six orders of magnitude difference in affinity between the protein-protein interaction and the protein-phospholipid interaction explains why the interaction of the PLA_2 and its target protein governs the pharmacological specificity. However, phospholipids near the target proteins could also contribute to these specific interactions. So far, several specific target proteins for presynaptic PLA_2 toxins have been identified:

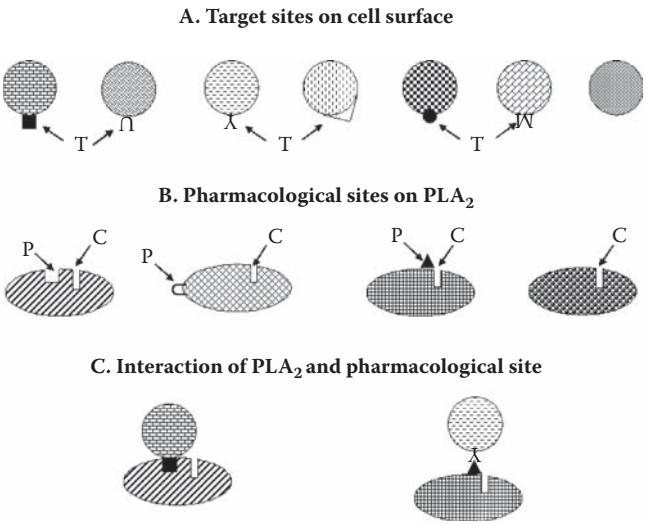
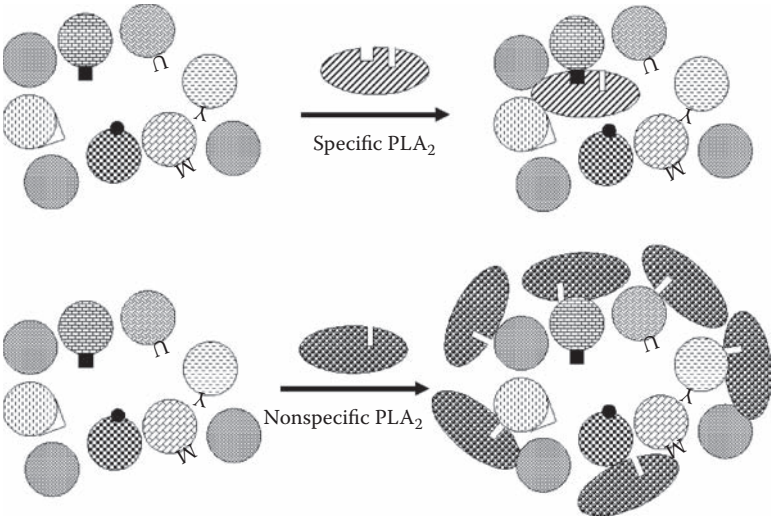


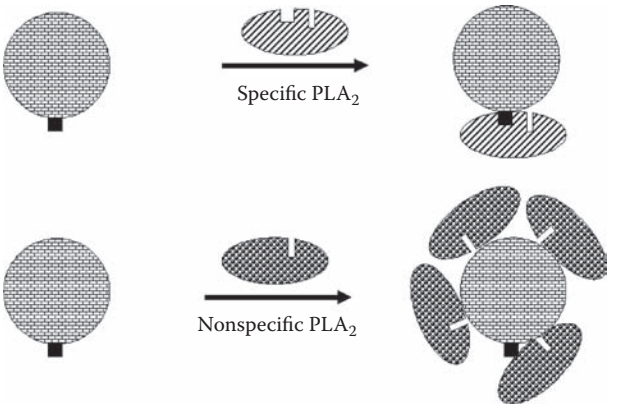
FIGURE 8.6 Schematic diagram of the target model to explain the pharmacological effects of venom PLA_2 enzymes based on Kini and Evans (1989b). (A) A target cell differs from a nontarget cell (extreme right) by the presence of a protein or glycoprotein target site (T). (B) PLA_2 molecule has a specific pharmacological site (P) on the surface of the molecule, in addition to the catalytic site (C). A nonspecific PLA_2 (extreme right) lacks the pharmacological site. (C) The PLA_2 pharmacological site interacts with the target site on the surface of a cell. The binding of the target site to the PLA_2 pharmacological site results in the pharmacological effects. (D) When PLA_2 enzymes are administered *in vivo*, the specific PLA_2 binds preferentially to the target cell because of its high affinity for the target site; a nonspecific PLA_2 will bind to many different types of cells. (E) In *in vitro* or *ex vivo* studies, isolated cells, tissues, or organs are incubated with PLA_2 enzymes, which can attack target cells and hence exhibit pharmacological effects. This is particularly true when enzymatic activity plays a major role in inducing the pharmacological effect. However, higher amounts (or catalytically higher activities) of nonspecific enzymes are required to induce effects similar to those of the specific PLA_2. (F) Specific PLA_2 enzymes bind to the target (or acceptor) protein in the plasma membrane (PM) with high affinity (~10^{-9} M or better). However, they also show low affinity (~10^{-4} to 10^{-6} M) binding to phospholipids, and specific binding studies always indicate both high- and low-affinity binding sites. The number of high-affinity binding sites per cell is low compared to low-affinity binding sites. Treatments to destroy the target proteins result in the loss of high-affinity binding, but not low-affinity binding. On the other hand, nonspecific PLA_2 binds to phospholipids with low affinity, and it does not bind to the target site. (G) The target site is a good fit for the pharmacological site in susceptible species. In the nonsusceptible species, either a mutation (M) or a posttranslational modification (PTM), such as glycosylation, is sufficient to alter the binding affinity of the PLA_2 to the target cell. This explains the observed species specificity in the ability of PLA_2 enzymes to exhibit pharmacological effects.

1. Voltage-sensitive K^+ channel for β-bungarotoxin (Black et al., 1988; Parcej and Dolly, 1989; Scott et al., 1990)
2. 85–88 kDa and 36–51 kDa proteins from rat brain synaptic membranes for OS_2 (Lambeau et al., 1989)
3. Neuronal pentraxin and taipoxin-associated Ca^{2+} binding protein (TCBP-49) for taipoxin (Dodds et al., 1995, 1997; Kirkpatrick et al., 2000)
4. Crocalbin, which binds crotoxin (Hseu et al., 1999) and crotoxin acceptor protein from *Torpedo* (CAPT), which also binds crotoxin (Križaj et al., 1996b; Faure et al., 2003)
5. A 53–56 kDa protein from bovine brain synaptic membranes for ammodytoxin A (Križaj et al., 1995)
6. 70 and 20 kDa proteins from *Torpedo* presynaptic membranes for ammodytoxin C (Križaj et al., 1997)

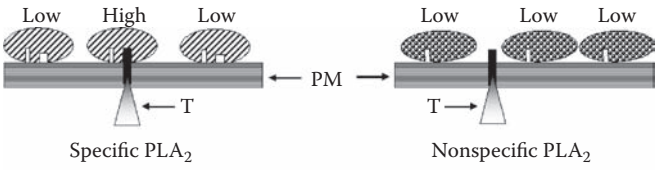
D. *In vivo* administration of PLA_2



E. *In vitro or ex vivo* studies of PLA_2



F. Binding affinity of PLA_2



G. Species specificity

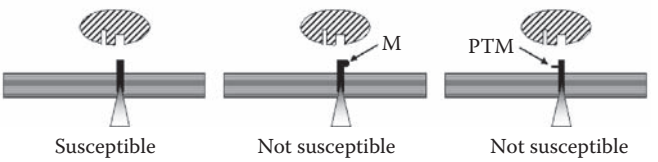


FIGURE 8.6 (continued).

7. Calmodulin (Vucemilo et al., 1998; Sribar et al., 2001), M-type receptor (Copic et al., 1999; Vardjan et al., 2001; Prijatelj et al., 2006), 14-3-3γ and ε proteins (Sribar et al., 2003) from porcine cerebral cortex for ammodytoxin C
8. N-type receptors from rat brain, identified to be the binding protein for SO_2, which might also play a role in central neurotoxicity (Lambeau et al., 1989)

An M-type receptor from skeletal muscle has been identified as the target protein for OS_1 and OS_2 (Lambeau et al., 1990). R180, isolated from the porcine brain cortex, is a high-affinity M-type receptor for ammyodotoxin (Prijatelj et al., 2006). Blood coagulation factor Xa is the target protein for the strongly anticoagulant PLA_2 enzymes (Stefansson et al., 1989, 1990; Evans and Kini, 1997; Mounier et al., 1998; Kerns et al., 1999). Target proteins that determine other pharmacological effects have not yet been identified.

A number of target proteins that have been identified are intracellular. Although internalization of PLA_2 enzymes and interference in the intracellular signaling are possibilities, such protein acceptors may not be important in the initial targeting of the PLA_2 enzymes to the specific target cell, as these proteins are not accessible at the cell surface.

Identification of the target proteins contributes significantly to our understanding of the mechanism of toxicity of PLA_2 enzymes. In addition, it clarifies the physiological role of the target protein and describes the susceptibility of a specific physiological system. This is particularly useful in developing new pharmaceutical agents affecting specific functions (for details on the methods used in identification, see Lambeau et al., 1997; Kini, 1997).

It is important to note that several PLA_2 enzymes can exhibit the same pharmacological effect through different mechanisms. For example, the neurotoxic effect can be induced by distinctly different mechanisms. Different neurotoxic PLA_2 enzymes could bind to different target proteins and exhibit similar presynaptic neurotoxic symptoms. The mechanism of some neurotoxic PLA_2 enzymes could be dependent on the enzymatic activity, while that of other neurotoxic PLA_2 enzymes could be independent of the enzymatic activity. This necessitates the presence of different neurotoxic sites responsible for the pharmacological effect. Therefore, care must be taken in generalizing the results from one PLA_2 enzyme to another.

IX. ROLE OF ENZYMATIC ACTIVITY IN PHARMACOLOGICAL EFFECTS

PLA_2 enzymes induce their pharmacological effects through mechanisms that are either dependent on or independent of enzymatic activity. In the mechanisms that are dependent on enzymatic activity, either the hydrolysis of intact phospholipids or the released products, such as lysophospholipids and fatty acids, can cause the pharmacological effect (Figure 8.7) (Kini and Evans, 1989b). Hydrolysis of phospholipids might change the membrane fluidity, disrupting the phospholipid packing and making it selectively permeable to ions and drugs. Lysophospholipids and fatty acids generated during hydrolysis also serve as precursors for lipid-derived mediators with a wide range of biological activities (Gelb et al., 1995, 1999; Dennis, 2000). Lysophospholipids may be metabolized to platelet-activating factors, which are potent inflammatory mediators (Kume and Shimizu, 1997; Jackson et al., 1998). They might also cause cell lysis, leading to the release of the inner content of the target cell. Fatty acids, particularly arachidonic acid, act as a precursor in the synthesis of secondary messengers such as prostaglandins and thromboxanes. The hydrolysis products could also alter the target cell behavior by interacting with various ligands, receptors, or enzymes (Figure 8.7).

In the mechanisms that are independent of enzymatic activity (Figure 8.8), binding to the target protein can cause the pharmacological effect by acting as an agonist or an antagonist. In antagonist-type interactions, PLA_2 could compete with the natural ligand or primary messenger for the target receptor, leading to the decrease in production/release of secondary messengers (such as cAMP, Ca^{2+}). In agonist-type interactions, PLA_2 could lead to signal transduction and increase in the secondary messengers in the target cell (Kini and Evans, 1989b). However, even in these cases, the

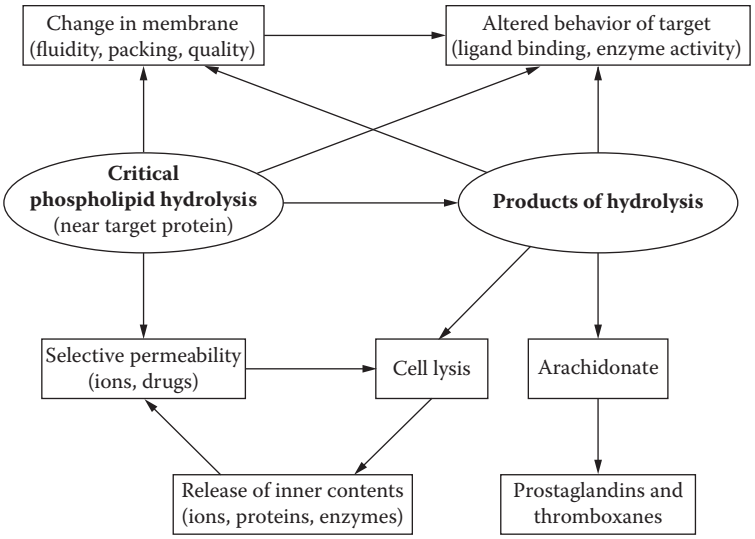


FIGURE 8.7 Enzymatic contribution of a PLA_2 enzyme to observed pharmacological effects.

enzymes would hydrolyze the phospholipids in the neighborhood of their target proteins, but this hydrolysis is inconsequential relative to the observed pharmacological effect (Stefansson et al., 1990; Evans and Kini, 1997; Mounier et al., 2000). It is important to note that some of the pharmacological effects may be due to the combination of both enzymatic and nonenzymatic mechanisms (Kini and Evans, 1989b). Therefore, it is critical to determine the role of enzymatic activity in inducing the pharmacological effects.

The simplest way to determine the role of enzymatic activity is to study the effect of the increase of (pre)incubation time of the PLA_2 enzyme with the complete system (Kini and Evans, 1988; Stefansson et al., 1990). In the case where phospholipid hydrolysis contributes to the pharmacological effect, the intensity of the effect increases with the incubation time, as more phospholipids are hydrolyzed with time. On the other hand, the intensity will not increase with the (pre)incubation times in nonenzymatic mechanisms. The presence or absence of (free fatty acid) serum albumin may help in resolving whether it is the loss of intact phospholipids or the released products that cause the pharmacological effects (Fletcher and Rosenberg, 1997; Rosenberg, 1997b).

Since PLA_2 enzymes require Ca^{2+} for their activity, chelators such as EDTA inhibit the enzymatic activity (Kini and Evans, 1988). The removal of Ca^{2+} ions sometimes affects the normal functions of the system. On the other hand, Ca^{2+} could also be replaced by other metal ions, such as Ba^{2+} and Sr^{2+} (Kelly et al., 1979). In some cases, Sr^{2+} can replace Ca^{2+} and support the normal functions of the system, but not the phospholipolytic activity of the PLA_2 enzyme. Nonenzymatic mechanisms are not affected by EDTA or metal ions, whereas the enzymatic contribution to the effect is compromised significantly. Care must be taken to ensure that the additional components (EDTA or different metal ions) do not contribute to the effect. Both of the above methods are suitable only for *in vitro* systems.

His48 is conserved in PLA_2 enzymes, and it plays a significant role in phospholipid hydrolysis (Scott, 1997). Alkylation of His48 residue leads to complete loss of enzymatic activity. Thus, His-modified PLA_2 enzymes are suitable for both *in vitro* and *in vivo* systems for studying the role of enzymatic activity. There is a general tendency to correlate the *in vitro* phospholipid-hydrolyzing ability to the pharmacological effects of a PLA_2 enzyme. *In vitro* studies can provide excellent information about the ability of the enzyme to hydrolyze specific phospholipids, based on head group specificity or fatty acyl group selectivity. They also help in determining the catalytic efficiency of the enzyme. However, in the *in vivo* situation, the enzymatic activity is influenced greatly by several

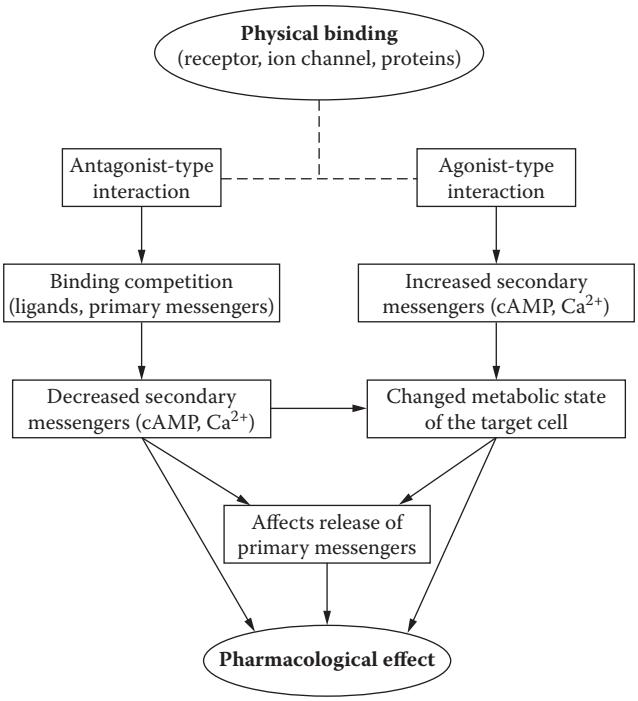


FIGURE 8.8 Nonenzymatic mechanism of a PLA_2 enzyme that produces pharmacological effects.

factors, such as targeting to specific proteins, availability of different classes of phospholipids in the neighborhood of the target protein, and the penetrating ability of the enzyme (see below). Rosenberg (1997a, 1997b) has stressed the importance of determining the phospholipid hydrolysis at the site of action.

Penetrability is another factor that plays an important role in inducing pharmacological effects (Kini, 1997). Phospholipids in plasma membranes are packed at higher densities than phospholipids in vesicles and liposomes, and PLA_2 enzymes hydrolyze phospholipids in the vesicles and liposomes more efficiently than those found in the plasma membranes. PLA_2 enzymes with higher penetrability cause more significant damage to the tightly packed cell membrane than those with lower penetrability. A PLA_2 enzyme may fail to exhibit its pharmacological effect, despite binding to the specific target protein, if it lacks penetrability. This is particularly important in the case of pharmacological effects that are dependent on phospholipid hydrolysis. However, an enzyme with lower penetrating ability can still cause phospholipid hydrolysis upon binding to its target protein, although at a slower rate. In these cases, a small amount of initial phospholipid hydrolysis could lead to membrane defects, due to both hydrolysis of intact phospholipids and accumulation of lysophospholipid and fatty acid products. Subsequently, these PLA_2 enzymes may be able to induce their pharmacological effect, and so PLA_2 enzymes with lower penetrability may exhibit an initial lag time. Penetrability is a critical factor in influencing the pharmacological potency of PLA_2 enzymes, and it is important to consider this factor during interpretation of the results of mutation and chemical modification studies. The loss of penetrability will significantly decrease the hydrolytic activity in both plasma membrane and liposomes.

The positive charges have been postulated to be responsible for penetrability in signal peptides (Verheij et al., 1980a) as well as for a large number of cytolytic proteins and peptides (Kini and Evans, 1989c). Most basic PLA_2 enzymes (but not all) have higher penetrability than neutral and acidic isoenzymes (Verheij et al., 1980a), probably because of the abundance of positive residues. This may explain why basic PLA_2 enzymes are generally more potent in inducing pharmacological

effects, such as tissue damage and toxicity, than neutral and acidic enzymes. However, it is important to bear in mind that basicity or penetrability alone does not confer pharmacological potency.

X. PHARMACOLOGICAL SITES

PLA_2 enzymes bind to target proteins through their specific pharmacological sites (Figure 8.6) (Kini and Evans, 1989b). The presence of pharmacological sites is supported by chemical modification studies (Yang and King, 1980; Yang et al., 1981; Babu and Gowda, 1994), studies using polyclonal and monoclonal antibodies, and analyses of interactions of inhibitors (Stiles and Choument, 1997; Gowda, 1997). Various chemical methods to modify specific amino acid residues have been used to identify pharmacologically relevant structural features. Despite systematic efforts, it has been difficult to pinpoint the residues or segments of PLA_2 enzymes that determine the pharmacological effects. As an alternative, various laboratories have used theoretical methods to identify specific pharmacological sites.

A. Presynaptic Neurotoxic Site

Several groups predicted the neurotoxic site of PLA_2 enzymes by theoretical methods. Dufton and his coworkers were the first to predict the site responsible for β-neurotoxicity by direct homology studies between neurotoxic and nonneurotoxic PLA_2 enzymes (Dufton et al., 1983; Dufton and Hider, 1983). Based on the hydropathic profiles of PLA_2 enzymes, Kini and Iwanaga (1986a) predicted that hydrophobic helix E may be important for presynaptic neurotoxicity (based on twenty-six amino acid sequences). More recent analysis of hydropathic profiles of forty single-chain neurotoxic PLA_2 enzymes showed a hydrophobic segment in the region of residues 80–110. However, several of the forty-seven nonneurotoxic PLA_2 enzymes also showed similar hydrophobicity, indicating a lack of correlation between neurotoxicity and hydrophobicity (Khan, 2002). Križaj and coworkers (1989) purified several PLA_2 isoenzymes from *Vipera ammodytes ammodytes* venom, which showed different neurotoxic potencies. From the amino acid sequences of three ammodytoxins they identified the neurotoxic site in the C-terminal segment of PLA_2 enzymes. Antibodies raised against the C-terminal peptide inhibited the neurotoxic effects of ammodytoxins, confirming the role of the C-terminal end in neurotoxic effects (Curin-Serbec et al., 1991). Site-directed mutagenesis studies reveal that Phe124 in the C-terminal end is important for neurotoxicity in ammodytoxin because substitution of Phe124 (aromatic) to Ile (aliphatic) produced a tenfold decrease in neurotoxicity. This indicated that the exposed aromatic amino acid in the C-terminal region might be important for the neurotoxicity of other similar toxins (Pungercar et al., 1999).

B. Myotoxic Region

The myotoxic site in presynaptically active PLA_2 enzymes has been identified by charge distribution (Kini and Iwanaga, 1986b). Myotoxic enzymes have a characteristic cationic site (residue charges +00+++00+), whereas nonmyotoxic enzymes lack this site. This cationic site is located toward the amino-terminal side of hydrophobic helix E (Kini and Iwanaga, 1986b), and together the hydrophobic and cationic sites form the complete myotoxic region. A similar combination of hydrophobic and cationic sites was also found in nonenzymatic myotoxins (Kini and Iwanaga, 1986b). Subsequently, a hydrophobic segment flanking the cationic site was found to occur commonly in various classes of cytolysins, including myotoxins, hemolysins, and antibacterial peptides (Kini and Evans, 1989c). The importance of this cationic site in cardiotoxins has been studied by chemical modification of Lys residues (Kini and Evans, 1989c). When the positive charges of the Lys residues are changed to negative or neutral charges by succinylation or carbamylation, respectively, cardiotoxin loses its cytolytic activity, but when guanidinated and the positive charges are retained, the cytolytic activity

remains intact (Kini and Evans, 1989a). A number of studies have supported this proposal of a specific cytolytic region (Blondelle and Houghten, 1991; Wade et al., 1992; Peck-Miller et al., 1994).

Lomonte and coworkers (2003) identified the C-terminal cationic/hydrophobic segment (115–129) as the heparin binding and cytolytic site of K49 PLA_2 enzymes as well as other myotoxins, with probable involvement of Lys36 and Lys38 (Lomonte et al., 2003). A peptide based on this site lysed endothelial cells, showed bactericidal effects, and induced skeletal muscle necrosis (Lomonte et al., 1994, 1999; Gutiérrez and Lomonte, 1997; Paramo et al., 1998; Nunez et al., 2001; Santamaria et al., 2005a, 2005b). The substitution of Tyr to Trp significantly increases the antibacterial activity, with minimal effect to mammalian cells (Paramo et al., 1998). The combined effect of ionic and hydrophobic interaction is responsible for the antibacterial activity, and the Trp residue plays a crucial role (Santamaria et al., 2005b). These studies also indicated that this site is responsible for cytolysis in muscle cells. Except for its location, the characteristics of this site resemble the predicted myotoxic site (Kini and Iwanaga, 1986b).

In their search for target proteins for myotoxic enzymes, Lambeau and coworkers (1990) showed that *Oxyuranus scutellatus* PLA_2 enzymes, OS_1 and OS_2, bind to M-type receptors from rabbit skeletal muscle. The residues of PLA_2 that are involved in binding M-type receptors were determined by using several mutants of porcine pancreatic enzymes. Their results indicated that the mutation of Gly30 and Asp49, which are present in the Ca^{2+} binding loop, drastically decreased the binding affinity. However, because many other PLA_2 enzymes that contain these residues do not bind to M-type receptors, there are probably other residues that are important for binding. The presence of an aliphatic or aromatic residue in the thirty-first position appears to be crucial for binding to M-type receptors, as mutating Leu31 to Arg31 in pancreatic PLA_2 resulted in decreased binding affinity (Lambeau et al., 1995). At present, the role of this region in myotoxicity is not clear.

C. Antimicrobial Region

The bactericidal activity of PLA_2 enzymes in Gram-positive bacteria requires recognition of anionic sites and subsequent hydrolysis of the phospholipid membrane. In Gram-negative bacteria, it requires the synergistic action of a bactericidal/permeability-increasing protein and depends on the hydrolytic activity (Weiss et al., 1979; Elsbach et al., 1985; Koduri et al., 2002). The antibacterial site of snake venom PLA_2 enzymes was determined by comparing the amino acid sequences and by chemical modification (Forst et al., 1986, 1987), while in the case of pancreatic and human inflammatory PLA_2 enzymes, it was determined by site-directed mutagenesis. The overall basicity and presence of a cluster of basic residues on the surface of the N-terminal helix is responsible for the bactericidal action of PLA_2 enzymes (Weiss et al., 1991, 1994).

D. Anticoagulant Region

By a systematic and direct comparison of the amino acid sequences of strong, weak, and nonanticoagulant enzymes, Kini and Evans (1997) identified the anticoagulant region to be between residues 54 and 77. The major difference between the strong anticoagulant and weakly or nonanticoagulant PLA_2 is the replacement of the residues in the anticoagulant region. Negatively charged Glu54 is replaced by neutral residue, positively charged Lys54 is replaced by negatively charged Glu, uncharged Gly57 is substituted by negatively charged Glu, positively charged Lys75 is replaced by Ser or Thr, and positively charged Lys77 is replaced by negatively charged Glu or Asp (Kini and Evans, 1987). Overall, this region is positively charged in strong anticoagulant and negatively charged in weak or nonanticoagulant PLA_2. All chemical modification studies have supported this prediction (Kini, 2006), as have site-directed mutagenesis studies (Inada et al., 1994; Mounier et al., 2000) and synthetic peptide studies (Mounier et al., 1998; Kini and Evans, unpublished observations).

XI. IMPORTANCE OF IDENTIFICATION OF PHARMACOLOGICAL SITES

The pharmacological sites of PLA_2 enzymes determine the affinity between the PLA_2 and target proteins. The identification of pharmacological sites helps in (1) understanding the structure-function relationships of PLA_2 enzymes, (2) developing strategies to neutralize the toxicity and pharmacological effects by targeting these sites, and (3) developing prototypes of novel research tools and pharmaceutical drugs. As these sites help in localizing or targeting the PLA_2 to a specific tissue or cell, they act as protein "postal addresses" that help in "delivering" the specific PLA_2 to a specific cell. These addresses could be exploited to develop a delivery system that is targeted to a specific tissue or cell. Since PLA_2 enzymes affect almost every vital organ or tissue, one could potentially obtain addresses to many types of cells and tissues. This possibility should provide a strong impetus for studying structure-function relationships of PLA_2 enzymes and identification of more pharmacological sites (Kini, 2003).

XII. ORIGIN AND EVOLUTION OF THE PLA_2 GENE

Phylogenetic analysis of mammalian pancreatic and venom PLA_2 genes reveals that they have a common origin from a nontoxic ancestral gene; however, human and Group I genes have followed a common pathway of evolution (Jeyaseelan et al., 2000), but Group II PLA_2 genes evolved separately after species diversification (Fujimi et al., 2002a, 2002b). Typically genes encoding Group I PLA_2 enzymes are about 4 kb, which follows a typical structural organization comprised of four exons interrupted by three introns (which resembles the human pancreatic PLA_2 gene). However, the size of intron 3 in the venom PLA_2 gene is smaller than the pancreatic counterpart (Jeyaseelan et al., 2000; Armugam et al., 2004). The smaller size of intron 3 is attributed to adaptive evolution of snake venom PLA_2 enzymes, and introns 3 and 4 might code for addition pharmacological properties (Jeyaseelan et al., 2000). Unlike the mammalian PLA_2 gene, which contains a propeptide sequence and is processed during maturation, some of the snake venom PLA_2 enzymes retain this, while in others it is removed. The characteristic feature of the mammalian PLA_2 enzyme is the presence of a pancreatic loop, which is encoded by exon 3; in some snake venom PLA_2 enzymes this loop is present, for example, in king cobra (Huang et al., 1997), Brazilian coral snake (Francis et al., 1997), and Australian elapid venoms, but it is absent from most of the other venom PLA_2 enzymes. The loss of this loop has been interpreted as providing an adaptive advantage for the development of toxic properties among the venom PLA_2 enzymes (Davidson and Dennis, 1990). It was postulated that with the loss of the pancreatic loop from exon 3, additional toxicity and enhanced enzymatic activity have been acquired by some snake venom PLA_2 enzymes (Armugam et al., 2004). Particularly in the case of *N. sputatrix* PLA_2 enzymes, the loss of the pancreatic loop has followed a Darwinian type of accelerated evolution, while the mammalian PLA_2 enzyme has undergone neutral evolution, retaining the pancreatic loop and showing a reduced rate of mutation in the gene. However, comparison of PLA_2 enzymes of Australian and king cobra venoms with those of the more derived cobra species *N. sputatrix* and *N. atra* reveals that the PLA_2 enzymes from the former two species appear earlier in the evolution of these enzymes, demonstrating that the presence of the pancreatic loop is an ancestral property (Jeyaseelan et al., 2000).

Group II PLA_2 enzymes, though sharing similarities in catalytic activity, are structurally different from Group I enzymes. The gene organization of Group II is also different from that of Group I, as it has five exons and four introns (Kordis and Gubenšek, 1996), similar to human synovial PLA_2 gene (Nakashima et al., 1993). The first exon encodes for the 5′ UTR (untranslated region), the second exon encodes for most of the signal peptide, and the matured protein is encoded by exons 3–5 (Kordis and Gubenšek, 1996). However, the Crotalinae Group II genes have the same organization as Group I genes (four exons and three introns), but have the same protein sequence as that of Group II PLA_2 enzymes. This irregular structure in the Crotalinae gene is due to the deletion of 40 bp in exon 1, which resulted in abolishing the splicing site of intron I (Kordis and Gubenšek, 1996).

Fujimi and coworkers (2002b) analyzed the invading sequences in the Group IA gene and showed that it is responsible for divergence of groups in the phospholipase A_2 gene. In the case of the Group I gene, integration of two types of CR1-like line sequences occurred before the divergence of Group IA and Group IB (Fujimi et al., 2002b). In the case of the Group II genes, such insertions were also observed, but the position is different from that of the Group I gene, suggesting that CR1-like sequences are conserved among PLA_2 genes, and that duplication events occurred after the integration. Further, it has been observed that Bov-B line and CR1-like sequences were found in some species of Group II genes, but their insertion positions were different (Kordis and Gubenšek, 1998). This observation suggests that integration events occurred independently after species diversification (Fujimi et al., 2002b).

Snake venoms contain a large number of isoenzymes that exhibit different physiological functions, although they share similarities in their amino acid sequence and three-dimensional structure. Venom PLA_2 isoenzymes are products of multiple genes and are known to evolve through gene duplication, followed by accelerated evolution to acquire diverse physiological functions (Ogawa et al., 1992, 1995, 1996; Nakashima et al., 1993, 1995; Kordis and Gubenšek, 1996; Nobuhisa et al., 1996; Chuman et al., 2000; Ohno et al., 2003). The first instance of accelerated evolution was observed in the cDNA encoding PLA_2 isoenzymes from *Protobothrops flavoviridis* venom gland (Ogawa et al., 1992). Accelerated evolution is due to more rapid changes in the protein-coding regions than in the UTRs. In the protein-coding regions, nonsynonymous substitutions are close to or greater than synonymous substitutions. Studies have also shown that the exons in PLA_2 genes mutate faster than introns (Ogawa et al., 1992; Nakashima et al., 1993, 1995; Nobuhisa et al., 1996; Chuman et al., 2000; Chijiwa et al., 2003). Such an accelerated evolution is proposed to result in the production of diverse pharmacological properties (Ogawa et al., 1996; Chijiwa et al., 2003). This phenomenon of accelerated evolution of PLA_2 enzymes is well documented in venoms of crotaline (*Crotalus scutulatus scutulatus*, *Ovophis okinavensis*, *Protobothrops flavoviridis*, and *Trimeresurus gramineus*), viperine (*Daboia palaestinae* and *Vipera ammodytes*), and elapid (*Austrelaps superbus* and *Naja kaouthia*) snakes (Kihara et al., 1992; Nakashima et al., 1993; Ogawa et al., 1995, 1996; Shimohigashi et al., 1996; Nobuhisa et al., 1996; Kordis et al., 1998; Ohno et al., 1998; Chuman et al., 2000). Kini and Chan (1999) compared the structures of 127 snake venom PLA_2 enzymes and showed that mutational hot spots occur on the surface of the molecule. They showed that substitutions occurred about 2.6 to 3.5 times greater in surface residues than in the buried residues. The surface residues are important in molecular recognition of target proteins, so substitutions in the surface residues would contribute directly toward modifying the molecular surface. PLA_2 enzymes interact with the target sites through the surface residues, and thus these residues form a scaffold for adaptive modification through substitution to induce different pharmacological effects via a process of neofunctionalization (Lynch, 2007).

By comparing the large number of nucleotide and protein sequences of PLA_2 enzymes, Lynch (2007) concluded that in Group I PLA_2 enzymes, gene duplication and diversification occurred after speciation. In contrast, functional diversification in Group II occurred before the diversification of the species (Lynch, 2007). Thus, the accelerated evolution of exons and surface substitutions plays a significant role in the evolution of new isoenzymes by altering target specificity. Accelerated evolution continued until it acquired a stable function and then evolved with lowered mutation rates, favoring functional conservation (Ohno et al., 2003). However, in the case of a unique sea snake (*Aipysurus eydouxii*), the venom has been found to be evolving at a much slower rate than other terrestrial and sea snake venoms, and PLA_2 enzymes also followed a decelerated mode of evolution. Although multiple cDNAs of PLA_2 isoenzymes were found in the venom gland, the number of mutations (two single synonymous mutations) were far lower than in other species. However, even functionally important residues have been found to be mutated. Since this snake feeds exclusively on fish eggs, these mutations do not affect their ability to obtain food (prey capture). Therefore, decelerated evolution in *A. eydouxii* PLA_2 enzymes is primarily due to a lack of selective pressure on venom composition, and secondarily to morphological changes, the loss of fangs, and a highly atrophied venom gland (Li et al., 2005).

XIII. FUTURE PROSPECTS

Snake venom PLA_2 enzymes are small enzymes that exist as isoenzymes and interfere in various physiological processes of a victim. These enzymes provide a great challenge to protein chemists as subtle and complex puzzles in structure-function relationships. A better understanding will contribute to our knowledge of protein-protein interactions, protein targeting, and protein engineering, and to the development of better-targeted delivery systems. Further research in identifying target proteins will help determine details of the mechanisms of the pharmacological effects at the cellular and molecular levels. Studies in these areas will result in new, exciting, and innovative opportunities and avenues in the future, both in finding answers to the toxicity of PLA_2 enzymes and in developing proteins with novel functions.

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9 Snake Venom Acetylcholinesterase

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CONTENTS

Acetylcholinesterase (AChE) belongs to a distinct family of serine hydrolases and is found in both synaptic and nonsynaptic locations. At the synapses, it plays a major role in the hydrolysis of the neurotransmitter acetylcholine (ACh), while in nonsynaptic tissue its function is unclear. Snake venom, particularly from the family Elapidae, is a common nonsynaptic source of AChE. In the Elapidae, the genus *Bungarus* is well known as a prominent source, as it contains the highest levels of AChE detected in snake venoms (close to 8 mg/g of dried venom; 0.8% w/w). A review of the literature shows that no other tissues or biological fluids exhibit such a high level of AChE activity. Comparative amino acid sequence studies have revealed that venom AChE exhibits significant homology with the catalytic domain of cholinesterases from other sources but contains unique monomeric properties. Venom AChE from all species assayed has a similar sensitivity to different inhibitors, such as edrophonium, tacrine, propidium, BW284C51, and decamethonium; one exception is fasciculin, which varies in its inhibitory potency depending on the source of the venom AChE. From an evolutionary point of view, the enhanced complexity of venoms may provide a selective advantage to snakes. In this context, either the presence of large amounts of AChE or the presence of fasciculins is an important feature. In fact, the existence of elevated amounts of AChE or fasciculin in snake venom may be related to disruption of cholinergic transmission in the central nervous system and at the neuromuscular junction of the prey. Furthermore, the presence of AChE in snake venom, because of its monomeric form and high level of activity, provides an important source of the enzyme for biochemical studies.

I. INTRODUCTION

Acetylcholinesterase (AChE) can be found in high amounts in the venom of most Elapidae, with the notable exception of mambas, which possess fasciculins, inhibitors of AChE (Karlsson et al., 1984; Bawaskar and Bawaskar, 2004; Frobert et al., 1997; see also Chapter 15, this volume). The Elapidae includes species such as the cobras (*Naja* and other genera) of Asia and Africa, the mambas (*Dendroaspis*) of Africa, the kraits (*Bungarus*) of Asia, the coral snakes (*Micrurus*) of the Americas, and the Australian elapids such as the coastal taipan (*Oxyuranus scutellatus*), tiger snakes (*Notechis*), king brown snake (*Pseudechis australis*), and death adders (*Acanthophis*), and the sea kraits and sea snakes. The enzyme AChE is very important for the communication between excitable cells, particularly between nerves and muscle (Massoulié et al., 1993). The existence of elevated amounts of AChE or fasciculin in snake venom may be related to selective pressures favoring the evolution of compounds that disrupt cholinergic transmission in the central nervous system and at the neuromuscular junction of the prey. Here we will briefly review the role of ACh and AChE in the mammalian body and present several points of evidence suggesting that although snake venom AChE shares similarities with mammalian AChE, it has unique biochemical properties and functions. In fact, the presence of AChE or fasciculin may confer unexpected properties to the venom and probably has contributed to the evolutionary adaptation of the snakes' feeding habits and prey capture strategies.

A. Acetylcholine

Acetylcholine was the first chemical agent reported to establish a communication link between two distinct mammalian cells, and it acts by propagating an electrical stimulus across the synaptic junction. The structure of acetylcholine is displayed in Figure 9.1. At the presynaptic neural terminus, an electrical impulse triggers the release of ACh, which accumulates in the synaptic cleft via exocytosis from vesicles. After its release into extracellular space, ACh binds to ACh receptors (AChRs) on the postsynaptic surface (commonly the motor endplate; Figure 9.2), and the ACh-AChR binding induces transmission of neural impulses to the postsynaptic site (endplate or neuron). Finally, ACh is rapidly degraded by the enzyme AChE, abundant in the synaptic cleft, resulting in the formation of choline and acetic acid (Kelly et al., 1979; Dunant et al., 1980; MacIntosh, 1981) (Figure 9.2). Moreover, ACh hydrolysis can be carried out by a related but less specific enzyme, butyrylcholinesterase (BChE), also known as serum cholinesterase or pseudocholinesterase (Dave et al., 2000; Li et al., 2000).

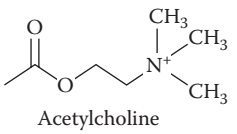

FIGURE 9.1 Chemical structure of acetylcholine.

B. Acetylcholinesterase

Acetylcholinesterase is present in all vertebrates, particularly in the muscle and nervous tissue, and plays a fundamental role in the hydrolysis of the neurotransmitter acetylcholine:

$$\text{acetylcholine} \xrightarrow{\text{AChE}} \text{acetate} + \text{choline}$$

Structurally and functionally, both AChE and BChE are serine hydrolases that belong to the esterase family within the higher eukaryotes. The efficacy with which AChE controls neurotransmitter lifetime in the synaptic cleft depends not only on its enzymatic activity but also on its density and location relative to acetylcholine receptors (Martinez-Pena y Valenzuela, 2005). The enzyme is also found in nonsynaptic locations, such as in blood cells, notably erythrocytes and lymphocytes (Kawashima and Fujii, 2000; Thiermann et al., 2005). Different types of snake venoms, such as

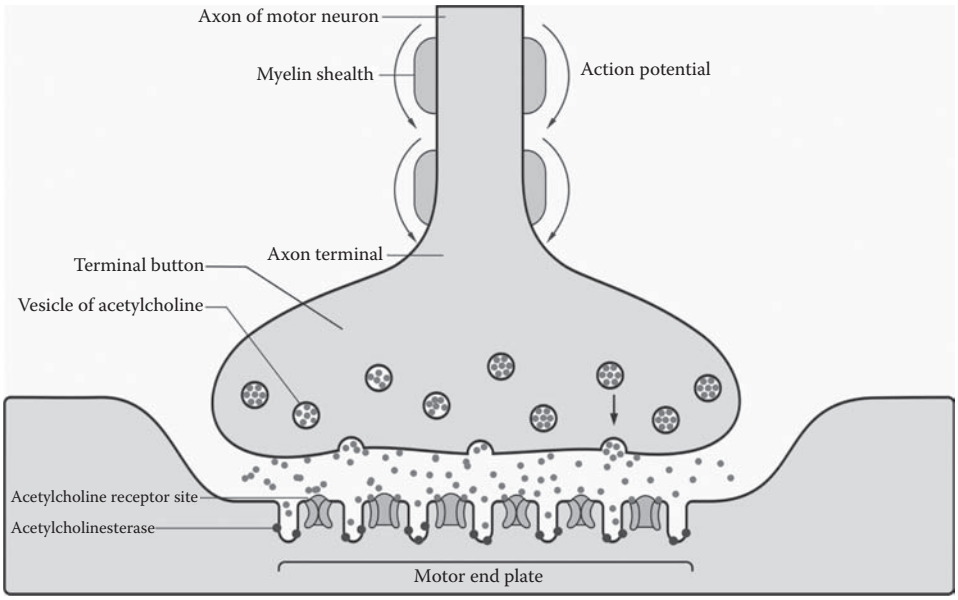

FIGURE 9.2 General components of the neuromuscular junction.

venoms from the genera *Bungarus*, *Hemachatus*, *Naja*, and *Ophiophagus* (family Elapidae), are a common nonsynaptic source of AChE (Iwanaga and Suzuki, 1979; Frobert et al., 1997), though the function of AChE during envenomation remains unclear.

C. Snake Families with Venoms That Possess AChE

The actual distribution of AChE in various snake venoms is poorly known due to the limited number of published accounts. However, snake venoms are the richest source of AChE known and contain only the soluble globular form of AChE. Of the approximately 2,500 different species of snakes, about 20% are venomous, and these are divided into numerous subfamilies of the families Elapidae and Viperidae (Figure 9.3) and the polyphyletic family Colubridae. Among these, the Elapinae is the main subfamily that possesses significant AChE activity. The Crotalinae and Viperinae subfamilies lack this enzyme (Frobert et al., 1997), and the presence of AChE is unknown in the Hydrophiinae. AChE activity has also been demonstrated in rear-fanged snakes of the family Colubridae, such as the Puerto Rican racer (*Alsophis portoricensis*), brown treesnake (*Boiga irregularis*; Mackessy et al., 2006), and *B. blandingi* and *B. dendrophila* (Broaders and Ryan, 1997; Mackessy, 2002). In the Elapinae, all venoms assayed contain AChE, except those from the genus *Dendroaspis* (mambas). In *Dendroaspis*, instead of AChE, venoms contain very potent reversible inhibitors of AChE, the fasciculins, which bind at the peripheral site of the enzyme (see Chapter 15, this volume). Venom of the coral snakes (*Micrurus*) is also a rich source of AChE (Tan and Ponnudurai, 1992). The catalytic characterization of affinity-purified AChEs from the venoms of four elapid genera showed that they share features of other vertebrate AChEs, such as inhibition by eserine, preferential hydrolysis of acetyl rather than of propionyl or butyryl esters, and substrate inhibition (Frobert et al., 1997).

II. GENERAL STRUCTURE OF AChE

AChE was first discovered in the 1930s and was found to be one of the most efficient enzymes, with an exceptionally high turnover number. Structure-activity studies have shown several major domains within the protein (Figure 9.4): a catalytic active site composed of two subsites, the aromatic gorge

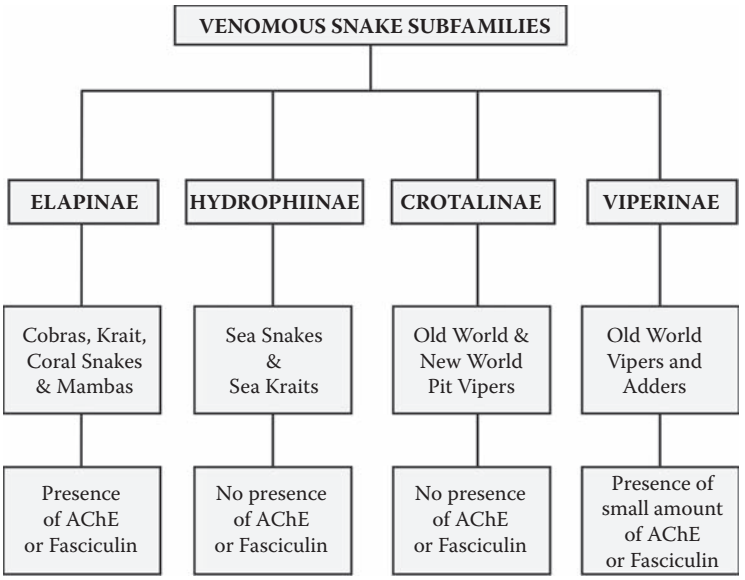

FIGURE 9.3 Presence of AChE or fasciculin in the front-fanged venomous snake subfamilies. Note that some rear-fanged members of the Colubridae also produce venoms with AChE.

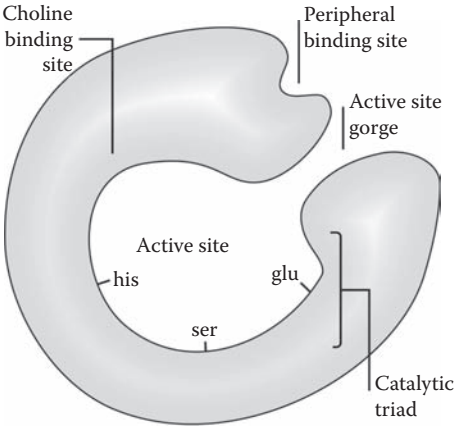

FIGURE 9.4 Structural features of acetylcholinesterase. (Adapted with permission from Soreq and Seidman, Nature Publishing Group, 2001.)

in which the catalytic active site lies, and a peripheral anionic site, distinct from the catalytic site (Rosenberry et al., 2005). Structure-activity studies have also shown that there are probably two binding domains in the active site: an ionic site containing a glutamate residue that can bind with the cationic head of ACh (van der Waals interactions likely contribute significantly) and an esteratic site that contains serine and histidine residues, which function as the acid/base catalyst domain (Kabachnik et al., 1970; Soreq and Seidman, 2001; Rosenberry et al., 2005).

The hydrolysis of the neurotransmitter ACh occurs at the esteratic site, which is functionally coupled to a substrate binding area containing a distinct anionic locus (Forede and Wilson, 1971; Rosenberry, 1975; Quinn, 1987; Massoulié et al., 1993). The ester group of acetylcholine allows hydrogen bonding with an asparagine residue of AChE. The hydrophobic area of AChE is responsible for binding the alkyl substituents of acetylcholine and is located close to the esteratic and

anionic site (Rosenberry, 1975; Quinn, 1987; Massoulié et al., 1993). The negative charged group in the active site of cholinesterase, which is at a distance of 4.5 to 5 Å from the active site serine residue, accommodates the quaternary ammonium moiety of the substrate via ion pair formation between the cationic side chain of the substrate and the anionic amino acid side chain (Forede and Wilson, 1971; Rosenberry, 1975; Quinn, 1987).

A most interesting aspect of this enzyme is the peripheral anionic site (PAS) on its surface. Site-directed labeling and mutagenesis studies indicate the location of the PAS at the entrance of an active center gorge (Barak et al., 1994). This site has the ability to bind diverse types of ligands. Six residues have activity within this site: Trp286, Tyr72, Tyr124, Glu285, Asp74, and Tyr341, which are located on the opposite side of the gorge entrance. This arrangement of residues exhibits flexibility, which accommodates many different ligands, and also allows for their conformational mobility (Ordentlich et al., 1995).

The crystal structure of *Torpedo californica* AChE confirmed the previous hypothesis about the structure and mechanism of substrate hydrolysis via the catalytic triad (Sussman et al., 1991, 1992). The esteratic serine (Ser200) is assisted by His440 and Glu327 at an appropriate hydrogen bonding distance located near the bottom of a 20 Å deep narrow gorge. Interestingly, the catalytic triad of AChE includes glutamate rather than aspartate, which is the most important residue of the catalytic triad of serine proteases. The three-dimensional structure of AChE indicates that the active site gorge contains negative ionic charges. Two anionic residues within the gorge, Glu327 and Asp443, are essential for the catalytic machinery of the enzyme. Glu327 is a member of the catalytic triad, while Asp443 is involved in hydrogen bonding with a water molecule to keep the water molecule in a position suitable for the chemical reaction. Removal of the Asp443 reduced the catalytic activity of the enzyme (Neville et al., 1992). A molecular modeling study revealed that ACh binds with some of fourteen aromatic residues located near the active site gorge, instead of binding to the negatively charged anionic site (Sussman et al., 1991; Radic et al., 1992).

It has been suggested that Glu199 binds with catalytically essential water molecules (Sussman et al., 1991). It had been speculated that Glu199 was involved in substrate inhibition, but this speculation was proved incorrect when similar results were obtained after replacement of Glu199 by glutamine. Thus, it was shown that the presence of an anionic charge at this location is not responsible for substrate inhibition (Radic et al., 1992). One important feature that was pointed out from the substitution of Trp84 by alanine in human AChE is that it causes a 3,000-fold decrease in the rate of hydrolysis of ACh (Soreq et al., 1992; Ordentlich et al., 1995). Additionally, an essential role for Trp84 was confirmed from experiments with mice, where mutation of Trp84 to alanine caused a 50-fold decrease in the hydrolysis of acetylcholine (Radic et al., 1992).

A. Mechanism of Acetylcholine Hydrolysis

The catalytic triad of AChE consists of serine, histidine, and glutamate. The serine and histidine residues at the catalytic site are involved in the hydrolysis of acetylcholine (Figure 9.5). The serine residue acts as a nucleophile, while histidine acts as an acid/base catalyst in the hydrolysis process. Serine alone is unable to catalyze hydrolysis of an ester, but the dual function (acid/base catalyst) of histidine increases the reactivity (nucleophilicity) of the serine $^{-}$OH and overcomes this chemical impediment. The hydrolysis of the substrate (ACh) is very fast. Within 100 μs, AChE catalyzes hydrolysis of acetylcholine by passing through the following steps:

Step 1: Acetylcholine binds at the active site through ionic and hydrogen bonding. The oxygen from the serine residue has one pair of electrons that acts as a nucleophile and forms a bond with the ester from acetylcholine. Thus, the nucleophilic addition opens up the carbonyl group of the ester.

Step 2: Histidine acts as a base and removes the proton, making serine more nucleophilic.

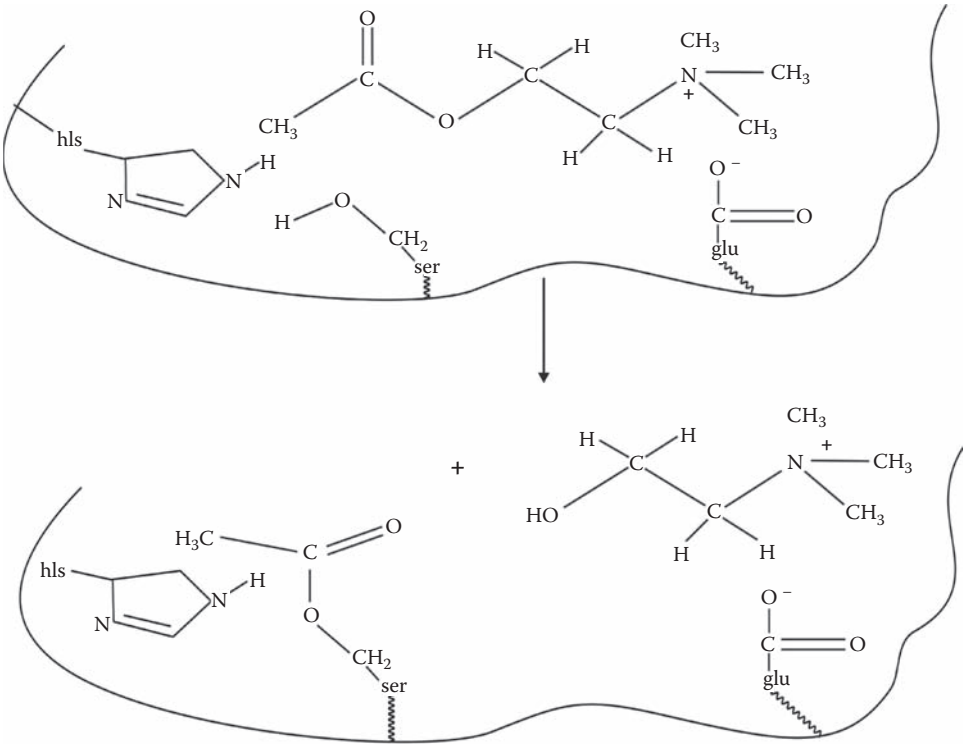

FIGURE 9.5 Binding of acetylcholine to the active center of acetylcholinesterase. (Adapted with permission from Soreq and Seidman, Nature Publishing Group, 2001.)

Step 3: Histidine acts as an acid by protonating the oxy-R group, making it a better leaving group.
Step 4: The carbonyl group reforms and repels the alcohol portion of the ester (choline).
Step 5: Choline leaves the active site and is replaced by a water molecule.
Step 6: The water oxygen has a single pair of electrons and acts as a nucleophile by attacking the acyl group.
Step 7: Histidine acts as a base and removes the proton, which makes serine more nucleophilic.
Step 8: Histidine acts as an acid by protonating the intermediate, making it a better leaving group.
Step 9: The protonation causes the serine residue to release the acetic acid in which the carbonyl group is reformed.
Step 10: Acetic acid leaves the active site and the cycle can be repeated, depending upon the availability of another molecule of acetylcholine.

This series of reactions is summarized in Figure 9.6.

B. Molecular Forms of Snake Venom Acetylcholinesterase

In *Torpedo* and mammalian AChE genes, the catalytic domains are coded by common exons, followed by alternative spliced sequences encoding C-terminal peptides, characterized as T (tailed), H (hydrophobic), and R (read-through) subunits. H subunits generate amphiphilic glycolipid-anchored dimers, while T subunits produce both amphiphilic (G1, G2, and G4 forms) and non-amphiphilic asymmetric forms. Interestingly, all of these subunits have the same catalytic property

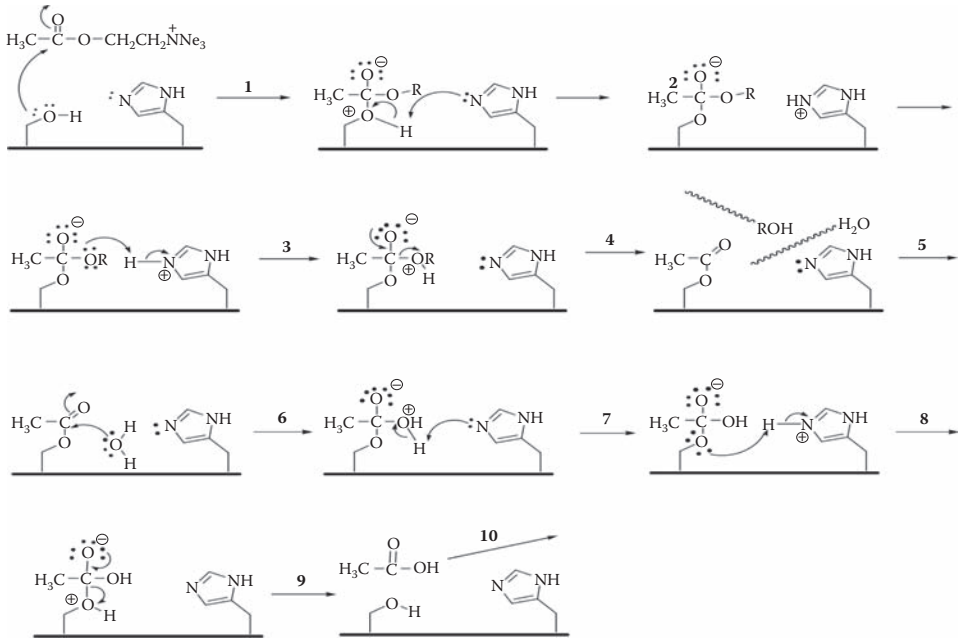

FIGURE 9.6 Mechanistic steps of acetylcholine hydrolysis catalyzed by AChE. (Modified from Patrick, 2001.)

for substrate hydrolysis but produce different molecular enzyme forms. A number of different forms, which are the oligomers of subunits H and T, have been reported (Massoulié et al., 1993). The snake AChE gene possesses an alternative spliced sequence of T and a new type of S-exon that encodes the C-terminal peptide. The H-exon, which is commonly found in mammalian and *Torpedo* AChE, is absent in snake AChE. Snakes of the genus *Bungarus* possess a single AChE gene containing a novel alternative exon, S, located downstream from the T-exon. The alternative splicing generates S-mRNAs in the venom gland, while in the liver and muscle both S-mRNAs and T-mRNA are produced. In *Bungarus* muscle, AChE, which is derived from an $AChE_T$ subunit, is mainly present in globular forms (G_1 and G_4) and in smaller amounts in asymmetric forms (A_8 and A_{12}). The snake liver possesses both AChE and BChE. In the liver, AChE is found in a nonamphiphilic monomeric form (G_1), while BChE is in a nonamphiphilic tetrameric form (G_4) (Massoulié et al., 1999).

A comparison of amino acid sequences revealed that snake venom AChE is different from both human ($AChE_H$) and *Torpedo* ($AChE_T$) AChE, primarily due to the C-terminal sequence. Venom AChE has a typical C-terminal sequence that is given the name of SARA due to the last four amino acids of the sequence, which give the terminus a hydrophilic characteristic (Massoulié et al., 1999). However, snake venom AChE has the same catalytic triad as that of other AChEs and hydrolyzes the substrate in the same way (as shown in Figures 9.4 and 9.5).

C. Molecular Weights and Isoelectric Points

AChE purified from snake venom consists of soluble, hydrophilic monomers. In the Central Asian cobra (*Naja oxiana*, formerly *N. n. oxiana*) venom, AChE has a molecular weight ranging from 65 to 69 KDa. The formation of isoforms is caused by the posttranslational deamidation of asparagine and glutamine residues (Raba and Aaviksaar, 1982). The different isoelectric points are caused by the difference in the number of free carboxyl groups of glutamic acid or aspartic acid. The molecular weight of desert cobra (*Walterinnesia aegyptia*) AChE, 67 ± 3 KDa, is essentially the same as that of *N. oxiana*. However, in other sources of AChE it is possible to find a number of

subunits connected through disulfide bonds, and the enzymes have a different molecular weight (Raba et al., 1979; Li and Bon, 1983; Al-Saleh et al., 1994). The isoelectric points of *W. aegyptia* AChEs range between pH 7.4 and 7.9, differing from other venom AChEs, which have isoelectric points ranging from pH 6.3 to 7.3 (Duhaiman et al., 1996). These data indicate that the *W. aegyptia* AChE isoforms have more basic amino acid residues than does the enzyme from other snake venoms. An investigation on purified AChEs from *Bungarus* revealed that this venom contains a monomeric form of AChE with a molecular weight of 70 KDa, similar to the monomeric structure of AChE from *N. oxiana* venom. The isoelectric points of *Bungarus* AChEs range from 5.2 to 5.8 (Cousin et al., 1996b).

III. ENZYMATIC AND BIOCHEMICAL PROPERTIES

Elapid AChEs exhibit biochemical properties similar to those of membrane-bound AChEs, in terms of both substrate hydrolysis and inhibition by excess substrate (Kesvatera et al., 1979; Kumar and Elliot, 1975; Agbaji et al., 1984; Frobert et al., 1997). The elapid AChE enzyme is stereospecific in the hydrolysis of acetyl-β-methylcholine and does not hydrolyze butyryl, propyonil, or benzoylcholine (Frobert et al., 1997). Venom AChE differs from other vertebrate tissue AChEs in that it is soluble and composed of nonamphiphilic monomers.

Snake venom AChE is also more stable than other AChEs. Among snake venom AChEs, *Bungarus* AChE is quite stable compared with those of *Hemachatus*, *Ophiophagus*, and *Naja* (Frobert et al., 1997). Rochu et al. (2001) studied the thermal stability of monomeric *Bungarus* AChE using capillary electrophoresis and observed that the wild-type is a stable enzyme under standard conditions. Furthermore, we found in our laboratory that the Sindhi krait (*Bungarus sindanus*) venom AChE is stable at 45°C. The enzyme lost only 5% of its activity after incubation at 45°C for 40 min (unpublished data).

In our laboratory we have observed a significant increase in the substrate inhibition of the Sindhi krait (*Bungarus sindanus*) venom AChE by using a high ionic strength buffer. In a lower concentration buffer (10 mM phosphate, pH 7.5), the enzyme was inhibited by 1.5 mM AcSCh, while in a higher ionic strength buffer (62 and 300 mM phosphate, pH 7.5), the enzyme was inhibited by 1 mM AcSCh. Our result corroborates the observations of Frobert et al. (1997), who noted that a high ionic strength buffer increased inhibition by excess substrate.

A. Venom AChE Turnover Number

Snake venom AChE exhibits the highest turnover number for ACh, ranging from 6,100 to 7,800 s^{-1}, except for *Ophiophagus hannah* AChE (Table 9.1), which has a turnover number of 14,711 ± 500 s^{-1}. These values are lower than those of *Electrophorus* AChE (Vigny et al., 1978). Interestingly, in the crude venom as well as in the purified form, the turnover number for AChE was the same, demonstrating that the presence of another serine hydrolase does not affect venom AChE (Frobert et al., 1997). Among the Elapidae, *Bungarus* venom has perhaps the highest content of AChE (8 mg/g dry venom), with an activity of about 654,000 ± 120,000, which is almost four times the activity of the *Naja* venom, about 150,000 ± 108,000 Ellman units/g of dry venom (Frobert et al., 1997).

B. Inhibition of Venom AChE

Venom AChEs vary in terms of sensitivity to natural anticholinesterase inhibitors called fasciculins (FAS; sixty-one amino acid peptides) isolated from the venom of *Dendroaspis* (mambas). *Ophiophagus* AChE is more sensitive to FAS ($IC_{50} = 10^{-10}$ M) than are *Naja*, *Bungarus*, and *Hemachatus* AChEs ($IC_{50} = 10^{-6}$, $IC_{50} = 10^{-8}$, and $IC_{50} = 10^{-6}$ M, respectively). The main mechanism of AChE inhibition by FAS is related to its binding to the peripheral binding site near the rim of the gorge, sterically occluding the ligand access to the active site, or by allosteric influence,

TABLE 9.1
Acetylcholinesterase Levels in Snake Venoms from Various Species: Activity and Turnover Number

	Elapidae	
Species	**Activity (EL units/g)**	**Turnover Number (s^{-1})**
Bungarus fasciatus	505,000–890,000	6,130–6920
Bungarus multicinctus	666,000	7,570
Bungarus caeruleus	747,000	7,340
Dendroaspis sp.	6	ND
Hemachatus haemachatus	157,000	6,430
Naja haje	282,000–331,000	7,470–7,800
Naja kaouthia	72,900–79,700	7,520–7,770
Naja nigricollis	380–22,000	ND–7,550
Naja naja naja	89,200	7,410
Naja naja atra	82,000	7,690
Naja nivea	147,000–238,000	7,600–7,690
Ophiophagus hannah	41,800–84,800	4,350–6,960
	Viperidae	
Species	**Activity (EL units/g)**	
Bitis gabonica	0	
Bitis arietans (formerly *B. lachesis*)	6–146	
Bothrops atrox	0	
Bothrops lanceolatus	0	
Echis carinatus	0	
Crotalus durissus terrificus	0–6	
Vipera aspis	0–6	
Vipera russelli	6	
Vipera ammodytes	0	

Source: Frobert et al., 1997. With permission from Elsevier.

locking the enzyme in a closed conformation (Radic et al., 1994, 1995, 2005). However, AChE from different snake venoms seems to have a similar affinity toward other inhibitors.

In our laboratory, we have studied the effect of several compounds on *Bungarus* venom AChE activity. Snake venom AChE, like human serum BChE, is inhibited by several commonly used pesticides and herbicides (Ahmed et al., 2007). Venom AChE exhibited a mixed type of inhibition for the pesticides malathion and carbofuran, and the herbicide paraquat, while human serum BChE showed a mixed inhibition for malathion and paraquat and an uncompetitive inhibition for carbofuran. In addition, this enzyme showed high sensitivity to tacrine (Ahmed et al., 2006), which is known to inhibit synaptic AChE. We observed that tacrine caused a mixed type of inhibition in *Bungarus sindanus* venom as well as in human serum BChE. Venom AChE was also affected by antidepressants such as paroxetine, imipramine, clomipramine, and sertraline (unpublished data). Paroxetine and sertraline caused a mixed type of inhibition, while imipramine and clomipramine exhibited a competitive inhibition in *Bungarus* venom. Moreover, the well-known chemical N,N,N′,N′-tetramethylethylene diamine (TEMED), which is used for initiating the polymerization of polyacrylamide gel for electrophoresis, caused a mixed type of inhibition in *Bungarus* venom as well as in horse serum BChE (unpublished data). We also observed that *B. sindanus* venom AChE was inhibited by $ZnCl_2$, $CdCl_2$, and $HgCl_2$. The IC_{50} values of tacrine, edrophonium, fasciculin, propidium, BW284C51, and decamethonium for *Bungarus* AChE are given in Table 9.2.

TABLE 9.2
IC_{50} Values of Different Inhibitors of AChE

Species	Fasciculin	BW284C51	Propidium	Tacrine	Edrophonium	Decamethonium
Bungarus fasciatus (lot 6)	1.2×10^{-8}	4×10^{-9}	3.6×10^{-5}	4.5×10^{-8}	2.3×10^{-6}	7×10^{-6}
Bungarus fasciatus (lot 8)	1.5×10^{-8}	3.6×10^{-9}	2.7×10^{-5}	5×10^{-8}	2.4×10^{-6}	7×10^{-6}
Bungarus fasciatus (China)	1.6×10^{-8}	3.2×10^{-9}	2.6×10^{-5}	5×10^{-8}	2.2×10^{-6}	12×10^{-6}
Bungarus multicinctus	8.5×10^{-9}	4×10^{-9}	6.2×10^{-5}	5×10^{-8}	2.2×10^{-6}	13×10^{-6}
Bungarus caeruleus	1.3×10^{-8}	5.2×10^{-9}	3×10^{-5}	4×10^{-8}	2.7×10^{-6}	7×10^{-6}
Hemachatus haemachatus	$\gg 10^{-6}$	7.3×10^{-9}	2.8×10^{-5}	4.6×10^{-8}	3.8×10^{-6}	18×10^{-6}
Naja haje	$\gg 10^{-6}$	7.5×10^{-9}	2.5×10^{-5}	7.3×10^{-8}	2×10^{-6}	15×10^{-6}
Naja kaouthia	$\gg 10^{-6}$	6.4×10^{-9}	1.9×10^{-5}	7×10^{-8}	3×10^{-6}	13×10^{-6}
Naja naja	$\gg 10^{-6}$	9×10^{-9}	2×10^{-5}	8×10^{-8}	3.5×10^{-6}	15×10^{-6}
Naja nivea	$\gg 10^{-6}$	6.7×10^{-9}	2.5×10^{-5}	8×10^{-8}	3.5×10^{-6}	13×10^{-6}
Ophiophagus hannah	5×10^{-11}	4.5×10^{-9}	4.5×10^{-5}	4.2×10^{-8}	2.7×10^{-6}	11×10^{-6}

Source: Frobert et al., 1997. With permission from Elsevier.

C. Toxicity

Most elapid venoms exhibit AChE activity, and *Bungarus* venom contains ~750,000 Ellman units of AChE-like activity per gram of dry venom, making this venom one of the richest sources of AChE activity. However, the cloned enzyme from *Bungarus fasciatus* is nontoxic even when tested at a high concentration (80 mg/kg, intravenously). Furthermore, it does not affect the other components of the venom, nor does it enhance or potentiate their activities (Cousin et al., 1996b). It is important to emphasize that in this study, the authors used a recombinant toxin and a route of injection of the venom (IV) that is not the most common for envenomation. In fact, when *Bungarus* feeds on prey, the venom is typically injected into the musculature and not directly into the blood.

One hypothesis about its toxicity is that the poisons and toxins that attack AChE, such as fasciculins, cause acetylcholine to accumulate in the nerve synapse, negatively affecting the muscle, particularly at the neuromuscular junction. On the other hand, in venoms where AChE is present, the reverse can be observed. In fact, either the depletion or accumulation of ACh could be deleterious for the prey, resulting in either flaccid or tetanic paralysis, respectively. In this sense, these venom components function as cholinotoxins.

D. Functional Role of Residues 70 and 285

Snake venom AChE is different from AChEs of other sources in its sensitivity to peripheral site ligands. Labeling and mutagenesis studies have indicated that the peripheral site is located at the mouth of a catalytic gorge (Sussman et al., 1991) about 20 Å from the active site (Berman et al., 1980; Kreienkamp et al., 1991; Harel et al., 1995). Comparing the amino acid sequence of venom AChE with that of mammalian and *Torpedo* AChEs reveals that the difference is at positions 70 and 285, where there are methionine and lysine residues instead of tyrosine and glutamic/aspartic acid, respectively. A site-directed mutagenesis study indicated that any modification of one or both of these two residues changes the enzymatic properties at the peripheral site level of the venom (*Bungarus*) AChE. Furthermore, these changes make the enzyme less sensitive to ligands such as

propidium, gallamine, and fasciculin, which also bind at the peripheral site of *Bungarus* AChE (Cousin et al., 1996a).

E. Sequence Similarity of Snake Venom and Other AChEs

Bungarus fasciatus venom AChE shows a very close resemblance to that of *Torpedo* and mammalian AChEs; the catalytic domain of venom AChE shows more than 60% identity and 80% homology with these enzymes. *Bungarus* venom AChE has four *N*-glycosylation sites, which correspond to the glycosylated position in *Torpedo* and mammalian AChEs. The six cysteine residues, which play an important role not only in the formation of intermolecular disulfide loops but also in the catalytic triad (Ser200, Glu327, and His440), and the tryptophan residue (Trp84), are present in all types of cholinesterases (Weise et al., 1990). The aromatic amino acid near the active gorge of *Torpedo* AChE (Sussaman et al., 1991) is also conserved in the *Bungarus* AChE. The only differences observed were at the peripheral site, where tyrosine 70 was replaced by methionone and lysine 285 was replaced by aspartic/glutamic acid. The C-terminal region of *Bungarus* AChE has a short hydrophilic peptide of fifteen residues, which contains six arginine and two aspartic acid residues.

IV. CONCLUSIONS

Snake venoms contain a monomeric soluble form of AChE, a useful source for further detailed studies of the catalytic mechanism and functional role of the peripheral site of this enzyme. The C-terminus is completely different from those of H and T peptides because of the presence of the novel S-exon in snake venom (*Bungarus*) AChE, which now opens up a number of evolutionary questions. It is likely that the S-exon independently generates the production of AChE in the venom gland. This S-exon is also expressed in the snake liver and muscles. More detailed studies are needed to confirm whether the S-exon is connected with the expression of the AChE in snake venom, particularly in relation to the venoms of the *Dendroaspis*, which contain fasciculin but not AChE. Thus, the presence of S-exon and the absence of H-exon in snakes, as well as in other vertebrates, need further studies. Moreover, the monomeric form of venom AChE has a behavior similar to that of other cholinesterases and can be a useful source for evaluating the effect of electric dipole movement of the protein (substrate and product) on the catalytic gorge, without the confounding influences of additional subunits.

From an evolutionary point of view, enhanced complexity of venoms due to the presence of large amounts of AChE or fasciculin may have functional importance during feeding. Mackessy et al. (2006) pointed out that it appears likely that the evolution of resistance mechanisms by prey is countered by an evolutionary response of the snakes. Thus, it is quite possible that the resulting secreted, soluble form of AChE in venom may have a functional role by catalyzing hydrolysis of ACh in muscles (Massoulié et al., 1999), facilitating prey capture, while the presence of fasciculin would lead to accumulation of ACh and tetanic paralysis. In both cases, the cholinergic system of the prey is affected, and these venoms can be considered a cholinotoxic.

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10 Snake Venom L-Amino Acid Oxidases

Nget-Hong Tan and Shin-Yee Fung

CONTENTS

L-amino acid oxidase (LAAO) occurs widely in snake venoms. The enzyme is highly specific for L-amino acids, and generally hydrophobic amino acids are the best substrates. LAAO is a flavoprotein and consists of two identical subunits, each with a molecular weight of 57–68 kDa. The purified enzymes are glycoproteins with 3 to 4% carbohydrate. Deglycosylation of the enzyme does not alter the enzymatic activity but appears to alter its pharmacological activities. The amino acid

sequences of snake venom LAAOs show a high degree of homology. X-ray structural analysis of LAAO revealed a dynamic active site and the presence of three domains: an FAD-binding domain, a substrate-binding domain, and a helical domain. LAAOs were reported to exhibit moderate lethal toxicity. Recent studies showed that LAAOs are multifunctional enzymes exhibiting edema-inducing, platelet aggregation-inducing or -inhibiting, apoptosis-inducing, as well as antibacterial, anticoagulant, and anti-HIV effects. These effects are mostly mediated by the H_2O_2 liberated in the oxidation process, but direct interactions between LAAO and the target cells may play an important role. High-resolution x-ray structural analysis of the enzyme revealed the presence of a channel that would direct the H_2O_2 product to the exterior surface of the protein, near the glycan moiety at Asn 172, which is thought to be involved with LAAO–target cell interaction. This may explain the ability of LAAO to localize H_2O_2 to the targeted cells. A better understanding of the pharmacological actions of LAAOs will facilitate the application of snake venom LAAOs in the design of anticancer and anti-HIV drugs, as well as drugs for the treatment of infectious diseases caused by parasites such as *Leishmania*.

I. INTRODUCTION

L-amino acid oxidase (L-amino acid: O_2 oxidoreductase, EC 1.4.3.2) is a flavoenzyme that catalyzes the oxidative deamination of an L-amino acid to form the corresponding α-ketoacid and ammonia:

$$RCH(NH_3^+)COO^- + O_2 + H_2O \rightarrow RCOCOO^- + NH_4^+ + H_2O_2$$

L-amino acid oxidase (LAAO) occurs widely in nature (Iwanaga and Suzuki, 1979), and snake venoms are perhaps the richest sources of this enzyme. Snake venom LAAOs are generally very active and have been used widely in preparation of α-keto acids because of their chemo- and stereospecificity (Szwajcer et al., 1982; Findrik et al., 2006). α-Keto acids of essential amino acids are useful nutraceuticals as well as therapeutic agents for certain diseases. Recently, snake venom LAAO has become an interesting object for biomedical studies because of its antimicrobial, anti-HIV, anticoagulant, platelet aggregation-inducing and -inhibiting, apoptosis-inducing, as well as anticancer activities. Snake venom LAAO is recognized as a multifunctional protein with promising biomedical application. Several reviews on snake venom L-amino acid oxidases have been published (Meister and Wellner, 1963; Bright and Porter, 1975; Tu, 1977; Iwanaga and Suzuki, 1979; Curti et al., 1992; Tan, 1998; Du and Clemetson, 2002).

II. ASSAY METHODS

Many methods of L-amino acid oxidase assay are available (Iwanaga and Suzuki, 1979). The O_2 electrode technique has been widely used, particularly in kinetic studies. A commonly used spectrophotometric method was described by Bergmeyer (1983) that followed the rate of oxidation by measuring the rate of formation of color complex between the hydrogen peroxide produced and o-dianisidine. Based on the same principle, a spectrophotometric microplate assay has been developed suitable for processing large numbers of samples (Kishimoto and Takahashi, 2001).

Assay Procedure

The reaction mixture consists of 0.05 ml of enzyme, 0.05 ml of 0.0075% horseradish peroxidase (100 purpurogalin units/mg), 67.5 μg of o-dianisidine, and 10 μM L-leucine in 0.9 ml of 0.1 M Tris-HCl, pH 8.5, and the initial rate was measured as the increase in absorbance at 436 nm. The molar absorption coefficient in this assay system is $8.31 \times 10^3\ M^{-1}cm^{-1}$. One unit of enzyme activity was defined as the oxidation of 1 μmole of L-leucine per minute.

III. OCCURRENCE IN SNAKE VENOMS

L-amino acid oxidase can be found in venoms from most genera of snakes (Tan and Ponnudurai, 1992). The richest sources of L-amino acid oxidase are crotaline venoms. The enzyme usually constitutes 1 to 4% of the venom by weight, but in *Calloselasma rhodostoma* (Malayan pit viper) it constitutes up to 30% by weight of the dried venom (Tan, 1998). Venoms from mambas and sea snakes contain either no or only trace amounts of L-amino acid activity. LAAO activity is typically absent from colubrid venoms (Mackessy, 2002).

IV. PURIFICATION OF SNAKE VENOM LAAOs

Since the 1990s, many authors have reported the purification and characterization of L-amino acid oxidases from various snake venoms (Table 10.1). In some snake venoms, the enzymes present in many isoforms. Hayes and Wellner (1969), for example, reported that there were at least eighteen isoforms of the L-amino acid oxidase in *Crotalus adamanteus* venom, and that glycosylation contributes to the microheterogeneity for the enzyme. However, microheterogeneity was not observed for L-amino acid oxidases isolated from most other venoms. In general, it is relatively easy to obtain homogenous LAAO from snake venom. For example, the LAAO from *C. rhodostoma* venom can be obtained using a simple two-step procedure: Sephadex gel filtration chromatography followed by Mono-Q high-performance ion exchange chromatography (Ponnudurai et al., 1994).

Crude venom was dissolved in the appropriate buffer and fractionated by Sephadex G-200 gel filtration chromatography. Fractions exhibiting high LAAO activity were pooled and further fractionated by Mono-Q HR 5/5 high-performance anion exchange chromatography to yield the purified enzyme (Figure 10.1).

TABLE 10.1
Physiochemical Properties of Purified Venom L-Amino Acid Oxidases

Venom Source	Some Physical Properties	Reference
Agkistrodon contortrix laticinctus	120 kDa (dimer), 60 kDa, pI 4.4	Souza et al. (1999)
A. halys blomhoffii	60 kDa, pI 4.9	Takatsuka et al. (2001)
Bothrops alternatus	123 kDa (dimer), 66 kDa, pI 5.37	Stabeli et al. (2004)
B. moojeni	140 kDa (dimer), 69 kDa, pI 4.8	Tempone et al. (2001)
B. pirajai	66 kDa	Izidora et al. (2006)
Calloselasma rhodostoma	132 kDa (dimer), 66 kDa, pI 4.4, carbohydrate 4.4%	Ponnudurai et al. (1994)
Crotalus adamanteus	58.7 kDa, carbohydrate 3–4%	Raibekas and Massey (1998)
C. atrox	100 kDa (dimer), 55 kDa, pI 6.0–6.5	Torii et al. (1997)
C. durissus cascavella	120 kDa (dimer), 68 kDa, pI 5.43	Toyama et al. (2006)
Eristocophis macmahoni	58.7 kDa	Ali et al. (2000)
Naja naja kaouthia	112 kDa (dimer), 57.4 kDa, pI 8.12	Tan and Swaminathan (1992)
Ophiophagus hannah	135 kDa (dimer), 65 kDa, pI 4.5, carbohydrate 3.8%	Tan and Saifuddin (1989), Li et al. (1994)
Pseudechis australis	142 kDa (dimer), 56 kDa	Stiles et al. (1991)
Vipera berus berus	126 kDa (dimer), 55.7 kDa	Samel et al. (2006)
V. lebetina	140 kDa (dimer), 60.9 kDa	Tonismagi et al. (2006)
Trimeresurus flavoviridis	55 kDa	Abe et al. (1998)
T. mucrosquamatus	140 kDa (dimer), 70 kDa, pI 5.6	Ueda et al. (1988)
T. stejnegeri	120 kDa (dimer), 58 kDa,	Zhang et al. (2003)

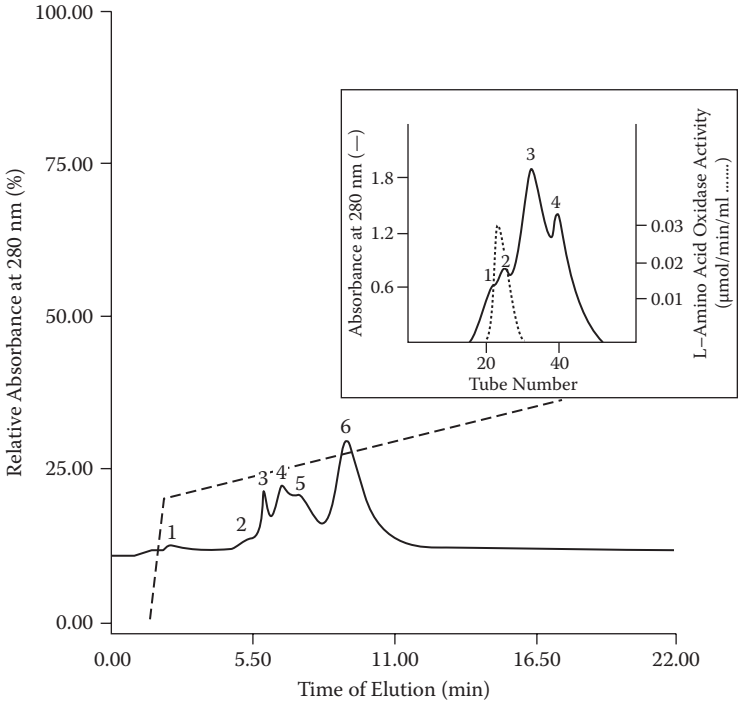


FIGURE 10.1 Purification of *C. rhodostoma* LAAO. The LAAO fraction obtained from Sephadex G-200 gel filtration chromatography (1 × 90 cm) of the crude venom (100 mg in 2 ml of 0.02 M Tris-HCl, pH 8.5; insert, top right) was injected into the column equilibrated with 0.02 M Tris-HCl, pH 8.5, and a linear 0.2 to 0.4 M sodium chloride gradient was started 2 min after injection of the sample. Flow rate was 1 ml/min. Peak 6 was the purified LAAO. ----, sodium chloride gradient.

V. CLONING AND EXPRESSION OF SNAKE VENOM LAAOs

Several snake venom LAAOs have been cloned and sequenced (Raibekas and Massey, 1998; Torii et al., 2000; Macheroux et al., 2001; Takatsuka et al., 2001; Zhang et al., 2003; Kommoju et al., 2007; Franca et al., 2007). The expression of LAAOs from several animal species has been attempted in various expression hosts, but the levels of expression were generally rather low, presumably due to the toxic effects of LAAO to the host cells. Torii et al. (2000), however, reported that LAAO from *Crotalus atrox* could be expressed in an active form in mammalian cells. Kommoju et al. (2007) reported the successful expression of active recombinant LAAO in the methylotropic yeast *Pichia pastoris*. The catalytic properties and substrate specificity of the recombinant LAAO are similar to those of the native enzyme.

VI. PHYSICAL PROPERTIES OF SNAKE VENOM LAAOs

A. General Physical Properties of Snake Venom LAAOs

Snake venom LAAOs generally have a molecular mass ranging from 112 to 140 kDa as determined by gel filtration chromatography, and 57 to 68 kDa by SDS–polyacrylamide gel electrophoresis, indicating that the enzymes are dimers, usually with identical subunits (Table 10.1). Snake venom LAAOs have a wide range of isoelectric points, ranging from 4.4 to 8.12 (Tan, 1998).

LAAO is a flavoprotein with two molecules of flavin coenzymes. The flavins, which exhibit absorption maximums at 275, 390, and 462 nm, are responsible for the yellowish color of the enzyme

as well as for the venoms. Most authors reported that the flavin coenzymes are both FAD, though some earlier reports suggested FMN as the coenzymes in some LAAOs (Tan, 1998). Snake venom LAAOs are stable at room temperature and at 4°C (Tan 1998). *Ophiophagus hannah* LAAO, for example, retained 100% and 80% of activity after incubating at 37°C for 5 days or 14 days at pH 7.4, respectively. Many LAAOs, however, are unstable under alkaline conditions.

Some snake venom LAAOs have a highly sensitive active site. For example, *Crotalus adamanteus* LAAO undergoes reversible pH or temperature-dependent inactivation, accompanied by structural changes in the flavin-binding site, though retaining its overall secondary structure (Coles et al., 1977). Earlier, Curti et al. (1968) reported that *C. adamanteus* LAAO was inactivated by storage at –5°C and –60°C, and by freeze-drying. Many other snake venom LAAOs are also inactivated by freezing. Generally, the inactivated enzyme can be reactivated completely by heating at pH 5. The inactivation was accompanied by shifts in the absorption spectrum and optical rotary dispersion spectrum, and reactivation reverses the spectra changes completely. The inactivation was believed to be due to a limited conformational change of the enzyme structure, presumably also in the vicinity of the flavin-binding site (Soltysik et al., 1987). This has been substantiated by x-ray structural studies (see below). Some snake venom LAAOs (for example, LAAOs from *O. hannah* and *C. rhodostoma*), however, are not inactivated by freezing (Tan, 1998).

B. Reconstitution of LAAO

As a result of the high sensitivity of many snake venom LAAOs to their microenvironment, it was not possible to prepare reconstitutable apoprotein, as reconstitution with the FAD coenzyme often resulted in an inactive protein, with a perturbed conformation of the flavin-binding site. However, Raibekas and Massey (1996) reported near complete activation of the reconstituted apoprotein and the restoration of its native flavin-binding site in the presence of 50% glycerol. Glycerol as a cosolvent plays a special role in this restorative process by induction of rearrangement in the protein structure. The authors suggested that hydrophobic effect appears to be the dominating force in this *in vitro*–assisted restorative process.

VII. THE CHEMICAL STRUCTURE OF LAAOs

A. N-terminal Sequences

The N-terminal amino acid sequences of many LAAOs have been elucidated, and they are highly similar, except for LAAO from *O. hannah* venom (Figure 10.2). There is the presence of a highly conserved Glu-rich motif. The structure of LAAO from *C. rhodostoma* revealed that residues 5–25 constituted one part of the substrate-binding domain (see below). The conserved amino acids may therefore play an important role in the substrate binding.

B. Amino Acid Sequences

Raibekas and Massey (1998) reported the cDNA and deduced amino acid sequence of *C. adamanteus* venom LAAO. Protein similarity search and analysis showed that the enzyme possesses 37% homology with a protein encoded by the mouse B cell interleukin 4–induced protein (Figure 10.1), the role of which is yet to be determined. The N-terminus of the protein contains a common fingerprint or βαβ-fold for binding of the adenylate moiety of FAD (Du and Clemetson, 2002).

Takatsuka et al. (2001) reported that the full-length cDNA sequence of *Agkistrodon halys blomhoffii* LAAO encodes a putative a signal peptide with 18 amino acid residues and a 486-residue subunit (Figure 10.3). Franca et al. (2007) reported the cDNA sequences of LAAO from *B. moojeni* and *B. jararacussu* venom, and phylogenetic analysis showed sequence identities within the range 83 to 87% being closely related to LAAO from *A. h. blomhoffii* (Takatsuka et al., 2001) and *Trimeresurus*

Calloselasma rhodostoma	**A D D** - **R N P L** A **E C F** Q E N **D Y E E F L**
Crotalus atrox	**A** H **D** - **R N P L E E C F R E T D Y E E F L**
C. adamanteus	**A** H **D** - **R N P L E E C F R E T D Y E E F L**
Sistrurus catenatus edwardsii	**A D D** – **R N P L E E C F R E T D Y E E F L**
Bothrops pirajai	**A D D** - K **N P L E E** - **F R E T** N **Y E** V **F L**
Agkistrodon h. blomhoffi	**A D D** - **R N P L E E C F R E T D Y E E F L**
Trimeresurus stejnegeri	**A D D** - **R N P L E E C F R E T D Y E E F L**
Vipera lebetina	**A D D** - K **N P L E E C F R E** D **D Y E E F L**
Notechis scutatus	**A D D** R **R** R **P L E E C F** Q **E** A **D Y E E F L**
Naja naja kaouthia	**D D** R **R** S **P L E E C F** Q Q N **D Y E E F L**
Ophiophagus hannah	H V I - **N** - **L E E** S **F** Q **E** P E **Y** X N H **L**

FIGURE 10.2 The N-terminal amino acid sequence of some snake venom L-amino acid oxidases. Residues in bold are the same in most enzymes. X, uncertain. The sequences are taken from Ponnudurai et al. (1994), Sakurai et al. (2001), Izidoro et al. (2006), Tonismagi et al. (2006), and Pahari et al. (2007).

MNVFFMFSLLFLAALGSCADDRNPLEECFRETDYEEFLEIARNGLKATSNPKHVVIVGAG
MSGLSAAYVLSGAGHQVTVLEASERAGGRVRTYRNDKEGWYANLGPMRLPEKHRIVREYI
RKFGLQLNEFSQENDNAWYFIKNIRKRVGEVKKDPGVLKYPVKPSEEGKSAGQLYEESLG
KVVEELKRTNCSYILNKYDTYSTKEYLLKEGNLSPGAVDMIGDLMNEDSGYYVSFPESLR
HDDIFAYEKRFDEIVGGMDKLPTSMYRAIEEKVHLNAQVIKIQKNAEKVTVVYQTPAKEM
ASVTADYVIVCTTSRATRRIKFEPPLPPKKAHALRSVHYRSGTKIFLTCTKKFWEDEGIH
GGKSTTDLPSRFIYYPNHNFTSGVGVIIAYGIGDDANFFQALDFKDCADIVINDLSLIHQ
LPREEIQTFCYPSMIQKWSLDKYAMGGITTFTPYQFQHFSEPLTASVDRIYFAGEHTAEA
HGWIDSTIKSGLRAARDVNRASEQ

FIGURE 10.3 The deduced amino acid sequence of *Agkistrodon halys blomhoffii* L-amino acid oxidase (Takatsuka et al., 2001). The signal peptide portion is underlined.

stejnegeri (Zhang et al., 2003) venoms. The complete cDNA sequence of *Sistrurus catenatus edwardsii* LAAO has also been reported recently (Pahari et al., 2007).

C. The Glycan Structure

LAAO is a glycoprotein with 3 to 4% carbohydrate, and it has been shown that glycosylation contributes to the microheterogeneity reported for some LAAOs (Hayes and Wellner, 1969). Glycosylation appears to be important for the secretion and solubility of the protein. Removal of the glycan moieties from *C. rhodostoma* LAAO leads to insoluble proteins (Geyer et al., 2001). The glycan moiety also appears to play an important role in the pharmacological actions of the enzyme. It has been suggested that LAAO docks to the cell surface, leading to the generation of high local concentrations of H_2O_2 (Suhr and Kim, 1999), which mediates many pharmacological actions of LAAOs (see

below). Deglycosylation of the enzyme, however, did not alter the enzyme activity (Stabeli et al., 2004; Izidoro et al, 2006).

Geyer et al. (2001) reported on the glycosylation of LAAO from *C. rhodostoma*. Its glycosylation is remarkably homogeneous, with the major oligosaccharide accounting for approximately 90% of the total sugar content. The glycan is identified as a bis-sialylated, biantennary, core-fucosylated dodecasaccharide. An interesting possibility with regard to the homogeneity of the glycan moiety is that it is a functional requirement connected with the biological activities ascribed to LAAO.

VIII. THREE-DIMENSIONAL STRUCTURE OF LAAOs

A. X-ray Structure of *Calloselasma rhodostoma* LAAO

The x-ray structure of *Calloselasma rhodostoma* LAAO has been elucidated (Pawelek et al., 2000), and the topography of the enzyme most closely resembles that of polyamine oxidase. The data indicate that it is functionally a dimer consisting of two 55 kDa monomers. Each monomer consists of fifteen α-helices and twenty-two β-strands that fold into three well-defined domains: an FAD-binding domain, a substrate-binding domain, and a helical domain. The main structural feature of the FAD-binding domain (consists of residues 35–64, 242–318, and 446–486) is a six-stranded β-pleated sheet sandwiched between three α-helices and a four-stranded β-pleated sheet. This motif makes up the classical nucleotide-binding fold seen in many FAD-binding enzymes. The substrate-binding domain is made up of residues 5–25, 73–129, 233–236, and 323–420. The helical domain consists of residues 130–230 and comprises one side of a funnel-shaped entrance to the active site of the enzyme. The interface between the substrate-binding and helical domains forms a 25 Å long funnel, which provides access to the active site. Comparison of the LAAO with the structure of mammalian D-amino acid oxidase reveals significant differences in their modes of substrate entry.

Moustafa et al. (2006) reported a high-resolution (1.8 Å) x-ray structure of *C. rhodostoma* LAAO with its substrate L-phenylalanine. The data reveal a dynamic active site, as conformational changes are apparent for the isoalloxazine ring. There is a Y-shaped channel system, extending from the external surface of the protein to the active site. The authors suggested that one portion of this channel may serve as the entry path for O_2 during the oxidative half-reaction. On the other hand, the second region, which is separated from the proposed O_2 channel by the N terminus (residues 8–16) of the protein, may play a role in H_2O_2 release. Presumably, the channel would direct the H_2O_2 product to the exterior surface of the protein, near the glycan moiety at Asn172, which was thought to anchor the enzyme to the host cell. This channel location may explain the ability of the enzyme to localize H_2O_2 to the targeted cell, thus inducing the apoptotic effect as well as other pharmacological activities (see below). The x-ray structure confirmed that the carbohydrate moieties are linked to Asn172 and Asn361. The authors speculated that the disialylated oligosaccharides at Asn172, which is located in the vicinity of the channel leading to the active site of the enzyme, may bind to siglecs (sialic acid-binding immunoglobulin superfamily lectins) of the target cells via its sialylated glycan moiety, resulting in production of a locally high concentration of H_2O_2 in or near the binding interface. This, in turn, could lead to oxidative damage to the siglec or another adjacent cell structural element.

B. Molecular Modeling of *Bothrops jararacussu* and *B. moojeni* LAAOs

Molecular modeling experiments with overlapping of *Bothrops jararacussu* and *B. moojeni* LAAO models demonstrated that these proteins are almost identical (Franca et al., 2007). In addition, the overall fold of the two models is very similar to that of LAAO from *C. rhodostoma* venom. All the essential residues in *C. rhodostoma* LAAO are conserved in the *B. moojeni* and *B. jararacussu* LAAO models, demonstrating the putative functional similarity between the models and the *C. rhodostoma* LAAO structure. It is possible that most snake venom LAAOs have a similar three-dimensional structure.

IX. THE ENZYMATIC PROPERTIES OF LAAOs

A. General Enzymatic Properties

LAAO required Mg^{2+} and was inhibited by Ca^{2+}, phosphate, or p-chloromercuribenzoate. Certain amino acids stabilize the enzyme, while at high concentration they become inhibitors. The enzyme is also competitively inhibited by various aliphatic and aromatic acids and had a pH optimum from 7 to 8.5. (Tan, 1998). LAAOs from different sources differ substantially in their specific activities. When L-leucine was used as the substrate, at pH 8.5, the specific activities of the enzyme isolated from *C. rhodostoma*, *N. kaouthia*, and *O. hannah* were 0.54, 4.59, and 20.9 μmole/min/mg, respectively. Substrate inhibition occurs at high substrate concentrations.

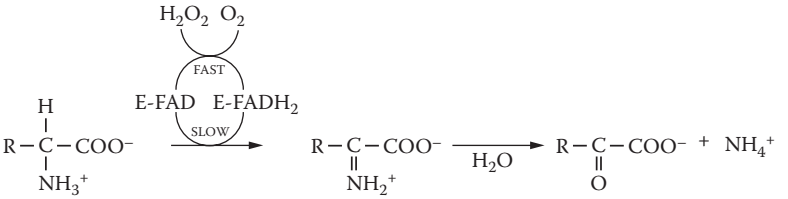
B. Substrate Specificity

Many authors have investigated substrate specificity of snake venom LAAO (Tan, 1998). LAAO did not oxidize any D-amino acid and was highly specific for the L-enantiomer of amino acids. Effective oxidation of L-amino acid by the enzyme requires the presence of a free primary α-amino group. Generally, the best substrates are L-Leu, L-Met, L-Phe, L-Tyr, and L-Trp, whereas L-Lys, L-Ser, L-Thr, L-Asp, and L-Glu were generally hydrolyzed slowly or not at all (Ponnudurai et al., 1994; Souza et al., 1999; Stabeli et al., 2004; Izidoro et al., 2006; Samel et al., 2006; Tonismagi et al., 2006). One exception is *O. hannah* LAAO, for which L-Lys is the best substrate (Tan and Saifuddin, 1991). It is interesting to note that the N-terminal sequence of the *O. hannah* (king cobra) enzyme is also quite different from the other snake venom LAAOs.

Examination of the substrate specificity data of snake venom LAAOs suggested the presence of an alkyl side chain–binding site that comprises at least four subsites, each accommodating a methyl/methylene carbon. *Ophiophagus hannah* LAAO, on the other hand, appears to have an additional amino-binding subsite (Tan, 1998). A similar alkyl-binding site was suggested to be present in D-amino acid oxidase (Dixon and Kleppe, 1965).

C. Mechanism of Catalysis

The oxidation of L-amino acid by the enzyme proceeds in two steps, forming α-imino acid as the intermediate product, as detected by borohydride trapping experiments (for a review of the kinetic mechanism, see Bright and Porter, 1975). During the reductive half-reaction, the α-hydrogen atom of the amino group is abstracted by FAD, producing the α-imino acid intermediate, which then reacts with water to form the α-keto acid. Two alternative mechanisms have been proposed for the reductive half-reaction: (1) the carbanion mechanism, in which the proton is transferred, leaving a negative charge on the alpha carbon atom, and (2) a hydride transfer mechanism, in which the hydrogen atom and the two electrons are transferred simultaneously. Recent data are consistent with a direct hydride transfer mechanism (Fitzpatrick, 2004).



Reaction mechanism of LAAO-catalyzed deamidation of an amino acid.

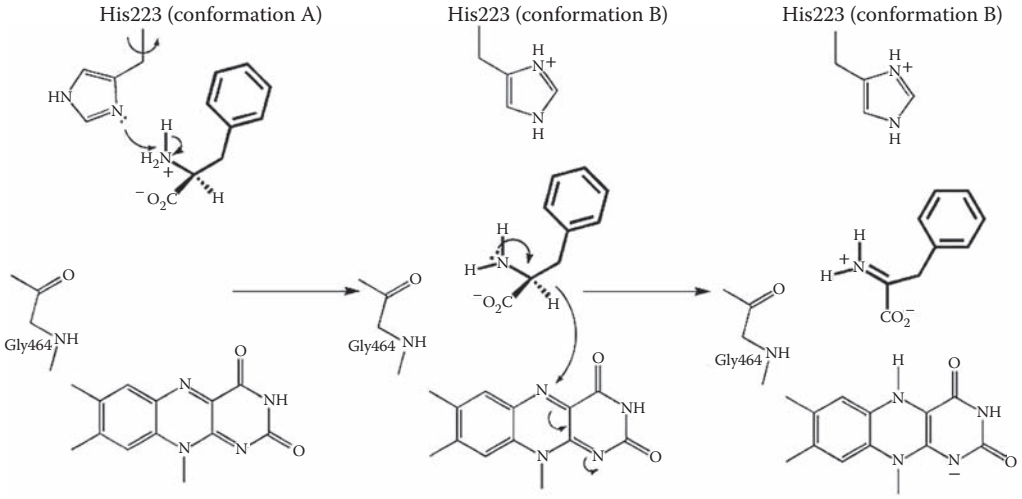


FIGURE 10.4 Reaction mechanism for the oxidation of L-phenylalanine by L-amino acid oxidase. The substrate is shown in thick bonds. Two conformations for His223 are included as labeled. (From Moustafa et al., 2006. With permission.)

According to data obtained from high-resolution x-ray structure of *C. rhodostoma* LAAO (Moustafa et al., 2006), conformational changes of the key active site components His223, Arg322, and the FAD cofactor can be related to the direct hydride transfer mechanism. Induced by the presence of the zwitterionic form of the substrate, His223 alters its side chain conformations (Figure 10.4, from His223A to His223B). The α-amino group of the substrate is then de-protonated by His223. The lone pair of electrons from the amino nitrogen atom then moves to the α-carbon atom, followed by hydride transfer to FAD to form the imine. The conserved water molecule at Lys326 may play a role in the reductive half-reaction or may assist in H_2O_2 formation from the flavin-hydroperoxy intermediate.

X. IMMUNOLOGICAL PROPERTIES OF LAAOs

Snake venom LAAOs are antigenically similar, but it has been reported that antibodies to *O. hannah* LAAO yielded indirect ELISA cross-reactions with neurotoxin, hemorrhagin, and phospholipase A_2 isolated from the same venom, implying that the LAAO shares common epitopes even with unrelated proteins (Tan et al., 1993). This was supported by recent studies by Stabeli et al. (2005). They reported that antibodies to a homoserine lactone derivative of the undecapeptide IQRWSLDKYAM (Ile^{1}-Hse^{11}) excised from *B. moojeni* LAAO cross-reacted with some serine proteases, phospholipase A_2 homologues, and LAAOs from venoms of snakes of genera *Bothrops*, *Lachesis*, *Crotalus*, and *Micrurus*. It was hypothesized that the cross-reactivity of the anti-Ile^{1}-Hse^{11} antibodies to unrelated venom proteins derives from their mechanism of antigen recognition, whereby complementarity is achieved through reciprocal conformational adaptation of the reacting molecules.

XI. THE PHARMACOLOGICAL ACTIVITIES OF LAAOs

Several LAAOs from snake venoms were reported to exhibit moderate lethal toxicity, with i.v. LD_{50}s of approximately 5–9 μg/g in mouse. The LD_{50} of LAAO is usually higher than that of the corresponding venom, and hence the enzyme is not a major lethal component of the venom, as the enzyme usually constitutes less than 5% of the venom dry weight (Tan and Saifuddin, 1989).

Over the last 15 years, LAAO has become an interesting object for biomedical studies because of its apoptotic, cytotoxic, platelet aggregation, anticoagulant, and other physiological effects. These effects are thought to be mediated by the chemically reactive H_2O_2 generated in the oxidation process, because H_2O_2 scavengers such as catalase neutralize the effects. Sometimes the toxic effects cannot be attributed to H_2O_2 liberated alone, and direct interactions between LAAO and the target cells may play an important role (Zhang et al., 2003).

A. Edema-Inducing and Hemorrhagic Activities

Several authors reported that venom LAAO was able to induce extensive edema in the mouse paw, some with slight hemorrhage (Tan and Choy, 1993; Du and Clemetson, 2002; Stabeli et al., 2004; Izidoro et al., 2006). Tan and Choy (1993) reported that *O. hannah* LAAO exhibited strong edema-inducing activity, and the enzyme elicited a delayed-type time course of edema formation, indicating that the edema formation caused by LAAO was not mediated through release of amines subsequent to mast cell degradation, which usually elicited a rapid type of edema formation. The edema-inducing activity of the enzyme was not inhibited by diphenhydramine or dexamethasone. Izidoro et al. (2006) suggested that edema formation is due to activation of the inflammatory response by the H_2O_2 generated, as administration of glutathione to the mouse paw inhibited the edema-inducing activity of the enzyme. The hemorrhagic effect of LAAO results from complex effects, and may involve apoptosis of endothelial and other vascular cells.

B. Anticoagulant Effects

Sakurai et al. (2003) reported that LAAO purified from *A. h. blomhoffii* venom possesses anticoagulant activity. The enzyme significantly delayed the onset and progress of blood coagulation, prolonged the activated partial thromboplastin time, but had little effect on the prothrombin time. The results indicated that LAAO interferes primarily with the intrinsic blood coagulation pathway, and further studies by the authors indicated that the anticoagulant effect of LAAO is due to its inhibitory action on clotting factor IX.

C. Effects on Platelet Aggregation

Reports on the effects of snake venom LAAOs on platelet aggregation seem to be contradictory: some authors reported that LAAOs induce platelet aggregation, whereas other authors reported that LAAOs have an inhibitory action on platelet aggregation (Du and Clemetson, 2002). Catalase, an H_2O_2 scavenger, inhibited both platelet aggregation-inducing and -inhibiting effects, indicating that both effects are due primarily to the action of H_2O_2 produced by the enzyme during the oxidation.

LAAOs from venoms of *C. durissus cascavella*, *E. macmahoni*, *B. alternatus*, *B. pirajai*, and *O. hannah* induce platelet aggregation (Li et al., 1994; Ali et al., 2000; Stabeli et al., 2004; Toyama et al., 2006, Izidoro et al., 2006). Toyama et al. (2006) reported that the platelet aggregation-inducing activity was inhibited by cyclooxygenase pathway inhibitors such as aspirin and indomethacin, suggesting that the H_2O_2 liberated leads to activation of inflammatory enzymes. Du and Clemetson (2002) suggested that H_2O_2 production promoted a rapid increase of thromboxane A_2 synthesis and consequently the platelet aggregation. Conversely, LAAOs from *A. h. blomhoffi*, *V. lebetina*, and *N. naja kaouthia* dose-dependently inhibited both agonist-induced platelet aggregation and shear-induced platelet aggregation (Sakurai et al., 2001; Takatsuka et al., 2001; Tonismagi et al., 2006). One mechanism for platelet aggregation inhibition may be connected with a reduced binding of ADP in platelets exposed to H_2O_2, or the interference of the peroxide in the interaction between the activated platelet integrin GPIIb/IIIa and fibrinogen (Takatsuka et al., 2001; Samel et al., 2006).

It is still not clear why some LAAOs induce and others inhibit platelet aggregation. Sakurai et al. (2001) suggested that the controversies may be connected with differences in the experimental procedure or preparation of blood samples. Other possibilities include the difference in specific activity of the enzyme, or the involvement of mechanisms other than H_2O_2 liberation that are present only in certain LAAOs. Takatsuka et al. (2001), for example, suggested that only LAAOs with very high specific activities would induce platelet aggregation, as initiation of aggregation requires relatively high peroxide concentration (millimolar range). However, since LAAOs generally liberate H_2O_2 in only micromolar concentrations, it has been further suggested that these enzymes may bind to the platelet and be able to generate high concentrations of H_2O_2 locally to induce platelet aggregation.

D. Apoptosis-Inducing Effect

Many snake venoms are known to exhibit apoptosis-inducing effects. Apoptosis is the programmed cell death characterized by a distinct pattern of cellular events, including cleavage of nuclear DNA into fragments that produce a typical nucleosomal DNA ladder upon agarose gel electrophoresis. Suhr and Kim (1996) and Torii et al. (1997) reported that the snake venom component that induced apoptosis was an LAAO, and that LAAO induced apoptosis in human umbilical vein endothelial, human promyelocytic leukemia HL-60, human ovarian carcinoma A2789, and mouse endothelial KN-3 cells. Since then, many snake venom LAAOs were reported to also exhibit apoptosis-inducing activity (Souza et al., 1999; Takatsuka et al., 2001; Sun et al., 2003; Ande et al., 2006; Izidoro et al., 2006; Samel et al., 2006), and apoptosis was usually demonstrated by the DNA fragmentation gel pattern. The apoptosis-inducing activity was abolished by catalase and other H_2O_2 scavengers, indicating that the H_2O_2 generated by LAAO action plays an important role in the apoptosis. Tempone et al. (2001) suggested that cells exposed to oxidative stress induced by LAAO generated H_2O_2 that could activate heat shock proteins and initiate cell membrane disorganization, DNA fragmentation, apoptosis, and therefore cell death. Sun et al. (2003) suggested that the generated peroxide activates the transcription of such factors as the nuclear factor B, the activator protein 1, Fas/Apo-1, and p53.

Suhr and Kim (1999), however, demonstrated that the LAAO-induced apoptotic mechanism was clearly distinguishable from the one stimulated directly by exogenous H_2O_2, suggesting that the LAAO-induced apoptosis was not solely triggered by the peroxide produced by oxidation reactions. Takatsuka et al. (2001) demonstrated that venom LAAOs directly bind to cell surface, thereby increasing the local peroxide concentration. On the other hand, Torii et al. (2000) reported that the venom LAAO did not associate with human embryonic kidney cells. The reason for these discrepancies is not clear.

Ande et al. (2006) and Samel et al. (2006), using Jurkat and K562 (human chronic myeloid leukemia) cells, respectively, reported that at low concentration LAAO induced apoptosis, but caused necrosis of the cells at higher concentrations. According to Ande et al. (2006), the factors contributing to apoptosis are: (1) generation of toxic intermediates from fetal calf serum and (2) binding and internalization of LAAO, which appears to be mediated by the glycan moiety of the enzyme, as desialylation of the enzyme reduces cytotoxicity. D-amino acid oxidase, which lacks glycosylation, also triggers necrosis by the H_2O_2 liberated, but it does not cause apoptosis. Thus, just like the effect on platelet aggregation, induction of cell death by LAAO also appears to involve both the generation of H_2O_2 and the molecular interaction of the glycan moiety of the enzyme with structures at the cell surface.

E. Antibacterial Activity

Stiles et al. (1991) reported that two LAAOs from the venom of *Pseudechis australis* (mulga snake) have a powerful antibacterial effect against Gram-positive and Gram-negative bacteria. Compared to tetracycline, the *in vitro* antibacterial effects of the enzymes were 18–70 times more effective, on

a molar basis. Recently, many authors reported LAAO from other snake venoms also exhibited similar antibacterial activity (Izidoro et al., 2006; Stabeli et al., 2004; Tonismagi et al., 2006; Toyama et al., 2006). It is believed that the antibacterial effect of LAAO is also due to the H_2O_2 liberated, as addition of catalase completely suppressed the antibacterial activity. Electron microscopic studies suggested that the H_2O_2 generated in the oxidation process induced bacterial membrane rupture and then cell death (Toyama et al., 2006). Zhang et al. (2004) reported that the *A. halys* LAAO was able to bind to the surfaces of bacteria and generate high concentrations of H_2O_2 locally, which enables the enzyme to inhibit bacterial growth at low concentrations. It is not clear whether this happens with other snake venom LAAOs.

F. Leishmanicidal Activity

Leishmaniasis, caused by several protists in the genus *Leishmania*, includes a spectrum of human infectious disease ranging from self-healing cutaneous ulceration to a progressive and lethal visceral infection. It is a disease that affects approximately 12 million people and is prevalent in eighty-eight nations throughout the world. Tempone et al. (2001) and Toyama et al. (2006) reported that snake venom LAAO possesses strong leishmanicidal activity, as the H_2O_2 generated by the enzyme was a strong inducer of apoptosis in promastigotes of *Leishmania* ssp. cells.

At present, few drugs are available for treatment of leishmaniasis. The understanding of the mode of action of LAAO upon parasites may lead to the design of new drugs or therapeutic approaches for leishmaniasis. For example, if one were able to target an H_2O_2 generator (such as snake venom LAAO) toward the intracellular parasitophorous vacuole occupied by *Leishmania*, this would represent a highly specific treatment not only for leishmaniasis but also for other intracellular parasites.

G. Anti-HIV Activity

Zhang et al. (2003) reported that LAAO isolated from *T. stejnegeri* venom possesses antiviral activity. The enzyme exhibited dose-dependent inhibition of HIV-I infection and replication at concentrations that showed little effect on cell viability. Under the same experimental conditions, no anti-HIV-1 activity was observed by exogenous addition of H_2O_2. Furthermore, the presence of catalase caused a decrease in its antivirus activity but resulted in an increase of its antiviral selectivity. The authors suggested that while liberated H_2O_2 is involved in the anti-HIV-1 activity of the LAAO, the dosages of H_2O_2 and relative molecular pathways mediating suppression in virus infection and replication are independent of or different from those causing cell death. Presumably, the mechanism of the anti-HIV-1 effect of LAAO involves specific binding of the enzyme to cell membrane, which helps to generate high local concentrations of H_2O_2 and trigger specific signal reactions and activation of host cells, resulting in the inhibition of HIV infection or replication.

XII. CONCLUSIONS

Prior to the 1990s, studies of snake venom LAAO dealt mainly with their enzymatic properties and industrial applications. In the past 15 years, there has been considerable progress in the studies of the structure and mechanism of the enzyme, but the focus has shifted to the investigations of the pharmacological actions of the enzyme and its potential biotechnological and medical applications.

Snake venom LAAOs are interesting multifunctional enzymes exhibiting edema-inducing, platelet aggregation-inhibiting or -inducing, apoptotic-inducing, and anti-HIV-1 activities, as well as anticoagulation effect. Their toxicological actions are due mainly, but not entirely, to the H_2O_2 liberated during the oxidation. The exact mechanism of the toxicological actions of snake venom LAAO awaits further studies.

Sun et al. (2003) suggested that LAAO may be applied clinically in glioma therapy by cloning the cDNA of the enzyme and transfecting the tumor cells of patients to induce apoptosis in the

target tumor cells. Many authors have demonstrated the apoptotic effect of snake venom LAAO on various malignant cells. There is therefore potential in the application of LAAO in cancer therapy. The understanding of the LAAO mode of action upon parasites may also lead to the design of new drugs or therapeutic approaches for leishmaniasis as well as other intracellular parasites. In addition, investigation on the anti-HIV activity of LAAO would also provide valuable information on the therapeutic development of new generations of anti-HIV drugs.

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11 Hyaluronidases, a Neglected Class of Glycosidases from Snake Venom
Beyond a Spreading Factor

K. Kemparaju, K. S. Girish, and S. Nagaraju

CONTENTS

Efficient diffusion of systemic toxins into circulation from the bite site results in successful envenomation, and fragmentation of megadalton hyaluronan in the extracellular matrix by snake venom hyaluronidase is a key factor in this diffusion process. Recent studies have demonstrated that hyaluronidase not only augments the lethal potency of the venom, but also damages the bite site, resulting in severe morbidity. Despite its critical role in lethal toxicity, the enzyme has been less extensively studied in snake venoms than many other proteins. In this chapter, an effort has been made to provide a consolidated overview of the developments in the recent past and the future challenges required to understand the role of this glycosidase in snake venom pathophysiology. Furthermore, the beneficial effects of inhibition of this enzyme are highlighted, underscoring its significance as a therapeutic target in the more efficient and efficacious management of snakebite.

I. INTRODUCTION

Venomous snakes efficiently capture their prey through immobilizing them, and rapid dissemination of target-specific systemic toxins into the blood and tissues is a prerequisite (Kini, 1997;

Gutiérrez and Rucavado, 2000). This process is facilitated primarily by hyaluronidases and metzincin family enzymes, which degrade the constituents of extracellular matrix (ECM) and connective tissue surrounding the blood vessels (Anai et al., 2002; Gutiérrez et al., 2005; Girish et al., 2004b; Girish and Kemparaju, 2006). Metzincin family enzymes are Zn^{2+}-dependent hemorrhagic metalloproteases that degrade the protein scaffold of the ECM, which includes collagen, elastin, fibronectin, laminin, and other proteins, while hyaluronidases are endoglycosidases and degrade primarily hyaluronan, a glycosaminoglycan (GAG) of ECM (Gutiérrez et al., 2005; Kemparaju and Girish, 2006). Metzincin family enzymes are extensively studied, and hence are not discussed in detail in this chapter (see Chapter 4 this volume; Bjarnason and Fox, 1994; Gutierrez and Rucavado, 2000; Fox and Serrano, 2005). In contrast, hyaluronidases are perhaps the least studied among the snake venom enzymes. Nevertheless, hyaluronidase is popularly called a spreading factor (Tu and Hendon, 1983; Girish et al., 2004b; Girish and Kemparaju, 2006). In contrast to the toxic principles of snake venoms (Kini, 1997), hyaluronidase is claimed to be nontoxic in nature (Girish et al., 2004b). This presumption might be the reason for the lack of interest, and hence few attempts have been made to characterize this enzyme. Indeed, the enzyme is now gaining much attention due to the fact that it appears to play a significant role in venom-induced systemic and local toxic effects. Therefore, the primary concern of this chapter is to address systematically the significance of this neglected glycosidase in venom toxicity, in addition to its spreading property.

II. SPREADING PROPERTY

The structural collapse of ECM due to the fragmentation of megadalton hyaluronan by the snake venom hyaluronidase (Figure 11.1) paves the easy diffusion of systemic toxins, which were otherwise diffused much more slowly into the circulation for distribution to their targets. For example, hyaluronidase from *Heloderma horridum horridum* enhanced the local hemorrhagic effect of a toxin from *Trimeresurus flavoviridis* venom (Tu and Hendon, 1983). Similarly, hyaluronidase from *Naja naja* venom potentiated the myotoxicity of VRV-PL-VIII myotoxic phospholipase A_2 (Figure 11.2A) and hemorrhagic activity of a hemorrhagic complex-I (Figure 11.2B) of *Daboia* (formerly *Vipera*) *russellii* venom (Girish et al., 2004b). Nevertheless, the history of hyaluronidase as a spreading factor dates back to Duran-Reynals (1928), who first showed the aggravated diffusion of injected India ink, drugs, antiviral vaccines, and bacterial invasion by a factor that was found in an extract of mammalian testes, and hence described it as a spreading factor (Hoffman and Duran-Reynals, 1931; Duran-Reynals, 1931; see review by Meyer, 1971). The role of hyaluronidase as a spreading factor has subsequently become widely accepted. Eventually, hyaluronan, the chief substrate of the enzyme, was isolated from bovine vitreous humor and several other sources. Karl Meyer, in 1940, coined the term *hyaluronidase* for the enzyme that caused the degradation of hyaluronan (Meyer, 1971). Interestingly, all secretory hyaluronidases are probably spreading factors, but not all spreading factors are hyaluronidases. For example, jararafibrase-I, a hemorrhagic metalloprotease from *Bothrops jararaca* venom, still facilitated the diffusion of systemic toxins even though its inhibition by antibodies prevented the venom-induced systemic coagulopathy (Anai et al., 2002).

III. DISTRIBUTION AND CLASSIFICATION

Hyaluronidases are endo-β-glycosidases and are distributed widely in nature (Manzel and Farr, 1998; Girish and Kemparaju, 2007). They are found in various human organs and body fluids, and in external secretions of bacteriophages and other viruses, bacteria (Stern and Jedrzejas, 2006), fungi (Shimizu et al., 1995), nematode, and leeches (Frost et al., 1996), and in the venoms of snakes (Xu et al., 1982; Kudo and Tu, 2001; Girish et al., 2004b; Girish and Kemparaju, 2005a), scorpions (Ramanaiah et al., 1990; Pessini et al., 2001; Morey et al., 2006), bees (Gmachl and Kreil, 1993;

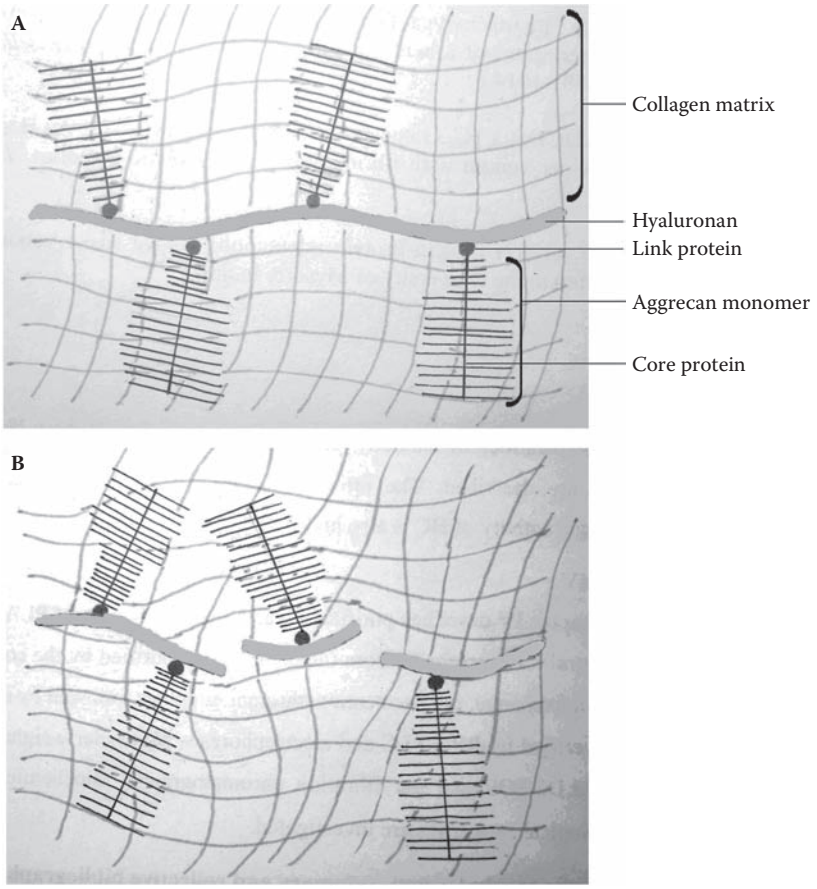


FIGURE 11.1 Schematic diagrams of (A) intact ECM and (B) disseminated ECM after hyaluronidase activity. (Girish, 2004.)

Markovic-Housley et al., 2000), wasps (Kreil, 1995), hornets (Lu et al., 1995), spiders (Rash and Hodgson, 2002; Nagaraju et al., 2006), caterpillars (da Gouveia et al., 2005), fishes (Poh et al., 1992; Ng et al., 2005), and lizards (Tu and Hendon, 1983). Three major groups of hyaluronidases have been classified based on their mechanism of action and end product analysis (Meyer, 1971):

1. Hyaluronate 4-glycanohydrolases/hyaluronoglucosaminidases/endo β-N-acetylhexosaminidases (EC 3.2.1.35) that hydrolyze β-1,4 glycosidic bonds, yielding tetrasaccharides and hexasaccharides as major end products, with N-acetyl glucosamine and glucuronic acid placed in reducing and nonreducing ends, respectively, of the products. Examples include hyaluronidases from lysosomes, spermatozoa, snake venoms, etc.
2. Hyaluronate 3-glycanohydrolases/hyaloglucuronidases/endo β-glucuronidases (EC 3.2.1.36) that hydrolyze β-1,3 glycosidic bonds, yielding tetrasaccharides and hexasaccharides (GlcNAc-GlcUA-GlcNAc-GlcUA) as major end products, with glucuronic acid and N-acetyl glucosamine placed in reducing and nonreducing ends, respectively, of the products. Examples include hyaluronidase from salivary gland of leeches, crustaceans, etc.
3. Hyaluronate lyases/endo β-N-acetylhexosaminidases (EC 4.2.99.1) that hydrolyze β-1,4 glycosidic bonds, yielding predominantly disaccharides as end products, with N-acetyl glucosamine and glucuronic acid placed in reducing and nonreducing ends, respectively, of the products. Examples include microbial hyaluronidases.

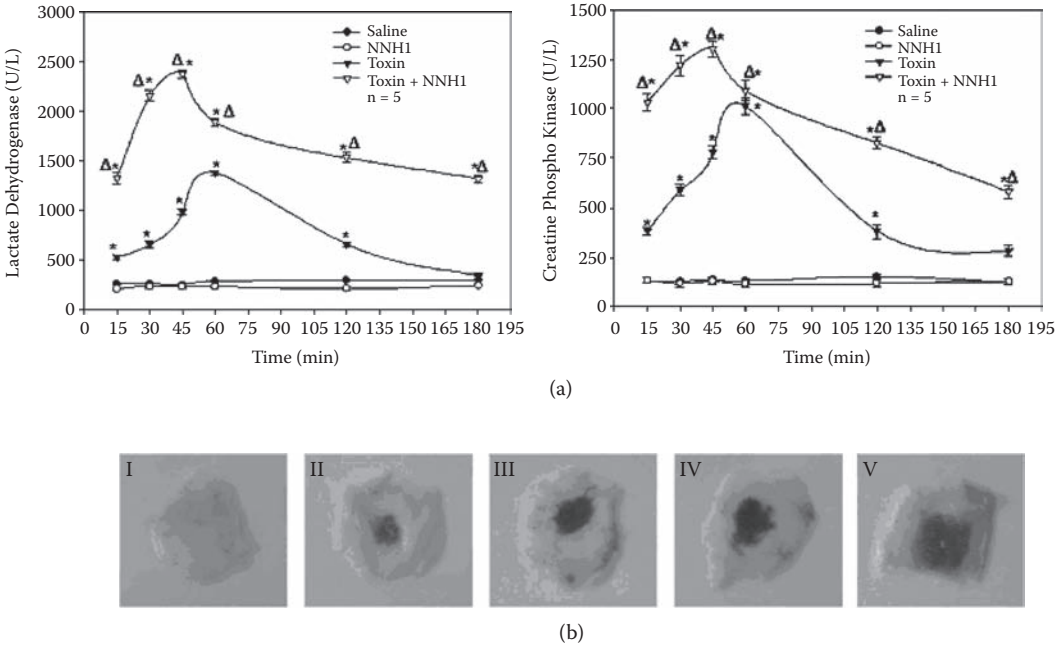


FIGURE 11.2 Spreading property of *Naja naja* venom hyaluronidase NNH1. (a) Myotoxicity of VRV-PL-VIII myotoxin in presence of NNH1: serum LDH and CPK activities of mice injected with VRV-PL-VIII myotoxin in the presence and absence of NNH1. Mice were injected with saline (●—●), NNH1 (○—○), VRV-PL-VIII myotoxin (▼—▼), or VRV-PL-VIII myotoxin + NNH1 (▽—▽). *, $p < .001$ to control; Δ, $p < .001$ to toxin. Data represent mean ± SEM. (Girish, 2004; Girish et al., 2004. With permission.) (b) Hemorrhagic activity of hemorrhagic complex-I in presence of NNH1: (I) NNH1 alone (25 μg), (II) hemorrhagic complex-I alone (at MHD dose of 2 μg), and hemorrhagic complex-I (at MHD dose of 2 μg) co-injected with 2 μg (III), 4 μg (IV), or 6 μg (V) of NNH1. (Girish, 2004.)

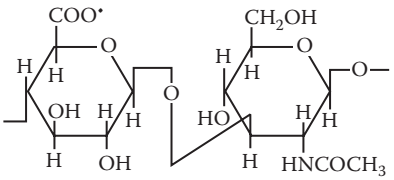
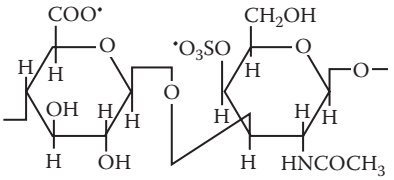
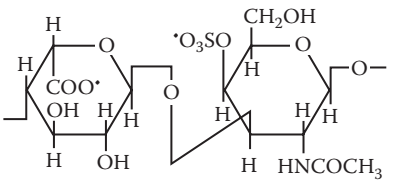
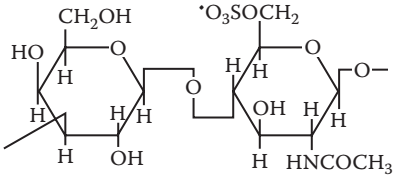
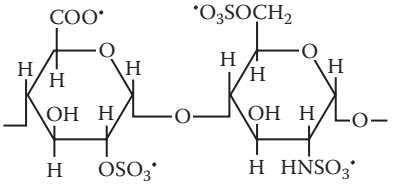
The first two classes are hydrolases, as the mechanism involves hydrolytic cleavage, while the last class is an eliminase/lyase due to its β-elimination with the introduction of a double bond, yielding Δ4,5 unsaturated glucuronic acid–containing disaccharides. All hyaluronidases predominantly degrade hyaluronan; however, some also cleave, to a lesser extent, other GAGs, such as chondroitin and chondroitin sulfates (Table 11.1) (Baker et al., 2002; Stern and Jedrzejas, 2006). Strikingly, at pH 7.0, bovine testicular hyaluronidase catalyzes the formation of a glycosidic bond through a transglycosylation reaction, while at pH 5.0 it will cleave the glycosidic bond by its glycosidase activity (Saitoh et al., 1995). However, a transglycosylation reaction (if any) has not been studied for any of the snake venom hyaluronidases.

In addition to the above classification, based on pH activity profile, hyaluronidases are loosely classified into two groups: acidic enzymes, active between pH 3 and 4 (e.g., human liver and serum hyaluronidases), and neutral enzymes, active between pH 5 and 8 (e.g., ovine testicular, snake, and other venom hyaluronidases) (Frost et al., 1996; Kemparaju and Girish, 2006).

IV. HYALURONAN

Hyaluronan is a high molecular weight, nonsulfated, linear, acidic GAG found throughout the animal kingdom, especially in the ECM of soft connective tissues. It is composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine linked through β-1,3 and β-1,4 glycosidic. It is thought also to participate in such physiological processes as embryogenesis, cell

TABLE 11.1
Disaccharide Unit and Some Properties of Glycosaminoglycans (GAGs)

Glycosaminoglycan	Disaccharide Unit	Linkage	Sulfation	Protein Core
Hyaluronan (HA) GlcA/GlcNAc		β (1,3)	–	–
Chondroitin sulfate (CS) GlcA/GalNAc		β (1,3)	+	+
Dermatan sulfate (DS) GlcA or IdoA/GalNAc		β (1,3)	+	+
Keratan sulfate (KS) GlcA or IdoA/GlcNAc		β (1,4)	+	+
Heparin/heparan sulfate GlcA or IdoA/GlcNAc		α (1,4)	+	+

migration, wound healing, tissue turnover, and malignancies (Spicer and McDonald, 1998; Laurent and Fraser, 1992; Lee and Spicer, 2000; Toole, 2004; Girish and Kemparaju, 2007).

Hyaluronan from bovine vitreous humor was purified in 1934 (Meyer and Palmer, 1934) and its structure was solved in 1954 (Weissman and Meyer, 1954). In contrast to other GAGs, it is a nonsulfated, megadalton molecule with a molecular mass range of ~2×10^5 to ~10×10^7 Da, with an extended length of 2–25 μm (Toole, 2004). All GAGs, except hyaluronan, are sulfated and are bound covalently to any of the core proteins. Hyaluronan acts like a glue to hold aggrecan monomers and other proteins throughout its linear structure to provide mechanical support and defined volume to the ECM (Matsushita and Okabe, 2001; Toole, 2004). The formation of hyaluronan has also been related to the evolution of the notochord due to its prevalence in this structure. The notochord in modern protovertebrates is seen first in the free-swimming larva of tunicates (Urochordata), but disappears in the sessile adult. However, genome analysis revealed no hyaluronan synthase or

synthase-like genes. In vertebrates, the notochord is essentially a prerequisite for the development of spinal column. It has been presumed that hyaluronan appeared later in evolution than chondroitin (Hay and Meier, 1974; Solursh et al., 1979).

Hyaluronan is synthesized at the inner face of the plasma membrane as a free linear polymer without anchoring to any core protein, while resident Golgi enzymes synthesize other GAGs that are anchored covalently to core proteins (Toole, 2004). In humans, three transmembrane glycosyltransferases, HAS1, HAS2, and HAS3, perform cell- and tissue-specific regulated synthesis of hyaluronan (Weigel et al., 1997; Itano et al., 1999). Although the amino acid sequences of these isozymes are 50 to 71% identical, the gene sequences are located on different chromosomes (hCh19-HAS1, hCh8-HAS2, and hCh16-HAS3) and encode three different proteins with distinct enzymatic properties (Itano et al., 1999). HAS3 synthesizes shorter forms of HA molecules ($<3 \times 10^5$ Da) and is thought to be more active than HAS1 and HAS2, both of which produce high molecular mass HA molecules (3.9×10^6 Da) (Adamia et al., 2005). Hyaluronan degradation in mammals is mediated by coordinated activity of three separate enzymes: a hyaluronidase, an endo-glycosidase, and two other exoglycosidases, β-glucuronidase and β-N-acetyl hexosaminidase (Stern, 2003).

V. EXTRACELLULAR MATRIX AND HYALURONAN

Extracellular matrix (ECM) is a diverse, complex structural entity of animal systems. The structure and function vary greatly with the nature of the tissue with which it is associated, that is, bone and cartilage, connective tissue surrounding glands and blood vessels, and intercellular scaffolds. Despite this diversity, the ECM consists of three common classes of molecules: (1) structural proteins such as collagens and elastins, (2) protein-glycosaminoglycan complexes (proteoglycans), and (3) adhesive glycoproteins such as fibronectins, entactin, laminin, and nidogen. The protein-glycosaminoglycan complexes form a matrix, embedding structural proteins in it (Spicer and Tien, 2004; Toole, 2004). Table 11.1 illustrates the disaccharide portion and lists some properties of each GAG.

The GAG hyaluronan is highly hydrated, and because of its negative charge, it can hold a large number of cations, serving as a reservoir of extracellular water and various cations. The role of hyaluronan in numerous physiological and pathological phenomena, such as embryo development, migration, adhesion, proliferation and differentiation of cells, immune surveillance, inflammation, wound healing, angiogenesis, malignant transformation, and viscoelastic functions, is well documented (Toole, 2004).

As a cementing material, hyaluronan interacts with various groups of proteins that bind hyaluronan. These proteins can be grouped into extracellular and intracellular hyaladherins based on location. The extracellular hyaladherins are collectively termed hyalectins, and include aggrecan, neurocan, versican, and brevican (Spicer et al., 2003; Girish and Kemparaju, 2007). The intracellular hyaladherins include CD44, RHAMM (receptor for hylauronic acid–mediated motility), LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1), TSG-6 (tumor necrosis factor stimulated gene-6), and layilin (Day and Prestwich, 2002; Toole, 2004; Knudson and Knudson, 2004). Hyaluronan and hyaladherin interaction are important for several vital functions, such as retention of hyaluronan-rich ECM, cell-ECM interaction, cell-cell communication through the ECM, and induction of a complex intracellular cascade of events through direct stimulation (Toole, 2004; Girish and Kemparaju, 2007).

VI. ASSAYS FOR HYALURONIDASE ACTIVITY

A variety of assay methods have been employed to measure hyaluronidase activity. The commonly used assays to measure hydrolase types of hyaluronidases (venom and leech enzymes) are based on monitoring the reduction in viscosity of a medium (Dorfman and Ott, 1948), reduction in turbidity of a medium (Dorfman and Ott, 1948; di Ferrante, 1956), colorimetric estimation of a

reducing terminal of β-N-acetyl D-glucosamine/β-N-acetyl hexosamine after formation of product with p-dimethyl aminobenzaldehyde (Reissig et al., 1955), and a microtiter-based enzyme-linked immunosorbent assay (ELISA). The latter utilizes a specific high-affinity biotinylated hyaluronan binding peptide (HABP), derived from tryptic digests of proteoglycan core protein of bovine nasal cartilage. After incubation with enzyme, the remaining hyaluronan is quantified using an avidin-biotin-enzyme complex (Stern and Stern, 1992). This ELISA appears to be the most sensitive method currently available.

A truly quantitative estimation of hyaluronidase activity is difficult and complicated. In addition to the limitations of the assays employed, the substrate hyaluronan itself is a highly dynamic molecule in solution, and it has a great propensity to form varied structural aggregates as a function of its molecular mass. Crystallography reveals a variety of structures for the short-chain form of hyaluronan (Winter et al., 1975). Hence, the preferential cleavage of a specific structural aggregate by a hyaluronidase cannot be ignored. Although less sensitive and nonspecific for β-N-acetyl D-glucosamine, the method of Reissig et al. (1955) provides an accurate estimation of the formation of reducing β-N-acetyl hexosamine. Interestingly, the lyase type of microbial hyaluronidases may be quantified spectrophotometrically at 232 nm, using an assay that measures the introduction of the double bond between C4 and C5 of the glucuronic acid portion of hyaluronan during catalysis (Baker et al., 2002; Stern and Jedrzejas, 2006). On the other hand, a copolymerized substrate–polyacrylamide gel assay technique (zymography), based upon incorporation of hyaluronan in the gel, provides a qualitative measure of the activity and also information on the presence of possible isoforms of the enzyme (Cevallos et al., 1992; Guntenhoner et al., 1992; Girish et al., 2002). Further, the presence of inhibitors of the enzyme may be demonstrated using reverse zymography (Mio and Stern, 2000).

VII. EVOLUTIONARY ASPECTS

The roundworm *Caenorhabditis elegans* (Nematoda) and the fruit fly *Drosophila melanogaster* (Insecta) genomes contain single vertebrate hyaluronidase-like sequences, but this may be a chondroitinase, as neither of them contain hyaluronan but do contain chondroitin (Toyoda et al., 2000). However, the first chordate to possess hyaluronan is found to be Amphioxus (*Branchiostoma*), which is a small, flattened marine chordate (subphylum Cephalochordata). Hence, Amphioxus may be the most basal chordate to contain hyaluronidase and hyaluronan synthase sequences (Stern and Jedrzejas, 2006). Additionally, the vertebrate hyaluronidase has been postulated to evolve from preexisting chondroitinase (Stern and Jedrzejas, 2006).

The human genome contains six hyaluronidase sequences (HYAL1, HYAL2, HYAL3, HYAL4, PH-20/SPAM1 (sperm adhesion molecule 1), and pseudogene PHYAL1). The first three genes are found clustered on chromosome 3p21.3, and the latter three genes on chromosome 7q31.3. These genes are thought to have evolved from two distinct gene duplication events and later by duplication of each cluster of genes to produce a pseudogene and five gene products with distinct sequences and expression patterns (Csoka et al., 1999, 2001). The corresponding gene products hyaluronidases 1 and 2 are involved in hyaluronan degradation in somatic tissues. Hyaluronidase 3 is widely expressed, but its precise function is not known. Hyaluronidase 4 has been shown to act on chondroitin, while PH-20/SPAM1 is essential for fertilization. The pseudogene PHYAL 1 is transcribed but the corresponding hyaluronidase is not detected. Jedrzejas and Stern (2005) proposed three-dimensional model structures for human hyaluronidase enzymes, suggesting that the catalytic cleft and the active sites of these enzymes are conserved; however, the C-terminal domains differ in both sequence and structural functionality.

For snake venom hyaluronidase, Harrison et al. (2007) identified a single hyaluronan glucosaminidase activity matching sequence using their expressed sequence tag (EST) database for the saw-scaled viper (*Echis ocellatus*; Nigeria) venom gland. This sequence showed great homology (over 95%) with the venom gland cDNA sequences of another saw-scaled viper species, *Echis pyramidum leakeyi* (Kenya), the Sahara horned viper, *Cerastes cerastes cerastes* (Egypt), and the

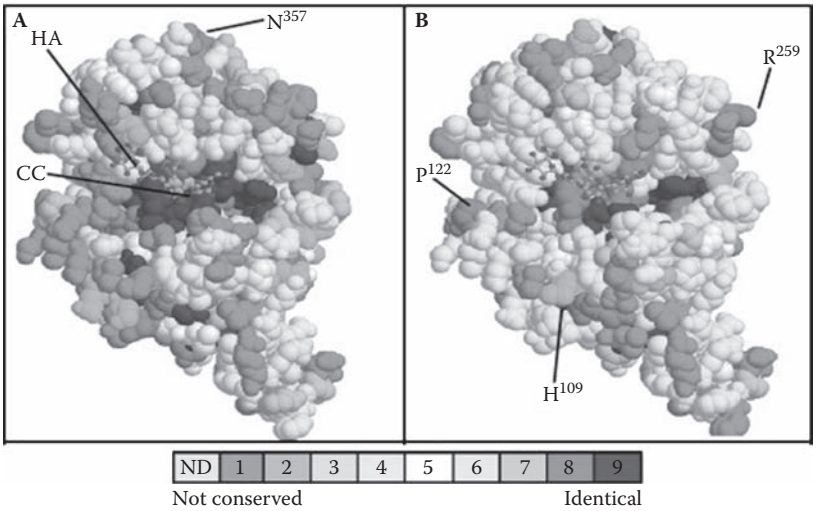


FIGURE 11.3 Modeled three-dimensional structure of (a) mammalian hyaluronidases and (b) venom hyaluronidases based on the crystal structure of *Apis mellifera* hyaluronidase. The images are oriented to show the catalytic cleft (cc) binding hyaluronan (HA), and shading indicates the extent of residue conservation. (Modified from Harrison et al., 2007. With permission.)

puff adder, *Bitis arietans* (Nigeria). The modeled structures of hyaluronidases from these venoms revealed a conserved folding pattern, including the catalytic, positional, and cysteine scaffolds. Hence, a "universal" viper venom hyaluronidase has been proposed (Figure 11.3). With the limited information available on viper venom cDNAs encoding hyaluronidase and hyaluronidase-like molecules, it is too early to comment on the presumed evolutionary history of the enzyme. However, gene duplication is an important evolutionary mechanism that results in the formation of functionally distinct isomers of snake venom metalloproteases (Moura da Silva et al., 1996), PLA_2s (Ohno et al., 2003), C-type lectins (Tani et al., 2002), Kunitz/bovine pancreatic trypsin inhibitors (Zupunski et al., 2003), and serine proteases (Deshimaru et al., 1996), and it likely plays a role in the evolution of hyaluronidase isomers as well.

VIII. PROPERTIES OF SNAKE VENOM HYALURONIDASE

Hyaluronidase is ubiquitously distributed in all venoms, including snake venoms, and multiple isoforms may exist (Girish et al., 2002, 2004b). Hyaluronidase activity is likely common in viperid venoms, as these are rich in high molecular weight toxins, and hyaluronidase activity is considered important to facilitating the spread of these toxins. Elapid venoms commonly show little or no detectable activity, but some of the elapid venoms show higher activity than viperid venoms (Figure 11.4) (Girish et al., 2002). *Naja naja* venom exhibited marked intraspecific variation of isoforms as a function of geographic distribution of the snake within the Indian subcontinent (Shashidharamurthy et al., 2002). These observations suggest that the functions for snake venom hyaluronidase may extend beyond its spreading factor property.

The limited number of studies on venom hyaluronidases indicates a lack of interest in this enzyme from snake venoms. The enzyme has been isolated from the venoms of several snakes, such as *Deinagkistrodon* (formerly *Agkistrodon*) *acutus* (Xu et al., 1982) and *Agkistrodon contortrix contortrix* (Kudo and Tu, 2001). *Deinagkistrodon acutus* venom hyaluronidase is a glycoprotein. The apparent molecular size of hyaluronidase ranges from 33 to 110 kDa, and the isoelectric pH is over 9.0. These are optimally active between pH 5.0 and 6.0, and therefore belong to neutral active class of enzymes. In the recent past, two isoforms of the enzyme, NNH1 and NNH2, were isolated and

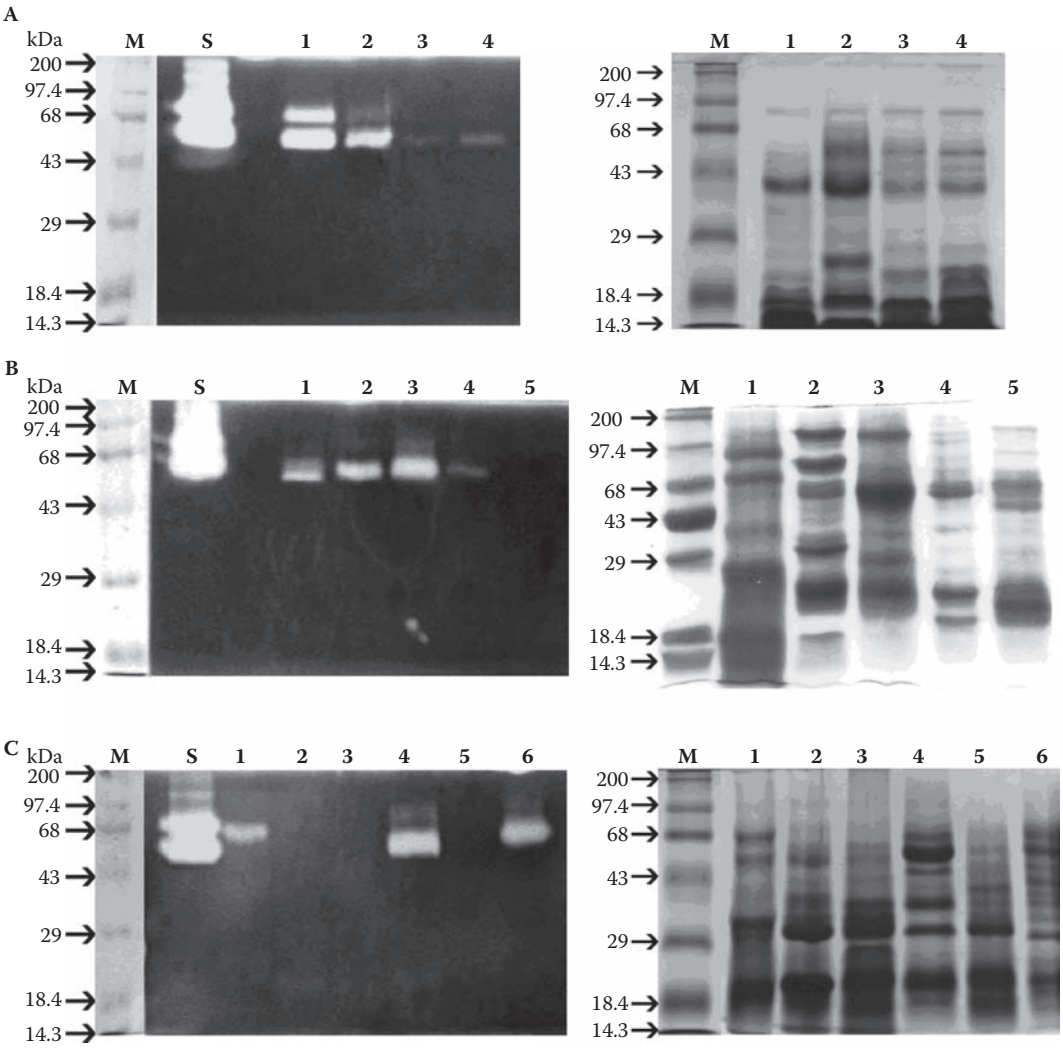


FIGURE 11.4 Hyaluronidase activity zymograms and SDS-PAGE patterns of snake venoms belonging to three different taxa. Venom samples (80 and 50 μg each) were prepared under nonreducing conditions for zymogram gel assay and SDS-PAGE, respectively. (A) Elapid venoms: lane 1, *Naja naja*; lane 2, *Naja kaouthia*; lane 3, *Naja melanoleuca*; lane 4, *Naja naja sputatrix*. (B) Viperine venoms: lane 1, *Daboia russellii*; lane 2, *Vipera palaestinae*; lane 3, *Echis carinatus*; lane 4, *Echis carinatus leakeyi*; lane 5, *Echis carinatus sochureki*. (C) Crotaline venoms: lane 1, *Trimeresurus flavoviridis*; lane 2, *Trimeresurus elegans*; lane 3, *Trimeresurus mucrosquamatus*; lane 4, *Crotalus atrox*; lane 5, *Bothrops asper*; lane 6, *Agkistrodon halys blomhoffii*. In all the cases, M represents molecular weight markers (in kDa). For zymogram assays, ovine testicular hyaluronidase (0.5 U) was used as a positive control (lane S). (Girish, 2004; Girish et al., 2004.)

characterized from the Indian cobra (*Naja naja*) venom (Girish et al., 2004a; Girish and Kemparaju, 2005a). Both isoforms are negative for periodate-Schiff base staining. NNHI is comparatively larger in size, with a molecular weight of 70.4 kDa, while NNH2 was found to be 52 kDa (Figure 11.5). NNH1 is slightly less basic than NNH2, with pI values of 9.2 and 9.7, respectively. NNH1 showed two fluorescence emission maxima of 310–320 nm and 340–350 nm, while NNH2 showed an emission maximum at 345 nm when excited at 280 nm. Both isoforms are maximally active at pH 5.0 and 37°C. Both are inactivated below pH 4.0 and above pH 8.0, and temperatures above 60°C, but are stable for over 30 days in assay buffer (200 mM sodium acetate buffer containing 150 mM

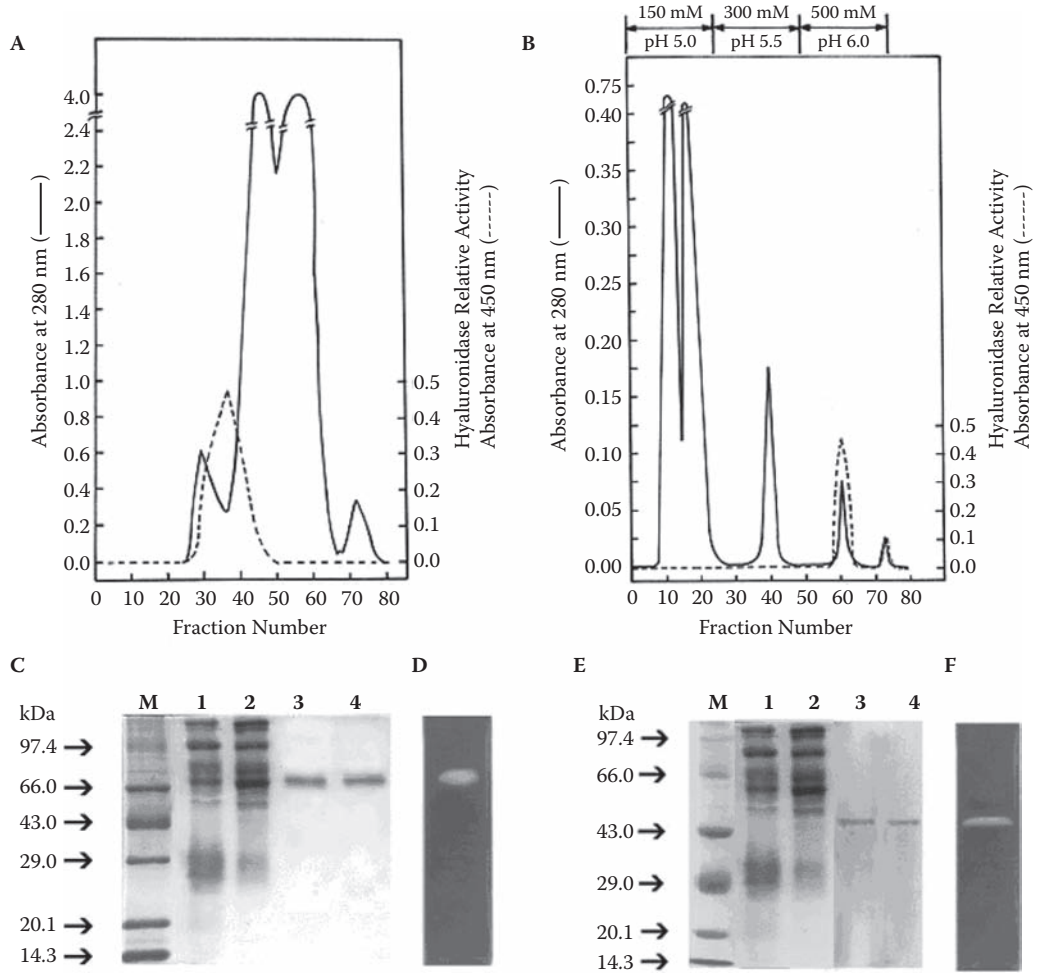


FIGURE 11.5 Isolation of hyaluronidase isoforms NNH1 and NNH2 from *Naja naja* venom. (A) Elution profile from Sephadex G-75 column (1.6 × 98 cm). Fractions having enzyme activity (dotted line) were pooled, concentrated, and applied to a CM-Sephadex C-25 column for further fractionation. (B) Elution profile from CM-Sephadex C-25 column (1.6 × 35 cm). Fractions showing enzyme activity (dotted line) were pooled. (C–F) Purity of NNH1 (C, D) and NNH2 (E, F) as shown by SDS-PAGE (12.5%) under nonreduced conditions (C, E) and hyaluronidase zymography (D, F). (Girish, 2004; Girish and Kemparaju, 2005a.)

NaCl and 0.2 mg/ml bovine serum albumin, pH 5.0). Sodium chloride has been shown to influence activity significantly; 150 mM enhanced the activity, while concentrations above 500 mM were inhibitory for both isoforms. NNH1 is a hydrolase type of endo-β-N-acetylhexosaminidase and released tetrasaccharides with N-acetyl glucosamine and glucuronic acid residues at reducing and nonreducing terminals (respectively) as final end products (Girish et al., 2004a). In addition to snake venoms, hyaluronidases have also been isolated and studied from the venoms of several chordates and nonchordates; their properties are summarized in Table 11.2.

IX. STRUCTURE AND MECHANISM OF ACTION

The structure of snake venom hyaluronidase was only recently elucidated. A study by Harrison et al. (2007), based on venom gland cDNA sequences from *Echis ocellatus*, provides the first full-length sequence of a snake venom hyaluronidase. The enzyme has five conserved N-linked glycosylation

TABLE 11.2
Biochemical and Biophysical Properties of Venom Hyaluronidases

Venom	Molecular Weight (kDa)	*p*I	Optimum pH	Optimum Temperature (°C)
		Fish		
Synanceja horrida	62	9.2	6	37
		Reptiles		
Agkistrodon acutus	33	10.3	5	37
A. contortrix contortrix	59.2	9	6	37
N. naja (isoforms)	70.4	9.2	5	37
	52	9.7	5	37
H. horridum horridum	63	5.1	5	Temperature sensitive
		Arthropods		
Heterometrus fulvipes	82	ND	4	30
Tityus serrulatus	51	ND	6	40
Palamneus gravimanus	52	ND	4.5	37
Apis mellifera	41	9	6	37
Lonomia oblique (isoforms)	49	ND	6–7	37
	53	ND	6–7	37
Loxoceles reclusa (isoforms)	33	ND	5–6.6	37
	63	ND	5–6.6	37
Dolichovespula maculata	39	ND	5–6	37
Vespula germanica	42	ND	5–6	37
Centruroides l. limpidus	47.5	ND	5–6	37
P. regosus	36	ND	5–6	37
Vespula vulgaris	43	ND	5–6	37

Note: PAS = periodic acid Schiff staining; ND = not determined.

sites, three of which appeared to have high potential for glycosylation. The final product appeared to be glycosylated, with a predicted mass of well over 50 kDa. This hyaluronidase showed high homology (over 95%) with enzymes from *Echis pyramidum leakeyi*, *Bitis arietans*, and *Cerastes cerastes cerastes* venoms. Each has conserved residues in the catalytic (Glu^{135}), positional (Asp^{133}, Tyr^{206}, Tyr^{253}, and Trp^{328}), and cysteine scaffold (Cys^{340}, Cys^{211}, Cys^{227}, Cys^{365}, Cys^{370}, Cys^{376}, and Cys^{429}) regions. In contrast, the *E. ocellatus* enzyme showed only 31 to 40% homology with hyaluronidases from venoms of honey bee (*Apis mellifera*), wasp (*Vespula vulgaris*), stonefish (*Synanceja horrida*), and puffer fish (*Tetraodon nigroviridis*). When compared with mammalian enzymes, it showed highest identity (50%) with dog (*Canis familiaris*) hyaluronidase 4.

The only hydrolase class of hyaluronidase with an established three-dimensional structure was from honeybee venom. The structure was established in its native form and also in complex with the hyaluronan oligomer. It is a globular single-domain protein with approximate core dimensions of $52 \times 44 \times 39$ Å. It has ten α helices, eleven β strands, and six 3_{10} helices. It contains four potential glycosylation sites and two disulfide bridges stabilizing the structure. The overall topology resembles a classical eight-stranded $(\beta/\alpha)_8$ triose phosphate isomerase (TIM) barrel motif, except that the hyaluronidase barrel contains only seven strands (Markovic-Housley et al., 2000). Interestingly, when viper hyaluronidase structure is modeled and superimposed onto the known structure of *Apis mellifera*, general conservation of the three-dimensional structural scaffold is observed (Figure 11.3) (Harrison et al., 2007). The regular $(\beta/\alpha)_8$ TIM barrel motif has been found in most of the well-known glycosidases, including α-amylase, β-amylase, and β-glucuronidase enzymes (Rigden et al., 2003).

Although the precise mechanism of hyaluronan degradation by vertebrate hyaluronidase is not clear, the crystal structure of bee venom enzyme (which is a vertebrate-like enzyme) provides insight into the mode of substrate binding and the catalytic site of the enzyme (Markovic-Housley et al., 2000). The enzyme has a substrate binding cleft that is large enough to bind the hexasaccharide unit of the polymer. Substrate binding appears to induce an allosteric modification of the enzyme secondary structure. The mechanism seems to be an acid/base-catalyzed reaction, in which a double displacement event at the anomeric carbon atom of N-acetyl-D-glucosamine of the polymer has been proposed. This process does not appear to involve a covalent intermediate (enzyme-substrate complex) formation; however, an oxocarbonium ion-like transition state occurs, and retention of the β-configuration of C1 of N-acetyl-D-glucosamine in the product is seen. Two carboxylic acid/acidic residues form the catalytic site, one donating the proton and the other acting as the nucleophile. Asp111 and Glu113 are strictly conserved acidic residues in the hyaluronan binding groove of the enzyme. Glu113 appears to act as proton donor, as its side chain carboxylic group is positioned within 2.6 Å of the glycosidic oxygen, while the side chain of Asp111 is placed more than 5 Å away. For steric reasons, Glu113 and Asp111 side chains cannot approach the anomeric carbon atom of the susceptible glycosidic bond of the substrate from opposite sides. Hence, a substrate-assisted reaction mechanism has been considered, with the N-acetyl carbonyl group at C2 of the N-acetyl-D-glucosamine of the substrate acting as a nucleophilic base, which helps position correctly the N-acetyl side chain. The reaction terminates by splitting water and incorporating the OH^- on C1 of N-acetyl-D-glucosamine and H^+ on Glu 113. A three-dimensional model of bovine PH-20 has also been deduced in an attempt to clarify the mechanism of glycosidic bond cleavage, and a functional analysis of mutants indicated the greater importance of Glu113 over Asp111. Substitution of Gln for Glu113 resulted in no detectable activity in the enzyme, while substitution of Asn for Asp111 resulted in 3% residual activity (Arming et al., 1997). As the active site residues and the general three-dimensional scaffold of mammalian and venom hyaluronidases are conserved, a common catalytic mechanism has been proposed (Figure 11.6). Detailed information

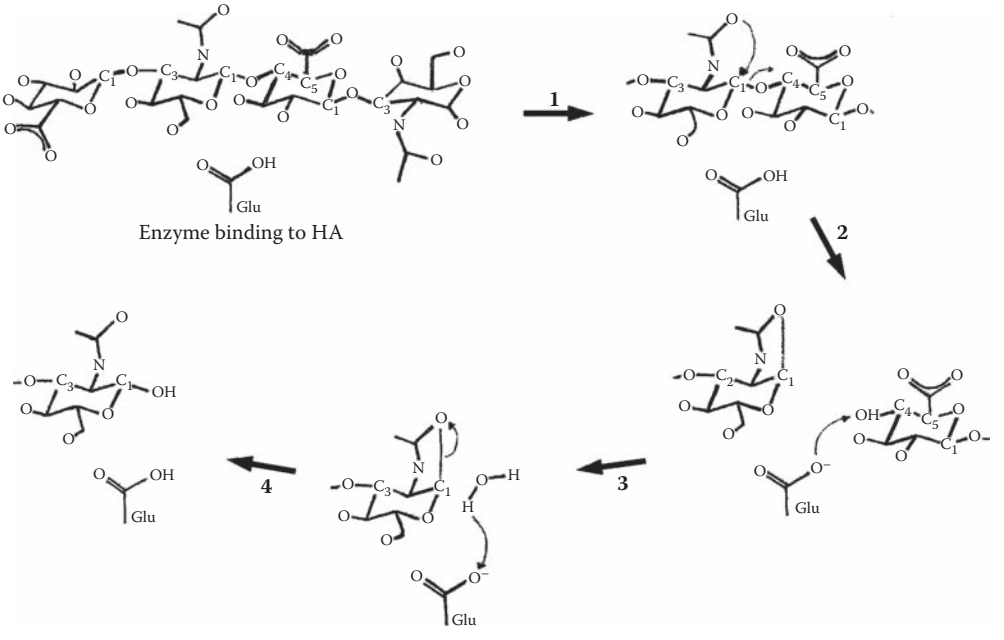


FIGURE 11.6 Proposed catalytic mechanism of hyaluronan hydrolysis by snake venom hyaluronidase. (Stern and Jedrzejas, 2006. With permission.)

on the structure of snake venom hyaluronidase could help solve several unexplained properties of animal hyaluronidases, including the mechanism of substrate hydrolysis.

X. HYALURONIDASE AND VENOM TOXICITY

Envenomation results in a complicated pathology consisting of both local and systemic toxicity. Systemic toxicity includes pre- or postsynaptic neurotoxicity, myotoxicity, pro- or anticoagulant activity, and hypotensive, and rarely hypertensive, effects (e.g., Kini, 1997; Shashidharamurthy et al., 2002; Aird, 2002). The local effects include edema, hemorrhage, dermonecrosis, and myonecrosis at the bite site (e.g., Gutierrez et al., 2005; Fox and Serrano, 2005). Two key factors, namely, dose of the venom injected during the bite and rate of diffusion of injected venom into the blood from the bite site, will determine the magnitude of systemic toxicity. Sudden flux of target-specific systemic toxins into the circulating blood will result in the rapid manifestation of toxic effects. Toxins that would otherwise diffuse much more slowly will diffuse rapidly due to hyaluronan fragmentation by venom hyaluronidase (Yingprasertchai et al., 2003; Kemparaju and Girish, 2006). Furthermore, histochemical studies revealed *in vivo* selective degradation of hyaluronan in human skin and muscle, and chick embryo limb tissue sections. The data strongly suggested that the ECM structure integrity has collapsed, and hence facilitated diffusion of toxins (Figure 11.7). Therefore, it is appropriate to consider hyaluronidase as a toxicity-potentiating factor, in addition to its designated spreading property. Furthermore, damage caused at the bite site due to uncontrolled fragmentation of hyaluronan may result in severe morbidity. As with many other regulatory systems in vertebrates, hyaluronan homeostasis, which is stringently controlled by the hyaluronidase-hyaluronan synthase system, is critical to normal functioning of the organism. Hyaluronidase activity of snake venom disrupts this critical structural component of tissues and likely increases overall toxic effects of venom, a subtle point that has been largely ignored. Tissue necrosis at the bite site is likely due to the cumulative effects of myonecrotic phospholipase A_2 enzymes (Kini, 1997), metzincin family enzymes (Anai et al., 2002; Gutierrez et al., 2005; Fox and Serrano, 2005), and hyaluronidases. Although the relative contribution of each component might vary with relative abundance in different snake venoms (Girish et al., 2002), the potential synergistic effects may explain much of the potent and variable symptoms seem clinically in envenomation cases. Hyaluronidase thus may be an essential mediator of venom toxicity, both systemic and local.

XI. POSSIBLE PATHOLOGICAL EFFECTS OF SNAKE VENOM HYALURONIDASE

Despite the destructive effects on tissue hyaluronan at the envenomed region (Girish et al., 2002, 2006), snake venom hyaluronidase is commonly considered a nontoxic component of venoms. However, the fact that it is present in low amounts in snake venoms, yet acts with high specific activity, and that it occurs in many venoms, strongly suggests an important role in envenomation. Furthermore, entry of this enzyme into circulation and the degradation of circulatory hyaluronan have the potential to cause circulatory collapse, and its activity (or released metabolites) in kidney, liver, and other vital organs may cause life-threatening complications. In arthropods, venom hyaluronidase is a known major allergen that can induce serious and occasionally fatal systemic immunoglobulin E–mediated anaphylactic reactions in humans (Kolarich et al., 2005; Girish and Kemparaju, 2007). Determination of the structural moieties responsible for its allergic potency will have great importance with clinical implications in arthropod stings, and it may be important to reptile envenomations as well.

In addition to the direct effects, the indirect effects of hyaluronidase may be quite important. The process of fragmentation of hyaluronan and generation of fragments of varied molecular mass by mammalian enzymes, and their role in a wide range of pathophysiological effects, are the topics of current research (Stern et al., 2006; Girish and Kemparaju, 2007). The intact high molecular weight hyaluronan present in the ECM is antiangiogenic and immunosuppressive, while smaller fragments

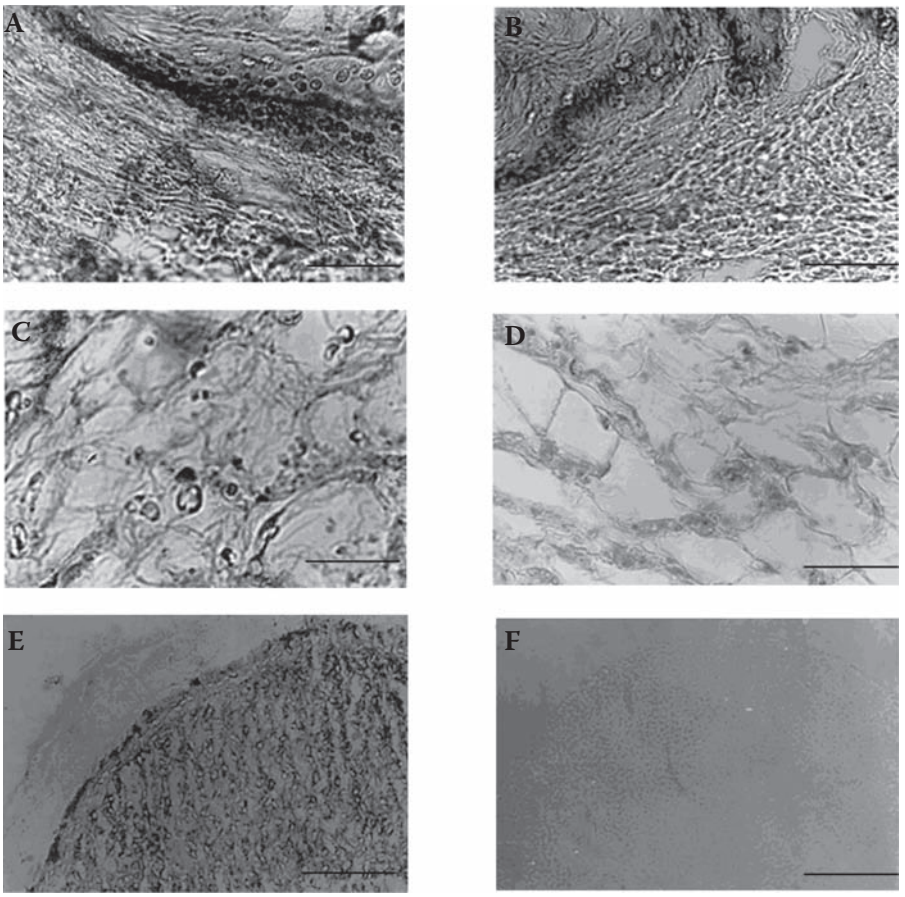


FIGURE 11.7 Human skin, muscle, and chick embryo limb sections stained for hyaluronan with biotinylated hyaluronic acid binding protein (HABP). Control sections: skin (A), muscle (C), and chick embryo limb (E). Sections of NNH1-treated tissues: skin (B), muscle (D), and chick embryo limb (F). (Girish et al., 2002. With permission.)

are inflammatory, immunostimulatory, and angiogenic in property (Noble, 2002; Toole, 2004; Day and de la Motte, 2005). Snake venom hyaluronidase is expected to generate a broad size range of hyaluronan fragments, and these likely participate in the acute pharmacological effects, including inflammatory responses, which often accompany envenomations. Further, activation of endogenous tissue hydrolases such as proteases and glycosidases may also account for some of the pharmacological effects of the venom. Extracellular-generated hyaluronan fragments at the site of inflammation upregulated the expression of matrix metalloproteases (Fieber et al., 2003; Stern et al., 2006; Iacob and Knudson, 2006). It is therefore tempting to speculate that venom hyaluronidase and its catalytic end products might play a role in cellular death at the bite site.

XII. INEFFICACY OF ANTIVENOM TO PROTECT FROM LOCAL TISSUE DESTRUCTION

Antivenoms are the most effective treatment for bites by venomous snakes (see Section IV, this volume). Despite the high rate of success in saving lives, they provide much less than desirable protection against local tissue damage (Lomonte et al., 1996; Leon et al., 1999, 2000). Local damage despite antivenom is due to the speed with which tissues are degraded and to the failure of systemically

administered antivenom to reach the bite site. Local tissue damage may continue uncontrolled even after the successful neutralization of systemic toxicity; this is seen particularly in viper bites. The continued local tissue destruction appears to be complex, and there might be involvement of several factors, including effects of ECM-degrading venom enzymes, activation of ECM-degrading tissue enzymes, or microbial degradation due to opportunistic microbial infection. We consider hyaluronidase to be one of the prime agents responsible for the snakebite-induced deformation of local tissue, and hence hyaluronidase is an important target for minimizing envenomation-associated morbidity. Inhibition of hyaluronidase not only prevents local tissue destruction but also retards the diffusion of toxins into the blood, resulting in delay in time to death in severe cases of envenomation (Yingprasertchai et al., 2003; Girish and Kemparaju, 2005b, 2006). As antivenoms are often not readily available in many rural areas worldwide, and time to treatment is critical, slowing diffusion of venom components could make the difference between life and death. Inhibition of snake venom hyaluronidase therefore has significant therapeutic relevance in preventing local tissue damage and in managing snakebite more effectively. This is supported by the observations that small-sized antibody fragments (Fab and $F(ab)_2$), which should be relatively rapidly diffusing, fail to protect against local tissue damage caused by a venom metalloprotease (Lomonte et al., 1996; Leon et al., 1997, 2000). Thus, identification and characterization of hyaluronidase inhibitors would be valuable for developing strategies that increase efficacy of antivenoms, antitoxins, and antimicrobial agents.

XIII. SNAKE VENOM HYALURONIDASE INHIBITORS

A wide range of compounds belonging to several different chemical classes (proteins, glycosaminoglycans, polysaccharides, alkaloids, antioxidants, anti-inflammatory agents, and synthetic organic compounds) have been investigated for their potential to inhibit hyaluronidase activity (Mio et al., 2000; Mio and Stern, 2002; Khanum et al., 2005; Salmen et al., 2005; Machiah et al., 2006; Girish and Kemparaju, 2007) (Table 11.3).

Alkaloids such as aristolochic acid, ajmaline, and reserpine inhibited *Naja naja* venom hyaluronidase activity. In contrast to the partial inhibition by ajmaline and reserpine, aristolochic acid completely inhibited the activity through a noncompetitive mechanism (Figure 11.8). Aristolochic acid inhibited the spreading property of the hyaluronidase, and it also inhibited the hyaluronidase-potentiated toxicity of a myotoxic phospholipase A_2 and a hemorrhagic complex from *Daboia russelli* venom (Girish and Kemparaju, 2005b, 2006).

The glycosaminoglycan (GAG) heparin is a well-known inhibitor of mammalian hyaluronidases. It inhibited enzymatic activity via a noncompetitive mechanism by interacting with surface amino groups of the enzyme (Wolf et al., 1984; Maksimenko et al., 2001). At lower concentrations, heparin

TABLE 11.3
List of Different Classes of Snake Venom Hyaluronidase Inhibitors

Type	Compounds
Alkaloids	Aristolochic acid, ajmaline, reserpine
Antioxidants	Ascorbic acid, NDGA, N-propyl gallate, BHT, chlorogenic acid, curcumin, tannic acid
Anti-inflammatory drugs	Dexamethasone, indomethacin, fenoprofen, oxyphenbutazone, sodium cromoglycate, salicylates, tranilast, sodium aurothiomalate
Terpenoids/flavonoids	Flavone, quercetin, apigenin, kaempferol, silybin, luteolin, hesperidin, triterpenes, rutin, myricetin, glycyrrhizin
Synthetic compounds	Diphenyl propionic acids, indole derivatives, chalcone derivatives, benzoyl phenyl benzoates
Glycosaminoglycans	Heparin, heparan sulfate, dermatan sulfate
Polysaccharides	Chitosans
Other proteins	*Withania somnifera* glycoprotein

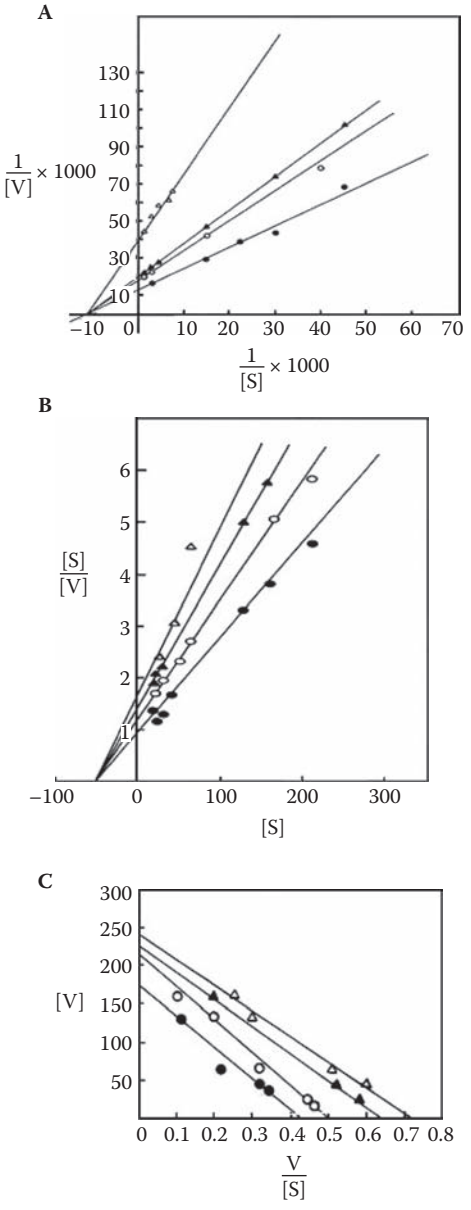


FIGURE 11.8 Noncompetitive inhibition of *Naja naja* venom hyaluronidase NNH1 by aristolochic acid. (A) Lineweaver-Burk plot. (B) Hanes-Woolf plot. (C) Woolf-Augustinsson-Hofstee plots. Initial velocities (V) are expressed as millimoles of N-acetyl glucosamine released at 37°C. Control (NNH1 alone ●—●) or with 5 μM (○—○), 10 μM (△—△), or 20 μM (▲—▲) aristolochic acid. (Girish and Kemparaju, 2006.)

inhibited venom enzyme more efficiently than bovine enzyme, while leech and *Streptomyces* enzymes were not inhibited (Mio and Stern, 2002). The GAGs dermatan sulfate and heparan sulfate inhibited snake venom as well as human serum hyaluronidase (Afify et al., 1993; Girish and Kemparaju, 2005b). There appears to be an electrostatic interaction between the negatively charged GAGs and positively charged NNH1 (basic in property with a pI value of 9.2), and this might prohibit the binding of the hyaluronan substrate, resulting in inhibition of activity. Melo and Ownby (1999) demonstrated complex formation between a basic myotoxic PLA_2 and the acidic GAG heparin,

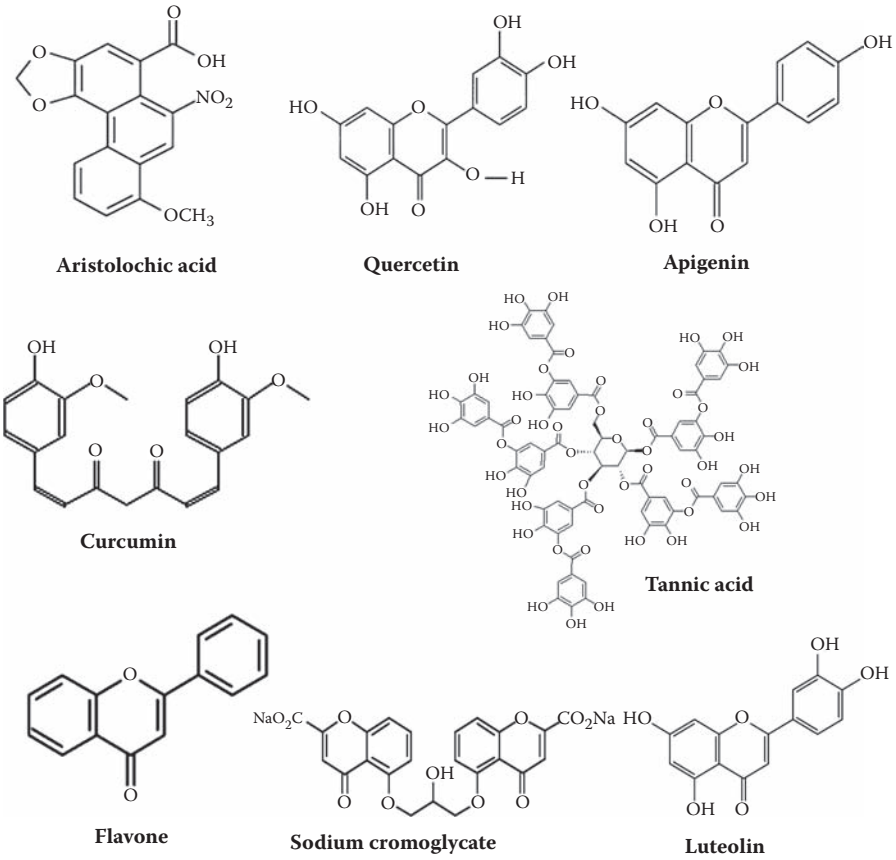


FIGURE 11.9 Structures of several biologically derived compounds that inhibit snake venom hyaluronidase activity.

which resulted in partial neutralization of myotoxicity of *Bothrops jararacussu* venom. Diccianni et al. (1990) reported the inhibition of a PLA_2 enzyme by negatively charged GAGs and established that a complex formed with heparin.

The polysaccharide chitosan, which is a positively charged deacetylated form of chitin, inhibited both snake venom and bovine testicular hyaluronidases. Level of inhibition was found to be in proportion to molecular weight, with high molecular weight forms being more effective inhibitors. Complex formation between cationic and anionic functionalities of chitosan and hyaluronan, respectively, limits the availability of the hyaluronan substrate for the enzyme, and this could be the reason for *in vitro* inhibition by chitosan (Denuziere et al., 2000; Girish and Kemparaju, 2005b).

Several anti-inflammatory drugs (including salicylates, indomethacin, fenoprofen, phenylbutazone, oxyphenbutazone, dexamethasone, sodium aurothiomalate, and sodium cromoglycate) inhibited hyaluronidase activity of various snake venoms (Figure 11.9). Indomethacin was found to be a more potent inhibitor than dexamethasone against *Naja naja* venom hyaluronidase NNH1 (Girish and Kemparaju, 2005b). Sodium aurothiomalate and sodium cromoglycate completely inhibited the hyaluronidase activity of *Naja kaouthia* and *Calloselasma rhodostoma* snake venoms. In addition, these drugs neutralized the local (edema, hemorrhage) and systemic (myotoxicity, survival time) toxicities exhibited by these venoms (Yingprasertchai et al., 2003).

Flavonoids are biodynamic compounds and are known to possess anti-inflammatory and anti-allergic properties and inhibit PLA_2 (Havsteen, 1983; Mors et al., 2000) and hyaluronidase activities (Kuppusamy et al., 1990; Kuppusamy and Das, 1991; Girish and Kemparaju, 2005b). Some

flavonoids and tannins were reported to prolong the survival time of mice following subcutaneous injection of venom (Kakegawa et al., 1985). Among these, quercetin completely inhibited the hyaluronidase enzyme activity. Flavone and tannic acid exhibited similar types of inhibition patterns (Girish and Kemparaju, 2005b), while kaemferol and apigenin partially inhibited the hyaluronidase activity of *Naja kaouthia* and *Calloselasma rhodostoma* venoms (Yingprasertchai et al., 2003).

Antioxidant compounds such as NDGA (nordihydroguaiaretic acid), curcumin, n-propyl gallate, BHT (butylated hydroxytoluene), chlorogenic acid, and ascorbic acid inhibited the *Naja naja* venom hyaluronidase NNH1 activity to a varied extent. Maximum inhibition was seen with NDGA and curcumin, while ascorbic acid was found to be least inhibitory (Girish and Kemparaju, 2005b). In contrast, ascorbic acid and its analogs are more specific inhibitors of the bacterial lyases (Spickenreither et al., 2006).

Various natural products are also hyaluronidase inhibitors. A glycoprotein from *Withania somnifera* (Machiah et al., 2006), polyphenols from the fruit of blackberry (*Rubus fruticosus*) (Marquina et al., 2002), water extract of *Pavo cristatus* feathers (Murari et al., 2005), aqueous root extract of *Mimosa pudica* (Girish et al., 2004a), and ethanol extract of *Tamarindus indica* seeds (Ushanandini et al., 2006) neutralized the hyaluronidase activity of *Echis carinatus*, *Daboia russellii*, *N. naja*, and *Trimeresurus malabaricus* venoms. Benzoyl phenyl benzoates inhibited *N. melanoleuca*, *D. russelii*, and *N. naja* venom hyaluronidase activity (Khanum et al., 2005).

The compounds above inhibited crude snake venom hyaluronidase activity as well as the purified enzyme to a varying degree, but the nature of interactions and the pattern of inhibition have not been established for most inhibitors. Aristolochic acid was found to inhibit *Naja naja* venom hyaluronidase NNH1 through a noncompetitive mechanism (see Figure 11.8), and the interaction was found to alter the three-dimensional structure of the enzyme, as it caused dose-dependent quenching of fluorescence emission of the enzyme (Girish and Kemparaju, 2006). Figure 11.9 shows some of the bioactive compounds that inhibit snake venom hyaluronidase activity.

XIV. CONCLUSIONS

This chapter provides a broad perspective of structure, activity, and functions of snake venom hyaluronidases and their potential role in venom toxicity. There is limited information available on snake venom hyaluronidases, and we hope that this chapter stimulates further investigations on this neglected enzyme. The spreading property of the enzyme promotes distribution of other venom components and generalized tissue destruction. The importance of venom hyaluronidases as a therapeutic target is stressed, and identification of nontoxic inhibitors of the enzyme could play an important role in the efficient management of snakebite.

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12 Natural Inhibitors
Innate Immunity to Snake Venoms

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CONTENTS

Modern understanding of natural inhibitors of snake venom toxins has led to a diversified classification of these compounds, and the primary structure of antihemorrhagic proteins allowed them to be assigned to the immunoglobulin supergene, ficolin/opsonin P35, or to the cystatin families. Phospholipase inhibitors were classified as C-type lectin-like proteins (α-PLIs), molecules bearing leucine-rich repeats (β-PLIs), or presenting a three-fingered conformation (γ-PLIs); a single PLI member of the immunoglobulin supergene family has also been described. This chapter reviews recent developments in understanding the biological, chemical, and physicochemical properties of the isolated inhibitors and their mechanisms of action, as well as the structure-function relationships of these inhibitors. Several classical and state-of-the-art methods for assaying the inhibition of toxin activity and characterizing inhibitors' composition and interactions are provided. Perspectives on the use of natural inhibitors in the neutralization of venom metalloproteinases and phospholipases A_2 are also discussed.

I. INTRODUCTION

In tropical and subtropical countries of Latin America, Asia, Africa, and Oceania, envenomations inflicted by snakebites are a significant public health hazard. Unfortunately, public authorities at

the community, state, and national levels neglect the study of this health problem (Gutiérrez et al., 2006b). Incident data are few and not reliable, and figures for the global mortality due to snakebites vary according to the report chosen. They may range from fifty thousand to one hundred thousand per year, but because the majority of victims use traditional treatments at home, there are likely a significant number of unrecorded deaths, and these figures may be considerably higher (Chippaux, 1998). For instance, population-based studies performed in Senegal, Kenya, Nigeria, and West Bengal revealed rates ranging from 2 to 16 deaths per 100,000 per year. Snakebite envenoming can be classified as an occupational disease because the most affected group is young male agricultural workers. For every death, there are unreported sequelae of survivors (morbidity rates), such as chronic ulceration, renal failure or pituitary-adrenal insufficiency, thrombosis, osteomyelitis with malignant transformation, neurological damages resulting from intracranial hemorrhages, and amputations (Theakston et al., 2003).

Local effects of snakebites by viperids often include swelling, due to permeability-enhancing factors and necrosis, which results from the action of metallo- and serine proteinases, myotoxins, cytotoxins, thrombotic factors, and secondary infection. Systemic envenoming, on the other hand, leads to spontaneous hemorrhage in the brain, gastrointestinal tract, or elsewhere, as well as incoagulable blood and thrombosis of capillaries and larger blood vessels due to antihemostatic and thrombotic effects attributed to venom procoagulant enzymes, snake venom metalloproteinases, phospholipases A_2, and platelet factors. Other venom effects, particularly from elapid bites, are paralysis caused by venom neurotoxins acting pre- and postsynaptically and hypotension resulting from hypovolemia, caused by the loss of circulating volume into the swollen limb. Venom phospholipases (PLA_2) and other myotoxins also cause generalized rhabdomyolysis, which contributes to muscle weakness and fatal hyperkalemia and may also induce intravascular hemolysis (Theakston et al., 2003).

Treatment of this plethora of deleterious effects relies upon animal antiserum (antivenoms), primarily produced in horse or sheep, against antigenic toxins present in single or pooled snake venoms. Three different types of products are used: whole immunoglobulin G (IgG) molecules, $F(ab')_2$ fragments obtained from IgG by digestion with pepsin, or Fab fragments from papain digestion. The main problem associated with a lack of efficacy of antiserum therapeutics involves local tissue damage. The rapid action of toxins in the development of local pathologies contributes to difficulties in fast neutralization by antibodies before irreversible damage occurs at the injury site. Antivenom safety is also a strong concern, and its administration may provoke adverse reactions such as direct complement activation, effects of contaminating pyrogens, and reactions to immune complexes. Other important problems for the use of antivenom are transportation to remote villages and storage that demands specific conditions for preservation of its neutralizing titer, a problem that is solved by lyophilization but which is not yet commonly practiced (Gutiérrez et al., 2006b).

Thus, it is clear that other sources of therapeutic tools must be explored and developed if they show prospective for circumventing flaws with antivenom use. Proteins that are natural inhibitors of snake venoms are such an example. Historically, their study began with Felice Fontana (considered the father of modern toxinology) following the publication in 1781 of his classic text. Over 140 years later, another important book, *Animaux Venimeux et Venins*, by M. Phisalix, reviewed the field and helped disseminate information on the natural immunity to venoms of an incredible number of animals, including fishes, batrachians, invertebrates, and mammals (Domont et al., 1991). Forgotten for a long time, in the last 30 years this area has experienced a rather slow development of the understanding of the phenomenon of innate or natural immunity, which can de described as the natural resistance to snake venoms expressed by some animals or the genetically inherited resistance that certain animals possess against the deleterious action of snake venoms.

Modern analysis of these inhibitors has led to a diversified classification. They are proteins of low or high molecular mass, may be present in body fluids or tissues, and are homologous with different protein families: antihemorrhagic proteins (metalloproteinase inhibitors), for example,

TABLE 12.1
SVMPIs Isolated from Plasma or Serum of Snakes

Name	Species	pI	Molecular Mass (kDa) Native	Molecular Mass (kDa) Subunits	Stoichiometry[a] (Subunit:Toxin)	Glycosylated	pH[b]	Temperature (°C)[c]
				Cystatin Superfamily				
BJ46a[1]	*Bothrops jararaca*	4.6	79[d]	46	1:2[e]	Yes	nd	nd
HSF[2]	*Trimeresurus flavoviridis*	4.0	70	48	1:1	Yes	1.0–13.0	100
				Undetermined Protein Family				
—[3]	*Agkistrodon c. mokasen*	4.6	nd	62–79	nd	nd	nd	nd
BaSAH[4]	*Bothrops asper*	5.2	66	nd	2:1	nd	1.5–9.0	60
—[5]	*Crotalus atrox*	nd	nd	65–80	nd	Yes	1.3–11.5	85
—[6,f]	*Dinodon semicarinatus*	nd	59	52	nd	nd	2.0–11.0	60
NtAH[7,f]	*Natrix tessellata*	4.5	880	70, 100, 150[g]	4:1	nd	5.5–11.7	50
TMI[8, h]	*Trimeresurus mucrosquamatus*	nd	nd	47	nd	Yes	nd	nd
—[9]	*Vipera palaestinae*	4.7	nd	80	nd	Yes	4.0–9.5	85

[1] Valente et al., 2001.
[2] Omori-Satoh et al., 1972; Omori-Satoh, 1977; Yamakawa and Omori-Satoh, 1992; Deshimaru et al., 2005.
[3] Gloyd, 1933; Weinstein et al., 1991.
[4] Borkow et al., 1995.
[5] Weissenberg et al., 1991, 1992.
[6] Tomihara et al., 1988.
[7] Borkow et al., 1994.
[8] Huang et al., 1999.
[9] Ovadia, 1978.
[a] Subunit:toxin (mol:mol).
[b] Inhibitory activity maintained for at least 1 h within this range.
[c] Inhibitory activity maintained for at least 15 min up to this temperature.
[d] Dimer.
[e] Inhibitor monomer:enzyme (mol:mol).
[f] Nonvenomous snake.
[g] Not subunits, but polypeptide chains separated by SDS-PAGE after reduction.
[h] N-terminal sequence only (18 residues).
Note: nd, not determined.

can be members of the immunoglobulin supergene family, the ficolin/opsonin P35 family, or the cystatin superfamily (Tables 12.1 and 12.2 and references therein). Inhibitors of toxic PLA_2 (PLIs) may contain C-type lectin-like domains (α-PLIs), exhibit leucine-rich repeats similar to human α_2-glycoprotein (β-PLIs), or display disulfide-defined loops with a three-finger appearance, which is also found in mammalian cell-surface Ly-6 antigens, elapid neurotoxins, and urokinase-type plasminogen activator receptor (γ-PLIs). One PLI member of the immunoglobulin supergene family has also been described (Table 12.3 and references therein).

This chapter reviews current understanding of the biological, chemical, and physicochemical properties of the isolated inhibitors and their mechanisms of action. Structure-function relationships of these inhibitors and their use in the neutralization of the action of venom metalloproteinases and phospholipases A_2 will be discussed.

TABLE 12.2
SVMPIs Isolated from Mammal Plasma, Serum, or Muscle

Name	Species	pI	Molecular Mass (kDa)		Stoichiometry[a] (Subunit:Toxin)	Glycosylated	pH[b]	Temperature (°C)[c]
			Native	Subunits				
Ig Supergene Family								
DM43[1]	*Didelphis (marsupialis) aurita*	<3.5	72[d]	43	1:1	Yes	nd	nd
DM40[1,f]	*Didelphis (marsupialis) aurita*	<3.5	95[d]	40	1:1	Yes	nd	nd
Oprin[2,e]	*Didelphis virginiana*	3.5	nd	52	nd	Yes	2.5–11.5	70
DA2-II[3, f]	*Didelphis albiventris*	nd	nd	43	nd	nd	nd	nd
AHF-1[4,g]	*Herpestes edwardsii*	nd	65	—	nd	Yes	2.0–11.0	60
AHF-2[4,f]	*Herpestes edwardsii*	nd	65	—	nd	Yes	2.0–11.0	60
AHF-3[4,f]	*Herpestes edwardsii*	nd	65	—	nd	Yes	2.0–11.0	60
—[5, f]	*Lutreolina crassicaudata*	nd	nd	49	nd	Yes	nd	nd
PO41[6,f]	*Philander opossum*	<3.5	82–85[d]	41	1:1	Yes	nd	nd
Ficolin/Opsonin P35 Family								
Erinacin[7,h]	*Erinaceus eurapaeus*	nd	1090[i] (α_{10}·2β_{10})	α = 38[f] β = 35[j, k]	1:1	nd	nd	nd
Undetermined Protein Family								
β-macroglobulin[8]	*Erinaceus eurapaeus*	nd	700[i]	34 and 39	nd	nd	nd	nd
—[9]	*Neotoma micropus*	4.1	54	nd	nd	nd	3.0–10.0	56
—[10]	*Sigmodon hispidus*	5.4	90	nd	nd	nd	3.0–10.0	55
—[11]	*Spermophilus mexicanus*	4.9	nd	52	nd	nd	2.0–12.0	70

[1] Perales et al., 1986; Neves-Ferreira et al., 2000, 2002. [2] Catanese and Kress, 1992. [3] Farah et al., 1996. [4] Tomihara et al., 1987; Qi et al., 1994, 1995. [5] Perales et al., 1994. [6] Perales et al., 1994; Jurgilas et al., 2003. [7] Mebs et al., 1996; Omori-Satoh et al., 2000. [8] de Wit and Westrom, 1987a, 1987b. [9] Garcia and Pérez, 1984. [10] Pichyangkul and Pérez, 1981. [11] Martínez et al., 1999. [a] Subunit:toxin (mol:mol). [b] Inhibitory activity maintained for at least 1 h within this range. [c] Inhibitory activity maintained for at least 5 min at this temperature. [d] Dimer. [e] Partial sequence, ca. 80%. [f] N-terminal sequence only (15 to 32 residues long). [g] Partial sequence, ca. 76%. [h] Inhibitor isolated from muscle sample. [i] Molecular mass of the oligomer. [j] Molecular mass of the polypeptide chain after reduction of the 350 kDa subunit. [k] Partial sequence, ca. 75%.

Note: nd, not determined.

TABLE 12.3
PLIs Isolated from Plasma or Serum of Snakes and Mammals

Name	Species	Family	pI	Molecular Mass (kDa)		Stoichiometry[b] (Inhibitor:Toxin)	Glycosylated	PLA_2 Group Specificity[c]	Function
				Native	Subunit[a]				
					α-Type PLI[d]				
GbPLIα[1]	*Gloydius brevicaudus* (*Agkistrodon blomhoffii siniticus*)	Viperidae	nd	75	20	1 trimer:1 PLA_2	Yes (irrelevant to activity)	II (acidic)	nd
PLI-IV/V[2]	*Protobothrops* (*Trimeresurus*) *flavoviridis*	Viperidae	4.5	75	22 (A) and 21 (B)	1 trimer:1 PLA_2	Yes (irrelevant to activity)	II (acidic)	nd
BaMIP[3]	*Bothrops asper*	Viperidae	4.0	120	23–25	1 monomer:1 PLA_2	Yes	II (basic)	Antimyotoxic
BmjMIP[4]	*Bothrops moojeni*	Viperidae	4.2	120	23–25	1 monomer:1 PLA_2	Yes (irrelevant to activity)	II (basic)	Antimyotoxic
CgMIP-II[5]	*Cerrophidion godmani* (fomerly *Bothrops godmani)*	Viperidae	4.0	180	20–25	nd	Yes	II (basic)	Antimyotoxic
AnMIP[6]	*Atropoides nummifer*	Viperidae	4.1–4.7	92	22.2–22.3	nd	Yes	II (basic)	Antimyotoxic
					β-Type PLI[e]				
GbPLIβ [7]	*Gloydius brevicaudus*	Viperidae	nd	160	43.8	1 monomer:1 PLA_2	Yes	II (basic)	nd
EqPLIβ[8,i]	*Elaphe quadrivirgata*	Colubridae	nd	150	50 (A and B)	nd	Yes	II (basic)	nd
					γ-Type PLI[f]				
GbPLIγ[9]	*Gloydius brevicaudus*	Viperidae	4.4	100	20 and 25	nd	Yes (25 kDa subunit)	I, III, and II (acidic/basic/neutral)	nd

(continued on next page)

TABLE 12.3 (continued)
PLIs Isolated from Plasma or Serum of Snakes and Mammals

Name	Species	Family	pI	Molecular Mass (kDa)		Stoichiometry[b] (Inhibitor:Toxin)	Glycosylated	PLA_2 Group Specificity[c]	Function
				Native	Subunit[a]				
CgMIP-I[10]	*Cerrophidion* (*Bothrops*) *godmani*	Viperidae	4.0	110	20–25	nd	Yes	II (basic)	Antimyotoxic
CNF/CICS[11]	*Crotalus durissus terrificus*	Viperidae	nd	160 (CNF); 130 (CICS)	23.6 (CNF) 23–25 (CICS)	1 monomer:1 PLA_2 (CNF) 1 oligomer:1 PLA_2 (CICS)	Yes	II (acidic and basic)	Antineurotoxic[h]
PLI-I[12]	*Protobothrops* (*Trimeresurus*) *flavoviridis*	Viperidae	4.0–4.5	nd	27	nd	Yes (irrelevant to activity)	II (basic)	nd
NnkPLIγ[13]	*Naja naja kaouthia*	Elapidae	4.5	90	31 and 25	nd	Yes (31 kDa subunit)	I and II	nd
NAI[14]	*Notechis ater*	Elapidae	nd	110	22.5 (α) and 19.8 (β)	nd	Yes (α subunit)	I, III, and II acidic/basic/neutral	nd
NSI[15]	*Notechis scutatus*	Elapidae	nd	110	22.5 (α) and 19.8 (β)	nd	Yes (α subunit)	I and II (acidic/basic/neutral)	Antineurotoxic
LsPLIγ[16]	*Laticauda semifasciata*	Elapidae	4.4	100	25 (A) and 20 (B)	nd	Yes (A subunit) (irrelevant to activity)	I, III, and II (acidic/basic/neutral)	nd
PIP[17,i]	*Python reticulatus*	Pythonidae	nd	140	23.3	nd	Yes (irrelevant to activity)	I, II, and III	Antilethal and anti-inflammatory
EqPLIγ[18,i]	*Elaphe quadrivirgata*	Colubridae	4.5	130	30 (A) and 29 (B)	nd	Yes	I, III, and II (acidic/basic/neutral)	nd

Ig Supergene Family[g]									
DM64[19]	*Didelphis* (*marsupialis*) *aurita*	Didelphidae	4.5	110 (dimer)	63.6	nd	Yes	II (basic)[j]	Antimyotoxic

[1] Ohkura et al., 1993, 1997; Okumura et al., 1999b, 2005. [2] Kogaki et al., 1989; Inoue et al., 1991, 1997; Nobuhisa et al., 1997, 1998. [3] Lizano et al., 1997. [4] Soares et al., 2003. [5] Lizano et al., 2000. [6] Quirós et al., 2007. [7] Ohkura et al., 1997; Okumura et al., 1998. [8] Okumura et al., 2002. [9] Ohkura et al., 1997. [10] Lizano et al., 2000. [11] Fortes-Dias et al., 1991, 1994; Perales et al., 1995; Fortes-Dias et al., 1999; Faure et al., 2000. [12] Nobuhisa et al., 1997, 1998. [13] Ohkura et al., 1994b, 1994a. [14] Hains and Broady, 2000; Hains et al., 2000. [15] Hains and Broady, 2000. [16] Ohkura et al., 1999. [17] Thwin et al., 2000. [18] Okumura et al., 1999a. [19] Rocha et al., 2002.

[a] Hyphenated values indicate the range within the molecular mass of the subunit (blurred protein bands); values separated by "and" indicate two subunits with different molecular masses (named either A and B or α and β). [b] Inhibitor:toxin (mol:mol). [c] PLA_2 group against which the inhibitor is (more) effective. [d] α-type PLI, inhibitors of PLA_2 classified as C-type lectin-like proteins. [e] β-type PLI, PLA_2 inhibitors bearing leucine-rich repeats. [f] γ-type PLI, PLA_2 inhibitors presenting a three-fingered conformation. [g] Antimyotoxic protein from the immunoglobulin supergene family. [h] Binds to the phospholipase subunit CB of crotoxin, but not to the acidic chaperon subunit CA. [i] Nonvenomous snake. [j] Inhibits PLA_2-induced myotoxicity without inhibiting its catalytic activity.

Note: nd, not determined.

II. SNAKE VENOM METALLOPROTEINASES (SVMPs)

SVMPs, also known as hemorrhagins, play an important, if not the primary, role in the hemorrhagic syndrome following envenomation by members of the family Viperidae. Effects often occur most intensely at the site of venom inoculation (local effects), contributing to local ischemia and poor tissue regeneration (Gutiérrez et al., 1995, 2005; Chapter 5, this volume). Systemic effects, leading to complications such as hemorrhagic shock and intracranial hemorrhage, may also occur (Warrell, 1996; White, 2005). SVMPs belong to the reprolysins subfamily (Bjarnason and Fox, 1995) of the M12 family of metalloproteinases and are synthesized as latent precursor pro-proteinases, which are subsequently processed, yielding active metalloproteinases (Hite et al., 1992; Shimokawa et al., 1997). The mature proteins were classified into four classes (P-I to P-IV) according to the increasing number of different domains they possessed: metalloproteinase (P-I) + disintegrin/disintegrin-like (P-II) + cysteine-rich (P-III) + lectin-like (P-IV); only members of the P-II class have a true disintegrin domain (Bjarnason and Fox, 1994, 1995). Recently, a modified classification scheme has introduced P-II and P-III subclasses due to the existence of dimeric structures as well as the speculation of differences in potential for proteolytic processing (Fox and Serrano, 2005; Chapter 4, this volume).

III. SVMP INHIBITORS (SVMPIs)

Several SVMPIs have been described and characterized to varying extents, and these inhibitors have been isolated from the plasma/serum of several snakes (both venomous and nonvenomous) and from plasma/serum or muscle of mammals such as opossums, hedgehogs, mongooses, hispid cotton rats, wood rats, and squirrels. Most interesting, in the case of *Didelphis* (*marsupialis*) *aurita* (Cerqueira and Lemos, 2000), the presence of these inhibitors was confirmed in the plasma of laboratory newborn animals (indicating a natural immunity phenomenon) (Perales et al., 1986) and also in opossum's milk, which could provide additional protection for the neonates against snake envenomation (Jurgilas et al., 1999).

Tables 12.1 and 12.2 list all purified SVMPIs published to date as well as some of their physicochemical and structural properties. In this review we will describe only some of these inhibitors (those that have been more thoroughly characterized), and we refer the reader to previous reviews on the subject (Domont et al., 1991; Thwin and Gopalakrishnakone, 1998; Perales and Domont, 2002; Perales et al., 2005).

Generally speaking, SVMPIs are acidic glycoproteins whose mechanism of interaction involves the establishment, through noncovalent bonds, of a complex with the venom enzyme, rendering it inactive. These molecules maintain their inhibitory properties even after being submitted to high temperature and broad pH range. They can be found as monomeric or dimeric molecules, with the exception of erinacin, a very complex structure of several polypeptide chains linked by disulfide bonds and subunits linked by noncovalent interactions. Regarding their structural characteristics, only two SVMPIs isolated from snakes have had their complete primary structure determined, and these were classified as members of the cystatin superfamily (Rawlings and Barrett, 1990), although they are not able to inhibit cysteine proteinases. Some authors have proposed a new classification scheme for peptidase inhibitors, and these snake SVMPIs would belong to clan IH, family I25C of peptidase inhibitors (Rawlings et al., 2004). SVMPIs isolated from mammals have been assigned to two protein families: the Ig supergene (Hood et al., 1985) and the ficolin/opsonin P35 (Sugimoto et al., 1998) families.

A. Cystatin Superfamily

The first SVMPI belonging to this family was purified from the serum of the habu snake, *Trimeresurus flavoviridis*, in 1972. The purified 70 kDa protein, named HSF (habu serum factor),

was able to inhibit hemorrhagic activities of two autologous SVMPs: HR1 (class P-III) and HR2 (class P-I). In the same work the authors demonstrated that the crude habu serum was able to inhibit hemorrhagic activities, to different extents, of several homologous Viperidae venoms. Surprisingly, an exception was reported for *Bothrops jararaca* venom, for which inhibition was poor (Omori-Satoh et al., 1972), although another antihemorrhagic factor (named BJ46a), very similar to HSF (85% sequence identity), is fully effective in inhibition of *B. jararaca* venom hemorrhagic activity; more details regarding BJ46a will be described later. Additional work revealed that HSF was able to inhibit an autologous class P-I SVMP (H_2-proteinase) proteolytic activity through formation of a noncovalent inhibitor:enzyme complex (Omori-Satoh, 1977). In 1992, the complete primary structure of HSF, determined by Edman degradation chemistry, was published and the 323 amino acid long molecule displayed two contiguous (double-headed) N-terminal cystatin domains and one His-rich C-terminal domain. Furthermore, three oligosaccharide-N-linked chains were confirmed at positions 123, 185, and 263 (Yamakawa and Omori-Satoh, 1992). This inhibitor has a molecular mass of 47,810 Da as measured by MALDI-TOF mass spectrometry (Deshimaru et al., 2003), indicating, when compared with the native molecular mass (~70 kDa), that HSF could have a homodimeric structure (although the authors do not mention this possibility). Moreover, stoichiometric determination by molecular exclusion assay indicated a one-to-one interaction between HSF and H6-proteinase, a class P-III SVMP isolated from *Gloydius halys brevicaudus* venom. HSF was subjected to chemical modifications at specific amino acids (Trp, Tyr, His, Lys, and Arg), which demonstrated that Lys and Trp residues seemed to play a crucial role in HSF's inhibitory mechanism (Deshimaru et al., 2005). In a recently published work, Aoki and co-workers (2007) have demonstrated that upon complex formation between HSF and autologous metalloproteinases, three lysines (at positions 15, 41, and 103) are shielded from chemical modification, indicating their involvement in the interaction. Additionally, several active fragments of HSF were generated by papain digestion and cyanogen bromide cleavage, and it was clearly demonstrated that the N-terminal half (residues 1–89) of the first cystatin domain (D1) is indispensable for HSF's antihemorrhagic activity. As a result of this elegant protein chemistry experimental approach, the data generated were compared with a speculative molecular model of HSF's first cystatin-like domain, suggesting that Trp^{17} and Trp^{48}, along with Lys^{15} and Lys^{41}, are located in a cluster on the surface of HSF that is involved in this molecule's inhibitory activity (Aoki et al., 2007).

Another SVMPI belonging to the cystatin superfamily that has been well characterized is BJ46a (46 kDa isoform *a* from *Bothrops jararaca* serum). It was the first inhibitor of this class to have its full-length cDNA reported, and 81% of the sequence was confirmed by protein sequencing. It is an acidic glycoprotein (17% of carbohydrate by weight), containing four putative N-glycosylation sites spread along its 322 amino acid primary structure and sharing 85% sequence identity with HSF. The inhibitor is expressed with a signal peptide, and the processed mature protein is a homodimer with a glycosylated subunit molecular mass of 46,101 Da. Upon complex formation with class P-I (atrolysin-C) or P-III (jararhagin) SVMPs, the homodimer dissociates, and each subunit is able to interact with two molecules of metalloproteinase. Furthermore, the interaction seems to be by noncovalent binding to the SVMP metalloproteinase domain, since there was no complex formation with jararhagin-C (a protein with a sequence identical to that of jararhagin but devoid of the metalloproteinase domain) even at a 3:1 excess inhibitor subunit to jararhagin (Valente et al., 2001). Collectively, these data for HSF and BJ46a suggest that the inhibitors interact with SVMPs specifically at their metalloproteinase domain, blocking the catalytic site (Bode et al., 1993), perhaps through the as yet speculative Lys/Trp cluster exposed at the N-terminal D1 domain of the inhibitor.

B. Immunoglobulin Supergene Family

The first SVMPI representative to be classified in this family was oprin (opossum proteinase inhibitor) isolated from the serum of the North American opossum, *Didelphis virginiana*. It is an acidic

glycoprotein (26% carbohydrate by weight) that inhibited snake venom metalloproteinase activity but showed no such effect against different metalloproteinases of bacterial origin, cysteine proteinases, or snake venom serine proteinases. The N-terminal sequence (26 residues) and partial cDNA sequence (211 residues) accounted for approximately 69% of the expected complete sequence, based on amino acid analysis data. Available sequence data for oprin displayed a sequence identity of 41% to human α_1B-glycoprotein (Catanese and Kress, 1992). The latter protein contains five repeating structural domains (ninety-two to ninety-eight residues each) that show significant homology to the variable and the constant regions of certain immunoglobulins, as well as sequence similarities to other members of the immunoglobulin supergene family (Ishioka et al., 1986), a group of proteins with Ig-like domains that are related to the vertebrate immune response (Hood et al., 1985). A possible function that has been assigned to human α_1B-glycoprotein consists of protecting the organism against potential harmful effects triggered by free circulating cysteine-rich secretory protein 3 (CRISP-3), through the formation of an equimolar complex between α_1B-glycoprotein and CRISP-3 (Udby et al., 2004).

From the serum of the mongoose (*Herpestes edwardsii*), three antihemorrhagic factors were isolated (AHF-1 to -3) that inhibited the hemorrhagic activities of HR1 and HR2, metalloproteinases purified from *T. flavoviridis* venom (Tomihara et al., 1987). Further studies demonstrated that AHF-1 is a glycoprotein (4.2% carbohydrate by weight) that exists in a monomeric 65 kDa form. The inhibitor was able to inhibit completely the hemorrhagic activity of HR2a (a subfraction of HR2) at a fivefold molar excess. However, the proteolytic activity of this SVMP was reduced by only 50% at a twentyfold molar excess of AHF-1. No complex formation was observed by molecular exclusion or ultracentrifugation techniques, though there was a suggestion of very weak complex formation by ion-exchange chromatography, and a possible interaction (no K_D determined—qualitative data only) was detected by surface plasmon resonance (Qi et al., 1994). Finally, N-terminal sequencing of the native molecule and thirteen internal fragments (generated by chemical and enzymatic treatment) revealed that AHF-1 displayed ~46% identity with α_1B-glycoprotein, and that the partial sequence of AHF-1 obtained accounted for ~76% of the whole molecule (Qi et al., 1995).

To date, the best-characterized member of the SVMPIs belonging to the Ig supergene family of proteins is DM43, a 43 kDa form from *Didelphis marsupialis* (now *D. aurita*) (Cerqueira and Lemos, 2000). Besides its antihemorrhagic nature, it also showed antilethal, antiedematogenic, and antihyperalgesic activities against *Bothrops jararaca* venom and fully inhibited the hydrolysis of casein, fibrinogen, and fibronectin by this same venom (Neves-Ferreira et al., 2000). DM43 was able to inhibit fully the fibrinogenolytic activities induced by bothrolysin and jararhagin (P-I and P-III SVMPs, respectively, from *Bothrops jararaca*), but failed to inhibit atrolysin C and atrolysin A activities, P-I and P-III metalloproteinases from *Crotalus atrox* venom. Moreover, DM43 was able to form complexes with bothrolysin or jararhagin (as visualized by size exclusion chromatography) but not with atrolysin C or A (Neves-Ferreira et al., 2002), indicating possible co-evolution between resistant animals and the snakes that occur in their habitat. DM43 is a 291 amino acid long acidic glycoprotein (21% carbohydrate by weight) that displays 51% overall similarity (37% identity plus 14% conservative substitutions) to α_1B-glycoprotein. Analysis of the glycan moiety indicated the presence of *N*-acetylglucosamine, mannose, galactose, and sialic acid at 4:3:2:2 molar ratios. Size exclusion chromatography experiments determined that in its native state, DM43 is a homodimer consisting of 43 kDa subunits. Upon interaction (complex formation) with the metalloproteinase, the dimer dissociates and one subunit binds to one SVMP. The binding seems to be at the metalloproteinase domain, as DM43 did not form a complex with jararhagin-C (Usami et al., 1994). Modeling studies of DM43 based on the crystal structure of KIR2, a killer cell inhibitory receptor (Valiante et al., 1997), corroborated *in silico* that the DM43 subunits exist as a three-domain (D0, D1, and D2) protein. A region composed of six exposed loops, between domains D1 and D2, is hypothesized to be the metalloproteinase-binding site, by analogy with other molecules for which crystal structures of the ligand-receptor complexes are known (de Vos et al., 1992; Somers et al., 1994). Another important observation resulting from modeling was the presence of exposed hydrophobic

clusters on the inhibitor's subunit D2 domain, which could be involved in the dimerization process of the subunits, resulting in the native structure of DM43 when not in contact with SVMPs (Neves-Ferreira et al., 2002).

C. Ficolin/Opsonin P35 Family

The only known member of this family is erinacin, a protein initially purified from the muscle of the hedgehog *Erinaceus europaeus* (Mebs et al., 1996); this work was extended in a later publication (Omori-Satoh et al., 2000). Another inhibitor, purified from the plasma of this species, had been isolated previously (de Wit and Westrom, 1987a, 1987b), but due to a lack of any structural data, it was not possible to confirm if this inhibitor is also erinacin. Erinacin has a mass of approximately 1,040 kDa and displays a very complex structure. It is composed of two subunits, α and β, present at a molar ratio of 1:2, respectively. The α subunit is a 370 kDa homodecamer (interacting by noncovalent bonds), while the β subunit (350 kDa) is composed of ten 35 kDa polypeptide chains interacting via covalent bonds. The final assembly, $\alpha_{10}{\cdot}2\beta_{(10)}$, when visualized by electron microscopy, displays a flower bouquet-like structure, just like plasma ficolin (Ohashi and Erickson, 1997) and the Hakata antigen, members of the ficolin/opsonin P35 family (Sugimoto et al., 1998). The inhibitor was able to inhibit completely the hemorrhagic and proteolytic activities of an undisclosed purified SVMP from *B. jararaca*, through the establishment of an inactive equimolar complex, but no inhibition of serine proteinases such as trypsin or chymotrypsin was observed. Incomplete sequencing of α (seventeen N-terminal residues) and β subunits (ca. 75% of the complete sequence) revealed that this inhibitor belongs indeed to the ficolin/opsonin P35 family of molecules, characterized by a conserved amino terminus followed by collagen-like GXY triplet repeats and fibrinogen-like domains. The authors speculated that the mechanism of inhibition could be occurring in two ways: (1) the fibrinogen-like domain could recognize an *N*-acetylglucosamine residue present in the metalloproteinase molecule (based on Matsushita et al., 1996), and (2) because SVMPs exhibit activity toward collagen substrates, the collagen-like domain could act as a mimic substrate for the SVMP molecule (Omori-Satoh et al., 2000).

IV. SNAKE VENOM PHOSPHOLIPASES A_2 (PLA_2s)

Snake venom phospholipases A_2 (EC 3.1.1.4) are carboxylic ester hydrolases that excise the fatty acid residue at the sn-2 position of susceptible phosphoglycerides in monomeric, micellar, or lipid bilayer phases, also releasing the corresponding lysophospholipid (Kini, 2003; see also Chapter 8, this volume). They are very abundant in snake venoms, typically as secreted, compact 13–15 kDa enzymes containing six or seven disulfide bonds. Whether classified in group IA (Elapidae PLA_2) or group IIA/B (Viperidae PLA_2), they share significant sequence and structural similarities and contain the highly conserved His^{48}/Asp^{99} dyad and the Ca^{2+}-dependent catalytic mechanism (Six and Dennis, 2000; Schaloske and Dennis, 2006). PLA_2s from elapid and viper venoms seem to have evolved from different nontoxic phospholipase ancestors by a process of repeated gene duplication followed by functional divergence (Lynch, 2007).

Venom PLA_2s are synthesized and stored in the venom glands as catalytically active enzymes. They may show little or no toxicity, being mainly involved in predigestive processes of the venom, or they can be highly toxic, inducing a spectrum of pathological symptoms in animals. Some PLA_2s are pharmacologically active only when complexed with other venom components, either covalently (e.g., β-bungarotoxin) or noncovalently (e.g., crotoxin), while others function as single-chain molecules (e.g., notexin, Asp^{49} and Lys^{49} myotoxic PLA_2s) (Kini and Evans, 1989; Harris, 1998; Kini, 2003).

In an attempt to explain the wide variety of effects that can be induced by these highly homologous snake venom PLA_2s, Kini and Evans proposed in 1989 that these enzymes bind to soluble or membrane-bound protein acceptors through specific pharmacological sites, which can be

independent of or overlapping with the active site of the enzyme. This high-affinity interaction with target sites (in the low nanomolar range) would determine the toxic effect of the PLA_2 *in vivo* (Kini and Evans, 1989). Indeed, a few target proteins for neurotoxic and anticoagulant PLA_2s have already been described, such as N- and M-type receptors in mammalian tissues (Lambeau and Lazdunski, 1999) and factor Xa of the coagulation cascade (Kini, 2005), respectively. On the other hand, mapping the pharmacological sites on the surface of each PLA_2 seems to be a much bigger challenge, with several groups working to understand this puzzling issue (Chioato and Ward, 2003; Lomonte et al., 2003a; Rouault et al., 2006; Chioato et al., 2007).

V. SNAKE VENOM PLA_2 INHIBITORS (PLIs)

Most PLIs have been isolated from the serum or plasma of reptiles. They are acidic oligomers of 75–180 kDa composed of three to six subunits (either identical or different) ranging from 20–50 kDa, some of which are glycosylated (Lizano et al., 2003). Based on the PLA_2 inhibitors isolated from the Chinese mamushi *Agkistrodon blomhoffii siniticus* (now *Gloydius brevicaudus*) serum, Ohkura and coworkers have proposed the existence of three structurally distinct classes (Ohkura et al., 1997), described below.

PLIαs show sequence homology to the carbohydrate recognition domain (CRD) of C-type lectins, a structural element also found in mammalian PLA_2 receptors. To date, they have only been identified in the blood of viperid snakes, where they may be composed of one or two types of subunits (Lizano et al., 2003) that specifically inhibit group II acidic (Inoue et al., 1997) or basic (Lizano et al., 1997, 2000) PLA_2s.

PLIβs are glycoproteins whose amino acid sequence has 33% identity with human leucine-rich α_2-glycoprotein, a serum protein of unknown function. They have a molecular mass of 160 kDa, are formed by three identical ~50 kDa subunits, and have nine leucine-rich repeat (LRR) domains, each with twenty-four amino acid residues. At present, only two members of this group have been isolated from the viperid snake *Gloydius brevicaudus* (Ohkura et al., 1997; Okumura et al., 1998) and from the nonvenomous colubrid snake *Elaphe quadrivirgata* (Okumura et al., 2002). PLIβs inhibit only class II basic crotaline PLA_2s and have not been detected in plasma of elapid snakes. Since LRRs are motifs usually involved in protein-protein interactions, it may be that they are responsible for the specific binding to PLA_2.

PLIγ subunits show internal sequence repeats with a pattern of cysteine residues similar to that of the typical three-finger motifs of u-PAR- and Ly-6-related proteins, which include postsynaptic neurotoxins. They are the most widely distributed class of PLIs, having been found in the blood of Viperidae, Elapidae, Pythonidae, and Colubridae snakes. Based on biochemical and structural characteristics, Lizano and coworkers proposed a further subclassification of these inhibitors in PLIγI and PLIγII (Lizano et al., 2003). The first subgroup is composed of two different types of subunits with a broader spectrum of inhibition; they may be active against PLA_2s from classes I, II, and III and have been isolated from elapids, viperids, and colubrids. This relative lack of specificity suggests that they recognize a common structural element, such as the calcium-binding loop, which is conserved among all groups of PLA_2s (Ohkura et al., 1997). On the other hand, the PLIγII type of inhibitor is composed of identical monomers, seems to be more specific against group II PLA_2, and has been isolated from viperid snakes. All reptile PLIs isolated so far fall in one of these three classes, PLIα, β, or γ. A fourth class contains only DM64, the unique PLI isolated from a mammal, the marsupial *Didelphis aurita*, which shows sequence homology to members of the immunoglobulin supergene family (Rocha et al., 2002).

Further classifying these PLIs according to their biological properties is not an easy task. A review of the literature shows that most PLIs were simply described as "phospholipase inhibitors" (Perales and Domont, 2002), meaning that they were able to inhibit the catalytic activity of these enzymes, without specifying which pharmacological effects were analyzed, if any. As already described, snake venom PLA_2s can interfere with several physiological processes, leading to serious

toxic consequences such as neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant effects, platelet aggregation induction/inhibition, and convulsant activity. To complicate this even further, these toxic effects are not always directly related to the catalytic activity of the enzyme, and sometimes were even demonstrated to be completely independent phenomena (Kini, 2003). For example, in some group IIA myotoxic PLA_2s, a critical Asp^{49} residue at the calcium-binding loop is replaced by Lys^{49}, thus impairing the enzymatic activity (Lomonte et al., 2003a). Recently it has been proposed that, although catalytically defective, Lys^{49} PLA_2 would still be able to bind fatty acids in its active site, which is expected to induce a conformational change within its C-terminal cationic-hydrophobic cytolytic region, leading to an ideal conformation for membrane insertion and induction of muscle damage (Ambrosio et al., 2005). Another example comes from a recent study by Rouault and co-workers (Rouault et al., 2006). Using the β-neurotoxin OS2 from the Australian taipan snake venom as a model, they have convincingly demonstrated that the catalytic activity of the PLA_2 is a minor factor determining central neurotoxicity, and that its N-terminal portion is critical for the observed pharmacological effect, i.e., inhibition of acetylcholine release from peripheral cholinergic synapses.

Therefore, just describing a protein as being able to inhibit venom PLA_2 catalytic activity *in vitro* does not contribute much to the understanding of its antitoxic properties. In this review, we focus on those PLA_2 inhibitors whose biological activities have been systematically analyzed, and thus ended up with two main groups of PLIs: antimyotoxic proteins and antineurotoxic proteins. Nevertheless, to further enrich the discussion, data on several PLIs with undefined biological function will be compared with antimyotoxic and antineurotoxic PLIs. For more detailed information on these undefined PLIs, refer to several reviews available in the literature (Domont et al., 1991; Thwin and Gopalakrishnakone, 1998; Faure, 2000; Dunn and Broady, 2001; Fortes-Dias, 2002; Perales and Domont, 2002; Lizano et al., 2003; Marcussi et al., 2007).

A. Antimyotoxic PLIs

The first well-characterized natural antimyotoxic protein was described in 1997 (Lizano et al., 1997). It was isolated from plasma of *Bothrops asper* and was named BaMIP (*B. asper* myotoxin inhibitor protein). Similar to other PLA_2 inhibitors (e.g., Domont et al., 1991; Lizano et al., 2003), under native conditions, BaMIP is an oligomeric acidic glycoprotein (120 kDa made of five subunits of 23–25 kDa linked by noncovalent bonds). The N-terminal sequence of BaMIP indicated an α-type PLI nature; i.e., its primary structure bears motifs characteristic of the carbohydrate recognition domains (CRDs) of C-type lectins. By both ELISA and Western blot analysis, it was shown that BaMIP binds all four basic myotoxin isoforms isolated from *B. asper* venom. Furthermore, it inhibited the catalytic activity of myotoxins I and III from *B. asper* venom as well as their anticoagulant and hemolytic properties *in vitro*. When assayed *in vivo*, BaMIP clearly neutralizes the myotoxicity, the cytotoxicity, and the edema-forming property of all four myotoxins tested. Based on the inhibition experiments, it has been suggested that one 25 kDa subunit of BaMIP inactivates one myotoxin molecule (Lizano et al., 1997).

In the last several years, two other α-type PLIs with antimyotoxic properties were isolated by Lomonte's group at the University of Costa Rica: CgMIP-II, from the plasma of the snake *Cerrophidion* (*Bothrops*) *godmani* (Lizano et al., 2000), and AnMIP, from *Atropoides nummifer* (Quirós et al., 2007). A fourth α-type antimyotoxin was isolated from *Bothrops moojeni* snake plasma (Soares et al., 2003). As with BaMIP, these α-type PLIs are all acidic with glycosylated 20–25 kDa subunits that form oligomeric structures ranging from 92 to 180 kDa. These oligomers are able to inhibit the myotoxicity of the basic group II PLA_2 from viperid snake venoms. Although no mapping experiments with these antimyotoxins have been performed to date, the authors believe that the CRD-like domain of the inhibitors might be the main region of interaction with myotoxic PLA_2. This hypothesis was based on the assumption of analogy with mammalian PLA_2 M-type

receptors (Higashino et al., 1994; Nicolas et al., 1995; Lambeau and Lazdunski, 1999) and with PLI-IV/V from *Protobothrops* (*Trimeresurus*) *flavoviridis* (Nobuhisa et al., 1998), whose CRD-like domains were shown to be involved in binding to PLA_2. However, a very interesting study by Okumura and co-workers (Okumura et al., 2005) recently showed that the region of the α-type PLI from the serum of the venomous snake *Gloydius brevicaudus* (*Gb*PLIα) responsible for PLA_2 inhibition was distinct from its C-terminal CRD-like domain (residues 49–147). They used a very elegant approach by analyzing the inhibitory and binding activities of various chimeric proteins (between *Gb*PLIα and the inactive PLIα from *Elaphe quadrivirgata*) and various point-mutated inhibitors. The authors suggested that the central pore formed by the trimerization of the inhibitor would be the PLA_2-binding site. One trimeric *Gb*PLIα stoichiometrically binds one PLA_2 molecule, and residues 13–36 were critical for trimerization, and thus for the inhibition of acidic PLA_2.

For BmjMIP, circular dichroism spectroscopy indicated that no significant alterations in the secondary structure of either inhibitor or PLA_2 were observed upon binding. Moreover, the PLI's carbohydrate moiety did not seem to participate in the interaction with the enzyme, as the inhibitor (2 μg) was still active against PLA_2 even after 18 h of incubation with 10 mU of N-glycosidase F, although no evidence of complete deglycosylation was given in the paper (Soares et al., 2003). A different study with a homologous α-type PLI from *Protobothrops flavoviridis* showed more clearly that glycosylation of the inhibitor does not seem crucial for the interaction with PLA_2 (Nobuhisa et al., 1998). The full-length inhibitors and a few of their fragments, expressed in fusion with glutathione S-transferase (GST) in *Escherichia coli* (thus not glycosylated), were still able to interact with PLA_2 isoenzymes immobilized in magnetic beads. Unfortunately, the authors did not evaluate if the pharmacological effects of bound PLA_2 were also inhibited by GST-fused recombinants. However, another interesting study showed that the α-type PLI from *A. b. siniticus* was able to inhibit acidic venom PLA_2 in a carbohydrate-independent fashion, while this nonprotein moiety seemed essential for the inhibitory activity against basic PLA_2. According to the authors, αPLI might electrostatically interact with basic PLA_2 through sialic acids in the carbohydrate chains of the glycosylated inhibitor (Okumura et al., 1999b). The few examples above illustrate the peremptory need for more detailed structure-function studies on these α-type antimyotoxic proteins.

An antimyotoxic γ-type PLI named CgMIP-I was isolated from the same biological source (plasma of *Cerrophidion godmani*) as the α-type CgMIP-II PLI (Lizano et al., 2000). Interestingly, these two PLIs present in the blood of a single snake species showed subtle differences in their pharmacological specificities toward the same structural group II basic PLA_2. The γ-type CgMIP-I was more specific against the enzymatically active myotoxin I from *C. godmani* venom, inhibiting its catalytic, myotoxic, anticoagulant, intracerebral lethality, cytotoxic, and edematogenic activities. The α-type CgMIP-II was very effective in neutralizing the myotoxic, cytotoxic, and edematogenic effects of the catalytically inactive Lys^{49} myotoxin II from this same venom. These results suggested that several endogenous snake proteins have evolved to neutralize a wide array of PLA_2 components present in snake venoms (Lizano et al., 2000). At present, only a meager amount of structural data are available on the PLIγ domains that participate in the interaction with PLA_2s. In a binding study using truncated recombinant forms of PLI-I from *T. flavoviridis*, it was shown that only one of the two three-finger motifs was able to bind to PLA_2 isozymes (Nobuhisa et al., 1998). It was also demonstrated that recombinant soluble uPAR, structurally similar to PLIγ but containing three intramolecular repeats of the cysteine-rich domain, also bound to crotoxin and its catalytically active subunit CB (Faure, 2000). These results indicate that the three-finger motif is important not only for the interaction between PLIγ and PLA_2 enzymes, but also probably for the interaction between the receptors and agonists that might have this motif. However, a peptide based on a proline-flanked cluster between amino acid residues 89 and 109 of PIP (phospholipase inhibitor from *Python*), which is absent in other three-finger motif-containing proteins (such as the prototype mammalian uPAR receptor/Ly-6 superfamily of proteins), also exhibited antitoxic and anti-inflammatory properties, and was able to bind various venom PLA_2s in a pattern similar to that of native PIP (Thwin et al., 2002).

Only one antimyotoxic PLI has been isolated from the blood of a mammal, the Neotropical black-eared opossum (*Didelphis aurita*) from Rio de Janeiro, a marsupial naturally resistant to viperid snake venoms. This PLI was named DM64 to indicate a protein from *Didelphis marsupialis* (the former name; Cerqueira and Lemos, 2000) with subunits of approximately 64 kDa (Rocha et al., 2002). DM64 is an acidic homodimeric glycoprotein that effectively inhibits both the *in vivo* myotoxicity and the *in vitro* cytotoxicity of Asp^{49} myotoxin I and Lys^{49} myotoxin II, two group II basic PLA_2s isolated from *Bothrops asper* venom. It was shown by native PAGE and affinity chromatography that this PLI forms noncovalent soluble complexes with myotoxins I and II. Interestingly, DM64 did not inhibit the PLA_2 activity, anticoagulant activity, or intracerebroventricular lethality of myotoxin I effects directly dependent on the catalytic activity of this enzyme (Gutiérrez and Lomonte, 1995). This was clear evidence of the lack of association between the catalytic activity and the pharmacological effect (myotoxicity) induced by myotoxin I, in accordance with previous results (Lomonte et al., 1992). It had been much easier to establish this lack of association for the catalytically inactive Lys^{49} myotoxins (Lomonte et al., 2003a). The C-terminal region of these PLA_2 variants, comprising residues 115–129, is believed to play a central role in toxicity (Lomonte et al., 2003b; Chioato et al., 2007). Accordingly, DM64 immobilized on an affinity column was able to "fish out" the C-terminal peptide of *B. asper*'s myotoxin II (produced by cleavage after Glu^{98}) from a mixture of peptides obtained after digesting the enzyme with endoproteinase Glu-C (unpublished results). DM64 was cloned, fully sequenced, and classified as a member of the immunoglobulin supergene family, just like the inhibitors of snake venom metalloproteinases (DM40 and DM43) isolated from this same mammal, although no anti-SVMP activity was observed (Rocha et al., 2002). It is important to point out that this type of binding protein is completely different from all known ligands of secreted PLA_2 (either from venom or not) already described in the literature (Lambeau and Lazdunski, 1999; Valentin and Lambeau, 2000). As previously mentioned, homology modeling of DM43 suggested the existence of three Ig-like domains, each one with approximately ninety-seven residues, homologous to α_1B-glycoprotein domains. Considering the molecular mass and primary structure of DM64, it has been suggested that this antimyotoxin contains two additional Ig-like domains. However, although homologous, DM43 and DM64 have two completely different inhibitory specificities: the former is a metalloproteinase inhibitor, while the latter is an antimyotoxin with no antiproteolytic activity. The authors have hypothesized that the two extra domains at DM64 C-terminal side may partially explain this functional shift. Another striking difference between these two molecules is a gap of four residues in the third domain of DM64. This same region in DM43 was predicted to bear one of the main loops responsible for the interaction with metalloproteinases (Rocha et al., 2002). Both working hypotheses are currently under analysis by our group.

B. Antineurotoxic PLIs

The first report of a natural antineurotoxin was made by Ovadia et al. (1977). An acidic protein factor of 56 kDa that protected mice against a neurotoxin preparation of the same venom was isolated from the serum of *Vipera palaestinae*. Unfortunately, this inhibitor was not further investigated, but the authors suggested that it was an albumin-like or α_1-globulin fraction rather than an immunoglobulin. The only fully characterized antineurotoxic PLI was described by Fortes-Dias et al. (1991, 1994) and Perales et al. (1995), working independently: the inhibitor was named CNF (standing for *Crotalus* neutralizing factor) or CICS (for crotoxin inhibitor from *Crotalus* serum), respectively. Since the overall characteristics of the two molecules are very similar, throughout this text the inhibitor will be referred to as CNF/CICS. This oligomeric glycoprotein of 130–160 kDa had a subunit molecular mass of about 23 kDa and a theoretical pI of 5.45, and subunits associated via noncovalent interactions. As determined by cDNA sequencing, the subunit is composed of a single polypeptide chain of 181 amino acid residues and has been classified as a member of the γ-type PLI. CNF/CICS antineurotoxic effects against *Crotalus* venom and crotoxin were demonstrated

in vivo by lethality assays. It also effectively inhibited the catalytic activity of crotoxin and its PLA_2 component CB. A very unique and interesting mechanism of inhibition was described: CNF/CICS binds to CB, but not to the acidic chaperon CA, thus inducing the dissociation of the crotoxin complex. In other words, the interaction mechanism between the inhibitor and the toxin resembles that of crotoxin and its cellular acceptor. Therefore, it has been suggested that CNF/CICS acts physiologically as a false crotoxin acceptor and retains the toxin in the vascular system, preventing its action at the neuromuscular junction (Perales et al., 1995). The only discrepancy between the two groups' analyses concerned the interaction between the inhibitor and crotoxin/CB. Size exclusion chromatography on Superdex 200 and PAGE under nondenaturing conditions indicated that the interaction occurs between one oligomeric CICS (130 kDa) and one 12 kDa CB subunit (Perales et al., 1995). Based on a series of experiments with increasing concentrations of crotoxin and a fixed amount of CNF (applied to a Superdex 75 column and followed by the separation of these ligands by C_4 RP-HPLC), it was suggested that one CNF oligomer (160 kDa) forms a stable complex with eight CB subunits (Fortes-Dias et al., 1994). Further systematic investigation is needed to determine unequivocally which interpretation of toxin:inhibitor stoichiometry is correct.

Concerning specificity, CNF was able to inhibit group II PLA_2, either basic or acidic (Fortes-Dias et al., 1999). The interaction of CICS with several neurotoxic and nontoxic PLA_2s was studied in real time by surface plasmon resonance (Faure et al., 2000). These latter authors confirmed the specificity of this inhibitor toward group II PLA_2s from viperid venom and showed that in the presence of CICS, no significant changes were observed in enzymatic activity of porcine pancreatic PLA_2, elapid β-neurotoxins (group I), human secretory PLA_2s (group II), or bee venom enzymes (group III). These conclusions are in contrast with the wider specificity of other members of the γ-type PLIs isolated from Elapidae and Viperidae plasmas, which inhibit PLA_2 not only from group II but also from groups I and III (see Table 12.3).

VI. ASSAY METHODS

A. Inhibition of Toxin Activity

For practical reasons, most inhibition assays are performed by mixing venom and inhibitor and incubating (usually for 30 min at 37°C) before assessing the neutralizing capacity. Venom activity can then be measured either *in vitro* or *in vivo*, even though this timing is strikingly different from the clinical situation. In the interest of complying with the 3Rs (reduce, refine, and replace) relating to animals in research (Sells, 2003), the use of *in vitro* assays has being increasingly encouraged by the World Health Organization (Theakston et al., 2003); these assays require much smaller amounts of sample, are less expensive than *in vivo* assays, and do not cause animal suffering. A disadvantage is that the correlation between venom pharmacological effects *in vivo* and its behavior *in vitro* is not always straightforward. Some of the most commonly used *in vitro* and *in vivo* methods for assaying venom toxicity and neutralization will be briefly described below.

For testing hemorrhagic activity, the method originally described by Kondo and coworkers (Kondo et al., 1960) is still largely employed, using rats instead of rabbits. According to Theakston and Reid (1983), the minimum hemorrhagic dose (MHD) is defined as the least amount of venom (micrograms dry weight) that, when injected intradermally, results in a hemorrhagic lesion of 10 mm diameter 24 h later. Another *in vivo* method for assessing hemorrhage was first published 10 years ago (Sells et al., 1997) but has not been very widely adopted. It employs fertile hens' eggs no more than 10 days old, which have a vascularized yolk sac membrane with normal blood circulation, together with an incomplete nervous system that guarantees a pain-free procedure. In this system, a hemorrhagic halo induced by the venom on the vitelline vein can be easily measured. This method correlates very well with the rodent test described above and has also been successfully used for

estimating venom lethality (Sells et al., 1998) in place of the standard murine median lethal dose (LD_{50}) assay (Theakston and Reid, 1983).

Hemorrhage induced by snake venom metalloproteinases is directly dependent on the hydrolysis of basement membrane components in microvessels (Gutiérrez et al., 2005), although recently it has been demonstrated that blood flow (and the biophysical forces associated with it) is absolutely required for observing hemorrhage *in vivo* (Gutiérrez et al., 2006a). Several *in vitro* assays for measuring the proteolytic degradation of laminin, fibronectin, collagen IV, gelatin, and fibrinogen, using general substrates such as (azo)casein or synthetic peptides, were used to infer evidence of the antihemorrhagic activity of various inhibitors (Sells, 2003; Perales et al., 2005). These methods are based on SDS-PAGE (Neves-Ferreira et al., 2000), zymography (Hasson et al., 2004), ELISA (Bee et al., 2001), or fluorometry (Bjarnason and Fox, 1995). The use of SILAC (stable isotope-labeled amino acids in culture) technology for exploring SVMP proteolytic activity *in vitro* was recently proposed (Pinto et al., 2007). Human fibroblasts in culture were labeled with heavy lysine ([U-$^{13}C_6$]-L-lysine) and then incubated with atrolysin A. This SVMP-treated culture medium supernatant was next combined with the supernatant of untreated cells labeled with light lysine ([$^{12}C_6$]-L-lysine). The combined supernatants were then analyzed by SDS-PAGE followed by *in gel* digestion and MS/MS sequencing. Peptides from extracellular matrix proteins (fibronectin, collagen VI, fibulin 2, and annexin V) were observed with only heavy-labeled lysine, indicating that they were generated as a result of the action of atrolysin A (either direct or indirect) on fibroblasts.

In vivo, PLA_2 neurotoxic activity is usually assayed by inoculating mice intravenously or intraperitoneally and registering the mortality rate due to asphyxia following paralysis of respiratory muscles (Theakston and Reid, 1983). For assessment of neurotoxicity *in vitro*, preparations of chick biventer cervicis or rodent hemidiaphragm phrenic nerves can be employed (Sells, 2003). Purely cholinergic nerve terminals (synaptosomes) isolated from the electric organ of *Torpedo marmorata* have also been used to study the neurotoxic action of β-neurotoxins on the neuromuscular junction. In this model, induced acetylcholine release is continuously monitored by chemiluminescence (Faure et al., 2000).

Myotoxic activity *in vivo* can be determined in a qualitative manner histologically, in mouse gastrocnemius muscle injected with myotoxic PLA_2. Quantitative estimations are easily obtained measuring plasma levels of creatine kinase in the above-mentioned mice by a colorimetric endpoint assay (Gutiérrez et al., 1984). An *in vitro* method for evaluating the cytolytic activity of myotoxic PLA_2 has been described (Lomonte et al., 1999). Skeletal muscle myotubes (C2C12) were used as targets, and the release of lactic dehydrogenase (LDH) as a measure of cell damage was quantified using a colorimetric assay similar to that described above.

A convenient *in vitro* method for measuring snake venom PLA_2 catalytic activity and its inhibition is fluorometry, using 1-palmitoyl-2-pyrenedecanoylphosphatidylcholine as substrate (Radvanyi et al., 1989). Upon phospholipid hydrolysis in the presence of BSA, the generation of 10-pyrenyldecanoic acid can be easily monitored by measuring the increase in the fluorescence intensity at 398 nm (excitation at 345 nm). Alternatively, hens' egg yolk emulsions containing phospholipids can be employed as substrates for directly measuring PLA_2 activity using the pH-stat method or a dye to indicate pH variation (Sells, 2003). The enzymatic activity can also be estimated by indirect hemolysis, using egg yolk lecithin supplemented with red blood cells (Gutiérrez et al., 1988).

B. Inhibitor Composition and Interactions

As described earlier, naturally occurring snake venom inhibitors exist in their native states as homo- or hetero-oligomers (same or different subunits associated by noncovalent bonds) or as single or double polypeptide chain molecules. Hence, a multiple technique approach is necessary to assess

these molecules' structural composition and arrangement. Initially, purified inhibitors should be submitted to native PAGE and SDS-PAGE under reducing and nonreducing conditions. Preferably, subunit molecular masses should be determined by mass spectrometry (Cole, 1997; Hillenkamp and Peter-Katalinic, 2007), if available. These data are analyzed along with native molecular weight determination using techniques such as native PAGE coupled to Ferguson plot analysis (Ferguson, 1964; Butterman et al., 1988), size exclusion chromatography (Andrews, 1964), dynamic laser light scattering (Valente et al., 2001), or analytical ultracentrifugation (Hensley, 1996). These various strategies have been employed to different extents by several researchers (Qi et al., 1994; Neves-Ferreira et al., 2000; Omori-Satoh et al., 2000; Valente et al., 2001; Rocha et al., 2002).

Complex formation and inhibitor:toxin stoichiometry can be determined by the use of simple techniques like size exclusion chromatography, native PAGE, and dynamic laser light scattering (Neves-Ferreira et al., 1997, 2002; Valente et al., 2001). The characterization of these interactions, i.e., the determination of equilibrium constants (K_A and K_D) and kinetic rate constants (k_a and k_b), is a more complicated matter. Here we propose the use of surface plasmon resonance technique, commonly referred to as BIAcore (the trade name of a leading instrument in this field), as the technique of choice. In simple terms, this method allows the monitoring of the interaction, with no labeling requirements, of an analyte to its binding partner (immobilized on an inert surface) in real time (Jonsson et al., 1991), and the resulting binding curve is used to generate the kinetic parameters of the interaction (Karlsson et al., 1991; O'Shannessy et al., 1993; Roden and Myszka, 1996). To obtain reliable data, experimental design, implementation, and interpretation should follow strict rules (Karlsson and Falt, 1997; Myszka, 1997, 1999a, 2000). Surface plasmon resonance has been validated for different applications in the biological sciences in more than five thousand scientific articles published from 1989 to 2005 (Myszka, 1999b; Rich and Myszka, 2000, 2001, 2002, 2003, 2005a, 2005b, 2006). It requires small amounts of proteins (nano- to microgram range), can be coupled to mass spectrometry (Krone et al., 1997), is highly reproducible (Katsamba et al., 2006), generates equilibrium, thermodynamic, and kinetic constants that match the values obtained by traditional solution-based techniques (Day et al., 2002), and is on the verge of satisfying the higher-throughput requirements for many contemporary scientific approaches (Rich and Myszka, 2007).

To date, only three articles have been published using surface plasmon resonance to explore interactions between venom proteins and potential inhibitors. A qualitative experiment was done to evaluate the inhibitor:toxin interaction between AHF-1 (see Table 12.2) and HR2b, a class P-I SVMP from *T. flavoviridis* venom, which could not be determined by size exclusion chromatography or analytical ultracentrifugation (Qi et al., 1994). Another report used BIAcore technology to demonstrate that CICS (Table 12.3) is a specific inhibitor of the monomeric and multimeric β-neurotoxins from viperid snakes; the K_D (equilibrium dissociation constant) for the CICS:CB (crotoxin basic component, a PLA_2) interaction was 13 nM (Faure et al., 2000). Finally, equilibrium and rate constants for the interaction between G*b*PLIα (Table 12.3) and an acidic PLA_2 were determined, in addition to qualitative data regarding the interaction between truncated forms of the inhibitor and the same toxin, in an effort to map the region of this α-type PLI that is responsible for its inhibitory activity (Okumura et al., 2005).

VII. PERSPECTIVES

Many systems within vertebrates provide defense against the hostile external environment via physical barriers, specialized cells such as natural killer lymphocytes, phagocytic cells, and blood-borne molecules such as those of the complement system. However, snake venom toxins transgress these barriers and cause physiological disturbances in the recipient that, if not neutralized in a short time, may lead to extensive morbidity and, eventually, death. To be considered innate or natural, molecular defense mechanisms must satisfy three requirements: presence before exposure to the antigen, no enhancement because of this exposure, and no discrimination between foreign invaders. Natural inhibitors, such as soluble acceptors of metalloproteinases or PLA_2, satisfy most of

these conditions (Perales and Domont, 2002). Whether they also play any specific physiological function in the resistant animals remains unknown. Having reviewed major recent advances in our understanding of natural inhibitors, we now identify several areas that need to be addressed in the near future in order to enhance their use as biochemical and pharmacological tools, to acquire new knowledge on the biochemical behavior of these molecules in some areas that remain obscure, and to use these molecules as antidotes against snake envenoming.

The first is that new knowledge to deepen the understanding of their mechanism of action is badly needed. This should include the identification on both the toxin and the inhibitor of their interacting residues, the chemical and physicochemical nature of the interaction, the determination of association constants, the elucidation of the minimum structural size of the polypeptide chains needed for interaction, and their conformation. Once we are aware of the molecular basis of these interactions, it will be possible to design synthetic peptides to learn more about the structure and the dynamics of the toxin-inhibitor complexes.

In this direction, three general experimental strategies have been employed: molecular biology, classical protein chemistry, and proteomics techniques. Through the construction of chimeric recombinant proteins of inactive and active PLIs, Okumura and coworkers have located candidate regions responsible for the inhibitory activity of an active PLI (Okumura et al., 2005). Site-directed mutagenesis was also used to confirm the participation of putative critical residues. It was clearly demonstrated that in the α-type PLI from *G. brevicaudus*, the region comprised of residues 13–36, a peptide stretch different from its CRD-like region, is important for the inhibitory activity. Trinitrophenylation of Lys residues followed by papain digestion and cyanogen bromide cleavage showed that HSF, a metalloproteinase inhibitor of the cystatin superfamily, present in the serum of *T. flavoviridis*, had its critical inhibitory domain located in residues 5–89 and 312–317 of two polypeptide chains linked by a disulfide bond (Deshimaru et al., 2005; Aoki et al., 2007). For both approaches, we envisage BIAcore on line with MS/MS sequencing as a very promising complementary technique. After real-time monitoring of the interaction between a toxin and its inhibitor (or fragments of it), one could *in situ* digest the molecules bound to the sensor chip, capture the peptides in a capillary column, and sequence them on line by MS/MS. This high-throughput BIAcore-MS/MS methodology would give not only quantitative data concerning the interaction but also data on the primary structure of the ligands (Natsume et al., 2000). A third approach to be fully developed takes advantage of the dramatic advances made in the last decade in our ability to characterize complex biological samples using proteomics techniques. It is expected that these new tools will change the scope of the questions that can be addressed in natural immunity studies, greatly extending the classical knowledge about venom complexity and inhibition as well as the physiopathological consequences of envenomation.

A second question is to ask whether these natural acceptors can be exploited as models for the design of new drugs. The prospects are tempting, as long as nonantigenic peptides can be synthesized. The design of peptides for therapeutic trials will depend on the knowledge of the molecular basis of toxin-inhibitor interactions and of the physicochemical parameters that govern these interactions. Considering that small peptide regions are responsible for the inhibitory activity, it is hoped that use of synthetic compounds designed after these critical regions could help overcome the problem of local tissue damage, avoid the adverse antibody reactions seen following immunotherapy, and solve drug transportation and storage problems.

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Section III

Reptile Venom Toxins

13 Snake Venom Three-Finger Toxins

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CONTENTS

Snake venoms are complex mixtures of pharmacologically active proteins and polypeptides, and they often contain over one hundred proteins, both enzymatic and nonenzymatic. However, these toxins belong to a very small number of superfamilies of proteins. One such family of nonenzymatic proteins is the three-finger toxin family. All three-finger toxins share a common structure of three β-stranded loops extending from a central core. However, they bind to different receptors/acceptors and exhibit a wide variety of biological effects. Thus, the structure-function relationships of this group of toxins are complicated and challenging to elucidate. Based on their biological activity, three-finger toxins can be classified into different categories. This chapter deals with the overall functions and structure-function relationships of each of the different categories of three-finger toxins.

I. INTRODUCTION

Snakes use a complex mixture of toxins and enzymes, venom, for incapacitating, immobilizing, and digesting their prey, as well as for defense against predators (Mackessy, 1988; Hider et al., 1991). Consequently, the toxins in snake venom have evolved to target a wide variety of receptors

and ion channels with high affinity and specificity. Some of these toxins produce debilitating or lethal effects as an outcome of neurotoxic, cardiotoxic, hemorrhagic, and tissue necrotizing effects, whereas others induce various pharmacological effects but are of a lower toxicity. All these protein toxins target various physiological processes at specific sites. Hence, research on snake venom toxins has provided not only new tools to decipher molecular details of various physiological processes, but also inspiration to design and develop a number of therapeutic agents.

Snake venoms commonly contain over one hundred protein toxins; however, these toxins belong to a very small number of superfamilies of proteins. Some of the well-recognized families of venom proteins include the three-finger toxins, proteinase inhibitors, C-type lectins, phospholipase A_2 enzymes and toxins, serine proteinases, and metalloproteinases. The members in a single family show remarkable similarities in their primary, secondary, and tertiary structures. At times, however, they differ from each other in their biological targeting, and hence their pharmacological effects (Kini, 2002). This review will discuss aspects of a broadly distributed and functionally important family of toxins, the three-finger toxins.

II. THREE-FINGER TOXIN FAMILY

The three-finger toxins (3FTXs) are nonenzymatic proteins containing sixty to seventy-four amino acid residues. This family of proteins is found abundantly in the venoms of elapids (including cobras, kraits, mambas, and sea snakes) (Fry et al., 2003b; Pahari et al., 2007a). However, its presence in the venoms of Colubridae (Fry et al., 2003a; Lumsden et al., 2005; Pawlak et al., 2006, 2008) and Crotalinae (rattlesnakes) snakes (Junqueira-de-Azevedo et al., 2006; Pahari et al., 2007b) has been revealed only recently. The characteristic feature of all 3FTXs is their distinct protein fold—three β-stranded loops extending from a small, globular, hydrophobic core that is cross-linked by four conserved disulfide bridges (Ménez, 1998; Tsetlin, 1999). The three loops that project from the core region resemble three outstretched fingers of the hand (Figure 13.1), and hence the name *three-finger toxin*.

All 3FTXs have structurally conserved regions that contribute to the proper folding and structural integrity of the polypeptide chain (Figures 13.1 and 13.2). In addition to eight conserved cysteine residues found in the core region, Tyr25 or a homologous aromatic residue Phe27 is also conserved in most toxins and is required for proper folding (Dufton and Hider, 1983; Antil et al., 1999) and stability of the antiparallel β-sheet structure (Torres et al., 2001). Some charged amino acid residues (e.g., Arg39 in erabutoxin-a and Asp60 in α-cobratoxin) have also been conserved, and they stabilize the native conformation of the protein by forming a salt link with the C- or N-terminus of the toxin (Endo and Tamiya, 1991).

Despite the overall similarity in structure, these proteins differ from each other in their biological activities. Members of this family include short- and long-chain α-neurotoxins, which antagonize muscle nicotinic acetylcholine receptors (nAChRs) (Changeux, 1990; Tsetlin, 1999); long-chain α-neurotoxins, which target neuronal α7 nAChR (Tsetlin, 1999); κ-bungarotoxins, which recognize neuronal nicotinic receptors (Grant and Chiappinelli, 1985); muscarinic toxins, which are selective agonists/antagonists of distinct subtypes of muscarinic acetylcholine receptors (Jerusalinsky and Harvey, 1994); fasciculins, which inhibit acetylcholinesterase (Eastman et al., 1995); calciseptine and related toxins, which block the L-type Ca^{2+} channels (de Weille et al., 1991; Albrand et al., 1995); cardiotoxins/cytotoxins, which exert their toxicity by forming pores in cell membranes (Bilwes et al., 1994); and dendroaspins, which are antagonists of various cell adhesion processes (McDowell et al., 1992). The three-finger fold is not restricted to snake venom toxins, because several other non-venom proteins and polypeptides also belong to this superfamily (Fleming et al., 1993; Ploug and Ellis, 1994; Gumley et al., 1995; Miwa et al., 1999; Cordero-Erausquin et al., 2000; Georgaka et al.,

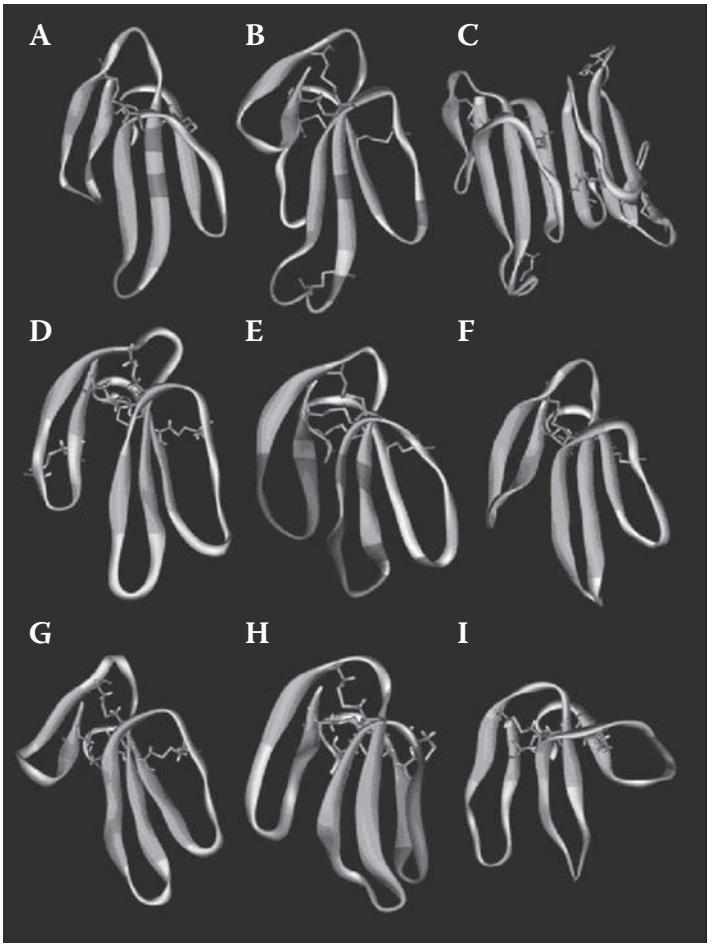

FIGURE 13.1 ***(A color version of this figure follows page 240.)*** Similar structures, diverse functions. Three-dimensional structures of 3FTXs from representative snake venoms are shown. (A) Erabutoxin (3EBX). (B) α-cobratoxin (2CTX). (C) Dimer observed in κ-bungarotoxin (1KBA). (D) Candoxin (1JGK). (E) Fasciculin (1FSS). (F) Muscarinic toxin 2 (1FF4). (G) Cardiotoxin (2CRT). (H) FS_2 (1TFS). (I) Dendroaspin (1DRS). The cysteine residues are shown in stick representation, and disulfide bonds are shown in yellow. Important functional residues, in toxins with known structure-function relationships, are shown in red.

2007; Palmer et al., 2007). In the following sections we discuss the overall functions and structure-function relationships of each of these categories of venom 3FTXs.

A. Neurotoxins

Neurotoxins interfere with cholinergic transmission at various postsynaptic sites in the peripheral and central nervous systems (Changeux, 1990). Based on their receptor selectivity, they can be broadly classified as curaremimetic or α-neurotoxins, κ-toxins, and muscarinic toxins that target muscle nAChR, neuronal nAChR, and various subtypes of muscarinic receptors, respectively. For many years, α-neurotoxins have contributed significantly to isolation and characterization of muscle (α1) nAChR, making it one of the best-characterized receptors (Grutter and Changeux, 2001). Similarly, muscarinic and κ-neurotoxins have also helped us understand molecular details of various muscarinic and neuronal AChR subtypes and their role in neurotransmission.

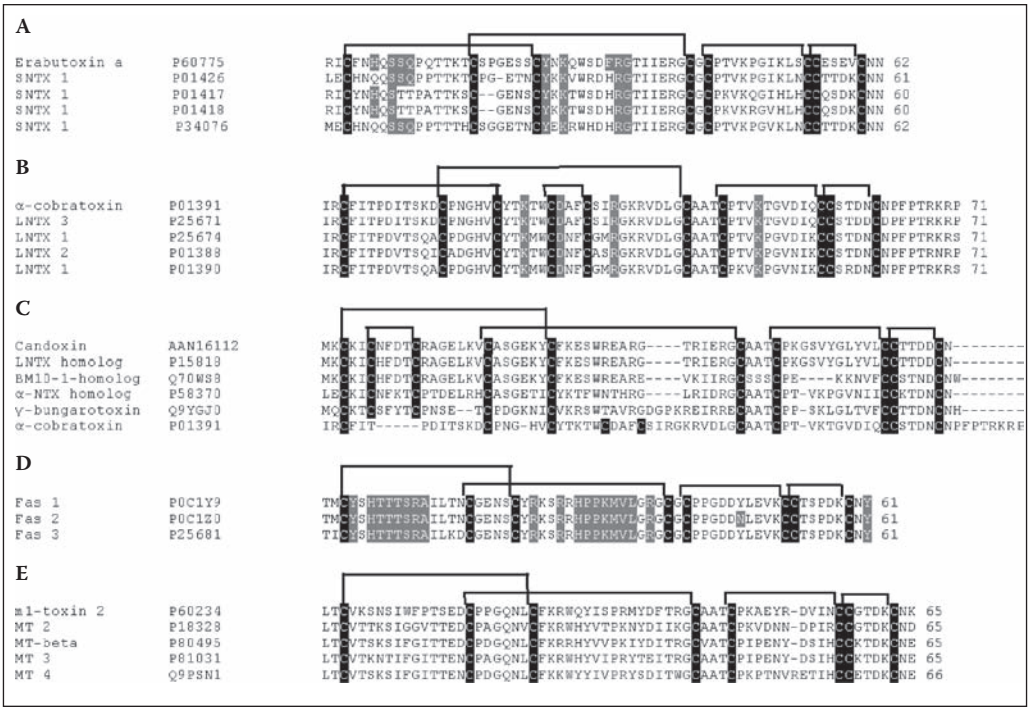

FIGURE 13.2 Sequences from various classes of 3FTXs. (A) Short-chain neurotoxins. (B) Long-chain neurotoxins. (C) Candoxin and other nonconventional toxins. (D) Fasciculins. (E) Muscarinic toxins. (F) Cardiotoxins. (G) Calciseptine and FS_2 toxins. (H) Dendroaspin or mambin. (I) colubrid 3FTXs. The conserved Cys residues have been highlighted in black, and the disulfide pairing pattern is indicated on top. The functional site residues are highlighted in red; in (A) peripheral residues surrounding the functional site are highlighted in gray.

1. α-Neurotoxins

Snake venom α-neurotoxins bind to postsynaptic nAChRs at the skeletal neuromuscular junction and inhibit acetylcholine from binding to the receptor, resulting in blockade of neuromuscular transmission (Changeux, 1990; Chang, 1999; Servent and Ménez, 2001). The discovery nearly 40 years ago of α-bungarotoxin from the venom of *Bungarus multicinctus* enabled the isolation and study of the nAChR from the electric organ of the electric eel and other sources (Changeux et al., 1970; Lee, 1972). These toxins mimic the neuromuscular blocking effects of the plant alkaloid (+)-tubocurarine, but with approximately 15- to 20-fold greater affinity and poor reversibility of action, and hence are also referred to as curaremimetic neurotoxins (Chang, 1999). To date, more than one hundred three-finger α-neurotoxins have been isolated and sequenced from elapid snake venoms (Endo and Tamiya, 1991). Depending on their amino acid sequence and Cys pattern, α-neurotoxins can be classified into short-chain α-neurotoxins (sixty to sixty-two amino acid residues and four conserved disulfide bonds) and long-chain α-neurotoxins (sixty-six to seventy-four amino acid residues and four conserved disulfide bonds, with an additional disulfide bond in the tip of loop II) (Servent and Ménez, 2001).

The structure-function relationships of α-neurotoxins have been thoroughly studied using both chemical modification and genetic engineering approaches (Tremeau et al., 1995; Antil et al., 1999). Using a mutagenesis approach, Tremeau and coworkers (1995) identified the functional site of erabutoxin-a, a short-chain neurotoxin isolated from the venom of the sea krait *Laticauda semifasciata*. The functional site is formed by numerous residues forming a homogeneous surface of

approximately 680 Å^2, the most important of which are Ser8, Glu10, Lys27, and Arg33. There is a set of peripheral residues surrounding this functional site and playing a minor role in determining the affinity to the receptor; these are His6, Ser9, Tyr25, Phe32, and Gly34. Thus, the functionally important residues are distributed on the tips of all three loops in the toxin (Figure 13.1A) (Tremeau et al., 1995). Extensive studies on the structure-function relationships of α-cobratoxin, a long-chain neurotoxin isolated from the venom of *Naja kaouthia*, have revealed a functional site of approximately 880 Å^2 residing mostly in the central second loop of the toxin. The most important residues are Lys23, Asp27, Arg33, and Lys49 (Figure 13.1B). All of these residues have structurally equivalent counterparts in erabutoxin. There are three major points in which the functional sites of these two toxins vary:

1. The first loop is not involved in any way in α-cobratoxin function, while the tip of the first loop in erabutoxin is critical for its binding with the receptor.
2. The C-terminal tail is critical in α-cobratoxin function, but it has no role to play in erabutoxin (Antil et al., 1999).
3. The extra fifth disulfide linkage found in the tip of the second loop in α-cobratoxin cyclizes a helix-like conformation and has been reported to be important for long-chain α-neurotoxin binding to neuronal α7 nAChR (Servent et al., 1997).

Therefore, the functional sites of long- and short-chain neurotoxins are not identical. They share several common features, but there are many significant differences leading to the observed difference in specificities.

2. κ-Neurotoxins

Unlike α-neurotoxins, which bind to α1 nAChRs, κ-neurotoxins bind specifically to neuronal α3β4 nAChR (Grant and Chiappinelli, 1985). κ-bungarotoxin was isolated from the venom of *Bungarus multicinctus* (many-banded krait). It is a protein with five disulfide bonds, and the Cys arrangement is similar to that for long-chain α-neurotoxins. However, it has a short C-terminal tail like the short α-neurotoxins, and an intermediate number of sixty-six amino acid residues (Grant and Chiappinelli, 1985; Fiordalisi et al., 1994). This is the first 3FTX reported to exist as a homodimeric complex (Figure 13.1C) (Dewan et al., 1994). Six main chain–main chain hydrogen bonds and three other hydrogen bonding interactions involving side chains are observed at the dimer interface. Residues Phe49 and Leu57, found in κ-bungarotoxin, but not in α-neurotoxins, form van der Waals interactions across the dimer interface. The two subunits of the dimer are not identical in folding, with the major difference between them occurring at the tip of the central loop (Cys 27–Pro 36). Residue Arg 34, which is essential for the activity of both α- and κ-neurotoxins, occurs at the tip of the central loop in each subunit, with guanidinium groups that are ~44 Å apart (Dewan et al., 1994). However, there is no clear evidence for the role played by the dimer in binding to the receptor.

3. Nonconventional Toxins

Nonconventional toxins are a poorly understood group of 3FTXs. They consist of sixty-two to sixty-eight amino acid residues and five disulfide linkages. However, unlike the long-chain α-neurotoxins and κ-neurotoxins, the fifth disulfide linkage is present in loop I (Figure 13.1D). Members from this group were initially identified from the venom of *Naja melanoleuca* (forest cobra) (Carlsson, 1975), and hence were initially named the *melanoleuca*-type toxins (Joubert and Taljaard, 1980). The nonconventional toxins show low and wide-ranging lethality (LD_{50} ~5–80 mg/kg) compared to the highly lethal α-neurotoxins (LD_{50} ~0.04–0.3 mg/kg). This group of toxins has also been referred to as weak due their low lethality (Utkin et al., 2001). However, this convention does not hold true for a few cases, such as γ-bungarotoxin, isolated from the venom of *Bungarus multicinctus* (many-banded krait), which has an LD_{50} value of 0.15 mg/kg (Antil et al., 1999), which is comparable to that of α-neurotoxins. Taking the above-mentioned points into account, this functionally

heterogeneous group of 3FTXs has been renamed nonconventional toxins (Servent and Ménez, 2001; Nirthanan et al., 2003b).

Candoxin (Figure 13.1D), isolated from the venom of *Bungarus candidus* (Malayan krait), is a well-studied member of the nonconventional toxin family (Nirthanan et al., 2002, 2003a, 2003b). Unlike short- and long-chain α-neurotoxins that produce irreversible blockage of nerve-muscle preparations, candoxin-caused neuromuscular blockade was readily and completely reversible. The solution structure of candoxin (Figure 13.1D) shows the presence of some of the invariable functionally important residues, demonstrating that the same site is involved in binding to muscle nAChR. Candoxin also irreversibly blocks the rat neuronal α7 receptors at nanomolar range (Nirthanan et al., 2002). Long-chain α-neurotoxins possess a cyclized helix-like conformation at the tip of loop II, formed by the fifth pair of disulfide bond, and this segment is postulated to be important for their binding to the neuronal α7 receptors (Servent et al., 1997). Interestingly, in candoxin the fifth disulfide bond is located in loop I and not in the tip of loop II. Thus, candoxin shares a common scaffold with long-chain α-neurotoxins but might possibly use additional determinants that assist in recognizing neuronal α7 receptors (Nirthanan et al., 2003a).

4. Fasciculins

Fasciculins (see also Chapter 15) are potent acetylcholinesterase inhibitors isolated from the venom of mambas (*Dendroaspis* sp.). They are structurally similar to short-chain neurotoxins and have sixty-one amino acid residues with four disulfide bridges. Fasciculins 1 and 2 were first purified from the venom of green mamba (*Dendroaspis angusticeps*) (Karlsson et al., 1984). Fasciculins interfere in neuromuscular transmission by binding to acetylcholinesterase present in the neuromuscular junction (Eastman et al., 1995). Due to this binding, acetylcholine accumulates in the neuromuscular junction and causes fasciculations, hence the name fasciculins. They are noncompetitive inhibitors of acetylcholinesterase, with a K_i in the picomolar range. They bind at the peripheral anionic site of acetylcholinesterase and block the entry of the acetylcholine to the active site of the enzyme (Karlsson et al., 1984). The functional site involved in this interaction was identified by designing cyclic synthetic peptides derived from the second loop (Falkenstein and Pena, 1997) and by site-directed mutagenesis (Marchot et al., 1997). Mutagenesis studies revealed that this functional site is present in the second and third loops of the molecule, and Thr8, Thr9, Gln11, Arg24, Arg27, His29, Pro30, Pro31, and Met33 are the residues that are involved in interaction (Figure 13.1E).

5. Muscarinic Toxins

The first protein toxins that bind to muscarinic acetylcholine receptors (mAChRs) were isolated from the venom of *Dendroaspis angusticeps* (African green mamba), and they were named muscarinic toxins (Adem et al., 1988). Unlike nAChRs, which form an ion channel, the mAChRs belong to the large family of cell surface proteins called G protein-coupled receptors (GPCRs), which act through GTP binding proteins (G proteins) to stimulate or inhibit intracellular effector systems (Jerusalinsky and Harvey, 1994; Segalas et al., 1995; Karlsson et al., 2000). There are five subtypes of mAChRs (M1 to M5), which control a large number of physiological processes, such as smooth and cardiac muscle contraction, glandular secretions, release of neurotransmitters, gene expression, arousal, attention, rapid eye movement in sleep, temperature control, modulation of stress, and cognitive functions, such as learning and memory (Segalas et al., 1995; Karlsson et al., 2000). mAChRs have been implicated as important drug targets in disorders like schizophrenia, depression, and Parkinson's and Alzheimer's diseases (Segalas et al., 1995; Bradley, 2000). Although there are nearly one hundred conventional small molecular muscarinic antagonists currently available, not one of these is specific for just one subtype of mAChR, and most have nearly equal affinity for two or three of the muscarinic subtypes (Bradley, 2000). Because of this promiscuity of conventional antagonists, it was difficult to assess the functional significance of the individual subtypes of mAChRs in different tissues, as more than one subtype of the receptor may exist simultaneously

in many organs (Jerusalinsky and Harvey, 1994; Adem and Karlsson, 1997; Karlsson et al., 2000). Muscarinic toxins were the first subtype-specific agonists/antagonists, isolated from various African mamba venoms (Figure 13.1F). They vary in length from sixty-three to sixty-six amino acid residues and possess eight Cys residues forming four disulfide linkages. A possible explanation for the good selectivity of muscarinic toxins for various subtypes of mAChRs is that the large size of the toxin enables it to interact with the highly variable extracellular loop regions of the GPCRs (Karlsson et al., 2000). This has made them invaluable research and diagnostic tools for biomedical applications (Jerusalinsky and Harvey, 1994; Adem and Karlsson, 1997; Karlsson et al., 2000; Bradley, 2000).

B. Cardiotoxins

Snake venom cardiotoxins (CTXs) are a group of 3FTXs that are found abundantly in the venoms of most elapid snakes (Boffa et al., 1983), particularly cobras (Figure 13.1G). CTXs may constitute about 50% of the dry weight of some cobra venoms, and they are the leading cause of death and morbidity, as they are highly lethal (Boffa et al., 1983). There are about two hundred CTX genes in the sequence databases, and the toxins are purported to induce various pharmacological effects on prey that precipitate the death of the animal. Some of the reported activities of CTXs are:

- Inhibition of ACTH-stimulated lipolysis in isolated fat cells and steroidogenesis in isolated adrenal cells (Keung et al., 1975)
- Deactivation of Na^+-K^+-activated ATPase of axonal membranes (Vincent et al., 1976)
- Contracture induction in skeletal muscle of humans and rats (Fletcher and Lizzo, 1987)
- Increase of Ca^{2+} influx via nonspecific calcium channels, Na^+-Ca^{2+} exchange, and mobilization of intracellular Ca^{2+} in electrically induced single ventricular myocytes (Wang et al., 1999)
- Contraction of smooth muscle (Hider and Khader, 1982)
- Activation of tissue phospholipase C and arachidonic acid–associated phospholipase A_2 (Fletcher and Jiang, 1993)
- Anticoagulation effects (Boffa et al., 1983; Kini and Evans, 1988)
- Hemolytic activity (Louw and Visser, 1978; Osorio e Castro and Vernon, 1989)
- Induction of platelet aggregation (Teng et al., 1984)
- Inhibition of platelet aggregation (Kini and Evans, 1988)
- Decrease in force of contraction and increase in heart rate due to ventricular tachycardia in perfused dog hearts and anaesthetized rats (Slotta and Vick, 1969; Sun and Walker, 1986)
- Lysis of various cell types, such as epithelial cells, fetal lung cells, and certain types of tumor cells (Kumar et al., 1997)
- Inhibition of bone resorption by binding to $\alpha_v\beta_3$ integrin (Wu et al., 2006)

Although much information about the various pharmacological activities of CTXs is known, a molecular target or mechanism of action is yet to be determined. However, most of the CTXs studied also show potent hemolytic/cytolytic activity, and the lipid membrane interaction and penetrating capabilities of CTXs are suspected to be the prime causes of this property. The proposed functional site of CTXs is the continuous hydrophobic patch, starting from the tip of loop I and covering large parts of loops II and III (covering nearly 40% of the molecular surface) (Bilwes et al., 1994). Conserved basic residues form a border around this hydrophobic patch and help in the recognition of negatively charged head groups of phospholipids in membranes, and subsequently, the hydrophobic tip of loop I interacts with hydrophobic parts of the membrane and leads to membrane insertion of the CTX molecule (Kini and Evans, 1989; Bilwes et al., 1994) (Figures 13.1G and 13.3).

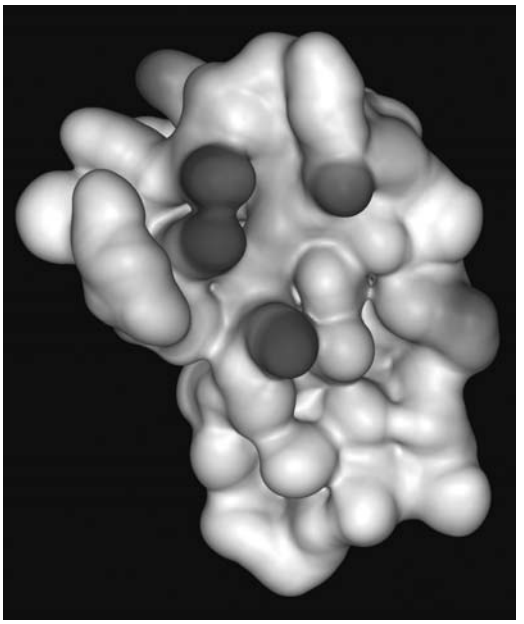

FIGURE 13.3 ***(A color version of this figure follows page 240.)*** Surface charge view of cardiotoxin structure. Positive, negative, and hydrophobic residues are in blue, red, and white, respectively.

C. Anticoagulant Proteins

We have isolated and characterized two unique, synergistically acting anticoagulant proteins, hemextins A and B, from the venom of the elapid snake *Hemachatus haemachatus* (African Ringhals cobra) (Banerjee et al., 2005a, 2005b, 2007a, 2007b). Hemextin A (but not B) exhibits mild anticoagulant activity. However, hemextin B forms a complex with hemextin A (hemextin AB complex) and synergistically enhances its anticoagulant potency. Using a dissection approach (Kini and Banerjee, 2005), we determined that hemextin A and hemextin AB complex prolong clotting time by inhibiting TF-FVIIa (tissue factor–factor VIIa) activity. This site of anticoagulant effects was supported by their inhibitory effect on the reconstituted TF-FVIIa complex. Therefore, we named it hemextin AB complex (*Hemachatus* extrinsic tenase inhibitor), and the individual proteins as hemextins A and B, respectively. This is the first synergistic anticoagulant complex. Further, we demonstrated the specificity of inhibition by studying their effects on twelve serine proteases; hemextin AB complex potently inhibited the amidolytic activity of FVIIa in the presence or absence of soluble TF. Kinetic studies showed that hemextin AB complex is a noncompetitive inhibitor of soluble TF-FVIIa amidolytic activity, and isothermal titration calorimetric studies showed that hemextin AB complex binds directly to FVIIa. Hemextin AB complex is the only known natural anticoagulant that specifically inhibits factor VIIa enzymatic activity in the absence of factor Xa (Banerjee et al., 2005a). As the initial formation of TF-FVIIa complex is crucial to clot initiation, it is considered an ideal target for the treatment of thromboemboloic disorders. Hence, the hemextin AB complex may be of great interest in this area.

Since hemextin AB complex is the only known heterotetrameric complex of two 3FTXs, we investigated the molecular interactions in its formation (Banerjee et al., 2007b). CD studies of individual hemextins A and B indicate that both hemextin A and hemextin B have β-sheet structure. A titration CD experiment to study the complex formation between the two proteins suggests that there is an increase in β-sheet content after complex formation. Hemextin AB complex has an

identical molecular diameter in both the gas and solution phases. Isothermal titration calorimetry (ITC) studies reveal that the complex formation is entropically unfavored, which indicates the reduced structural flexibility of the complex. Hemextin AB assembly is an enthalpically driven process with some conformational changes accompanying the complexation. The tetrameric complex breaks into dimers in buffers with higher ionic strength or glycerol. However, these dimers differ in size and anticoagulant activity (for details, see Banerjee et al., 2007b). A complex interplay of electrostatic and hydrophobic interactions drives the formation and stabilization of this novel anticoagulant complex. We also examined the binding of individual heavy and light chains of FVIIa to the anticoagulant proteins. The results indicated that the binding site of hemextin AB complex and hemextin A is localized in the heavy chain of FVIIa (unpublished observation). We have also isolated and characterized a number of other anticoagulant proteins of the 3FTX family, and the details of the structure, function, and mechanism are in progress.

D. β-Cardiotoxin

β-cardiotoxin is the first reported protein showing β-adrenergic receptor antagonism (Rajagopalan et al., 2007). β-adrenergic receptor antagonists, commonly referred to as beta-blockers, are the drugs of choice for treatment of cardiovascular diseases (Thom et al., 2006; Rosamond et al., 2007). β-cardiotoxin, isolated from the venom of *Ophiophagus hannah* (king cobra), was nonlethal up to a dose of 10 mg/kg. It induced reduction in heart rates (bradycardia) in anesthetized animals and also in isolated perfused rat hearts. We showed by competitive binding assays that this reduction of heart rates was due to the binding of β-cardiotoxin to the β-adrenergic receptors that are responsible for the rapid regulation of the cardiac contraction (Rajagopalan et al., 2007). All currently used β-adrenergic receptor antagonists are small molecules belonging to a class known as aryloxypropanolamines (Griffith, 2003). However, these drugs have shown many adverse side effects in patients, and newer and more specific beta-blockers are actively pursued (Waldo et al., 1996). Thus, β-cardiotoxin holds great promise for the future, as a better understanding of its structure and structure-function relationships will enable us to design novel therapeutics for the treatment of cardiovascular diseases.

E. Calciseptine and FS_2 Toxin

Calciseptine, isolated from the venom of *Dendroaspis polylepis polylepis* (black mamba), is the only natural peptide known that specifically blocks L-type Ca^{2+} channels in heart and skeletal muscles (de Weille et al., 1991; Garcia et al., 2001). It has sixty amino acid residues with four disulfide bridges and structural features similar to those of other 3FTXs. Another toxin, known as FS_2, is similar to calciseptine in action and has been isolated from same snake venom (Yasuda et al., 1994). The binding of calciseptine to L-type Ca^{2+} channels has been studied using rat brain synaptosomal membranes, which have specific binding sites for L-type Ca^{2+} channel ligands (Yasuda et al., 1993). The binding site of this toxin to L-type Ca^{2+} channels was determined by using the proline bracket hypothesis (Kini and Evans, 1995). The functional site in both calciseptine and FS_2 was predicted to be in between Pro42 and Pro47, and a synthetic peptide derived from this region inhibited activity in a manner similar to that of the native molecule (Kini et al., 1998). The functional site in this molecule is located on the third loop, in contrast to other toxins, which have functional sites found primarily in the first or second loop.

F. Dendroaspin or Mambin

Dendroaspin, also referred to as S_5C_1 (Joubert and Taljaard, 1979) and mambin (McDowell et al., 1992), was isolated from both *Dendroaspis jamesoni kaimosae* (eastern Jameson's mamba) and

D. viridis (western green mamba) venoms (Williams, 1992). It contains fifty-nine amino acid residues and four disulfide bonds (Figure 13.1I). Dendroaspin contains the Arg-Gly-Asp (RGD) motif with which it interacts with the platelet glycoprotein GP IIb-IIIa (also called $\alpha_{IIb}\beta_3$), causing potent inhibition (IC_{50} ~170 nM) of platelet aggregation (Figure 13.2H) (McDowell et al., 1992). Integrins are a family of heterodimeric class I transmembrane receptors that mediate cell-matrix and cell-cell adhesion processes. $\alpha_{IIb}\beta_3$ is a member of the β_3 integrin subgroup, which is essential for platelet aggregation and controls platelet function in thrombosis and hemostasis (Wattam et al., 2001). The RGD tripeptide motif is important for the binding of many extracellular matrix proteins, such as fibrinogen and fibronectin, to $\alpha_{IIb}\beta_3$ and other integrin receptors. Dendroaspin and the disintegrins (a large family of proteins from viperid snake venoms; see also Chapter 17, this volume) compete with the binding of these proteins to their receptors, and hence act as potent inhibitors of cell-cell and cell-matrix interactions. However, dendroaspin and disintegrins do not share any structural or sequence similarity except for the presence of the RGD tripeptide motif (Sutcliffe et al., 1994).

G. Hannalgesin

Hannalgesin is a novel protein isolated from the venom of *Ophiophagus hannah* (king cobra) that exhibits neurotoxicity and potent analgesic effects in mice (Pu et al., 1995). It has structural features similar to those of other neurotoxins and is seventy-two amino acid residues long. The analgesic effect of hannalgesin has been determined by the hot plate assay at a dose of 24 ng/g (administered intraperitoneally). Other routes, such as intravenous, periocular, and intracerebroventricular (i.c.v.), also exhibited analgesic effects, and i.c.v. injections revealed that such action might be mediated via the central nervous system. The functional site of this molecule has been predicted using the proline bracket hypothesis, and a synthetic peptide designed using this predicted functional site showed potent analgesic effects. This functional site has been found to reside in the C-terminal end of the molecule, demonstrating the different location of a functional site even though it is structurally similar to other 3FTXs.

H. 3FTXs from Colubridae Snake Venoms

It was widely believed that 3FTXs were present in the venoms of elapid snakes only. However, α-colubritoxin, the first 3FTX from a nonelapid source, was isolated and characterized from the Asian ratsnake *Coelognathus radiatus*, a member of the polyphyletic family Colubridae (Figure 13.2I). α-colubritoxin is a potent postsynaptic neurotoxin displaying readily reversible competitive antagonism at the nicotinic acetylcholine receptor (Fry et al., 2003a). Interestingly, *C. radiatus* is considered a nonvenomous snake and has been commonly available in the pet trade for many years, and bites to humans are asymptomatic. Taxon-specific effects of colubrid venoms also suggested that many colubrid snakes (such as the brown treesnake, *Boiga irregularis*) likely had potent toxins in their venoms, but because they are not highly toxic to mammals, they have been overlooked pharmacologically (Mackessy et al., 2006).

Boigatoxin-A is a unique 3FTX isolated from the venom of *Boiga dendrophila* (mangrove catsnake). Detailed pharmacological characterization of this protein has shown that it causes a weak postsynaptic neurotoxicity in skeletal muscle. Surprisingly, this protein also exhibited prejunctional neurotoxic activity in the smooth muscle of the rat vas deferens and inhibited the release of neurotransmitters (Lumsden et al., 2005).

Denmotoxin (*B. dendrophila* monomeric toxin) was isolated from the venom of *B. dendrophila* (mangrove catsnake) (Figure 13.2I). Denmotoxin is seventy-seven amino acid residues long and has five disulfide bonds. It produced potent and irreversible neuromuscular blockade of chick biventer cervicis nerve-muscle preparations. However, it showed a 100-fold weaker and reversible inhibition

of electrically induced twitches in mouse hemidiaphragm nerve-muscle preparations. These data indicate that denmotoxin has a bird-specific neurotoxicity. *B. dendrophila* live in Southeast Asian lowland rainforests and mangrove swamps, and they primarily feed on birds. Thus, denmotoxin illustrates the relationship between toxin specificity and the feeding habits of the snake, and denmotoxin is the first taxon-specific 3FTX (Pawlak et al., 2006). The crystal structure of this protein shows a nonconventional three-finger fold with the fifth disulfide bond present in the first loop.

A new class of covalently linked heterodimeric dimeric neurotoxins, which also show extreme taxon-specific toxicity, has recently been described from the venom of *B. irregularis* (Pawlak et al., 2009). This new toxin, irditoxin (*B. irregularis* dimeric toxin), is very toxic to birds and lizards but is apparently nontoxic to mammals (mice). Like *B. dendrophila*, *B. irregularis* primarily feeds on birds and lizards, and this prevalent (~10% of total venom protein) toxin further illustrates the link between venom toxin evolution and prey preferences of snakes. Irditoxin is also a member of the nonconventional 3FTX group, and each subunit has an extra cysteine that participates in the sole disulfide bond covalently linking the subunits.

I. 3FTXs from Crotalinae Snake Venoms

Recently we and others have reported the presence of 3FTX molecules from crotaline (pit viper) snake venom glands (Junqueira-de-Azevedo et al., 2006; Pahari et al., 2007b). The presence of these molecules in Crotalinae venom gland transcriptomes suggests that the repertoire of toxins could be more conserved between families than has been considered previously, at least at the transcriptome level. Also, evolutionary processes such as multiple recruitments of important scaffolds might have maintained the minimalist nature of toxin structures, as opposed to nontoxin protein structures.

III. CONCLUSIONS

Our understanding of the structure and functions of 3FTXs has improved remarkably in the past few years. The three-finger protein fold, with a highly conserved globular core consisting of four disulfide bonds, forms a highly stable, compact structure that (speaking teleologically) has been exploited by nature for developing ligands that perform a wide variety of functions. As is the case with other superfamilies of toxins, the robust three-finger protein scaffold has evolved in snake venoms to result in a group of toxins with wide variations in function and pharmacology, involving just a few subtle changes in the functional sites. Further, the various activities appear to be conserved and may result from cassette-like switching of highly conserved regions (ASSET: accelerated segment switch in exons to alter targeting), as has been suggested by a recent analysis of the 3FTX toxin genes present in the viperid *S. c. edwardsii* (Doley et al., 2008). In fact, there is no single designated location for the functional sites. This capacity of adaptation no doubt offers the flexibility of functionality against a variety of different receptor subtypes, allowing snakes to capture effectively (via chemical means) many species of prey at their disposal. Their intended interests notwithstanding, snake venom toxins provide us with ample challenging opportunities to decipher the subtleties in their functional sites such that we may better understand the plasticity of protein structure and function. Moreover, they will also tremendously enhance our potential to use their molecular architecture to design and develop mini-proteins with novel functions of scientific and therapeutic interest.

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14 Sarafotoxins, the Snake Venom Homologs of the Endothelins

Avner Bdolah

CONTENTS

The sarafotoxins (SRTXs) are unique products of burrowing asps, snakes belonging to the genus *Atractaspis*. The venom of *A. engaddensis*, the most toxic species, was found to be highly cardiotoxic, and the cardiotoxic fraction of this venom contains four peptides of twenty-one amino acid residues, SRTX-a, -b, -c, and -d/e. Their primary structure and their conserved cysteine scaffold showed high homology with the endothelins (ETs). SRTX-like peptides have been isolated from venoms of three other *Atractaspis* species: bibrotoxin, a twenty-one amino acid peptide from *A. bibroni*; SRTX-m1-5 from *A. m. microlepidota*; and SRTX-i1-3 from *A. irregularis*. Most SRTX isoforms of the last two groups have molecular sizes longer than the typical twenty-one amino acid SRTXs. cDNAs of SRTX precursors, which were cloned from the venom glands of *A. engaddensis* and *A. m. microlepidota*, indicated that genes encoding these peptides have a polycistronic structure, whereas a cDNA from the venom gland of *A. irregularis* revealed only one stretch encoding a single SRTX-like peptide. Both SRTXs and ETs induce cardiovascular disturbances in experimental animals; both groups of peptides are potent vasoconstrictors and affect also smooth muscle function in nonvascular systems. Binding studies have shown that the two families of peptides share common receptors and activate similar cellular signal pathways. However, ETs have been identified and found to be active in a variety of vertebrate tissues. It became evident that the densities of different SRTX/ET receptor subtypes and their subtle pharmacological properties vary between different animals and tissues.

I. INTRODUCTION

The sarafotoxins (SRTXs) are unique venom products of burrowing asps belonging to the snake genus *Atractaspis* (family Atractaspididae). The fifteen or sixteen species of this genus are widely distributed, from the Cape of South Africa, through the entire breadth of Central Africa, and along the Rift Valley to Arabia, Sinai, and extending to the north along the Jordan valley (Gasperetti, 1988). Both information on bites by the different species of *Atractaspis* (Table 14.1) and experimental work

TABLE 14.1
Reported Cases of Bites by *Atractaspis* Species

Atractaspis sp.	No. of Reported Cases	Deaths
A. engaddensis	11	4
A. dahmyensis	7	—
A. aterrima	1	—
A. m. microlepidota	16	4
A. m. andersoni	1	1
A. irregularis	11	1
A. bibroni	22	—
A. corpulenta	3	—

Note: More than 300 bites were reported in Sudan, but no details on their effects or species of *Atractaspis* were given (see Ismail et al., 2007).

Source: According to Warrell et al. (1976), Kochva (2004), and Ismail et al. (2007).

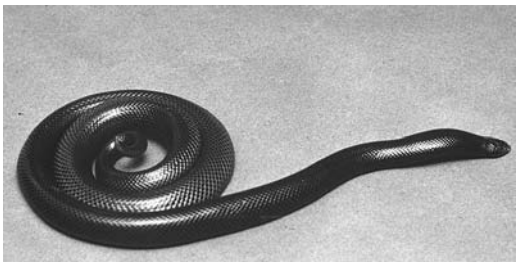

FIGURE 14.1 *Atractaspis engaddensis.*

on *Atractaspis* venom are rather limited. Because of the rapid clinical course in the fatal cases and some reported symptoms following envenomation (including loss of consciousness), it had been suggested by several investigators (e.g., Warrell et al., 1976) that the venom of *Atractaspis* probably contains a powerful neurotoxic component.

Reports of the clinical manifestations following envenomation by the Israeli burrowing asp, *A. engaddensis* (Figure 14.1), have described both local and systemic symptoms, including loss of consciousness and cardiac disorders (Alkan and Sukenik, 1974; Chajek et al., 1974). However, up to the early 1980s there had been no agreement on whether the venom of *A. engaddensis* directly affects the heart, or rather that these phenomena reflect the indirect effects of the venom. In order to resolve these questions, Weiser et al. (1984) examined the effect of crude venom of *A. engaddensis* on the heart and on nerve-muscle preparations. The most prominent effect of the venom in anesthetized mice was manifested in the function of the heart. Interestingly, the changes observed in the ECG of mice that led to atrioventricular block were similar to those recorded in human victims (see Figure 9 in Kochva et al., 1993). The venom had no pre- or postsynaptic neurotoxicity.

II. ISOLATION AND IDENTIFICATION OF SARAFOTOXINS IN THE VENOM OF DIFFERENT SPECIES OF *ATRACTASPIS*

The first fractionation of *A. engaddensis* venom revealed it to have a unique molecular sieving pattern, with a low molecular weight peak containing highly toxic proteins (Kochva et al., 1982). This

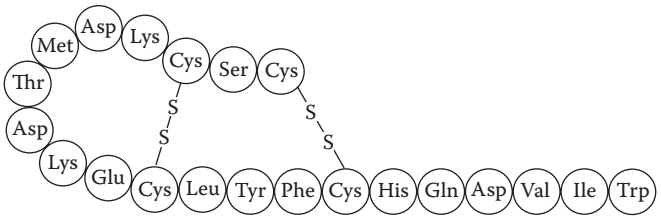

FIGURE 14.2 Primary structure of sarafotoxin-b.

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1                5                  10                  15                  20
Cys-Ser-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Asn-Phe-Cys-His-Gln-Asp-Val-Ile-Trp   SRTX-a
Cys  *  Cys  *   *   *   *   *   *   *  Cys  *  Tyr  *  Cys  *   *   *   *   *   *    SRTX-b
Cys-Thr-Cys-Asn  *   *   *   *  Glu  *  Cys  *   *   *  Cys  *   *   *   *   *   *    SRTX-c

Cys  *  Cys-Ser-Ser-Leu-Met  *   *   *  Cys-Val-Tyr  *  Cys  *  Leu  *  Ile  *   *    ET-1
Cys  *  Cys-Ser-Ser-Trp-Leu  *   *   *  Cys-Val-Tyr  *  Cys  *  Leu  *  Ile  *   *    ET-2
Cys-Thr-Cys-Phe-Thr-Tyr-Lys  *   *   *  Cys-Val-Tyr-Tyr-Cys  *  Leu  *  Ile  *   *    ET-3
1                5                  10                  15                  20
```

FIGURE 14.3 Amino acid sequences of sarafotoxins and endothelins (ETs). *, Residues identical to SRTX-a.

main toxic peak comprises 30 to 40% of the total proteins in the venom. The venom also demonstrates enzymatic activities as well as the hemorrhagic activity found in many other snake venoms. Further fractionation of the highly toxic fractions resulted in the isolation and characterization of the first three sarafotoxins: SRTX-a, -b, and -c (Wollberg et al., 1988; Takasaki et al., 1988). The SRTXs are twenty-one amino acid polypeptides with two disulfide bridges, between Cys 1–15 and 3–11 (Figures 14.2 and 14.3). To our surprise, the first report on the most potent vasoconstrictor produced by endothelial cells, the endothelin (Yanagisawa et al., 1988), and the amino acid sequences of endothelin-1, -2, and -3 (Inoue et al., 1989), revealed a high homology between the two groups of peptides (Figure 14.3). Peptides of both groups have the same cysteine skeleton, all have a hydrophobic carboxy-terminal tail, His16-Trp21, and the most important differences between the various peptides of the SRTX/endothelin family reside within the sequences of the inner loop Cys3–Cys11.

In an attempt to identify SRTX fractions in other species of *Atractaspis*, several venom samples were subjected to molecular sieving fractionation (Figure 14.4). This analysis did not reveal the presence of SRTX fractions in venom samples of *A. microlepidota* (originated from East Africa) and *A. micropholis*. However, the most recent report of Ismail et al. (2007) on fractionation of *Atractaspis microlepidota* venom from Saudi Arabia showed a molecular sieving pattern with a prominent toxic peak of SRTXs, similar to the pattern obtained for the Israeli *A. engaddensis* venom. Indeed, according to Gasperetti (1988), there are two subspecies of *Atractaspis* in Arabia: *A. microlepidota engaddensis* and *A. microlepidota andersonii*. However, there is no available information on the venom composition of each of the two subspecies.

Using a highly potent rabbit antiserum against SRTX-b, immunoreactive SRTX, albeit at a low level, could be demonstrated in venom samples of the Central African species, *A. microlepidota* and *A. micropholis*. A much higher level of immunoreactive SRTX was revealed in the venom of *A. bibroni*, from which an SRTX-like peptide, bibrotoxin, which differs from SRTX-b in only one position (Figure 14.5), was isolated (Becker et al., 1993). It is interesting to note that the levels of immunoreactive-SRTX-b that were found in these venoms are in correlation with their toxicities (Table 14.2).

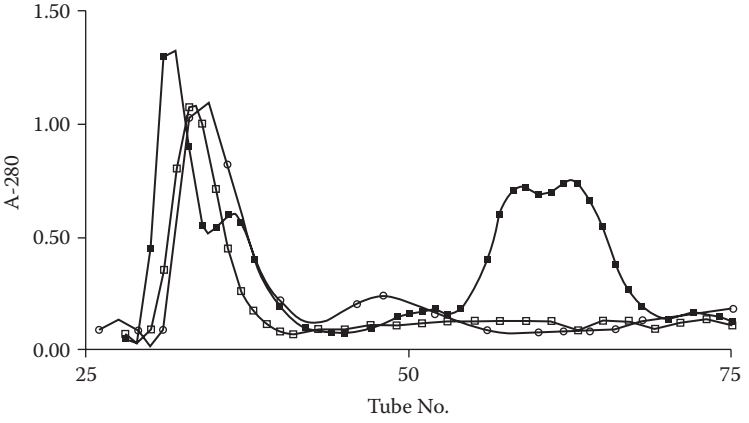

FIGURE 14.4 Sephadex G-50 gel permeation profiles of *A. engaddensis* (■), *A. microlepidota* from East Africa (□), and *A. micropholis* (○).

1				5					10					15					20			
Cys	Ser	Cys	Lys	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Asn	Phe	Cys	His	Gln	Asp	Val	Ile	Trp		SRTX-a
Cys	*	Cys	*	*	*	Ser	*	*	*	Cys	*	*	*	Cys	*	*	*	*	*	*		SRTX-a1
Cys	*	Cys	*	*	*	*	*	*	*	Cys	*	Tyr	*	Cys	*	*	*	*	*	*		SRTX-b
Cys	*	Cys	*	*	*	Ser	*	*	*	Cys	*	Tyr	*	Cys	*	*	*	*	*	*		SRTX-b1
Cys	*	Cys	Ala	*	*	*	*	*	*	Cys	*	Tyr	*	Cys	*	*	*	*	*	*		BTX[a]
Cys	Thr	Cys	Asn	*	*	*	*	Glu	*	Cys	*	*	*	Cys	*	*	*	*	*	*		SRTX-c
Cys	Thr	Cys	*	*	*	*	*	*	*	Cys	*	Tyr	*	Cys	*	*	Gly	Ile	*	*		SRTX-d/e[b]
Cys	*	Cys	Asn	*	Ile	Asn	*	*	*	Cys	Met	Tyr	*	Cys	*	*	*	*	*	*	Asp-Glu-Pro	SRTX-m
Cys	*	Cys	Asn	*	Met	Asn	*	*	*	Cys	Met	Tyr	*	Cys	*	*	*	*	*	*	Asp-Glu-Pro	SRTX-m1
Cys	*	Cys	Asn	*	Ile	Asn	*	*	*	Cys	Met	Tyr	*	Cys	*	*	*	Ile	*	*	Asp-Glu-Pro	SRTX-m2
Cys	*	Cys	Asn	*	Met	Asn	*	*	*	Cys	Val	Tyr	*	Cys	*	Leu	*	Ile	*	*	Asp-Glu-Pro	SRTX-m3
Cys	*	Cys	Asn	Asn	Met	Asn	*	*	*	Cys	Leu	*	*	Cys	Asn	Leu	*	Ile	*	*	Glu-Asn-Val	SRTX-m4
Cys	*	Cys	Asn	*	Met	Asn	*	*	*	Cys	Met	Tyr	*	Cys	*	Gln	*	Ile	*	*	Asp-Glu-Pro	SRTX-m5
Cys	*	Cys	Thr	*	*	Ser	*	Leu	*	Cys	Met	*	*	Cys	*	Lys	*	*	*	*	Ile-Asn-Arg-Asn	SRTX-i1
Cys	*	Cys	Ala	*	*	Ser	*	Leu	*	Cys	Met	*	*	Cys	Arg	Leu	*	*	Met	*	Val-Asn-Arg-Asn	SRTX-i2
Cys	*	Cys	Thr	*	*	Ser	*	Leu	*	Cys	Met	*	*	Cys	*	Lys	*	*	*	*	Val-Asn-Arg-Asn	SRTX-i3
1				5					10					15					20			

FIGURE 14.5 Amino acid sequences of the sarafotoxin isopeptides. Only the most abundant isotoxins from *A. m. microlepidota* (twenty-four amino acids long) and *A. irregularis* (twenty-five amino acids long) are included in this figure. (After Kochva et al., 1993; Ducancel, 2005.) *, Residues identical to SRTX-a; [a], BTX = bibrotoxin. [b], Reexamination of SRTX-d (Bdolah et al., 1989b) revealed that this isopeptide is identical to SRTX-e (unpublished) identified in the cDNA of the SRTXs from *A. engaddensis* (Ducancell et al., 1993).

During the last decade a combination of mass spectrometry analysis and molecular cloning has led to the identification of two more families of SRTX-like peptides from the venoms of *A. microlepidota microlepidota* (*A. m. microlepidota*) and *A. irregularis* (Hayashi et al., 2004; Quinton et al., 2005; Ducancel, 2005). The SRTX-like peptides of these two *Atractaspis* species have two conserved disulfide bridges and several invariant residues, including Trp21, similar to the SRTXs from *A. engaddensis* (Figure 14.5). However, the mass spectrometry analysis of the venom from these two species revealed the presence of SRTX isoforms with molecular sizes longer (and also a few shorter) than the typical twenty-one amino acid SRTXs of *A. engaddensis*. Most of the SRTX isoforms isolated from the venom of *A. m. microlepidota* are twenty-four amino acid peptides with a C-terminal sequence of Asp-Glu-Pro; they are designated SRTX-m1-5. The SRTX isoforms

TABLE 14.2
Toxicity of *Atractaspis* Venoms

Atractaspis sp.	LD_{50} (ng/g body weight)	Immunoreactive SRTX-b (fmol/μg venom)
A. engaddensis	75	120,000
A. bibroni	500	300
A. dahomeyensis	2,000	
A. microlepidota[a]	>2,000	6
A. micropholis[b]	>3,000	6

[a] From East Africa.
[b] Low molecular weight fraction of the venom.

isolated from the venom of *A. irregularis*, designated SRTX-i1, -i2, and -i3, include peptides of a wide range of molecular sizes (fifteen to thirty amino acids); the most abundant molecular species are twenty five amino acid long peptides with four additional C-terminal residues as compared with the SRTXs from *A. engaddensis*. This C-terminal extension is unrelated to the C-terminals of the SRTX-m isopeptides.

III. BIOSYNTHESIS OF THE SRTXs

Isolation of the complementary DNA encoding SRTXs was first achieved in the venom gland of *Atractaspis engaddensis* (Ducancel et al., 1993). This cDNA comprises 1,948 base pairs (bp), including an open reading frame of 1,629 bp coding for a long pre-pro-polypeptide of 543 amino acids. The deduced amino acid sequence of this cDNA revealed a unique rosary-type organization of the gene encoding the SRTXs in the venom gland of *A. engaddensis*. It consists of 12 successive stretches of 40 residues (39 in the first stretch), each of which begins with a spacer of 19 invariant residues (18 in the first one), which is followed by a sequence of an SRTX isoform. Six SRTX isoforms were identified within a single precursor molecule (Figure 14.6).

It is interesting to note that this polycistronic organization of this SRTX gene differs completely from the organization of the genes encoding the ETs. A separate gene, which is located on a separate

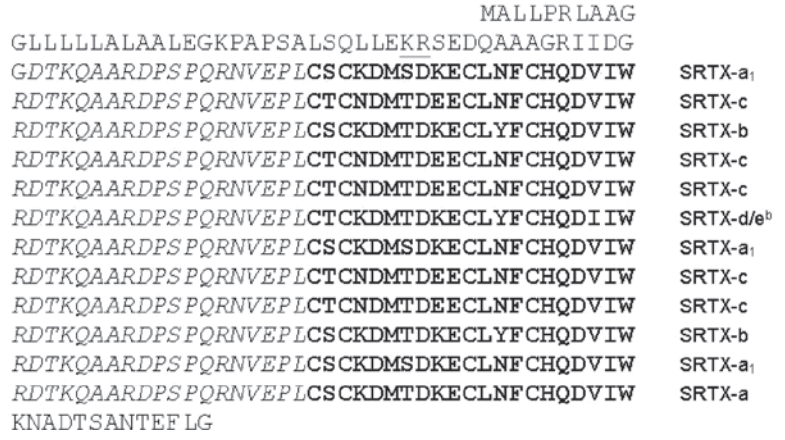

FIGURE 14.6 Precursor of the SRTXs in *A. engaddensis,* deduced amino acid sequence of the cDNA reading frame (according to Ducancel et al., 1993). Sequences of the mature SRTXs are **boldfaced** (see text); the spacer domains are in *italics*. The underlined dibasic is the cleavage site of trypsin-like enzyme. [b], See note in Figure 14.5.

```
                                                        IWDEP
VVVSARDTEEAARVPSPQKRSQPLCSCNDINDKECMYFCHQDVIWDEP        SRTX-m
VVVSVRDTEEAARVPSPQKRPQPRCSCNDMNDKECMYFCHQDVIWDEP        SRTX-m1
VVVSVRDTEEAARVPSPQKRSQPLCSCNDMNDKECMLFCHQDIIWDEP        SRTX-m3
VVVSVRDTEEAARVPSPQKRSQPLCSCNDINDKECMYFCHQDIIWDEP        SRTX-m2
VVVSVRDTEEATRVPSPQKRSQPLCSCNDINDKECMYFCHQDIIWDEP        SRTX-m2
VVVSARDTEEAARVPSPQKRSQPLCSCNDINDKECMYFCHQDVIWDEP        SRTX-m
VVVSVQDTEEAARVPSPQKRSQPLCSCNDINDKECNLFCHQDIIWENV        SRTX-m4
DTSADPEFLG
```

FIGURE 14.7 Precursor of the SRTXs in *A. m. microlepidota,* deduced amino acid sequence of the incomplete cDNA reading frame. See notes in Figure 14.6.

chromosome, encodes each ET isopeptide (Inoue et al., 1989; Arinami et al., 1991), and each of the genes contains a single copy of a pre-pro-polypeptide. The study of Landan et al. (1991) on the evolutionary history the gene families of the ETs and the SRTXs indicated that the SRTXs' lineage diverged from a common ancestral gene of the SRTX/ET superfamily prior to the first endothelin gene duplication event. This notion is further supported by the unique organization of the SRTXs' gene as compared to the ET genes.

A second precursor encoding the SRTXs in the venom gland of *A. m. microlepidota* was identified by Hayashi et al. (2004). The longest cDNA fragment that was isolated from this species is 1228 bp long, which corresponds to the C-terminal of the precursor. This incomplete cDNA contains an open reading frame that encodes a 351 amino acid long fragment (Figure 14.7). The structure of this precursor indicates that the SRTX gene of *A. m. microlepidota* has a polycistronic organization similar to that found in *A. engaddensis*, but not identical. It comprises seven tandem stretches of forty-eight residues corresponding to five different SRTX isoforms (see Figure 14.5). Each contains the sequence of twenty-four amino acid long SRTX followed by a highly conserved twenty-four residue long spacer (Figure 14.8). Another isoform of the SRTX-m series (SRTX-m5) was identified within another incomplete precursor (not shown).

Two precursors of SRTX-like peptides were cloned from the venom gland of *A. irregularis* (Quinton et al., 2005). Two cDNAs, which included open reading frames of 118 amino acids, were isolated. The deduced amino acid sequences of the two clones, which show high sequence identity, include only one stretch of twenty-five amino acid long mature SRTX, SRTX-i1, or SRTX-i2. No cDNA clone that codes for SRTX-i3 was identified. Since SRTX-i1 and SRTX-i3 differ by only one amino acid, at position 22, following the conserved Trp21, it was suggested (Ducancel, 2005) that SRTX-i3 could have derived from an edited mRNA (cf. Liu et al., 1998).

To date, SRTX precursors have been identified in only three species of *Atractaspis*. Despite the different overall organization of genes encoding these precursors, they display a high degree of sequence identity in specific domains of the genes. This includes the domain encoding the signal peptide as well as the sixty-one to sixty-nine amino acid sequences that precede the stretches that encode the mature SRTXs (Figure 6, Ducancel, 2005).

A. enqaddensis						**R**	**D**	**T**	*K*	*Q*	**A**	**A**	**R**	*D*	**P**	**S**	**P**	**Q**	*R*	*N*	*V*	*E*	**P**	**L**
A. m. microlepidota	*V*	*V*	*V*	*S*	*V*	**R**	**D**	**T**	*E*	*E*	**A**	**A**	**R**	*V*	**P**	**S**	**P**	**Q**	*K*	*R*	*P*	*Q*	**P**	**L**
	V	*V*	*V*	*S*	*V*	**R**	**D**	**T**	*E*	*E*	**A**	**A**	**R**	*V*	**P**	**S**	**P**	**Q**	*K*	*R*	*S*	*Q*	**P**	**L**
	V	*V*	*V*	*S*	*V*	**R**	**D**	**T**	*E*	*E*	**A**	*T*	**R**	*V*	**P**	**S**	**P**	**Q**	*K*	*R*	*S*	*Q*	**P**	**L**
	V	*V*	*V*	*S*	*A*	**R**	**D**	**T**	*E*	*E*	**A**	**A**	**R**	*V*	**P**	**S**	**P**	**Q**	*K*	*R*	*S*	*Q*	**P**	**L**
	V	*V*	*V*	*S*	*V*	*Q*	**D**	**T**	*E*	*E*	**A**	**A**	**R**	*V*	**P**	**S**	**P**	**Q**	*K*	*R*	*S*	*Q*	**P**	**L**

FIGURE 14.8 Amino acid sequences of the spacer domains in the SRTX precursors of *A. engaddensis* and *A. m. microlepidota*. Identical residues in the spacers are **boldfaced**.

The structure of two of the precursors that were identified in the venom glands of *A. engaddensis* and *A. m. microlepidota* revealed a unique polycistronic gene organization. The genes comprise tandem repeats of stretches that include amino acid sequences of the mature SRTXs and spacers. Even though the lengths of these repeats are different in the two precursors, their SRTX domains as well as the spacer domains (Figure 14.8) are highly homologous. This may suggest that the SRTX precursors of *A. engaddensis* and *A. m. microlepidota* have evolved from a common ancestor. Indeed, Underwood and Kochva (1993), analyzing morphological characters in most species of *Atractaspis*, claim that there is evidence for two distinct lineages in this genus: the *microlepidota* group, which includes *A. engaddensis* and *A. microlepidota*, and the *bibroni* group, which includes *A. bibroni* and *A. irregularis*. It is interesting to note that members of the *microlepidota* group have relatively long venom glands, whereas those of the *bibroni* group have glands that are confined to the head region. However, much more information on the SRTXs and their gene structure in the different species of *Atractaspis* is required in order to determine whether the organization of these genes is in correlation with the subgrouping of *Atractaspis*.

IV. TOXINOLOGY OF THE SARAFOTOXINS

A. Cardiotoxicity

As mentioned above, the venom of *A. engaddensis* has a prominent cardiotoxic effect. Isolated SRTX-b was examined *in vivo* in anesthetized mice and *in vitro* in Lagendorff's preparations and on rat and human cardiac muscle preparations (Wollberg et al., 1988). The effects of the isolated SRTXs on the heart were similar to those described for crude *A. engaddensis* venom (Kochva et al., 1993; Lee et al., 1986; Weiser et al., 1984). Changes in the ECG of the anesthetized mice appeared within 10 s of injection of a lethal dose of the toxin (Figure 14.9). They included an increase of amplitude of the R wave and transient elevation of the ST segment. At about 20 to 30 s a prolongation of the PR interval was evident, culminating at about 2 to 3 min in a complete atrioventricular dissociation (AV block). In the intact perfused rat heart SRTX-b induced a rapid bradycardia followed by an AV block. A reduced coronary perfusion was observed, simultaneously with the changes in the electrical activity, implying vasoconstriction of the coronary vessels. A positive inotropic response induced by the toxin could be observed in the Lagendorff system as well as with rat muscle preparations. These results may suggest that SRTX-b induces three, apparently separate, effects on the heart: coronary vasoconstriction, a

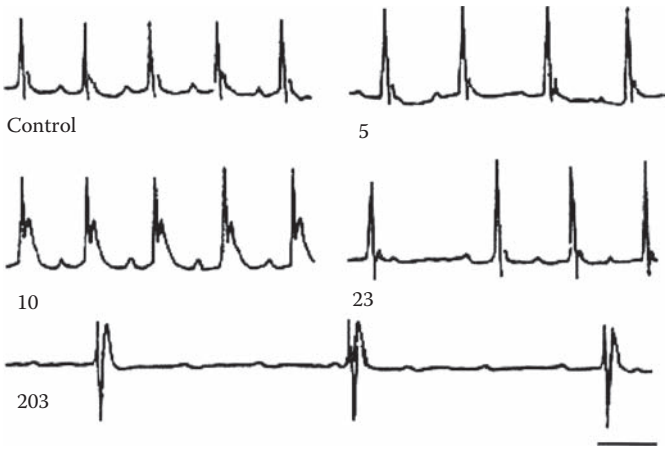

FIGURE 14.9 Effect of SRTX-b on the ECG of anesthetized mice. Selected samples of continuous recording following intravenous administration of a lethal dose. Numbers below the tracing indicate time (seconds) following the toxin administration. Scale: 0.1 s.

TABLE 14.3
Toxicity of SRTXs and ET-1

Peptide	LD_{50} (ng/g body weight)[a]
A. engaddensis venom	75
SRTX-a	10
SRTX-b	10
SRTX-c	300
SRTX-d/e	>2,000
SRTX-m	20
ET-1	15

[a] i.v. LD_{50} in ICR 20 g mice.

direct effect on the conducting system of the heart, and a positive inotropic effect (Wollberg et al., 1988).

SRTX-m (from *V. m. microlepidota*) has a toxicity comparable to that of SRTX-b (Table 14.3). The effects of SRTX-m on the ECG in mice were similar to those induced by SRTX-b; however, the latency period before the disturbances of the ECG were visible were much longer in comparison to those induced by SRTX-b (Hayashi et al., 2004).

The effects of SRTX/ET peptides have been examined in several nonmammalian systems. Zigdon-Arad et al. (1992) studied the effects of SRTX-b on isolated heart preparations of fish (*Tilapia* and *Torpedo*), toad, lizard (*Agama*), and water snake. The toxin reduced the rate of spontaneous contraction in the *Torpedo* and lizard preparations at 0.05 μg/ml, whereas much higher concentrations of SRTX-b were required for a similar effect in the *Tilapia* and water snake preparations. ECG deteriorations that led to cardiac arrest were also evident in *Agama* after administration of 15 μg SRTX-b, whereas in toad higher doses of SRTX-b induced only transient disturbances in the ECG.

Interestingly, administration of ET-1 (1 μg/kg) into anesthetized rats induced comparable electrocardiographic deteriorations (Yorikane and Koike, 1990), which included a transient elevation of the ST segment that coincided with maximal hypertension. Indeed, we have shown that ET-1 has a comparable lethal capacity in mice (Table 14.3) and affects the ECG in mice at doses similar to those of SRTX-b (Bdolah et al., 1989a).

These results indicate that the SRTXs and ETs activate similar cellular targets (see below). Thus, the main difference between these two peptide subfamilies is their concentrations in different tissues and animals. The concentration of the SRTXs in the venom glands of *A. engaddensis* is about five orders of magnitude higher than the concentration of endothelin in the inner medulla of the rat kidney (9 pg/mg wet tissue), in which the highest concentration of ET-1 was found (Kitamura et al., 1989). It may thus be suggested that the ETs evolved, at least in most vertebrates, as regulatory peptides, which are needed in trace quantities. In the venom gland of *Atractaspis*, however, multiple replication of a gene (see above) of a common ancestor dramatically increased the production of SRTXs, resulting in the yield of "toxic concentrations" of the peptides in the *Atractaspis* venom gland.

B. Effects of SRTXs on Smooth Muscle Systems

Yanagisawa et al. (1988), in their first report on the isolation of endothelin, indicated that this peptide is the most potent vasoconstrictor. Our *in vivo* and *in vitro* studies on the cardiotoxic effects of the SRTXs (see above) also indicated that SRTX-b and other SRTXs are potent vasoconstrictors (Kochva et al., 1993). In order to further explore the effects of the SRTXs on smooth muscle systems, we examined the induced contraction or motility by these peptides in three isolated systems. As depicted in Figure 14.10a–c, cumulative application of SRTX-b to isolated segments of the aorta, uterus, and ileum induced a dose-dependent increase in contraction. Maximal effects were attained at around 10^{-7} M of the toxin (Wollberg et al., 1989, 1991, 1992). The patterns of the vasoconstriction effect of SRTX-a, -b, and ET-1 were similar. SRTX-d/e, which is practically nontoxic to mice, showed very low contraction efficacy and potency in the rabbit aorta system (Bdolah et al., 1989b). SRTX-c, however, which has a low toxicity, was much less effective as a vasoconstrictor, and at high concentrations a prolonged relaxation was evident (Wollberg et al., 1989). In this context it is interesting to note that intravenously injected ETs induce an initial decrease in the systemic blood pressure, which is followed by an extremely prolonged pressor response. ET-1 and ET-2 are

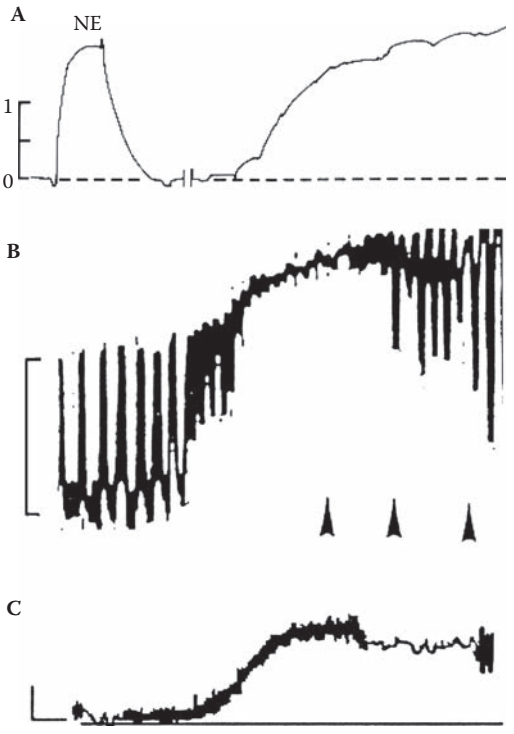

FIGURE 14.10 Isometric responses of isolated smooth muscle system to cumulative applications of SRTX-b. (Modified from Wollberg et al., 1989, 1991, 1992.) (A) Response of a rabbit aortic strip after testing the effect of 6 μM norepinephrine (NE, first trace). (B) Response of a segment of rat uterus at the proestrous stage. Vertical scale, tension force 4g. Arrowheads, washouts. (C) Response of a guinea pig ileal segment. Vertical scale, tension force 0.5 g.

much more potent than ET-3 as pressor agents, whereas ET-3 is much more potent as a vasodilator (for review, see Kanaide, 1996).

In the isolated uterine segments SRTX-b evoked three apparent effects (Figure 14.10b): (1) a gradual increase in the peak tension of the spontaneous rhythmic contraction, (2) a suppression of the relaxation phase of rhythmic contraction, and (3) an increase in the rate of these contractions. The response elicited in the isolated ileum segments was mainly a concentration-dependent increase of the basal tone, with some increase in the amplitude of the spontaneous rhythmic contractions, but their rate was not affected (Figure 14.10c).

Olsen et al. (1991) and Poder et al. (1991) investigated the effects of ET-1 on nonmammalian cardiovascular systems. Profound increases in the tension of arterial preparations were demonstrated in catfish and trout. The recent highly interesting report by Borgheresi et al. (2006) demonstrates *in vivo* and *in vitro* effects of SRTX/ET peptides on the cardiovascular system of *Bothrops jararaca*. SRTX-b and ET-1 induced comparable cumulative concentration response in isolated segments of snake aorta. Both SRTX-c and ET-3 showed lower efficacy and potency.

V. SRTX/ET RECEPTORS AND THEIR ROLE IN TRANSMEMBRANE SIGNALING

The use of ^{125}I-labeled SRTX-b made it possible to identify and characterize high-affinity binding sites (K_d = 3–4 nM, B_{max} ~ 100 fmol/mg protein) in heart and brain preparations. These earlier experiments also showed that the SRTXs are potent activators of phosphoinositide hydrolysis (Kloog et al., 1988). Competition binding experiments between the labeled SRTX-b and other SRTXs, as well as with ET-1 and ET-3, indicated the existence of SRTX/ET receptor subtypes (Kloog et al.,

1989). Indeed, the first reports of the cloning and characterization of the genes encoding ET receptors appeared simultaneously 2 years after the first discovery of the ET by Yanagisawa et al. (1988). The first ET receptor, designated ET_A, was cloned from bovine lung cDNA library and expressed in *Xenopus* oocytes. ET_A showed high selectivity to ET-1 and ET-2, whereas the affinity of ET-3 to this receptor was much lower (Arai et al., 1990). The second receptor subtype, designated ET_B, was cloned from rat lung and then expressed in COS-7 cells. This receptor subtype showed similar affinities to ET-1, -2, and -3 (Sakurai et al., 1990). Hydropathicity analysis indicated that both receptors belong to the family of heptahelical G-protein-coupled receptors. Human cDNAs of ET_A and ET_B, which show considerable homology, predict 427 and 442 amino acids, respectively, with the most conserved regions occurring within the transmembrane domains (Haendler et al., 1992). Of the SRTXs, SRTX-c usually binds with high affinity to the ET_B receptor subtype and is used as a selective ET_B agonist (Williams et al., 1991). On the other hand, SRTX-b, like ET-1, does not distinguish between these two receptor subtypes. SRTX-c is a very poor vasoconstrictor (Wollberg et al., 1989). ET_B receptors are widely distributed throughout the vascular tissues, mediating vasodilation (Kanaide, 1996). ET_B receptors are also present in many nonvascular tissues, including the lung, where they mediate bronchial smooth muscle contraction (e.g., Noguchi et al., 1993). A third subtype of ET receptor, ET_C, which is specific for ET-3, was described in the dermal melanophores of *Xenopus laevis* (Karne et al., 1993). This receptor mediates induction of pigment movement. An ET_A subtype receptor was cloned from the South American snake *Bothrops jararaca* (Borgheresi et al., 2006), which appears to be the predominant one in the vascular system. ET_A-selective antagonists, such as BQ-123 (Nambi et al., 1994), potently inhibit the vasoconstriction response. Evidence for the presence of ET_A receptors in the vascular system of a colubrid snake was recently presented (Mesquita et al., 2008).

Since the discovery of ET in cultured porcine endothelial cells, ETs have been identified and found to be active in a variety of vertebrate tissues (for a review, see Masaki, 2001). The densities of the SRTX/ET receptors and their subtle pharmacological properties may vary between different animals and tissues. For example, binding studies in the mongoose, which is resistant to *A. engaddensis* venom and SRTX-b, revealed SRTX-specific binding in brain and cardiovascular preparations. However, SRTX-b failed to induce contraction in aortal preparations of the mongoose, whereas ET-1 did induce contraction in the same preparation (Bdolah et al., 1997). It was suggested that the SRTX/ET receptors in the mongoose have some structural features that enable them to differentiate between the two peptides. Among other examples of different effects of SRTX/ET peptides are the effects of SRTX-b and ET-1 on the saphenous vein (Maguire et al., 1996). Binding studies with the two isopeptides, using membrane preparation, revealed similar affinities, with a difference in their maximal binding. This discrepancy was attributed to the existence of two populations of receptors. Another example is the difference in the affinities of SRTX-c in human and rat left ventricle, whereas no such difference was found for ET-3 (Russell and Davenport, 1996). The above-mentioned examples of pharmacological studies with the different SRTX/ET isopeptides, as well as many others (for a review, see Henry and Goldie, 2001), raised the possibility that more than the two (or three) conventional endothelin receptor subtypes exist.

The ET/SRTX receptors are able to activate different signal cascades, including phospholipase A_2, phospholipase C, and phospholipase D (Abdel-Latif et al., 1996), as well as to increase production of cyclic nucleotides and other cell signaling systems (for review, see Simonson, 2001). The different signal responses have been attributed to differences in the receptor activation, which probably occur at the level of ligand-receptor-G-protein coupling (see Sokolovsky and Shraga-Levine, 2001, and references therein). In rat atrial slices, ET-1 stimulated or inhibited cAMP production, depending on its concentration. SRTXs showed a different pattern of behavior. ET-1 and SRTX-b also modulated the guanylate cyclase pathway. The stimulation or inhibition of cGMP production was also dependent on peptide concentration. Interestingly, the stimulation of cGMP production via the NO pathway or the CO pathway was found to be different in different tissues and was activated specifically by the different SRTX/ET peptides that interacted with different receptor subtypes. A study

by Shraga-Levine and Sokolovsky (2000) examined the interaction between different SRTX/ET peptides and ET_A or ET_B receptor subtype and their coupling with different G-proteins. It was concluded that upon binding of the peptide agonist with a specific receptor subtype, the appropriate G-protein activates a specific intracellular signal pathway.

VI. CONCLUDING REMARKS

The discovery of the ETs and the expanded research on the structure, metabolism, and pharmacology of these peptides have stimulated a worldwide interest among the biomedical scientific community and the pharmaceutical industry. Isolation of the highly potent snake toxins, the SRTXs, revealed them to possess a remarkable similarity in structure and pharmacology to the ETs. However, whereas the vast research on ETs over a wide spectrum of biomedical fields has supplied a wealth of information on the gene structure, biosynthesis, and pharmacology of the ETs, research on the SRTXs has been rather limited. Of the fifteen or sixteen *Atractaspis* species, the producers of the SRTXs, our local species, *A. engaddensis*, has been most intensively investigated. The limited information on the structures of SRTX-like peptides that were isolated from three other species has provided some idea of the variability of these peptides. cDNAs were isolated from three species; the structures of two of these indicate that their SRTX genes have a polycistronic structure, which is different from the gene organization of the ETs.

Studies on the venom glands of the different *Atractaspis* species and their venoms should provide us with information on the variety of the SRTX-like peptides produced by these species. Their structure and pharmacological properties, including their interactions with the known SRTX/ET receptor subtypes or other subtypes of receptors, may even provide us with a source of natural antagonists of SRTX/ET peptides. The different species of *Atractaspis* have been divided into two groups (see Underwood and Kochva, 1993), on the basis of morphological characters. It will be interesting to determine whether this grouping is also consistent with the venom gland structure and the profile of the secretory products of these snake species.

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15 Fasciculins
Toxins from Mamba Venoms That Inhibit Acetylcholinesterase

Alan L. Harvey

CONTENTS

Fasciculins are small proteins found in the venoms of mambas (*Dendroaspis*; family Elapidae). Three sequences have been determined for fasciculins 1–3: they have sixty-one amino acid residues in a single polypeptide chain that is held together in a three-fingered shape by four disulfide bridges. The fasciculins inhibit acetylcholinesterase with high (picomolar) potency and selectivity by binding to the peripheral anionic site of the enzyme. The toxins have been extensively studied as probes for the enzyme, and they have been used as pharmacological tools to inhibit acetylcholinesterase selectively.

I. INTRODUCTION

Venoms of mambas (*Dendroaspis*; family Elapidae) contain several components that do not seem to occur in venoms of other snakes, including those of other elapid snakes. These include the potassium channel-blocking dendrotoxins (Harvey, 2001; Harvey and Robertson, 2004) and the calcium channel-blocking calciseptine (de Weille et al., 1991) and calcicludine (Schweitz et al., 1994). Mamba venoms also contain unique polypeptide toxins that inhibit the activity of acetylcholinesterase—the fasciculins, which are the subject of this chapter.

Three fasciculins have been isolated, sequenced, and studied to a greater or lesser extent: fasciculins 1 and 2 from the venom of the eastern green mamba, *Dendroaspis angusticeps* (Viljoen and Botes, 1973; Rodríguez-Ithurralde et al., 1983), and fasciculin 3 (also called toxin C) from black mamba, *Dendroaspis polylepis* (Joubert and Taljaard, 1978), and western green mamba, *Dendroaspis viridis* (Marchot et al., 1993), venoms. From isolation studies, it is thought that other fasciculins are present in mamba venoms, although these components have not yet been characterized (Harvey et al., 1984).

As with other components from mamba venoms (Harvey et al., 1984), the fasciculins were isolated and their sequences determined before their biological activity was characterized. Early studies with whole venom and venom fractions from *Dendroaspis angusticeps* indicated that some components might enhance muscular activity (Osman et al., 1973; Viljoen and Botes, 1973), but it was several years later before the anti-acetylcholinesterase activity was noted (Rodríguez-Ithurralde

et al., 1981). The name *fasciculin* was introduced because an easily observed effect after injection of the peptides into mice is the extensive muscular fibrillations or fasciculations (Viljoen and Botes, 1973; Rodríguez-Ithurralde et al., 1983).

II. CHEMISTRY AND STRUCTURE-ACTIVITY RELATIONSHIPS

Fasciculins have sixty-one amino acid residues in a single polypeptide chain that is cross-linked by four disulfide bonds. The fasciculins belong to the large family of three-fingered peptides (Harvey, 2006). They are basic molecules with a net charge of +4. The three well-characterized fasciculins are very similar in amino acid sequence. Fasciculin 2 differs from fasciculin 1 in only one position (Tyr instead of Asp at position 47), while fasciculin 3 has four differences from fasciculin 1: Ile for Met at position 2, Lys for Thr at position 15, Asp for Asn at position 16, and Tyr for Asn at position 47.

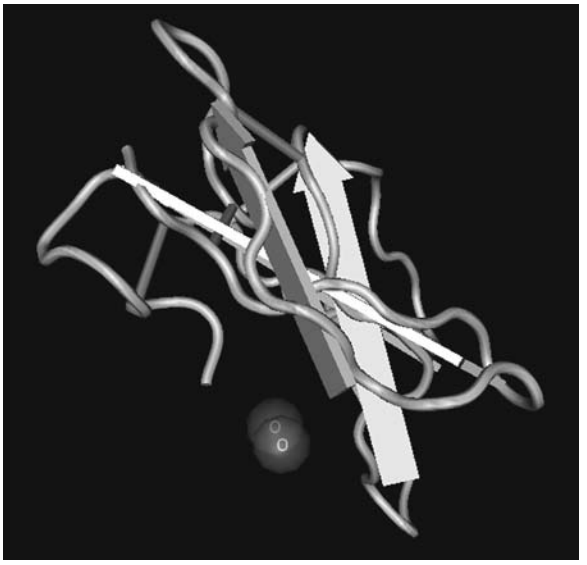
The crystal structures of fasciculin 1 and fasciculin 2 have been solved (le Du et al., 1992, 1996), and it was suggested that parts of the protein are extremely flexible, which might contribute to the tight binding of the toxins to the enzyme. Molecular dynamics simulations with fasciculin 2 (Figure 15.1A) indicated that the tips of loops 1 and 2 are flexible, suggesting that these regions might be important for binding to acetylcholinesterase (Baker et al., 1999).

Loop 2 in fasciculins differs from that in structurally related α-neurotoxins (which bind to the receptors for acetylcholine) in having six positively charged amino acids. Two residues in particular (Arg27 and Lys32) are at the tip of loop 2. Fasciculins also differ from other three-finger toxins in having Arg at position 11. It can be speculated that the side chains of these conserved basic residues may be important for the interaction with acetylcholinesterase. However, fasciculins also have another cluster of positive side chains on the opposite face of the molecule: those of Arg24, Arg37, Lys51, and Lys58.

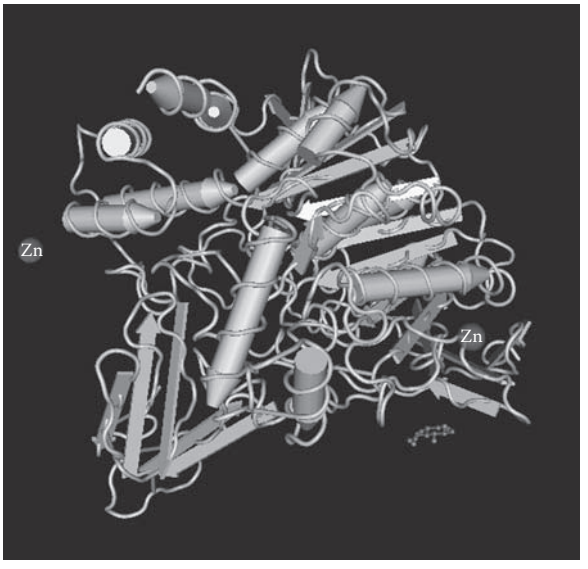
Selective chemical modifications of the positively charged residues on fasciculin 2 have been carried out in order to probe which are important for activity (Cerveñanský et al., 1994, 1995). Acetylation of individual Lys residues was achieved, and the derivatives were found to have lost 60 to 70% of their anti-acetylcholinesterase activity. However, there were some changes to the three-dimensional structure of some of the derivatives, as revealed by circular dichroism (CD) spectra. The Lys25 derivative appeared to retain the native structure, suggesting that this residue should be involved in binding to the enzyme. Modification of the guanidine functional groups of some Arg residues was also successful. The Arg11 and Arg27 derivatives had normal CD spectra but had lost 70 to 80% of their activity against acetylcholinesterase (Cerveñanský et al., 1995). These three residues (Arg11, Lys25, and Arg27) are on the same side of the three-dimensional structure of fasciculin 2, suggesting that they might be part of the interacting surface of the toxin for binding to acetylcholinesterase.

Such studies are necessarily limited to chemically accessible residues, and the addition of the modifying group may disrupt the native structure or introduce steric hindrances. They can be extended through site-directed mutations of individual residues, as has been done with fasciculin 2 (Marchot et al., 1997). Recombinant fasciculin 2 was transiently expressed in Chinese hamster ovary cells and fourteen mutants prepared. Surprisingly, replacement of Arg11 by Gln led to an increase in inhibitory activity, in contrast to what was found after chemical modification of Arg11. The introduction of a bigger side chain in the chemically modified version may have disrupted binding through steric hindrance. Mutation of Lys 25 with Leu had very little effect on inhibitory activity, but when Arg27 was mutated to Trp there was a 50-fold loss of activity. However, Marchot et al. (1997) noted that additional changes to shape and size of the binding site may have been introduced with such a dramatic substitution.

Site-directed mutagenesis provides the ability to probe residues that are not easy to modify by direct chemical reaction. In the study of Marchot et al. (1997), major changes in the ability to inhibit acetylcholinesterase were found to result from substitution of Pro residues at positions 30 and 31—both these residues are at the tip of loop 2. Two other mutations in loop 2 (Arg24Thr and



(A)



(B)

FIGURE 15.1 (A) Ribbon diagram of fasciculin 2 from eastern green mamba (*Dendroaspis angusticeps*) venom. Structure is from the NCBI Structure Molecular Modeling Database (MMDB) and was visualized using Cn3D software (Chen et al., 2003) at the NCBI site (http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=49131). (B) Mouse acetylcholinesterase–fasciculin 2 complex, also from NCBI database and based on the crystal structure determined by Harel et al. (1995).

Met33Ala) also resulted in about tenfold loss of activity. Mutations of residues Thr 8 and 9 to Ala actually increased the inhibitory activity of fasciculin 2 (Marchot et al., 1997), although modeling studies focused on loop 1 suggested that Thr 8 would be critical to the binding interaction (Wang et al., 2005, 2006). Further insights into critically important residues in fasciculins would be gained from x-ray determinations of the structures of fasciculin co-crystallized with acetylcholinesterase (see Section III).

Attempts were also made to probe the binding residues of fasciculins by means of synthetic peptides that resembled one or another of the loops in native fasciculins. Loop 2 mimics retained some activity (Falkenstein and Peña, 1997), but at about 10,000-fold lower activity, indicating the critical importance of the precise three-dimensional structure of the fully folded peptide. Mimics of loop 1 were virtually without activity against acetylcholinesterase (Falkenstein and Peña, 1999). A further attempt to make a synthetic peptide that retained the structural features of the triple-stranded β-sheet of fasciculins (between residues 22 and 27, 34 and 39, and 48 and 53) also resulted in a peptide with only very weak inhibitory activity (Falkenstein et al., 2004).

The importance of the overall three-fingered shape was confirmed by studies on chimeric toxins in which the assumed functionally important parts of loops 1 and 2 of fasciculin 2 were transferred to a short-chain α-neurotoxin (Ricciardi et al., 2000). One such chimeric molecule was only fifteen times less active than the native fasciculin. This study also highlighted the importance of the C-terminal Tyr61 residue for the interaction of fasciculins with acetylcholinesterase.

III. INTERACTIONS WITH ACETYLCHOLINESTERASE

A. Sensitivity to Fasciculins

Acetylcholinesterase (EC 3.1.1.7) is one of a family of cholinesterases that also includes butyrylcholinesterase (EC 3.1.1.8). The primary function of acetylcholinesterase is to hydrolyze the neurotransmitter acetylcholine, and hence it is found in cholinergic synapses in the peripheral and central nervous systems. Butyrylcholinesterase is found in blood plasma and in the brain, although its physiological role is uncertain.

There are many small-molecule inhibitors of acetylcholinesterase, including drugs like neostigmine and galanthamine. Such agents are not particularly selective for acetylcholinesterase. In contrast, fasciculins are much more powerful inhibitors of acetylcholinesterase activity than butyrylcholinesterase activity (for a review, see Cerveñanský et al., 1991). Fasciculins can inhibit mammalian acetylcholinesterases with apparent Kis of below 1 nM, whereas around 1 μM is needed to inhibit butyrylcholinesterases. Avian acetylcholinesterase is exceptionally insensitive to fasciculins, however. Acetylcholinesterase activity is also found in many elapid and some colubrid snake venoms, and it was shown that cobra venom acetylcholinesterase is extremely insensitive to inhibition by fasciculins (Durán et al., 1996). A more extensive study of the acetylcholinesterase activities of different snake venoms confirmed that the enzyme from cobra venoms was resistant to fasciculin, but acetylcholinesterases from *Bungarus* and *Ophiophagus* species were sensitive to fasciculin (Frobert et al., 1997).

On susceptible acetylcholinesterases, fasciculins act as tightly binding noncompetitive inhibitors (Cerveñanský et al., 1991). They bind away from the enzyme's catalytic site at a distinct site on the enzyme, the so-called peripheral anionic site. This type of binding was implied from a variety of biochemical observations (Cerveñanský et al., 1991), but shown more directly by experiments involving chemical modifications to residues in the peripheral anionic site of acetylcholinesterase (Durán et al., 1994). Modification with DPA (N,N-dimethyl-2-phenylaziridinium) made the enzyme one million times less sensitive to fasciculin.

B. Structural Studies

With the ability to crystallize acetylcholinesterase and determine its structure by x-ray techniques (Sussman et al., 1991), it was possible to co-crystallise fasciculin bound to acetylcholinesterase and to determine the toxin's binding site directly. These studies of fasciculin 2 bound to acetylcholinesterase from mouse (Bourne et al., 1995; 2003) and *Torpedo californica* (Harel et al., 1995) confirmed the importance of the loop 2 residues Pro30-Pro31-Lys32-Met33 for binding and the role of loop 1. They also revealed the role of the Tyr residues at positions 4 and 61 of fasciculin. Figure 15.1B

presents the binding of fasciculin with mouse acetylcholinesterase (based on the crystal structure of Harel et al., 1995).

In particular, Met 33 was seen to form hydrophobic bonds with Tyr residues in the peripheral anionic site. Since these residues are absent from avian acetylcholinesterase, this may explain why such enzymes are virtually resistant to inhibition by fasciculins (Harel et al., 1995; Durán et al., 1996).

Similar structural studies have been performed with fasciculin 2 bound to human acetylcholinesterase (Kryger et al., 2000). Binding of fasciculin appears to be very similar to that found with mouse and *Torpedo* enzyme. It is predicted from molecular dynamics simulations that fasciculin 2 undergoes a conformational change on binding to acetylcholinesterase (Bui and McCammon, 2006; Bui et al., 2006).

Since fasciculins are not binding directly at the enzyme's active site, the inhibition of the hydrolysis of acetylcholine by fasciculin has to be indirect. It may be through occlusion of the entry to the active site "gorge," so that acetylcholine cannot reach the active site, or it may be through an allosterically induced change in the conformation of the active site. Analysis of the crystal structures does not resolve these two possibilities. Kinetic studies suggested that fasciculin primarily affected the conformation of the active site (Eastman et al., 1995), although there was also evidence for an additional steric blockade (Rosenberry et al., 1999). Molecular dynamics simulations reveal both a conformational change to the active site residues in mouse acetylcholinesterase and steric effects in the gorge (Tai et al., 2002).

Direct experimental evidence for the changes induced by fasciculin was obtained from time-resolved fluorescence anisotropy with three different fluorescently labeled derivatives of mouse acetylcholinesterase (Boyd et al., 2004); fasciculin was found to have mainly localized effects near its binding site, although additional probes may be needed to get a complete picture of its effects on the enzyme. The active site has been probed by use of electron paramagnetic resonance and a spin-labeled organophosphate compound that bound to the serine in the catalytic site (Sentjurc et al., 1999). The active site residue was still accessible after binding of fasciculin to the peripheral anionic site, although at a much reduced rate. Moreover, there was evidence for fasciculin having caused an allosteric alteration of the active site gorge.

A more detailed kinetic study of the effects of fasciculin binding on the ability of mouse acetylcholinesterase to interact with different substrates and inhibitors was undertaken (Radić and Taylor, 2001). Fasciculin 2 was found not only to slow access of substrates to the catalytic site, but also to have an allosteric effect on the active center. Similar conclusions were reached for the interaction of fasciculin 2 with electric eel acetylcholinesterase (Golicnik and Stojan, 2002).

IV. USE AS EXPERIMENTAL PROBES

Fasciculin could be used as a pharmacological tool to study the effects of localized inhibition of acetylcholinesterase following microinjection into specific brain regions. It does not diffuse far from the site of injection, and its inhibitory effects are long-lasting (see Cerveñanský et al., 1991). Since fasciculin can be labeled with ^{125}I (Marchot et al., 1993; Anglister et al., 1998), it can also be used as a probe for acetylcholinesterase in quantitative autoradiography.

Fasciculin can be fluorescently labeled, and such a molecule was used to study the turnover of acetylcholinesterase at the neuromuscular junction of anaesthetized mice (Martinez-Pena y Valenzuela et al., 2005; Krejci et al., 2006). An activity-dependent insertion of acetylcholinesterase was demonstrated, as well as an acceleration of the loss of acetylcholinesterase following binding of fasciculin. It was noted that the unbinding of fasciculin from acetylcholinesterase in intact muscle took days rather than hours as found with solubilized enzyme (Martinez-Pena y Valenzuela et al., 2005; Krejci et al., 2006). The authors speculate that this might reflect differences between monomeric and multimeric forms of acetylcholinesterase *in vitro* and *in vivo.*

Fasciculin has been used to investigate the role of acetylcholinesterase in outgrowth of neurites from nerve cells (Giordano et al., 2007), an effect thought to be unrelated to the enzyme's hydrolytic

action on acetylcholine. Acetylcholinesterase may act as an adhesion factor, and it was suggested that local charges in the peripheral anionic site may be involved. Neuroblastoma cells transfected to express acetylcholinesterase grow longer neurites than control cells. Treatment of transfected cells with fasciculin caused a significant decrease in both the number of neurites and their length. This was not a reflection of the inhibition of acetylcholinesterase activity, because cells exposed to high concentrations of the reversible anticholinesterase edrophonium (which binds to the catalytic site) did not show decreased neurite growth or number.

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16 Cysteine-Rich Secretory Proteins in Reptile Venoms

William H. Heyborne and Stephen P. Mackessy

CONTENTS

Cysteine-rich secretory proteins (CRISPs) are found in a wide variety of animal tissues, particularly the epididymis of mammals, and most reptile venoms appear to contain at least one isoform. Although several venom CRISPs have been assigned specific functions, many have not, and the biological significance of this family of proteins in venoms is not clear. In many colubrid venoms, they are major protein constituents, suggesting that they have an important role in envenomation. Like many other families of reptile toxins, CRISPs show a highly conserved molecular scaffold, and the sixteen cysteines and eight disulfides they form are 100% conserved. Because they are widely distributed among reptile venoms, show structural conservation, and many have been sequenced, they may have utility as phylogenetic markers. In general, venom CRISP relationships reflect established phylogenetic relationships among the species from which they are derived. By analogy with the three-finger toxins of reptile venoms, which also have a highly conserved protein scaffold stabilized by disulfides, one can expect that venom CRISPs will also show myriad pharmacological activities. Future efforts should be directed toward the elucidation of these activities, as they are an excellent protein family for structure-activity studies.

I. INTRODUCTION

Cysteine-rich secretory proteins (CRISPs) are a widely distributed family of proteins that have been isolated from numerous animal tissues, including reptile venoms (e.g., Hill and Mackessy, 2000; Yamazaki and Morita, 2004; Sanz et al., 2006; Fry et al., 2006), a cone snail venom (Milne et al., 2003), tissues of several other invertebrates (e.g., Schreiber et al., 1997; Ookuma et al., 2003), and a suite of nonreptilian vertebrate tissues. Nonreptilian vertebrate tissues represent the source from which CRISPs were originally discovered and described. The list of tissues containing CRISPs continues to grow, and now includes tissues such as the pancreas, the mammalian male reproductive tract, and the salivary glands (Kierszenbaum et al., 1981; Haendler et al., 1993; Kratzschmar et al.,

1996; Schambony et al., 1998, 2003; Roberts et al., 2006). Although the function of many of the CRISPs is not known, some venom CRISPs have shown a diverse array of biological activities, including inhibition of several types of ion channels (Brown et al., 1999; Nobile et al., 1994, 1996; Yamazaki et al., 2002a; Wang et al., 2005), induction of hypothermia in prey animals (Mocha-Morales, 1990), and specific proteolysis (Milne et al., 2003).

CRISP proteins were originally described from the mammalian male reproductive tract (e.g., Kierszenbaum et al., 1981), but they have since been found in a number of vertebrate tissues such as neutrophils, plasma, salivary gland, pancreas, ovary, thymus, and colon (Kratzschmar et al., 1996, and references therein; Udby et al., 2002, and references therein). Several reviews (Kratzschmar et al., 1996; Udby et al., 2002; Jalkanen et al., 2005) provide summaries of the types and expression locations of nonreptilian CRISPs. In brief, CRISPs were historically placed into one of three primary types: CRISP-1, CRISP-2, and CRISP-3. CRISP-1, also known as sperm-coating glycoprotein DE or acidic epididymal glycoprotein (AEG), is expressed primarily in the epididymis and is thought to be involved in gamete fusion. CRISP-2, also known as *Tpx-1*, is produced in the testes and is likely involved in the interaction of spermatogenic and Sertoli cells. CRISP-3 is the most widely distributed of the three types and has been hypothesized to be involved in innate immune response. A fourth type of nonreptilian CRISP, known as CRISP-4, has been discovered in the mammalian epididymis (Jalkanen et al., 2005; Nolan et al., 2006).

Previously characterized venom CRISPs have shown a wide variety of functionalities, including inhibition of several types of ion channels (Brown et al., 1999; Nobile et al., 1994, 1996; Yamazaki et al., 2002a; Wang et al., 2005), induction of hypothermia in prey animals (Mocha-Morales, 1990), and specific proteolysis (Milne et al., 2003). However, for the majority of CRISPs isolated from reptile venoms, the function and biological role in venom are unknown.

II. DISTRIBUTION OF CRISPs AMONG REPTILE VENOMS

Venom CRISPs have been isolated and characterized from the venoms of all three major venomous snake families (for a review see Yamazaki and Morita, 2004)—Colubridae (Hill and Mackessy, 2000; Mackessy, 2002; Mackessy et al., 2006; Yamazaki et al., 2002b; Fry et al., 2006), Elapidae (Brown et al., 1999, 2003; Osipov et al., 2001, 2005; Yamazaki et al., 2002a, 2002b, 2003; Jin et al., 2003; Wang et al., 2004a, 2004b, 2005; Fry et al., 2006), and Viperidae (Chang et al., 1997; Yamazaki et al., 2002b, 2003; Jin et al., 2003; Wang et al., 2004b; Guo et al., 2005; Shikamoto et al., 2005; Fry et al., 2006)—and from lizard venom and saliva (Mocha-Morales et al., 1990; Fry et al., 2006). Interestingly, some species appear to have a suite of different CRISP isoforms that are expressed simultaneously (Jin et al., 2003; Osipov et al., 2005; Fry et al., 2006). As mentioned in a review by Mackessy (2002), CRISPs are likely more widely distributed in the secretory products of venomous reptiles than has been previously recognized.

III. BIOLOGICAL ACTIVITY OF VENOM-DERIVED CRISPs

Venom CRISPs demonstrated a wide variety of biological effects, including blockage of potassium currents in neurons (Nobile et al., 1994), blockage of calcium currents in neurons (Nobile et al., 1996), binding to cyclic nucleotide-gated ion channels in both photoreceptor cells and olfactory neurons (Brown et al., 1999, 2003; Yamazaki et al., 2002a), blockage of BK_{Ca} channels (Wang et al., 2005), blockage of Ca^{2+} release from the sarcoplasmic reticulum (via ryanodine receptors) of both cardiac and skeletal muscle (Morrissette et al., 1995), blockage of vascular smooth muscle contraction (Yamazaki et al., 2002b), specific proteolysis (Milne et al., 2003—a cone snail venom toxin), induction of hypothermia in prey animals (Mocha-Morales et al., 1990), and lethality (Mocha-Morales et al., 1990). Despite the diversity of functionalities listed above, many venom

CRISPs currently have no identifiable function and apparently no acutely toxic effects (Chang et al., 1997; Yamazaki et al., 2002b; Jin et al., 2003; Osipov et al., 2005; Heyborne and Mackessy, unpublished data).

Several reptile venoms have contained multiple CRISP isoforms (Jin et al., 2003; Osipov et al., 2005; Fry et al., 2006). With this in mind, it would be interesting to examine the venom of *Heloderma horridum* more thoroughly. The CRISP from the venom of this species (helothermine) has very diverse functionalities, including the blockage of multiple types of ion channels (Nobile et al., 1994, 1996) and the induction of hypothermia in prey (Mocha-Morales et al., 1990). Given the diversity of biological activities reported for helothermine, one might hypothesize there to be more than a single CRISP isoform in the venom of this species, each with a slightly different sequence and thus biological activity.

IV. CRISP STRUCTURE

Despite the lack of functional data for many of the CRISPs, the structural chemistry of the venom CRISPs is quite well understood, following the recent crystallization of three such molecules (Guo et al., 2005; Shikamoto et al., 2005; Wang et al., 2005). These venom CRISP structures have shown this family of proteins to have a highly conserved primary, secondary, and even tertiary structure. Due to the high levels of structural conservatism, new members of this family are easily identifiable based on their primary structure alone.

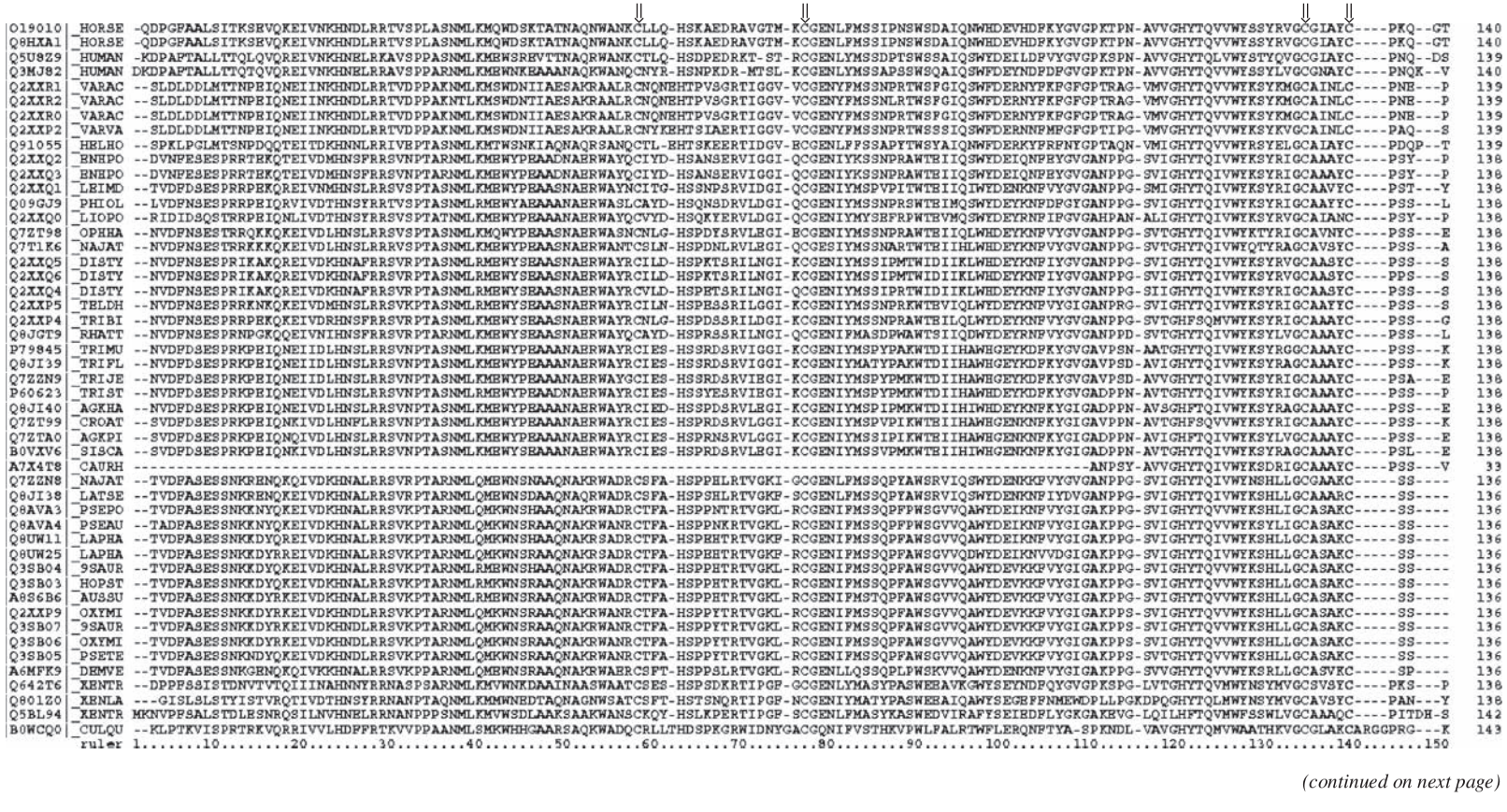
A. Primary Structure

Cysteine-rich secretory proteins were first named because of the large number of cysteine residues found in the C-terminal portion (the cysteine-rich domain—see below). However, because many venom proteins contain numerous cysteines and disulfides, Kini et al. (2001) suggested the name *helveprin* (derived from *helothermine-like venom protein*) to distinguish venom CRISPs from other cysteine-rich venom proteins. Like the phospholipases A_2 (PLA_2s) and three-finger toxins (3FTxs) (see Chapters 5 and 10, this volume), venom CRISPs have a constrained structure defined by sixteen cysteines participating in eight highly conserved disulfide bonds (Table 16.1).

B. Crystallography

The first comprehensive structural analysis of a venom CRISP was conducted on the protein stecrisp from the venom of *Trimeresurus stejnegeri* (Guo et al., 2005). Crystallization of this molecule showed stecrisp to be comprised of two distinct regions connected by a folded hinge or bridge (Figure 16.1). The first of these regions, from the N-terminus of the molecule, was called the PR-1 domain due to its structural homology to the plant pathogenesis group 1 protein family. Known PR-1 crystal structures, including P14a described by Fernández et al. (1997), have shown a characteristic α/β/α sandwich element, which was also seen in stecrisp. The second region, from the C-terminal portion of stecrisp, was called the cysteine-rich domain (CRD) due to the high proportion of cysteine residues in this part of the molecule. Previous work on venom CRISPs had shown a strictly conserved set of sixteen cysteine residues throughout the molecule (Yamazaki and Morita, 2004). Guo et al. (2005) showed these sixteen residues form eight paired disulfide bonds in stecrisp. Three of these were found in the PR-1 domain, two in the hinge or bridge, and three in the cysteine-rich domain. Subsequent crystallization of two additional venom CRISPs (natrin from the venom of *Naja atra*, Wang et al., 2005, and triflin from *Trimeresurus flavoviridis*, Shikamoto et al., 2005) confirmed the presumed structural homology of venom CRISPs, as natrin and triflin also showed the two bridge-connected domains, as well as the α/β/α sandwich element in the PR-1 domain and the eight conserved disulfide bonds.

TABLE 16.1
Alignment (ClustalX v1.81) of 49 Cysteine-Rich Secretory Proteins from Venoms and Other Sources



(continued on next page)

TABLE 16.1 (continued)
Alignment (ClustalX v1.81) of 49 Cysteine-Rich Secretory Proteins from Venoms and Other Sources

Note: Conserved (100%) cysteine residues are indicated by an arrow. The PR-1 domain, hinge region, and cysteine-rich domain (based on stecrisp; Guo et al., 2005) are also indicated. Individual CRISPs are listed by their GenBank accession numbers; see appendix for species names. Sequences were aligned using ClustalX v.1.81 (Thompson et al., 1997).

C. Structure-Function Relationships

The broad distribution and variable functionality of CRISP proteins found in reptile venoms has been equated with those of PLA_2s (Mackessy, 2002), which also have a broad range of functions and taxonomic distribution. Kini (2003) suggested that the range in functionalities seen among the PLA_2s may be due to separate pharmacological sites and active sites on each of the molecules. The pharmacological site is thought to be responsible for cell- or molecule-specific binding in the target tissue, while the active site is responsible for actual catalytic activity. Perhaps a similar model would be applicable to the venom CRISPs. Based on x-ray crystallography with a venom CRISP, Guo et al. (2005) commented on the possibility of "functional separation" between two main domains of the molecule, thus strengthening the argument for a similarity in diverse functionality on a conserved structural scaffold, as seen in the PLA_2s and 3FTxs.

The functional sites of venom CRISPs have not been absolutely determined for any molecule. The wide variety of functionalities, as discussed above, would suggest that there may be multiple functional sites or residues. Yamazaki et al. (2002a) described the CRISP toxins pseudechetoxin and pseudecin (both from Australian elapids) that were found to block cyclic nucleotide-gated ion channels. Based on differences in blocking affinity between these two molecules, the researchers

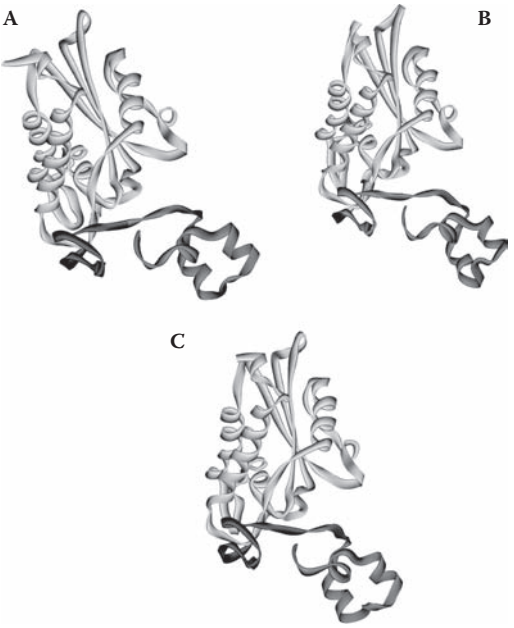


FIGURE 16.1 ***(A color version of this figure follows page 240.)*** Snake venom CRISP structures generated by SWISS-MODEL (Guex and Peitsch, 1997; Schwede et al., 2003). (A) Stecrisp (GenBank/EBI accession number 1RC9A). (B) Triflin (1WVRA). (C) Natrin (1XTAA). Ribbon color is used to differentiate structural domains as described by Guo et al. (2005). Pale grey, N-terminal PR-1 domain; dark grey, bridge region; grey, C-terminal cysteine-rich domain. Note the high degree of structural homology in all three molecules.

contended that a string of basic residues (Lys167, Lys174, Arg175), which were found in pseudechetoxin but were lacking (except for Arg166) in pseudecin, may interact electrostatically with the CNG channels, leading to the observed blockage. Yamazaki et al. (2003) proposed that the region near and including residues 184–189 may be the functional region in snake venom CRISPs found to block smooth muscle contraction (ablomin, triflin, latisemin, piscivorin, and catrin). Based on differences in the binding affinity of these venom CRISPs, residues Phe189 and Glu186 were considered likely candidates for the observed channel blocking activity. Wang et al. (2005) and Guo et al. (2005) both conducted crystallization studies of snake venom CRISPs (natrin and stecrisp, respectively) and noted that previously suggested functional residues (Lys175, Arg176, Glu186, and Phe189—see above) are all part of "exposed solvent loop I" (Wang et al., 2005) ("N-terminal loop"; Guo et al., 2005) in the cysteine-rich domain of the molecules. They suggested that this loop may prove to be the region of interface between CRISPs and other molecules due to the high levels of variability found here (which would help explain the diverse functionalities observed) and its presumed ease of interaction with other molecules because of its exposed conformation. In studies of the only known venom CRISP protease, Milne et al. (2003) suggested that residues Ser80, Glu115, and His130 may be part of a catalytic triad (analogous to those of known serine proteases) due to their proximity and location within an electronegative cleft. Catalytic triads of this type not only act enzymatically, but also likely charge-stabilize the intermediate stages of the molecular substrate (Carter and Wells, 1988).

V. USES IN PHYLOGENETIC HYPOTHESIS TESTING

Due to the wide distribution of the CRISP family among reptilian oral secretions (Mackessy, 2002), including some lizard species that have not previously been considered venomous (Fry et al., 2006), this protein family may be especially useful in the study of venom evolution. Other researchers

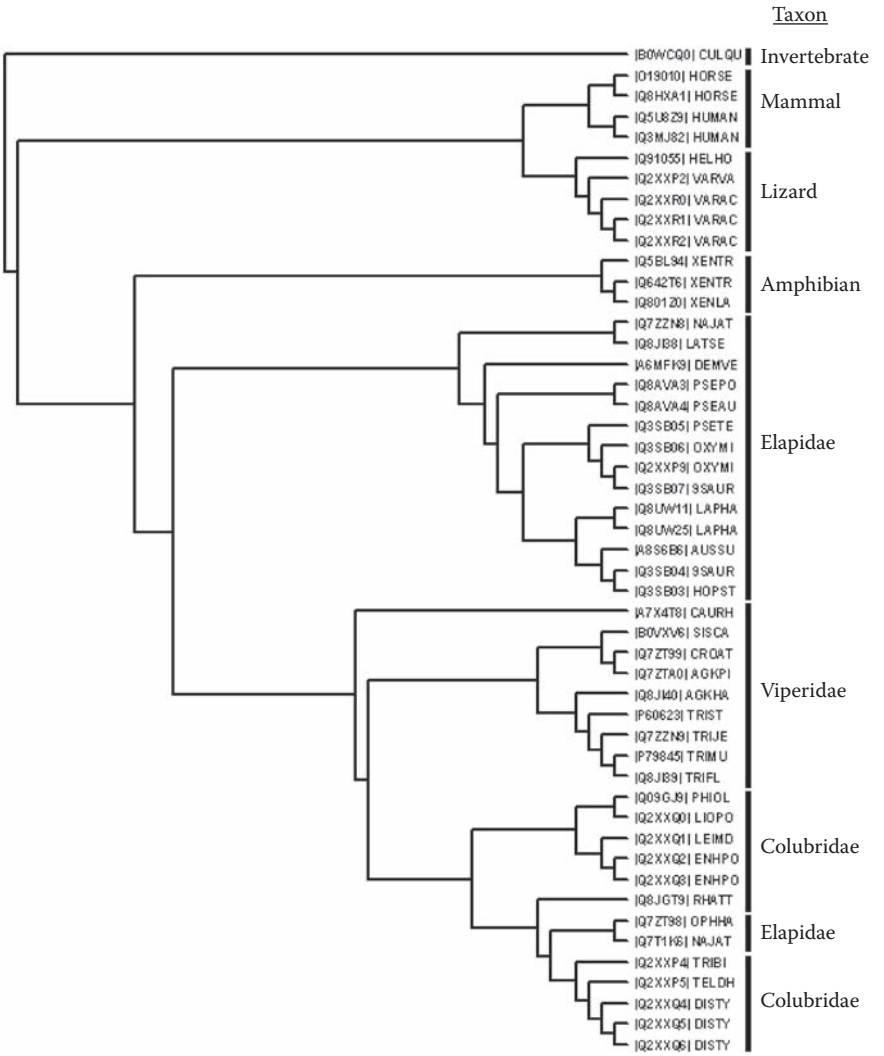


FIGURE 16.2 Phylogram of sequence similarity relationships between venom and other CRISPs. The neighbor-joining tree was drawn using ClustalX v.1.81 alignment (Thompson et al., 1997) and TreeView 1.6.6. Note that toxin clades follow generally accepted taxonomic groups, with the exception of two elapid taxa, which cluster with the Colubridae.

(Yamazaki et al., 2003; Fry and Wüster, 2004) have recognized the potential use of CRISPs and have used CRISP sequence data in phylogenetic analyses. However, the number of CRISP sequences now available has grown significantly. A BLAST search of available sequences (2007) revealed forty-nine CRISP sequences, most of which are derived from reptile venom gland DNA sequences (see Table 16.1 and appendix). These forty-nine sequences were aligned and a neighbor-joining tree was drawn using ClustalX 1.81 and TreeView 1.6.6. (Figure 16.2). In general, sequence similarities follow phylogenetic affinities, with an exception that two elapid taxa (*Ophiophagus* and *Naja atra*) cluster within the Colubridae.

There are two additional reports of CRISP antibody screening of crude venoms. The first of these reports (Yamazaki et al., 2002b) gives few details regarding methodology or diversity of species screened. However, the authors do note the detection of CRISP-like proteins in the venoms of *Agkistrodon blomhoffi*, *Trimeresurus flavoviridis*, and *Laticauda semifasciata* when screened (via

Western blotting or ELISA) with anti-tigrin antibodies. The second study, conducted by the same group (Yamazaki et al., 2003), documents much wider screening (via ELISA) of snake venoms using anti-triflin antibodies. A total of fifteen species were screened, with all but two (*Notechis scutatus* and *Oxyuranus scutellatus*) showing positive reactivity. Of note is the fact that a second *Notechis* species, *N. ater*, was screened as part of the current study and also showed no reactivity toward anti-triflin, but did show positive activity toward anti-tigrin antibodies. Finally, because these fifteen species were screened using ELISA, the results should be interpreted with caution due to the high levels of apparent nonspecific interactions that we have observed.

Yamazaki et al. (2003) also performed a phylogenetic analysis of CRISP sequences known at that time. As this analysis was performed a number of years ago, the data set they utilized was much smaller than the data set presented here. They also utilized a different method (unweighted pair group method) to construct a phylogenetic tree, but their results were similar to those reported here. They reported the identification of a monophyletic viperid clade and a monophyletic elapid clade. One notable exception was the genus *Ophiophagus*, which grouped with the viperids instead of the elapids. Because they only used a single colubrid sequence, from *Rhabdophis tigrinus tigrinus*, it was not possible to make conclusions regarding the taxonomic status of this group.

VI. CONCLUSIONS

There has been some confusion regarding the naming of CRISP venom proteins. The name cysteine-rich secretory protein is not specific enough to identify unambiguously members of this group, as many other families of secreted proteins are also cysteine-rich, such as the three-finger toxins and phospholipases A_2. Some authors (Chang et al., 1997; Jin et al., 2003) have proposed calling venom CRISPs cysteine-rich venom proteins (CRVPs), but this name is no less ambiguous for the same reasons stated above. Kini et al. (2001) advocated the use of the term *helveprins* as an alternative to either of the above. While this name is more descriptive, as it refers to the first venom CRISP isolated (helothermine) and indicates the source (venom), it has not been adopted generally.

Ohno et al. (1998) commented on the "functional prodigality associated with a structural economy" often seen among well-characterized snake venom toxins such as PLA_2s and 3FTxs. With increasing frequency, researchers have noted that venom toxins with very different biological functions often have a remarkably similar protein fold. Evolutionarily, the modification of a stable and biologically successful molecular scaffold for additional or novel functionalities, perhaps via a cassette-like exchange mechanism of specific toxin segments (ASSET; Doley et al., 2008) rather than the evolution of molecules *de novo*, is a more parsimonious mechanism for toxin diversification. It is apparent that CRISP venom proteins will similarly adhere to this model of structural conservatism. Undoubtedly, future studies of CRISP molecules will help to provide answers to structural and functional questions regarding this family of diverse proteins.

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APPENDIX: SPECIES SOURCES OF CRISPs USED IN ALIGNMENTS AND PHYLOGRAM

GenBank accession number, trivial name, and source species are provided for each CRISP.

Invertebrates

B0WCQ0	Catrin	*Culex quinquefasciatus* (southern house mosquito)

Vertebrates

Amphibians

Q642T6	Crisp2 protein	*Xenopus tropicalis* (western clawed frog)
Q5BL94	MGC108118 protein	*Xenopus tropicalis* (western clawed frog)
Q801Z0	Cysteine-rich secretory protein	*Xenopus laevis* (African clawed frog)

Reptiles

Lizards

Q91055	Helothermine precursor (HLTx)	*Heloderma horridum horridum* (Mexican beaded lizard)
Q2XXP2	CRISP-VAR10 (fragment)	*Varanus varius* (lace monitor)
Q2XXR2	CRISP-VAR3 (fragment)	*Varanus acanthurus* (ridge-tailed monitor)
Q2XXR1	CRISP-VAR4 (fragment)	*Varanus acanthurus* (ridge-tailed monitor)
Q2XXR0	CRISP-VAR5 (fragment)	*Varanus acanthurus* (ridge-tailed monitor)

Snakes

Colubridae

Q8JGT9	Tigrin precursor	*Rhabdophis tigrinus tigrinus* (tiger keelback snake)
Q2XXP4	CRISP-TRI1 (fragment)	*Trimorphodon biscutatus* (lyre snake)
Q2XXQ6	CRISP-DIS1	*Dispholidus typus* (boomslang)
Q2XXQ5	CRISP-DIS2	*Dispholidus typus* (boomslang)
Q2XXQ4	CRISP-DIS3	*Dispholidus typus* (boomslang)
Q09GJ9	Cysteine-rich secretory protein precursor (CRISP-PHI1) (CRISP-PHI2)	*Philodryas olfersii* (green snake)
Q2XXQ3	CRISP-ENH1	*Enhydris polylepis* (Macleay's water snake)
Q2XXQ2	CRISP-ENH2	*Enhydris polylepis* (Macleay's water snake)
Q2XXP5	CRISP-TEL1 (fragment)	*Telescopus dhara* (Egyptian catsnake)
Q2XXQ1	CRISP-LEI1 (fragment)	*Leioheterodon madagascariensis* (Malagasy giant hognose snake)
Q2XXQ0	CRISP-LIO1 (fragment)	*Liophis poecilogyrus* (water snake)

Viperidae

A7X4T8	CRISP-Cau1 (fragment)	*Causus rhombeatus* (rhombic night adder)
Q8JI40	Ablomin precursor	*Agkistrodon halys blomhoffi* (mamushi) (*Gloydius blomhoffii*)
Q8JI39	Triflin precursor	*Trimeresurus flavoviridis* (Habu) (*Protobothrops flavoviridis*)
P60623	Cysteine-rich secretory protein precursor (Stecrisp)	*Trimeresurus stejnegeri* (Chinese green tree viper)
P79845	Cysteine-rich venom protein precursor (TM-CRVP)	*Trimeresurus* (*Protobothrops*) *mucrosquamatus* (Taiwan habu)
Q7ZZN9	Cysteine-rich venom protein precursor (TJ-CRVP)	*Trimeresurus* (*Protobothrops*) *jerdonii* (Jerdon's pit-viper)
Q7ZT99	Catrin-1/2 precursor	*Crotalus atrox* (western diamondback rattlesnake)
B0VXV6	Cysteine-rich secretory protein isoform 2	*Sistrurus catenatus edwardsii* (desert massasauga)
Q7ZTA0	Piscivorin precursor	*Agkistrodon piscivorus piscivorus* (eastern cottonmouth)

Elapidae

Q7ZT98	Ophanin precursor (Opharin)	*Ophiophagus hannah* (king cobra)
Q7T1K6	Natrin-1 precursor (cysteine-rich venom protein 1) (NA-CRVP1) (protein G2a)	*Naja atra* (Chinese cobra)
Q8JI38	Latisemin precursor	*Laticauda semifasciata* (broad-banded blue sea snake) (Erabu sea snake)
Q3SB03	Pseudechetoxin-like protein precursor	*Hoplocephalus stephensii* (Stephens' banded snake)
Q8UW11	Cysteine-rich venom protein 2 precursor (CRVP)	*Lapemis hardwickii* (Hardwick's sea snake)
Q8UW25	Cysteine-rich venom protein 1 precursor (CRVP)	*Lapemis hardwickii* (Hardwick's sea snake)
Q3SB04	Pseudechetoxin-like protein precursor	*Notechis scutatus*
Q3SB05	Pseudechetoxin-like protein precursor	*Pseudonaja textilis* (eastern brown snake)
Q8AVA4	Pseudechetoxin precursor (PsTx)	*Pseudechis australis* (mulga snake) (king brown snake)
Q8AVA3	Pseudecin precursor	*Pseudechis porphyriacus* (red-bellied black snake)
Q3SB07	Pseudechetoxin-like protein precursor	*Oxyuranus scutellatus*
Q2XXP9	CRISP-OXY1	*Oxyuranus microlepidotus* (Inland taipan)
Q3SB06	Pseudechetoxin-like protein precursor	*Oxyuranus microlepidotus* (inland taipan)
A8S6B6	CRISP precursor	*Austrelaps superbus* (Australian copperhead)
Q7ZZN8	Natrin-2 precursor (cysteine-rich venom protein 2) (NA-CRVP2) (protein G2b)	*Naja atra* (Chinese cobra)
A6MFK9	Cysteine-rich secretory protein precursor	*Demansia vestigiata* (lesser black whip snake)

Mammals

Q8HXA1	Cysteine-rich secretory protein 3	*Equus caballus* (horse)
O19010	Cysteine-rich secretory protein 3 precursor (CRISP-3)	*Equus caballus* (horse)
Q3MJ82	Cysteine-rich secretory protein 3	*Homo sapiens* (human)
Q5U8Z9	Testis-specific protein TPX1 e isoform (cysteine-rich secretory protein 2, isoform CRA_a) (CRISP2 protein) (testis-specific protein TPX1 d isoform) (testis-specific protein TPX1 b isoform)	*Homo sapiens* (human)

17 Snake Venomics and Disintegrins

Portrait and Evolution of a Family of Snake Venom Integrin Antagonists

Juan J. Calvete, Paula Juárez, and Libia Sanz

CONTENTS

Disintegrins represent a family of polypeptides released in the venoms of Viperidae (vipers and rattlesnakes) by the proteolytic processing of multidomain metalloproteinases, which selectively block the function of β_1 and β_3 integrin receptors. Research on disintegrins not only is relevant for understanding the evolution and biology of viper venom toxins, but also provides information of new structural determinants involved in integrin recognition that may be useful in both basic and clinical research. Here, we describe the application of proteomics ("snake venomics") for the detailed characterization of the toxin content of snake venoms, and review our current view and hypotheses on the emergence and the structural and functional diversification of disintegrins by accelerated evolution and the minimization of the structure of both the duplicated genes (loss of intronic sequences) and the venom expressed proteins (selective loss of disulfide bonds). The role of the composition, conformation, and concerted dynamics of the integrin inhibitory loop and the C-terminal tail in determining the selective inhibition of integrin receptors is discussed.

I. EVOLUTION OF THE ADVANCED SNAKES AND THEIR VENOM SYSTEMS

The suborder of snakes (Serpentes, order Squamata) includes about 3,000 extant species placed in approximately 400 genera and 18 families (cf. http://www.reptile-database.org/). The timing of major events in snake evolution is not well understood, however, owing in part to a relatively patchy and incomplete fossil record (Scanlon and Lee, 2000; Apesteguía and Zaher, 2006). Nevertheless, after more than 100 years of research, the most generalized phylogenetic view is that the group evolved from a family of terrestrial lizards during the time of the dinosaurs in the Jurassic Era, about 200 million years (Myr) ago (Vidal and Hedges, 2005). At the end of the nonavian dinosaurs' reign,

near the Cretaceous-Tertiary boundary 65 Myr ago (Alvarez et al., 1980), the Boidae (the ancestors of boas and pythons) were the dominant snake family on earth. During the Cenozoic Era, advanced snakes (Colubroidea) arose as long ago as the Oligocene epoch (35–25 Myr). Regarded today as typical snakes, they remained a small taxon until biogeographic and climatic events excluded boids from many ecological niches. Colubrids quickly colonized these empty habitats, and this family today comprises over two-thirds of all the living snake species (Greene, 1997).

Colubroidea encompasses Viperidae (30 genera, 230 species of vipers and pit vipers), Elapidae (63 genera, 272 species of corals, mambas, cobras, and their relatives), Atractaspididae (14 genera, 65 species of stiletto snakes and mole vipers), and the polyphyletic Colubridae (290 genera, almost 1,700 species of rear-fanged and "harmless" snakes) (http://www.reptile-database.org). Higher-level relationships of snakes, inferred from nuclear and mitochondrial gene sequences, indicate that vipers diverged from the ancestral boid stock, underwent one of the earliest advanced snake radiations, and developed afterward independently of and in parallel to other advanced snake groups (Heise et al., 1995; Vidal and Hedges, 2002; Vidal, 2002; Vidal et al., 2007). Elapids, on the other hand, represent one of the more recent derivations (Vidal et al., 2007). Noteworthy, the front-fanged venom delivery system appeared three times, independently in Viperidae, Elapidae, and Atractaspididae (Vidal and Hedges, 2002).

The presence of a venom-secreting oral gland is a shared derived character of the advanced (Caenophidia) snakes. All venomous squamates, snakes, and lizards such as the Gila monster and beaded lizard may share a common venomous ancestor (Fry et al., 2006). Given the central role that diet has played in the adaptive radiation of snakes (Greene, 1983), venom thus represents a key adaptation that has played an important role in the diversification of these animals. Venoms represent the critical innovation in ophidian evolution that allowed advanced snakes to transition from a mechanical (constriction) to a chemical (venom) means of subduing and digesting prey larger than themselves, and as such, venom proteins have multiple functions, including immobilizing, paralyzing, killing, and digesting prey. Venoms of snakes in the family Viperidae are produced in paired specialized venom glands located in the upper jaw, ventral and posterior to the eyes (e.g., Mackessy, 1991), and venom is introduced deeply into prey tissues via elongate, rotatable fangs. Viperids and elapids possess the most widely studied types of animal toxins (Meier and White, 1995; Ménez, 2002; Fox and Serrano, 2005a). These snake venoms contain complex mixtures of hundreds of important pharmacologically active molecules, including mineral and organic components (histamine and other allergens, polyamines, alkaloids, etc.), small peptides, and proteins (Markland, 1998; Fry, 1999; Ménez, 2002). The biological effects of venoms are complex because different components have distinct actions and may, in addition, act in concert with other venom molecules. The synergistic action of venom proteins may enhance their activities or contribute to the spreading of toxins.

The major toxic effects of snake venoms in an envenomed animal often manifest as neurotoxic or hemorrhagic disorders. Snakes of the family Elapidae (mambas, cobras, and particularly the Australian venomous snakes) contain venoms rich in three-finger toxins (often neurotoxins), while snakes of the family Viperidae (vipers and pit vipers) typically produce venoms rich in proteins that interfere with the coagulation cascade, the hemostatic system, and tissue repair. Human envenomations by viperids are frequently characterized by clotting disorders, hypofibrinogenemia, and local tissue necrosis (Markland, 1998; Ménez, 2002; Fox and Serrano, 2005a), while neuropathologies often dominate elapid envenomations. Section IV (this volume) contains further details on envenomations and their treatments.

The existence in the same venom of a diversity of proteins of the same family, but differing from each other in their pharmacological effects, reflects an accelerated positive Darwinian evolution of venom constituents. Venom toxins likely evolved from proteins with a normal physiological function and appear to have been recruited into the venom proteome before the diversification of the advanced snakes, at the base of the Colubroid radiation (Vidal, 2002; Fry and Wüster, 2004; Fry, 2005; Fry et al., 2006). Gene duplication followed by functional divergence is a main source of molecular novelty. Gene duplication creates redundancy and allows a gene copy to be selectively expressed in

the venom gland, escaping the pressure of negative selection and evolving a new function through positive selection and adaptative molecular evolution (Lynch, 2007). The occurrence of multiple isoforms within each major toxin family evidences the emergence of paralogous groups of multigene families across taxonomic lineages where gene duplication events occurred prior to their divergence, and suggests an important role for balancing selection (Richman, 2000) in maintaining high levels of functional variation in venom proteins within populations. The mechanism leading to this mode of selection is unclear, but we (Sanz et al., 2006a) have speculated that it may be related to unpredictability, with which a sit-and-wait predator like a rattlesnake encounters different types of prey, each of which is most efficiently subdued with different suites of venom proteins. Thus, to deal with this uncertainty, a variety of proteins are available in their venom at all times to deal with different prey. The selection pressure leading to high levels of variation in venom genes may parallel the selection pressures acting by the birth-and-death model of protein evolution (Nei et al., 1997), which promotes high levels of variation (neofunctionalization) in the genes involved in the vertebrate adaptive immune response. Some examples include those that encode major histocompatibility complex proteins of vertebrates (Hedrick and Kim, 2000) and proteins expressed by host plant defense genes in response to herbivores (Stahl et al., 1999; Bergelson et al., 2001; Tian et al., 2002).

In addition to understanding how venoms evolve, characterization of the protein/peptide content of snake venoms also has a number of potential benefits for basic research, clinical diagnosis, development of new research tools and drugs of potential clinical use, and antivenom production strategies (Ménez et al., 2006). Snakebite is still a serious threat in both developed and underdeveloped countries. Snake envenomation accidents represent a sociomedical problem of considerable magnitude, with about 2.5 million envenomations annually worldwide and more than 100,000 fatalities. Paradoxically, although bites can be deadly, snake venoms also contain components of therapeutic value. The only effective treatment for systemic envenomation is the intravenous administration of an antivenom. Although antivenoms have gone a long way to reduce mortality, many of them do not achieve optimal protective effects. This is in part due to the fact that conventional antivenoms are prepared from sera of animals that are hyperimmunized with whole venom. The resulting polyclonal antisera include numerous antibodies with specificities not confined to the toxic target molecules. Hence, knowledge of the toxin composition of venoms could be used to generate immunization protocols to elicit toxin-specific antibodies with greater specificity and effectiveness than conventional systems.

On relatively rare occasions, toxins represent potential therapeutic agents that have been used for the treatment of pathophysiological conditions in homeopathy, in folk remedies, and in Western and Chinese traditional medicine (Harvey et al., 1998; Koh et al., 2006). Moreover, the fact that members of a single family show remarkable structure similarity but differ in their biological targeting makes them valuable biotechnological tools for studying physiological processes, and poses exciting challenges in delineating structure-function correlations. In order to explore the putative venom components, several laboratories have recently carried out transcriptomic analyses of the venom glands of viperid (*Bothrops insularis*: Junqueira-de-Azevedo and Ho, 2002; *Bitis gabonica*: Francischetti et al., 2004; *Bothrops jararacussu*: Kashima et al., 2004; *Bothrops jararaca*: Cidade et al., 2006; *Agkistrodon acutus*: Qinghua et al., 2006; *Echis ocellatus*: Wagstaff and Harrison, 2006; *Lachesis muta*: Junqueira-de-Azevedo et al., 2006; and *Sistrurus catenatus edwardsii*: Pahari et al., 2007) and colubrid (*Philodryas olfersii*: Ching et al., 2006) snake species. Transcriptomic investigations provide catalogs of partial and full-length transcripts that are synthesized by the venom gland. However, transcriptomes include translated and nontranslated mRNAs, transcripts encoding nonsecreted, housekeeping, and cellular proteins in addition to toxin precursor genes. Moreover, toxins may undergo posttranslational processing, and this event will not be evident in a transcriptomic analysis. Thus, outlining the full map of native toxins that actually constitute the venom requires a combined biochemical and proteomic approach. To address the need for detailed proteomic studies of snake venoms, we have initiated a project whose long-term goal is a detailed analysis of viperid venomes.

II. SNAKE VENOMICS

Comprehensible studies of their toxin repertoire may contribute to a deeper understanding of the biological effects of the venoms, and may also serve as a starting point for studying structure-function correlations of individual toxins. For a detailed characterization of the toxin content of snake venoms, the approach that we have coined "snake venomics" (Juárez et al., 2004) (Figure 17.1) starts with the fractionation of the crude venom by reverse-phase HPLC, followed by the initial characterization of each protein fraction by a combination of N-terminal sequencing, SDS-PAGE, and mass spectrometric determination of molecular masses and cysteine (SH and S-S) content (Figure 17.1a–d). Protein fractions showing a single electrophoretic band, molecular mass, and N-terminal sequence can be straightforwardly assigned to a known protein family, indicating that representative members of most snake venom toxin families are present among the 923 viperid protein sequences deposited to date in the SwissProt/TrEMBL database (Knowledgebase release 10.0 of March 2007). Protein fractions showing heterogeneous or blocked N-termini were analyzed by SDS-PAGE, and the bands of interest are subjected to in-gel tryptic digestion. The resulting tryptic peptides are analyzed by MALDI-TOF mass fingerprinting, followed by amino acid sequence determination of selected doubly and triply charged peptide ions by collision-induced dissociation tandem mass spectrometry (CID-MS/MS) (Figure 17.1e, f). As expected from the rapid amino acid sequence divergence of venom proteins evolving under accelerated evolution (Ménez, 2002; Ohno et al., 1998; Ogawa et al., 1995, 2005), with a few exceptions, neither the tryptic mass fingerprints nor the product ion spectra match any known protein using the ProteinProspector (http://prospector.ucsf.edu) or MASCOT (http://www.matrixscience.com) search programs (Figure 17.1g). The CID-MS/MS spectra must therefore be manually interpreted, and the deduced peptide ion sequences submitted to BLAST (http://www.ncbi.nlm.nih.gov/BLAST) sequence similarity searches (Figure 17.1h). Although the lack of snake genome sequences is a serious drawback for the identification of venom proteins by MALDI-TOF mass fingerprinting, MS/MS fragmentation usually yields sufficient amino acid sequence information derived from almost complete series of sequence-specific b- or y-ions to identify unambiguously a homologous protein in the current databases. The outlined snake venomics approach allows us to assign unambiguously all the isolated venom toxins (representing >0.05% of the total venom proteins) to known protein families. In addition, comparison of the apparent molecular masses of proteins run under nonreducing and reducing SDS-PAGE conditions (Figure 17.1c) provides valuable information regarding the aggregation state and subunit composition of the toxins.

To date, we have applied snake venomics for elucidating the protein composition of the venoms from the North American rattlesnakes *Sistrurus miliarius barbouri* (Juárez et al., 2004; Sanz et al., 2006a), *Sistrurus catenatus catenatus*, *tergeminus*, and *edwardsii* (Sanz et al., 2006a), *Crotalus atrox*, and the copperhead *Agkistrodon contortrix contortrix* (manuscript in preparation); the Tunisian vipers *Cerastes cerastes cerastes*, *Cerastes vipera*, and *Macrovipera lebetina transmediterranea* (Bazaa et al., 2005); the African species *Bitis arietans* (Ghana) (Juárez et al., 2006a), *Bitis gabonica gabonica* (Calvete et al., 2007a), *Bitis gabonica rhinoceros*, *Bitis nasicornis*, *Bitis caudalis*, and *Echis ocellatus*; and the South American bushmaster (*Lachesis muta*) (manuscripts in preparation). As a whole, our results show that, in spite of the fact that viperid venoms may contain well over one hundred protein components (Serrano et al., 2005), venom proteins belong to only a few protein families (Table 17.1), including enzymes (serine proteinases, Zn^{2+}-snake venom metalloproteases, L-amino acid oxidase, group II PLA_2) and proteins without enzymatic activity (disintegrins, C-type lectins, natriuretic peptides, myotoxins, cysteine-rich secretory protein (CRISP) toxins, nerve and vascular endothelium growth factors, cystatin, and Kunitz-type protease inhibitors). The molecular scenario, however, is more complex because this primary classification of the venom proteins does not take into consideration the existence of

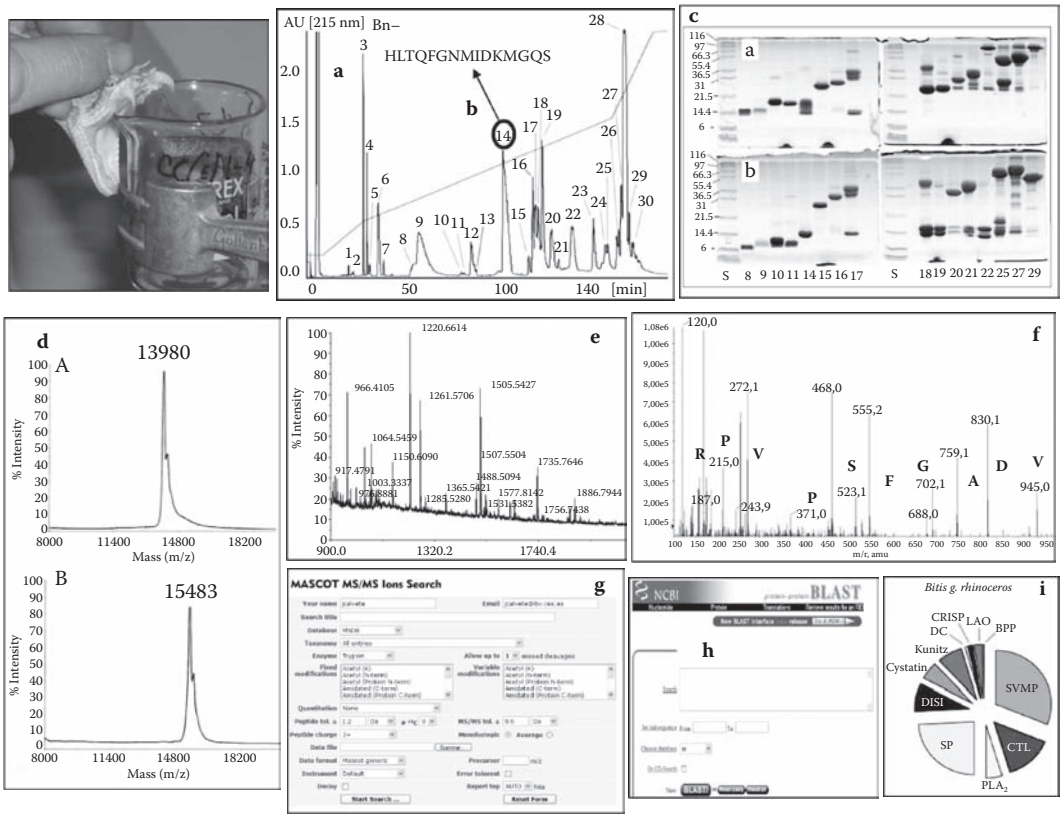

FIGURE 17.1 ***(A color version of this figure follows page 240.)*** Snake venomics. Schematic representation of the steps typically followed in a snake venomics project. (a) Reverse-phase chromatographic separation of the venom proteins. (b) N-terminal sequencing of the isolated protein fractions. (c) SDS-PAGE of the RP-HPLC isolated proteins run under nonreduced (upper panel) and reduced (lower panels) conditions. (d) Determination of the molecular masses of the proteins isolated in (a), and quantitation of their sulfydryl group and disulfide bond contents. (e) MALDI-TOF mass fingerprints of in-gel digested protein bands excised from SDS-polyacrylamide gels run as in (c). (f) Amino acid sequence determination by nanospray-ionization CID-MS/MS of doubly and triply charged tryptic peptide ions obtained as in (e). (g) Database searches using MS/MS spectra in MASCOT. (h) MS/MS-derived amino acid sequence through Basic Local Alignment Search Tool (BLAST). (i) Summary of the relative amounts of toxin families using a pie chart representation (see also Table 17.1).

multiple isoforms and subfamilies within the major toxin families that exhibit a wide range of pharmacological effects (see below and other chapters in this volume). Furthermore, snake venoms depart from each other in absolute composition or the relative abundance of specific toxins (Table 17.1). The availability of detailed proteomic information on individual proteins makes possible detailed estimates of the similarity and differentiation of the venom proteomes of different taxa, which are then useful for understanding the biology and ecology of snakes and in revealing broad-scale evolutionary patterns.

Since the late 1980s, our laboratory has been particularly interested in the biological effects and the structural and functional evolution of disintegrins, a family of antagonists of integrin receptors exclusively found in viperid venoms since its discovery 20 years ago in Prof. Stefan Niewiarowski's laboratory (Huang et al., 1987). Below, we outline an overview of the evolution of structure and function of this family of snake venom integrin antagonists.

TABLE 17.1
Overview of the Relative Occurrence of Proteins (in Percentage of the Total HPLC-Separated Proteins) of the Toxin Families in the Venoms of Viperid Snakes

	Species															
	SCC	**SCT**	**SCE**	**SMB**	**CCC**	**CV**	**MLT**	**BA**	**BGG**	**BGR**	**BN**	**BC**	**EO**	**LM**	**CA**	**ACC**
Protein Family	**% of Total Venom Proteins**															
Disintegrins																
Long	—	—	—	—	—	—	—	17.8	—	—	—	—	—	—	—	—
Medium	2.5	4.2	0.9	7.7	—	—	—	—	—	—	—	—	—	—	6.5	—
Dimeric	—	—	—	—	8.1	<1	6.0	—	3.4	8.5	3.5	—	4.2	—	—	1.5
Short	—	—	—	—	—	—	<1	—	—	—	—	—	2.6	—	—	—
Myotoxin	0.4	<0.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C-type BPP/NP	—	—	<0.1	<0.1	—	—	<1	—	2.8	0.3	—	—	—	14.7	2.1	<0.1
Kunitz-type inhibitor	—	—	<0.1	<0.1	—	—	—	4.2	3.0	7.5	—	3.2	—	—	—	—
Cystatin	—	—	—	—	—	—	—	1.7	9.8	5.3	4.2	—	—	—	—	—
DC fragment	<0.1	<0.1	<0.1	1.3	—	—	1.0	—	0.5	0.6	<0.1	—	1.7	—	—	<0.1
NGF/svVEGF	<0.1	<0.1	<0.1	<0.1	—	—	2.1	—	1.0	—	—	—	—	—	—	—
Ohanin-like	—	—	—	—	—	—	—	—	—	—	—	—	—	—	<0.1	
CRISP	0.8	1.3	10.7	2.9	—	—	—	—	2.0	1.2	1.3	1.2	1.5	1.8	4.2	—
PLA_2	29.9	31.6	13.7	**32.5**	20.0	21.1	4.0	4.3	11.4	4.8	20.1	**59.8**	12.6	8.7	16.3	18.5
Serine proteinase	18.2	20.4	24.4	17.1	9.1	20.0	9.2	19.5	**26.4**	23.9	21.9	15.1	2.0	**31.2**	10.1	13.8
C-type lectin-like	<0.1	<0.1	<0.1	<0.1	24.0	0.9	10.1	13.2	14.3	14.1	4.2	4.9	7.0	8.1	1.6	—
L-amino acid oxidase	4.2	1.6	2.5	2.1	12.0	9.0	—	—	1.3	2.2	3.2	1.7	1.4	2.7	8.0	2.2
Zn^{2+}-metalloproteinase	**43.8**	**40.6**	**48.6**	**36.1**	**37.0**	**48.1**	**67.1**	**38.5**	**22.9**	**30.8**	**40.9**	11.5	**67.0**	**31.9**	**51.1**	**63.6**

Species: *Sistrurus catenatus catenatus* (SCC), *Sistrurus catenatus tergeminus* (SCT), *Sistrurus catenatus edwardsii* (SCE), *Sistrurus miliarius barbouri* (SMB), *Cerastes cerastes cerastes* (CCC), *Cerastes vipera* (CV), *Macrovipera lebetina transmediterranea* (MLT), *Bitis arietans* (BA), *Bitis gabonica gabonica* (BGG), *Bitis gabonica rhinoceros* (BGR), *Bitis nasicornis* (BN), *Bitis caudalis* (BC), *Echis ocellatus* (EO), *Lachesis muta* (LM), *Crotalus atrox* (CA), and *Agkistrodon contortrix contortrix* (ACC).

III. SNAKE VENOM Zn^{2+}-METALLOPROTEINASES AND THE EMERGENCE OF DISINTEGRINS

Snake venom hemorrhagic metalloproteinases are multidomain proteins that have been classified according to their domain structure into four classes (Fox and Serrano, 2005b; also see Chapter 4, this volume). P-I metalloproteinases (25–30 kDa) are single-domain proteins with relatively weak hemorrhagic activity. The class P-II metalloproteinases (30–45 kDa) contain a disintegrin domain at the carboxyl terminus of a metalloproteinase domain structurally similar to that in the class P-I. Hemorrhagins of the P-III class are large toxins (50–100 kDa) with the most potent activity, and comprise multidomain enzymes consisting of an N-terminal metalloproteinase domain and C-terminal disintegrin-like and cysteine-rich domains. The P-IV class of SVMPs has a similar domain structure as the P-III class, but with additional disulfide-linked C-type lectin-like domains.

Snake venom hemorrhagic metalloproteases (SVMPs) represent the most abundant toxins in the majority of viperid venoms so far investigated (Table 17.1). SVMPs are clustered with mammalian matrix-degrading metalloproteinases and proteins of the ADAM family in a monophyletic evolutionary tree (Moura da Silva et al., 1996). The monophyletic distribution of the mammalian and snake venom proteins indicates that SVMPs have evolved relatively late during evolution from a common ancestor gene by both speciation (after mammals and reptiles diverged) and gene duplication, followed by divergence of the copies through positive Darwinian selection. Fry and coworkers have investigated the evolution of the venom system in advanced snakes and helodermatid lizards (Fry and Wüster, 2004; Fry, 2005; Fry et al., 2006). These studies showed that all lineages possessing toxin-secreting oral glands apparently form a clade, suggesting a single early origin of the venom system in lizards and snakes. The mapping of toxin types over the revised squamate phylogeny provided additional insights into the evolution of the reptile venom chemical arsenal. Nine toxin types (AVIT, B-type natriuretic peptide (BNP), CRISP, cobra venom factor, crotamine, cystatin, kallikrein, nerve growth factor, and vespryn) were recovered from both lizard and snake cDNA libraries (Fry et al., 2006). SVMPs, on the other hand, appeared to have been recruited after the divergence of lizards and Caenophidia snakes, and therefore represent Serpentes-specific toxins (Figure 17.2). In addition, the presence of SVMPs in Viperidae (vipers and pit vipers) (Fox and Serrano, 2005b; Lu et al., 2005), Elapidae (cobras, kraits, and coral snakes) (Tan and Saifuddin, 1990; Ward et al., 1996; Ito et al., 2001; Genbank entry AF063190), Colubridae (colubroid snakes) (Kamiguti et al., 2000; Ching et al., 2006; Peichoto et al., 2007), and Atractaspididae (mole vipers and stiletto snakes) (Ovadia, 1987; Genebank entry AF186368) strongly indicates that the evolution of P-III hemorrhagins occurred before the radiation of the advanced snakes. Structural and functional diversification of P-III SVMP genes continued after the colubroid radiation, creating paralogous multigene families.

P-II SVMPs occur only in viperid venoms and cluster with P-III SVMPs into a monophyletic cladogram (Moura-da-Silva et al., 1996) (Figure 17.3). P-II SVMPs may therefore represent a derivation from ancestral P-III SVMP genes subsequent to the split of the Viperidae as a distinct taxonomic group of advanced snakes. P-II SVMPs undergo limited proteolysis releasing the metalloproteinase and disintegrin domains into viperid venoms (Kini and Evans, 1992; Shimokawa et al., 1996), which act separately. Disintegrins exhibit a wide range of relative amounts in different viperid venoms (Table 17.1).

IV. STRUCTURAL DIVERSIFICATION OF SNAKE VENOM DISINTEGRINS

Disintegrins, a family of small (forty to one hundred amino acids), cysteine-rich polypeptides (Calvete et al., 2005; Calvete, 2005), were first described as potent inhibitors of the platelet fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$ (Huang et al., 1987). The isolation and characterization of disintegrins that target other integrin receptors and do not inhibit platelet aggregation (i.e., non-RGD-containing dimeric disintegrins) was achieved with the development in the late 1990s of cell adhesion

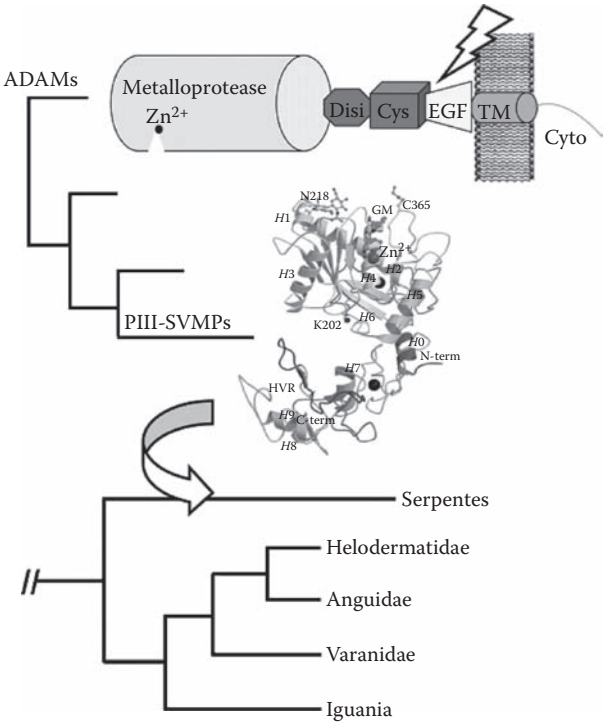

FIGURE 17.2 Evolution of SVMPs. Cartoon of the proposed emergence of P-III SVMPs by duplication of a gene coding for a multidomain ADAM integral membrane protein ancestor. A key event in this transition was the deletion (thunderbolt) from the duplicated ancestor of the regions coding for the EGF-like domain, the transmembrane region, and the cytosolic tail. It is hypothesized that the duplicated gene was recruited by (arrow), and selectively expressed in, the venom gland after the evolutionary divergence of the advanced snakes from the clade of venomous lizards, and before the Colubroid radiation. Phylogenetic relationships between advanced (Caenophidia) snakes (Serpentes) and venomous lizards (Iguania, Varanidae, Anguidae, and Helodermatidae) have been adapted from Fry et al. (2006). The depicted P-III SVMP model corresponds to the 2.5 Å resolution crystal structure of one subunit of the homodimeric P-III metalloproteinase vascular apoptosis-inducing protein-1 (VAP1) from *Crotalus atrox* venom (Takeda et al., 2006).

inhibition assays using cell lines expressing specific integrins (Marcinkiewicz et al., 1999a, 1999b) (see below). Structurally, disintegrins can be conveniently divided into five different groups according to the length and number of disulfide bonds of the polypeptides (Calvete et al., 2003). The first group includes short disintegrins composed of forty-one to fifty-one residues and four disulfide bonds. The second group is formed by the medium-size disintegrins, which contain about seventy amino acids and six cysteine bonds. The third group includes long disintegrins with an ~84-residue polypeptide cross-linked by seven disulfide bridges. The fourth group is composed of homo- and heterodimers. Dimeric disintegrins contain subunits of about sixty-seven residues with ten cysteines involved in the formation of four intrachain disulfides and two interchain cysteine linkages (Calvete et al., 2000; Bilgrami et al., 2004, 2005). Homo- and heterodimeric disintegrins have been reported in a number of viperid snakes. Dimeric disintegrins are built by restricted combinations of an α-type subunit (coded for by a short-coding message lacking the metalloproteinase domain) and a β-type (P-II structure) subunit. Homo- and heterodimeric disintegrins often coexist in the same venom and in many cases share a common subunit (Marcinkiewicz et al., 1999a, 2000; Calvete et al., 2002, 2003). Sharing of subunits may represent an effective mechanism of structural and functional diversification of dimeric disintegrins. In addition, the occurrence of precursors of the α- and β-subunits of dimeric disintegrins differing in their domain structure immediately suggests a mechanism for regulation

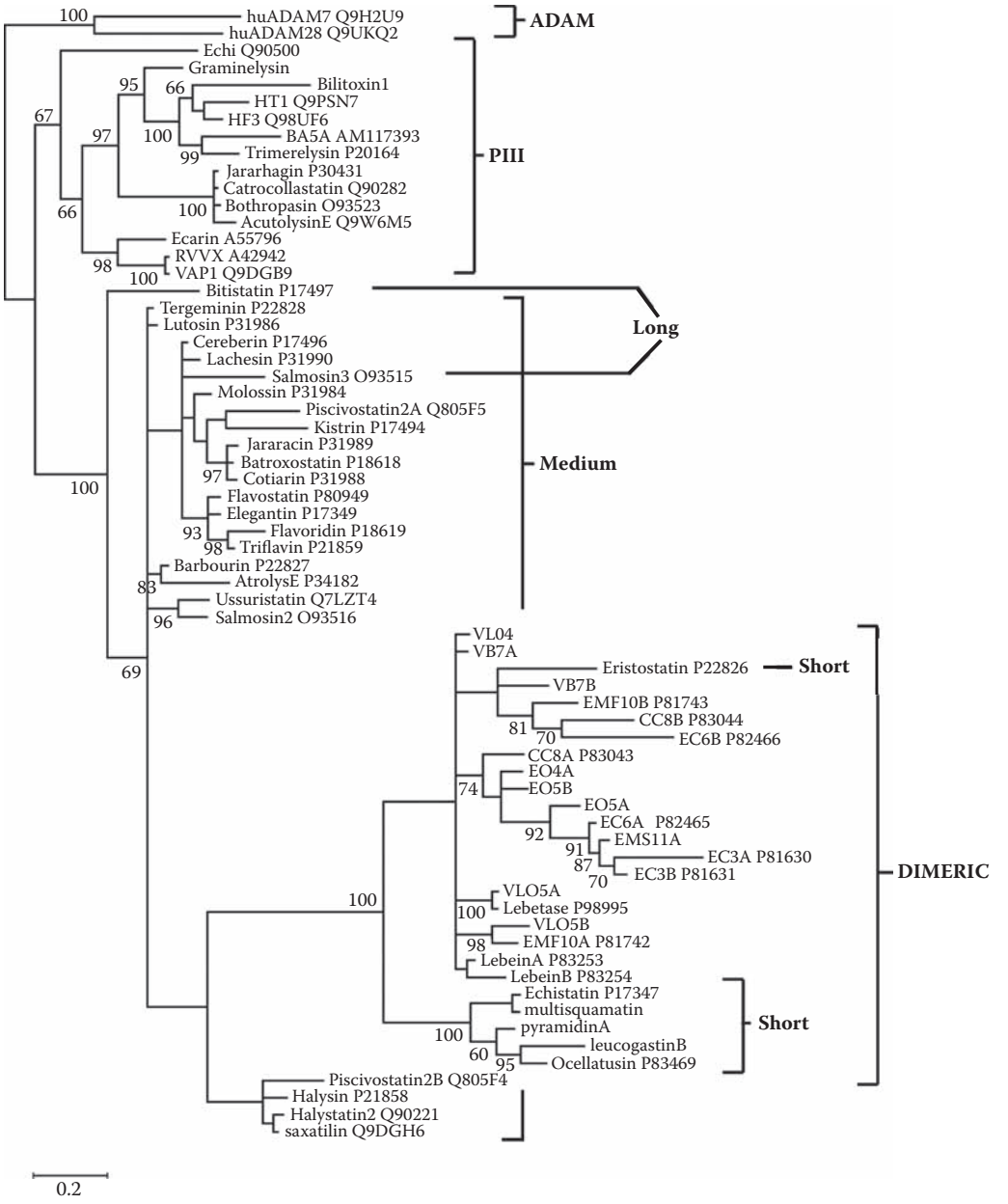

FIGURE 17.3 Evolution of the disintegrin family. Evolutionary relationships among disintegrins were inferred using maximum-likelihood and Bayesian methods. For rooting the tree, the disintegrin-like domains of human ADAM 7 and 10 were used as the outgroup. Nodes with confidence values greater than 60% are indicated.

of dimerization. Homodimers may result from disulfide bonding of two α-type subunit precursors, whereas the association of two β-type subunit precursors might be sterically hindered by the large N-terminal multidomain structure. On the other hand, heterodimers may be generated by α-type/α-type and α-type/β-type subunit associations (Calvete, 2005; Calvete et al., 2005).

It is worth noting that although disintegrins in each group usually have similar structures, polypeptides with new disulfide scaffolds can occasionally evolve within a group. Thus, albolabrin

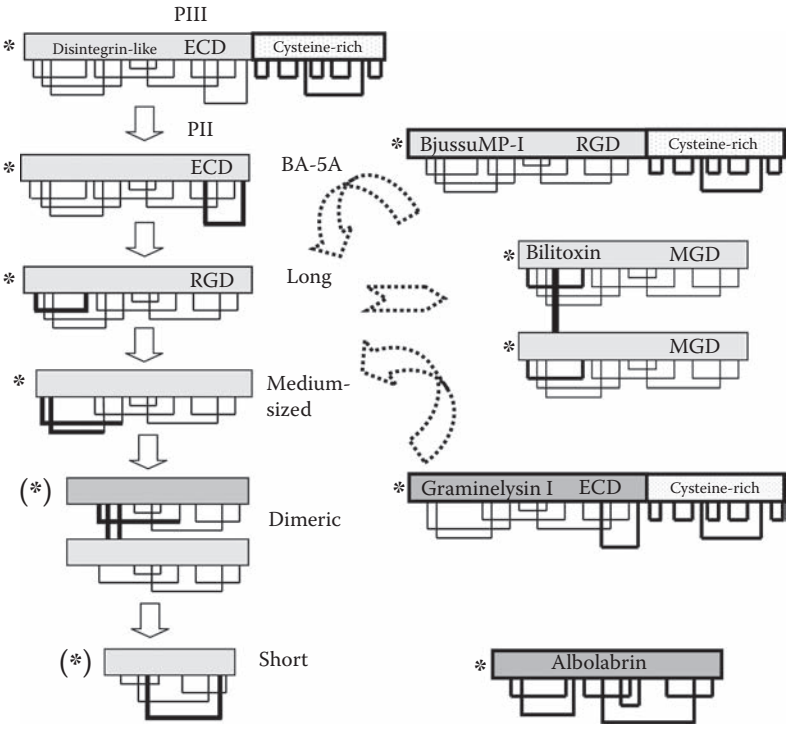

FIGURE 17.4 Minimization of structure along the evolutionary diversification pathway of disintegrins. Scheme of the domain organization, disulfide bond patterns, and proposed evolutionary pathway from the P-III disintegrin/cysteine-rich proteins to short disintegrins. Structural features (the cysteine-rich domain of P-III disintegrin-like molecules, and class-specific disulfides) lost along the canonical disintegrin diversification model depicted at the left are highlighted with thick lines. An asterisk indicates that the precursor protein is synthesized with an N-terminal metalloproteinase domain. (*), some, but not all, precursors of dimeric disintegrin subunits and short disintegrins contain the metalloprotease domain; the majority of proteins from these groups are synthesized from short-coding mRNAs coding for α-type subunits lacking the metalloproteinase domain (Okuda et al., 2002; Juárez et al., 2006b). Although most disintegrins may have followed the canonical diversification pathway, the evolutionary scenario might be more complicated: graminelysin (Wu et al., 2001) represents a P-III metalloprotease from *Trimeresurus gramineus* venom lacking the CysI–CysIV disulfide, and might thus represent an alternative intermediate in the evolution of a medium-size disintegrin; BjussuMP-I is a P-II metalloprotease bearing a C-terminal cysteine-rich domain isolated from the venom of *Bothrops jararacussu* (Mazzi et al., 2007); and bilitoxin-1, a long disintegrin from *Agkistrodon bilineatus*, possesses an extra cysteine residue between CysIII and CysIV involved in the formation of a disulfide-linked homodimer (Nikai et al., 2000). Furthermore, albolabrin is a medium-size disintegrin displaying a noncanonical disulfide bond arrangement.

(Calvete et al., 1991) represents a medium-size disintegrin displaying a noncanonical disulfide bond arrangement (Figure 17.4). This alternative S–S bonding pattern has also been described in saxatilin (Hong et al., 2002) and salmosin (Shin et al., 2003). Studies on recombinant saxatilin demonstrated that the disulfide bonds involving the four N-terminal cysteine residues do not appear to be critical for the biological function (inhibition of platelet aggregation) of saxatilin, whereas deletion of any of the other disulfide bonds (C5–C8, C6–C11, and C10–C12) abolished the inhibitory activity of the disintegrin. This evidence provides support to our current view that structural diversification of P-II disintegrins was driven through a disulfide bond engineering mechanism involving the selective loss of pairs of N-terminal cysteine residues engaged in the formation of disulfide bonds and minimization of the protein size (Calvete et al., 2003; Juárez et al., 2006a, 2006b) (Figure 17.4). The molecular details underlying such mechanism have been recently outlined for the emergence of a

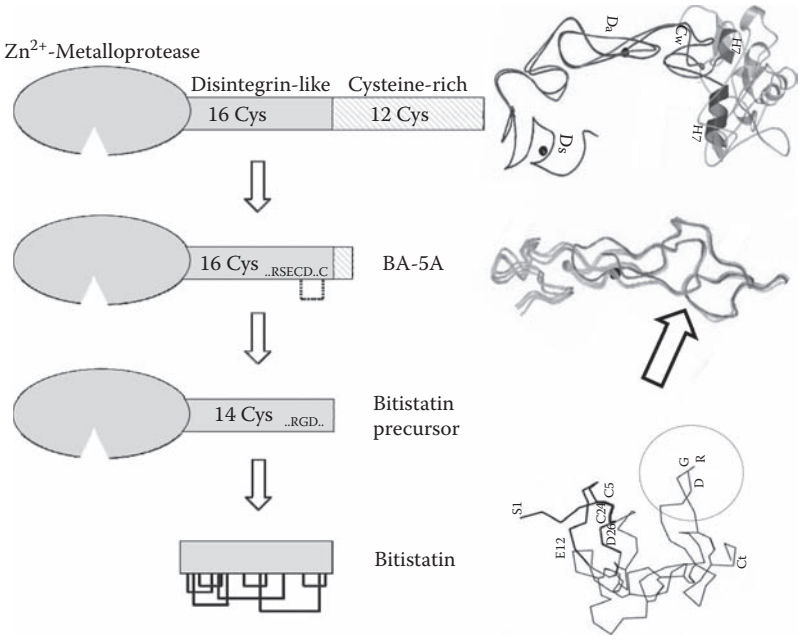

FIGURE 17.5 Hypothetical mechanism for the P-III–P-II disintegrin domain transition. Right: Cartoon of the proposed evolutionary pathway of the long disintegrin bitistatin, which includes the stepwise removal of (1) the cysteine-rich domain from a P-III metalloproteinase precursor gene, yielding the BA–5A messenger, (2) the loss of the P-III disintegrin-like domain-specific CysXIII–CysXVI linkage, and (3) the emergence of the RGD motif. The mature bitistatin structure, showing its seven disulfide bonds as connecting lines, is depicted. Left, from top to bottom: Structure of the disintegrin-like/cysteine-rich domain of a P-III metalloprotease (Takeda et al., 2006), a model of the disintegrin-like domain of BA–5A, and a model of the long disintegrin bitistatin (Calvete et al., 1997). The arrow points to the region topologically constrained by the CysXIII–CysXVI bond, whose removal is hypothesized to have paved the way for evolving the integrin inhibitory activity at the tip of the solvent exposed and mobile loop of the disintegrins proper. The RGD sequence of bitistatin is encircled.

long disintegrin from a P-III precursor (Juárez et al., 2006a), and for the transition from a dimeric to a short disintegrin (Juárez et al., 2006b). These authors have cloned BA–5A from a venom gland cDNA library of the puff adder, *Bitis arietans*, a cDNA encoding a novel ECD disintegrin-like domain that contains the sixteen cysteine residues conserved in all known disintegrin-like domains of P-III snake venom metalloproteinases, but lacks the cysteine-rich domain. These features suggest that BA–5A may represent an intermediate in the evolutionary pathway of the long disintegrin bitistatin, and that removal of the cysteine-rich domain and loss of the P-III-specific disulfide bond were separate events along the structural diversification pathway of disintegrins, the former predating the latter (Figure 17.5). We hypothesize that deletion of the P-III-specific CysXIII–CysXVI cysteine bond was a crucial event in the evolution of disintegrins proper because removal of this structural constraint may have paved the way for evolving the integrin inhibitory activity at the tip of a solvent exposed and mobile loop (Figure 17.5).

The transition from a dimeric disintegrin subunit to the short disintegrin ocellatusin can be achieved by a minimum of two nucleotide mutations (Cys→Tyr and Ser→Cys) (Juárez et al., 2006b) (Figure 17.6). The single amino acid substitution Cys7→Tyr, generated through a single nucleotide mutation TGT→TAT or TGC→TAC, is the simplest molecular solution to hinder dimerization. Moreover, the exquisite molecular economy of the transformation of a dimeric disintegrin subunit message into another coding for a short disintegrin is highlighted by the fact that substitution of a serine residue (..**S**DCD..) for the short-disintegrin-specific cysteine (..**C**DCD..) can also be

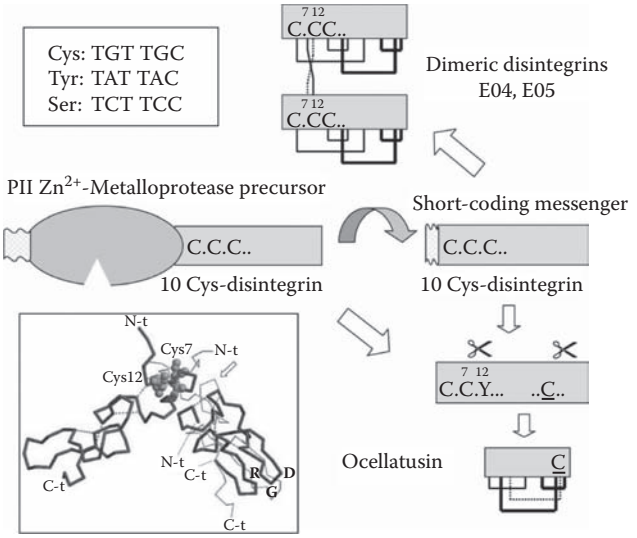

FIGURE 17.6 Proposed mechanism for the emergence of the short disintegrin, ocellatusin, from a dimeric disintegrin subunit precursor. Key events in the emergence of ocellatusin were (1) the substitution of the second N-terminal cysteine residue (Cys7 in the dimeric disintegrin subunit precursor) by tyrosine, thereby impairing dimerization through homologous CysA7–CysB12 and CysA12–CysB7 linkages; (2) the appearance of a novel cysteine residue between the ninth and tenth cysteines of the precursor (**C**) enabling the short-disintegrin-specific disulfide bond depicted by a broken line; and (3) the proteolytic processing of the N- and C-terminal regions of the expressed protein (scissors). Notice that the two key amino acid changes needed to convert a dimeric disintegrin subunit into a short disintegrin can be accomplished by two nucleotide mutations. The insert at the left lower corner shows the superposition of the structures of the short disintegrin ocellatusin (modeled based on echistatin; Monleón et al., 2005) and schistain (Bilgrami et al., 2004). N-t, N-terminus; C-t, C-terminus. Intrachain disulfide bonds are shown in one subunit of schistatin as broken lines, and the two interchain cysteine linkages of the homodimer are depicted in the space-filling model. Disulfide bonds of ocellatusin are shown as broken lines, and the RGD motif of the short disintegrin is labeled. The arrow indicates the position of the N-terminal proteolytic cleavage that yields mature ocellatusin.

accomplished by a single C→G mutation (Figure 17.6). On the other hand, changing threonine for cysteine needs a minimum of two mutations (i.e., ACT→TCT→TGT). This may in part explain the observation that short disintegrins are commonly found in the venoms of species from genera like *Echis*, which also express dimeric disintegrins containing the sequence **S**XDC, but that have not been reported in the venoms of snakes like *Bitis gabonica*, which express dimeric disintegrins with **T**PDC sequences (Francischetti et al., 2004; Calvete et al., 2007a).

V. STRUCTURE-FUNCTION CORRELATIONS OF DISINTEGRINS: A FAMILY OF INTEGRIN RECEPTOR ANTAGONISTS

A broad spectrum of integrin receptor antagonists has evolved among snakes. Thus, with the exception of the $\alpha_2\beta_1$ integrin, which is targeted by a number of C-type lectin-like proteins (Horii et al., 2004; Ogawa et al., 2005), inhibitory motifs toward β_1 and β_3 integrins have evolved in different members of the disintegrin family. Like many other venom toxins, the integrin inhibitory activity of disintegrins critically depends on the appropriate pairing of cysteines (Calvete et al., 1991), which determines the conformation of the inhibitory loop that harbors an active tripeptide located at the apex of a mobile loop protruding 14–17 Å from the protein core (Calvete, 2005; Calvete et al., 2005). Among the integrin inhibitory motifs, RGD blocks the β_1 and β_3 integrins $\alpha_8\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$,

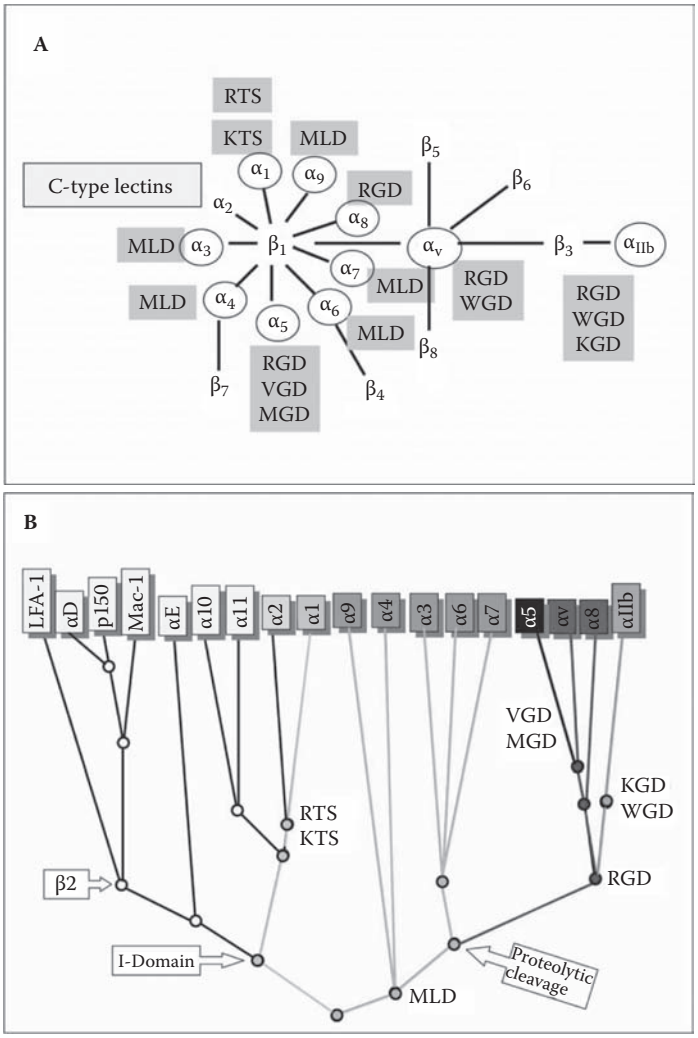

FIGURE 17.7 Evolutionary adaptation of disintegrin inhibitory motifs to the ligand-binding sites of integrins. (A) Diagram of the integrin family and the different disintegrin tripeptide motifs that block specific integrin-ligand interactions. Integrin heterodimers antagonized by snake venom disintegrins are encircled. $\alpha_1\beta_1$ is a receptor for collagen IV (CollIV); $\alpha_2\beta_1$ binds collagen I; $\alpha_4\beta_1$ interacts with fibronectin and VCAM-1; $\alpha_4\beta_7$ binds the same ligands as $\alpha_4\beta_1$ and in addition is a receptor for MdCAM; $\alpha_5\beta_1$ represents the major fibronectin (FN) receptor; integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_7\beta_1$ represent major laminin (LM) receptors; $\alpha_8\beta_1$ and $\alpha_9\beta_1$ bind tenascin (TN); $\alpha_v\beta_1$ and $\alpha_v\beta_3$ are major vitronectin (VN) receptors; and $\alpha_{IIb}\beta_3$ is the platelet fibrinogen (FB) receptor involved in platelet aggregation. (B) Segregation of disintegrin inhibitory motifs within the phylogenetic tree of the integrin α-subunits is highlighted by identical branch line colors. The phylogenetic relationships of the integrin α-subunits have been adapted from Evans et al. (2005) and Huhtala et al. (2005). Branches are not scaled according to evolutionary distance. Branch points linked with the emergence of the proteolytic cleavage of integrin α-subunits, and the acquisition of an I-domain, are indicated.

$\alpha_v\beta_3$, and $\alpha_{IIb}\beta_3$; MLD targets the $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, and $\alpha_9\beta_1$ integrins; VGD and MGD impair the function of the $\alpha_5\beta_1$ integrin; KGD inhibits the $\alpha_{IIb}\beta_3$ integrin with a high degree of selectivity; WGD has been reported to be a potent inhibitor of the RGD-dependent integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_{IIb}\beta_3$; and KTS and RTS represent selective $\alpha_1\beta_1$ inhibitors (Calvete, 2005; Calvete et al., 2005; Sanz et al., 2006b) (Figure 17.7A). Whereas the majority of single-chain (long, medium-size,

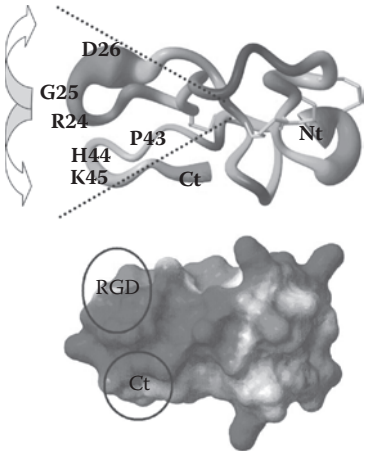

FIGURE 17.8 ***(A color version of this figure follows page 240.)*** NMR structure of the short disintegrin echistain. The upper panel shows a "sausage" model of the solution structure of echistatin (PDB code 1RO3) highlighting the topology of the active tripeptide and the C-terminal region, which form a conformational epitope engaged in extensive interactions with the target integrin receptor, and display concerted lateral movements. The lower panel shows the surface electrostatic potential of echistatin. The molecule is depicted in the same orientation as the sausage model. The positively and negatively charged areas are colored blue and red, respectively. The integrin recognition motifs (RGD) and the C-terminal tail (Ct) are labeled and circled.

and short) P-II disintegrins express the RGD sequence, dimeric disintegrins exhibit large variability in their integrin recognition motifs. It is worth mentioning that non-RGD disintegrins are usually present in venoms that also contain RGD disintegrins (Calvete et al., 2003). The coexistence in the same snake species of disintegrins with conserved RGD motif and disintegrins with variable non-RGD sequences supports the hypothesis that, following gene duplication, one copy of the gene (i.e., that coding for an RGD disintegrin) divergently evolved under selective pressure dictated by the ancestral function (i.e., blocking of platelet aggregation). The duplicate gene (non-RGD disintegrin), now unencumbered by a functional role, is free to mutate to new physiological roles, such as inhibition of non-RGD-dependent integrin receptors.

Figure 17.7B shows the clear segregation of the different disintegrin inhibitory motifs with defined branches on the phylogenetic tree of the integrin α-chains. This highlights that an efficient, though restricted, panel of integrin-blocking sequences has evolved among disintegrins, and indicates an evolutionary adaptation of the snake venom antagonists to the ligand-binding sites of integrin receptors.

The crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ in complex with an RGD ligand (Xiong et al., 2002) showed that the peptide fits into a crevice between the α_V propeller and the β_3 A-domain. The Arg side chain is held in place by interactions with α_V carboxylates 218 and 150, the Gly residue makes several hydrophobic interactions with α_V, and the Asp ligand interacts primarily with βA residues. Hence, with the exception of the KTS and RTS $\alpha_1\beta_1$ inhibitory motifs, the conserved aspartate residue might be responsible for the binding of disintegrins to integrin receptors that share a β-subunit, while the two other residues of the integrin-binding motif (RG, KG, MG, WG, ML, and VG) may dictate the integrin specificity through interaction with the α-subunit. Other structural features, such as integrin-binding motif-flanking residues and the C-terminal tail, have been reported to modulate the fine specificity and affinity of the disintegrin-integrin recognition process (McLane et al., 1998; Wierzbicka-Patynowski et al., 1999; Marcinkiewicz et al., 1999b; Calvete et al., 2002, 2005). Determination of the NMR solution structures and internal dynamics of the short disintegrins obtustatin (Moreno-Murciano et al., 2003; Monleón et al., 2003) and echistatin (Monleón et al., 2005) (Figure 17.8) have revealed that the integrin recognition loop exhibits a global, lateral hinge motion within a range of 30° (echistatin) to 35° (obtustatin) and a maximum displacement of about 5 Å. In line with the concept that fast recognition and fitting processes are typically brought about by mobile segments in protein structures (Burgen et al., 1975; Williams, 1989), the loop movement occurs in the 100–300 ps timescale and is articulated at residues located at the base of the integrin recognition loop. In both short disintegrins, this loop and the C-terminal tail display concerted motions and form a conformational epitope engaged in extensive interactions with the target integrin receptor, providing a structural basis for the functional synergy between

these two structural elements. Similarly, Senn and Klaus (1993) and Fujii et al. (2003) have reported that the C-terminal residues are in close proximity of the corresponding RGD loops of the medium-size disintegrins flavoridin and trimestatin, and may represent functional epitopes. The architecture, dynamics, and surface potential of the integrin-interacting conformational epitope (Figure 17.8) may endow disintegrins with selective inhibition activity toward integrin receptors.

VI. LOSS OF INTRONS DURING THE ACCELERATED EVOLUTION OF DISINTEGRIN GENES

The large sequence and structural diversity exhibited by the different subfamilies strongly suggest that disintegrins, like toxins from other venoms (Duda and Palumbi, 1999; Kordis et al., 2002; Ohno et al., 2003), have evolved rapidly by adaptive evolution. The birth-and-death model of protein evolution has been proposed to underpin the evolution of elapid three-finger toxins (Fry et al., 2003). Details of the molecular events leading to snake venom toxin diversification remain largely to be disclosed, but a model of accelerated exon segment exchange for viperid three-finger toxins (3FTxs) has been proposed that could lead to rapid diversification of function (Doley et al., 2008). To investigate the molecular mechanism underlying the structural diversification of disintegrins, we have initiated the analysis of the genomic organization of their genes (Bazaa et al., 2007) (Figure 17.9). Among the three topologically conserved introns within the disintegrin-like domains of vertebrate ADAM genes, only introns 1 and 2 have been conserved in the genes coding for the medium-size disintegrins halystatin 2 and 3 (Figure 17.9). Introns 1 and 3 have been removed from, whereas the insertion position of intron 2 is conserved in, genes coding for dimeric disintegrin subunits. On the other hand, short disintegrins are encoded by intronless genes. As a whole, a comparative analysis of currently available disintegrin-like genes outlines the view that a minimization of the gene organization underlies the evolution of the disintegrin family (Figure 17.9).

To test the hypothesis that functional diversification of disintegrin genes could be driven by positive Darwinian selection, we determined the strength and direction of selection by estimating the

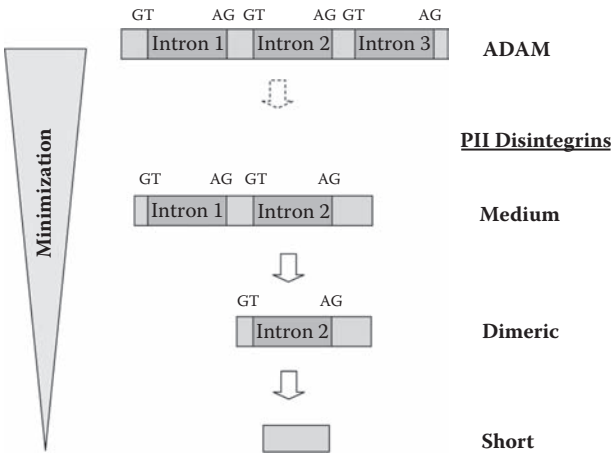

FIGURE 17.9 Minimization of the gene organization of disintegrins. Cartoon of the conserved exon-intron organization of known disintegrin-like domains of vertebrate ADAM proteins and of the medium-size disintegrin halystatin 2 and 3 (GenBank accession code D28871), the dimeric disintegrin subunits Ml_G1, Ml_G2, and Eo_D3, and the short-disintegrins Ml_G3 and Eo_C3 (Bazaa et al., 2007). *GT…AG* denotes the 5′-*GT*AAG (donor)/3′-*AG* (acceptor) consensus intron splice site signature conserved in all known disintegrin-like and disintegrin genomic DNAs. Structures of genes coding for P-III disintegrin-like domains and long disintegrins are not available. The concept of minimization of both the protein and the gene structures along the diversification pathway of disintegrins is highlighted.

TABLE 17.2
K_A/K_S Values for Pairs of Disintegrin Genes from *Echis ocellatus* (Eo) and *Macrovipera lebetina transmediterranea* (Ml)

Gene Pairs	K_S	K_A	K_A/K_S
Eo_C3 vs. Eo_D3	0.15	0.26	1.69
Eo_C3 vs. Eo_RTS	0.18	0.48	2.59
Eo_C3 vs. Ml_G1	0.20	0.21	1.05
Eo_C3 vs. Ml_G2	0.17	0.23	1.32
Eo_C3 vs. Ml_G3	0.18	0.44	2.59
Eo_D3 vs. Eo_RTS	0.29	0.44	1.50
Eo_D3 vs. Ml_G1	0.14	0.15	1.03
Eo_D3 vs. Ml_G2	0.18	0.07	0.38
Eo_D3 vs. Ml_G3	0.29	0.44	1.50
Eo_RTS vs. Ml_G1	0.23	0.44	1.90
Eo_RTS vs. Ml_G2	0.17	0.43	2.48
Ml_G1 vs. Ml_G2	0.06	0.10	1.61
Ml_G1 vs. Ml_G3	0.23	0.44	1.90
Ml_G2 vs. Ml_G3	0.17	0.43	2.48

Note: K_S and K_A values were calculated with MEGA 3.1.

nonsynonymous-to-synonymous substitution rate ($K_A/K_S = \omega$), with $\omega = 1$, <1, and >1 indicating neutral evolution, purifying selection, and directional selection, respectively. Within the framework of Ohno's model of postduplication accelerated divergence, which predicts an increase in the nonsynonymous substitution rate following duplication, as positive Darwinian selection drives the fixation of mutations that confer new or modified functions on gene duplicates (Ohno, 1970; Ogawa et al., 1995, 1996, 2005; Ohno et al., 1998), our data (Table 17.2) support the conclusion that snake venom disintegrins have been subjected to selection favoring functional diversification (Juárez et al., 2008).

VII. CONCLUDING REMARKS

Evolution of the structural diversification pathway of disintegrins involved minimization of both the genomic and protein structures, including, respectively, the stepwise loss of introns and pairs of cysteine linkages. Functionally, disintegrins have evolved by accelerated evolution and adaptation of a conformational epitope (the integrin recognition motif and the C-terminal tail) to the active site of the targeted integrin receptor. P-II disintegrins block the function of integrin receptors with a high degree of selectivity. Selective blockade of integrins is a desirable goal for the therapy of a number of pathological conditions, including acute coronary ischemia and thrombosis ($\alpha_{IIb}\beta_3$), tumor metastasis, osteoporosis, restenosis and rheumatoid arthritis ($\alpha_v\beta_3$), bacterial infections and vascular diseases ($\alpha_5\beta_1$), inflammation and autoimmune diseases ($\alpha_4\beta_1$, $\alpha_7\beta_1$, $\alpha_9\beta_1$), and tumor angiogenesis ($\alpha_1\beta_1$, $\alpha_v\beta_3$). The relevant integrin receptors involved in these pathologies are among the targets of many disintegrins (cf. Figure 17.7), and the potential clinical relevance of blocking specific integrin receptors using snake venom disintegrins has been reviewed recently (Marcinkiewicz, 2005). More specifically, the possible therapeutic approach toward tumor neovascularization by targeting the $\alpha_5\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_3$ integrins with RGD-bearing disintegrins, and the $\alpha_1\beta_1$ with RTS and KTS disintegrins, has been explored in a number of laboratories (Marcinkiewicz et al., 2003; Sanz et al., 2005; Swenson et al., 2005; Minea et al., 2005; Calvete et al., 2007b). In addition, chemically modified synthetic RGD peptides and RGD disintegrins with nuclides emitting γ radiation (^{99m}Tc, ^{125}I), β particles (^{64}Cu), positrons (^{18}F), or infrared radiation are being used as tools for visualizing

$\alpha_v\beta_3$-dependent tumor angiogenesis *in vivo* (reviewed in Calvete et al., 2007b). Understanding how toxins evolve in an accelerated fashion may hold the key not only to understanding speciation at a molecular level, but also to learning how to use deadly toxins as therapeutic agents.

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18 Reptile C-Type Lectins

Xiao-Yan Du and Kenneth J. Clemetson

CONTENTS

Snake venoms contain two types of C-type lectins based on the structural features and functional properties: snake C-type lectin-like proteins (CLPs) and sugar binding snake lectins. There is some degree of sequence homology between the two proteins. Most snake lectins are 26–28 kDa homodimers that agglutinate erythrocytes only via binding to carbohydrates. On the other hand, CLPs are composed of homologous heterodimers existing as monomeric or oligomeric forms $(\alpha\beta)_x$ and contribute to the disruption of hemostasis in envenomed prey by targeting a wide range of plasma components or blood cell types (especially platelets), either activating or inhibiting these. Snake CLPs, with a relatively conservative and homologous structure (primary, secondary, and tertiary), are unique in their diverse biological targets and have become useful tools in studies of protein structural and functional relationships. The thorough characterization of convulxin, a snake-derived CLP, has contributed greatly to the cloning and identification of glycoprotein (GP) VI and helped to open the field of platelet signaling transduction pathways via GPVI. Botrocetin, a von Willebrand Factor (VWF) modulator, has been broadly applied in clinical diagnostics. In this work, we will report recent findings on snake venom–derived CLPs and sugar binding lectins, as well as their targets and interacting mechanisms.

I. STRUCTURE AND FUNCTION OF SNAKE C-TYPE LECTIN-LIKE PROTEINS AND SNAKE LECTINS

Snake venoms are complex mixtures of biologically active proteins and peptides that belong mainly to the serine protease, metalloprotease, phospholipase, disintegrin, and C-type lectin families. Snake C-type lectins are subclassed into snake lectins that are classic sugar binding proteins and

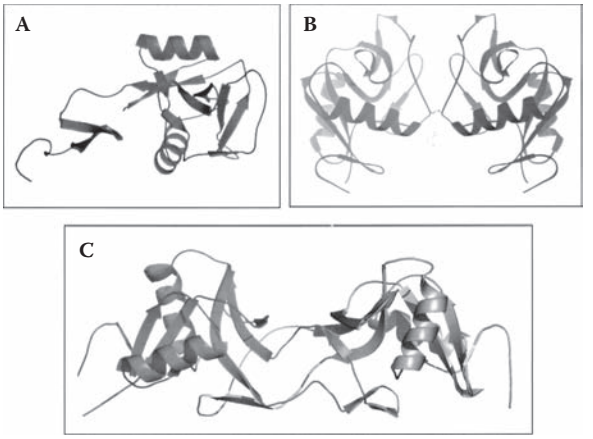

FIGURE 18.1 Structure of lectins. (A) A canonical C-type lectin. (B) Dimeric sugar binding C-type lectin. (C) Heterodimeric snake venom C-type lectin-like protein.

snake C-type lectin-like proteins (CLPs) that have a typical fold resembling that in classic C-type lectins, but are usually unable to recognize carbohydrate because they lack the Ca^{2+} binding loop involved in sugar binding, and snake CLPs consist of heterodimers formed by loop swapping. The basic structure of most snake lectins is a 26 to 28 kDa homodimer, whereas CLPs are composed of heterodimers with homologous α- and β-subunits, each with a molecular mass ranging from 13 to 18 kDa. Each globular subunit has two α-helices, five β-strands, and a long loop that interacts with the other subunit to form a swapped loop dimer linked by a disulfide bond (Figure 18.1). CLPs have been found to exist as either αβ, $(\alpha\beta)_2$, or $(\alpha\beta)_4$ oligomeric forms and have highly diverse targets. While CLPs, regardless of target, share considerable sequence similarity, a large part of this consists of the highly conserved segments forming the core fold structure. This is also a major reason for the degree of sequence similarity between α- and β-subunits. The amino acids exposed at the surface of the protein, particularly those at the surface of the concave domain formed by the swapped loops, are much more variable, either because they provide the specificity of the binding site(s) or because for structural reasons they are not under evolutionary pressure to remain conserved.

Snake CLPs are important components in the highly hemorrhagic venoms of the Viperidae. They contribute to the disruption of hemostasis in envenomed prey by targeting a wide range of plasma components and blood cells, either activating or inhibiting these. Recent studies on the purification of ophioluxin (Du et al., 2002b) from king cobra (*Ophiophagus hannah*) venom, and the cloning of cDNAs encoding CLPs from *Bungarus fasciatus* and *Bungarus multicinctus* (Zha et al., 2001), revealed that snake CLPs exist in the family Elapidae, venoms that had been thought to have primarily neurotoxic effects on prey. It is not yet known whether CLPs also occur in the mainly myotoxic sea snake venoms (family Elapidae, subfamilies Hydrophiinae and Laticaudinae), but they were recently reported to exist in the less well-characterized family Colubridae (Ching et al., 2006). Although there are some reports of venom components in reptiles other than snakes, none of these included CLP (Fry et al., 2006; Tu, 1991). Thus, in this chapter, we describe the structure of CLPs and how they differ from snake lectins. We also summarize recent discoveries about snake CLPs that interact with platelets and plasma proteins, as well as the snake lectins that bind sugars.

II. SNAKE C-TYPE LECTIN-LIKE PROTEINS TARGETING PLATELETS

A. Platelet Receptors Involved in Hemostasis

Platelets have critical roles in maintaining blood flow in an uninjured vessel by repairing gaps caused by minor endothelial cell defects and arresting blood loss in an injured vessel. The structure

and function of major platelet surface glycoproteins (GPs) that are involved in thrombosis and hemostasis, such as GPIb-V-IX, αIIbβ3, α2β1, and GPVI, have been extensively studied. GPIb-V-IX is a complex of leucine-rich repeat family glycoproteins. The N-terminal 45 kDa domain of the GPIbα subunit contains binding sites for von Willebrand factor (VWF) and α-thrombin, as well as an increasing number of other ligands (Solum and Clemetson, 2005). Upon exposure of VWF after endothelium injury, platelets rapidly adhere to the site of injury through the GPIb-VWF axis and establish a prothrombotic surface for further platelet accumulation. In humans, both GPIbα deficiency (Bernard-Soulier syndrome) and deficiencies or defects in VWF (von Willebrand's disease) lead to severe bleeding complications. GPVI, an immunoglobulin family protein expressed on platelets as a complex with Fc receptor γ-chain (FcRγ), is indispensable for platelet activation induced by collagen (Moroi et al., 1989; Polgar et al., 1997; Tsuji et al., 1997). GPVI-dependent platelet activation induces amplifying signaling transduction, releasing mediators (e.g., ADP, TXA_2, and serotonin) and adhesive proteins (e.g., fibrinogen and P-selectin). Mice lacking either GPVI or FcRγ (in which GPVI is not expressed because of the absence of FcRγ) do not form a normal thrombus in the ferric chloride injury model even though both collagen and VWF are exposed (Nieswandt et al., 2001; Kato et al., 2003). The α2β1 integrin, another collagen receptor, plays a complementary role in mediating platelet adhesion to a collagen surface (Holtkotter et al., 2002). Platelet integrin αIIbβ3 becomes activated by either outside-in or inside-out signals and binds to fibrinogen. Fibrinogen then cross-links αIIbβ3 and leads to the formation of platelet aggregates. Mice lacking β3 subunit develop significantly smaller thrombi and serious bleeding (Smyth et al., 2001), whereas humans with inherited deficient or defective αIIbβ3 suffer from a bleeding disorder known as Glanzmann's thrombasthenia. When platelets aggregate, a procoagulant surface is exposed, which initiates the coagulation cascade, leading to the activation of prothrombin to thrombin. Thrombin then activates platelets by interacting with GPIb and cleaving protease-activated receptors (PARs) expressed on the platelet surface (Sambrano et al., 2001), and further propagates thrombus formation. Snakes have evolved to produce CLPs that target the major platelet glycoprotein receptors to either promote or inhibit events and thus influence hemostasis.

B. Snake C-Type Lectin-Like Proteins Binding GPIb

It has been long recognized that the GPIb complex is composed of four protein chains, GPIbα, Ibβ, IX, and V, in a ratio of 2:2:2:1. Very recently, Luo and colleagues (2007) showed that each GPIbα subunit is actually linked to two Ibβ molecules via covalent disulfide bonds, and the ratio is therefore 2:4:2:1. The presence of two GP Ibß subunits rather than one may help the complex better withstand the forces involved in platelet rolling. The GPIb complex is one of the major receptors on platelets and has a role in many aspects of platelet function, particularly platelet activation by VWF and thrombin through binding to the outer domains of GPIbα. The other subunits, such as GPIbß, and the cytoplasmic domain of GPIbα anchor the complex to the cytoskeleton and, when clustered, activate signaling pathways (Clemetson, 2007). There are a large number of snake GPIb binding CLPs with many common features that either inhibit VWF binding to GPIb or induce VWF-dependent platelet aggregation. However, they have a wide variety of subunit composition. Some heterodimeric snake CLPs are functionally monomeric, whereas others are either dimeric or tetrameric. Thus, these venom proteins have very different biological effects on platelets even though they share GPIb as their common target (Morita, 2004; Lu et al., 2005a).

Echicetin, from *Echis carinatus* venom, is a classic example of a GPIb binding CLP. It consists of two subunits, α and β, with 131 and 123 amino acid residues, respectively. The two subunits share 50% amino acid sequence similarity. Echicetin strongly inhibits the aggregation induced by thrombin and VWF in washed platelets by binding directly to platelet GPIb (Peng et al., 1993). Both agkicetin (Chen and Tsai, 1995) and agkistin (Yeh et al., 2001) are monomeric heterodimers from *Deinagkistrodon* (formerly *Agkistrodon*) *acutus* venom. Agkicetin consists of 15 and 14 kDa subunits and is a potent antagonist of VWF-induced platelet agglutination. Reduction and alkylation

of agkicetin caused most of its inhibitory effect on VWF-induced platelet agglutination to be lost, implying that both subunits (and secondary and tertiary structures) are necessary for its activity. Agkistin is composed of 16.5 and 15.5 kDa subunits giving a molecular mass of 32.5 kDa based on SDS-PAGE and mass spectrometry. It blocked ristocetin-induced human platelet agglutination and aggregation in the presence of VWF in a dose-dependent manner. The GPIb binding specificity of agkistin was confirmed by monoclonal antibodies against GPIb, such as AP1 and 6D1, as they completely prevented ^{125}I-agkistin binding to platelets. Intravenous administration of agkistin in mice significantly prolonged the bleeding time, induced transient thrombocytopenia, and inhibited platelet thrombus formation in irradiated mesenteric venules of fluorescein sodium–treated mice *in vivo*. CHH-A, CHH-B (Andrews et al., 1996), GPIb-BP (*Bothrops jararaca*) (Fujimura et al., 1995), tokaracetin (Kawasaki et al., 1995), lebecetin (Sarray et al., 2003), and dabocetin (Zhong et al., 2006) are all low molecular mass, functionally monomeric, heterodimeric snake CLPs targeting GPIb and inhibiting VWF-dependent platelet aggregation.

A number of CLPs recognizing GPIb also activate platelets via this receptor. These CLPs target the GPIb complex by binding solely to the N-terminal 45 kDa fragment and inducing platelet signaling. However, the mode of action through GPIb is not always identical. Alboaggregin B (Peng et al., 1991) from *Cryptelytrops* (*Trimeresurus*) *albolabris* venom is a monomeric heterodimer and the first snake CLP that was identified as a platelet agonist via GPIb. It weakly agglutinated platelets in a calcium-independent manner and failed to activate αIIbβ3. Mamushigin (Sakurai et al., 1998) from *Gloydius* (*Agkistrodon*) *halys blomhoffii* venom induced platelet agglutination at low-shear stress, whereas at high-shear stress, mamushigin blocked platelet aggregation in a dose-dependent manner. TSV-GPIb-BP (Lee and Zhang, 2003) has a structure and function like alboaggregrin B. In particular, the α-subunit of TSV-GPIb-BP is identical to that of alboaggregin-B, and its β-subunits are 94.3% identical in sequence. It directly agglutinated washed human platelets via GPIb in the absence of additional calcium or other cofactors at dosages higher than 5 μg/ml. Mucrocetin (Huang et al., 2004) affected platelets like alboaggregin B, although it has a high molecular mass of 121 kDa and consists of 4 (αβ) subunits. Mucrocetin purified from *Protobothrops* (*Trimeresurus*) *mucrosquamatus* venom targets specific sites on GPIb distinct from flavocetin A (149 kDa) despite being 94.6% identical in sequence. Flavocetins A and B (Taniuchi et al., 1995) were isolated from the habu snake venom (*Protobothrops* (*Trimeresurus*) *flavoviridis*) as platelet antagonists binding to GPIb. Flavocetin A is made up of $(\alpha\beta)_4$ heterodimers. The crystal structure was the first obtained for a multimeric CLP and demonstrated that the tetramerization is mediated by an interchain disulfide bridge between cysteine residues at the C-terminus of the α-subunit and at the N-terminus of the β-subunit in the neighboring αβ-heterodimer (Fukuda et al., 2000). These sequence differences are characteristic for this group of tetrameric CLPs. Taniuchi et al. (2000) reported that flavocetin A induced small platelet agglutinates with washed platelets, a process requiring extracellular calcium. Structural data indicate that mucrocetin and flavocetin A have distinct binding sites on platelets that are probably due to a unique positively charged patch on the binding surface of mucrocetin distinct from flavocetin A. Purpureotin (63 kDa) is a dimer of αβ-heterodimers isolated from *Cryptelytrops* (*Trimeresurus*) *purpureomaculatus* venom. It induced platelet aggregation without any cofactor, and this was totally blocked by echicetin (Li et al., 2004). Agglucetin, a platelet agglutination inducer isolated from *Deinagkistrodon acutus* snake venom, is a 58.8 kDa tetramer composed of 2(αβ) subunits (Wang and Huang, 2001). It induced platelet agglutination in the absence of VWF in a dose-dependent way. Furthermore, αIIbβ3 activation was also detected in platelets treated with agglucetin, which is dependent on GPIb, as crotalin, a GPIb cleaving metalloprotease, could suppress this (Wang et al., 2003).

Unlike the GPIb binding CLPs mentioned above, mucetin, also known as TMVA (Wei et al., 2002), a multimeric CLP from *Protobothrops* (*Trimeresurus*) *mucrosquamatus* venom, bound to and agglutinated platelets via GPIbα, leading to αIIbβ3 activation and platelet aggregation. Mucetin induced strong signaling events, including tyrosine phosphorylation of Syk, LAT, PI3K,

PLCγ2, and weak phosphorylation of FcRγ. Inhibition of αIIbβ3 strongly reduced platelet activation and the signaling events, indicating that activation of αIIbβ3 and binding of fibrinogen are involved in mucetin-induced platelet aggregation (Lu et al., 2004). In platelets stimulated by mucetin, GPIb localized to the Triton-insoluble cytoskeleton fraction considerably more than in resting platelets. FcRγ was also more associated with the cytoskeleton of platelets activated by mucetin (Lu et al., 2005b) than that of resting platelets. Recently, Jennings et al. (2005) reported that Ba25, a CLP from *Bitis arietans* venom, interacted with platelets via GPIb-V-IX, as well as αIIbβ3, and increased fibrinogen binding to platelets. Whether Ba25 binds directly to αIIbβ3 or activates αIIbβ3 indirectly will require further investigation.

C. Snake C-Type Lectin-Like Proteins Binding GPVI

GPVI, a 62–65 kDa glycoprotein, is the major signaling receptor for collagen on platelets. GPVI belongs to the immunoglobulin (Ig) receptor superfamily member closely related to human FcαR and natural killer cell receptors (Clemetson et al., 1999). GPVI forms a complex with FcRγ chain via a positively charged arginine in its transmembrane region. Upon GPVI clustering, FcRγ is phosphorylated by the Src kinases (Fyn and Lyn) on its immunoreceptor tyrosine-based activation motif (ITAM), and transmits signals farther to the tandem SH2 domain-containing tyrosine kinase, Syk. This leads to activation of a downstream signaling cascade to enzymes, including PLCγ2 and phosphoinositide-3 kinase and small G-proteins (Nieswandt and Watson, 2003).

A number of multimeric snake venom CLPs induce platelet activation by clustering GPVI. No venom proteins that bind to and inhibit GPVI are known yet. Convulxin, isolated from the South American rattlesnake (*Crotalus durissus terrificus*), was the first toxin found to activate platelets powerfully via GPVI and induced a signaling cascade similar to that of collagen and collagen-related peptide (CRP) (Polgar et al., 1997; Leduc and Bon, 1998). The binding site for GPVI on convulxin is probably located on the concave surface between the two subunits. Convulxin, a 85 kDa molecule, has a cyclic tetrameric structure $(\alpha\beta)_4$ and also associates noncovalently, which allows it to activate platelets strongly by large-scale clustering GPVI molecules (Murakami et al., 2003; Batuwangala et al., 2004). Convulxin is also known to bind weakly to GPIb, and this could contribute to cross-linking of platelets, although it may only have a minor role in overall signal transduction at the low concentrations normally used to activate platelets (Du et al., 2002b; Kanaji et al., 2003). Alboaggregin A (50 kDa) (Kowalska et al., 1998; Dormann et al., 2001) and alboluxin (120 kDa) (Du et al., 2002a), two CLPs from *Trimeresurus albolabris* venom, also bound to a second surface receptor—GPIb—in addition to GPVI. They both induced powerful signaling to cause strong platelet activation. Interestingly, alboaggregin A also activated Bernard-Soulier platelets and cell lines transfected with GPVI, suggesting that the interaction with GPIb is less essential for activation. However, signal amplification by cross-linking GPIb and GPVI may play a role in the overall platelet activation. Two additional 120 kDa tetrameric $(\alpha\beta)_4$ CLPs from snake venoms, ophioluxin (Du et al., 2002b) from *Ophiophagus hannah* venom and stejnulxin (Lee et al., 2003b) from *Viridovipera* (*Trimeresurus*) *stejnegeri* venom, seem to be more specific for GPVI in activating platelets. The characterization of convulxin directly enabled the cloning and molecular characterization of GPVI and helped to open the field of platelet signal transduction pathways via GPVI. Further investigation on the dual receptor properties of convulxin and convulxin-like CLPs may help to understand better the synergistic mechanisms between GPVI and GPIb in platelet aggregation (Andrews et al., 2003).

D. Snake C-Type Lectin-Like Proteins Binding Integrin α2β1

Besides GPVI, the α2β1 integrin is another major collagen receptor on platelets, mainly mediating adhesive interactions and generating intracellular signals that help to stabilize the thrombus. Snake venom CLPs that interact with α2β1 are rhodocetin, EMS16, bilinexin, and aggretin

(rhodocytin). EMS16 is a heterodimer isolated from *Echis multisquamatus* venom with a molecular mass of 33 kDa, and is a selective inhibitor of integrin α2β1 (Marcinkiewicz et al., 2000). The crystal structure of EMS16 complexed with the integrin α2-I domain showed that EMS16 completely occupies the collagen binding site on the α2-I domain without interacting with the manganese ion and residues of the metal ion-dependent adhesion site (MIDAS). It also revealed that the collagen binding site of α2-I domain does indeed lie on the concave surface in EMS16, and direct binding sites are located at both ends of the surface (Horii et al., 2004). Rhodocetin, isolated from *Calloselasma rhodostoma* venom, is a 29 kDa CLP that functions as a platelet antagonist via α2β1 (Wang et al., 1999). It is the only one characterized so far where the α- and β-subunits are noncovalently associated and not attached via a disulfide bond. Bilinexin is a 110 kDa protein from *Agkistrodon bilineatus* venom with multiple hetero subunits that agglutinates fixed platelets, washed platelets, and platelet-rich plasma, without obvious activation, via α2β1 as well as GPIb (Du et al., 2001). Aggretin (rhodocytin), also identified from *Calloselasma rhodostoma* venom, is a 29 kDa heterodimeric CLP. It was first reported to activate platelets through α2β1 as well as GPIb, but not GPVI (Chung et al., 1999; Navdaev et al., 2001b). However, Bergmeier et al. (2001) observed later that aggretin could activate murine platelets lacking all three receptors, α2β1, GPVI, and GPIb. Recently, Suzuki-Inoue et al. (2006) using aggretin affinity chromatography and mass spectrometry identified a novel receptor, CLEC-2, in platelets. CLEC-2 is a 32 kDa C-type lectin receptor, first found expressed in human liver cells, that has a single carbohydrate recognition domain and a single cytoplasmic YXXL domain. Whether CLEC-2 alone is enough for platelet activation by aggretin/rhodocytin is still unclear. The discovery of CLEC-2 in platelets and its potential in signaling pathways could be important for understanding platelet activation during thrombosis. The physiological ligand for CLEC-2 has not yet been identified.

E. Snake C-Type Lectin-Like Proteins Interacting with Platelets via Undefined Receptors

There are a few CLPs that activate platelets where the receptors involved were not characterized. Agkaggregin (Liu et al., 2002), from the venom of *Deinagkistrodon acutus*, is a functionally monomeric platelet aggregation inducer with a simple αβ-dimer structure. It is not clear which receptors it bound to on platelets and whether it induced αIIbβ3 activation. Wang et al. (2001) reported that rhodoaggretin, a di-heterodimeric CLP, is a potent platelet activator, without indicating which receptor was involved. Crotacetin (Radis-Baptista et al., 2006), a 70 kDa CLP from *Crotalus durissus terrificus* venom, is also able to promote platelet aggregation by an undefined mechanism. Table 18.1 lists snake C-type lectins interacting with platelets and some of their properties.

III. SNAKE CLPs TARGETING PLASMA PROTEINS

A. Snake C-Type Lectin-Like Proteins Binding VWF

VWF is a multimeric, multidomain protein that is required for arresting platelets via GPIb on injured endothelium under high shear. In general, the adhesive properties of VWF are strictly regulated, and plasma VWF does not interact with circulating platelets unless ultralarge multimers are present. VWF interacts with its platelet receptor GPIb *in vitro* only in the presence of the antibiotic ristocetin or botrocetin-like snake toxins. Botrocetin from *Bothrops jararaca* venom was the first CLP identified to induce platelet aggregation in a VWF-dependent manner like ristocetin (Usami et al., 1993). The crystal structure of botrocetin together with the earlier biochemical data indicated that it is a disulfide-linked heterodimer. Although the β-chain of botrocetin has a Mg^{2+} binding site, botrocetin still modulates VWF binding to GPIb and induces platelet agglutination in the presence of EDTA (Sen et al., 2001). Crystallography studies of the botrocetin–VWF A1 complex demonstrated that botrocetin indeed binds to A1 domain through its concave region of the molecule; however, it does

TABLE 18.1
Snake C-Type Lectin-Like Proteins That Bind to Platelets

Protein Name	Target	Roles +/–	Subunits	Species	References
Echicetin	GPIb	–	αβ	*Echis carinatus*	Polgar et al., 1997; Navdaev et al., 2001a
Agkicetin	GPIb	–	αβ	*Agkistrodon acutus*	Chen and Tsai, 1995
Agkistin	GPIb	–	αβ	*Agkistrodon acutus*	Yeh et al., 2001
CHH-A and -B	GPIb	–	αβ	*Crotalus horridus horridus*	Andrews et al., 1996
Tokaracetin	GPIb	–	αβ	*Trimeresurus tokarensis*	Kawasaki et al., 1995
Lebecetin	GPIb	–	αβ	*Macrovipera lebetina*	Sarray et al., 2003
Dabocetin	GPIb	–	αβ	*Daboia russellii siamensis*	Zhong et al., 2006
GPIb-BP	GPIb	–	αβ	*Bothrops jararaca*	Fujimura et al., 1995
Agglucetin	GPIb	+	2(αβ)	*Agkistrodon acutus*	Wang et al., 2001
Alboaggregin B	GPIb	+	αβ	*Trimeresurus albolabris*	Peng et al., 1991
Mamushigin	GPIb	+	αβ	*Agkistrodon halys blomhoffii*	Sakurai et al., 1998
TSV-GPIb-BP	GPIb	+	αβ	*Trimeresurus stejnegeri*	Lee and Zhang, 2003
Mucrocetin	GPIb	+	4(αβ)	*Trimeresurus mucrosquamatus*	Huang et al., 2004
Flavocetin A	GPIb	+	4(αβ)	*Trimeresurus flavoviridis*	Fukuda et al., 2000; Taniuchi et al., 2000
Purpureotin	GPIb	+	2(αβ)	*Trimeresurus purpureomaculatus*	Li et al., 2004
Mucetin	GPIb	+	4(αβ)	*Trimeresurus mucrosquamatus*	Wei et al., 2002; Lu et al., 2004
Ba25	GPIb	+	αβ	*Bitis arietans*	Jennings et al., 2005
Convulxin	GPVI, GPIb	+	4(αβ)	*Crotalus durissus terrificus*	Polgar et al.,1997; Murakami et al., 2003
Alboaggregin A	GPVI, GPIb	+	2(αβ)	*Trimeresurus albolabris*	Dormman et al., 2001; Kowalska et al., 1998
Alboluxin	GPVI, GPIb	+	4(αβ)	*Trimeresurus albolabris*	Du et al., 2002a
Ophioluxin	GPVI	+	4(αβ)	*Ophiophagus hannah*	Du et al., 2002b
Stejnulxin	GPVI	+	4(αβ)	*Trimeresurus stejnegeri*	Lee et al., 2003b
EMS16	α2β1	–	αβ	*Echis multisquamatus*	Marcinkiewicz et al., 2000; Horri et al., 2004
Rhodocetin	α2β1	–	αβ	*Calloselasma rhodostoma*	Wang et al., 1999
Bilinexin	α2β1, GPIb	+	**	*Agkistrodon bilineatus*	Du et al., 2001
Aggretin	CLEC2	+	αβ	*Calloselasma rhodostoma*	Navdaev et al., 2001b; Bergmeier et al., 2001; Suzuki-Inoue et al., 2006
Agkaggregin	ND	+	αβ	*Agkistrodon acutus*	Liu et al., 2002
Rhodoaggretin	ND	+	2(αβ)	*Calloselasma rhodostoma*	Wang et al., 2001
Crotacetin	ND	+	2(αβ)	*Crotalus durissus terrificus*	Radis-Baptista et al., 2006

**, multihetero subunits; ND, not determined.

not alter the A1 conformation to a high-affinity state to increase GPIb binding, as most researchers had previously thought (Fukuda et al., 2002). So it raised the question of whether botrocetin could also bind directly to GPIb and form a trimeric botrocetin–GPIb–VWF A1 domain complex, which was subsequently confirmed by a crystal structure of all three components (Fukuda et al., 2005). Botrocetin has developed into a standard reagent for diagnosing von Willebrand disease

and GPIb-associated diseases like Bernard-Soulier syndrome. Obviously, a better understanding of the mechanism of botrocetin interacting with VWF and GPIb has benefited platelet studies and has broad applications in clinical diagnostics. So far, bitiscetin (Hamako et al., 1996) and bitiscetin-2 (*Bitis arietans* venom) (Obert et al., 2006) are the only other two CLPs that induce platelet agglutination via GPIb and VWF. The crystal structure of the bitiscetin–VWF A1 complex clearly showed that the α-helix 5 of the A1 domain binds to the concave domain of bitiscetin. Like botrocetin, bitiscetin promotes VWF binding to GPIb by interacting with both proteins and not by causing conformational changes in VWF A1 (Maita et al., 2003). In contrast, bitiscetin-2 mimics type III collagen by binding to the VWF A3 domain and inducing a conformational change in the A1 domain, which favors GPIb binding, therefore inducing platelet agglutination (Obert et al., 2006). However, collagen interacts with VWF only under high shear force. Thus, bistiscetin-2 interacting with VWF A3 and A1 domains could be a good *in vitro* model for interpreting collagen-VWF-GPIb interactions upon vessel injuries and the initiation of platelet aggregation.

B. Echicetin Binding IgMκ

Echicetin, a heterodimeric snake CLP from *Echis carinatus* venom, is known to bind specifically to platelet GPIb and prevent platelet activation, as mentioned earlier in this chapter. On the other hand, Navdaev et al. (2001a) showed that echicetin agglutinates platelets in plasma, and IgMκ is the specific protein that bridges echicetin and GPIb. Echicetin itself does not agglutinate washed platelets unless purified IgMκ is added. This mechanism may be due to the pentameric structure of IgM, which can bind up to five echicetin molecules, and therefore can cluster several echicetin molecules bound to the same platelet or different platelets, causing platelet agglutination via GPIb (Figure 18.2). This hypothesis is supported by the fact that adding avidin and biotinylated echicetin to washed platelets also induces platelet agglutination through a similar clustering mechanism. Platelet agglutination by echicetin and either IgMκ or avidin induced P-selectin expression and activation of αIIbβ3, as well as tyrosine phosphorylation of Lyn, Syk, and several proteins around 70, 90, and 120 kDa. This may explain why echicetin induces thrombocytopenia *in vivo*. This exciting result indicates that clustering GPIb is sufficient to induce platelet agglutination and, depending on the degree of clustering, also aggregation. However, heterodimeric echicetin, unlike some other CLPs that are naturally multimeric, has to use a plasma component to cause platelet aggregation. In addition, this work also indicates us that it is necessary to study CLPs interacting with platelets in both washed systems and platelet-rich plasma, as well as with the commonly used fixed platelets. Even when CLPs do not aggregate PRP but cause thrombocytopenia in small animals, they may have mechanisms involving other blood cells or endothelial cells.

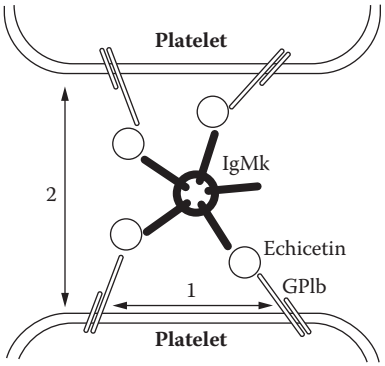

FIGURE 18.2 Diagram of cross-linking of platelet GPIb by echicetin-IgMκ complexes. 1 indicates clustering in the membrane of one platelet; 2 indicates cross-linking between platelets.

C. Snake C-Type Lectin-Like Proteins Binding Thrombin

Bothrojaracin is a selective thrombin inhibitor from the venom of *Bothrops jararaca*. It is a 27 kDa protein composed of 15 kDa and 13 kDa subunits. Each subunit has a high degree of identity with other snake CLPs. Bothrojaracin forms a noncovalent 1:1 complex with thrombin in a Ca^{2+}-independent manner. Heparin and the C-terminal peptide of hirudin compete for bothrojaracin binding to thrombin, indicating that it targets both anion binding exosites 1 and 2 of thrombin. Surprisingly, it does not interact with the catalytic site of thrombin, so that it does not change its catalytic activity on small peptide substrates. Bothrojaracin is able to block a number of thrombin functions, such as clotting of fibrinogen, thrombomodulin binding, and factor V, protein C, and platelet activation (Zingali et al., 1993; Arocas et al., 1996). Bothrojaracin also binds to the thrombin precursor, prothrombin, and prevents its activation to thrombin, which is mediated by proexosite 1 (Monteiro et al., 2001). Using a thrombin affinity column, Phe-Pro-Arg-chloromethylketone-thrombin-Sepharose, followed by analysis of bound materials by Western blot and ELISA with anti-bothrojaracin serum, Castro et al. (1999) found that bothrojaracin-like proteins were widely distributed among venoms of *Bothrops* species. Among them, bothroalternin from *Bothrops alternatus* venom was found to have one-third of the inhibitory effect toward thrombin-induced platelet activation of bothrojaracin at equivalent concentrations (Castro et al., 1998). Natural thrombin antagonists such as the bothrojaracin family are interesting models for candidate drugs in thrombotic diseases.

D. Snake C-Type Lectin-Like Proteins Binding Factor IX or X

Snake venom contains components that target coagulation factors extensively and have either activating or inhibitory roles in blood clot formation. Before the primary structure of IX/X binding protein (IX/X-bp) from *Protobothrops* (*Trimeresurus*) *flavoviridis* venom was determined to be a C-type lectin-like protein (Atoda et al., 1991), serine proteases and metalloproteinases were the only protein families thought to interact with coagulation components. IX/X-bp was the first snake CLP that was identified as anticoagulant via tight binding to gamma-carboxyglutamic acid (Gla) domains of factors IX, IXa, X, and Xa (Atoda et al., 1994). Blocking the Gla domain stops its association with phospholipids exposed on the cell (especially platelets) surfaces and results in the loss of all enzymatic activities. IX/X-bp is a typical heterodimeric CLP with both subunit sequences homologous to each other and also to sugar binding lectins. In addition, the crystal structures of the IX/X-bp/IX-Gla (one to forty-six amino acids) complex clearly showed that the Gla domain of FIX binds to the concave domain of IX/X-bp, and that Mg^{2+} ions greatly enhance the affinity between the Gla domain and IX/X-bp by bridging the two molecules (Shikamoto et al., 2003). Other IX/X binding CLPs identified so far include Jararaca IX/X-bp, ECLV IX/X-bp, and halyxin (Sekiya et al., 1993; Chen and Tsai, 1996; Koo et al., 2002). They have similar structures and the common ability to bind factor IX or X, but not other vitamin K–dependent coagulation factors in a Ca^{2+}-dependent manner.

In spite of the similarities in the primary and tertiary structures with IX/X binding proteins, TSV-FIX-BP (*Viridovipera* (*Trimeresurus*) *stejnegeri* venom) and AHP IX-bp (*Gloydius* (*Agkistrodon*) *halys* Pallas venom) are specific inhibitors of factor IX only, not factor X (Lee et al., 2003a; Zang et al., 2003). Nonetheless, Atoda et al. (1998) found an anticoagulation CLP, X-bp in *Deinagkistrodon acutus* venom, acting by binding the Gla domain of factor X. The crystal complex of X-bp with the Gla-domain (amino acid residues 1–44) of factor X revealed that the important structures in the Gla domain for membrane binding are buried in the complex, which inhibits factor X binding to the phospholipid membrane and prevents factor X activation (Mizuno et al., 2001). In addition, Xu et al. (2000) reported that ACF I and ACF II, two CLPs from *Agkistrodon acutus* venom, are specific inhibitors of factor X/Xa. A tentative explanation for the substrate specificity of factor IX/X, IX, or X binding CLPs is that the different amino acid residues on the concave surface provide the specific binding surface for each coagulation factor.

IV. CLASS P-IV SNAKE VENOM METALLOPROTEASES CONTAINING C-TYPE LECTIN-LIKE DOMAINS

The snake venom metalloprotease (SVMP) family is distributed in almost all venomous snakes and has evolved to affect numerous physiological activities of prey. The SVMP family is classified into four categories according to their primary structures and domain composites. P-I class metalloprotease has a single zinc-metalloprotease domain typical of HEXXH (His-Glu-X-X-His) sequence, while P-II contains P-I and a disintegrin-like domain in the C-terminus. P-III is composed of P-II and an additional cysteine-rich domain. Lastly, P-IV has an extra heterodimeric C-type lectin-like domain that is linked to the metalloprotease by a disulfide bridge during posttranslational processing (Jia et al., 1996; Ramos and Selistre-de-Araujo, 2006).

SVMPs were first identified as EDTA-inhibitable fibrinogenases due to the presence of metalloprotease domain. Snake disintegrins are proteins of about 7 kDa that are specific inhibitors of αIIbβ3 or αVβ3 involving an RGD (Arg-Gly-Asp) or similar sequence (Huang, 1998; McLane et al., 2004). Disintegrin-like domains usually contain a SECD (Ser-Glu-Cys-Asp) sequence, which may be a better ligand for α2β1 based on the fact that some disintegrin-like/cysteine-rich proteins mainly prevent collagen-induced platelet aggregation (Shimokawa et al., 1997; Liu et al., 2000). Serrano et al. (2006) discovered recently that the cysteine-rich domain of jararhagin, a P-III SVMP, is a ligand for the A1 domain of VWF. In addition, snake CLPs are known to target major platelet receptors, including GPIb, GPVI, α2β1, and some plasma components (Clemetson et al., 2005). As a result, multidomain SVMPs either powerfully inhibit or activate platelet functions or coagulation cascades.

RVV-X from *Daboia russellii* venom is the best-characterized P-IV SVMP and acts as a factor X activator. RVV-X is composed of a 60 kDa heavy chain responsible for the catalysis and two light chains homologous to CLPs and thought to be regulatory. RVV-X activates factor X in a Ca^{2+}-dependent manner. As previous findings indicated that C-type lectin-like proteins can bind factor IX or X, it is likely that the light chains of RVV-X recognize some portion of the zymogen factor X (Takeya et al., 1992; Tans and Rosing, 2001).

CA-1 is a prothrombin activator purified from *Echis carinatus* venom (Yamada et al., 1996). It is also a P-IV SVMP consisting of three subunits, 62, 17, and 14 kDa, under reducing conditions. The two small subunits have amino acid sequences like snake CLPs. The enzyme primarily recognizes the Ca^{2+}-bound conformation of the Gla domain in prothrombin via the regulatory subunit, and the subsequent conversion of prothrombin to active thrombin is catalyzed by the 62 kDa subunit. Multactivase is a novel prothrombin-activating enzyme isolated from *Echis multisquamatus* venom (Yamada and Morita, 1997), and multisquamase (Petrovan et al., 1997) and ecamulin (Solovjov et al., 1996) are also prothrombin activators isolated from the same venom by different groups. Since they are all P-IV-like metalloproteases, activate prothrombin-required Ca^{2+}, and have a similar molecular mass, substrates, and mode of action, they are probably the same protein. Ecarin is a P-III SVMP, therefore lacking C-type lectin-like domains, but activates prothrombin in a Ca^{2+} independent way (Nishida et al., 1995). Ecarin must recognize prothrombin in a different way from the SVMPs containing a C-type lectin-like domain, which is worth further investigation. SVMP containing a C-type lectin-like domain or CLPs that target the coagulation cascade are shown in Figure 18.3.

V. SUGAR BINDING SNAKE LECTINS

Snake venoms also contain galactose/lactose binding lectins. Such snake lectins, in general, are not very toxic. They are only found to agglutinate erythrocytes, which are highly calcium dependent and mediated by the carbohydrate recognizing domain (CRD) of the lectins. The pathophysiological consequences of agglutinating erythrocytes are unclear, and other roles of snake lectins are still

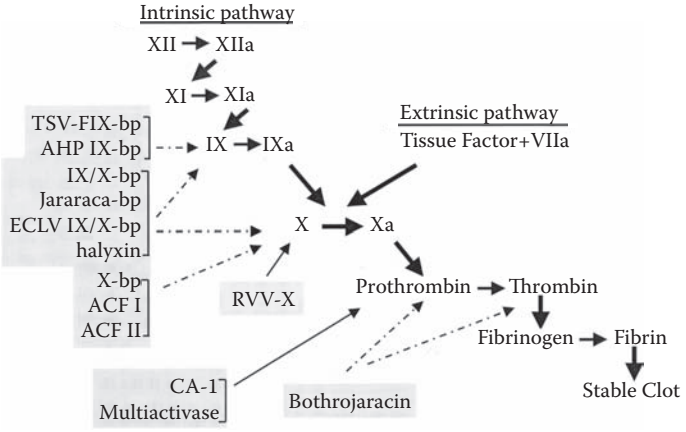

FIGURE 18.3 SVMPs containing C-type lectin-like domain or CLPs (shaded in gray) targeting the coagulation cascade. Broken arrows represent inhibitory effects, whereas solid arrows represent activating effects.

to be found (Du et al., 2006). Recently, BjcuL (*Bothrops jararacussu* venom) has been shown to inhibit tumor cell lines and endothelial cell growth; however, the detailed molecular mechanism was not explained (de Carvalho et al., 2001). In contrast, some lectins in animals play important roles in host defense by recognizing carbohydrates that exist exclusively on invading pathogens, so perhaps the snake lectins contribute to the antibacterial effects of snake venom, important for preventing infection during the slow digestive process.

Snake venom lectins are homodimers. Each monomer is made up of ~135 amino acids and four pairs of intrachain disulfide bridges. Cys86–Cys86 is an intermolecular disulfide link for most snake lectins; however, Cys86 is not conserved in BfL1 and BfL2, where they are replaced by arginine and serine residues, respectively (Zha et al., 2001; Abreu et al., 2006). Even though disulfide bonds are important for the biological activities of snake lectins, LmsL (*Lachesis muta stenophrys* venom) is an exception since addition of dithiothreitol (DTT) to reduce disulfide bonds did not abolish its sugar binding properties (Aragon-Ortiz et al., 1996). Most of the snake lectins that were investigated recognize galactose/lactose specifically, except for BfL2, which is reported to be a mannose binding protein. This may be due to replacement of the well-preserved QPD (Gln-Pro-Asp) sequence in the CRD in other lectins by the EPN (Glu-Pro-ASN) motif in BfL2, as well as the Tyr100/Phe100 substitution for Ser100. Snake lectins have been recognized for years primarily as saccharide binding proteins, but RSL (*Crotalus atrox* venom) is found to induce platelet aggregation as well (Wilson-Byl et al., 1991). RSL is similar in size to other snake lectins; its primary structure has a 70 to 90% homology to other snake lectins, including BAL, TSL, LSL, APL CaL, and BiL (Abreu et al., 2006). Recently, the x-ray crystal structure of the RSL-lactose complex revealed that RSL is a decamer, with two pentamers arranged symmetrically back to back (Walker et al., 2004). Each monomer contains a Ca^{2+} ion where lactose binds. This multimeric structure of RSL favors cell aggregation by receptor cross-linking, which may be why it induces platelet aggregation. How the crystal structure of RSL is related to that of other snake lectins awaits more information.

VI. FUTURE PROSPECTS

Snake CLPs have many diverse targets in the blood system. The detailed studies on convulxin led to the cloning and molecular characterization of GPVI and helped to open the field of platelet signal transduction pathways via GPVI. It is likely that the newly discovered platelet receptor CLEC2 characterized through studies with aggretin (rhodocytin) will also contribute to a better understanding of platelet activation mechanisms. Further investigations on the GPVI-GPIb dual receptor targeting

properties of several CLPs may help to understand synergistic mechanisms involving GPVI and GPIb in platelet activation. Besides, several researchers are looking for novel roles of CLPs beyond platelets and coagulation. For example, lebectin was found to be a potent angiogenesis inhibitor, binding to α5β1 and αv-containing integrins (Pilorget et al., 2007); crotacein, a convulxin-like lectin, exhibits antimicrobial activities (Radis-Baptista et al., 2006). Snake venom CLPs are powerful tools in platelet function and hemostasis studies (Clemetson, 1998; Morita, 2005), and undoubtedly will have more applications with growing understanding of their other functions.

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Nerve growth factor (NGF) is a member of the neutrotrophin family of proteins that plays a significant role in the maintenance and survival of neuronal cells. Thus, it is somewhat incongruous that a protein designed to enhance survival is a component of snake venom, a specialized fluid responsible for the immobilization and killing of prey. However, it is also evident that members of this family of proteins, in shaping the development of the nervous system, are capable of inducing apoptosis. In addition, the spectrum of effects of NGF on PC12 cells in culture may well point to different roles for snake venom NGF. Half a century has now passed since crude venom was first shown to contain a substance that promoted fiber outgrowth in spinal ganglia, subsequently identified as an NGF-like molecule and shown to be present in the major families of venomous snakes. In the interim, an important body of information has accumulated on the purification, characterization, and role of NGF in snake venom. Recent advances in gene technology and proteomics have further assisted our understanding of snake venom NGFs, and these advances are the primary focus of this review.

I. INTRODUCTION

A nerve growth-stimulating factor was first isolated from sarcomas by Cohen and Levi-Montalcini (1954). These findings were inspired by earlier observations that transplantation of mouse sarcomas into chick embryos accelerated ganglia differentiation and caused an atypical distribution of nerve fibers. Cohen and Levi-Montalcini (1954) demonstrated that cell-free homogenates of the sarcomas duplicated the *in vivo* observations. The assay involved a hanging drop preparation containing plasma chick extract and the material to be tested. In attempting to isolate and identify the growth-promoting component of the tumor, crude snake venom, as a source of phosphodiesterase, was added, which increased the activity of the tumor extracts. They subsequently showed that the snake venom was a source of a potent growth-promoting agent (Cohen and Levi-Montalcini, 1956). Indeed, on a dry weight basis, the venom was $3–6 \times 10^3$ times more active than the tumor extracts in promoting nerve fiber outgrowth in spinal ganglia *in vitro*. While the factor was not identified at this time, it was shown to be heat labile, nondialyzable, and most likely protein in nature. The factor was

present in two distinct species of snakes, *Agkistrodon piscivorus* (eastern cottonmouth) and *Crotalus adamanteus* (eastern diamondback rattlesnake). The presence of a nerve growth-promoting factor in a modified salivary gland in the snake led Levi-Montalcini and Booker (1960) to the submaxillary gland of the mouse, which proved to be a source of more potent material. Injection of this factor into mice caused a marked increase in sympathetic ganglia. However, injection of a highly purified form also resulted in moderate toxicity, which may be relevant to the presence of this factor in snake venom (Cohen, 1960). The specific activity of the mouse protein was tenfold greater than the most purified of the snake venom preparations and appeared to be greater in size as determined by ultracentrifugation. Cross-reactivity was also observed with antisera raised against the mouse protein. McDonald et al. (1991) reported the crystal structure of the murine NGF dimer at 2.3 Å resolution. The protomer structure consisted of three antiparallel pairs of β-strands forming a flat surface through which two subunits associate. Variability between different NGF-related molecules was largely confined to four loop regions that were suggested to influence receptor specificity.

II. NGF ISOLATION AND CHARACTERIZATION

As indicated above, snake venom featured prominently as an early source of a nerve growth factor (Cohen and Levi-Montalcini, 1956). Once this factor had been characterized from the submaxillary gland of the mouse (Cohen, 1960; Bocchini and Angeletii, 1969; Pearce et al., 1972) it was inevitable that a similar approach would be employed with snake venom, such as that from the Indian cobra, *Naja naja*, a source of the second most potent form of the growth factor. A series of chromatographic steps, including size separation and cation and anion exchange, were employed to purify *N. naja* NGF. The sedimentation velocity of the protein was 2.37S, which corresponded well to the value for the mouse protein (2.42S), and the molecular size was estimated to be between 25 and 28 kDa. The chemical and biological properties of the *N. naja* NGF were similar to those of the mouse protein, and there was evidence for the presence of carbohydrate in the *N. naja* protein. This very likely represents post-translational modification (discussed in more depth later). In keeping with the known activities of NGF, the purified *N. naja* protein was devoid of *in vitro* or *in vivo* toxic effects. Further characterization of the *N. naja* NGF revealed that the molecular size was 28 kDa, and under reducing conditions was approximately half this size, providing evidence that NGF was present as a dimer, similar in subunit structure to mouse NGF (Hogue-Angeletti et al., 1976). Analysis of *N. naja* NGF allowed for the construction of a tentative primary structure revealing 64% identity with mouse NGF (which agrees well with the value of 66% derived from the cDNA precursor sequence).

Subsequent to those pioneering studies with *N. naja* NGF, a series of reports on NGF purified from different snake venoms were described. The development of monoclonal antibodies against NGF from *Macrovipera lebetina* provided a useful reagent for comparing NGFs in different snake species (Arumae et al., 1987). These antibodies recognized two partially overlapping epitopes of *M. lebetina* NGF that are not involved directly in its biological activity. This was demonstrated by a failure of the antibodies to interfere with the capacity of NGF to induce neurite outgrowth in sympathetic ganglian chick explants and in PC12 cells. Both epitopes were also present in β-NGF from the mouse, as well as NGFs from several snake venoms (*Vipera ursini*, *Vipera berus berus*, *Echis carinatus*, *Bungarus caeruleus*, *Gloydius halys* (formerly *Agkistrodon halys)*, *Naja naja oxiana*, *Naja naja atra*, and *N. naja*). These data pointed to a high degree of relatedness between NGFs from the different snakes, but the approach also revealed considerable variation in the size of the cross-reacting NGFs. NGF has also been purified and characterized from the venom of other snake species, such as *Bothrops atrox* (Glass and Banthrope, 1975), *Crotalus adamentus* (Perez-Polo et al., 1978), and *Naja sputatrix* (Koh et al., 2004). A comparison of the characteristics of the different snake venom NGFs investigated to date appears in Table 19.1. It can be seen that NGFs have been purified from a range of snakes, including those from viper genera, *Vipera* and *Echis*, found in Europe and Asia; the pit viper *Gloydius*, found in Europe and Asia; and the largely Asian elapids,

TABLE 19.1
Properties of Purified Human, Mouse, and Snake Venom NGFs

Name	Species	Accession Number (β-subunit)	Yield (mg/g venom)	Structure	Activity	Mol. Wt. (Da)	Glycosylation	Reference
NGF	*Homo sapiens*	P01138	—	Complex	Yes	130,000	Unknown	Goldstein et al., 1978
β-NGF	*Mus musculus*	NP_038637	—	Part of complex	Yes (3–16 ng/biol. unit)	13,000	Unknown	Bocchini and Angeletti, 1969
α-NGF	*Mus musculus*	NP_035045	—	Part of complex	None	27,000	Unknown	Ronne et al., 1984
γ-NGF	*Mus musculus*	NP_032719	—	Part of complex	Serine protease	26,400	Unknown	Ronne et al., 1984
NGF complex	*Crotalus adamanteus*	AAG30924	—	χβ2 complex	Yes (8–16 ng/biol. unit)	54,000	Unknown	Perez-Polo et al., 1978
β-NGF	*Daboia* (*Vipera*) *russellii*	AAA03282	3.6	—	Yes	17,500	Yes	Koyama et al., 1992; Siigur et al., 1987
NGF complex	*Vipera lebetina*	AAV64846	3.9	Multimer	Yes	32,500	Yes	Siigur et al., 1985, 1987
NGF complex	*Vipera berus berus*	—	2.3	Multimer	Yes	15,000	Unknown	Siigur et al., 1986
NGF complex	*Echis multisquamatus*	—	—	Multimer	Yes	37,000	Yes	Khamidov et al., 1989
NGF complex	*Echis carinatus*	—	2.5		Yes	32,500	Unknown	Siigur et al., 1987
NGF complex	*Gloydius halys*	—	2.0		Yes	32,500	Unknown	Siigur et al., 1987
β-NGF	*Naja naja*	P01140	1.5	Dimer	Yes	14,000	Yes	Hogue-Angeletti et al., 1976
NGF complex	*Naja naja oxiana*	—	3.1	Dimer	Yes	32,500	Unknown	Siigur et al., 1987
NGF complex	*Naja naja atra*	P61898	5.5	Dimer	Yes	32,500	Unknown	Siigur et al., 1987
β-NGF	*Naja sputatrix*	AAS94268	—	Monomer	Yes	14,000	Unknown	Koh et al., 2004

Naja. The yield of purified NGF from these venoms was between 1 and 5 mg per gram, demonstrating that these proteins are of low abundance in the venoms. Activity was demonstrated using the neurite outgrowth assay for all the purified snake venom NGFs, and many have been shown to be glycosylated. It is evident that snake venom NGFs are all related and have similar biological characteristics, but differ in subunit structure from one another and in comparison to human and mouse NGF.

III. BIOLOGICAL PROPERTIES OF SNAKE VENOM NGF

In order to investigate the biological activity of snake venom NGF, it is important first to appreciate how NGF functions in a well-described system. The majority of analyses on NGF have been carried out in the mouse. Considerably less data are available on human NGF, although the subunit structure appears to be similar to that of mouse NGF. However, the human form of NGF has been shown to be present as a high molecular weight (130,000 Da) complex (Goldstein et al., 1978). The 7S precursor form of mouse submandibular NGF is different from other forms of NGF in that it is composed of α- and γ-subunits as well as the β-chain (Table 19.1). The 7S form is processed to the mature, dissociated β-form, which binds to cell surface receptors on target cells (Bradshaw et al., 1994). The target cells for NGF are restricted to cholinergic neurons in the central nervous system and to neural crest-derived cells of sympathoadrenal origin in the peripheral system (Levi-Montalcini et al., 1996). NGF binds through high- and low-affinity receptors, p75 and $p140^{trk}$, respectively (Johnson et al., 1986; Kaplan et al., 1991). NGF binding to $p140^{trk}$ activates positive signals, whereas binding to p75 is known to activate both positive and negative signals. Binding of NGF to $p140^{trk}$ (TrkA) occurs with an affinity of 10 nM, inducing NGF-dependent tyrosine phosphorylation of TrkA and initiating downstream signaling, leading to neurite survival and differentiation (Kaplan et al., 1991). Two other Trk receptors, TrkB and C, respond to other members of the neurotrophin family (Klein et al., 1991; Lamballe et al., 1991). Conversely, NGF binding to p75 has been demonstrated to initiate neuron cell death (Barrett, 2000; Casaccia-Bonnefil et al., 1999; Friedman, 2000). However, it has been established that p75 enhances the ability of TrkA to respond to NGF in neurons (Barker and Shooter, 1994; Hantzopoulos et al., 1994), and that binding of NGF to p75 may initiate pro-survival pathways independent of TrkA (Brann et al., 1999; Dobrowsky and Carter, 1998; Roux et al., 2001; Mamidipudi et al., 2002).

While neuronal cells are the major targets for NGF, there is also evidence that non-neuronal cells respond to this factor. NGF triggers respiratory burst activity in monocytes, increases the proliferative response in splenocytes, is chemotactic for human polymorphonuclear leukocytes, and leads to mast cell degranulation (Kostiza and Meier, 1996). NGF has also been implicated in wound healing and vascular permeability (Li et al., 1980; Otten et al., 1984).

These data suggest that non-neuronal cells possess receptors for NGF. Indirect evidence for receptors was demonstrated by the observation that release of granules and histamine from mast cells was enhanced by phospholipid and prevented by albumin (Bruni et al., 1982). More direct evidence was presented by Pearce and Thompson (1986), who showed that this release was Ca^{2+} dependent and was abrogated by anti-NGF antibodies. It seems likely that the receptor involved is TrkA, since both mRNA for TrkA and the protein were detected in mast cells responding to NGF (Horigome et al., 1993). Evidence of intracellular signaling was obtained using a phosphotyrosine antibody and downstream serotonin release from mast cells.

These observations with mammalian NGF point to a broader role for this protein and also provide explanations for the potential effects of venom NGFs on mammalian systems. Kostiza and Meier (1996) have estimated an approximate yield of 0.5% (w/w) for NGF in snake venom and an injection amount of 50 μg at the bite site. For the genus *Oxyuranus* (taipan), the average amount of venom injected is approximately 20 mg (Morrison et al., 1982, 1984), high when related to other venomous snakes and accountable for the extreme toxicity associated with envenomation by these snakes. A rough estimate of the amount of NGF in *Oxyuranus* venom, based on the intensity of spots from

two-dimensional gel electrophoresis (2DE) and identified by mass spectrometry, is 0.1 to 0.5% of total venom protein (which translates to 20–100 μg of NGF; Earl et al., 2006). This is of the same order as estimated by Kostiza and Meier (1996). While some NGF would be lost or sequestered at the bite site, a significant amount would nevertheless appear in the circulation and have the potential to exert some physiological effects on nonneuronal cells or tissues. The capacity of cobra NGF to cause plasma extravasation at the injection site could be an indication that this protein enhances the vulnerability of different tissue to other toxic components in the venom. Another possibility, as suggested by Levi-Montalcini (1987), is that NGF acts as a carrier or chaperone protein to deliver various toxins (e.g., phospholipases) to target cells that lack receptors for these toxins. However, there is no substantive evidence to support this. It has been known for some time that mammalian muscle tissue is capable of producing significant quantities of NGF that can alter the plasticity of neuromuscular junctions (Bennett, 1983). It is curious that although snake venom NGF has been shown to be lacking in toxicity, the neuromuscular junctions on which they are active are also the site of action of the post-synaptic neurotoxins that are abundant in elapid venoms.

IV. CLONING OF VENOM GLAND NGF cDNA

Cloning of the cDNA for a variety of genes has relied directly or indirectly on the primary sequence of the corresponding protein. In the case of NGF, the complete amino acid sequence of the 2.5S form from the male mouse submaxillary gland was established by digestion into peptides with a variety of proteases, followed by Edman degradation (Hogue-Angeletti and Bradshaw, 1979). These data revealed a protein of 108 amino acids. Tryptic peptide mapping, cyanogen bromide cleavage, and peptide sequencing had provided the basis for the construction of a tentative primary structure of *N. naja* NGF (Hogue-Angeletti et al., 1976). This derived sequence showed 64% identity with mouse NGF. This degree of identity allowed Selby et al. (1987) to utilize a mouse NGF gene probe to isolate NGF clones from a *Naja naja siamensis* cDNA library and clone a full-length cDNA sequence. The peptide sequence derived from this cDNA predicted a 241 amino acid protein, with a molecular mass of 27 kDa. The organization of this precursor was similar to that of the mouse and is consistent with other NGF cDNAs identified since. These NGF precursors contain a presumptive 18 amino acid signal sequence, which would allow translocation of the nascent protein into the endoplasmic reticulum or secretion from the gland (Figure 19.1). This is followed by a propeptide (proNGF) of approximately 109 amino acids, which has been suggested to facilitate folding of the mature NGF. Proteolytic processing to remove the propeptide from the precursor protein at the predicted Lys-Arg motif yields a mature β-NGF with an estimated molecular size of 13 kDa. The *Naja* cDNA sequence was used to verify the peptide sequence obtained previously. In all, nineteen residues were corrected based on the cDNA sequence predictions. The major differences were lack of a glycine residue previously detected at position 23 by peptide sequencing and the insertion of an additional lysine near the C-terminus. Sequence identity with the mature β-NGF protein from the mouse was 66%, whereas the proNGF peptide was less well conserved (45%).

The use of immunoblotting by Arumae et al. (1987) revealed NGF species varying in size from 13 to 44 kDa, which they suggested might be due to the presence of precursors or aggregates of the monomeric form. Among the Elapidae, *B. caeruleus* NGF migrated as an 18 kDa band, whereas the venom NGFs of three other species were 13 kDa. In order to distinguish between particularities in NGF sequence and a specific processing pattern, Danse and Garnier (1993) cloned and sequenced a full-length cDNA from a *Bungarus multicinctus* cDNA library. The nucleotide sequence revealed an open reading frame of 729 bp predicted to code for a 243 amino acid protein. This sequence had a high degree of similarity (93%) with *N. naja siamensis* prepro-β-NGF (Selby et al., 1987). The *B. multicinctus* NGF cDNA was 994 bp, composed of a 66 bp 5′ UTR and a 154 bp 3′ UTR. A high degree of similarity (96%) in the 3′ UTR with other known NGF homologs suggested that this region possessed a functional role that has limited sequence divergence, perhaps related to regulation of transcription or translation. The presence of a consensus Kozak initiation signal close

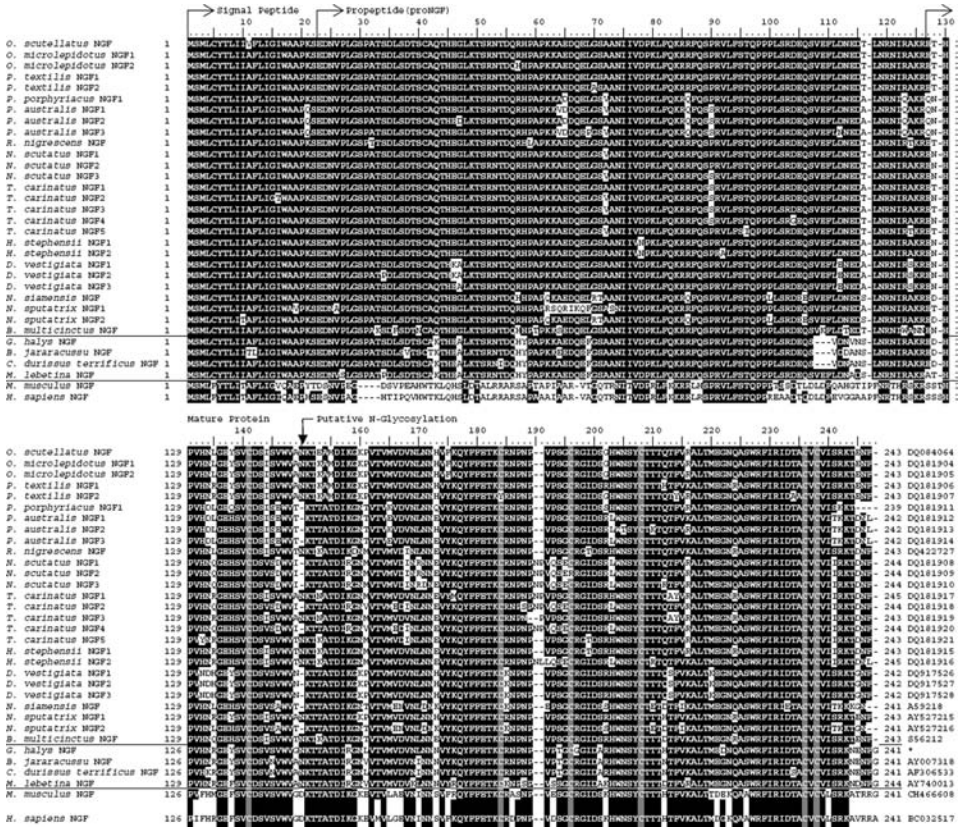

FIGURE 19.1 Alignment of NGF protein sequences deduced from cDNA clones identified from the venom gland of a number of viperid and elapid snakes. Elapidae (above first horizontal line) and Viperidae families are distinguished, and human and mouse NGFs are listed below the second line. Arrows denote the sites of cleavage for the signal, propeptide, and mature protein sequences, and conserved cysteine residues involved in putative disulfide bond formation are shaded gray. A putative N-linked glycosylation site is also indicated, and Genbank accession numbers are included at the end of each sequence. (Sequences for comparison taken from Guo et al., 1998.)

to the first methionine predicted a 243 amino acid precursor of 27.5 kDa. The organization of the *B. multicinctus* prepro-NGF was closely related to that from other snakes, human, bovine, and other species (Danse and Garnier, 1993).

Based on cDNA sequence for NGF from *N. naja siamensis* and *B. multicinctus* and the high degree of relatedness observed across NGFs from a variety of species, it was possible to amplify NGF sequences from other snakes. Guo et al. (1998) used reverse transcriptase polymerase chain reaction (RT-PCR) with primers complementary to the highly conserved 5′ and 3′ UTRs to clone NGF cDNA from *G. halys*. The cDNA coded for a prepro-NGF of 241 amino acids containing a mature NGF molecule of 119 amino acids. cDNA homology to *B. multicinctus* NGF was 91%, 89% with *N. naja siamensis* NGF, and 63% with human NGF. The *G. halys* NGF cDNA was cloned into a pcDNA vector and expressed in COS-7 cells; the resulting supernatant was capable of rescuing PC12 cell survival in serum-free medium, indicative of biologically active snake NGF.

The first cDNA from a South American snake, *Bothrops jararacussu*, was described by Kashima et al. (2002). In this case, a cDNA library was also prepared using RNA from the venom gland of the snake to facilitate the cloning of NGF cDNA. This cDNA, 726 bp in length, coded for a 241 amino acid prepro-NGF containing a 118 amino acid mature NGF. As expected, the mature NGF

from *B. jararacussu* had a high degree of sequence similarity with NGFs from other snakes, human, and mouse. A molecular model of *B. jararacussu* NGF, based on a monomeric form of human NGF, revealed that the core region was formed by a pair of two-stranded, twisted β-sheets and three β-hairpin loops at one end, and a reverse turn at the other. A short α-helix composed of four amino acids was located near the N-terminus.

Australian elapid snakes are well represented among the most dangerously venomous snakes worldwide, but descriptions of their venom components and the genes responsible have not kept pace with those of many other species of snakes. More recently, a variety of studies involving genomics, transcriptomics, and proteomics have assisted in addressing this deficiency (St. Pierre et al., 2005a,b; Birrell et al., 2007). St. Pierre et al. (2005a) described the use of a cDNA microarray from the venom gland of *Oxyuranus scutellatus* to identify venom gland-specific transcripts. One of the clones expressed in the venom gland was a partial cDNA, including 3′ UTR and 139 nucleotides of coding sequence, homologous to venom NGF from *B. multicinctus* (Danse and Garnier, 1993). A complete cDNA sequence with specific primers based on the *O. scutellatus* and *B. multicinctus* sequences was generated. The open reading frame was 730 nucleotides and coded for a 243 amino acid precursor protein. This amino acid sequence was identified as an NGF, with greater than 90% identity to *B. multicinctus* NGF. Based upon a conserved pro-NGF cleavage site, the mature β-NGF from *O. scutellatus* was estimated to have a molecular size of 13.4 kDa. NGF cDNAs from nine other Australian elapid snakes were also amplified using the same set of primers, and subsequently cloned and sequenced (see Figure 19.1). The signal and propeptide sequences are highly conserved among the different snake species but show significant differences from the corresponding mammalian sequences. However, there is a high degree of relatedness between the snake and mammalian proteins in the mature β-NGF region, accounting for observations of similar activity between the snake venom and mammalian proteins. A list of all NGF cDNA clones identified from snake venoms is given in Table 19.2.

Phylogenetic analysis of representative full-length precursor NGF sequences derived from cDNA clones demonstrates a distinct pattern of clustering according to families (Figure 19.2). NGF sequences from Elapidae and Viperidae families fall into two distinct clades. Furthermore, associations between species within these clades reflect those previously observed by both morphological and molecular phylogenetic means (Slowinski et al., 1997). This includes clustering of the two *Oxyuranus* species with *Pseudonaja textilis*, as well as grouping of the *Pseudechis* species and two *Naja* species (St. Pierre et al., 2005a,b). The early evolutionary split of *Demansia vestigiata* from other elapid snakes as observed with other toxin families, including the factor X–like prothrombin activators and the cysteine-rich secretory proteins, is also supported by the phylogenetic analysis of venom NGF sequences (St. Pierre et al., 2007).

V. PROTEOMIC IDENTIFICATION AND ANALYSIS OF SNAKE NGF

As discussed earlier in this chapter, classical protein separation techniques were successfully employed to isolate and characterize NGF from snake venoms 40 years ago. In the intervening period, the methodologies have changed and now include a proteomics approach and more sophisticated methods of analysis. Progress in proteomic analysis to identify and quantitate protein components of snake venom allows for the sensitive detection of individual proteins among complex mixtures. This methodology has been applied to provide proteomic profiling for snake venom proteins from Elapidae and Viperidae families (e.g., Nawarak et al., 2003; Li et al., 2004; Serrano et al., 2005; Birrell et al., 2006, 2007; Calvete et al., 2007).

In order to detect the presence of NGF proteins in venom, Earl et al. (2006) carried out 2DE of venom proteins from six Australian elapid snakes (*O. scutellatus*, *Oxyuranus microlepidotus*, *Pseudechis australis*, *Acanthophis antarcticus*, *Hoplocephalus stephensii*, and *Rhinoplocephalus nigrescens*). Separation of these venoms using 2DE yielded between one and two hundred individual

TABLE 19.2
Table of All Known Venom Nerve Growth Factor cDNA Clones Currently Identified from the Venom Glands of Snakes

Species	No. Clones	cDNA Size	Genbank Accession No.	Reference
		Elapidae		
Oxyuranus scutellatus	1	732 bp	DQ084064	St. Pierre et al., 2005a
Oxyuranus microlepidotus	2	732 bp	DQ181904–DQ181905	Earl et al., 2006
Pseudonaja textilis	2	732 bp	DQ181906–DQ181907	Earl et al., 2006
Pseudechis porphyriacus	1	720 bp	DQ181911	Earl et al., 2006
Pseudechis australis	3	729 bp	DQ181912–DQ181914	Earl et al., 2006
Rhinoplocephalus nigrescens	1	732 bp	DQ422727	Earl et al., 2006
Notechis scutatus	3	735 bp	DQ181908–DQ181910	Earl et al., 2006
Tropidechis carinatus	5	732–738 bp	DQ181917–DQ181921	Earl et al., 2006
Hoplocephalus stephensii	2	732–738 bp	DQ181915–DQ181916	Earl et al., 2006
Demansia vestigiata	3	729 bp	DQ917526–DQ917528	St. Pierre et al., 2007
Naja sputatrix	2	726–732 bp	AY527215–AY527216	Koh et al., 2004
Naja naja siamensis	1	741 bp	A59218	Selby et al., 1987
Bungarus multicinctus	1	732 bp	S56212	Danse and Garnier, 1993
		Viperidae		
Gloydius (*Agkistrodon*) *halys*	1	726 bp	Not submitted	Guo et al., 1998
Macrovipera lebetina	1	735 bp	AY740013	Direct submission
Crotalus durissus terrificus	1	726 bp	AF306533	Direct submission
Bothrops jararacussu	1	726 bp	AY007318	Kashima et al., 2002
Azemiops feae	1	672 bp (partial)	DQ139929	Fry et al., 2006

protein spots for each sample. These spots varied in molecular size from approximately 5 kDa to over 100 kDa and had pIs from 3 to 10. A typical two-dimensional gel separation is represented in Figure 19.3. Excision of spots from gels, digestion with trypsin, and analysis of the resulting peptides by mass spectrometry (MS) identified a number of different venom proteins, including NGF with apparent molecular masses of 20 kDa for *O. scutallatus* and *O. microlepidotus*. Two distinct forms of NGF were located for *A. antarcticus*, with molecular masses of 14 and 20 kDa, respectively and that for *R. nigrescens* had a mass of 29 kDa. The NGFs from *H. stephensii* and *P. australis* were shown to resolve at an apparent molecular mass of 14 kDa. A comprehensive proteomic analysis of Australian elapid snake venoms has subsequently identified spots corresponding to NGF from two-dimensional maps in additional species such as *Notechis ater serventyi*, *Pseudonaja nuchalis*, *Pseudechis porphyriacus*, *Pseudechis guttatus*, *Pseudechis colletti*, and *Tropidechis carinatus* (Birrell et al., 2007).

Other proteomic studies have also identified NGF as a venom component from various snake species. Sanz et al. (2006) examined the venoms of four closely related rattlesnake species with divergent diets (*Sistrurus catenatus catenatus*, *Sistrurus catenatus tergeminus*, *Sistrurus catenatus edwardsii*, and *Sistrurus miliarius barbouri*) using a combination of N-terminal sequencing, HPLC separation, and MS analysis. NGF was identified in all four venoms, but was observed to be in low abundance, with a relative occurrence of 0.1%. An analysis of *Naja naja kaouthia* venom using 2DE and MS identified a spot at approximately 15 kDa as NGF (Nawarak et al., 2003). Similarly, a proteomic analysis of *Crotalus atrox* venom identified an SDS-PAGE band of approximately 16 kDa as NGF by MS (Fox et al., 2007).

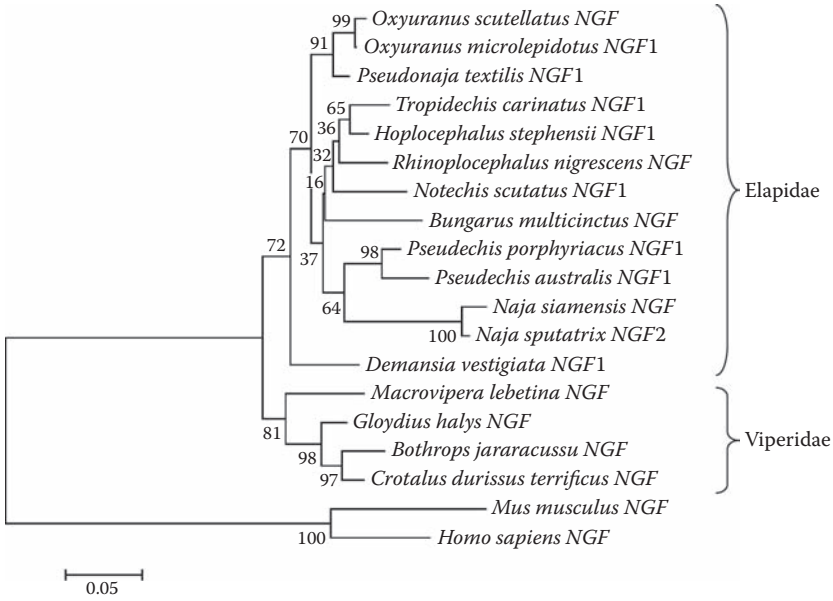

FIGURE 19.2 Phylogenetic relationship of precursor NGF protein sequences isolated from the venom of Elapidae and Viperidae snakes. A single representative NGF clone was selected from each species for the comparison. Analysis was performed using the neighbor-joining method (pairwise deletion) with one thousand bootstrap replicates, with the human and mouse NGF sequences selected as outgroups (MEGA 2.1 software). Note that the phylogenetic relationship of Australian snake NGF sequences reflects the previously described evolutionary relationship for these species, and that the viperid and other elapid sequences cluster as well.

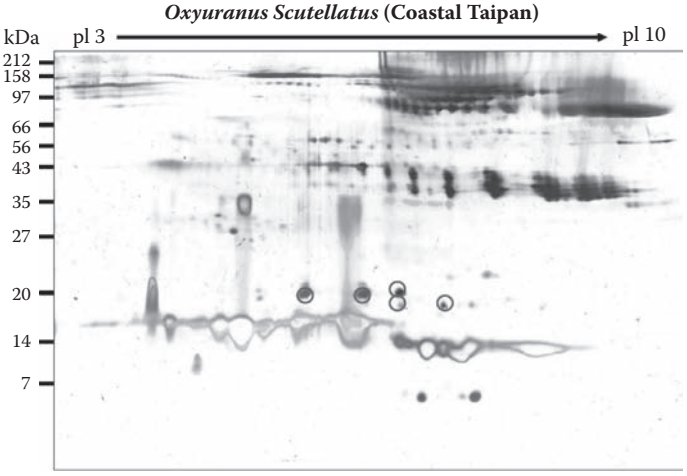

FIGURE 19.3 Two-dimensional SDS-PAGE of crude venom from *Oxyuranus scutellatus*. 100 µg sample of crude venom was subjected to isoelectric focusing on 11 cm, pH 3–10, IEF strips and subsequently separated by 12% SDS-PAGE and silver-stained. Protein spots were excised for identification by mass spectrometry. Circled spots represent those identified as NGF.

VI. POST-TRANSLATIONAL MODIFICATION OF SNAKE NGF

Varied forms of NGF in the same venom (Earl et al., 2006) and in the venom from different snakes (Koyama et al., 1992; Birrell et al., 2007) might be explained by dimeric structure or different genes. However, the failure to detect significantly different isoforms at the level of cDNA suggested that it was more likely to be explained by post-translational modifications. On the other hand, it is possible that spots at positions corresponding to dimeric forms could be explained by incomplete denaturation. The NGFs of a series of *Naja* species possess a molecular size of approximately 25 kDa, are composed of two subunits of 13 kDa each, and appear to be devoid of carbohydrate (Hogue-Angeletti and Bradshaw, 1979). Similarly, as discussed above, NGFs from *C. adamanteus* and *V. berus berus* were also found to be devoid of carbohydrate (Perez-Polo et al., 1978; Siigur et al., 1986). In contrast, NGFs from *B. atrox*, *Agkistrodon rhodostoma*, *Vipera ammodytes*, and *Daboia* (*Vipera*) *russellii* were shown to have a molecular size of 35 kDa; this difference in size can be accounted for by the presence of up to 20% carbohydrate (Kostiza and Meier, 1996). A variety of other venom proteins from both elapid and viperid snakes are also known to be glycosylated (Birrell et al., 2006, 2007; Nawarak et al., 2004).

It has been postulated that Asn23 of the mature β-chain is the glycosylation site in venom NGFs, since it is part of an N-glycosylation consensus sequence (NXS/T) (Kornfeld and Kornfeld, 1985; Gavel and von Heijne, 1990). Additionally, Asn23 is not present in mammalian and cobra NGFs, which are not glycosylated. In order to investigate the mechanism of glycosylation in venom NGFs, Earl et al. (2006) examined the Australian elapid NGF amino acid sequences deduced from cDNAs and identified the presence of Asn23 in most species. However, this proposed N-glycosylation site is missing from *N. scutatus*, *P. australis*, and *P. porphyriacus* (Earl et al., 2006). No other N-glycosylation consensus sequence is present in the Australian elapid NGF cDNAs. Subsequent 2DE and enzymatic deglycosylation studies supported these observations from the cDNA sequences.

The exact identity of the carbohydrate moieties is currently not known for any venom NGF. However, exposure of *O. scutellatus* venom to PNGase F, an enzyme that removes N-linked carbohydrates, caused NGF to resolve from a train of spots at 20 kDa to two discrete spots at 13 kDa, consistent with the size of the mature β-NGF predicted from the cDNA. Conversely, incubation with O-glycosidase, an enzyme that cleaves O-linked carbohydrate groups, had no effect on the location of the NGF isoforms. In addition, the lectin wheat germ agglutinin (WGA) was observed to react with the venom NGF spots from *O. scutellatus*, further supporting the presence of glycosylation. These results strongly suggest that NGF from *O. scutellatus* is glycosylated via an N-linked mechanism. Additional evidence for the presence of N-linked glycosylation of NGF is observed with *A. antarcticus* and *R. nigrescens* venoms after treatment with PNGase F, which also changes the 2DE location of NGF spots, and NGF spots from these two venoms also demonstrated reactivity with WGA. Asn23 is absent from the *P. australis* NGF cDNA, and accordingly, the NGF protein from *P. australis* migrates at the expected molecular size of 13 kDa, is not altered by PNGaseF treatment, and shows no reactivity with WGA. This suggests that the NGF from *P. australis* is not glycosylated. Taken together, these data suggest that Asn23 in venom NGFs is the site of N-linked glycosylation. On the other hand, *H. stephensii* NGF cDNAs contain Asn23, but the protein migrates at the expected (non-glycosylated) molecular weight of 14 kDa, indicating that the glycosylation site is unmodified in this NGF. This is similar to *N. sputatrix* venom NGF, which migrates at 14 kDa by SDS-PAGE despite the presence of Asn23 in the corresponding cDNA sequence (Koh et al., 2004).

WGA binds to N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) carbohydrate groups (Peters et al., 1979; Monsigny et al., 1980), and one or both of these sugar groups may be present on venom NGFs from the Australian elapid species. The presence in *O. scutellatus* venom of multiple NGF spots with the same molecular weight but different pIs could be explained by differential charge states caused by minor variations in terminal sialic acid groups. Faint reactivity with Con A was also observed in the 20 kDa *A. antarcticus* NGF isoforms, suggesting the presence of glucose or mannose groups. The functional significance of these different types and amounts of

glycosylation present in Australian elapid venom NGFs, and indeed in other snake venom NGFs, remains unclear and requires further study. Both glycosylated and non-glycosylated snake venom NGFs show a similar ability to induce neuronal outgrowth, suggesting that the carbohydrate is not essential for activity (Katzir et al., 2002). It has been shown previously that glycosylation of some serum proteins increases stability and prolongs time in circulation (Marshall, 1972). Thus, it is hypothesized that glycosylation of the NGF proteins and other venom proteins functions to increase their stability in the venom gland or after envenomation.

VII. SNAKE VENOM NGF AS A THERAPEUTIC CANDIDATE

Venoms are a unique source of molecules that may be of therapeutic potential, as they derive from a non-mammalian source yet specifically target functional mammalian systems in a highly systematic manner. They are similarly useful in the drug discovery process due to their high potency and selectivity, and have the advantage that many components are relatively small peptides that may act as stable three-dimensional scaffolds to deliver specific pharmacological effects (Harvey et al., 1998). Venom peptides have an inherent degree of stability, an advantage for a therapeutic agent, due to the necessity to resist degradation by host enzymes, as well as by other components within the venom itself. This stability often arises from the heavily disulfide-bonded structures of many toxins, or from unique post-translational modifications (Lewis and Garcia, 2003).

Factors isolated from snake venoms have been used for therapeutic purposes for a number of decades. The first clinically controlled experiments using snake venom were performed in 1934, when the crude venom of a Russell's viper was used as a clotting agent for the treatment of hemophilia (MacFarlane, 1961). More recently, thrombin-like enzymes have been used as anticoagulants for the treatment of venous thrombosis, a condition responsible for significant morbidity, including venous thromboembolism. An example of such a thrombin-like enzyme is batroxobin from *B. atrox* venom (Stocker and Barlow, 1976). Examples of the putative application of an Australian snake venom toxin as a therapeutic lie in the textilinins, which are being studied as an antibleeding agent (Filippovich et al., 2002).

The advent of transcriptomics and proteomics has assisted in the cDNA cloning and expression of a number of these proteins. Improved yields of correctly folded proteins with functional activity pave the way for the use of recombinant proteins in pharmacological applications. The demonstrated ability of snake venom NGF to stimulate neurite outgrowth and differentiation in PC12 cells, together with availability of cDNA for several snake NGFs, is encouraging for the use of NGF in therapy for neurological disorders.

NGF is an important trophic factor for basal forebrain cholinergic neurons. Its capacity to increase choline acetyltransferase and prevent atrophy of basal forebrain neurons provides the potential for treatment of disorders that cause atrophy of these neurons, including aging and neurodegenerative disease (Koliatsos et al., 1993; Markowska et al., 1996). However, the size of this molecule reduces its usefulness as a "brain therapeutic." Nevertheless, several studies have been conducted that are designed to prevent neuronal degeneration in Alzheimer's disease and other neurodegenerative disorders (Blesch and Tuszynski, 2004). Currently, treatment of these disorders is restricted by available methods of delivery to the appropriate region of the brain. Studies with animals demonstrate that adeno-associated viral and lentiviral vectors expressing NGF are effective in preventing cholinergic neuronal degeneration (Blömer et al., 1998; Mandel et al., 1999). Gene therapy using different gene expression constructs or engraftment of cells capable of secreting NGF is a promising approach. Capsoni et al. (2002) have circumvented the problem of delivery to the brain using an intranasal route of administration in a mouse model for Alzheimer's disease. In this model, NGF is abrogated with anti-NGF antibodies, and as a consequence, these mice show a progressive neurodegeneration characterized by atrophy of forebrain cholinergic neurons, accumulation of phosphorylated insoluble tau protein, and deposition of β-amyloid. Intranasal route delivery of NGF rescues all of the histological markers of the Alzheimer's disease–like neurodegeneration (Capsoni

et al., 2002). The same group subsequently showed that intranasal administration of NGF reversed recognition memory deficits in this animal model (De Rosa et al., 2005).

It has been reported that NGF from *N. sputatrix* venom exhibits similar neurite differentiation activity to its mammalian homolog and also possesses additional beneficial effects. For example, it was shown that *N. sputatrix* NGF, unlike the mammalian protein, is capable of upregulating endogenous expression of NGF in PC12 cells and also upregulates expression of pro-survival cell surface receptors and ion channels (Koh et al., 2004). Given these data and the high level of sequence similarity between the *N. sputatrix* and *O. scutellatus* venom NGFs, Earl et al. (2006) undertook a functional analysis of *O. scutellatus* NGF and confirmed that NGF from *O. scutellatus* venom has neurite differentiation activity. As mentioned previously, a variety of other snake venom NGFs have also demonstrated ability to induce neurite differentiation in the PC12 assay (see Table 19.1). These data provide the basis for a more detailed analysis of snake venom NGF proteins as potential therapeutic agents in the treatment of neuronal injury. These analyses may provide further insights into the mechanism of action of snake venom NGF. Similarly, testing the ability of snake NGFs to treat neurodegeneration in an animal model would provide proof of principle data to enable further development. One potential animal model, in addition to the currently available rodent models of Alzheimer's disease, is the GFAP luciferase transgenic mouse, in which neuronal damage can be monitored by *in vivo* luminescence emission (Kadurugamuwa et al., 2005).

In summary, the biological role of NGF as a venom component remains largely unknown despite recent advances in the field (increasing numbers of cDNA sequences and proteomic analyses). However, it is apparent that NGF is present in a large number of different snake venoms, and those molecules investigated have neurite differentiation activity similar to that of the mammalian protein. Given this information and the fact that NGF from *N. sputatrix* venom also demonstrates additional beneficial effects over mammalian NGF, these proteins may have potential application as therapeutic agents in the fight against neurodegenerative disorders.

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20 The Role of Purine and Pyrimidine Nucleosides in Snake Venoms

Steven D. Aird

CONTENTS

Purine nucleosides were first discovered as constituents of snake venoms more than 50 years ago. Despite the fact that relatively little attention has been paid to them, nucleosides are among the most elegant and versatile toxins. They exert synchronous effects upon virtually all cell types, contributing simultaneously to prey immobilization via hypotension, prey immobilization via paralysis, and prey digestion. Because purine nucleosides have similar pharmacological roles in all vertebrates, they are useful against all prey classes, and it is impossible for any prey organism to develop resistance to them. In a sense, purine nucleosides represent the trump card in the competitive predator-prey relationship (increasing toxicity vs. increasing resistance). The pyrimidine nucleosides cytidine and uridine are also widely distributed among snake venoms, but thymidine has not been found in any to date. In contrast to viperine and elapid venoms, crotaline venoms are virtually devoid of nucleosides, suggesting that pit vipers rely upon the enzymatic release of endogenous prey nucleosides. Quantities of venom injected by various venomous snakes in predatory strikes may be used to predict the potential plasma levels of circulating nucleosides achievable as a result of injected venom nucleosides. These levels are more than adequate to activate physiological processes

consonant with snake envenomation strategies. Deoxyribonucleosides, such as would be liberated from DNA, by phosphodiesterase and 5′-nucleotidase, have been little explored pharmacologically; however, current data suggest that deoxyribonucleosides have biological activities similar to those of ribonucleosides, although their potencies vary.

I. HISTORICAL BACKGROUND

The first report of purine nucleosides as snake venom constituents appeared more than 50 years ago when Fischer and Dörfel (1954) found adenosine in venoms of the puff adder (*Bitis arietans*) and the eastern green mamba (*Dendroaspis angusticeps*). They suggested that adenosine might contribute to the hypotension promoted by these venoms. The hypotensive effects of adenosine had been first reported by Drury and Szent-Györgyi (1929). Doery (1956) reported the presence of adenosine, adenosine 3′-phosphate, and "guanine compounds" in venoms of several dissimilar elapids, *Acanthophis antarcticus*, *Notechis scutatus*, *Dendroaspis angusticeps*, and *Denisonia superba* (*Austrelaps superbus*), and also in that of *Bitis arietans*. Doery (1957) later isolated guanosine, inosine, and hypoxanthine from *Notechis scutatus* venom. No further reports of venom purines appeared until Wei and Lee (1965) reported that guanosine constitutes slightly over 1% of crude *Bungarus multicinctus* venom, but found no evidence that it influenced blood pressure in cats. Lo and Chen (1966) found adenosine, guanosine, and inosine in the venom of *Naja atra*. Lin and Lee (1971) suggested that nucleoside contamination might explain the detection of carbohydrate (ribose) in many apparently pure elapid neurotoxins. Eterovic et al. (1975) reported that *Bungarus multicinctus* venom contains large amounts of guanosine, which accounted for 10% of the 280 nm absorbance of the crude venom. Takasaki et al. (1991) also identified adenosine, guanosine, and inosine in venom of the long-glanded coral snake (*Maticora bivirgata*). Finally, Francis et al. (1997) discovered that venom of the Brazilian coral snake, *Micrurus frontalis*, a South American elapid, contains significant quantities of guanosine.

II. ROLE OF PURINES IN ENVENOMATION

The functional significance of snake venom purines remained enigmatic until Aird (2002) outlined three fundamental ophidian envenomation strategies that appear to be employed by all advanced venomous snakes. Aird (2002) suggested that purines act as multifunctional toxins, exerting synchronous effects upon virtually all cell types, and further proposed that exogenous and enzymatically released endogenous purines are central components in the envenomation strategies of all venomous snakes, contributing to prey immobilization via hypotension, prey immobilization via paralysis, and prey digestion. Adenosine contributes to prey immobilization by activation of neuronal adenosine A_1 receptors (Reddington et al., 1982; Prestwich et al., 1987; Higgins et al., 1994; Lovinger and Choi, 1995; Di Iorio et al., 1996; Burgdorf et al., 2001; Arrigoni et al., 2006), suppressing acetylcholine release from motor neurons (Ginsborg and Hirst, 1971; Ribeiro and Dominguez, 1978; Silinsky, 1984; Marks et al., 1993; Huang et al., 2002; Silinsky, 2004) and excitatory neurotransmitters from central sites (Dolphin and Prestwich, 1985; Prince and Stevens, 1992; Dunwiddie and Masino, 2001). It also exacerbates venom-induced hypotension by activating A_2 receptors in the vasculature and by depressing cardiac output and causing atrioventricular block (Fukunaga et al., 1982; Barraco et al., 1986; Fredholm and Sollevi, 1986; Simpson et al., 1989; Belloni et al., 1992; Stella et al., 1993; Pennanen et al., 1994; Lo et al., 1998). Inosine potentiates the coronary vasodilatory effects of adenosine (Saito et al., 1993; Ko et al., 1990). Adenosine and inosine both activate mast cell A_3 receptors, liberating vasoactive substances and increasing vascular permeability (Ramkumar et al., 1993; Hannon et al., 1995; Reeves et al., 1997; Jin et al., 1997; Sawynok et al., 2000).

Adenosine also has other pharmacological actions that would benefit snakes during prey capture. Acting at central A_1 receptors, adenosine induces sedative effects (Snyder et al., 1981; Dunwiddie

and Worth, 1982; Barraco et al., 1983), anxiolytic activity (Jain et al., 1995), operant response suppression (Coffin and Spealman, 1985, 1987), aggression inhibition (Palmour et al., 1989; Ledent et al., 1997; Navarro et al., 2000), alterations of cognitive functioning (Winsky and Harvey, 1986), and locomotor depression (Karlsten et al., 1990; Nikodijevic et al., 1991; Barraco et al., 1993; Jain et al., 1995). Adenosine administered via either an i.c.v. route (Radulovacki et al., 1985) or i.p. (Sarda et al., 1986) induces sleep in rats. All of the aforementioned pharmacological activities would limit prey flight and suppress aggressive actions against the snake.

Prey preoccupied with pain might also be less likely to respond aggressively. In mammals, analgesia and hyperalgesia are mediated by adenosine A_1 and A_2 receptor activation, respectively, depending upon the adenosine concentration (Taiwo and Levine, 1990; Sollevi, 1997; Khasar et al., 1995; Sawynok, 1998). Additionally, activation of mast cell A_3 receptors produces pain via the liberation of histamine and 5-HT (Ramkumar et al., 1993; Sawynok, 1998), which activate histamine H_1 and 5-HT receptors, respectively, located on sensory nerves (Ninkovic et al., 1982; Taiwo and Levine, 1992; Pierce et al., 1996). Adenosine levels resulting from envenomation would almost certainly trigger hyperalgesia rather than analgesia.

Adenosine and inosine promote inflammation and plasma extravasation via activation of mast cell A_3 receptors (Ramkumar et al., 1993; Marquardt, 1994; Fozard et al., 1996; Kohno et al., 1996a; Walker et al., 1997; Fan and Jamal Mustafa, 2006). Such activities would serve to enhance tissue access for other venom constituents. Adenosine inhibits platelet aggregation (Born et al., 1964; Michal and Thorp, 1966; Rozenberg and Holmsen, 1968). Incoagulable blood also facilitates tissue access of hydrolytic venom components.

Apoptosis, or programmed cell death, is induced in HL-60 cells and U-937 lymphoma cells by high (≥10 μM) concentrations of adenosine and other A_3 receptor agonists (Tanaka et al., 1994; Abbracchio et al., 1995; Wakade et al., 1995; Kohno et al., 1996b; Yao et al., 1997; Barbieri et al., 1998; Kulkarni et al., 1998; Shneyvays et al., 1998; Saitoh et al., 2004). According to Ralevic and Burnstock (1998), apoptosis may occur during pathological conditions, resulting in cell damage and release of high levels of purines (e.g., envenomation). Clearly, induction of apoptosis or fast necrotic cell death would contribute significantly to prey digestion.

III. THE ELEGANCE OF PURINE NUCLEOSIDES AS TOXINS

Because purine nucleosides serve as endogenous regulatory compounds, it is impossible for prey organisms to develop resistance to them. In a sense, purine nucleosides represent the trump card in the escalation of predator-prey competition (increasing toxicity vs. increasing resistance), and as such, they are evolution-proof. The elegance of using purines as toxins is also apparent in their broad taxonomic specificity. Because purines have similar pharmacological roles in all vertebrates (Burnstock, 1975, 1996), they are useful as toxins against all prey classes (fish: Quintana, 1985; Murray and Siebenaller, 1987; Poli et al., 1993; Sundin et al., 1999; Pellegrino et al., 2005; amphibians: Ribeiro and Dominguez, 1978; Silinsky, 1980; de Mendonça and Ribeiro, 1989; Knight and Burnstock, 1996; Huang et al., 2002; reptiles: Perez-Pinzon et al., 1993; Buck and Bickler, 1995; Knight and Burnstock, 1995, 2001; Milton and Lutz, 2005; birds: Barnes and Thampy, 1982; Bennett and Ho, 1992; Kattmann and Klempnauer, 2002; mammals: Drury and Szent-Györgyi, 1929; Ginsborg and Hirst, 1971; Gazelius et al., 1984; Hori and Kitakaze, 1991). The purine strategy is therefore expected to be important to juvenile venomous snakes as well as to adults.

IV. PURINE GENERATION FROM ENDOGENOUS PRECURSORS

Aird (2002) also proposed that purine generation from endogenous precursors in the prey explains the presence of many previously unexplained enzyme activities in snake venoms: 5′-nucleotidase (5NUC), endonucleases (including ribonuclease), phosphodiesterase (PDE), ATPase, ADPase, phosphomonoesterase (PME), and NADase. Phospholipases A_2, cytotoxins, myotoxins, and heparinase

also participate indirectly in purine liberation, in addition to their better-known functions. Consistent with the purine hypothesis, several of the aforementioned enzymes are among the most ubiquitous in snake venoms. Aird (2002) also proposed that envenomation utilizes the prey's own ectoenzymes (especially ecto-PDE and ecto-5NUC) as weapons against itself to convert released nucleotides (ATP, ADP, AMP cAMP) to adenosine. Dunwiddie et al. (1997a) reported that in the hippocampal extracellular space, AMP, ADP, and ATP were all rapidly converted to adenosine, with a $T_{1/2}$ for ATP conversion to adenosine of 200 msec. Interestingly, they found that the transformation of AMP to adenosine by 5NUC seemed to be the rate-limiting step. Snake venoms have vastly higher levels of 5NUC than of PDE (Table 20.1), although because such data involved the use of synthetic substrates, activity levels may also reflect the affinities of these enzymes for their respective substrates.

It remains to be seen whether the ontogenetic shifts in venom composition reported for *Crotalus ruber* proteases (Mackessy, 1985) also apply to venom purines or venom enzymes involved in purine liberation. Mackessy (1988) reported that exonuclease (PDE) tended to increase with size in juvenile *Crotalus viridis helleri* and *C. v. oreganus*. It could be that as juvenile snakes begin to eat larger prey with smaller surface-to-volume ratios, the digestive functions of purines become more important, in which case, one would also expect ontogenetic increases in PDE, 5NUC, PME, NADase, heparinase, and hyaluronidase activities.

In some venomous snake taxa, significant size differences have been reported between the sexes, with concomitant differences in prey preferences (Nogueira et al., 2003). The purine strategy is sufficiently flexible to accommodate such differences without necessitating different venom genetics for the two sexes.

V. RECENT STUDIES

Lumsden et al. (2004) reported that in the prostatic segment of the rat vas deferens, *Boiga dendrophila* venom induced concentration-dependent inhibition of electrically evoked (0.2 Hz, 0.3 ms, 70–100 V) twitches. The inhibitory effect of venom (100 µg/ml) was attenuated by 20 µM concentrations of the adenosine A_1 receptor antagonists 8-phenyltheophylline and 8-cyclopentyl-1,3-dipropylxanthine. The inhibitory effect of venom (100 µg/ml) was eliminated by adenosine deaminase, and was amplified by 10 µM dipyridamole, a PDE inhibitor and an antagonist of adenosine transporters. In anesthetized rats, venom (5–7.5 mg/kg i.v.) caused a hypotensive effect. These results indicate that *Boiga dendrophila* venom contains adenosine. This constitutes the first report of purines in a colubrid venom, indicating that the purine strategy did not originate with more advanced venomous snakes.

Lumsden et al. (2004) discount the possible contribution of venom PDE to the hypotension induced in the prey, citing the hypertensive effects of smooth muscle PDEs (Karsten et al., 2003; Mehats et al., 2003; Rybalkin et al., 2003; Oger et al., 2004). In this regard they are probably mistaken. The references cited by Lumsden et al. (2004) involve intracellular PDE4 and PDE5, which downregulate intracellular concentrations of the second messengers, cAMP and cGMP, respectively. They act in opposition to adenylate and guanylate cyclases, respectively, and are hypertensive by virtue of their specificity for cyclic nucleotides. In contrast, venom PDEs function in the extracellular fluid and have broad substrate specificities (Boman, 1959; Razzell and Khorana, 1959; Hadjiolov et al., 1966; Petrova et al., 1975; Pritchard et al., 1977; Laskowski, 1980; Stoynov et al., 1997). Venom PDEs cleave many oligonucleotide substrates to release mononucleotides, which in turn would be hydrolyzed to nucleosides by venom and endogenous 5NUC. Broaders and Ryan (1997) mention that PDE activity is only weakly present in *Boiga dendrophila* venom. Low PDE activity was detected in several venoms by Hill and Mackessy (2000). Accordingly, colubrid PDE may not play a significant role in envenomation, although this is uncertain at present. To date I am unaware of any studies that have assayed 5NUC activity in colubrid venoms, but its presence would not be surprising.

TABLE 20.1
Enzymatic Activities of the Venom Phosphodiesterase, 5′-Nucleotidase, and Alkaline Phosphomonoesterase Expressed as nmol Substrate Cleaved min^{-1} mg^{-1} of Venom

Species	PDE	5′-NUC	PME	Reference
Family Elapidae: Acanthophiinae				
Acanthophis antarcticus	1	669	1	Tan and Ponnudurai, 1990b
Austrelaps superbus	2	1,275	1	Tan and Ponnudurai, 1990b
Notechis ater	2	3,111	0	Tan and Ponnudurai, 1990b
Notechis scutatus	2	1,810	2	Tan and Ponnudurai, 1990b
Oxyuranus microlepidotus	2	2,384	119	Tan and Ponnudurai, 1990b
Oxyuranus scutellatus	2	3,349	193	Tan and Ponnudurai, 1990b
Pseudechis australis	1	2,607	27	Tan and Ponnudurai, 1990b
Pseudechis collettii	1	1,665	70	Tan and Ponnudurai, 1990b
Pseudechis guttatus	1	609	83	Tan and Ponnudurai, 1990b
Pseudechis porphyriacus	2	1,775	42	Tan and Ponnudurai, 1990b
Pseudonaja textilis	0	1,249	260	Tan and Ponnudurai, 1990b
Mean	**1**	**1,864**	**73**	
Family Elapidae: Bungarinae				
Bungarus caeruleus	2	1,517	4	Tan and Ponnudurai, 1990a
Bungarus candidus	1	185	3	Tan and Ponnudurai, 1990a
Bungarus fasciatus	6	3,426	3	Tan and Ponnudurai, 1990a
Bungarus multicinctus	1	43	10	Tan and Ponnudurai, 1990a
Mean	**3**	**1,293**	**5**	
Family Elapidae: Elapinae				
Dendroaspis angusticeps	2	391	5	Tan et al., 1991
Dendroaspis jamesoni	1	670	7	Tan et al., 1991
Dendroaspis polylepis	1	1,717	1	Tan et al., 1991
Dendroaspis viridis	1	182	7	Tan et al., 1991
Micrurus corallinus	0	83	23	Tan and Ponnudurai, 1992a
Micrurus dumerilii	5	759	129	Tan and Ponnudurai, 1992a
Micrurus frontalis	1	251	143	Tan and Ponnudurai, 1992a
Micrurus fulvius	7	739	4	Tan and Ponnudurai, 1992a
Micrurus lemniscatus	1	309	15	Tan and Ponnudurai, 1992a
Micrurus nigrocinctus	3	516	4	Tan and Ponnudurai, 1992a
Naja atra	71	312	22	Tan and Tan, 1988
Naja haje	110	530	62	Tan and Tan, 1988
Naja kaouthia	75	446	59	Tan and Tan, 1988
Naja melanoleuca	79	712	57	Tan and Tan, 1988
Naja mossambica mossambica	50	680	166	Tan and Tan, 1988
Naja mossambica pallida	39	532	57	Tan and Tan, 1988
Naja nigricollis	52	547	92	Tan and Tan, 1988
Naja nivea	93	874	64	Tan and Tan, 1988
Naja oxiana	87	368	59	Tan and Tan, 1988
Naja naja	78	201	82	Tan and Tan, 1988
Naja sputatrix	62	326	26	Tan and Tan, 1988
Ophiophagus hannah	14	3,042	144	Tan et al., 1991
Mean	**38**	**645**	**56**	

(continued on next page)

TABLE 20.1 (continued)
Enzymatic Activities of the Venom Phosphodiesterase, 5′-Nucleotidase, and Alkaline Phosphomonoesterase Expressed as nmol Substrate Cleaved min^{-1} mg^{-1} of Venom

Species	PDE	5′-NUC	PME	Reference
		Family Elapidae: Hydrophiinae		
Astrotia stokesii	0	18	0	Tan and Ponnudurai, 1991a
Hydrophis cyanocinctus	1	28	4	Tan and Ponnudurai, 1991a
Lapemis curtus	1	45	7	Tan and Ponnudurai, 1991a
Mean	**0**	**30**	**4**	
		Family Elapidae: Laticaudinae		
Laticauda colubrina	0	32	2	Tan and Ponnudurai, 1991a
Laticauda laticaudata	0	23	5	Tan and Ponnudurai, 1991a
Laticauda semifasciata	0	14	3	Tan and Ponnudurai, 1991a
Mean	**0**	**23**	**3**	
		Family Elapidae: Notechinae		
Hoplocephalus stephensii	1	788	1	Tan and Ponnudurai, 1990b
Tropidechis carinatus	2	1,042	1	Tan and Ponnudurai, 1990b
Mean	**2**	**915**	**1**	
		Family Viperidae: Crotalinae		
Agkistrodon b. bilineatus	20	6,867	27	Tan and Ponnudurai, 1990c
Agkistrodon contortrix	0	4,646	4	Tan and Ponnudurai, 1990c
Agkistrodon piscivorus	13	5,133	6	Tan and Ponnudurai, 1990c
Bothriechis schlegelii	48	8,153	6	Tan and Ponnudurai, 1991b
Bothriopsis bilineatus	51	6,521	6	Tan and Ponnudurai, 1991b
Bothrops alternatus	13	1,090	3	Tan and Ponnudurai, 1991b
Bothrops asper	33	2,750	7	Tan and Ponnudurai, 1991b
Bothrops atrox	28	1,878	4	Tan and Ponnudurai, 1991b
Bothrops cotiara	10	3,071	1	Tan and Ponnudurai, 1991b
Bothrops jararaca	19	1,726	3	Tan and Ponnudurai, 1991b
Bothrops jararacussu	17	2,321	3	Tan and Ponnudurai, 1991b
Bothrops moojeni	33	1,983	2	Tan and Ponnudurai, 1991b
Bothrops neuwiedi	18	3,052	3	Tan and Ponnudurai, 1991b
Bothrops pradoi	43	2,442	2	Tan and Ponnudurai, 1991b
Calloselasma rhodostoma	18	2,967	11	Tan and Ponnudurai, 1990c
Crotalus adamanteus	44	7,040	4	Tan and Ponnudurai, 1991c
Crotalus atrox	42	5,279	3	Tan and Ponnudurai, 1991c
Crotalus horridus	31	7,351	4	Tan and Ponnudurai, 1991c
Crotalus durissus	37	3,083	4	Tan and Ponnudurai, 1991c
Crotalus basiliscus	49	4,739	5	Tan and Ponnudurai, 1991c
Crotalus cerastes	15	1,814	3	Tan and Ponnudurai, 1991c
Crotalus molossus	26	2,233	2	Tan and Ponnudurai, 1991c
Crotalus ruber	31	3,388	4	Tan and Ponnudurai, 1991c
Crotalus scutulatus	27	2,765	27	Tan and Ponnudurai, 1991c
Crotalus viridis cerberus	17	1,757	3	Tan and Ponnudurai, 1991c
Crotalus viridis concolor	50	9,188	6	Tan and Ponnudurai, 1991c
Crotalus viridis helleri	13	3,910	4	Tan and Ponnudurai, 1991c
Crotalus viridis lutosus	50	3,353	4	Tan and Ponnudurai, 1991c

TABLE 20.1 (continued)
Enzymatic Activities of the Venom Phosphodiesterase, 5′-Nucleotidase, and Alkaline Phosphomonoesterase Expressed as nmol Substrate Cleaved min^{-1} mg^{-1} of Venom

Species	PDE	5′-NUC	PME	Reference
Crotalus viridis oreganus	24	4,272	3	Tan and Ponnudurai, 1991c
Crotalus viridis viridis	52	7,932	9	Tan and Ponnudurai, 1991c
Deinagkistrodon acutus	16	2,850	5	Tan and Ponnudurai, 1990c
Gloydius blomhoffii	15	2,600	7	Tan and Ponnudurai, 1990c
Gloydius halys ussuriensis	16	2,600	3	Tan and Ponnudurai, 1990c
Hypnale hypnale	6	2,700	12	Tan and Ponnudurai, 1990c
Ovophis okinavensis	*	409	*	Tan et al., 1989
Porthidium lansbergi	8	4,921	1	Tan and Ponnudurai, 1991b
Porthidium nasutum	14	3,501	4	Tan and Ponnudurai, 1991b
Porthidium nummifer	4	5,340	4	Tan and Ponnudurai, 1991b
Sistrurus catenatus tergeminus	67	2,023	3	Tan and Ponnudurai, 1991c
Sistrurus miliarius barbouri	7	1,415	3	Tan and Ponnudurai, 1991c
Trimeresurus albolabris	*	1,499	*	Tan et al., 1989
Trimeresurus elegans	*	2,480	*	Tan et al., 1989
Trimeresurus flavoviridis	*	1,959	*	Tan et al., 1989
Trimeresurus macrops	*	1,956	*	Tan et al., 1989
Trimeresurus mucrosquamatus	*	1,949	*	Tan et al., 1989
Trimeresurus popeorum	*	1,022	*	Tan et al., 1989
Trimeresurus purpureomaculatus	*	1,178	*	Tan et al., 1989
Trimeresurus sumatranus	*	2,336	*	Tan et al., 1989
Trimeresurus stejnegeri	*	2,863	*	Tan et al., 1989
Trimeresurus tokarensis	*	2,306	*	Tan et al., 1989
Mean	**26**	**3,412**	**6**	
	Family Viperidae: Viperinae			
Bitis arietans	6	3,288	5	Tan and Ponnudurai, 1992b
Bitis gabonica	12	5,559	5	Tan and Ponnudurai, 1992b
Bitis nasicornis	19	1,705	3	Tan and Ponnudurai, 1992b
Causus rhombeatus	19	692	0	Tan and Ponnudurai, 1992b
Cerastes cerastes	5	2,912	3	Tan and Ponnudurai, 1992b
Cerastes vipera	5	3,700	2	Tan and Ponnudurai, 1992b
Daboia russellii	45	3,271	3	Tan and Ponnudurai, 1990d
Echis carinatus	14	3,586	6	Tan and Ponnudurai, 1992b
Eristocophis macmahoni	9	5,606	2	Tan and Ponnudurai, 1992b
Pseudocerastes persicus	10	3,111	4	Tan and Ponnudurai, 1992b
Vipera aspis	5	1,250	2	Tan and Ponnudurai, 1990d
Vipera ammodytes	8	3,833	4	Tan and Ponnudurai, 1990d
Vipera berus	6	1,500	7	Tan and Ponnudurai, 1990d
Vipera lebetina	12	1,825	3	Tan and Ponnudurai, 1990d
Vipera latastei	7	1,450	2	Tan and Ponnudurai, 1990d
Vipera palaestinae	9	2,133	3	Tan and Ponnudurai, 1990d
Vipera xanthina	9	2,600	4	Tan and Ponnudurai, 1990d
Mean	**12**	**2,825**	**3**	

Note: Units reported by Tan et al. (1989) for PDE and PME were different from those reported in other publications and could not be directly compared. *, data unavailable.

Aird (2005) published the first systematic, quantitative investigation of purine and pyrimidine nucleosides in snake venoms. Venoms of thirty-five species—twenty-one elapids, seven viperines, and seven crotalines—were examined by gel filtration and reverse phase chromatography for the presence of adenosine, AMP, inosine, IMP, guanosine, GMP, hypoxanthine, uridine, cytidine, and thymidine. Thymidine was not found in any venom, indicating that nucleosides in snake venoms are not simply incidental by-products of degradation of gland cell DNA.

A. Venom Mononucleotides

GMP was not found in any venom, and only one possible instance of IMP was encountered in *Dendroaspis viridis* venom. AMP was tentatively identified in venoms of *Vipera palaestinae*, *Crotalus adamanteus*, *Micrurus corallinus*, *Lapemis curtus*, and *Laticauda semifasciata* (Table 20.2). The possible presence of nucleoside monophosphates in venoms is not surprising, because they are also hypotensive, though less so than nucleosides (Drury and Szent-Györgyi, 1929); thus, their presence in venoms would be consistent with snake envenomation strategies (Aird, 2002). Moreover, they could be readily degraded to nucleosides by venom 5NUC or prey ecto-5NUC. Nucleoside di- and triphosphates are hypertensive in many preparations and hypotensive in others; hence, they are less likely venom constituents.

B. Venom Pyrimidines

Cytidine and uridine were found in almost all elapid venoms and most viperine venoms, but no crotaline venoms (Aird, 2005). This was the first report of pyrimidine nucleosides as venom constituents. Uridine-positive viperine venoms had uridine concentrations approximately tenfold higher than those of uridine-positive elapid venoms. In the sixteen species in which both cytidine and uridine were present, uridine was more abundant in thirteen.

1. Cytidine Pharmacology

The literature offers few insights into pharmacological activities of cytidine that are potentially relevant to envenomation. Nayler and McCulloch (1959) reported that cytidine triphosphate (20 μM) has a positive inotropic effect on isolated, perfused toad hearts, but they offered no data regarding inotropic activity of cytidine. Krooth et al. (1978) reported that cytidine and uridine both acted as central nervous system (CNS) depressants, reducing activity in mice by 80% at doses of ~16.5 and ~11 μmol/g, respectively. Bushma and Lukienko (1977) found that cytidine, at 2 to 4% of the LD_{50}, reduced body temperature in rats, but reported that it had no detectable effect on cardiac function or respiration in cats. Likewise, Nuki et al. (1961) found that cytidine had no effect on blood pressure in cats or rabbits. Neither did it alter carotid or coronary blood flow in dogs.

While it is purely conjectural at this point, the primary physiological target of venom pyrimidines may be nucleoside transporters, which are classified as either equilibrative or concentrative transporters. The first can function bidirectionally, depending upon the concentration gradient. Concentrative transporters utilize the Na^+ gradient (140 mM extracellular vs. 5–10 mM intracellular) to pump nucleosides into the cells against their concentration gradients (Wang et al., 1997). These are currently classified into five subclasses. N1 transporters are selective for purines, but they also transport uridine. N2 transporters are selective for pyrimidines, but also accept adenosine (Wang et al., 1997). Subtypes N3–N5 have broader specificity and have been defined on the basis of relatively few tissue types. Differentiating among these subtypes has been difficult owing to the fact that some cell types express more than one type of transporter (Wang et al., 1997).

Wu et al. (1992) reported that in rabbit choroid plexus, pyrimidine and purine nucleosides are transported by both equilibrative and active Na^+-dependent concentrative transport systems. The concentrative system can be saturated (i.e., different nucleosides act as competitive inhibitors of one another) and is selective for naturally occurring purine and pyrimidine ribo- and

TABLE 20.2
Purine Levels in the Venoms of 32 Elapid, Viperine, and Crotaline Species

Taxon		AMP	HYP	CYT	URI	INO	GUA	ADN	G/I	G/A
Family Elapidae: Acanthophiinae										
Acanthophis antarcticus		0	0	30	50	590	3,550	5,638	6	1
Aspidelaps scutatus		0	1	0	0	264	1,154	466	4	2
	Mean					427	2,352	3,052		
	SEM					163	1,198	2,586		
Family Elapidae: Bungarinae										
Bungarus fasciatus		0	0	0	5	13	3,239	0	249	
Bungarus multicinctus		0	0	13	61	132	3,306	0	25	
	Mean					132	3,273			
	SEM					0	34			
Family Elapidae: Elapinae										
Dendroaspis polylepis		0	0	70	76	879	107	37,706	0	0
Dendroaspis viridis		0	57	47	40	24,919	227	21,019	0	0
Micrurus altirostris		0	0	2	13	10	1,407	7	141	201
Micrurus corallinus		32	0	2	29	217	3,396	3,780	16	1
Micrurus nigrocinctus		0	0	10	63	684	2,604	7,912	4	0
Naja atra		0	0	10	93	401	1,397	328	3	4
Naja mossambica pallida		0	3	0	0	1,262	386	314	0	1
Naja nigricollis		0	16	0	36	1,857	708	302	0	2
Naja oxiana		0	0	10	6	84	2,007	73	24	27
Naja sputatrix		0	5	0	0	199	290	128	1	2
Ophiophagus hannah		0	0	18	45	50	2,185	98	44	22
	Mean					3,802	1,338	8,936		
	SEM					3,023	333	4,821		
Family Elapidae: Hydrophiinae/Laticaudinae										
Hydrophis cyanocinctus		0	47	2	56	232	801	723	3	1
Lapemis curtus		42	4	0	35	152	104	131	1	1
Laticauda semifasciata		20	0	0	21	43	830	149	19	6
	Mean					192	578	334		
	SEM					40	237	194		
Family Viperidae: Crotalinae										
Agkistrodon c. contortrix		0	0	0	0	0	0	0		
Agkistrodon p. conanti		0	0	0	0	0	0	0		
Bothrops moojeni		0	0	0	0	0	0	0		
Calloselasma rhodostoma		0	0	0	0	0	0	0		
Crotalus adamanteus		6	0	0	0	12	0	0		
Crotalus durissus terrificus	0	6	0	0	0	0	0			
Crotalus mitchellii pyrrhus	0	0	0	0	0	0	0			
Family Viperidae: Viperinae										
Bitis gabonica		0	43	61	360	38,206	5,844	42,617	0	0
Bitis nasicornis		0	14	35	435	13,444	3,428	36,038	0	0
Cerastes cerastes		0	0	0	0	28	0	0	0	
Daboia russellii siamensis	0	29	93	436	4,611	2,585	36	1	72	

(continued on next page)

TABLE 20.2 (continued)
Purine Levels in the Venoms of 32 Elapid, Viperine, and Crotaline Species

Taxon		AMP	HYP	CYT	URI	INO	GUA	ADN	G/I	G/A
Echis coloratus		0	5	118	104	114	71	0	1	
Pseudocerastes fieldi		0	0	3	187	2,789	1,325	37,126	0	0
Vipera palaestinae		398	0	0	0	62	0	0	0	
	Mean					11,832	3,295	38,593		
	SEM					6,962	953	2,036		

Note: Immense variation in purine content is seen at all taxonomic levels (e.g., Crotalinae vs. Viperinae or *Dendroaspis polylepis* vs. *D. angusticeps*). Guanosine/inosine and guanosine/adenosine ratios are also calculated, although the significance of these ratios, if any, is unclear.

deoxyribonucleosides. Mercader et al. (1996) reported that cytidine is a weak inhibitor of uridine uptake via the concentrative transporter in rat liver parenchymal cells, although inosine and adenosine also inhibited it in a dose-dependent manner. Guanosine also inhibited uridine uptake, but with some residual activity. Patil and Unadkat (1997) found evidence for two types of nucleoside transporters in human jejunum brush border membrane vesicles. Possibly, cytidine could help to elevate plasma levels of purine nucleosides. The cytidine levels seen in most venoms are probably inconsequential, but cytidine and 2′-deoxycytidine levels resulting from degradation of RNA and DNA by venom and host tissue enzymes could be significant.

2. Uridine Pharmacology

In addition to its possible role as a competitive inhibitor of concentrative purine transporters, uridine exhibits other pharmacology that is pertinent to envenomation. As mentioned above, uridine promotes CNS depression in mice with roughly 50% greater potency than cytidine (Krooth et al., 1978). Guarneri et al. (1983) reported that uridine displays anticonvulsant activity, inhibiting [^{3}H] GABA binding to receptors on rat cerebellar membranes (IC_{50} = 0.1 mM). The IC_{50} decreases tenfold in membrane preparations treated with Triton X-100. Guarneri et al. (1985) showed that uridine also inhibits [^{3}H]GABA binding to membrane preparations from the frontal cortex, hippocampus, and thalamus, and it antagonizes bicuculline-induced seizures. Kimura et al. (1993, 1996) found evidence that uridine derivatives interact with the benzodiazepine receptor in synaptic membranes. Kardos et al. (1999) suggested that uridine should be considered a neurotransmitter, and Dobolyi et al. (1999) showed that 50 to 100 μM uridine decreases the firing rate of hippocampal, but not thalamic neurons, while adenosine suppressed both.

Kimura et al. (2001a, 2001b) have proposed the existence of a uridine receptor that has hypnotic and sleep-inducing activity. According to Honda et al. (1984) and Inoue et al. (1984), when infused nocturnally into the third cerebral ventricle of conscious rats, a dose of 10 pmol of uridine enhances non-REM and REM sleep, but Inoue et al. (1984) found that the same dose had no effect when infused diurnally. Radulovacki et al. (1985) likewise concluded that uridine (1–100 nmol, ICV) had no effect on sleep. Thus, the possible relevance of uridine's soporific qualities to envenomation is unclear.

Uridine also affects vascular tension, but existing data suggest that it promotes vasoconstriction, not vasodilation, contrary to what one might have expected from its presence in venoms. Sakai et al. (1979) reported that injections of uridine into rat femoral artery induced vasoconstriction; however, even adenosine, ATP, guanosine, and GTP induced vasoconstriction in this preparation. MacDonald et al. (1984) reported that uridine, uridine monophosphate, and uridine diphosphate increased blood pressure in anaesthetized rats and increased the perfusion pressure in isolated, perfused rat kidneys. However, in everted rat portal vein, uridine had no discernible effect. UTP generally induces vasoconstriction (Hillaire-Buys et al., 1995; Welsh and Brayden, 2001), but in some preparations it promotes smooth muscle relaxation (Sakai et al., 1979; Martin et al., 1985; Hardebo et al., 1987). That

latter study showed that in pial vessels, UTP and UDP were powerful vasodilators (0.1–10.0 μM), but at higher concentrations, they behaved as vasoconstrictors. Uridine and UMP were without effect (Hardebo et al., 1987). The picture of uridine pharmacology with regard to vascular tension is far from conclusive.

C. Venom Purines

All elapid venoms contained inosine and guanosine, and all but the two species of *Bungarus* also contained adenosine (Table 20.2). These three nucleosides were absent from all crotaline venoms, except for that of *Crotalus adamanteus*, which had only 12 μg/g of inosine. Viperine venoms all contained inosine, but *Cerastes cerastes*, *Echis coloratus*, and *Vipera palaestinae* had low titers. These three species also lacked adenosine, and *V. palaestinae* and *C. cerastes* also lacked guanosine (Table 20.2). On average, nucleoside-positive viperine venoms had 4.8-fold more inosine, 1.8-fold more guanosine, and 6.4-fold more adenosine than elapid venoms (all subfamilies) that included those nucleosides. Only *Dendroaspis polylepis* and *D. viridis* had inosine, guanosine, and adenosine levels comparable to those of viperines (Table 20.2). Venoms of the piscivorous marine elapids, *Hydrophis cyanocinctus*, *Lapemis curtus* (Hydrophiinae), and *Laticauda semifasciata* (Laticaudinae), had significantly lower levels of all three major purines than other elapid subfamilies.

Attempts to correlate purine levels with prey specificity are fraught with problems that stem chiefly from our ignorance of the prey preferences of venomous snakes, to say nothing of ontogenetic and geographic variation in such preferences, and corresponding ontogenetic variation in venoms. No studies to date have explored possible ontogenetic changes in purine levels or purine-releasing enzymes.

1. Hypoxanthine Pharmacology

Hypoxanthine was confirmed in seven elapid (all subfamilies examined) and five viperid venoms. It was absent from all crotaline venoms except for a sample of *Crotalus durissus terrificus*, which possessed only 6 μg/g. Only Doery (1957) had previously reported hypoxanthine as a venom constituent (*Notechis scutatus*). Aird (2005) found 57 μg/g in a sample of *Dendroaspis viridis* venom, while a sample of *D. polylepis* had none.

Aird (2005) concluded that the most likely explanation for the presence of hypoxanthine in some venoms is as a substrate for the plasma enzyme, xanthine oxidase, which generates hydrogen peroxide (Tachon, 1989; Dowell et al., 1993). Hydrogen peroxide stimulates nitric oxide synthase, which produces the potent vasodilator nitric oxide (Wolin and Burke, 1987; Mittal, 1993). Berman and Martin (1993) showed that hypoxanthine in the presence of xanthine oxidase increases plasma extravasation, and that it impairs lysosomal stability (Olsson et al., 1989). Thus, venom hypoxanthine may contribute to hypotensive immobilization of prey (Aird, 2002). Secondarily, it may also contribute to prey digestion via apoptosis, fast necrotic cell death, and cellular degradation promoted by other venom constituents (Suhr and Kim, 1996; Torii et al., 1997, 2000). The hypotensive contribution of hypoxanthine is probably minor, compared to that of L-amino acid oxidase, which also generates hydrogen peroxide.

A second possible explanation for venom hypoxanthine is its interaction with benzodiazepine receptors. Skolnick et al. (1978, 1979a), Marangos et al. (1978), and Asano and Spector (1979) have shown that hypoxanthine inhibits [^{3}H]diazepam binding to central benzodiazepine receptors. Benzodiazepine receptor agonists display anxiolytic, anticonvulsant, sedative, and myorelaxant activities (Haefely et al., 1993; Sanger et al., 1994).

2. Inosine Pharmacology

Inosine may be more important in elapid and viperine species that prey largely upon mammals or birds. It is greatly elevated in venoms of *Bitis gabonica*, *B. nasicornis*, *Daboia russellii*, and *Dendroaspis viridis*, but not *D. polylepis* (Table 20.2). Both *Dendroaspis* have massive amounts

of adenosine, as do the two *Bitis* species. Adenosine and inosine both activate mast cell A_3 receptors (Ramkumar et al., 1993; Metcalfe et al., 1997; Tilley et al., 2000), so it may be that either or both suffice equally well for mammal or bird predation. In addition, many effects of adenosine are potentiated by inosine. Komarek and Parish (1975) report that adenosine given intravenously to dogs (0.2 mg/kg) did not cause bradycardia, but the same dose administered with inosine (3.2–10.0 mg/kg) did slow the heart rate. Jones et al. (1981) reported that inosine relaxed canine arterial rings more completely and more consistently than did adenosine. Moreover, its effects were not blocked by the methylxanthine aminophylline, indicating that inosine relaxes canine coronary arteries at a site distinct from adenosine P1 receptors (Aviado, 1983). The additivity of the effects of adenosine and inosine may stem from inosine's activation of benzodiazepine receptors, an activity it shares with hypoxanthine (Skolnick et al., 1978, 1979a, 1979b; Marangos et al., 1978; Asano and Spector, 1979; MacDonald et al., 1979).

3. Guanosine Pharmacology

Like adenosine, extracellular guanosine is hypotensive (Vuorinen et al., 1991, 1992, 1994). Vuorinen et al. found that guanosine (10 μM–1 mM) relaxed rat mesenteric artery rings, with and without endothelium, and concluded that guanosine does not act at P1 or P2Y receptors. Rathbone et al. (1992a, 1992b) found that proliferation was induced in quiescent chick astrocytes, human brain capillary endothelial cells, chick meningeal fibroblasts, Swiss mouse 3T3 cells, and human astrocytoma cell lines by addition of guanosine to the culture medium. Stimulation of proliferation was blocked by the adenosine A_2 receptor antagonist, DPMX, but not by PACPX, an A_1 receptor antagonist. They hypothesized that guanosine and other ribo- and deoxyribonucleosides inhibited extracellular adenosine deaminase, thereby increasing the extracellular concentration of adenosine. Whether guanosine's mode of action is the same in the vasculature remains to be seen. Ciccarelli et al. (1999) reported that cultured rat astrocytes spontaneously released guanine nucleotides at a higher rate than that at which they released adenine nucleotides, such that the level of guanosine in the culture was continuously threefold higher than the level of adenosine. Exposure of the cultures to hypoxia/hypoglycemia for 30 min increased the extracellular concentration of adenine-based purines by 2.5-fold and of guanine-based purines by 3.5-fold.

Vinade et al. (2005) report that intraperitoneal or oral guanosine caused an amnesic effect that appeared related to suppression of glutamatergic neurotransmission, but unrelated to adenosine. They found that guanosine also weakened retention in an inhibitory avoidance task and reduced locomotor activity in an open field test. Schmidt et al. (2000) reported that guanosine has antiseizure activity, which they attributed to a suppression of glutamatergic neurotransmission. Traversa et al. (2002, 2003) showed that guanosine binds to its own receptor on rat brain membranes (cerebellum removed), distinct from P1 receptors.

Guanosine generally appears to be elevated relative to inosine and adenosine in venoms of species that prey largely upon fish, reptiles (especially snakes), or amphibians. For instance, guanosine/inosine ratios significantly greater than unity are seen in ophiophagous (snake-eating) species (*Ophiophagus hannah* (43.7), *Micrurus corallinus* (15.6), *M. altirostris* (140.7), *Bungarus fasciatus* (249.2), and *B. multicinctus* (25.1)). In *M. nigrocinctus* the guanosine/adenosine ratio is low despite elevated guanosine levels because this species also possesses a large amount of adenosine (Table 20.2).

D. Unidentifiable Purines

Aird (2005) reported that unidentifiable low molecular weight venom constituents fell into three categories:

1. Compounds having spectra identical (from 200 to 300 nm) to those of NAD and xanthosine were found in various venoms; however, their retention times varied sufficiently from those of the standards that they could not be identified.

2. Numerous other peaks were seen with spectra similar to those of adenosine, inosine, and guanosine, but with retention times so different from these nucleosides (in the same venoms) that they unquestionably represent derivatives of these three. Most of these were relatively minor components.
3. Several significant unknown peaks were seen in many venoms. These had spectra that bore no resemblance to those of the nucleosides examined or to peptides.

E. Venom Enzymes That Release Purines

Injecting exogenous (venom) nucleosides and releasing endogenous (prey tissue) nucleosides with venom enzymes are complementary envenomation strategies. For this reason, Aird (2005) also examined data from the literature regarding titers of venom enzymes responsible for purine liberation. The primary venom enzymes involved include 5NUC, PDE, and PME (Aird, 2002). On average, viperine and crotaline venoms have 3.0- and 3.7-fold more 5NUC, respectively, than elapid venoms, and these differences were statistically significant ($p < .001$). Elapids have more PDE than either crotalines (8.3-fold) or viperines (13.5-fold) ($p < .01$). Alkaline phosphomonoesterase levels are not so disparate. Crotalines have an average of 1.4-fold more PME than elapids and 2.2-fold more than viperines. Only the latter comparison was statistically significant ($p < .001$).

Graham et al. (2005) used LC-MS and CE-MS to identify adenosine in the venoms of *Bitis gabonica*, *Bitis arietans*, and *Bitis nasicornis*. They surveyed thirty different elapid and viperid venoms for adenosine, but did not mention the other twenty-seven species that they examined. Given the abundance of purines in elapid and viperine venoms, their failure to have found them in other venoms suggests some methodological problems, and several earlier reports demonstrated the presence of adenosine in *Bitis* venoms (Fischer and Dörfel, 1954; Doery, 1956). A role for adenosine and its secondary metabolite, inosine, in the hypotension observed in the prey was first hypothesized by Fischer and Dörfel (1954), based upon the earlier work of Drury and Szent-Györgyi (1929). Aird (2002) explored the function of exogenous venom purines in envenomation, and also the role of ubiquitous, nontoxic venom enzymes, the presence of which had previously been unexplained. This review highlighted and extended the earlier observations on the potential roles of adenosine on prey physiology. Graham et al. (2005) suggest that the concentrations of adenosine found in some *Bitis* venoms are sufficient to promote localized vasodilation. This is probably correct; however, localized vasodilation probably would have little impact upon prey flight. Rather, it is profound systemic hypotension leading to circulatory shock that is of strategic importance to snakes.

VI. PHARMACOLOGICAL SIGNIFICANCE OF THE QUANTITIES OF NUCLEOSIDES FOUND IN VENOMS

It is logical to assume that if a venom constituent is widespread among different venoms, and present in a significant quantity, it is also functionally significant. Nonetheless, it is possible to estimate the amounts of nucleosides injected into prey tissues during envenomation by some snake species. The nucleoside concentrations actually achieved in the prey vasculature are another matter. Without using injections of radiolabeled nucleosides, it would be difficult to estimate the speed with which intramuscularly or intraperitoneally injected purines might find their way into the bloodstream. This says nothing of the difficulty of estimating nucleoside liberation by venom and prey PDE, 5-NUC, PME, and NADase, though quantities of enzymatically liberated nucleosides could conceivably be much more significant than the quantities of exogenous nucleosides injected.

A number of studies have estimated the amount of venom injected into prey by venomous snakes (Morrison et al., 1982, 1983a, 1983b; Tun-Pe et al., 1991; Hayes et al., 1992, 2002; Hayes, 1992).

However, of the fifteen species studied, only *Acanthophis antarcticus*, *Daboia russellii*, *Daboia palaestinae*, and *Agkistrodon piscivorus* were also examined by Aird (2005). While *Agkistrodon piscivorus* venom has modest levels of PDE and a significant amount of 5NUC (Tan and Ponnudurai, 1990c), it is devoid of nucleosides. Predatory bites by *Daboia palaestinae* and *Daboia russellii* could achieve plasma inosine concentrations as high as 18 and 17 μM, respectively (Table 20.3A). While *Acanthophis antarcticus* probably injects less venom than the two foregoing species, it has a higher nucleoside content, and bites by *A. antarcticus* could induce prey plasma nucleoside levels as high as 100 μM inosine, 570 μM guanosine, and 960 μM adenosine (Table 20.3A). It should be noted that the estimated quantities of venom injected by *Daboia russellii* (Tun-Pe and Khin-Aung-Cho, 1986) are extremely low. Given the size of the snake, it is conceivable that actual values could be as much as tenfold higher. In contrast, values they report injected into a model human limb are comparable to those reported by other authors. If the quantities of venom injected by various elapids and crotalines are used to estimate the quantities of venom injected by the species studied by Aird (2005), blood concentrations of inosine and adenosine in mice could exceed 3 mM (Table 20.3B). These data assume no contribution to circulating nucleoside concentrations from nucleoside-releasing enzymes (PDE, endonucleases, 5NUC, PME, NADase); however, released endogenous nucleosides may be much more significant than exogenous nucleosides. These species were examined by Aird (2005), but the literature contains no studies of venom quantities injected. Instead, venom injected is estimated from other species with the most similar body size and proportions. The species used for the estimates are shown in parentheses (*Oxyuranus microlepidotus*: Morrison et al., 1984; *Notechis scutatus*: Morrison et al., 1982; *Oxyuranus scutellatus*: Morrison et al., 1982; *Agkistrodon piscivorus*: Hayes et al., 2002; *Crotalus viridis*: Hayes, 1992). Blood volumes for mice are assumed to be 6 to 8% of body mass (J. Crossland, *Peromyscus* Genetic Stock Center, University of South Carolina, personal communication; Animal Care Guidelines, McGill University Animal Care Committee, 2002; Mitruka and Rawnsley, 1981; Harkness and Wagner, 1989); thus, a 30 g mouse would have a maximum total blood volume of 2.4 ml. Using molecular weights of 267.2 g/mol (adenosine), 283.3 g/mol (guanosine), and 268.2 g/mol (inosine), maximal plasma molarities that could result from envenomation were calculated.

These numbers assume that all injected nucleoside finds its way into the circulation immediately. While this is an unlikely possibility, the projected concentrations are so large that they are probably still significant. These concentrations are several times higher than necessary to activate P1 receptors mediating physiological processes consonant with the strategies of snake envenomation (vasodilation, bradycardia, suppression of neurotransmitter release, smooth muscle relaxation, or inhibition of smooth muscle contraction, hyperalgesia, apoptosis) (Table 20.4) (Aird, 2002).

VII. PHARMACOLOGY OF DEOXYRIBONUCLEOSIDES

The pharmacology of deoxyribonucleosides, which would be liberated by PDE, 5′NUC, and PME from deoxyribonucleic acid substrates, has been relatively little studied. Available data suggest that deoxyribonucleosides have pharmacological effects similar to those of ribonucleosides. For instance, Darlington and Gann (2005) found that inosine, guanosine, adenosine, deoxyadenosine, and deoxyguanosine were all able to stimulate rat erythrocyte Na^+/K^+ ATPase in a dose-dependent fashion, and all were able to overcome the partial inhibition caused by ouabain. Both adenosine and deoxyadenosine promote apoptosis in human breast cancer cells (Hashemi et al., 2005). Wakade et al. (1998) showed that 100 μM deoxyadenosine, but not adenosine, induces apoptosis in rat chromaffin cells. Both adenosine and 2′-deoxyadenosine have negative inotropic and chronotropic activity on isolated guinea pig atria (Fuhrman and Fuhrman, 1982), but 2′-deoxyadenosine exhibited much lower potency. Skolnick et al. (1979b) found 2′-deoxyinosine more than twice as potent as inosine at delaying the onset of pentylenetetrazole-induced seizures.

TABLE 20.3A
Venom Purine Nucleoside Levels and Maximal Plasma Values Potentially Achievable during Envenomation (Species for Which Purine Concentrations and Quantities of Venom Injected Are Known)

		Venom				Injected			Plasma			
Species		Injected (mg)	INO (μg/g)	GUA (μg/g)	ADO (μg/g)	INO (μg)	GUA (μg)	ADO (μg)	INO (μM)	GUA (μM)	ADO (μM)	Reference
Acanthophis antarcticus	Min	3	590	3,550	5,638	2	11	17	3	16	26	Morrison et al. (1983b)
	Mean	42	590	3,550	5,638	25	149	237	38	219	369	
	Max	109	590	3,550	5,638	64	387	615	100	569	958	
Daboia palaestinae	Min	1	62	0	0	0	0	0	0	0	0	Allon and Kochva (1974)
	Mean	54	62	0	0	3	0	0	5	0	0	
	Max	188	62	0	0	12	0	0	18	0	0	
Daboia russellii	Min	1	4,611	2,585	36	6	3	0	9	5	0	Tun-Pe and Khin-Aung-Cho (1986)
	Mean	2	4,611	2,585	36	8	4	0	12	6	0	
	Max	2	4,611	2,585	36	11	6	0	17	9	0	

TABLE 20.3B
Species for Which Purine Concentrations Are Known, but Injected Quantities of Venom Have Not Been Determined

		Venom				Injected			Plasma		
Species		Injected (mg)	INO (μg/g)	GUA (μg/g)	ADO (μg/g)	INO (μg)	GUA (μg)	ADO (μg)	INO (μM)	GUA (μM)	ADO (μM)
	Min	1	879	107	37,706	1	0	38	1	0	59
Dendroaspis polylepis	Mean	18	879	107	37,706	16	2	679	25	3	1,058
(*Oxyuranus microlepidotus*)	Max	46	879	107	37,706	40	5	1,734	63	7	2,705
	Min	1	24,919	227	21,019	25	0	21	39	0	33
Dendroaspis viridis	Mean	18	24,919	227	21,019	449	4	378	697	6	590
(*Oxyuranus microlepidotus*)	Max	46	24,919	227	21,019	1,146	10	967	1,781	15	1,508

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TABLE 20.3B (continued)
Species for Which Purine Concentrations Are Known, but Injected Quantities of Venom Have Not Been Determined

		Venom				Injected			Plasma		
Species		Injected (mg)	INO (μg/g)	GUA (μg/g)	ADO (μg/g)	INO (μg)	GUA (μg)	ADO (μg)	INO (μM)	GUA (μM)	ADO (μM)
	Min	1	401	1,397	328	0	1	0	1	2	1
Naja atra	Mean	14	401	1,397	328	6	20	5	9	29	7
(*Notechis scutatus*)	Max	37	401	1,397	328	15	52	12	23	76	19
	Min		50	2,185	98	0	0	0	0	0	0
Ophiophagus hannah	Mean		50	2,185	98	0	0	0	0	0	0
(*Oxyuranus scutellatus*)	Max		50	2,185	98	0	0	0	0	0	0
	Min	0	38,206	5,844	42,617	0	0	0	0	0	0
Bitis gabonica	Mean	14	38,206	5,844	42,617	535	82	597	831	120	930
(*Agkistrodon piscivorus*)	Max	58	38,206	5,844	42,617	2,216	339	2,472	3,443	499	3,854
	Min	0	13,444	3,428	36,038	0	0	0	0	0	0
Bitis nasicornis	Mean	14	13,444	3,428	36,038	188	48	505	292	71	787
(*Agkistrodon piscivorus*)	Max	58	13,444	3,428	36,038	780	199	2,090	1,211	293	3,259
	Min	5	2,789	1,325	37,126	14	7	186	22	10	289
Pseudocerastes fieldi	Mean	15	2,789	1,325	37,126	42	20	557	65	29	868
(*Crotalus viridis*)	Max	25	2,789	1,325	37,126	70	33	928	108	49	1,447

Note: Estimates are made from literature data about related species of similar physiognomy (in parentheses).

TABLE 20.4
Purine Concentrations Necessary to Activate Adenosine and Other Receptors in Various Tissues

Nucleoside	Effect	Tissue/Organism	Route of Admin.	Nucleoside Sensitivity	Receptor Subclass	Reference
Adenosine	Inhibition of field excitatory postsynaptic potentials	Rat hippocampal slices	Bath	30 μM	A1	Dunwiddie et al. (1997b)
	Inhibition of evoked glutamate release	Rat cerebrocortical synaptosomes	Bath	10–1,000 μM	A2A	Marchi et al. (2002)
	Inhibition of glutamate and GABA release	Rat subthalamic nucleus	Bath	100 μM	A1	Shen and Johnson (2003)
	Inhibition of glutamate release	Rat hippocampal slices	Bath	30 μM		Corradetti et al. (1984)
	Suppression of noradrenaline release	Rabbit kidney	?	0.1–10 nM		Hedqvist and Fredholm (1976)
	Suppression of excitatory junction potentials	Isolated rabbit mesenteric artery	Bath	4.7 nM		Illes et al. (1988)
	Inhibition of spontaneous contractions	Longitudinal smooth muscles of mouse distal colon	Bath	100–3,000 μM	A1	Zizzo et al. (2006)
	Relaxation	Longitudinal smooth muscles of mouse proximal colon	Bath	100–3,000 μM	A1, A2B	Zizzo et al. (2006)
	Suppression of myocyte contraction	Isolated rabbit atrioventricular nodal myocytes	Bath	0.1–50 μM	A1	Martynyuk et al. (1995)
	Vasodilation	Trout coronary rings	Bath	0.1–10 μM		Mustafa and Agnisola (1998)
	Vasodilation	Canine coronary rings	Bath	0.89 μM		Jones et al. (1981)
	Vasodilation	Canine adipose tissue	Secretion	0.25–1 nM		Sollevi and Fredholm (1981)
	Vasodilation	Canine femoral artery	i.v.	4.2 μM		Cotterrell and Karim (1982)
	Bradycardia	Dogs	i.v.	0.1–200 nM		Belloni and Hintze (1991)
	Nociception (pain)	Rat hindpaw	i.d.		A2	Taiwo and Levine (1990)
	Nociception (pain)	Rat hindpaw	s.c.	0.005–10 nmol	A3	Sawynok et al. (1997)

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TABLE 20.4 (continued)
Purine Concentrations Necessary to Activate Adenosine and Other Receptors in Various Tissues

Nucleoside	Effect	Tissue/Organism	Route of Admin.	Nucleoside Sensitivity	Receptor Subclass	Reference
	Apoptosis	HL-60 cells and U-937 lymphoma cells	Bath	10 μM	A3	Yao et al. (1997)
	Inhibition of thrombin-induced platelet aggregation	Human platelets	Bath	1–1,000 μM		Vuorinen and Laustiola (1992)
Inosine	Relaxation	Guinea pig trachea	Bath	35–1,000 μM		Coleman (1976)
	Vasodilation	Canine coronary rings	Bath	224 μM	non-P1	Jones et al. (1981)
	Vasodilation	Rat pial arterioles in somatosensory cortex		1,000 μM		Ko et al. (1990)
Guanosine	Vasodilation	Rat mesenteric artery (endothelium intact and denuded)	Bath	10–1,000 μM		Vuorinen et al. (1994)
	Attenuation of hyperlocomotion promoted by dizocilpine	Mice	i.p.	380 μM		Tort et al. (2004)
	Attenuation of quinolinic acid-induced seizures	Mice	i.p.	380 μM		Schmidt et al. (2000)
	Relaxation	Guinea pig trachea	Bath	35–1,000 μM		Coleman (1976)
Cytidine	Central depression of spontaneous activity	Mice	i.p.	~16.5 μM		Krooth et al. (1978)
Uridine	Central depression of spontaneous activity	Mice	i.p.	~11 μM		Krooth et al. (1978)
	Inhibition of GABA binding	Rat cerebellar membranes	Bath	100 μM		Guarneri et al. (1983)
	Inhibition of GABA binding	Rat frontal cortex, hippocampal and thalamic membranes	Bath	1,000 μM		Guarneri et al. (1985)

Note: Where possible, the receptor subtypes are identified. Nucleoside concentrations potentially achievable by exogenous venom purines alone are well above the concentrations necessary for receptor activation. Nucleoside sensitivities (nmol/kg) were converted to nM based on assumption of 10% blood volume (University of Minnesota Guidelines for Collection of Animal Blood: http://www.ahc.umn.edu/rar/blood.html).

VIII. CONCLUSIONS

Since the idea was first advanced that purine nucleosides are central components in the envenomation strategies of elapids and viperids (Aird, 2002), evidence has continued to accumulate in support of the concept, both from studies of venoms (Aird, 2005; Graham et al., 2005) and from the nucleoside pharmacological literature. Lumsden et al. (2004) have shown that *Boiga dendrophila* venom also contains adenosine, although it is presently unclear how widespread purines might be among colubrid venoms. Pyrimidine nucleosides are also found in many venoms, although at much lower levels than purine nucleosides. Crotaline venoms appear to be essentially devoid of nucleosides, although they are rich in enzymes that could liberate endogenous nucleosides from prey tissues. Viperines and elapids apparently use a blend of both approaches. There is currently no explanation for the great diversity of nucleoside levels seen in different venoms, particularly among congeneric taxa, although it probably reflects the physiology of the array of preferred prey consumed by each species. The strategic significance of cytidine, uridine, and to a lesser extent, hypoxanthine is unclear.

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Section IV

Envenomation: Occurrence, Prevention, Treatment

21 Envenomation
Prevention and Treatment in Australia

Julian White

CONTENTS

Australia contains a diverse array of snakes, with a predominance of elapids, which account for all dangerous species, viperids being absent from the continent and adjacent New Guinea (Cogger, 1995). These dangerous elapids are clinically characterized by systemic envenoming with generally minor or trivial local effects (White, 1987a). Neurotoxic paralysis (pre- or postsynaptic), myolysis, coagulopathy (defibrination or anticoagulant), renal failure, and microangiopathic hemolytic anemia are the major systemic effects of envenoming, but each of the five major terrestrial dangerous snake groups causes a distinctive pattern of envenoming involving only some of these effects (White, 1991, 1995a; White and Fassett, 1983). These five groups are the brown snakes (*Pseudonaja* spp.), the tiger snake group (*Notechis* spp., *Tropidechis carinatus*, *Austrelaps* spp., *Hoplocephalus* spp.), the black snake/mulga snake group (*Pseudechis* spp.), the taipans (*Oxyuranus* spp.), and the death adders (*Acanthophis* spp.). Antivenoms are available for each group and a polyvalent covering all groups (White, 2001). In addition, there are numerous sea snake species in northern waters, with an antivenom available (White, 1995b, 2001).

I. INTRODUCTION

Snakebite is the most significant form of envenoming encountered in Australia, if potential lethality linked to numbers of cases is considered. However, if numbers of cases alone are considered, then snakebite is far less important than spiderbite and jellyfish stings, neither of which account for a significant number of deaths in twenty-first-century Australia. Australia is known internationally as having many of the world's most dangerous snakes. While technically true based on venom toxicity (Broad et al., 1979), many other factors must be considered, not least the rate of bites and the availability of high-quality health care to manage envenomation. As will be noted below, few Australians suffer venomous snakebites and very few die (White, 1995a).

Nevertheless, major envenoming by Australian snakes can pose significant management challenges for health professionals, and without optimum care, death rates would likely rise steeply. Management of Australian snakebite has been through a period of stability, but has now entered a period of significant change, as research refines and redefines management standards (Currie, 2006; Isbister et al., 2006b, 2007a; Isbister, 2006; White, 2007; O'Leary et al., 2007). Much of this work is ongoing and yet to be published.

II. EPIDEMIOLOGY

In common with nearly all other nations, Australia has poor collection of epidemiologic data for envenoming. Available sources for researchers are discharge diagnosis coding for hospital admissions, reports of antivenom use (voluntary, considered to capture <30% of cases of antivenom use), poison center inquiries, and records of causes of death. All of these pose major problems in capturing comprehensive data. Therefore, published rates of snakebite in Australia are at best informed guesstimates. Based on personal experience dealing with snakebites around Australia over the last 30 years, this author considers there are likely to be around 1,000 to 1,500 definite or suspected snakebite victims seeking treatment in Australia each year, of which between 100 and 200 have systemic envenoming sufficient to justify antivenom treatment.

In mainland Australia, where most of the population resides, bites by brown snakes (*Pseudonaja* spp.) predominate, reflecting the commonness of these snakes in both rural and urban environments (Sutherland and Lovering, 1979; White, 1983a, 1995a; Jamieson and Pearn, 1989; Jelinek et al., 1991; Sutherland, 1992; Sutherland and Leonard, 1995; Barrett and Little, 2003). Not surprisingly, brown snakes cause most fatal snakebites, with occasional deaths from envenoming by tiger snakes, taipans, and death adders, all other dangerous species rarely causing fatalities now. While actual numbers of fatalities fluctuate considerably year to year, there are around two to four deaths per year. In the most recent snakebite season (2006–07) there were at least three deaths following brown snake bite, with two other cases likely to be due to brown snake bite, though not absolutely

confirmed. Of these five cases, one was a child (9 years), one a youth (16 years), and the rest adults. All died as a result of prehospital collapse and cardiac arrest, and while some might have survived if immediate effective resuscitation had been available, it is doubtful all would have survived. From this it can be surmised that despite the best available health resources for managing snakebite, once a bite has occurred, some patients are likely to die. This indicates that prevention of bites is a vital element in the reduction of snakebite fatalities.

III. OVERVIEW OF SNAKE FAUNA

Australia has a diverse snake fauna, dominated by venomous species, with only a minority being nonvenomous, quite a contrast to most other nations, where nonvenomous species predominate (Cogger, 1995; White, 2001). However, only a minority of the venomous species are capable of inflicting a medically significant bite, and the majority of venomous species are small, cryptic, rarely encountered by the general populace, and rarely cause bites. Similarly, the nonvenomous species are an infrequent cause of bites in most regions, so snakebite in Australia is dominated by bites by potentially lethal species.

A. Elapids

Elapid snakes (family Elapidae) account for nearly all Australian venomous snakes and all species of medical significance. As front-fanged cobra-like snakes, envenoming could be expected to be similar to that of elapids in other regions. While there are some similarities with other elapid venoms, Australian terrestrial elapids have evolved rather more complex venoms that can present a clinical picture significantly different from classic elapid envenoming elsewhere.

There are five major groups of medically important Australian terrestrial elapids (Table 21.1), plus the marine sea snakes, which are considered to have evolved from their terrestrial cousins. Each of these groups is discussed in detail later in this chapter.

B. Colubrids

There are relatively few colubrid snakes (family Colubridae) in Australia, and none are considered medically significant, though several are venomous, most notably the brown treesnake (*Boiga irregularis*), a species known from an introduced population on Guam to cause significant envenoming in infants, a problem not seen in Australia (Fritts et al., 1990, 1994). Further, colubrid snakes have a limited distribution in Australia.

IV. OVERVIEW OF VENOMS AND CLINICAL EFFECTS

A detailed discussion of venoms can be found elsewhere in this book. In this section, some basic information on Australian elapid venoms will be used to guide an understanding of the clinical problems of envenoming that are encountered. Any discussion of venoms must note the issue of venom variability, which may affect the patterns of envenoming encountered clinically and the choices of venoms used in immunizing for antivenom production (Chippaux et al., 1991). There are few studies on venom variability among dangerous Australian elapids, but these indicate that rapid development of variability can be expected, yet key components are likely to be conserved and common throughout a taxon (Williams and White, 1987; Williams et al., 1988).

A. Neurotoxins and Neurotoxicity

Classic discussions of Australian snakebite have emphasized neurotoxicity as the leading clinical problem and cause of death. One hundred years ago this may have been true, but it is no longer

TABLE 21.1
Major Groups of Dangerous Australian Terrestrial Snakes

Scientific Name	Common Name	Clinical Effects[a,b]	Antivenom[c]
	Brown Snake Group		
Pseudonaja affinis	Dugite	DC (c), RF (o), MAHA (o), NT (r)	BSAV
Pseudonaja guttata	Spotted brown snake	DC (c), RF (o), MAHA (o), NT (r)	BSAV
Pseudonaja ingrami	Ingram's brown snake	DC (c), RF (o), MAHA (o), NT (r)	BSAV
Pseudonaja inframacula	Peninsular brown snake	DC (c), RF (o), MAHA (o), NT (r)	BSAV
Pseudonaja nuchalis	Western brown snake or gwardar	DC (c), RF (o), MAHA (o), NT (r)	BSAV
Pseudonaja textilis	Eastern brown snake	DC (c), RF (o), MAHA (o), NT (r)	BSAV
	Tiger Snake Group		
Notechis scutatus	Common tiger snake	DC (c), NT (c), MY (c), RF (o)	TSAV
Notechis ater	Black tiger snake (several important subspecies)	DC (c), NT (c), MY (c), RF (o)	TSAV
Notechis (*ater*) *occidentalis*	West Australian tiger snake	DC (c), NT (c), MY (c), RF (o)	TSAV
Tropidechis carinatus	Rough-scaled snake	DC (c), NT (c), MY (c), RF (o)	TSAV
Austrelaps superbus	Lowland copperhead	NT (c), MY (u)	TSAV
Austrelaps ramsayii	Highland copperhead	NT (c), MY (u)	TSAV
Austrelaps labialis	Pygmy copperhead	NT (r), MY (u)	TSAV
Hoplocephalus bungaroides	Broad-headed snake	DC (c)	TSAV
Hoplocephalus bitorquatus	Pale-headed snake	DC (c)	TSAV
Hoplocephalus stephensi	Stephen's banded snake	DC (c)	TSAV
Rhinoplocephalus nigrescens	Eastern small-eyed snake	MY (r)	TSAV
	Black Snake Group		
Pseudechis australis	Mulga snake or king brown	MY (c), AC (o), RF (o)	BLAV
Pseudechis butleri	Butler's mulga snake	MY (c), AC (o), RF (o)	BLAV
Pseudechis colletti	Collett's snake	MY (c), AC (o), RF (o)	BLAV
Pseudechis guttatus	Spotted black snake	MY (o)	TSAV
Pseudechis porphyriacus	Red-bellied black snake	MY (o)	TSAV
	Death Adder Group		
Acanthophis antarcticus	Common death adder	NT (c)	DAAV
Acanthophis praelongus	Northern death adder	NT (c)	DAAV
Acanthophis pyrrhus	Desert death adder	NT (c)	DAAV
Acanthophis wellsi	Pilbara death adder	NT (c)	DAAV
	Taipan Group		
Oxyuranus scutullatus	Common taipan	DC (c), NT (c), MY (o), RF (o)	TAAV
Oxyuranus microlepidotus	Inland taipan	DC (c), NT (c), MY (o), RF (o)	TAAV
Oxyuranus temporalis	Central ranges taipan	DC (c), NT (c), MY (o), RF (o)	TAAV

[a] Type: DC = defibrination coagulopathy; AC = anticoagulant coagulopathy; NT = neurotoxicity (flaccid paralysis); MY = myotoxicity; RF = renal damage/failure; MAHA = microangiopathic hemolytic anemia.

[b] Frequency: (c) = common clinical effect; (o) = occasional clinical effect; (r) = rarely seen clinical effect; (u) = theoretically possible but unknown if occurs clinically in humans.

[c] Antivenom type: BSAV = CSL brown snake AV; TSAV = CSL tiger snake AV; BLAV = CSL black snake AV; DAAV = CSL death adder AV; TAAV = CSL taipan AV (CSL polyvalent snake AV is equally acceptable for taipan bites, because volumes, risk profile, and cost are similar).

valid (White, 1984; Sutherland, 1992). However, neurotoxicity is still seen and, without appropriate treatment, can still be lethal. There are two principal classes of neurotoxins in Australian terrestrial snake venoms that are of clinical significance: presynaptic and postsynaptic neurotoxins (White, 1987b). Both act at the skeletal neuromuscular junction (NMJ), causing progressive flaccid paralysis, extending in severe cases to full respiratory paralysis and failure, a condition universally fatal unless external respiratory support is applied. Most Australian terrestrial elapid venoms contain a mixture of both types of neurotoxins, though a few species have only postsynaptic neurotoxins, and a few show no neurotoxicity in humans at all, though they may cause paralysis in some mammal species.

The presynaptic neurotoxins are of the β-bungarotoxin type, with some of large molecular size and extremely potent, while the postsynaptic neurotoxins are of the α-bungarotoxin type, generally smaller in size and far less toxic (relatively), though still able to cause paralysis.

The clinical effects of these types of neurotoxins are essentially the same, but with one important difference from a treatment perspective. Presynaptic neurotoxicity is associated with damage to the terminal axon at the NMJ, and therefore established paralysis will not be reversed by antivenom. Postsynaptic neurotoxicity is not associated with damage to either the terminal axon or the muscle endplate, and so is potentially reversible with antivenom therapy, dependent on the reversibility of binding between the toxin and the acetylcholine receptor on the muscle endplate.

First signs of flaccid paralysis are usually seen >1 hour postbite, sometimes as late as 24 hours postbite (Campbell, 1979; White, 1981, 1987a). Progression from first signs to full respiratory paralysis usually takes >6 hours, often >12 hours, though can occasionally occur more rapidly. Only some cases with early signs will show progression to full paralysis. First signs are seen in the cranial nerves, starting with ptosis, and then external ophthalmoplegia, progressing to fixed forward gaze, fixed dilated pupils, loss of facial expression, reduced tongue extrusion, and then loss of airway protection and onset of drooling. At this point intubation may be required to stabilize the airway, even though there is no respiratory paralysis. Paralysis may then progress to involve all limbs, with weakness and loss of deep tendon reflexes (DTRs). Respiration and diaphragmatic function are last to be affected.

In a few cases, even those with relatively minor paralytic features, there may be alteration in, or complete loss of, taste or smell, or both. If this problem develops, it may be months before any return of function, and in some cases, normal function never returns (White, 1987a).

B. Myotoxins and Myotoxicity

Myotoxicity is a prominent feature of envenoming by a limited subset of Australian elapid snakes, most notably the tiger snakes and rough-scaled snake (*Notechis* spp., *Tropidechis carinatus*) and mulga snake/Collett's snake subgroup (*Pseudechis* spp.) (White, 1987a; Nocera et al., 1998; Isbister et al., 2006b). The myotoxins, generally forms of phospholipase A_2 and closely related to the presynaptic neurotoxins, affect skeletal muscle. Clinically this presents as muscle pain, tenderness, pain on contraction against resistance, and myoglobinuria, often macroscopic. Onset is variable, but always >1 hour postbite, generally >6 hours postbite, and occasionally >24 hours postbite. In some cases the first indication of developing myolysis is myoglobinuria and a rising CK. CK is the definitive measure for presence and progression of myolysis. CK levels can reach extremes of >100,000 IU/l (normal, <250 IU/l). Two secondary complications can arise, renal failure and hyperkalemia, both, especially the latter, potentially lethal (hyperkalemia can cause cardiac arrest). However, the common textbook view that myoglobinuria causes renal failure is not supported by experience with Australian snakebite; this author has treated a number of cases of snakebite myolysis over the last 30 years, with high CK levels (>100,000 IU/l) and macroscopic myoglobinuria, sometimes severe, without evidence of renal impairment.

C. Hemostatic System Toxins and Coagulopathy

Snake venoms, notably viperid venoms, target diverse points in the complex hemostatic pathways, the endpoint usually being coagulopathy with increased bleeding, though a few species cause thrombosis (White, 1983b, 1987b, 2005). Elapid venoms do not feature prominently in this area of activity, the exception being Australian elapids. Many, but not all, dangerous Australian elapids have toxins targeting points in the hemostasis process. Indeed, it is current wisdom that the resultant coagulopathy is the leading cause of snakebite deaths in Australia (White, 1984, 2000; Sutherland and Leonard, 1995).

Australian venoms affecting coagulation fall into two broad categories: procoagulants and anticoagulants (White, 2005). Procoagulants are represented as group C and group D prothrombin converters and cause a frequently rapid defibrination syndrome and consequent propensity to bleed. This is the most common clinical problem encountered. Experiments in animals indicate that early in the process of defibrination there is thrombus formation that can occlude key vessels, notably coronary vessels (Tibballs et al., 1989). It is speculated that this may be one of the mechanisms behind the early cardiac collapse following brown snake bites, currently the leading cause of death from snakebite in Australia (White, 2000). The nature of such cases means that confirmation of this may be difficult to obtain, as soon after any thrombi are destroyed as hyperfibrinolysis is established. In a classic case of defibrination following Australian snakebite, coagulation parameters rapidly become grossly abnormal, with essentially absent fibrinogen, gross elevation of fibrin(ogen) degradation products, an INR of >12, an aPTT of >150 s, but normal platelet count. Once sufficient appropriate antivenom is given, the defibrination process shuts off, though hyperfibrinolysis remains active longer. A variable period, measured in hours, is required for fibrinogen to be produced in measurable amounts. In the past this has been estimated at around 3 hours (White, 1983b), but more recent evidence indicates it can take 6 or more hours for fibrinogen to rise (Isbister et al., 2006b, 2007a). This has important implications for timing of decisions to give further antivenom. These same ongoing studies indicate that even quite small doses of antivenom are associated with a rapid and permanent fall to zero of circulating procoagulant toxin levels.

The anticoagulants inhibit the clotting pathways and also increase the risk of bleeding, though generally not as severely as the procoagulants. However, such anticoagulant coagulopathy is limited to envenomations by only a few species: the mulga/Collett's snake subgroup (*Pseudechis* spp.) (White, 1995a, 2005; Isbister et al., 2006a).

D. Nephrotoxicity

There are no known primary nephrotoxins in Australian elapid venoms, but kidney damage is not rare in major snakebite, particularly with brown snake (*Pseudonaja* spp.) bites (White, 1987a, 1995a). There are cases that could be interpreted as evidence of primary nephrotoxicity in brown snake envenoming (Acott, 1988), but for most cases secondary renal damage is far more likely. Periods of renal hypotension and damage from either products of myotoxicity or coagulopathy are likely causes. As mentioned earlier, even severe myoglobinuria is not routinely associated with renal damage in snakebite cases. In severe defibrination coagulopathy the kidneys have to cope with clearance of large quantities of fibrin(ogen) breakdown products. There are also cases of microangiopathic hemolytic anemia (MAHA), mostly after brown snake bites (White and Fassett, 1983; Isbister et al., 2007b). This disease process, in the snakebite setting, usually is associated with early defibrination coagulopathy, which resolves with appropriate antivenom therapy, but is followed by progressive development over a day or more of often severe thrombocytopenia, hemolytic anemia with schistocytes, and renal failure. It has clear similarities to thrombotic thrombocytopenic purpura (TTP), but generally resolves after a matter of days to a week or more.

In cases of renal failure after snakebite, other than MAHA cases, the lesion appears to be acute tubular necrosis, with anuric renal failure lasting days to weeks, requiring hemodialysis, but with

subsequent return to normal renal function. Permanent kidney damage is rare; this author is aware of a single case of bilateral renal cortical necrosis, with permanent major impairment of renal function, following an otherwise comparatively minor envenoming by a taipan in a 72-year-old woman with concurrent influenza.

E. Cardiotoxicity

No primary cardiotoxins have been reported from Australian elapid venoms. However, secondary cardiotoxicity is known. There are two principal associations: myolysis and coagulopathy. In myolysis, with hyperkalemia and secondary renal failure, high potassium levels can cause cardiac arrhythmias, and even cardiac arrest and fatal outcomes are known. This severity of myolysis is only known after bites by tiger snakes (*Notechis* spp.) and mulga and Collett's snakes (*Pseudechis* spp.) (White, 1995a; Isbister et al., 2006a).

Coagulopathy can result in ECG changes, as seen commonly in Papua New Guinea (PNG) with taipan bites, less commonly reported in Australian snakebites, though this may reflect lack of reporting more than lack of occurrence. More concerning is the early cardiac collapse, usually prehospital, seen with brown snake bites, as this frequently results in a fatal outcome. Experimental work with dogs suggests the cause may be early temporary thrombosis in critical vessels (such as coronary vessels) in the initial stages of procoagulant envenoming, prior to establishment of hyperthrombolysis and defibrination (Tibballs et al., 1989). This is likely to remain speculative for human victims of snakebite.

F. Local Venom Effects

Australian elapid snakes do not cause the moderate to severe local tissue injury commonly seen outside Australia with some elapid bites (such as cobras, *Naja* spp., in Africa and Asia) and many viperid bites (White, 1981, 1983c, 1995a). This does not mean local effects are not observed in Australian snakebite. Some snakes, notably the mulga/Collett's snakes and black snakes (*Pseudechis* spp.) commonly cause mild to moderate, occasionally severe, swelling of the bite site, extending to part or all of the bitten limb and associated with pain and a chemical cellulitis that resolves over 5–10 days spontaneously (White, 1995a; Isbister et al., 2006b).

Tiger snakes (*Notechis* spp.) frequently cause mild local swelling, local pain, erythema, and bruising without bleb formation. Brown snakes generally cause either no local effects, not even pain, or minimal local pain and erythema (White, 1987a).

Local tissue injury does occur in a small minority of cases, most often associated with inappropriate first aid, such as use of a tourniquet for an extended period. In most of these cases, of which few are reported, the damage is limited to a small area of skin loss, often superficial, around the bite site. There have been at least two cases of more extensive injury, both following mulga snake (*P. australis*) bite in the Northern Territory. In one, use of a tight tourniquet around the base of a bitten thumb, left on for 2 hours, resulted in necrosis of the thumb, leading to amputation (White, 1981). In the other case, the patient sustained about twelve bites to the hand and arm and subsequently developed severe compartment syndrome in the bitten limb and gangrene, resulting in a forequarter amputation. The treating doctors considered that this was associated with severe myolysis in the muscles of the bitten limb (Currie, personal communication).

V. OVERVIEW OF MANAGEMENT OF ENVENOMATION

Snakebite is not a common diagnosis presenting to Australian hospitals, and maintaining management expertise among treating doctors is an issue best addressed by regular update training and availability of expert phone consultation services. The majority of patients presenting with a possible snakebite will ultimately prove to be either not envenomed or maybe not even bitten by

a snake. Because of the potentially fatal outcome and possibility of severe complications from an inadequately treated case, all suspected snakebites should be managed as potentially severe, requiring admission and full workup (White, 2001, 2007).

A. First Aid

First aid can be critical in determining outcome in Australian snakebite. Much attention is focused on specific snakebite first aid, in particular the application of a pressure immobilization bandage. While this technique is of value, recent experience reinforces the view that other aspects of first aid, particularly resuscitation, are of great importance. Specifically, early cardiac collapse is likely to be fatal if immediate and ongoing cardiac resuscitation is not instituted. Several deaths in the last year can be attributed to lack of effective resuscitation by onlookers in the vital minutes prior to paramedics arriving. Similarly, preventable deaths have occurred because onlookers have failed to provide effective respiratory support in patients with neurotoxic respiratory paralysis. While these situations will apply to only a small minority of snakebite cases, these are the very cases where effective first aid is crucial in determining outcome.

Specific first aid for snakebite is based on two principles: do no harm and immobilize venom in the bite area. The latter is important in Australian elapid envenoming because it is the systemic effects of venom that pose the major threats to life and health, not the local effects. This is clearly quite different than snakebite by some non-Australian species that cause major local venom effects.

Specific first aid for Australian elapid bites has evolved through many recommended methods, nearly all of which are now discredited as useless or dangerous. These include "cut and suck" and tourniquets (Trevett et al., 1993). By the middle of the last century there was evidence that simple immobilization, using a plaster cast or splint, of the bitten limb would greatly retard venom movement to the systemic circulation (Barnes and Trueta, 1941). Once circulating venom-level assays became available as an experimental tool (Coulter et al., 1978; Sutherland et al., 1975; Sutherland and Coulter, 1977), pioneering work in monkeys demonstrated that combining a splint with a firm bandage applied to the entire bitten limb was as effective as a tourniquet in immobilizing venom, but could be left on for hours without causing limb damage. This allowed development of the pressure immobilization method (PIM) of snakebite first aid (Sutherland et al., 1979, 1981). This technique, with sundry minor variations, has been the standard of care for snakebite first aid since 1979. Though still immersed in some controversy, and without clinical trials to prove effectiveness, it is widely accepted as the method of choice. Used correctly, it does no harm and anecdotally can be effective at retarding venom movement. It is therefore clearly better than any alternative known and should remain the recommended method until research demonstrates a better method.

The basis of the technique is immobilization of lymphatic flow, using local pressure and inhibition of the muscle pump through limb immobilization. It is known that major venom toxins reach the circulation principally via the lymphatic system (Barnes and Trueta, 1941). The basic technique for PIM consists of applying a broad bandage over the bite site, bound firmly as for a sprain, then extended over the rest of the bitten limb, including fingers/toes, and over clothing, followed by full limb immobilization using a splint (Sutherland et al., 1979). The patient should then remain immobile, as any activity compromises effectiveness of first aid. The bite site should not be washed or cleaned in any way, as this may interfere with later venom detection. Consumption of food and any fluid, other than water, should be avoided, as there is a risk of aspiration should collapse and vomiting occur. As noted earlier, in those few patients with respiratory or cardiac collapse, resuscitation must take precedence.

B. Diagnosis

The diagnosis of snakebite in Australia is often far from easy. It is common for the patient to be bitten either without identifying the snake or often without even seeing a snake, and the initial bite

may be painless and go unnoticed. In children it may be impossible to gain a history sufficient to include or exclude snakebite. Thus, the treating doctor may be faced with a patient with no history of snakebite, but with symptoms and signs that could have many causes, including snakebite. Conversely, the patient may present with a history of definite or possible exposure to a snake, but uncertainty if a bite occurred. From this it follows that doctors in Australia must include snakebite in the differential diagnosis of many types of presentation and must manage cases of uncertain bites as if they are potentially major snakebites. Particularly in children this will include the "stick bites," most of which never develop systemic envenoming and many of which were probably never snakebites, but without such a cautious policy, occasional severe bites will be missed and fatalities will occur.

C. History

A detailed history of the bite or, if a bite is uncertain, activities and potential exposure that occurred are core components. If a snake was seen, a description should be sought. The geographic location and activities of the patient may be helpful. If a snake was seen to bite, then the number of bites is important; multiple bites are far more likely to result in life-threatening envenoming.

Timing of application and type of first aid should be noted, along with activity prior to first aid. A patient who, on being bitten, immediately lay down, had effective first aid applied by an onlooker, remained immobile, and was promptly transported to the hospital may be symptom-free because the first aid is working. On removal, systemic envenoming may develop rapidly, if the bite was effective. Conversely, the patient who delayed first aid until he or she had either chased after the snake or walked/ran for help, and then presents hours later symptom-free, may well have a dry bite.

Onset of symptoms should be noted. In particular, has the patient had a period of collapse, convulsions, developed a severe headache, nausea, vomiting, or abdominal pain? All of these may indicate developing systemic envenoming, though anxiety reactions should also be considered. Has the patient had any symptoms suggesting developing neurotoxicity (droopy or tired eyes = ptosis, difficulty speaking or swallowing, visual disturbance, limb weakness, respiratory distress), myolysis (muscle pain, tenderness, red or brown/black urine = myoglobinuria), coagulopathy (continuous oozing of blood from the bite site, any wound, or the gums, red urine = hematuria), or renal damage (polyuria, oliguria, or anuria)? Relevant past history can be important, in particular past exposure to antivenom, past reaction to antivenom, allergy to horses, other severe allergic history, preexisting renal, respiratory, or cardiac disease, or bleeding abnormality.

Current medications should be noted. Anticoagulants such as warfarin will interfere with assessment for coagulopathy. Aspirin, NSAIDs, and similar agents may interfere with platelet function and cause increased bruising tendency. Some antihypertensives, such as ACE inhibitors and beta-blockers, may modify immune response and impair ability to treat anaphylactic reactions.

D. Examination

The bite site should be inspected, looking for evidence of fang marks, multiple bites, and local venom effects such as swelling and bruising (Figures 21.1–21.3). If first aid bandaging is in place, rather than removing it, cut a window in the bandage over the bite site; the bandage can be retained for later venom testing, if required. This is also an appropriate time to swab the bite area for venom detection.

Regional lymph nodes should be palpated; if tender or enlarged, this may indicate venom movement from the bite site. The patient should be examined for signs of systemic envenoming, including neurotoxicity (ptosis (Figure 21.4), external or total ophthalmoplegia (Figure 21.5), fixed dilated pupils, poor tongue extrusion, drooling, loss of facial expression (Figure 21.6), limb weakness, reduced or absent DTRs, respiratory effort, cyanosis); myotoxicity (muscle weakness, tenderness, pain on movement against resistance); coagulopathy (continuous oozing blood from the bite site, wounds, venipuncture and IV sites (Figure 21.7), gums); and nonspecific signs.

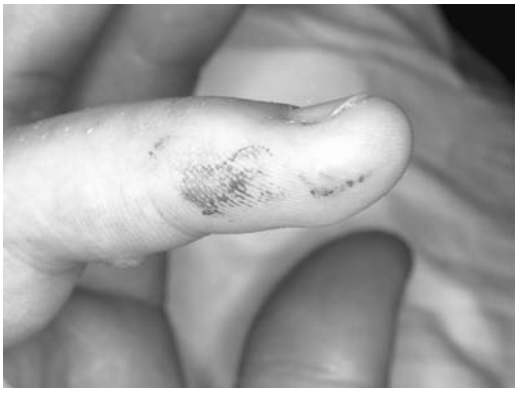

FIGURE 21.1 Brown snake bite to finger showing no significant swelling or bruising. (Original photo © Julian White.)

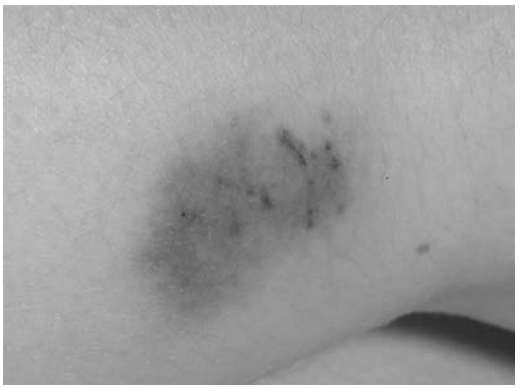

FIGURE 21.2 Tiger snake bite to calf; double bite, with local bruising. (Original photo © Julian White.)

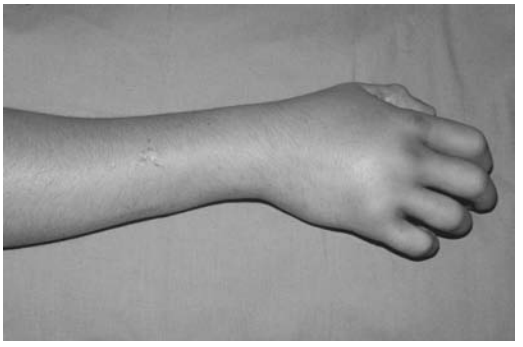

FIGURE 21.3 Mulga snake bite to base of thumb with extensive limb swelling. (Original photo © Julian White.)

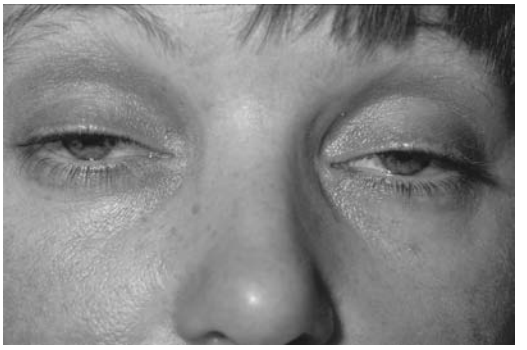

FIGURE 21.4 Tiger snake bite with early paralysis, showing bilateral mild ptosis. (Original photo © Julian White.)

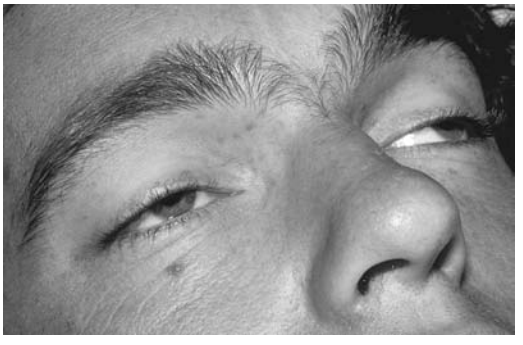

FIGURE 21.5 Death adder bite with early paralysis showing mild ptosis and external ophthalmoplegia. (Original photo © Julian White.)

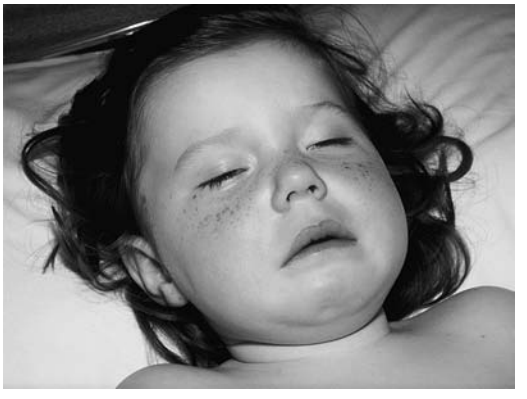

FIGURE 21.6 Tiger snake bite in a child showing expressionless facies typical of developing paralysis. (Original photo © Julian White.)

FIGURE 21.7 Inland taipan envenoming with full defibrination coagulopathy and persistent oozing blood from an IV site. (Original photo © Julian White.)

Envenoming can evolve over time. It is therefore essential that if initial examination is normal, frequent reexamination is performed, to detect later developing signs of paralysis (such as ptosis), myolysis, and coagulopathy.

E. Investigations

Because of the dominance of systemic effects, Australian snakebite diagnosis and assessment relies heavily on laboratory investigations. In particular, it is crucial to determine if there is coagulopathy, hemolysis, myolysis, or renal damage, all of which are largely reliant on laboratory tests.

The standard tests for any definite or suspected Australian snakebite are as follows:

1. Coagulation tests (prothrombin time/INR; aPTT, fibrinogen level; fibrin(ogen) degradation products, either FDP or d-dimer). It should be noted that recent studies have indicated fibrinogen level estimation is less helpful as performed by many labs, and so may be omitted (Isbister et al., 2006b).
2. Full blood examination (platelet count, white cell count and differential, red cell count and parameters, film examination for schistocytes).
3. Biochemistry (creatinine, urea, standard electrolytes, CK, which is essential in looking for myolysis; liver function tests are optional).
4. Urine should be examined and tested with a dipstix test for hematuria (which may actually indicate myoglobinuria (Figure 21.8)) and if positive, spun down and examined by microscope for red cell fragments (indicating true hematuria).

If there is respiratory failure from neurotoxic paralysis, then arterial blood gasses are relevant. In the past, some hospitals routinely grouped and matched snakebite patients. Transfusions are rarely required, so this test set is unnecessary as a routine and should be reserved for those with major bleeding or hemolysis.

Radiologic examination is only rarely indicated in snakebite patients, most notably if there is evidence of a head injury or intracranial bleed in the setting of snakebite coagulopathy, in which situation a CT head scan is most often used. In rare cases with clinical signs of pulmonary edema or other intrathoracic problem, plain chest x-rays may be indicated.

A single initial set of blood tests for coagulopathy, myolysis, hemolysis, and renal damage, as noted above, is inadequate in assessing snakebite patients. Envenoming can evolve over time, and a schedule of repeat testing is required. The most common schedule is to test first on arrival at

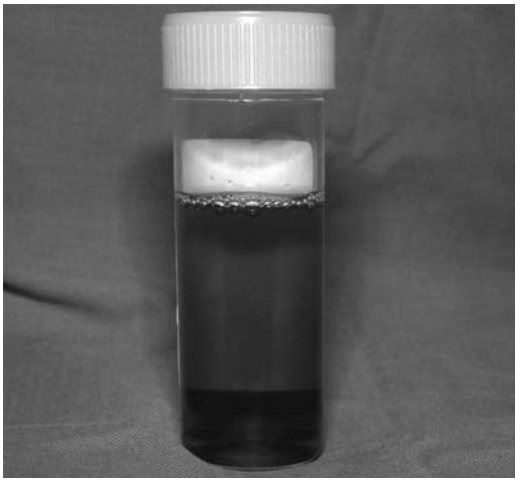

FIGURE 21.8 Mulga snake bite showing typical myoglobinuria—red urine that looks like hematuria and tests positive for hemoglobin. (Original photo © Julian White.)

hospital, then if initial tests are normal, retest about 2 to 3 hours later, and if still normal, a third time another 3 hours later. If first aid is in place, initial testing occurs on presentation, without removing first aid. Results are obtained urgently (usually takes 1–2 hours) and if normal (and the patient is symptom- and sign-free), first aid is removed and after a wait of about 1 hour, a second set of tests is taken.

F. Venom Detection

Venom detection is a standard test in snakebite patients, but is often overused. The test, an ELISA-based commercial kit (Figure 21.9), is designed to determine the most appropriate antivenom in a patient with significant systemic envenoming. It is not designed to determine if a snakebite has occurred (White, 2001). The best sample is a bite site swab. If this is unavailable and the patient has evidence of systemic envenoming, then urine can be tested. Blood is more likely to give unreliable results and is generally avoided as a test sample. In some areas, venom detection is only performed if the patient has systemic envenoming. It should never be used as a screening test for snakebite, as it is ineffective in this role, with significant false positive and false negative issues. If venom testing is performed at presentation, with first aid in place, then do not remove first aid, but cut a small window in the bandage over the bite site.

FIGURE 21.9 CSL snake venom detection kit. (Original photo © Julian White.)

G. Differential Diagnosis

Snakebite should be considered in the differential diagnosis of any patient presenting with unexplained coagulopathy, myolysis, flaccid paralysis, renal failure, collapse, or convulsions. Clearly the circumstances, potential exposure to snakebite, and past history will modify whether snakebite should be entertained as a likely diagnosis. Conversely, some patients will present with a speculative diagnosis of snakebite, without a clear history of a snake encounter. This may vary from a child who saw a "stick" bite them to a patient with apparent bite marks and some signs of systemic effects that might be due to snake envenoming. In addition to snakebite, envenoming by other organisms can cause systemic effects.

Paralysis ticks can cause progressive flaccid paralysis, developing usually over several days and often presenting first as ataxia rather than ptosis. A careful search will usually locate the tick. These ticks are limited to the eastern seaboard of mainland Australia, so if the patient has not been in this region, tick paralysis is not likely. Tick paralysis does not cause coagulopathy or significant myolysis.

Funnel web spiders can cause rapid severe systemic envenoming characterized by neuroexcitatory signs such as profuse sweating, salivation, lacrimation, and development of pulmonary edema and respiratory failure. Early collapse and convulsions can occur. They do not cause flaccid paralysis.

Cardiac arrest secondary to coronary artery occlusion can present in a manner similar to that of cardiac arrest secondary to snakebite, as the mechanisms are similar, and it may be initially difficult to differentiate, but in snakebite there will be a defibrination coagulopathy.

VI. APPROACH TO TREATMENT

Every suspected snakebite should be managed as a medical emergency, even though most cases will prove to be dry bites or minor envenoming only, or not even a snakebite (White, 2001, 2007). The potential for early catastrophic envenoming effects should not be ignored. When a patient presents with suspected or definite snakebite, initial assessment should be directed to stabilizing the patient, if symptomatic, then establishing if systemic envenoming is present and its nature and extent.

Good IV access should be established and, unless otherwise contraindicated, an initial IV fluid load administered, while assessing for envenoming. If the patient is stable, without significant flaccid paralysis or cardiac compromise, a window is cut in any first aid bandage over the bite site, and the wound is examined and swabbed for venom detection. Blood is taken for initial tests (see earlier section); then proceed to history and examination.

Once history, examination, and initial laboratory test results are available, it is possible to determine if there is systemic envenoming and its extent. At this point, before removing first aid, a decision is made on whether to give antivenom and, if positive, the type and dose of antivenom. If antivenom is not indicated at this time, then first aid is removed and close observation continued until the next set of blood tests are taken.

In patients presenting with advanced flaccid neurotoxic paralysis, it is important to realize that though apparently obtunded, the patients may well be awake and fully aware of what is said and done. Though they may be paralyzed, unable to move limbs, unresponsive to painful stimuli, with absent DTRs and fixed dilated pupils, they may feel pain when testing for pain response and be able to see when their eyes are opened to test pupil response. It is all too easy for staff to casually discuss the apparent brain death of the patient, while the actually conscious patient desperately wants to say, but cannot, that he or she can hear everything and is very much alive and wants to stay that way! Often, in such cases, careful examination will reveal some part of the body that can still be moved slightly, and this can be used to establish a simple yes and no communication system in response to questions to the patient.

Renal damage is not rare in Australian snakebite, and it is important to ensure adequate hydration and careful monitoring of fluid balance. However, a urinary catheter should be a last resort, as the addition of iatrogenic bladder infection to a severely envenomed patient is best avoided.

A. Specific Treatment—Antivenoms

The only true antidote for snake envenoming is antivenom, which in Australia is refined equine IgG F(ab)'$_2$ against major snake venoms. There are five major venom types, with corresponding specific antivenoms, plus a polyvalent covering all species (White, 2001). The specific antivenoms (Table 21.2) are listed as monovalent, but the realities of manufacture mean most have limited cross-reactivity with at least two and often all five of the venom types. However, they are all both lower volume and cheaper than the polyvalent antivenom, so wherever possible, should be used in preference to polyvalent. To assist in determining which antivenom to use, there are venom detection (see above) and diagnostic algorithms (White, 2001, 2007); both methods should be used together to ensure correct antivenom choice. If they give disharmonious results, expert advice should be sought from the manufacturer (CSL Ltd., Melbourne; +61-3-93891933). The dose of antivenom will depend on the type of snake, the extent of envenoming, and the type of antivenom (see Tables 21.1 and 21.2). Ongoing research is refining dose recommendations that are currently in a state of flux, with doses for the most commonly used antivenoms generally being reduced.

Antivenom is only indicated if there is significant systemic envenoming, manifested by features listed in Table 21.3. This will apply to only a minority of cases; most snakebite patients will not show significant envenoming and will not require antivenom. Because there are risks associated with antivenom therapy, it is not appropriate to give antivenom unless clearly indicated. Conversely, the risk of fatal outcome mandates the use of antivenom in patients with significant envenoming. Fear of an adverse reaction to antivenom is not a valid reason for withholding antivenom therapy.

If antivenom is to be given, it is important that resources are ready and on hand to treat an acute adverse reaction, such as an anaphylactoid reaction. This can occur *de novo* in a patient never previously exposed to antivenom. However, patients who have had antivenom previously are at greater risk of an adverse reaction. Adrenaline and resuscitation equipment should be immediately available. Neither premedication nor skin sensitivity testing is recommended. Indeed, the latter is a dangerous and nondiagnostic procedure that should never be used with any antivenom anywhere.

Snake antivenom should always be given IV, preferably diluted up to 1:10 in normal saline, Hartman's, or similar carrier. The degree of dilution will be determined by factors such as the volume of antivenom, the size/weight of the patient, and preexisting health issues. For large-volume antivenoms such as polyvalent, in small children, little dilution is possible because of volume overload issues. The dose of antivenom should be given reasonably quickly, starting the infusion slowly while watching for adverse reactions, then if no reaction, increasing the rate, aiming to give the entire dose over 15 to 20 minutes. If the dose is multiple vials, these can be mixed together in a single infusion.

TABLE 21.2
Australian Snake Antivenoms

Antivenom (AV)	Initial Dose[a]
CSL brown snake AV	2 vials
CSL tiger snake AV	2 vials
	4 vials for giant subspecies from Bass Strait
CSL black snake AV	1 vial
CSL death adder AV	1 vial
CSL taipan AV	1+ vial(s)
CSL polyvalent snake AV	Dose same as for specific antivenoms for bites from a given snake species
CSL sea snake AV	1+ vial(s)

[a] Initial dose varies between antivenoms and the type of snake and is also subject to clinical judgment about the severity of envenoming.

TABLE 21.3
Features of Significant Systemic Envenoming as Indicators for Antivenom Therapy

Clinical Feature	Indication for Antivenom
Flaccid paralysis	Any degree of flaccid paralysis, even ptosis only, indicates a requirement for antivenom therapy.
Myolysis	Myolysis, in general, indicates a requirement for antivenom therapy, but venom-induced myolysis is only clearly present if there is a significant rise in CK (>1,500 IU/l as a rough guide) or if there is myoglobinuria.
Defibrination coagulopathy	Defibrination coagulopathy, if other than very minor, indicates a requirement for antivenom therapy. In rough terms, if the INR > 2.0 with major elevation of fibrin(ogen) degradation products, then significant coagulopathy is or has developed.
Anticoagulant coagulopathy	Anticoagulant coagulopathy indicates a requirement for antivenom therapy.
Renal damage/failure	Any degree of renal insufficiency (elevated creatinine and urea) in the acute stages of snakebite (i.e., first 24 hours or so) indicates a requirement for antivenom therapy.
Microangiopathic hemolytic anemia (MAHA)	Presence of MAHA in the acute stages of snakebite indicates a requirement for antivenom therapy. However, MAHA more commonly develops later, in which setting the value of late antivenom therapy is uncertain.
Collapse/convulsions	A witnessed episode of collapse/convulsions in a known acute snakebite patient indicates a requirement for antivenom therapy.
General symptoms only	Snakebite patients with nonspecific symptoms, such as headache, nausea, vomiting, and abdominal pain only, where there is no evidence of paralysis, myolysis, coagulopathy, or renal damage (as noted above), do not, in general, require antivenom therapy. The usual exception would be intractable vomiting nonresponsive to antiemetic therapy.

If there is an adverse reaction to the antivenom, then the infusion should be stopped, the reaction controlled, usually with adrenaline, preferably by dilute-metered infusion, then the antivenom restarted. Sometimes in these situations it is necessary to titrate antivenom infusion rate against adrenaline infusion rate to maintain control of an adverse reaction. An initial dose of antivenom may often be sufficient to control envenoming (Isbister et al., 2006b, 2007a). The half-life of these antivenoms ensures high blood levels for days, so re-envenoming effects are not likely. However, the patient's response to antivenom should be monitored, and if the response is inadequate, follow-up doses may be required. Sufficient time for response to the initial dose must be allowed before considering such redosing. For defibrination coagulopathy, it may be necessary to wait 6 hours for a detectable rise in fibrinogen, which indicates reversal of the coagulopathy (Isbister et al., 2006b). This may be indicated by a fall in INR from >12 to a detectable figure, often <8.

Any patient given antivenom is at risk of late developing adverse effects, principally serum sickness. Every patient should be informed of the risk and symptoms of serum sickness prior to discharge. If considerable volumes of antivenom, approximately >25 ml, have been used, then a week-long course of oral prednisolone should be considered as prophylaxis.

B. Nonspecific Treatment

Antivenom, as a specific antidote, is a key part of the treatment process for systemic envenoming, but by no means the only treatment required. All cases of suspected or confirmed snakebite should have an IV line placed and be given an initial IV fluid load (crystalloid), then hourly IV fluid maintenance for at least the first 12 hours postbite, longer if there is envenoming, until this has resolved. Great care should be taken in insertion of IV lines, in case there is coagulopathy. In particular, IV access to the subclavian, jugular, and femoral vessels should be avoided if at all possible, as major bleeding can occur and be difficult to control in the context of snakebite coagulopathy. Oral intake should be restricted to limited clear fluids only until it is clear that either there is no significant envenoming or

acute envenoming effects have resolved. Urine output should be measured, and if the patient is unable to void, consider catheterization, but be especially careful if there is venom-induced coagulopathy.

If there is major neurotoxic paralysis with either imperiled airway or developing oxygenation issues, intubation is appropriate, and mechanical ventilation if respiratory muscle function is inadequate. Surgical access to the airway, such as tracheotomy, should be avoided, particularly if there is coagulopathy, though may be required in cases of prolonged respiratory paralysis unresponsive to antivenom (species with presynaptic neurotoxins). For those few species with only postsynaptic neurotoxins, anticholinesterases (neostigmine) can be a useful adjunctive treatment to reduce the extent of paralysis and avoid the need for intubation in cases where antivenom either is unavailable acutely or has failed to adequately reverse paralysis in a short time frame (Watt et al., 1986; Currie et al., 1988).

Bite site wound care is rarely needed, but in cases with significant limb swelling (such as mulga snake bites), elevation of the limb may be considered. Pain at the bite site is variable, but can occasionally be significant in the acute stages, requiring analgesia. Opiates/respiratory depressant analgesics are to be avoided. Secondary infection is uncommon with Australian snakebite, and antibiotics should only be used in proven infection. Tetanus is a potential risk after snakebite, but booster injections should be delayed until after resolution of any coagulopathy, to avoid unpleasant hematomas.

C. Complications

The major complications seen with Australian snakebite relate to the previously discussed principal systemic effects of envenomation. Thus, there can be severe prolonged flaccid paralysis, unresponsive to antivenom, requiring mechanical ventilation for extended periods, up to several months. Secondary complications of prolonged ventilation are then a further risk.

Apart from paralysis, long-term neurological effects can be seen, even after bites by species not likely to cause paralysis in humans (such as red-bellied black snakes). Most frequently seen, though still uncommon to rare, are alterations in sense of smell or taste, or both, ranging from partial to complete loss of the sensory modality, usually permanent.

Severe myolysis can result in major skeletal muscle damage and wasting, but regeneration is likely over a period extending beyond 1 month, with a likely requirement for ongoing physiotherapy to assist full return of muscle strength. Although unproven in humans, in experimental animals regenerated muscle after snakebite myolysis consists of slow fibers only, which may affect longer-term muscle function. Coagulopathy is generally short-term, but severe bleeding involving major organs can occur during the acute period, which if survived (often such bleeds prove lethal) may result in long-term deficits. Typically this might involve intracranial bleeding, resulting in permanent neurological dysfunction. Such cases are very rare. The renal damage seen after Australian snakebite is usually reversible, with return of normal renal function after a variable period, generally measured in days to weeks. There are rare isolated cases of permanent renal damage, generally renal cortical necrosis, requiring ongoing renal support.

Apart from the specific complications caused by particular toxins, there are nonspecific effects, generally poorly reported. In particular, patients who have had significant systemic envenoming sometimes report a vague malaise and generalized lethargy and nonparalytic weakness lasting months after the bite, though slowly resolving. Generally this will resolve within 3 to 6 months.

D. Prevention

Prevention of snakebite relies on common sense. The population should be made aware of the risk of snakebite, that snakes occur in urban as well as rural areas and frequently enter houses, that snakes may be active after dark on warm nights, and that if cornered, they are more likely to bite. Attempting to catch or kill snakes should be discouraged, as this is a high-risk behavior. Gardens should be kept free of habitat likely to encourage snake presence, such as rubbish and ground shelter

that may harbor both snakes and their prey. In dry areas, the presence of open fresh water may encourage snakes to visit. Education about correct first aid is also appropriate if a bite should occur, as application of effective first aid may improve outcomes.

E. Risk Groups

Essentially the entire Australian population, apart from those on a few isolated islands, is at potential risk of venomous snakebite. However, certain groups are at increased risk, including herpetologists, snake keepers and catchers, rural workers, venom researchers (allergy to inhaled venom), and field biologists. Individuals experiencing repeated bites run a double allergy risk: allergy to both the venom and the antivenom. Both can result in acute allergic reactions, including anaphylaxis. Anaphylaxis to antivenom, though potentially lethal, in reality is virtually always manageable in a hospital setting. Anaphylaxis to venom is likely to occur immediately after the bite, prehospital, and so is far more likely to prove lethal, and deaths from this cause have been recorded in Australia. The risk is not restricted to dangerous venomous species, but can occur following repeated bites by even species of lower venom toxicity.

VII. DETAILED CONSIDERATION OF IMPORTANT DANGEROUS SNAKES

A. Brown Snake Group

The following gives a brief overview of the known characteristics of envenoming by dangerously venomous Australian snakes. Brown snakes, of the genus *Pseudonaja* (Figures 21.10–21.13), are currently the leading cause of both bites and snakebite fatalities in Australia. A variety of species, with variable coloration, are distributed throughout mainland Australia and parts of New Guinea, but not on islands off the coast, including Tasmania. Brown snakes are active diurnal hunters, easily adapting to urban habitats, and are common in many Australian mainland towns and cities. On warm nights, like all other dangerous snakes, they can be active after dusk. They have small fangs, and some deliver only small amounts of venom, but it is highly potent.

While the majority of bites are dry (approximately 70–80%), when significant venom is delivered it can rapidly cause severe or lethal envenoming. In particular, early prehospital cardiac collapse is associated with brown snake bite and is frequently fatal. The bite may be painless and go unnoticed, with bite marks hard to detect, but systemic envenoming can develop in minutes, with defibrination coagulopathy the classic feature. Complete defibrination can occur in 15 to 30 minutes postbite, though can sometimes develop less rapidly. Major intraorgan bleeding is rare unless there is trauma, such as a fall with a blow to the head. As early collapse is common in severe cases, such trauma is always possible, though fortunately rare. Particularly in children, early collapse can

FIGURE 21.10 Common or eastern brown snake, *Pseudonaja textilis*. (Original photo © Julian White.)

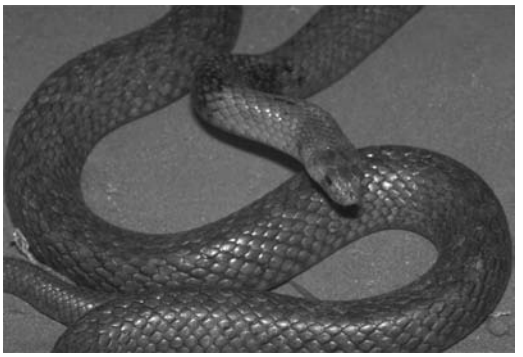

FIGURE 21.11 Western brown snake, *Pseudonaja nuchalis*. (Original photo © Julian White.)

FIGURE 21.12 Dugite, *Pseudonaja affinis*. (Original photo © Julian White.)

FIGURE 21.13 Approximate distribution of brown snakes, genus *Pseudonaja*, which occur throughout most of Australia. (Original photo © Julian White.)

be followed by generalized convulsions. Headache, nausea, vomiting, and abdominal pain are all common systemic symptoms, but in adults, there may be absence of symptoms even though there is complete defibrination.

In addition to defibrination coagulopathy, there may be renal failure and sometimes MAHA (see earlier discussion). Flaccid paralysis can occur, but is rare, most likely in patients with otherwise severe systemic envenoming and a long delay in initiating antivenom therapy. Myolysis does not occur, though occasionally very mild transient rises in CK are seen. For patients with significant systemic envenoming, notably defibrination-type coagulopathy, antivenom therapy is required. CSL brown snake antivenom is preferred, with an initial dose of two vials now considered adequate based on ongoing clinical research. This is a significant reduction from the previously advocated dose of five or more vials.

B. Tiger Snake Group

The tiger snake group contains a diverse collection of species spread across four genera. Tiger snakes, genus *Notechis* (Figures 21.14–21.16), are restricted, in general, to cooler, moister habitats in southern

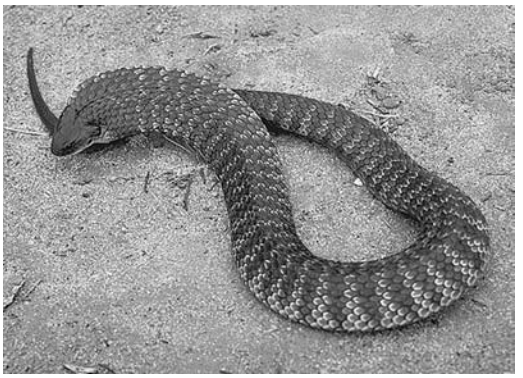

FIGURE 21.14 Mainland tiger snake, *Notechis scutatus*. (Original photo © Julian White.)

FIGURE 21.15 Western tiger snake, *Notechis occidentalis*. (Original photo © Julian White.)

and eastern Australia, including many southern offshore islands and Tasmania (Figure 21.17). They are less frequently encountered than previously throughout much of their range, but still cause significant numbers of bites. Their fangs are larger and they produce, on average, more venom than brown snakes, but the venom is slightly less toxic. They have a lower dry bite rate, and prior to antivenom development, had a 45% fatality rate for bites, compared to 8% for brown snakes. Bites cause local pain and often local erythema, mild swelling, and bruising. General systemic effects are similar to those for brown snakes, but specific effects include flaccid paralysis, which is common, often severe, predominantly presynaptic, and so not reversible with antivenom. Defibrination coagulopathy is seen in essentially all cases with systemic envenoming, but early cardiac collapse is not reported, unlike brown snake bites. However, early collapse (presumed noncardiac) with recovery, sometimes associated with generalized convulsions, does occur. Myolysis is common and can be severe. Renal failure is uncommon, but does occur occasionally, though not always linked to myolysis. MAHA is possible but rare. Long-term alteration or loss of taste/smell can occur, uncommonly, with tiger snake bites.

All cases with significant systemic envenoming require antivenom therapy, preferably CSL tiger snake antivenom, with an initial dose of two vials, possibly more for large specimens found on some Bass Strait islands. As with brown snake antivenom, this is a reduction in dose compared to previous recommendations.

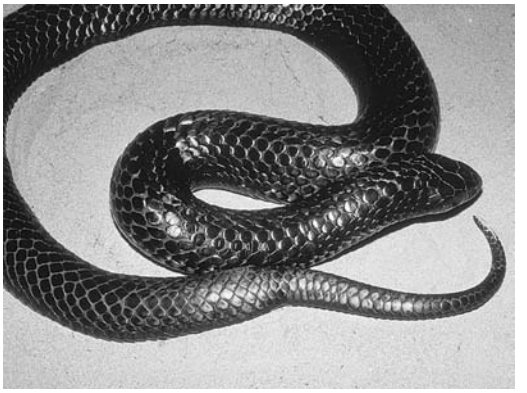

FIGURE 21.16 Black tiger snake, *Notechis ater*. (Original photo © Julian White.)

FIGURE 21.17 Approximate distribution of tiger snakes, genus *Notechis*. (Original photo © Julian White.)

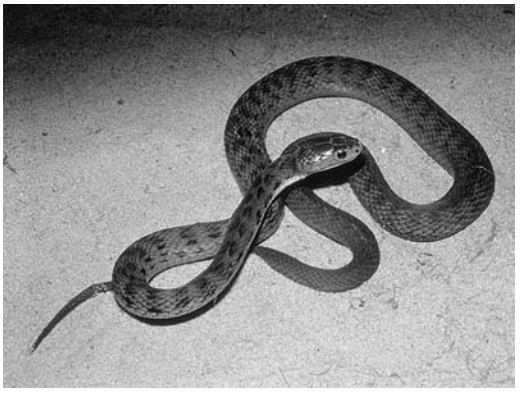

FIGURE 21.18 Rough-scaled snake, *Tropidechis carinatus*. (Original photo © Julian White.)

FIGURE 21.19 Approximate distribution of the rough-scaled snake. (Original photo © Julian White.)

The rough-scaled snake (*Tropidechis carinatus*) (Figure 21.18) is limited to pockets of distribution along eastern coastal Australia, from northeastern New South Wales (NSW) through to northeast Queensland (Figure 21.19). In clinical and treatment terms it is essentially identical to the tiger snakes.

Copperheads, genus *Austrelaps* (Figures 21.20 and 21.21), are restricted to southeast Australia and Tasmania and Kangaroo Island (Figure 21.22). Their venom and particularly their clinical effects are not well reported. They can cause flaccid paralysis. It is not clear if they are likely to cause coagulopathy or myolysis. Treatment is similar to that for tiger snakes.

The broad-headed snakes, genus *Hoplocephalus* (Figures 21.23–21.25), have restricted ranges in portions of eastern coastal NSW and Queensland (Figure 21.26). Clinically they are similar to brown snakes, causing defibrination coagulopathy, but not paralysis or myolysis, but their venom is closer to that of tiger snakes, and treatment is as for tiger snakes.

C. Black/Mulga Snake Group

The black snake and mulga snake groups, all within the genus *Pseudechis*, are widely represented throughout mainland Australia and into parts of New Guinea. The black snakes (*P. porphyriacus* and *P. guttatus*) (Figure 21.27) are restricted to eastern Australia, from NSW through Victoria and

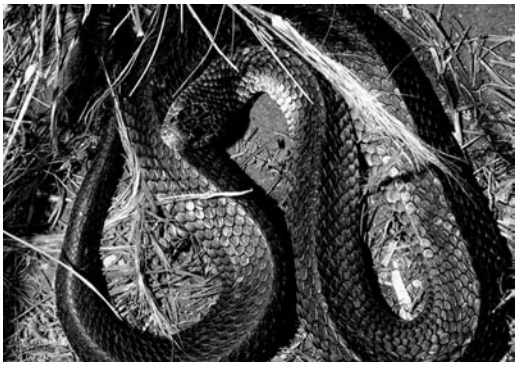

FIGURE 21.20 Lowland copperhead, *Austrelaps superbus*. (Original photo © Julian White.)

FIGURE 21.21 Pigmy copperhead, *Austrelaps labialis*. (Original photo © Julian White.)

FIGURE 21.22 Approximate distribution of copperheads, genus *Austrelaps*. (Original photo © Julian White.)

South Australia (*P. porphyriacus* only) (Figure 21.28). These snakes cause local pain, erythema and swelling, and nonspecific systemic symptoms, especially vomiting, abdominal pain, and sometimes diarrhea, but do not cause paralysis or major coagulopathy in humans. They uncommonly cause minor myolysis, rarely major myolysis. In most cases antivenom therapy is not required, but if it is used, CSL tiger snake antivenom is preferred, except if there is major myolysis, when CSL black snake antivenom should be considered. For either antivenom the initial dose is one vial.

The mulga snakes (*P. australis* and *P. butleri*) (Figures 21.29 and 21.30) and Collett's snake (*P. colletti*) (Figures 21.31 and 21.32) cause more severe envenoming, with marked local pain and swelling and sometimes severe systemic myolysis. Occasionally they also cause an anticoagulant (non-fibrinogen-consumptive) coagulopathy. Secondary renal failure is possible, but rare. Major paralysis is not reported, but minor paralytic effects, specifically ptosis, do occur in a few cases. Any case with significant systemic envenoming requires antivenom therapy and CSL black snake antivenom is preferred, with an initial dose of one vial. Rarely is more required.

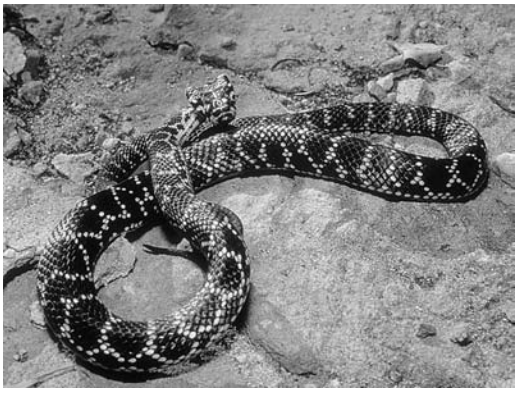

FIGURE 21.23 Broad-headed snake, *Hoplocephalus bungaroides*. (Original photo © Julian White.)

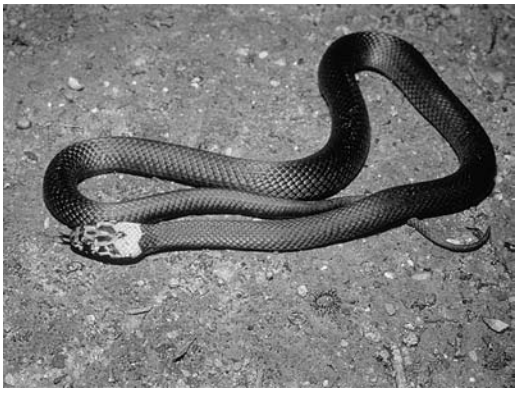

FIGURE 21.24 Pale-headed snake, *Hoplocephalus bitorquatus*. (Original photo © Julian White.)

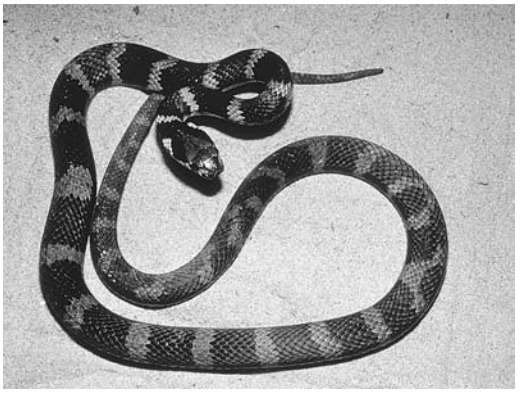

FIGURE 21.25 Stephen's banded snake, *Hoplocephalus stephensi*. (Original photo © Julian White.)

FIGURE 21.26 Approximate distribution of broad-headed snakes, genus *Hoplocephalus*. (Original photo © Julian White.)

FIGURE 21.27 Red-bellied black snake, *Pseudechis porphyriacus*. (Original photo © Julian White.)

FIGURE 21.28 Approximate Australian distribution of black snakes, *Pseudechis porphyriacus* and *P. guttatus*. (Original photo © Julian White.)

FIGURE 21.29 Mulga snake, *Pseudechis australis*. (Original photo © Julian White.)

FIGURE 21.30 Approximate Australian distribution of mulga snakes, *Pseudechis australis* and *P. butleri*. (Original photo © Julian White.)

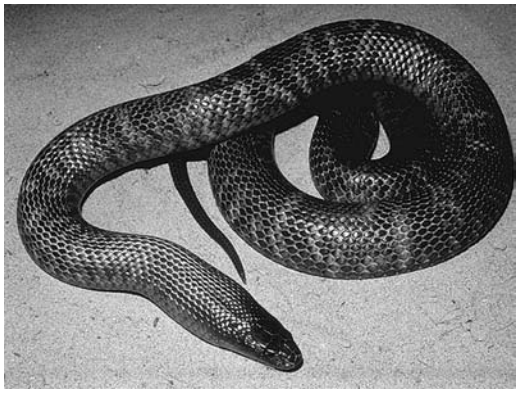

FIGURE 21.31 Collett's snake, *Pseudechis colletti*. (Original photo © Julian White.)

FIGURE 21.32 Approximate distribution of Collett's snake. (Original photo © Julian White.)

The Papuan black snake (*P. papuanus*) from portions of New Guinea is different again in clinical effects, as it can cause coagulopathy, paralysis, and myolysis. The preferred antivenom is CSL black snake antivenom, but in New Guinea this is rarely available, so the equally effective CSL polyvalent snake antivenom is used. The initial dose is one vial.

D. Taipans

Taipans are among the most feared venomous snakes (Figures 21.33 and 21.34). They are large, have very potent venom, delivered in large amounts through long fangs, with a very low dry bite rate and a preantivenom fatality rate of >80%. There are currently at least three recognized species, occupying niches in northern and eastern coastal Australia from NSW to Western Australia (WA) (*Oxyuranus scutellatus*), in New Guinea (*O. s. canni*), in central and eastern inland Australia (*O. microlepidotus*), and in western inland Australia (*O. temporalis*) (Figure 21.35). Taipan bites are associated with variable local effects, from minor to significant local pain and swelling, and rapid onset of severe systemic envenoming, with flaccid paralysis, defibrination coagulopathy, and sometimes secondary renal failure. Myolysis has been reported, but is uncommon to rare.

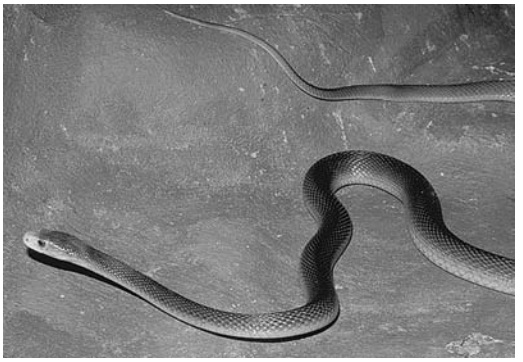

FIGURE 21.33 Common taipan, *Oxyuranus scutellatus*. (Original photo © Julian White.)

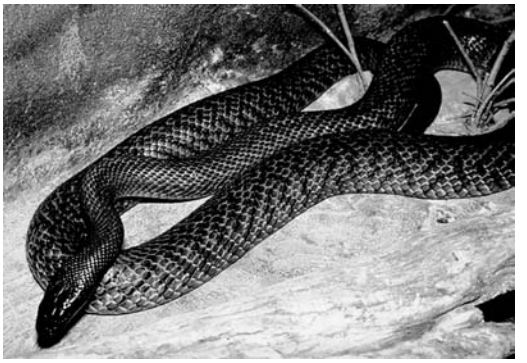

FIGURE 21.34 Inland taipan, *Oxyuranus microlepidotus*. (Original photo © Julian White.)

FIGURE 21.35 Approximate Australian distribution of taipans, genus *Oxyuranus*. (Original photo © Julian White.)

Virtually all Taipan bites will require antivenom therapy, using either CSL taipan or polyvalent snake antivenom at an initial dose of one or two vials.

E. Death Adders

Death adders, genus *Acanthophis* (Figure 21.36), used to be widespread across mainland Australia and throughout New Guinea, but in southern Australia they are increasingly rare as a cause of bites, except from captive specimens (Figure 21.37). They had a fearsome reputation, with a low dry bite rate and a preantivenom fatality rate of 50%. However, their principal venom components affecting humans are postsynaptic neurotoxins, so the predominant clinical feature of envenoming is flaccid paralysis, sometimes rapid in onset, but sometimes delayed >24 hours before onset. They do not cause coagulopathy, myolysis, or renal failure. Antivenom therapy is required in cases developing paralytic features, the preferred choice being CSL death adder antivenom at an initial dose of one vial, though severe paralysis may occasionally require substantially higher doses before reversal. Neostigmine has been used successfully as an adjunct to antivenom to reduce the severity of paralysis in death adder bites. Some death adders also possess presynaptic neurotoxins.

FIGURE 21.36 Common death adder, *Acanthophis antarcticus.* (Original photo © Julian White.)

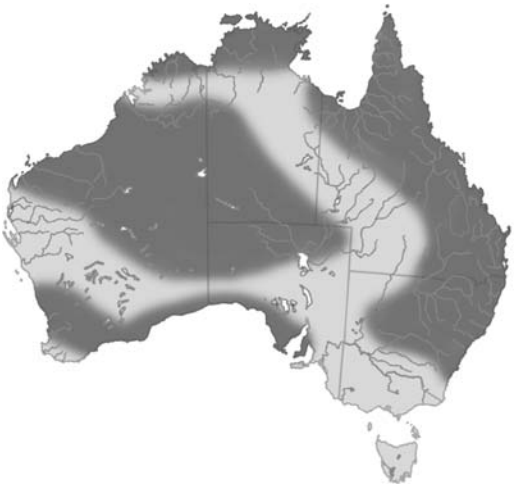

FIGURE 21.37 Approximate Australian distribution of death adders, genus *Acanthophis.* (Original photo © Julian White.)

F. Sea Snakes

Sea snakes represent a diverse lineage of elapid snakes that have adopted a marine lifestyle, radiating from their presumed evolutionary origins in the Australian region to inhabit tropical waters from the Middle East, across the Indian Ocean, and much of the western Pacific, with one pelagic species even extending across the Pacific to the Americas. Bites generally occur only when the sea snake is molested, notably when caught in fishermen's nets. Changing fishing practices has reduced the number of bites to fishermen in recent years. Many sea snakes have very potent venom with two distinct clinical effects: flaccid paralysis, postsynaptic, and systemic myolysis, sometimes with secondary renal failure. Most cases of sea snake bite result in either paralysis or myolysis, but rarely both together.

Cases with significant systemic envenoming require antivenom therapy, preferably CSL sea snake antivenom at an initial dose of one to three vials, depending on severity of envenoming. Neostigmine can be used as an adjunct to antivenom in cases with paralytic envenoming. In the past it has been recommended that if CSL sea snake antivenom was unavailable, CSL tiger snake or polyvalent snake antivenom could be used. Changes in antivenom manufacturing processes at CSL mean that this substitution is less certain to be effective now.

G. Other Venomous Snakes

Within Australia there are many other venomous snake species, ranging from tiny to moderate in size, but none are considered a major threat to humans. Antivenom therapy would not be used except in exceptional circumstances, and their importance is more related to potential confusion with truly dangerous species, either by visual appearance or errors in venom detection.

In New Guinea, there is at least one dangerous species unique to the region, the small-eyed snake (*Micropechis ikaheka*), which has caused a number of fatalities. Envenoming by this snake appears to respond to antivenom therapy using CSL polyvalent snake antivenom at an initial dose of one or two vials. In adjacent Pacific islands/nations there are several other elapid species, but none pose a major threat to humans.

VIII. FUTURE DIRECTIONS

Ongoing clinical research is delivering new data on envenomation profiles for major Australian snakes and the response to antivenom therapy. It is possible that further changes to antivenom type and dose recommendations will be made in the future. The extent of the dangerous Australian snake fauna is in flux. A new species of taipan was recently discovered (*O. temporalis*; Doughty et al., 2007), and several other important genera are the subject of ongoing taxonomic research and change. However, not all suggested taxonomic change is likely to withstand peer scrutiny (e.g., *Pailsus pailsii* is not accepted as a valid genus, though the species may be recognized within *Pseudechis*) (Hutchinson, personal communication, 2007).

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22 Snakebite in Africa
Current Situation and Urgent Needs

Jean-Philippe Chippaux

CONTENTS

Incidence of snakebite is high in Africa and is characterized by an amazing heterogeneity of factors, related to the great diversity of both the snake fauna and human activities. Composition of snake populations varies according to the climate and environment. Important anthropogenic modifications of the environment—increasing tremendously over the last 30 years—have led to rearrangements in both the composition of the snake populations and the demography of individuals. Some

species are attracted to human-modified environments, such as industrial plantations, while others are deterred. Thus, some locations show higher densities of venomous species than are represented in the natural environment. Several epidemiologic methods are used to evaluate the quantitative and qualitative importance of snakebites. Retrospective or prospective surveys in health centers evaluate the use of health facilities by the victims and the severity of the envenomations. However, a major underestimation of snakebite incidence occurs as a consequence of poor attendance of health centers, which are often very scarce. Many victims do not have the time to reach a hospital or prefer to consult traditional practitioners. Efficient household surveys, which consist of questioning the population directly, could correct the estimate of the incidence and mortality and give a more realistic evaluation of snakebite importance. Moreover, household surveys allow evaluation of level of dependence on traditional medicine (which involves ~40 to 80% of bites, depending on location). These types of surveys are now standardized. Envenomation represents a neglected public health problem in Africa. Actual incidence of snakebites varies from 100 to 650 bites for 100,000 inhabitants per year, and annual mortality can exceed 10 deaths per 100,000 inhabitants in rural areas, resulting in an estimation of more than 1 million bites and 25,000 deaths per year for the whole of sub-Saharan Africa. Delay of treatment is also a characteristic of African snakebites and can exceed 24 hours; for some victims, this delay may be 1 or 2 weeks. This is the consequence of complex care-seeking behavior, health center scarcity, and the lack of confidence of African populations generally toward equipment and capacity of health centers, which appear to them inappropriate for treating snakebites. Antivenom is typically of exceptionally limited availability, but the only effective treatment is antivenom administration. For the last decade, concerted efforts have been made to improve the accessibility of antivenom in Africa, and greater access will decrease morbidity and mortality tremendously.

I. INTRODUCTION

The geographic and climatic diversity of Africa results in a great variability of snake species, and different ecological and economic conditions occur from place to place. Circumstances leading to snake-human encounter—and thus the incidence of bites—are due to both the behavior of the snakes and human activities. The severity of the bite, i.e., morbidity and mortality, depends on the species involved, yield and composition of venom, and therapeutic recourse in all its components: choice of the doctor (traditional or modern) made by victims or their family, access to health centers, and finally, therapeutic capacity, which is related to the availability of the antivenom (AV) and the expertise of health care staff.

Difficulties that are characteristic of the epidemiology of African envenomations include four main factors:

1. Heterogeneity of the envenomation type, varying with the diversity of African snake fauna of a given area
2. Insufficient knowledge of the epidemiological situation
3. Inappropriate therapeutic management
4. The often severe economic context of envenomations

A better knowledge of the circumstances of snake-human encounters, based on studies performed in Africa concerning both herpetological and human socioeconomic activities, could help in identifying at-risk populations and the relative importance of factors involved in bites. Epidemiological studies undertaken since the 1970s led to the identification of specific therapeutic needs: geographical localization of the accidents, quantities of antivenins to supply, and level of required care. Clinical and therapeutic studies allow validating treatment protocols adapted to the needs and the conditions prevailing in African health centers, which are often poorly equipped and undersupplied

with drugs. Finally, proposals and recommendations will help identify solutions regarding snakebite problems that are in accord with the economic and sociocultural constraints present.

II. INDIVIDUAL AND SNAKE ENCOUNTER COMPONENTS

Africa is a continent that extends over the two hemispheres. All types of climates, except arctic cold, can be found there. Snakes occur, at very different densities and species compositions, in all of the biotopes represented in Africa: primary and secondary forests, savannas, sahels, deserts, mountains, managed perimeters, rural villages, cities, etc. Each biotope, including human habitations, has a distinct herpetofauna. As a consequence, this produces differential risks associated with specific places. Moreover, human activities, varying with place and season, bring together humans and snakes under a variety of different circumstances and conditions.

A. Description of Snake Fauna from Sub-Saharan Africa

Venomous snakes belonging to four families are present in Africa: Colubridae, Atractaspididae, Elapidae, and Viperidae. The first family, comprising approximately one hundred species in Africa, which may be harmless or rear-fanged venomous, typically does not constitute a serious risk for humans under most conditions (*Dispholidus* and *Thelotornis* are two notable exceptions).

The Atractaspididae, of which only the genus *Atractaspis* (seventeen species) has a complete solenoglyphous venom apparatus (long fangs, on a short maxilla that is mobile, connected to the venom glands by a duct), are endemic to Africa and the Middle East. Their venom is inflammatory, necrotizing, discreetly hemorrhagic, and highly cardiotoxic because of the presence of sarafotoxins (Warrell et al., 1976b; see also Chapter 6, this volume). These snakes are nocturnal and burrowing, which limits their potential danger. Currently, there is no AV that will neutralize their venom. Death occurs quickly, and case fatality rate remains high (10%), while specific incidence is low, except in some Soudanian savanna areas, where the frequency of Atractaspididae bites varies between 1 and 3%.

The Elapidae (eight genera and twenty-nine species) have proteroglyphous venom apparatus. Hollow fangs are short and located at the anterior end of a maxilla that is small and fixed (in the genus *Dendroaspis*, the maxilla is moderately mobile). Elapids live in all biotopes and habitats. They are burrowing (*Elapsoidea*), terrestrial (*Naja*), aquatic (*Boulengerina*), or arboreal (*Dendroaspis*). They can occur in desert or sahel (*N. haje*), savanna (*N. nigricollis*, *D. polylepis*, *D. angusticeps*), or forest (*N. melanoleuca*, *D. viridis*, *D. jamesoni*). *Naja nigricollis* and related species are, generally, ubiquitous species, living from the sahel and some Saharan oases to forested areas, where they may be found within human habitations. Some species also occur in large towns (*D. jamesoni*, *N. nigricollis*, and *N. melanoleuca*) and can be common. Generally, the relative abundance of elapids is low (less than 10% of the entire snake population). However, in some areas, it can be significantly higher, as in maritime Guinea (Baldé et al., 2000, 2002, 2005c), in the Democratic Republic of Congo (Malukisa et al., 2005), or in some industrial plantations (Stahel, 1980; Chippaux and Bressy, 1981).

The Viperidae (six genera and forty-eight species) are solenoglyphous, with long retractable fangs on a short maxilla that is erected at the time of the bite. *Cerastes* (North African sand vipers) lives in sandy areas. The genus *Echis* (five species) is present in all of the dry parts of tropical Africa (desert, sahel, and savanna). *Echis leucogaster* lives in arid regions (receiving 100–500 mm rain annually), while *E. ocellatus* lives in wet savanna (300–1,200 mm rain) (Roman, 1969). *Bitis* (ten species) live from the sahel (*B. arietans*) to primary forest (*B. gabonica*, *B. nasicornis*) and mountains (*B. atropos*). *Atheris* (six species) is an arboreal viper, exclusively from forests, and it can also colonize plantations. *Causus* (five species) is ubiquitous, living in both savanna and forest; *Causus* can become abundant in anthropogenic areas. *Causus maculatus* can be found in savanna as well as in forest, and represents less than 10% of snake species in natural habitat, but sometimes nearly 50% in some banana plantations (Chippaux and Bressy, 1981). Frequency of viper bites is higher

than bites by all of the other venomous snake families. In Africa, at least 60%—and in some places more than 85%—of the envenomations are due to vipers.

Snakes are often sedentary and gregarious. Their territory is generally small, from a few hectares up to 1 or 2 km^2 for large venomous species like *Bitis*, *Dendroaspis*, or *Naja*. In Africa, snake movements within this space are mostly related to three factors:

1. Hunting for food, generally according to specific schedules; the frequency and the duration of movements will depend on the abundance of prey, and a weekly or semimonthly cycle is common.
2. Mating, generally a seasonal (annual) activity; in most cases, males in search of females are more active.
3. Birth (or hatching), also a seasonal activity, which generally occurs at the end of the dry season or the beginning of the rainy season. Newborn snakes disperse in search of their own future territory, and during this period, there is a considerable increase of snake number (often ten to fifty or hatchlings per female, depending on species).

Each species shows preferences for a particular biotope; however, the preferences of most venomous species are still poorly known.

Two elements play an essential role in explaining the incidence and severity of snakebites: snake density, which is very difficult to determine precisely, and species composition within a given population. Cultivated areas and industrial plantations create attractive environments for both snakes and their prey, as well as providing favorable shelter for reproduction and development (Chippaux and Bressy, 1981; Oyaberu and Shokpeka, 1984; Malukisa et al., 2005), and several venomous species are more abundant in these areas than in natural habitat.

B. Human Activities Related to Snakebite Risk

In all of Africa, agricultural and pastoral activities constitute the main activities during which snakebites occur (Chippaux, 1999, 2000). Depending on the country, they represent from 50 to 60% of the circumstances of bites. Hunting, and collecting wood or water, which women can sometimes transport for several kilometers, are also frequent occasions of bites (approximately 20%). Dwellings can shelter many snakes, which look for cover and prey. They easily enter houses, and many bites occur during the night sleep, even within houses, in particular by the spitting cobras *N. nigricollis* and *N. mossambica* (Chippaux et al., 1978; Greenham, 1978). Children are commonly bitten in the hand while exploring burrows in search of small animals (rodents, lizards, or birds) to supplement their nutrition. Consequently, the site of the bite is often the hand or the arm.

In all countries, the majority of snakebites occur during the rainy season. This is due to the behavior of the snakes, which are generally more active at this period of the year, and human agricultural activities, which also increase during this season. In Moslem communities, men have been bitten on the penis while urinating, squatting against a low wall or a rock. This phenomenon remains a minimal but relatively constant occurrence risk (Warrell and Arnett, 1976; Chippaux, 2002b).

In certain parts of southern Côte d'Ivoire (Chippaux, 2002b), snakebite incidence is proportional to snake density and rainfall. Elsewhere, as in Benin (Chippaux, 2002c) or Cameroun (Chippaux et al., 2002) and Niger (Chippaux and Kambewasso, 2002), some agricultural activities paradoxically take place during the dry season, resulting in higher incidence of snakebites at this period. These phenomena remain relatively limited and correspond to a specific economic sector, particularly in plantations of sugar cane, cotton, and rice, and other specific contexts. A better knowledge of snake populations and epidemiological context could help to anticipate the risk and organize prevention measures.

C. Epidemiology of Snakebites

Two sources are available to gather basic epidemiological information regarding snakebites and envenomations: health center records and household surveys. Cartography using geographic information system (GIS) methods allows synthesis of results of the surveys and facilitates their analysis (Molesworth et al., 2003).

1. Surveys in Health Centers

These involve snakebite victims who attend a public or private health center. Information is reported in registers that collect various data: date of admission, age, sex, symptoms, severity, treatment, evolution, outcome, etc. The surveys can be retrospective, considering only patients recorded in hospital registers, or prospective, if consultation patients are included; the latter allows more precise and formalized data concerning snakebite incidence.

The number of victims treated in health centers can be determined and some factors may be analyzed, such as seasonal variation of the accidents and severity of cases—and eventually snake species involved—allowing health care personnel to begin to anticipate quantitative and qualitative needs related to the management of the snakebites.

2. Household Surveys

These surveys use standardized questionnaires administered to an entire population or a subset sample of a particular population. The survey consists of questioning the villagers on snakebites they or their relatives experienced during past years. Based on differences in memory, this type of investigation likely contains biases (e.g., lapse of memory, relatively limited in the case of snakebite, and multiple mentions of bites involving the same individual). However, it is relatively easy to avoid these difficulties during the preparation of the questionnaire.

These investigations obtain three fundamental pieces of information: incidence (morbidity), case fatality rate (mortality), and therapeutic choice (traditional or modern). Moreover, they allow defining of populations most at risk. Various studies have validated this method (e.g., Guyavarch and Chippaux, 2005).

3. Therapeutic Choice and Treatment of Snakebites

Use of traditional medicine is widespread and very common in Africa. In Senegal, household surveys have revealed that 95% of snakebite victims consulted a traditional practitioner first, 85% exclusively (Chippaux and Diallo, 2002; Chippaux et al., 2005b). Similar surveys have revealed that 80% of snakebite patients in Benin (Chippaux, 2002c) and Guinea (Baldé et al., 2005a), and 70% in Kenya (Snow et al., 1994), were treated by traditional practitioners. Baldé et al. (2005a) also uncovered (in the traditional practitioner's records) all cases that also attended a hospital. However, they found that a considerable number of patients only consulted the traditional practitioner. An in-depth study concerning traditional practitioners and health centers in a province in Burkina Faso (Somé et al., 2002) concluded that public health statistics reflected only 35% of actual envenomations and 20% of deaths resulting from snakebites. In Nigeria, where use of traditional medicine was utilized by 50% of patients (Pugh et al., 1979), only 8.5% of *Naja nigricollis* bites were treated in hospitals (Pugh et al., 1980). Since snakebites are frequently attributed to supernatural causes, such as revenge or punishments that make Western medicine irrelevant (and also because modern care is inaccessible), traditional practitioners are solicited much more frequently than modern health centers. However, worsening conditions of envenomation victims may lead to a change in the victim's mind and convince him or her to attend a health center or hospital.

Limited therapeutic choice and health center scarcity contribute to increased delay of consultation, which can be considerable (Table 22.1) and hence detrimental to treatment effectiveness. Such delays vary depending on infrastructure, reputation, and specific practices of health

TABLE 22.1
Delay to Hospital Consultation after Snakebite in Sub-Saharan Africa

Country	Delay (hours)	References
Benin	24	Chippaux et al., 2007
Burkina Faso	50	Somé et al., 2002
Cameroon	12	Chippaux et al., 1998, 1999b, 2002
Côte d'Ivoire	14	Chippaux, 2002b
Gambia	10	Enwere et al., 2000
Ghana	38	Visser et al., 2004
Natal	5–8	Coetzer and Tilbury, 1982
Nigeria	12–49	Idoko and Ikwueke, 1984; Meyer et al., 1997; Pugh et al., 1979; Pugh et Theakston, 1980; Warrell and Arnett, 1976

centers. Bleedings may appear several days after an *Echis* bite, and necrosis caused by *Bitis* and *Naja* or due to inappropriate first aid measures may evolve several weeks before the victim eventually seeks treatment at a hospital. According to Fayomi et al. (1997), in Benin 58% of patients consult less than 1 hour after the accident, and 12% do so 3 hours after the bite. Coetzer and Tilbury (1982) reported that in Natal, only 3% of patients reach a hospital in less than 1 hour after the bite, and 32% within 12 hours. Warrell and Arnett (1976) observed that in Nigeria, patients on average wait between 12 and 22 hours before seeking hospital treatment. Such delays are not necessarily correlated with the distance between the place of occurrence and the hospital location, but are also linked to other influencing factors, such as road conditions, vehicle availability, or therapeutic choice (Chippaux, 2002a). Also in Nigeria, in a series of 46 patients, the average delay between snakebite and arrival at a hospital was found to be 2.1 days (Meyer et al., 1998). During an AV trial in Benin, the mean of delay in consultation was 24 hours (Chippaux et al., 2007). This study also demonstrated the negative impact of delay of consultation on the efficacy of AV treatment.

Thus, information obtained in health centers is typically not representative of the actual incidence of snakebite, and this limitation should be carefully considered. Nevertheless, its validity for prediction of therapeutic needs, although vastly underestimated, is not contested. On an encouraging note, all of the improvements in snakebite management have led to a significant increase in the number of consultations (Chippaux et al., 1998; Visser et al., 2004).

4. At-Risk Populations

Active men from 15 to 50 years of age are twice as likely to be bitten as women or children. However, a few authors have observed an equal distribution of incident among sexes, such as in Guinea (Baldé et al., 2005a), Gabon (Tchoua et al., 2002), or Zimbabwe (Kasilo and Nhachi, 1993; Nhachi and Kasilo, 1994), and a higher bite frequency in women from Burkina Faso (Somé et al., 2002) and the Democratic Republic of Congo (Bokata, 2005). These differences may be explained by the differential assignment of tasks or biases when reporting cases. Paradoxically, children less than 15 years old constitute one-quarter of the victims in most of the studies, though they represent close to half of the general population. However, when considering body weight relative to the amount of venom injected (Enwere et al., 2000), and taking into account that the bite frequently takes place on the hands (Chippaux, 1999), the severity of envenomation is higher among children than adults. In children, amputations occur in approximately 10% of patients (Loro et al., 1994), which is likely higher than in adults. Studies focusing on envenomations in children are largely insufficient, as they likely underestimate the incidence and perhaps severity relative to those occurring in adults (Guyavarch and Chippaux, 2005). Finally, the severity of envenomation in pregnant

women is high, since hemorrhagic obstetrical complications lead to abortions or premature births, with a significant fatality rate in both mother and fetus (Dao et al., 1997).

5. Evaluation of Therapeutic Needs

A comparison of results indicates some important differences between the morbidity calculated on the basis of health center records and the incidence of bites given by household surveys. For example, in Kenya in 1992, 137 envenomations and 1 death were reported in the entire country by health authorities, while in this same year, a survey geographically limited to one district recorded much higher figures (more than 450 snakebites and 14 deaths) in the studied area alone (Coombs et al., 1997). Such difference may be related to several factors: (1) dry bites, i.e., bite without venom injection, and thus without need of medical care; (2) bites by nonvenomous snakes; and (3) therapeutic choice that would tend toward traditional medicine rather than modern health care. Asymptomatic bites represent a high proportion of accidents, estimated as follows: 25% in North Cameroon (Chippaux et al., 1998b, 1999b), 22% in North Nigeria (Pugh et al., 1979), 36% in the center of Nigeria (Idoko and Ikwueke, 1984), 45% and 40%, respectively, in forest and savanna of Côte d'Ivoire (Chippaux, 2002b; Chippaux and Bressy, 1981), 20% in Guinea (Baldé et al., 2002), 15% in Senegal (Chippaux and Diallo, 2002), and up to 80% in Kenya (Snow et al. 1994).

The incidence, i.e., annual number of snakebites with or without clinic symptoms, varies considerably depending on the regions as well as poor epidemiological data records (Table 22.2). In forests or arboreal savannas, the annual incidence may reach 200 to 600 bites per 100,000 inhabitants, while in dry savannas or in sahel regions, snakebite incidence is ~100 to 200 bites per 100,000 inhabitants. The underlying reasons for such a difference are snake density, which is low in drier regions, and practices of the human populations, which have lower agricultural activity. However, focused surveys show more impressive results, in both forest and savanna (see Table 22.2).

In Côte d'Ivoire, 25% of bites reported by health centers refer to industrial plantation workers, who represent 1.5% of the entire population (Chippaux, 2002b). In costal banana plantations, the incidence may reach 3,000 bites per 100,000 agricultural workers, while incidence varies from 200 to 400 in the surrounding village plantations (Chippaux and Bressy, 1981). In other industrial plantations, incidence appears to be less, though mostly higher than in surrounding plantations (Odio et al., 2005).

However, except in the sahel and savanna regions where *Echis* species are responsible for 70 to 90% of the envenomations (Chippaux et al., 1998, 2007; Einterz and Bates, 2003; Habib et al., 2001; Meyer et al., 1997; Pugh et al., 1979; Warrell and Arnett, 1976), assessing the specific risks in other African zones remains very difficult. Elapid bites appear comparatively less frequently in both dry and wet areas, except in forested Guinea, where the incidence of cobra envenomations may reach 30% (Baldé et al., 2000, 2002, 2005c).

Morbidity, in terms of the annual number of envenomations per one hundred thousand inhabitants, varies in similar proportions. Nevertheless, frequency of dry bites, as well as therapeutic choice made by victims that diverts them from health centers (where cases are registered), should also be taken into account. Thus, quite often, morbidity is the result of the accessibility of health systems—in a broad sense—rather than the actual snakebite risk.

Case fatality rate and mortality reflect, most of all, the adequacy and effectiveness (or lack thereof) of the health care system: drug availability, in particular AV as the actual etiological and effective treatment, and medical staff whose training remains insufficient (Chippaux, 2002a). An indirect indicator of morbidity and envenomation severity is the hospital occupation rate. In some regions of Nigeria, 7.6% of hospital beds are occupied by snakebite victims (Idoko and Ikwueke, 1984); this percentage may exceed 10% in rainy season (Pugh et al., 1979). In the center and north of Benin, 7.7% of beds are occupied by snakebite victims (Chippaux, 2002c), whereas at the national level, the hospital occupation rate for snakebite represents 0.9 and 1.5% of primary and secondary health centers, respectively (Massougbodji et al., 2002).

TABLE 22.2
Incidence and Severity of Snakebite in Some Countries of Sub-Saharan Africa

Country	Incidence (bites/100,000)	Morbidity (envenomations/ 100,000)	Hospital Case Fatality Rate (% deaths)	Mortality (deaths/ 100,000)	References
Benin	200–650	20–450	3–15	10.1	Chippaux, 2002c; Fayomi et al., 1997, 2002; Fourn et al., 2005; Massougbodji et al., 2002; Mensah et al., 2005; Tamou et al., 2005
Burkina Faso	600	7.5–120	1.9–11.7	0.3–2.4	Drabo et al., 1996; Lankoandé Salifou, 1981; Roman, 1969; Somé et al., 2002
Cameroon	—	75–200	5–15	—	Chippaux et al., 2002; Einterz and Bates, 2003
Congo	12–430	20	1–6.6	—	Carme et al., 1986; Akiana et al., 2005
Democratic Republic of Congo	—			—	Biézakala, 2005; Bokata, 2005; Odio et al., 2005
Côte d'Ivoire	—	100–400	2–5	—	Binlin-Dadié et al., 1995; Chippaux, 2002b; Chippaux and Bressy, 1983
Gabon	—	20–90	2.5	—	Tchoua et al., 2002
Gambia	—	—	14	—	Enwere et al., 2000
Ghana	—	50–150	1.3–11*	10.7**	Molesworth et al., 2003; Visser et al., 2004
Guinea	—	100–150	18		Baldé et al., 2000, 2002, 2005a, 2005b, 2005c
Kenya	150	5–70	2.6–9.4	0.5–6.7	Coombs et al., 1997; Snow et al., 1994
Mali	—	90	6.8–17.6	15.8**	Dabo et al., 2002; Dramé et al., 2005
Natal	—	100	—	—	Coetzer and Tilbury, 1982; Blaylock, 2004
Niger			5.1–6.9		Chippaux and Kambewasso, 2002
Nigeria	160 48–603	100–150	2.1–27	15.6**	Anonymous, 1995; Habib et al., 2001; Harries et al., 1984; Idoko and Ikwueke, 1984; Meyer et al., 1997; Pugh et al., 1979, 1980; Pugh and Theakston, 1980; Warrell and Arnett, 1976; Warrell et al., 1977
Senegal	15–195	20–150	7	1.5–14	Chippaux and Diallo, 2002; Chippaux et al., 2005b; Guyavarch and Trape, 2005; Trape et al., 2001, 2002
South Africa		35–100	5	0.5	Blaylock, 1982, 1994; Coetzer and Tilbury, 1982; Mcnally and Reitz, 1987; Rippey et al., 1976; Wilkinson, 1994
Togo		130	3	4.5	Gogovor, 2005
Zimbabwe		3.5	1.8–5		Kasilo and Nhachi, 1992, 1993; Muguti et al., 1994; Nhachi and Kasilo, 1994; Wapnick et al., 1972

*, before current use of the AV; **, in rural zone.

III. CLINICS AND TREATMENT OF ENVENOMATIONS

A. Symptoms

More than 80% of bites are on the lower limbs, mainly below the knee. Bites on the arm are more frequent in savannas than in forests. The use of an axe with a short handle (for agricultural work), which places the hand in dangerous proximity to the ground, as well as children's habit to search for animals in burrows with naked hands, largely explains this aspect of etiology.

Several factors explain severity of envenomations. The species responsible for the bite is certainly the most important factor, as is the snake's size, its overall condition, the quantity of venom injected, and the bite site. The victims' overall health, age, or circumstances (pregnancy in particular) can also play an important role in envenomation symptoms and outcome. Delay of treatment is one of the most important reasons leading to therapeutic failure. In fact, it seems that AV effectiveness is not the problem, but rather, the complications linked to venom action on coagulation factors, or those caused by bleeding, are most challenging (Chippaux et al., 2007).

1. Clinical Heterogeneity of Envenomations

African snake venoms contain many proteins that correspond to two groups, characteristics of which are very distinct at pharmacological, clinical, and therapeutics levels: toxins and enzymes (Chippaux, 2006). Toxins usually have a low molecular weight and bind to a specific cellular receptor, inhibiting or disrupting its function. The toxicological effects depend on the proportion of the injected quantity of toxins to that of the corresponding receptors, and they are dose dependent. Enzymes, generally of higher molecular weight, catalyze substrate transformation. Enzyme toxicity is proportional not only to injected quantity, though that remains an important factor, but also to the enzyme's persistence in the organism. These components influence the evolution of envenomation symptoms and are generally time dependent. However, clinical symptoms resulting from toxin or enzyme actions can appear immediately (Blaylock, 1983; Chippaux, 1999), as a consequence of the rapid spread of the venom in an individual.

2. Elapid Envenomations

Elapid snake venoms are rich in neurotoxins, which selectively bind to cholinergic receptors of the postsynaptic membrane or to ion channels of neurons. Neurotoxins, which have a low molecular weight, quickly bind their target with very high affiinity, blocking the nervous stimulus, and thus resulting in flaccid paralysis of involved skeletal muscles. A great variety of toxins can be found in mamba venoms. Dendrotoxins enhance the release of acetylcholine and potentiate its pharmacologic action; fasciculins inhibit cholinesterase (see Chapter 15). Finally, muscarine-like toxins bind specifically to muscarinic receptors of the motor endplate and prompt an early muscarinic effect (Chippaux et al., 1977). Cytotoxins provoke cellular lysis, which can induce the focal necrosis usually observed following spitting cobra bite (Chippaux et al., 1978; Greenham, 1978; Warrell et al., 1976a). Cobra envenomation is dominated by neurological symptomatology. Venom inoculation is usually painless, though certain cobra and mamba bites can be painful (Chippaux et al., 1977, 1978).

During the first minutes following a bite by an elapid snake, several paraesthesias signs are described by the victim: itching and formication at the bite site, and sometimes local anesthesia that rapidly extends along the bitten limb. Stress clearly dominates the clinical picture with epigastric pain, sensation of thirst and mucus membrane dryness, nausea, and hearing and visual disturbances. After 10 or 15 minutes, highly suggestive physical signs appear. Somnolence confirms the neurotoxic effects of the venom. Muscarinic troubles, like lacrimation, photophobia, abundant flow of saliva, hypersweating, muscle cramps, vomiting, and diarrhea, are particularly intense and early in mamba bites (Chippaux et al., 1977), although they may, to some extent, also appear as a consequence of cobra envenomation. Muscles can show trembling, as well as cramps and spasms.

Bilateral ptosis, typical of cobra envenomation, associated or not with trismus, appears rapidly and confirms central nervous system involvement, often necessitating assisted ventilation. A quiet coma, without the lost of consciousness, precedes death, which occurs when respiratory muscles cease functioning. Progression toward death may require 2 to 10 hours, depending on the quantity of venom injected and the victim's size.

The ocular globe dysfunction caused by venom spits (*Naja nigricollis* or related species such as *N. mossambica*, *N. katiensis*, *N. nubiae*, *N. nigricinta*, and in South Africa, *Haemachatus hemachatus*) provokes a painful conjunctivitis, but it is usually not severe if quickly and abundantly washed, but may worsen in cases of insufficient treatment or inappropriate topical applications (Warrell and Ormerod, 1976; Yaya and Danai, 2007).

African elapid envenomations primarily affect respiration, and neurological, cardiovascular, or kidney sequelae have never been reported after cobra envenomation that was correctly treated. Iatrogenic or nosocomial complications are the most commonly observed sequelae, except for local necrosis following cobra bites.

3. Viper Envenomations

African viper envenomations result in inflammation, bleeding, and necrosis. Processes observed with hemorrhagic syndrome are complex, resulting from wide and contradictory interactions. Two phenomena should be highlighted. First, hemorrhagins provoke lesions at the endothelium surface, which turn into local bleedings. Second, other factors that act on the coagulation are mainly thrombin-like enzymes (which partially mimic natural thrombin and transform fibrinogen into fibrin), glycoproteins that can convert the prothrombin into thrombin, and active substances acting on blood platelets (Chippaux, 2006). Simultaneous presence of these substances in a single venom can explain the various clinical manifestations and the difficulty of treatment. The hemorrhagic syndromes have been previously described as consumptive coagulopathies; however, they should be more rigorously redefined (Chippaux et al., 1999a).

Necrosis is essentially linked to the presence of proteases, enzymes that catalyze hydrolysis of structural proteins of many biological tissues. However, other factors can be involved in necrosis (Chippaux, 1982), notably postenvenomation infections and, quite often, inappropriate local first aid treatments such as incision, tourniquets, etc. (Pugh and Theakston, 1987a, 1987b).

Venom injection, which is usually deep as a result of the long fangs of vipers, is often very painful and indicates that venom was injected. Pain increases rapidly, radiating toward the root of the limb. Pain may also be resistant to treatment and may require local anesthesia. However, some viper bites, for example, *Echis ocellatus* envenomations, have been described by the victim as nonpainful or comparable to a thorn or needle prick, which can mislead the diagnosis. An inflammatory syndrome is always associated with viper bites. Edema appears at the bite site, usually within minutes, progressively extending to adjacent regions. It may spread to the half of the body in a few hours. Necrosis, quite often moist and oozing, spreads rapidly in surface and sometimes in depth.

Hemorrhagic syndrome appears progressively, and bleeding at the site of the bite can last for several days. Epistaxia, hematuria, massive purpura, and sometimes hemoptisia or digestive hemorrhage can occur. After *Echis* bites, hemorrhagic syndrome can appear with a delay from a few hours to several days, and be expressed as severe anemia, systemic bleedings, or meningeal hemorrhage, which can cause the death (Warrell and Arnett, 1976; Warrell et al., 1977). Clinically diagnostic signs appear earlier. Fibrinogen levels decrease quickly, followed by other coagulation factors and sometimes blood platelets.

Sequelae may occur that are related to either necrosis leading to amputation, or embolism that can induce a visceral infarction far from the site of the bite. Renal lesions are the most frequent, turning into anuria, which strikes up to several weeks after the bite. Renal ischemia may lead to early tubulus or cortical necrosis. Glomerulonephritis, which is pathogenically more complex, appears later. It has been described after *Bitis arietans* envenomation, and may be a consequence of extracapillary proliferative glomerulonephritis following hydrolysis of the glomerulus basement

TABLE 22.3
Prevalence (%) of Clinical Syndromes Observed after Snakebites in Sub-Saharan Africa

Country	Edema	Necrosis	Hemorrhage	Neurological	References
Benin	66–92	5–8	8–10	5–12	Fayomi et al., 1997 Massougbodji et al., 2002
Burkina Faso	100		55	3.5	Drabo et al., 1996
Cameroon	70	5	60	—	Chippaux et al., 1998, 1999b
Chad	95	—	22	—	Markwalder, 1980
Côte d'Ivoire	30–45	5–10	5–15	2–3	Chippaux, 2002b; Chippaux and Bressy, 1981
Gambia	86	9	13	—	Enwere et al., 2000
Guinéa	30	—	—	35	Baldé et al., 2000, 2002
Liberia	94	1.5	6	3	Stahel, 1980
Natal	70	12	5	5	Coetzer and Tilbury, 1982
Nigeria	64–80	—	26–60	—	Idoko and Ikwueke, 1984; Pugh et al., 1979; Warrell and Arnett, 1976
South Africa	85	13	—	2	Blaylock, 2004

membrane. This mechanism is completely independent of immunopathology, as revealed by the absence of deposits of immunoglobulins or complement on the glomerulus. This kind of complication also occurs with *Atractaspis* and *B. gabonica* bites. On the other hand, to our knowledge, renal complications are not documented after *Echis* envenomation, except in cases of hemorrhagic or allergic syndromes.

Inflammatory and hemorrhagic syndromes due to viper envenomations are more frequent than the neurological syndromes typical of elapid bites (Table 22.3), but actual prevalence is uncertain. On the other hand, cardiotoxic syndromes, for which *Atractaspis* are responsible, are relatively constant at 1 to 2%, independent of geographic region. However, clinical observations must be carefully interpreted considering both recruitment of health center and lack of standardization of clinical observations. Inflammatory and hemorrhagic syndromes are usually associated, but the first is predominant in forest areas, while the second is more frequent in the savanna. Necrosis occurs in 1 to 5% of the envenomations. The anatomical extent varies depending on the species involved and the care provided after the bite. Frequency may be artificially higher in major hospitals, because severe cases or those needing surgical care are evacuated to such facilities (Binlin-Dadié et al., 1995). Some iatrogenic measures such as tourniquet or incisions, often used in Africa, are responsible for negative sequelae. Deaths—attributable to either hemorrhagic syndromes or respiratory paralysis—are mostly due to poor management of envenomations.

B. Treatment of Envenomation

Traditional medicine is frequently used to treat human envenomations (Newman et al., 1997). It is a first-line treatment whose effectiveness is doubtful or clearly harmful (scarification, tourniquet, cauterization) (Chippaux, 1999, 2005, 2006). In addition, traditional treatment contributes to delay in health professional consultation. The only definitive therapeutic for snake envenomations is the administration of antivenom (Chippaux and Goyffon, 1998). In Africa, current conditions for its utilization vary considerably and are usually inappropriate and insufficient, and availability in health facilities is poor (Chippaux, 1998b, 2002a). In order to guarantee effective and rapid action, AV must be administered intravenously. Infusion or slow direct intravenous administration depends on the urgency of the bite and experience of the clinical staff. High doses can be necessary, although this point is controversial (Chippaux et al., 2007), and the venom is neutralized in the vascular compartment (Chippaux and Goyffon, 1998; Chippaux, 2006). Currently, $F(ab')_2$ antivenoms are

preferred because of slow elimination (providing long duration in the vascular compartment), high neutralization capacity, good patient tolerance, and low cost. However, symptomatic or adjuvant treatments are usually necessary.

Antihistamines seem to be particularly useful, and positive effects have not been explained but have been confirmed by experimental studies (Chippaux et al., 1997). This effect indicates that antihistamines could be used generally as an adjuvant to the AV to improve treatment.

Respiratory paralysis (in cases of severe cobra envenomation) requires artificial respiration, which must be maintained until natural respiration resumes, from several days to several weeks (Visser and Chapman, 1982). Tracheotomy should be avoided as much as possible. Some authors administer neostigmine, which seems to potentiate the effect of the AV (Warrell et al., 1983). Atropine has been shown experimentally to be effective against mamba venom (Chippaux et al., 1997; Lee et al., 1982). However, these treatments should not be used systematically, but rather employed upon proper indication as determined by experienced practitioners.

Hemorrhagic syndromes (systemic viper envenomation) necessitate resuscitation that is difficult to codify because they depend on specific etiology and clinical evolution of envenomation. Since African viperid venoms are insensitive to thrombin inhibitors such as heparin or hirudin, these are ineffective: in fact, the hemorrhagic syndrome is reinforced by heparin. Administration of fresh blood or coagulant fractions, which are difficult to obtain in African peripheral health centers, is useful only after AV treatment, because as long as venom remains in the patient, many of the coagulant factors are immediately consumed. However, severe anemia may necessitate rapid substitution therapy. It should also be mentioned that blood used must be free of the many common infectious diseases, such as HIV, hepatitis B and C, etc.

Medical treatment of edema and necrosis treatment should continue until the patient's condition is stabilized. Premature surgical interventions, frequently performed, are sources of hemorrhagic and septic complications (Chippaux, 1982), and the need for surgical intervention can be determined by ultrasound of soft tissues and, in particular, by monitoring intracompartmental pressures (Le Dantec et al., 2004). Surgical intervention may be justified in cases of nonstabilized edema or high intracompartmental pressures that can block vascular circulation in the bitten limb. Unless wounds are stabilized and inflammation is sufficiently controlled, the incision of necrotic tissue is not beneficial. However, after several days, when limb conditions have improved, surgical cleaning of the wound, assessing functional balance, and finalizing treatment are recommended (Chippaux et al., 1961; Chippaux, 1982; Pugh and Theakston, 1987a, 1987b).

In addition to necrosis, meningeal hemorrhage (probable cause of most deaths) and renal insufficiency are the two main complications occurring after a viper envenomation. The former is difficult to avoid if the initial hemorrhagic syndrome is not properly treated, and treatment may benefit from corticoid and mannitol administration. Renal insufficiency may be prevented by early improvement of diuresis with maintenance at approximately 50 ml per hour. Surveillance includes monitoring for proteinuria and microscopic hematuria. Kidney insufficiency treatment is based on peritoneal dialysis, which is more effective when started early.

C. Logistical and Socioeconomic Aspects

Shortage of AV during the last 30 years is the primary concern (Chippaux, 1998b; Theakston and Warrell, 2000). Annual sales of AV in sub-Saharan Africa decreased from two hundred thousand doses at the beginning of the 1980s to less than twenty thousand by the early 2000s (Chippaux, 2002a). The reason is definitively not a dramatic decrease of snakebite incidence, and the main reason given is the high cost of AV. During this period, the price has increased by more than 200%, while the economic crisis that affects Africa more than any other region in the world has effectively reduced the purchasing power of African rural populations. However, other reasons for decreased use exist, including AV poor accessibility (on top of prohibitive costs) and inappropriate use, which leads to distrust of this essential drug.

Very few hospitals or private pharmacies in Africa, particularly in rural areas, possess even one or two doses of AV, making effective treatment exceptionally difficult. Surveys carried out in Niger (Chippaux and Kambewesso, 2002), Senegal (Chippaux et al., 2005b), Cameroon (Diffo et al., 2005), and Benin (Chobli et al., 2005) showed that (1) national AV stocks remain, to a great extent, insufficient related to the number of patients visiting health centers; and (2) distribution is inadequate—AVs are usually found in the main towns, whereas they are unavailable in peripheral health centers, in areas where bites occur. It has also been observed that there is no coherent policy of supply or distribution in any countries of sub-Saharan Africa.

Since therapeutic regimes are not codified, they often vary drastically among health centers. Dosage is usually decided according to the victim's weight rather than the amount of injected venom (based on clinical symptoms), which leads to insufficient doses. Administration routes are usually incorrect. Additional treatment (symptomatic, adjuvant, or resuscitation) is at best ineffective and at worst dangerous for the patient, increasing the cost of the treatment without providing any clear benefit.

The above situation leads to therapeutic failure and to suspicion of the efficacy of AV and the capacity of peripheral health centers or hospitals to be able to treat envenomations.

Snakebites constitute accidents needing urgent treatment entailing important cost for care. Cost for AV may be up to US$100 for correct treatment, and additional treatment and hospitalization can reach US$120 or more; this represents several months' income for a rural family. Nevertheless, families accept spending these large amounts of income for the treatment of severe envenomation, calling upon communal aid, resorting to savings, or selling some goods (poultry, cattle). Thus, it is essential that such a payment result in effective treatment and represent the greatest chances for success in managing snakebite. However, quite often a lack of funds or AV availability means that only incomplete treatment will be obtained. Consequences of this insufficient treatment are catastrophic, resulting in a patient's death or permanent incapacitation and producing a lack of trust of AV and modern therapeutic treatments.

Social costs of envenomations are still poorly known. In Africa it is probable that more than 375,000 adults and 125,000 children are envenomated yearly, and in the absence of treatment, in particular AV, it is estimated that the number of deaths is at least 15,000 adults and 5,000 children. The number of disabled persons would be around 10,000 adults and 6,000 children, leading, respectively, to 250,000 and 240,000 annual disability-adjusted life-years (DALYs). Besides the DALYs, infectious complications, such as tetanus, are not uncommon (Ehui et al., 2007; Habib, 2003). On average, envenomations result in a loss in productivity of 10 days. This represents a loss of approximately 15,000 adult-days and 10,000 children-days per year per million rural people, i.e., more than 300,000 adult-days and 200,000 children-days per year.

IV. A PROPOSAL FOR A PLAN OF ACTION

The following discussion is based on several recent reports (Chippaux, 2002a; Chippaux et al., 2005a; Stock et al., 2007). The response to such a disaster should necessarily target the different problems encountered at each pertinent level. The strategy should be aimed at (1) recovering confidence in AV, (2) improving access to AV, and (3) ensuring distribution of relevant information to the public and health care staff. The following description of the different requirements does not assume any priority order: all interventions should be simultaneous.

A. Defining AV Quality Standards

AV quality should be as high as possible, with an attempt made to meet international standards. At Potters Bar (Theakston et al., 2003), four criteria for AV have been defined and recommended to the World Health Organization.

1. Effectiveness

Production of an effective AV requires high-quality, positively identified venoms from African species, as representative as possible of all toxicological variations observed (Nkinin et al., 1997). Neutralization capacities of AV can be confirmed by a variety of immunological and biological tests. These tests should be standardized and in accordance with international agreements.

2. Safety

Production of safe AV requires attention to selecting production animals that do not represent any risk of dangerous known infection for humans, in particular prion-based infections. Care must be taken to avoid any contamination during AV production (i.e., follow standardized good laboratory practices). Risk of allergic reactions to the AV should be reduced by elimination of all nontarget proteins (purified antibodies only) and by using immunoglobulin G fragment preparations (Fab, Fab_2, etc.), which retain antibody functionality. At this stage, it seems necessary to perform clinical confirmation of effectiveness through appropriate trials of AV.

3. Stability

Stability must take into account prevailing climatic conditions (heat, humidity), particularly in tropical regions. Addition of authorized chemical stabilizers and preservatives is the most frequently adopted solution. However, lyophilization of AV presents important advantages by allowing stable storage without the need for refrigeration, which may not be possible in many areas.

4. Accessibility

Accessibility includes two issues: price of the AV and availability of the product. Price of the AV is an essential issue that depends not only on the production costs but also on commercial constraints and usage modalities. Factors influencing AV cost should be studied to result in a consensus among the many parties involved in production and distribution. Manufacturers must be committed to reducing their costs as much as possible, including profit margins, and in return, a prequalification of AV would be guaranteed (assuming minimum quality assurances were met), facilitating its marketing and allowing for a significant increase in sale and distribution. Distributors and middlemen promoting accessibility of AV to rural areas should strive to reduce costs, receiving the same compensations as manufacturers. Health authorities who will supply the needs of health facilities, as well as local authorities, must act in the interest of the specific populations. Private companies should be encouraged (or required) to stock sufficient amounts of AV according to assessment of probable risk encountered by their employees and families; authorities can encourage companies, by incentive or appropriate legislation, to reinforce local supply of AV. A progressive price scale for snakebite victims unable to afford full treatment costs should be established, as this would encourage victims to seek appropriate medical treatment. Availability of the AV, at the locations where it is necessary, requires defining at-risk populations and having an appropriate and effective distribution network.

B. Identifying At-Risk Populations

Epidemiological studies must be conducted to inform health authorities of areas with high snakebite incidence, so that they may anticipate sufficient AV quantities needed. Health authorities must take into account the fact that snakebite morbidity is severely underestimated in most of Africa, and that there are currently numerous limitations to epidemiological surveys performed by health centers. In order to adapt an appropriate response to the epidemiological reality, it is necessary to carry out household surveys in many different rural zones to identify precisely snakebite incidence and mortality. An assessment of current estimated at-risk populations and cases treated per year is presented in Table 22.4.

TABLE 22.4
Assessment of Current Needs (Chippaux, 2005; updated) and Number of Treated Bites in the Sub-Saharan Countries of Africa

Country	At-Risk Population (in millions)	Treated Bites[a]
Benin	4	**4,500**
Burkina Faso	10	**6,500**
Cameroon	8.5	8,000
Central African Republic	2.5	2,000
Chad	7	5,000
Congo	1.5	1,500
Democratic Republic of Congo	39	30,000
Côte d'Ivoire	9	10,000
Gabon	0.3	200
Ghana	11	13,000
Guinéa	6	8,000
Kenya	35	25,000
Mali	9	9,000
Mauritania	1.5	1,500
Niger	10	8,000
Nigeria	90	50,000
Senegal	5.5	4,000
Togo	3.5	**5,000**

[a] Annual reported morbidity, representing approximately 20–40% of actual morbidity.

Note: **Bold**, data from health authorities (average of 5 to 10 years); other values, evaluation of partial data from health centers but not verified by multiple-year study.

C. Ensuring a Supply of AV

AV should be available and stored in peripheral health centers. It is assumed that AV use would be facilitated by establishing a simple and standardized therapeutic protocol and by appropriate training. Two distribution networks exist in Africa, with some differences between countries, namely, a public network managed by the Ministry of Health, which supplies drugs to public and agreed health centers, and private providers, which supply drugs to other health centers and pharmacies. There are some degrees of interaction between the two networks at present, but this should be expanded.

Most of the pharmacies are in towns and are not supplied with AV, except occasionally in response to circumstantial demands (tourists, hunters, etc.). Health facilities hesitate to purchase AV for the four following reasons:

1. Investment is at too high a price and represents a significant proportion of the pharmaceutical budget for an inaccurately assessed risk.
2. Preservation of AV requires effective cold storage at all stages, and expiry date is usually relatively short (less than 3 years from the purchasing date).
3. Use of AV is hazardous, because practitioners are poorly trained in its usage; patients may also refuse to pay for AV.
4. The high likelihood of allergic reactions has been broadly communicated, including in medical books, for the last 30 years, promoting hesitancy in use of AV.

D. Health Personnel Training

Health personnel training must contain simple and clear messages, communicating the definitive efficacy of AV if it is used correctly. High-quality modern AV is very well tolerated in patients as a result of purification and control of production techniques, and administration protocols are easy to follow. The algorithm of AV administration is based on a few well-codified diagnoses and prognostic criteria (edema, bleedings, coagulation tests without reagents or apparatus, muscular paralysis), and dose adjustment is adapted to the clinical symptoms based on the above criteria. However, general training must be incorporated into programs of medicine, pharmacy, and nursing schools.

E. Information to the Public

The public must be reassured about efficacy and safety of snakebite treatment. Confusion that prevails at health facilities, inaccessibility of AV, and competition of traditional medicine, whose foundations are more understandable and direct, mislead the victim who looks for help and appropriate treatment. A patient will accept the (relative) high costs, and can even accept therapeutic failure, if he or she is convinced that everything possible has been done to affect a cure and that the treatment performed was justified. It is therefore essential not to disappoint the patient. In turn, three points must be communicated to and understood by the patient:

1. Specific issues have been considered by health authorities and correct solutions exist.
2. Treatment, consisting of AV administration that can be carried out in any health center, must be performed quickly and efficiently.
3. The cost of the treatment, even if relatively high, is borne by several participants (including the patient).

Suggestions on collective (general sanitation, cleaning of surroundings of rooms or plantations in order to avoid proliferation of venomous snakes) or individual (wearing boots or gloves during agricultural work or wood collection, use of lighting while walking at night, etc.) protective actions should also be communicated. All communication media can be used, but the radio, in particular rural radio broadcasts, constitutes a more effective medium for dispensing information.

V. CONCLUSIONS

In Africa, over 500 million people live in rural settings, many in high-risk areas. According to current epidemiological estimations, approximately five hundred thousand envenomations occur annually, of which less than half receive appropriate medical care, resulting in more than twenty thousand deaths and an equal number of permanent disabilities (Chippaux, 1998a). Most snakebites occur in sub-Saharan Africa, whereas scorpion stings prevail in North Africa.

A typical patient's profile is represented by a young man, between 20 and 45 years old, with an agricultural or pastoral occupation. Bites occur mainly in the rainy season. Bites are typically located in the leg and are usually caused by snakes of the family Viperidae, resulting in inflammatory syndrome, which can turn into necrosis and hemorrhagic syndrome.

Three factors may influence distribution of occurrence and relative risk: snake population densities and species composition, human activities (mainly agricultural and pastoral), and the management of the bite, in turn dependent on the elected treatment (made by the victim and his or her family) and the quality of the treatment provided by health staff. The unacceptably high proportion of morbidity and mortality results from insufficient management of envenomations, due either to delay in or poor efficacy of the treatment.

In sub-Saharan Africa, snakebite prevalence is strongly underestimated by health authorities. The reason is twofold: on the one hand, the case reporting system is not reliable, and on the other,

most of the victims do not attend modern health centers. As a consequence, snakebite in Africa represents a poorly managed medical emergency because of a lack of analysis of the problem and inadequate therapeutic means.

The high prevalence of snakebites—and its socioeconomic impact—remains neglected by health authorities because of a feeble demand by the population that prefers traditional medicine. What is more, the lack of a simple and effective therapeutic protocol does not encourage intervention by health authorities. For rural populations, snakebite represents a moderate daily risk (about five bites a year and one death every 5 years in a village of one thousand inhabitants), which is regarded as a regular accident that is not foreseeable or avoidable, a source of fatality that cannot be defeated, and an incident that does not prompt health authority's attention. This is why it is crucial to develop epidemiological studies in order to achieve a better understanding of qualitative, quantitative, and geographical needs. At the same time, it is theoretically simple to improve snakebite treatment. Thanks to new technologies that facilitate the use of effective and well-tolerated AV, it is possible to provide definitive protocols of treatment even in rural health centers, which are at the "front line" of need. It is essential to promote AV accessibility by increasing production and distribution, to reduce costs and reach at-risk populations, and to develop new financial systems with a wider partnership to help diffuse costs to patients. Finally, training of health personnel and information to the public must restore confidence in the use of AV and its utility in improving outcomes for victims of snakebite.

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23 Envenomations by Reptiles in the United States

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CONTENTS

Venomous reptiles native to the United States include pit vipers (*Crotalus*, *Agkistrodon*, and *Sistrurus* species), coral snakes (*Micrurus* and *Micruroides* species), and Gila monsters (*Heloderma* species). Both coral snake and Gila monster bites are extremely rare, and the bulk of significant envenomations are due to the pit vipers (rattlesnakes, cottonmouths, and copperheads). Though it is illegal to keep them in many states, imported exotic venomous reptile bites also occur and can be difficult to assess and treat. Antivenom is the mainstay of treatment for pit viper envenomations and is readily available in most areas of the United States. Clinical symptoms may include local soft tissue damage, hematotoxicity, and neurotoxicity. Death is rare, and long-term morbidity is generally due to soft tissue damage. Both coral snake and Gila monster bites are generally managed with supportive care. Effects of coral snake venom are primarily neurotoxic, whereas Gila monster envenomations cause local pain as well as possible vasodilation and hypotension. Currently, no effective field first aid is available for any of these envenomations, so rapid transfer to a health care facility is critical in the event of a potentially serious bite. Careful preventive measures and avoiding intentional handling of venomous or unknown reptiles would likely eliminate the majority of bites that occur each year in the United States.

I. INTRODUCTION

A. Background and Occurrence

There are thousands of venomous reptile bites each year in the United States (Parrish, 1966; Norris, 2004), but a more precise number is difficult to estimate, as there is no systematic reporting for these incidents. Venomous reptiles native to the United States include three genera of pit vipers (family Viperidae, subfamily Crotalinae): rattlesnakes (*Sistrurus* and *Crotalus*), and copperheads and cottonmouths (*Agkistrodon*). Additionally, the family Elapidae is represented by two genera of coral snakes (*Micrurus* and *Micruroides*), and Helodermatidae by Gila monsters and beaded lizards (*Heloderma*). Of all the bites each year, the majority are delivered by rattlesnakes, with less than 1% by coral snakes (Norris, 2004). Gila monster bites are very rare and are likely largely unreported. Finally, around one hundred exotic bites are reported to the American Association of Poison Control Centers annually (Lai et al., 2006).

Envenomation syndromes vary by species and individual. Pit viper venom is complex and causes varied effects on the human body, including local tissue injury and systemic toxicity. Antivenom is generally available, and in most areas of the United States, patients have relatively rapid access to care. Because of this, mortality rates are extremely low, estimated at five or six deaths per year (Langley and Morrow, 1997; Lai et al., 2006). There is approximately 10% incidence of long-term morbidity (Dart et al., 1992), mostly from local tissue destruction. This does not account for complications of surgical procedures and does not include close follow-up for more subtle findings, and so is likely an underestimate (Gomez and Dart, 1995).

Coral snake bites are much less common. The snakes are timid, and most bites occur when a person is deliberately handling them (often because they've confused them with similar-appearing harmless snakes). Their venom delivery apparatus contains fangs of much shorter length than those of the pit vipers, which likely contributes to a lower incidence of human envenomations. Effects of coral snake venom are primarily neurotoxic, with minimal local tissue effects, but the most severe complications can include respiratory failure. Because dry bites are common and symptoms of envenomation may be delayed and then occur precipitously, treatment decisions can be difficult. An antivenom manufactured by Wyeth-Ayerst Laboratories in the United States is no longer being produced; a new antivenom for U.S. coral snakes is currently in development. While symptoms may be long-lived (days to weeks), most patients recover fully. Before antivenom, death rates from coral snake envenomations were reported to be around 10 to 20% (Norris, 2004). Very few deaths have been reported since the introduction of Wyeth's antivenom in the 1960s (Russell and Picchioni, 1983; Spinner, 2006).

Snakebites can be extremely variable and unpredictable even when familiar snakes are involved. But when a patient presents to a U.S. emergency department with an exotic snake bite, the situation can become particularly precarious. Roughly one hundred nonnative, venomous snake exposures are reported to U.S. poison centers each year, a third of which result in serious clinical effects, including deaths (Lai et al., 2006). Exotic snakes are easily obtained, both legally and illegally at trade shows or via the Internet. Poison Control's Toxic Exposure Surveillance System reported more bites from exotic venomous snakes than from native U.S. coral snakes in 2007. Antivenom may not always be available for all species. Supplies have limited shelf life, may be expensive, and several are no longer in production. The Food and Drug Administration (FDA) considers foreign antivenoms investigational or unapproved.

A relatively new resource has recently become available for assistance in managing exotic envenomations: the Online Antivenom Index (OAI), developed by the American Association of Poison Control Centers and the American Zoo and Aquarium Association. By calling poison control (1-800-222-1222), the OAI can be accessed, and antivenom stocked by zoos for exotic snakes can be rapidly located. This will facilitate determining, locating, and obtaining of exotic antivenoms in a timely manner, improving medical care of patients with these uncommonly encountered and often

very challenging envenomations. Additionally, zoos can continuously update the OAI with their antivenom stock and contact information, replacing an 8-year-old hardcopy reference.

Gila monster envenomations are rarest, and occur almost universally when a person is deliberately handling the animal. Such bites are rarely reported. These reptiles have a relatively inefficient venom delivery apparatus. Their venom may cause vasodilation and hypotension, as well as severe pain. They have strong jaws that may cause significant tissue damage from the bite itself. There is no commercially available antivenom. Local pain may last for weeks, but most victims make a full functional recovery. It is controversial whether or not the bite of a venomous lizard has ever caused death in an otherwise healthy individual (Norris, 2004).

The bites of venomous reptiles most commonly occur on the extremities, and in the United States usually occur in the late spring and summer, when both people and snakes are more active (Gold et al., 2004). Bites on the hands and arms are often the result of people intentionally interacting with the animal. Accidental bites more frequently occur on the feet and legs when an unwitting individual steps on a hidden reptile. Bites on the face and trunk are unusual and most often occur when attempting to handle a reptile.

B. Prevention

As urban sprawl leads humans to encroach on the native habitats of venomous reptiles, more interaction is inevitable. Careful preventive measures are necessary to reduce dangerous encounters. Clearing property of brush and log piles, where snakes like to hide, may be helpful. It is also advisable to remove bird feeders, which spill seeds that attract rodents and the snakes that feed on them. When walking or climbing, taking care to only place one's hands and feet where one can clearly see will help avoid accidentally stepping on or offending a snake. If a snake must be removed from personal property, it is best to call animal control or another trained professional to do so. If someone is not immediately available, use a long-handled object such as a rake to lift the snake into a large plastic trash can with a secure lid. The animal can be kept contained until someone arrives to relocate or otherwise dispatch it.

One should never attempt to handle a venomous (or unknown) reptile, even if it is thought to be dead. The decapitated head of a snake may retain biting ability for several minutes and possibly hours (Norris and Bush, 2007).

II. ENVENOMATION BY PIT VIPERS

A. Background and Venom Effects

Pit vipers have a sophisticated venom delivery apparatus. Venom is made and stored in paired venom glands located on either side of the head. These are connected via ducts to accessory glands that activate the venom. These glands drain into the hollow anterior fangs, from which venom is delivered during a bite. Pit vipers can strike at distances of half their body length or more and have been clocked at 8 feet per second (Wingert and Wainschel, 1975). They require only a brief, stabbing motion to effectively deliver venom, and can control the amount of venom delivered in each strike. The fangs are mobile and, when not in use, may be folded up against the roof of the mouth. Dry bites, in which no venom is delivered, occur roughly 25% of the time (range: <10 to 50; Parrish et al., 1966). It is estimated that approximately 35% of envenomations will be mild, 20% moderate, and 10 to 15% severe (Norris and Bush, 2007). In general, rattlesnakes tend to cause the most severe envenomations, followed by cottonmouths, and then copperheads.

The venom of pit vipers is complex and extremely variable, making it challenging to study. It affects the human body both locally and systemically. Locally, the venom causes tissue digestion and increased vascular permeability. The leaky vessels allow fluid and blood to extravasate into the tissues, producing swelling and bruising. If untreated, the swelling may progress to encompass

large areas of the body. It can be profound enough to cause hypovolemic shock as the intravascular volume is depleted. The tissue digestive process is usually painful and may cause severe tenderness in the affected areas. Patients often report that the first symptom they developed after the bite was an immediate burning pain starting at the bite site and spreading proximally. As the venom is absorbed and transported up the lymphatic system, tenderness along lymphatics and swollen lymph nodes may develop. As swelling progresses, blisters or blebs filled with clear or hemorrhagic material may develop and be extensive. Hematotoxicity may cause persistent bleeding or oozing from the bite site.

Although most bites occur on the extremities, local swelling becomes a much more significant problem in bites to the face or neck, where swelling can compromise a patient's ability to breathe and swallow (Brooks et al., 2002). With treatment, mortality is rare after pit viper envenomation in the United States, and local tissue destructive effects are the most common cause of morbidity (Norris and Bush, 2007).

Pit viper venom may also cause systemic effects. Victims will sometimes complain of a metallic or minty taste in the mouth, thought to be due to the metalloproteinases in the venom. Nonspecific symptoms such as shortness of breath, nausea/vomiting, and paresthesias may occur due to the venom, but can sometimes be difficult to differentiate from symptoms caused by anxiety. Some pit viper venoms cause systemic myotoxicity. When severe, the resultant muscle breakdown may lead to rhabdomyolysis and myoglobinuric renal failure. The venom's interaction with calcium or its binding sites may also cause uncontrolled muscle fasciculations known as myokymia. They generally start locally but often progresses to involve remote parts of the body. Myokymia may be refractory to treatment with antivenom.

Other pit viper venoms (such as among certain populations of *Crotalus scutulatus*) contain presynaptic neurotoxins. The syndrome caused by these includes hypotension, lethargy, ptosis (drooping of the eyelids), dysphagia (trouble swallowing), and dysphonia (trouble speaking). A feared complication of this weakness is respiratory failure. Some of these venoms lack the local tissue toxic effects and will not cause much, if any, significant bruising or swelling. Health care providers must be aware of this and be careful not to mistake it for a dry bite.

The final category of systemic effects that may be caused by pit viper venom is hematotoxicity. The findings range from mild to severe and include thrombocytopenia and consumptive coagulopathy. Though clinical bleeding is unusual even in the face of profound laboratory abnormalities, serious gastrointestinal and intracranial bleeds have occurred (Lai et al., 2006). Rare cases of clotting diathesis rather than bleeding have also been observed from rattlesnake bites. These may be difficult to treat given the simultaneous propensity for bleeding.

In addition to the commonly described above reactions to pit viper venom, rare, overwhelming systemic syndromes have been reported in the literature. There are several case reports of patients with distal extremity bites developing hypotension and swelling of the face or airway (Hogan and Dire, 1990; Hinze et al., 2001; Kerns and Tomaszewski, 2001). It is unclear whether these represent direct venom effects or some sort of anaphylactoid or anaphylactic (if previously sensitized) reaction to the venom. In either case, the clinical syndrome is extremely dynamic and health care providers should be ready for an acute decompensation at any time, no matter how trivial the initial bite may seem.

1. Prehospital Treatment

Many first aid measures have been proposed throughout the years as methods of treating pit viper envenomations. These include cutting the bite site and applying suction, applying tourniquets or constriction bands, cryotherapy, applying electric current, and various topical salves. These methods have fallen out of favor as more rigorous study proves they are not helpful and may cause harm. In the United States, calling 911 to arrange rapid transport to a facility possessing antivenom is the best course of action following a pit viper bite. It is prudent to have a plan when working with dangerously venomous animals or venturing deep into remote wilderness. Such a plan should include

a way to effectively call for help and evacuate to an area where help can reach you. Staying calm and monitoring the progression of symptoms may provide useful information to your health care providers. This includes marking the leading edge of tenderness with a marking pen and noting the time of measurement. Remove tight jewelry and clothing and keep the bitten extremity in a position of comfort (neither elevated nor below the level of the heart until treated and it becomes apparent whether the predominant injury will be local or systemic). When dealing with native venomous snakes, identification to species is not crucial for treatment, although it is important to be able to distinguish pit vipers from coral snakes and colubrids. It may be helpful to take a digital or Polaroid photo of the snake from a safe distance to help with identification purposes. When working with exotic venomous animals, identification is paramount, and this information should be kept in a safe and readily accessible location in case of a bite.

Extraction devices applied to intact skin to remove venom after a bite (such as the Sawyer Extractor) were until recently recommended by the Wilderness Medical Society. Initial uncontrolled rabbit studies suggested that if applied early, they might be able to remove a substantial amount of venom (Bronstein et al., 1985, 1986). However, subsequent animal and human studies refuted this effect. Only a miniscule portion of venom is extracted by these devices, and they may cause significant tissue damage themselves (Bush et al., 2000; Alberts et al., 2004). One study performed on pigs showed that after this device was used as directed, some animals developed slow-healing tissue lesions in the exact shape of the suction cup (Bush et al., 2000). A recent case report demonstrated the wound resulting from its use on a human patient's calf (Holstege and Singletary, 2006), and such devices are no longer recommended (Bush, 2004).

In parts of the world where snakes producing primarily isolated neurotoxidromes predominate and time to definitive care may be prolonged (such as Australia), the technique of pressure immobilization is sometimes used to delay systemic spread of venom (discussed in detail in Section III). However, in the United States, where pit vipers predominate and may cause serious local tissue destructive effects, such treatment severely worsens outcome (Bush et al., 2004), as most morbidity is due to permanent local tissue damage.

If one arrives to a scene where a patient has already applied a tourniquet, immediate removal of the band may precipitate a bolus dose of venom or a severe anaphylactoid reaction (McKinney, 2001). For this reason, it is recommended that the band be left in place until preparations have been made to manage an immediately life-threatening envenomation and allergic reaction.

Other first aid methods, such as the application of electric current (Guderian et al., 1986) and packing the extremity in ice (Gill, 1970), have never been validated as helpful and have often been found to be harmful (Johnson et al., 1987; Dart and Gustafson, 1991; Dart and Russell, 1992; Hardy, 1992). These treatments are not recommended.

2. Hospital Treatment

Antivenom is the most definitive treatment available. The first pit viper antivenom available in the United States was Wyeth's Antivenin (Crotalidae) Polyvalent (ACP) in 1954. The introduction of this antivenom along with other advances in medicine reduced mortality rates due to pit viper envenomation from an estimated 5 to 25% down to less than 0.5% (Norris and Bush, 2007). A whole immunoglobulin product, it is made from the serum of horses immunized to *Crotalus adamanteus*, *C. atrox*, *C. durissus terrificus*, and *Bothrops atrox*. It was indicated for North and South American pit viper bites. ACP was believed by some to be less effective at countering neurotoxic effects, and often large doses were used to treat these symptoms.

Its main criticism was the high risk of both immediate and delayed allergic reactions. Such reactions range from mild skin rash to hypotension, airway swelling, and life-threatening anaphylactic shock. Between 20 and 50% of patients developed immediate allergic reactions, and about half of these were serious (Norris and Bush, 2007). Close to 75% of patients developed a delayed, type 3 allergic reaction known as serum sickness (Norris and Bush, 2007). Serum sickness results from

the deposition of immune complexes in tissues and causes fever, rash, arthralgias, joint swelling, and renal problems. While symptoms might be at least partially treatable with steroids and antihistamines, this reaction can be extremely debilitating. The high risk of adverse reactions led to some hesitance in the medical community about administering the medication. There was need to carefully consider the risks versus benefits of its use. It was a difficult decision to make, especially when clinicians were inexperienced in treating pit viper bites and had not seen the severe complications that could result if left untreated. There are reports in the literature of patients with life-threatening envenomations who were successfully treated with ACP despite severe anaphylactic reactions (Bush and Jansen, 1995). In these cases, an epinephrine drip had to be run simultaneously with a diluted antivenom solution and titrated to mitigate the allergic effects. Wyeth stopped production of ACP antivenom in 2001, and any leftover supplies will probably have expired by the time of printing.

In 2000, Protherics, Inc. (Nashville, Tennessee) introduced Crotaline immune Fab polyvalent (ovine), or CroFab®. It is an ovine-derived product made from the serum of separate flocks of sheep immunized with the venom of *C. adamanteus*, *C. atrox*, *Agkistrodon piscivorus*, or *C. scutulatus*. The four groups of sera separately undergo digestion with papain to cleave off the Fab fragment of the immunoglobulin (Ig) G molecule. The Fc portion of the molecule is precipitated and removed. The four groups are then combined in equal portions to create a polyvalent finished product. It contains less than 3% Fc fragments, thought to be the portion of the IgG molecule responsible for activating the immune system and causing allergic reactions. Crotaline Fab antivenom is indicated for envenomations caused by any North American pit viper. Preliminary study suggests it is effective for copperhead envenomation (Lavonas et al., 2004). Because venoms of South American snakes are not used in its production, it is not currently indicated for bites by those species. However, preliminary studies suggest that it may offer some cross-protectivity (Richardson et al., 2005).

Initial studies added evidence to the effectiveness of Crotaline Fab antivenom but demonstrated a higher than expected rate of allergic reaction (Dart et al., 2001). It was later found that five of the six patients who had reactions had all received antivenom from the same batch, which had been improperly purified, and contained high levels of Fc fragments. Once this purification step was modified, lower rates of reaction were demonstrated (Ruha et al., 2002). It has been FDA-approved for use in both adults and children (Offerman et al., 2002).

Crotaline Fab antivenom is given in an initial starting dose of four to six vials, which is repeated until local and systemic progression have been halted. This dose should not be adjusted in children because it is based on estimated average venom load, not the size of the patient. The amount of fluid used may need to be adjusted in small children. After initial control has been achieved, a maintenance protocol is recommended where two additional vials of medication are given every 6 hours for the next 18 hours (Dart et al., 2001). There is currently some dispute as to whether these scheduled doses are necessary or whether patients should be observed for 18 hours and only given the doses as needed if their swelling or hematologic symptoms begin to recur. With this method, approximately 50% of patients will demonstrate recurrent progression of local pain and swelling and require the additional doses (Dart et al., 2001). Given the product's high cost, this may be a more cost-effective regimen as long as the patient can be closely observed for recurrence. Although the pharmacokinetics and dynamics are not fully elucidated, it is believed that local symptoms generally do not progress or recur after the first 24–36 hours (Seifert, 1997). While antivenom is most effective if given within 6 hours of the bite, case reports have shown effectiveness if given up to 52 hours after the bite (Rosen et al., 2000; Bebarta and Dart, 2004).

No discussion of the local tissue toxic effects after snakebite would be complete without mentioning compartment syndrome. Compartment syndrome occurs when swelling inside a closed muscle compartment causes the pressure to rise to a point at which circulation is compromised. The results of allowing this to progress untreated are catastrophic. The muscles and neurovascular bundles within the compartment will die and the limb will be left nonfunctional. Despite popular belief, it

is actually a rare manifestation of pit viper envenomation. It might be more likely to occur if treatment is delayed or if the patient is undertreated. It may pose a diagnostic dilemma because many of the clinical signs and symptoms used to diagnose compartment syndrome (pain with passive stretch, paresthesias, firm, tender compartments) are also present with moderate to severe pit viper envenomations. The most objective measurements of compartment pressures are therefore needed to determine whether the syndrome is actually present in these patients when clinical symptoms cannot be relied upon.

Management of snakebite-induced compartment syndrome is controversial. Compartment syndrome caused by other etiologies (such as crush injury, fracture, or burn) is invariably considered a surgical entity. Emergent fasciotomy should be performed to release the pressure and allow perfusion to return to the muscles and nerves. With snakebite, this may not always be the case. Animal studies and anecdotal evidence suggest that in the presence of significantly elevated compartment pressures, a trial of high-dose antivenom prior to surgical intervention may be merited and improve outcome (Hall, 2001; Tanen et al., 2003; Gold et al., 2003; Dart, 2004; Fulton and Hoffman, 2005). Reduction of compartment pressures and good long-term outcomes have been documented in humans in case reports (Rosen et al., 2000; Gold et al., 2003). Animal studies suggest that fasciotomy may worsen outcome (Tanen et al., 2004). At this time, most experts would recommend an aggressive trial of antivenom and rechecking the compartment pressure after medical therapy rather than proceeding directly to fasciotomy in the face of a true compartment syndrome. If antivenom does not lead to decreased compartment pressure, fasciotomy may be necessary.

With regard to neurotoxicity, Crotaline Fab antivenom appears to work very well to reverse its manifestations. This is likely due to the fact that the venom of *C. scutulatus*, which contains prominent neurotoxins, is used in its production. Resolution of muscular weakness, ptosis, dysphagia, dysphonia, and lethargy is seen rapidly after antivenom treatment. These symptoms tend not to recur once they have been treated.

While local and most systemic effects (including neurologic) generally begin within minutes to hours after a bite, hematologic effects may onset either early or late. Experience suggests that venom components may circulate in the body for 2 weeks or more after rattlesnake envenomation (Seifert and Boyer, 2001). Some patients present to the hospital with immediate evidence of hematotoxicity. This early phase generally responds quite well to antivenom treatment, and thrombocytopenia may begin reversing as early as 1 hour after antivenom infusion. This suggests a mechanism of binding or aggregation and subsequent release of platelets rather than destruction and *de novo* synthesis. The clotting cascade takes longer to recover, and improvements may be seen within 3 to 6 hours after sufficient antivenom infusion. Venom studies suggest that the coagulopathy is a consumptive one, and the factors need to be resynthesized, explaining the longer recovery time (White, 2005). A trend of the values toward normal indicates that venom effects have been sufficiently neutralized. Persistent abnormalities or continued worsening indicates that insufficient antivenom may have been given.

During the initial 24 to 36 hours, recurrent hematotoxicity, like local progression, generally responds well to two vial doses of Crotaline Fab antivenom. Several hypotheses exist to explain these early recurrence phenomena. The most likely entails a pharmacodynamic and pharmacokinetic mismatch between venom antigens and the Fab molecules (Seifert and Boyer, 2001). It is thought that the bite site may serve as a sustained release or depot source of venom for 2 weeks or more. In contrast, the half-life of unbound Fab antivenom is only 3 to 18 hours (Seifert and Boyer, 2001). These small fragments may be cleared rapidly from the body, and venom effects may start to recur as venom is released from the depot site once the neutralizing antivenom molecules are gone (Seifert, 1997).

In addition to early hematotoxicity, a phenomenon of late-onset hematotoxicity has also been described. Anywhere from 2 days to greater than 2 weeks after envenomation, patients may develop profoundly low platelets or fibrinogen, or a severely prolonged prothrombin and international

normalized ratio (INR). It has been observed that these abnormalities may respond well to antivenom initially, but as time progresses, the thrombocytopenia becomes refractory to treatment with even large doses of antivenom (Seifert, 1997; Miller et al., 2002; Offerman et al., 2003). This suggests a different mechanism than that responsible for the initial thrombocytopenia, which reverses rapidly with antivenom administration. It may be that platelets are being sequestered or destroyed at sites of tissue swelling and injury during the late phenomenon, and are not easily released with antivenom (Seifert and Boyer, 2001).

Treatment of recurrent or late-onset hematotoxicity is controversial. Some suggest that if the patient is asymptomatic, careful observation without further treatment is sufficient (Yip, 2002). There is risk associated with this method as the abnormalities may become severe enough to potentially cause spontaneous bleeding. Others suggest treating with additional antivenom if certain criteria are met (Boyer et al., 2001). For late recurrent or delayed symptoms (approximately 5–12 days after envenomation), antivenom alone may not seem to reverse the abnormalities, even when large doses are given. At this point, blood products, given after a small dose of antivenom to bind any circulating venom, might be more successful at correcting hematologic abnormalities, at least temporarily, especially if risk of serious spontaneous bleeding is high (e.g., platelets < 10–30). The effects of venom on the hematologic system usually resolve on their own after about 2 weeks or so, so if the patient can be safely maintained through this time period, the abnormalities will then resolve spontaneously without further therapy.

While antivenom is the most important part of therapy for pit viper bites in the United States, several other ancillary treatments are used as well. Intravenous fluid hydration is important in patients who develop hypotension or severe swelling. While most hypotension will resolve with fluid therapy and blood products as indicated, vasopressors are occasionally required to maintain blood pressure in severe envenomations. In the event of an anaphylactic or anaphylactoid reaction, epinephrine would be the vasopressor of choice.

Pit viper bites may become extremely painful. Opioid analgesics may be used, but great care should be taken in patients who've had hypotension or lethargy as part of their syndrome. It might be prudent to start with a short-acting agent that causes less hypotension, such as Fentanyl. Subsequently or alternatively, small doses of analgesia should be used judiciously with careful monitoring until it is known how the patient will respond to the medication in light of the envenomation. The medication can be titrated up to pain relief while optimizing hemodynamic stability. Any medication that could thin the blood, including aspirin or anything in the NSAID family (such as ibuprofen or naproxen), should be avoided to prevent compounding hematotoxicity from the venom.

Infection is quite rare after pit viper envenomation in the United States (Clark et al., 1993), and routine use of prophylactic antibiotics is not necessary. However, one should watch for signs of infection, and antibiotics may be needed should an infection develop. Updating tetanus immunization is recommended if indicated.

3. Outcomes and Follow-up

With rapid transport and adequate treatment, most patients recover fully from pit viper envenomations. The severity of the envenomation and the quality of the treatment largely dictate the likelihood of complications or long-term sequelae. It may take months to a year before a full recovery is made. Most sequelae are due to local tissue toxic effects.

Significant envenomations may cause permanent damage to the regional lymphatic system. This can lead to chronic swelling of the affected extremity. This seems to be more common in lower-extremity bites and those that had more severe initial swelling. Bites to the hands and fingers are prone to causing stiffness. Initially, pain and swelling may prevent a person from moving the fingers, and scarring results. Early range of motion (ROM) exercises and physical therapy may help prevent this complication or treat it after it has occurred. In general, mobilizing as early as possible is recommended. Occasionally, areas of necrotic tissue may develop and need to be debrided surgically.

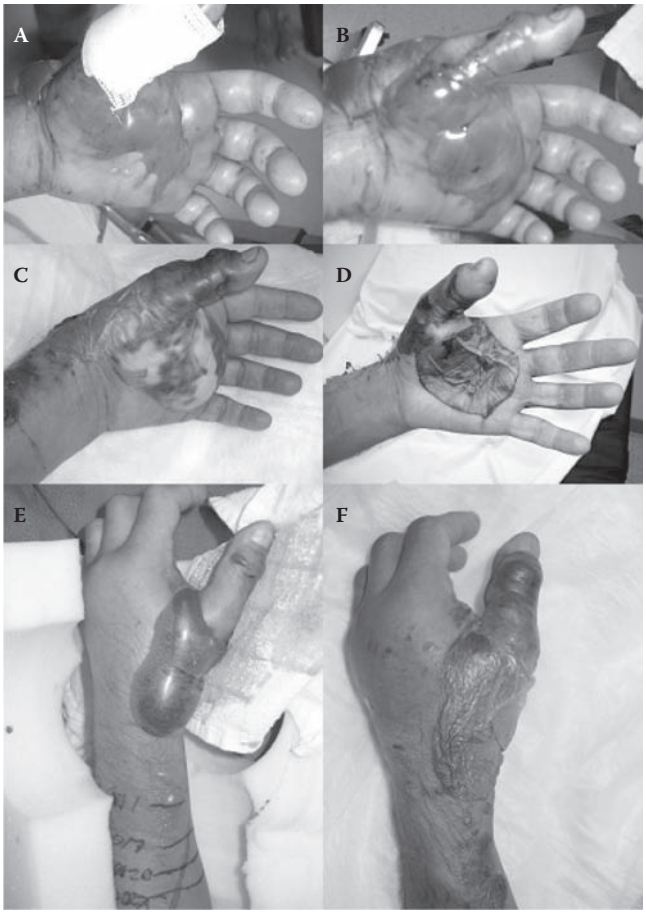

FIGURE 23.1 Progression of a pit viper bite. (A) Hand, day 1 of envenomation. (B) Hand, day 2. (C) Hand, day 6. (D) Hand, day 9. (E) Wrist, day 1. (F) Wrist, day 6.

If blisters or hemorrhagic blebs have formed, leaving them intact for 3 to 4 days is universally recommended. This allows the skin to provide a physiologic dressing to prevent contamination and decrease infection risk. Some experts recommend debriding the blisters at 3 to 4 days (Hall, 2001). However, good results and full healing have been documented without debriding blisters. Blisters generally begin to drain on their own within the first 3 to 5 days, and after that time can be kept clean and dry until healed (Figure 23.1).

Due to the risk of delayed or recurrent thrombocytopenia or coagulopathy, patients should have bloodwork (complete blood count with platelets, prothrombin, and partial thromboplastin times, and possibly fibrinogen assay) checked every 2 to 3 days for 14 days after the envenomation (more or less often may be appropriate depending on clinical circumstances), even if they are asymptomatic. Some studies suggest that in resource-limited areas, the prothrombin time may be used as a surrogate for fibrinogen levels, and that these levels do not need to be checked (Isbister et al., 2006). Current recommendations for when to treat hematologic recurrence with additional antivenom include: fibrinogen < 50 μg/dl, platelet count < 25,000/mm^3, INR > 3.0, aPTT > 50 s, multicomponent coagulopathy, worsening trend in patient with prior severe coagulopathy, high-risk behavior for trauma, and presence of comorbid conditions that increase hemorrhagic risk (Boyer et al., 2001). Blood products may be given in the event of serious or imminent risk of life-threatening bleeding, but antivenom should also be given to neutralize circulating venom and to avoid worsening the consumptive process.

III. ENVENOMATION BY CORAL SNAKES

A. Background and Venom Effects

Only about 1% of reported venomous snakebites reported in the United States each year are due to coral snakes. These snakes are small and shy, and rarely bite unless they are being intentionally handled. The United States has three native species of coral snakes. The eastern coral snake, *Micrurus fulvius*, is the most widespread. It is found in the southeastern United States from North Carolina to Florida, Arkansas, and Louisiana (east of the Mississippi River). The Texas coral snake, *Micrurus tener*, is native to southeast and central Texas, southwestern Arkansas, and Louisiana, west of the Mississippi River. The Arizona or Sonoran coral snake (*Micruroides euryxanthus*) is found from west Texas to Arizona. In general, the venom of the eastern coral snake is believed to be more dangerous than that of the Texas coral snake. The Arizona coral snake is smaller, with shorter fangs, and is considered to be the least dangerous. No human deaths have ever been reported after its bite (Norris, 2007).

Often, bites will occur when a person unsuspectingly picks up a snake thought to be harmless. There are several nonvenomous mimics that closely resemble coral snakes, such as the scarlet kingsnake (*Lampropeltis triangulum doliata*). In the United States, coral snakes have wide red and black bands separated by narrower yellow or cream-colored bands. The bands completely encircle their bodies. The snout is always black. Most mimics have adjacent red and black bands. This pattern becomes unreliable south of Mexico City. Rare specimens may be bicolor, albino, or melanistic.

The venom delivery apparatus of coral snakes is less sophisticated than that of pit vipers. Coral snakes have paired venom glands that lead into short, fixed fangs on the anterior part of the maxilla. They do not strike rapidly, but rather may need to hang on and "chew" to deliver venom effectively. In one case series of documented coral snake bites, 85% of patients reported the snake hung on and had to be manually removed (Kitchens and Van Mierop, 1987). However, a more recent series suggests that envenomation may still occur if the bite is only momentary (Morgan et al., 2007). Dry bites are estimated to occur 20 to 40% of the time (Kitchens and Van Mierop, 1987; Norris, 2007).

Coral snake venom differs from pit viper venom in that it has primarily neurotoxic effects and lacks significant local tissue toxicity. While the delivery is less efficient, the potency of the venom is greater than all pit viper venoms except *C. scutulatus*. The venom contains potent postsynaptic neurotoxins that block the neuromuscular junction at acetylcholine receptor sites. The clinical effects include burning pain, paresthesias, bulbar palsies (dysphagia, dysarthria, dysphonia, ptosis), muscle weakness, altered mental status ranging from drowsiness to euphoria, nausea and vomiting, respiratory failure, hypotension, and seizures.

Local swelling is generally absent or minimal. The bite itself may look like scratches from rows of small teeth. It may be subtle enough to go unnoticed, even when carefully sought (Norris and Dart, 1989). Clinicians and patients should be aware that venom effects may be delayed up to 13 hours after the bite (Kitchens and Van Mierop, 1987), but may then progress rapidly and be difficult to treat.

1. Prehospital Treatment

The pressure immobilization technique initially described for Australian elapid bites (Sutherland et al., 1979) may be helpful prehospital care for coral snake bites. It involves wrapping an elastic bandage around the bitten extremity at a tension of 55 to 70 mmHg (about the same as an ankle sprain) and then immobilizing the extremity. It has been shown to impair systemic absorption of the venom and may delay onset of symptoms (German et al., 2005).

Because many harmless snakes may closely resemble coral snakes, accurate identification is very important to determine whether antivenom is needed. The safest option is to take a digital or Polaroid photo of the snake. Attempting to capture the animal wastes time and risks additional bites.

2. Hospital Treatment

The first and only U.S.-produced antivenom for coral snake bite (antivenin *Micrurus fulvius*) was introduced by Wyeth in 1967. Prior to this time, mortality was estimated to be about 10%. Only one death has been reported since then (Spinner, 2006). While some stocks of this antivenom may still be available, production ceased in 2001 (German et al., 2005). An Fab fragment antivenom has been produced by Bioclon, a company in Mexico. While it is not currently FDA-approved for use in the United States, it may be available as an experimental therapy. Other antivenoms produced from Central and South American coral snakes may offer some cross-protectivity.

A patient with a possible coral snake bite may present a diagnostic and therapeutic dilemma for clinicians. Because of the possibility of delayed, progressive, and refractory symptoms, it was originally recommended that antivenom be given if there was any reasonable suspicion that an eastern or Texas coral snake bite occurred, even if the patient was asymptomatic (Russell, 1980; Kitchens and Van Mierop, 1987). These recommendations originated in the 1960s when supportive care was not as advanced as it is today, and there is no objective data to support them. We currently have the capacity to monitor patients closely and support them effectively if symptoms develop, so it may be reasonable to employ watchful waiting and only give antivenom in the event that symptoms develop. Because of the relative rarity of coral snake bites, neither approach has been well studied, and there is no clear consensus about which is superior. Bites by the Arizona coral snake require supportive care only because the *Micrurus fulvius* antivenom may not offer cross-protectivity, and because the bites are not generally severe enough to require antivenom. Helpful case history questions include the coloration pattern of the snake and whether it had to be manually removed. Fang or teeth marks should be sought, but their apparent absence does not definitely rule out a bite (Norris and Dart, 1989). The presence of any symptoms (other than mild local pain) at the time of presentation is an indication for antivenom treatment.

The starting dose for the Wyeth antivenom is three to seven vials, diluted in normal saline to a volume of 250 to 1,000 cc (20 cc/kg for children). Sometimes a second dose may be required, but the need for more than ten vials is rare. Since it is a whole IgG preparation derived from horse serum, pretreatment with H1 and H2 blockers and steroids is recommended. The infusion should be started slowly (1–2 cc over the first 3–5 minutes) and the patient should be watched closely for any evidence of allergic reaction. If no reaction occurs, the rate may be increased to deliver the remainder of the medication over approximately 1 to 2 hours (Norris, 2007; Anonymous, 2001). If a mild reaction develops, the antivenom may be further diluted and infused more slowly, and treatment with epinephrine may be considered. If the reaction is severe or life threatening, a risk-benefit analysis must be done. Since patients may be successfully treated with supportive care alone, it may be safest to withhold further antivenom in these cases (Norris and Bush, 2007). The Fab fragment antivenom may have a lower risk of acute or delayed allergic reaction.

All patients should be monitored closely for any neurologic symptoms, especially any evidence of impending respiratory weakness. Antivenom does not always completely neutralize or prevent progression of symptoms. Peak flows or arterial blood gas measurements may be followed. If any weakness develops, early elective intubation should be considered to prevent aspiration. Great care should be taken when administering any medications, such as opiates or benzodiazepines, which may further depress respiratory drive or muscle strength. Given judiciously and with close monitoring, they can be used safely (Morgan et al., 2007).

Routine laboratory testing is not useful in coral snake bites (Norris, 2007). Hematotoxicity has not been described in humans. The venom does contain a myotoxic component, so occasionally muscle breakdown may occur and creatinine kinase can be measured if this is suspected (Norris and Bush, 2007).

If antivenom is unavailable or withheld, asymptomatic patients should be closely monitored in an intensive care setting for at least 24 hours to watch for delayed symptoms (Norris, 2007; Norris and

Bush, 2007). In symptomatic patients treated with supportive care only, weakness may persist for weeks. If treated with antivenom, patients should be warned about the possibility of delayed serum sickness, which may require treatment with antihistamines or steroids. As a rule, patients generally make a full recovery and long-term morbidity has not been described.

IV. ENVENOMATION BY GILA MONSTERS

A. Background and Venom Effects

The Gila monster (*Heloderma suspectum*) is one of the world's two species of venomous lizard. It is native to Arizona and parts of California, New Mexico, Nevada, and Utah, and also occurs in parts of northwestern Mexico. However, it is very popular among collectors (though it is a protected species and possession is illegal without permit) and zoos, so it may be found anywhere. Throughout history, helodermatids have taken on legendary properties in folklore, leading to mistaken beliefs that they can kill with their poisonous breath, spit venom, and sting with their tails or tongues (Russell and Bogert, 1981). While such stories lead to the reasonable advice that these lizards should not be handled or molested, most of this exaggeration is not true.

The Gila monster is generally 300 to 400 cm in length, but specimens of up to 550 cm have been reported (Norris and Bush, 2007). While generally slow moving, it can become defensive quickly when threatened. The jaws are strong and muscular and are capable of inflicting severe injury by mechanical force alone. The venom glands are located at the anterior aspect of the lower jaw and deliver venom through the oral cavity and along grooved teeth via capillary action. A chewing motion is required to deliver the venom effectively, unlike pit vipers, which can deliver venom in one rapid strike. The longer the animal stays attached to the victim, the more venom it has the potential to deliver. It is estimated that the animal effectively delivers venom in 60 to 70% of bites (Norris and Bush, 2007).

Gila monster bites are unusual and probably underreported. They almost always occur during intentional handling of a captive specimen. The few reports in the literature to the contrary include a patient who had a motorcycle accident in the desert and, as he fell to the side of the road, accidentally stuck his hand in a Gila monster's mouth (Russell and Bogert, 1981). Another report involved a stuntman who was dragged through the desert attached to his parachute and was dragged directly onto a Gila monster (Russell and Bogert, 1981).

Gila monster venom contains multiple components, including serotonin, short peptides (exendins, etc.), hyaluronidase, protease, phospholipase A_2, and kallikrein-like protein (gilatoxin; Utaisincharoen et al., 1993). Gilatoxin causes release of bradykinins from circulating kininogen and is likely responsible for the hypotension that may be seen (Norris and Bush, 2007). Venom effects are generally mild, but there are reports of severe syndromes. The symptoms include weakness, dizziness, hypotension and shock, diaphoresis, nausea and vomiting, and severe pain. The pain generally starts almost immediately and reaches a maximum within an hour. The course then often subsides within the next 6 to 10 hours.

While permanent tissue destruction and necrosis are rare, swelling, tenderness, and erythema may be seen. Lymphangitis and lymphadenopathy may also develop. Infection is unusual, and the swelling usually resolves without sequelae in days to weeks. There are scattered case reports in the literature of facial and airway swelling after distal bites, consistent with an anaphylactoid reaction in patients who had not had previous exposure to Gila monster venom (Piacentine et al., 1986).

Transient EKG changes and myocardial infarction have also been reported (Roller, 1977; Bou-Abboud and Kardassakis, 1988). There are probably no confirmed reported cases of death due to Gila monster bite in an otherwise healthy individual. It is hypothesized that death could occur in a young child or an adult with significant comorbidities, especially if the lizard was large and the bite duration of contact was long.

1. Treatment and Outcomes

One of the first concerns in treating the victim of a Gila monster bite is making sure the lizard can be safely detached. They have a strong and tenacious grip, and while they generally have released by the time the victim arrives to a health care facility, this is not always the case. Several methods have been suggested for removal. Methods must prevent any further damage to the victim, and also avoid creating a second bite victim. Immersing the extremity and the lizard in cold water is one suggested method of getting the lizard to release its grip. If effective, this method is less likely to harm the lizard, which is protected. Prying the lizard's jaws apart with a strong object, such as a butter knife or cast remover, may be successful, but may also cause the lizard to bite down harder. Simply ripping the lizard off with brute force may cause substantial damage to the victim's tissue and is not recommended (Russell and Bogert, 1981; Norris and Bush, 2007).

Once the lizard has been removed and secured, attention should be turned toward the victim's airway, breathing, and circulation. Airway compromise is rare, but has been reported as part of an anaphylactoid reaction to the venom, or with bites to the face or neck (Piacentine et al., 1986). Bites may cause significant local tissue damage and bleeding. Bleeding should be controlled with direct pressure. Hypotension due to venom-induced release of bradykinins is usually responsive to a bolus of IV fluids. In rare cases, vasopressors are needed for circulatory support.

The teeth are only loosely attached to the animal's jaw, so it is common for them to break off and lodge in the wound. X-ray may identify some of them, but all wounds should be explored directly. After appropriate analgesia and irrigation, a 30-gauge needle can be used to probe the wound and search for teeth. Local infiltration with lidocaine is not recommended, as it may worsen tissue swelling and ischemia. If left in place, the teeth represent an increased infection risk, but will generally work their way out during the healing process. While damage to neurovascular structures is possible given the strength and force of the bite, tissue necrosis and infection are rare. Empiric antibiotic prophylaxis is probably not necessary, but it is recommended that the tetanus immunization be updated (Norris and Bush, 2007). Ice packs should be used only with great care, as they may worsen local vasospasm and tissue ischemia, and ice should not be placed directly on the wound.

Pain may be severe and require opioid analgesics. The pain generally peaks in the first hour and fades within 6 to 10 hours, but may persist in a mild form for weeks (Norris and Bush, 2007). Coagulopathy is uncommon following Gila monster bites, and this phenomenon has rarely been reported (Bou-Abboud and Kardassakis, 1988). It has been hypothesized as due to endothelial cell damage rather than direct venom effect (Norris and Bush, 2007). Appropriate screening laboratory tests include complete blood count, coagulation parameters, electrolyte panel with creatinine, urinalysis, and an EKG.

Patients who do not show any signs of envenomation at initial evaluation should be monitored for about 6 hours (Hooker and Caravati, 1994). At that time, if no symptoms other than local wound symptoms developed, the patient can be discharged with local wound care. The wound should be carefully evaluated for any neurovascular damage and for retained teeth prior to discharge. The patient should return for a recheck in 24 to 48 hours.

If any signs of envenomation develop, the patient should be admitted for at least 24 hours of observation. Screening labs and EKG, as noted above, should be obtained at this time. Treatment is supportive as there is currently no commercially available antivenom. Long-term morbidity and mortality are extremely rare.

V. RESEARCH AND DEVELOPMENT

While much research has been done over the years on venomous reptile bites in the United States, there is still much that has yet to be discovered. Great strides have been made in treating these bites, but new challenges are emerging. For example, while Crotaline Fab antivenom is a relatively safe

and effective medication, recurrence phenomena are one problem that needs to be further studied in order to be resolved. Suggestions have been made about modifying the molecule in ways that would increase its half-life (such as including Fab-2 or even whole IgG particles) and allow it to neutralize venom released in a delayed fashion from a depot site. This could lead to unexpected side effects, and such a product has yet to be fully developed or studied.

Additionally, Crotaline Fab antivenom contains the mercury-based preservative thimerosal, which in high doses can cause nerve and kidney toxicities in developing fetuses and small children. The manufacturer states that thimerosal will be eliminated in the near future.

While antivenom may be effective even when started days after an envenomation, patients respond best results when antivenom can be given quickly. One way to address this delay is to stock EMS vehicles with starting doses of Crotaline Fab antivenom that can be infused during transport. Problems with this approach include cost of medication, shelf life, and risk of precipitating a severe allergic reaction in a less controlled environment. Difficult decisions would have to be made if there was suspicion that the patient may have received a dry bite or has been bitten by a nonvenomous snake. As telemedicine technology improves, it may be possible for physicians to evaluate patients virtually during transport and help decide whether antivenom is indicated.

Coral snake bites pose a greater challenge to researchers because of their rarity. An open niche was left when Wyeth stopped production of antivenin *Micrurus fulvius.* Other countries are producing antivenoms made from their native coral snakes that may offer some cross-protectivity against coral snakes in the United States. While supportive care may be enough to save patients with severe coral snake bites, availability of a new commercial antivenom would be a valuable tool to treat these patients.

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24 Snakebite Envenomation in Central America

José María Gutiérrez

CONTENTS

The epidemiology, clinical features, and treatment of snakebite envenomation in Central America are reviewed. This pathology constitutes a relevant public health problem in the region, where most of the accidents are inflicted by species of the family Viperidae, primarily affecting young agricultural workers. Only 1 to 2% of snakebite cases are inflicted by coral snakes (genus *Micrurus*); these venoms do not induce local tissue damage, but provoke serious paralytic effects ending in respiratory paralysis in severe cases. In contrast, envenomations caused by viperid snakes, among which *Bothrops asper* (known as *terciopelo*, *barba amarilla*, or *equis*) is the most important, are characterized by a prominent local pathology including edema, blistering, hemorrhage, and necrosis, often associated with infection. In moderate to severe viperid snakebite cases, various systemic alterations appear, such as bleeding, coagulopathy, cardiovascular shock, and acute renal failure. After an initial diagnosis, based on the assessment of objective signs and symptoms of envenomation, the mainstay in the clinical management of these patients is the intravenous administration of either polyvalent antivenom (for pit viper bites) or anticoral antivenom (for coral snake bites), diluted in saline solution. Close monitoring of the patient is critical to detect the appearance of early

adverse reactions to antivenom therapy and to monitor the evolution of the case. In addition, tetanus prophylaxis has to be considered, together with the administration of antibiotics in moderate to severe pit viper bites. Finally, the management of complications derived from envenomations has to be considered, depending on the clinical manifestations of each case.

I. INTRODUCTION: THE VENOMOUS SNAKES OF CENTRAL AMERICA

Snakebite envenomations represent a relevant public health concern in Central America, inflicting morbidity and mortality, primarily on agricultural workers. Many species of snakes are abundant in this region, but most do not represent a medical problem because they are nonvenomous (families Typhlopidae, Leptotyphlopidae, Anomalepidae, Boidae, Loxocemidae, and Ungaliophidae; Solórzano, 2004). The most diverse group is the family Colubridae (*sensu lato*), containing many species possessing a venom gland and a venom injection system with rear maxillary fangs. However, Central American colubrid snakes do not inflict serious envenomations in humans; the reported cases mostly correspond to people that intentionally handle these snakes, and bites result in mild to moderate local signs and symptoms (Gutiérrez and Sasa, 2002).

The most serious cases of snakebite envenomations are inflicted by species of the families Elapidae and Viperidae. The former includes the coral snakes (genus *Micrurus*) and the pelagic yellow-bellied sea snake (*Pelamis platurus*, subfamily Hydrophiinae), truly venomous species possessing a short front maxillary fang (proteroglyph dentition). *Pelamis platurus* inhabits the Pacific Ocean, and there are very few reports of bites by this species in humans, probably due to anatomical constraints and behavioral features. In contrast, there are sixteen species of *Micrurus* in the region, common in all of the countries (Campbell and Lamar, 2004); *Micrurus nigrocinctus* is the most abundant species, and it is responsible for the majority of coral snake bites. However, the incidence of coral snake bites is low, representing 1 to 2% of the total number of cases in the region (Bolaños, 1984).

The vast majority of snakebites in Central America are caused by species of the family Viperidae. There are twenty-three species of pit vipers in the region, classified within the genera *Agkistrodon*, *Atropoides*, *Bothrops*, *Bothriechis*, *Cerrophidion*, *Crotalus*, *Lachesis*, and *Porthidium* (Campbell and Lamar, 2004). They are distributed in highly variable biotopes, being particularly abundant in tropical rain forests and tropical altered areas devoted to agriculture or cattle raising. By far, the species causing the highest number of accidents is *Bothrops asper*, locally known as *terciopelo*,

FIGURE 24.1 ***(A color version of this figure follows page 240.)*** *Bothrops asper*, locally known as *terciopelo*, *barba amarilla*, or *equis*, is the most dangerous venomous snake in Central America, being responsible for a large number of cases every year. Adult specimen collected in Costa Rica. (Photo courtesy of Dr. Mahmood Sasa and published in Gutiérrez et al., *PLoS Medicine* 3 (2006): e150.)

TABLE 24.1
Distribution of Venomous Snakes in the Different Countries of Central America

Species	Guatemala	Belize	Honduras	El Salvador	Nicaragua	Costa Rica	Panama
Micrurus alleni			X		X	X	X
M. ancoralis							X
M. browni	X						
M. clarki						X	X
M. diastema	X	X	X				
M. dissoleucus							X
M. dumerilii							X
M. elegans	X						
M. hippocrepis	X	X					
M. latifasciatus	X						
M. mipartitus							X
M. multifasciatus					X	X	X
M. nigrocinctus	X		X	X	X	X	X
M. ruatanus			X				
M. stewarti							X
M. stuarti	X						
Pelamis platurus	X		X	X	X	X	X
Agkistrodon bilineatus	X	X	X	X	X	X	
Atropoides mexicanus	X		X		X	X	X
A. occidus	X		X	X			
A. olmec	X						
A. picadoi						X	X
Bothriechis aurifer	X						
B. bicolor	X						
B. lateralis						X	X
B. marchi			X		X		
B. nigroviridis						X	X
B. schlegelii	X	X	X		X	X	X
B. supraciliaris						X	
Bothrops asper	X	X	X		X	X	X
Cerrophidion godmani	X		X	X	X	X	X
Crotalus durissus (*simus*)	X	X	X	X	X	X	
Lachesis acrochorda							X
L. melanocephala						X	
L. stenophrys					X	X	X
Porthidium lansbergi							X
P. nasutum	X	X	X		X	X	X
P. ophryomegas	X		X	X	X	X	
P. porrasi						X	
P. volcanicum						X	

Source: From Campbell and Lamar, 2004.

barba amarilla, or *equis* (Figure 24.1). This species is highly abundant in lowland areas of all countries except El Salvador (Campbell and Lamar, 2004). *Bothrops asper* is also highly tolerant of altered areas, where forests have been converted to agricultural or pasture areas, thus having close contact with agricultural workers. Table 24.1 summarizes the distribution of elapid and viperid snake species in Central America.

II. EPIDEMIOLOGY

In spite of efforts performed to obtain epidemiological data on snakebite envenomations in Central America, the information available is only partial, and there is probably a high underreporting, as occurs in other regions of the world (Gutiérrez et al., 2006a; see also Chapter 22, this volume). In Costa Rica, hospital statistics indicate that there were around 500 to 600 cases per year in the decade 1990–2000 (Sasa and Vásquez, 2003), which roughly corresponds to an incidence of 15 cases per 100,000 population per year. Epidemiologic data provided by health authorities in Panama indicate that over two thousand reported cases occur per year in this country, thus suggesting that Panama probably has the highest incidence of snakebites in Latin America. Nicaragua, Honduras, and Guatemala present a similar number of cases as Costa Rica, i.e., around five hundred per year, whereas in El Salvador the incidence is lower due to several factors, and the fact that *Bothrops asper* is not found in this country plays a determinant role. It is estimated that a total number of four to five thousand cases occur in Central America per year. However, there is an urgent need to perform a systematic effort to gather more reliable epidemiological information on snakebites in the region, by carrying out community-based studies. Mortality varies among countries, depending on the accessibility that snake-bitten people have to health centers and antivenom, as well as on the clinical management of these envenomations. In Costa Rica, owing to an interinstitutional effort carried out during several decades to confront this problem, the mortality has been reduced to rates of ~0.1 per 100,000 population per year (Rojas et al., 1997; Fernández and Gutiérrez, 2008). The impact of snakebite envenomation goes beyond mortality, since many affected people develop permanent sequelae due to tissue loss and disability secondary to viperid snakebites. Therefore, when analyzed in terms of disability-adjusted life years (DALYs) lost, the actual impact of this health problem becomes larger.

The majority of snakebite envenomations affect young agricultural workers, predominantly males, who are bitten while performing their duties (Gutiérrez, 1995). A significant percentage of cases occur in children and adolescents (Figure 24.2). In Costa Rica, Sasa and Vázquez (2003)

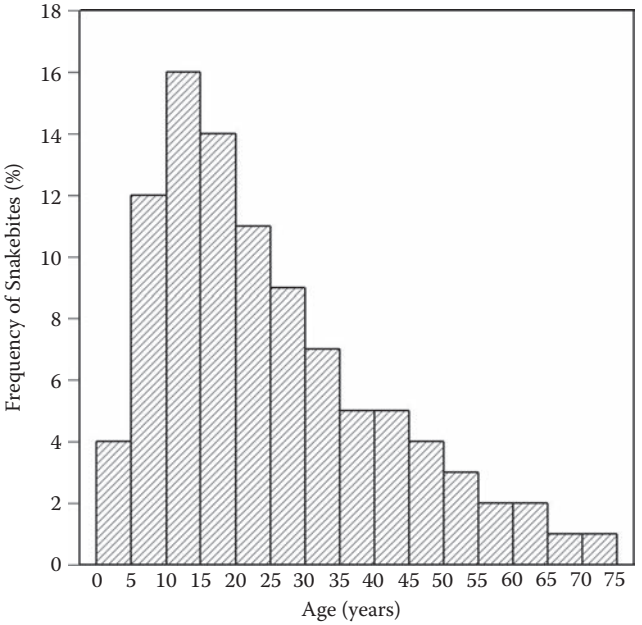

FIGURE 24.2 Frequency (%) of snakebite cases in Costa Rica according to the age of the victim. This figure was prepared from data of hospital statistics gathered by the staff of Instituto Clodomiro Picado.

reported two peaks of incidence, one in June–July, associated with the start of the rainy season and its associated agricultural activities, and the other in November–December, although there are cases throughout the year. Regarding the anatomical region of the bite, most cases occur in feet (50%) and hands (30%). Similar epidemiological trends have been reported in various studies performed in Costa Rica (Bolaños, 1982; DeFranco et al., 1983; Saborío et al., 1998; Arroyo et al., 1999; Sasa and Vázquez, 2003), as well as in unpublished results collected in other countries of Central America.

III. CLINICAL ASPECTS OF SNAKEBITE ENVENOMATION

A. General Considerations

Not all snakebites result in envenomation, since some of them are inflicted by nonvenomous snakes, and even in the case of bites by venomous species, a number of cases do not result in venom injection, thus constituting dry bites. Therefore, the correct diagnosis of envenomation must be based on the analysis of the objective signs and symptoms presented by the patient, together with the laboratory analyses performed. Such initial clinical examination, instead of the information provided by the victim or his or her companions, must guide the diagnosis and subsequent therapeutic interventions.

In the event of envenomation, the severity of each case is highly variable and depends on the following factors, particularly the amount of venom injected. *Bothrops asper* is able to inject a relatively large volume of venom, whereas other viperid species, such as those of the genus *Bothriechis*, inject much less venom. The route and anatomical site of injection are also important. In most cases, bites occur in feet or hands and venom is injected subcutaneously or intramuscularly, with the consequent slow systemic absorption of the venom. In contrast, bites to the head or trunk, or those in which venom is injected intravenously, result in more serious envenomations with rapid onset. Similarly, the physiological and anatomical characteristics of the victim affect severity. Bites in children tend to be more complicated than those in adults, owing to the lower body mass, i.e., a lower volume of distribution, and the velocity of systemic absorption in the former. The time lapse between the bite and the beginning of medical attention is also a factor, because rapid administration of antivenom guarantees that envenomation does not develop to severity, whereas prolonged delays in receiving medical attention, including antivenom administration, are associated with more complicated outcomes. Although there is a lack of clinical studies in which the identity of the offending snake species has been conclusively determined, evidence suggests that bites by bushmasters (*Lachesis stenophrys* and *L. melanocephala*) often cause severe envenomations (Bolaños et al., 1982), whereas bites by the Central American rattlesnake (*Crotalus durissus durissus*), which has recently been classified as *C. simus* (Campbell and Lamar, 2004), usually provoke mild or moderate envenomations (Bolaños et al., 1981). Moreover, bites by large (>1 m) *terciopelo* (*B. asper*) have a great risk of becoming severe cases (Otero et al., 1999).

B. Envenomations by Species of the Family Elapidae

There have been very few reports on bites by the yellow-bellied sea snake, *Pelamis platurus*, in Central America (Solórzano, 1995); therefore, there is no information on the clinical manifestations of these envenomations. However, based on reports of bites by other species of sea snakes in other regions, together with experimental studies on the effects of *P. platurus* venom, it is expected that an envenomation by this species would result in neurotoxic manifestations, due to the action of α-neurotoxins that act at the neuromuscular junction (Tu et al., 1976), provoking a flaccid paralysis of several muscles, including respiratory muscles. In addition, other sea snake venoms have been shown to induce systemic myotoxicity, i.e., rhabdomyolysis, associated with myoglobinuria and renal disturbances (White, 1995). Whether this effect also occurs in bites by *P. platurus* is unknown.

TABLE 24.2
Signs and Symptoms in Patients Envenomated by Coral Snakes (Elapidae: *Micrurus* sp.) or Pit Vipers (Viperidae) in Central America

Coral Snakes	Pit Vipers
Local pain	Pain
Paresthesias	Edema
Palpebral ptosis	Nausea
Dysarthria	Vomiting
Salivation	Necrosis
Ophthalmoplegia	Local bleeding
Diplopia	Ecchymosis
Fasciculations	Blistering
Dyspnea	Fever
Respiratory paralysis	Systemic bleeding
	Hypotension
	Oliguria or anuria

Bites by coral snakes (*Micrurus* sp.) constitute 1 to 2% of all snakebite cases in Central America (Bolaños, 1984). The species that induces the majority of coral snake bites is *M. nigrocinctus* (Gutiérrez, 1995). These bites usually occur in fingers, and it is common that the snake holds to the bite site, a feature that enables people to recognize the offending snake. Venom is injected subcutaneously and distributes systemically by absorption through the lymphatic vessels. Local manifestations are scant, characterized by pain and paresthesias, and there is no overt swelling, a feature that facilitates the differential diagnosis of these bites from those of viperid snakes. Thus, the extent of local alterations should not be used as a criterion of severity in the case of coral snake bites. Once systemically absorbed, the low molecular mass (6–9 kDa) α-neurotoxins present in these venoms (Alape-Girón et al., 1996a; Rosso et al., 1996) bind with high affinity to the cholinergic receptors present at the motor end plate of muscle fibers. As a consequence, flaccid paralysis ensues in various muscles. The onset of paralytic signs may appear as soon as 1 h after the bite, but in most cases it is delayed several hours, thus justifying that the patient remains under close observation at the health center for at least 12 h. One of the first clinical manifestations of paralysis is palpebral ptosis, followed by paralysis of ocular, facial, and deglutition muscles, and ending in the paralysis of respiratory muscles. There have been reports of paralysis of muscles of the upper and lower extremities as well. The main signs and symptoms of coral snake bite envenomation are depicted in Table 24.2.

From a therapeutic standpoint, it is important to consider that once the first neurotoxic manifestations appear, there is a cascade of neurotoxic events that may end in a severe envenomation in a relatively short time, thus requiring rapid and effective intervention. Experimentally, envenomations by coral snakes in mice have been shown to induce skeletal muscle damage with increases in plasma creatine kinase (CK) and myoglobin levels (Gutiérrez et al., 1986). However, with few exceptions (i.e., Kitchens and van Mierop, 1987), for envenomations by the North American coral snake (*M. fulvius*), this effect has not been demonstrated to occur in the clinical setting, although myalgia has been described in some cases by *M. nigrocinctus*, which suggests myotoxicity (unpublished observations).

C. Envenomations by Species of the Family Viperidae

Viperid snakebite envenomations are characterized by a complex series of local effects of rapid onset that develop in the vicinity of the site of venom injection (Figure 24.3). When treatment is

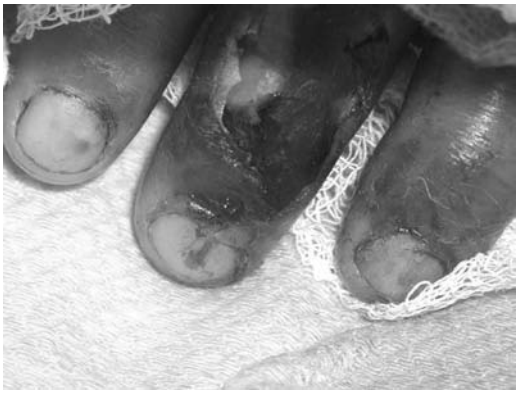

FIGURE 24.3 ***(A color version of this figure follows page 240.)*** Local necrosis in a finger of a child after a bite by *Bothrops asper* in Costa Rica. (Photo courtesy of Dr. María Luisa Avila-Agüero, Hospital Nacional de Niños, Costa Rica.)

not initiated rapidly, these local pathological effects may result in permanent sequelae secondary to tissue damage, with long-lasting consequences. In moderate and severe cases, the distribution of the venom produces systemic manifestations, such as hemorrhage, coagulopathy, and in some cases, acute renal failure and cardiovascular shock. The main clinical manifestations of viperid snakebites in Central America are depicted in Table 24.2, and have been described in a number of publications (Bolaños et al., 1981, 1982; Bolaños, 1984; De Franco et al., 1983; Gutiérrez, 1995; Saborío et al., 1998; Arroyo et al., 1999; Avila-Agüero et al., 2001a, 2001b). A possible exception to this common pathophysiological picture in Central America is the venom of newborn and juvenile specimens of the rattlesnake *Crotalus durissus durissus* (*C. simus*). The venom from adult specimens of this species induces the typical features described for viperid venoms (Bolaños et al., 1981). However, venoms from young specimens have a toxicological profile similar to that described for the venom of the South American subspecies *C. d. terrificus*. These venoms induce very little local pathology, but systemic manifestations with associated neurotoxicity, rhabdomyolysis, coagulopathy, and acute renal failure are observed (Gutiérrez et al., 1991; Saravia et al., 2002). Whether these effects occur clinically in bites by young Central American rattlesnakes is unknown, but clinicians in the region must be aware of this possibility. The differential diagnosis of these cases from a coral snake bite can be based on clotting tests, since rattlesnake venom provokes defibrin(ogen)ation, whereas coral snake venoms do not.

1. Local Effects

a. Edema and Pain

These effects are described in practically all cases of viperid snakebites when venom is injected (Arroyo et al., 1999); in envenomations in which large volumes of venom are injected, these effects are highly notorious. Edema is of multifactorial origin, since it is due to (1) the direct effect of venom components in the microvasculature, with the consequent extravasation, and (2) the generation and release of many inflammatory mediators, such as histamine, eicosanoids, nitric oxide, kinins, cytokines, and matrix metalloproteinases, which directly or indirectly induce increase in vascular permeability (Gutiérrez and Lomonte, 2003; Teixeira et al., 2003, 2005). Venom-induced edema has two main pathophysiological consequences: (1) net movement of fluid from the vascular compartment to the interstitial compartment, thus resulting in hypovolemia, which is a typical manifestation of these envenomations and contributes to cardiovascular shock; and (2) induction of an increase in the interstitial pressure in muscle compartments, thus provoking the development of compartmental syndrome, which is one of the most serious consequences of severe envenomations. On the other hand, pain is due to the action of various endogenous mediators released in the tissues

and acting on afferent nerve fibers, inducing hyperalgesia and allodynia (Chacur et al., 2001, 2004). The fact that edema and pain result from the action of endogenous mediators released in the tissues complicates their treatment, especially when antivenom administration occurs after the onset of these inflammatory cascades.

b. Hemorrhage and Blisters

Bleeding and the appearance of blisters at the site of venom injection are two common features of viperid snakebite envenomations. Both effects are the consequence of the action of venom zinc-dependent metalloproteinases (see Chapters 4 and 5, this volume). In the case of hemorrhage, these enzymes induce a selective hydrolysis of critical components at the capillary vessel basement membrane, thus weakening the mechanical stability of the capillary wall. As a consequence, the hemodynamic biophysical forces that normally operate in the circulation, i.e., hydrostatic pressure and shear stress, provoke the distention and eventual disruption of capillary vessel integrity, resulting in extravasation (Moreira et al., 1992; Gutiérrez and Rucavado, 2000; Gutiérrez et al., 2005). Metalloproteinases are also responsible for blistering, due to the hydrolysis of components of the dermal-epidermal junction, thus resulting in the separation of the epidermis (Rucavado et al., 1998).

c. Myonecrosis

Necrosis of skeletal muscle is another one of the serious local alterations induced by viperid venoms. This effect is mostly due to the action of myotoxic phospholipases A_2, which are abundant in these venoms and which cause direct damage to the integrity of the skeletal muscle plasma membrane (Lomonte et al., 1994, 2003; Gutiérrez and Lomonte, 1995; Gutiérrez and Ownby, 2003). In addition, muscle tissue is affected by the ischemia resultant from the action of hemorrhagic components in the microvasculature, as well as by the impairment in blood flow secondary to thrombosis, angionecrosis, and increases in intracompartmental pressure. A recent review (Gutiérrez and Lomonte, 2003) discussed the local pathological effects induced by viperid snake venoms in detail.

d. Local Infection

After passage through the venom duct and fangs, snake venoms become highly contaminated fluids, containing abundant bacteria. Thus, snakebite envenomations are often associated with local infection (Criales and Arguedas, 1994; Avila-Agüero et al., 2001a). The tissue-damaging action of myotoxins and hemorrhagic toxins greatly facilitates infection by *Staphylococcus aureus* (Saravia-Otten, 2004), and probably by other bacteria as well. In turn, such infection may complicate the local pathological effects initiated by the action of venom components.

2. Systemic Effects

a. Hemorrhage, Coagulopathy, and Hemodynamic Alterations

Snake venom metalloproteinases, especially those of the P-III class, induce systemic microvascular damage leading to hemorrhage (Gutiérrez et al., 2005), with diverse manifestations such as gingival bleeding, hemoptysis, hematemesis, hematuria, and bleeding in various organs, including the central nervous system (Otero et al., 2002; Warrell, 2004). The direct effect of metalloproteinases in microvessels is further complicated by the alterations that these venoms induce in hemostasis. Most viperid venoms are rich sources of thrombin-like serine proteinases and activators of prothrombin and factor X, which are metalloproteinases in viperid venoms (Markland, 1998). The venom of *B. asper*, for example, contains a thrombin-like serine proteinase and a metalloproteinase having prothrombin-activating effects (Aragón-Ortiz and Gubensek, 1978; Loría et al., 2003; Rucavado et al., 2004, 2005). The combined action of these enzymes *in vivo* results in the formation of microthrombi, with depletion in plasma fibrinogen levels, i.e., defibrin(ogen)ation, and increments in fibrin degradation products and D-dimer (Barrantes et al., 1985; Rucavado et al., 2005). In addition, viperid venoms affect platelet counts and their function, inducing thrombocytopenia and platelet hypoaggregation, thus compromising this branch of the hemostatic system as well (Rucavado et al., 2005).

There are interspecies variations in Central American viperid venoms concerning the alterations in hemostasis. All of these venoms are hemorrhagic (Gutiérrez et al., 1996), but not all of them induce defibrin(ogen)ation. For instance, the venoms of *Bothriechis lateralis* and *Porthidium nasutum* are not procoagulant and do not induce defibrin(ogen)ation *in vivo* (Gené et al., 1989). Clinicians must be aware of these findings, since no alterations in the clotting tests would be expected in patients bitten by these two species. The combined effect of hemorrhagic metalloproteinases and enzymes and toxins that affect hemostasis results in profuse bleeding (Rucavado et al., 2005), which may bring serious hemodynamic alterations, i.e., cardiovascular shock, the main cause of death by these envenomations in Central America.

b. Renal Alterations

A fraction of viperid envenomations, especially those having a moderate or high severity, present renal alterations, manifested by oliguria or anuria, elevations of urea and creatinine concentrations in serum, and presence of erythrocytes or hemoglobin in urine. When not treated properly, these renal alterations may end up in acute renal failure. The pathogenesis of renal effects in these envenomations has not been clearly established, but it is likely to be multifactorial, involving renal ischemia secondary to hypovolemia, degradation of glomerular basement membrane by venom metalloproteinases, direct cytotoxic effect of some venom components in tubular and glomerular cells, and in some cases, accumulation of hemoglobin in the renal tubules, with the consequent nephrotoxicity. Renal complications of snakebite may end up in chronic renal failure.

D. The Clinical Laboratory in Monitoring Snakebite Envenomation

Envenomations by coral snakes and sea snakes do not require a close laboratory monitoring, since the main clinical outcome is neurotoxic paralysis. In contrast, envenomations by viperid snakes are associated with pathophysiological alterations that can be monitored by diverse laboratory tests. The following tests are recommended on a routine basis:

1. Coagulation tests: Prothrombin time and partial thromboplastin time tests are often employed, together with the quantification of fibrinogen levels. In some health facilities, where these tests are not available, the whole blood clotting test can be performed and is useful to detect coagulopathy (Warrell, 1999; Otero et al., 1999).
2. Hemogram, including platelet count.
3. Determination of urea and creatinine concentration in serum.
4. Determination of the serum activity of enzymes, such as creatine kinase (CK) and lactate dehydrogenase (LDH), that are elevated as a consequence of tissue damage.

E. Complications in Viperid Snakebite Envenomations

Complications have been described in viperid snakebite envenomations, especially in severe cases in which antivenom treatment is delayed. The most frequent complications are systemic bleeding, especially in the brain, with the consequent neurological effects; local tissue necrosis; cardiovascular shock; acute and chronic renal failure; infection and sepsis; and abortion (Avila-Agüero et al., 2001a; Otero et al., 2002).

IV. PREVENTION OF SNAKEBITES

Following the epidemiological characteristics of snakebite envenomations in Central America, and taking into consideration some behavioral features of the snakes in this region, the following measures are recommended in order to prevent snakebites:

1. Always wear boots or shoes when walking in the field and performing agricultural work.
2. Avoid touching the ground, fallen trees, or rocks directly with the hands. Instead, first use a stick to check whether snakes are present.
3. In the event of an encounter with a snake, avoid getting too close to it, and walk away instead. Do not try to grab or handle a snake, even if it may look as if nonvenomous.
4. Be aware that some snake species, particularly *B. asper*, are often found near or inside rural houses. Use care when walking around the houses.
5. Pay attention when collecting crops from trees or bushes, such as coffee in Central America, since some venomous snakes are arboreal and are often found in these plants.
6. Organize and develop prevention campaigns in local communities, schools, and groups of agricultural workers. These activities should be tailored to the cultural features of the community, including the use of local indigenous languages when required. The local communal organizations should be actively integrated into these campaigns.

V. TREATMENT OF SNAKEBITE ENVENOMATION

A. First Aid Interventions and Antivenom Use in the Field

The basic first aid measures that should be implemented in the event of a snakebite are:

1. Reassure the bitten person, who is usually very anxious.
2. Immobilize the bitten extremity as much as possible, in order to delay the systemic absorption of the venom, which is favored by muscle contractions; a splint may be used for immobilization.
3. Transport the person to the nearest health facility as rapidly as possible.

A number of interventions that were promoted in the past as first aid measures are now strongly contraindicated, due to their inefficacy and especially to the harm that they induce. These include the use of a tourniquet, the use of suction devices, the application of ice bags (cryotherapy), the administration of synthetic or natural substances claimed to be effective against snakebite envenomations (there is no rigorous proof of efficacy of these products in the treatment of these envenomations), and the application of electric discharges. None of these measures ameliorate the manifestations of envenomation, and most of them are harmful. In addition, they delay the transportation of the patient to the health center. Above all, first aid interventions should not harm the patient. Unfortunately, some of these harmful measures are still employed in Central America; therefore, educational campaigns should include a clear message regarding the need to avoid these interventions. Communities, especially in rural areas, should be organized so as to ensure a rapid transportation of people bitten by snakes to the nearest health center.

The use of antivenom in the field, i.e., outside a health center, is not recommended, with very few exceptions. In such conditions, antivenom has to be administered intramuscularly. The bioavailability of antivenom by this route is low, around 40%, and absorption is delayed (Pepin et al., 1995; Ismail and Abd-Elsalam, 1996; Gutiérrez et al., 2003). In addition, these injections are painful and there is a risk of hematomas, since patients are often defibrin(ogen)ated; moreover, there is the risk of early adverse reactions (EARs) to antivenom, which are difficult to treat in field conditions. Taken together, these facts indicate that intramuscular injection of antivenom is inconvenient in terms of safety and efficacy, and should be left only for circumstances in which the transport of the patient to the nearest health center is excessively prolonged.

B. Management of Snakebite Envenomations in Health Centers

1. Diagnosis and First Interventions

The correct diagnosis of a snakebite case should be based on the assessment of clinical and laboratory findings, and not on the anecdotic information provided by the patient or his or her relatives or

companions. Envenomations by viperid species in Central America are characterized by the onset of local pain and swelling rapidly after the bite; the development of other local manifestations, such as bleeding, blistering, and necrosis, depends on the severity of the case. This allows a rapid diagnosis of such envenomations. There is no need to identify the viperid species responsible for the accident, since polyvalent antivenom is effective in envenomations by all viperid species in the region (Gutiérrez et al., 1996). The occurrence of laboratory-tested clotting disturbances should be assessed upon admission, and an assessment of the general condition of the patient has to be performed, in order to judge the severity of the case. However, it should be kept in mind that the severity of each case may vary rapidly, since these envenomations are highly dynamic clinical conditions characterized by a rapid evolution.

When patients do not present local manifestations of envenomation, there are three possibilities to consider: (1) the person was bitten by a nonvenomous snake, (2) the person was bitten by a viperid snake that did not inject venom, or (3) the person was bitten by a coral snake. In the first two cases, there is no need of antivenom administration. However, if there is evidence that the offending species was a coral snake, because the patient brought the snake and it could be identified, the administration of anticoral antivenom is indicated, even before the onset of neurotoxic signs and symptoms, due to the likelihood of envenomation and to the fact that once the neurotoxic signs appear, the case is likely to become severe rapidly. In any case, a patient suspected of having suffered a snakebite should be kept under observation for at least 12 h in the health center.

2. Antivenom Administration

Two antivenoms, manufactured by Instituto Clodomiro Picado (University of Costa Rica, Costa Rica), are widely distributed in Central America: polyvalent antivenom, effective against the venoms of all Central American viperid species, and anticoral antivenom, effective against the venoms of the most important elapid species. These are whole immunoglobulin (Ig) G equine antivenoms that have been shown to neutralize the venoms of Central American venomous snakes (Gutiérrez et al., 1996). Other antivenoms are also available in the region, although their neutralizing potency varies; therefore, antivenom dosage should be established for each particular antivenom, on the basis of preclinical studies and clinical trials (e.g., Saravia et al., 2001).

In health centers, antivenom administration has to be performed only by the intravenous route. Skin or conjunctival hypersensitivity tests are no longer recommended before the administration of antivenom, since they have a very poor prognostic value and represent a delay in the start of antivenom therapy (Warrell, 1999). In some health centers, antihistamines are administered before antivenom in order to prevent EARs; however, there is no evidence from clinical trials supporting this measure (Fan et al., 1999). In the case of antivenoms of Instituto Clodomiro Picado, the initial dose to be administered corresponds to ten vials, i.e., 100 ml antivenom, being the same for both children and adults; this initial dosage has been established through the clinical experience of many years (Gutiérrez et al., 2006b). This dose should be diluted in a volume of 400 to 500 ml of isotonic saline or 5% dextrose solutions, in the case of adults, and in 200 to 250 ml of these solutions in the case of children. Antivenom administration should be started as a slow infusion in order to detect possible EARs. If no reactions develop within the first 20 min of treatment, the velocity of infusion should be increased in order to administer the complete antivenom dose in 1 to 2 h. The frequency of EARs with these antivenoms ranges between 10 and 20% of the cases; they are usually mild, characterized predominantly by urticaria and itching (Arroyo et al., 1999; Otero et al., 1999), although other manifestations, such as nausea, fever, abdominal cholic, angioedema, bronchospasm, and hypotension, may appear in a few cases (Otero et al., 1999). In the event of an EAR, antivenom infusion should be suspended, and the patient must be treated with a combination of antihistamines (chlorpheniramine maleate: adults, 10 mg/kg; children, 0.2 mg/kg, by intravenous injection) and corticosteroids (hydrocortisone: adults, 100 mg/kg; children, 2 mg/kg, intravenous). Adrenaline (1:1,000), administered by the subcutaneous route, should also be considered (Warrell, 1999). Once the EAR is controlled, antivenom infusion has to be restarted, and the

complete antivenom dose should be administered in 1 to 2 h. Close observation of the patient is mandatory, in order to detect possible additional reactions to antivenom, as well as the appearance of complications of envenomation.

3. Additional Doses of Antivenom

In the vast majority of the cases, the administration of ten vials of antivenom results in the halting of the manifestations of envenomation. In the case of viperid snakebites, local and systemic bleeding should be controlled within the first 3 to 6 h of treatment. Regarding clotting disturbances, laboratory tests should be partially or totally corrected by 12 h, and completely corrected by 24 h (Otero et al., 1999). In addition, the general condition of the patient has to improve after antivenom administration. If by 12 h there is persistent bleeding, or there is no correction of clotting tests, or the general condition of the patient keeps worsening, then an additional dose of antivenom (five to ten vials, depending on each case) is required. Such additional dose of antivenom is also indicated when there is evidence of recurrence of envenomation, i.e., when clinical manifestations of envenomation appear after they have been controlled. Owing to the prolonged half-life of IgG antivenoms, recurrence of envenomations is infrequent in Central America. Figure 24.4 presents the algorithm used in Costa Rica for the diagnosis and treatment of snakebite envenomations.

4. Ancillary Therapeutic Interventions

a. Tetanus Prophylaxis and Treatment of Bacterial Infections

Patients must also receive tetanus prophylaxis, i.e., tetanus toxoid. In addition, in the case of moderate or severe viperid cases with evident local compromise, antibiotic therapy (usually a combination of penicillin or clindamycin, and a wide-spectrum antibiotic such as gentamicin) is recommended (Avila-Agüero et al., 2001b). Bacteriological cultures of local abscesses are required to identify the bacteria and select the antibiotics to be administered.

b. Management of Local Complications

The bitten limb should be carefully cleaned. Local pain may be intense, and therefore analgesics should be administered. Drainage of abscesses must be implemented, together with debridement of subcutaneous necrotic tissue. In severe cases, the increases in pressure in muscle compartments, such as in the anterior tibialis compartment, may evolve into a compartmental syndrome resulting in tissue ischemia. Monitoring of intracompartmental pressure is recommended, together with a close observation of the evolution of local alterations, in order to detect the onset of a compartmental syndrome. Nevertheless, the classical signs of compartmental syndrome are difficult to assess in a viperid snakebite victim. When such a syndrome develops, with increases in intracompartmental pressure higher than 40 mmHg, fasciotomy is indicated, although it has to be performed only after clotting disturbances have been corrected by antivenom, in order to avoid extensive bleeding.

c. Management of Cardiovascular Alterations

Fluid therapy, associated with administration of plasma expanders (colloids or crystalloids), is recommended to correct the hypovolemia characteristic of viperid snakebite envenomations. These interventions require a close monitoring of the central venous pressure, in order to avoid fluid overload and pulmonary edema. Fluid therapy must follow antivenom administration, since the priority intervention should be aimed at neutralizing circulating venom by antivenom. When hematocrit and hemoglobin values drop excessively, transfusions have to be considered.

d. Management of Renal Alterations

Renal alterations often occur in viperid envenomations, evidenced by oliguria or anuria and by increases in serum levels of urea and creatinine, as well as by clinical evidence of uremia syndrome.

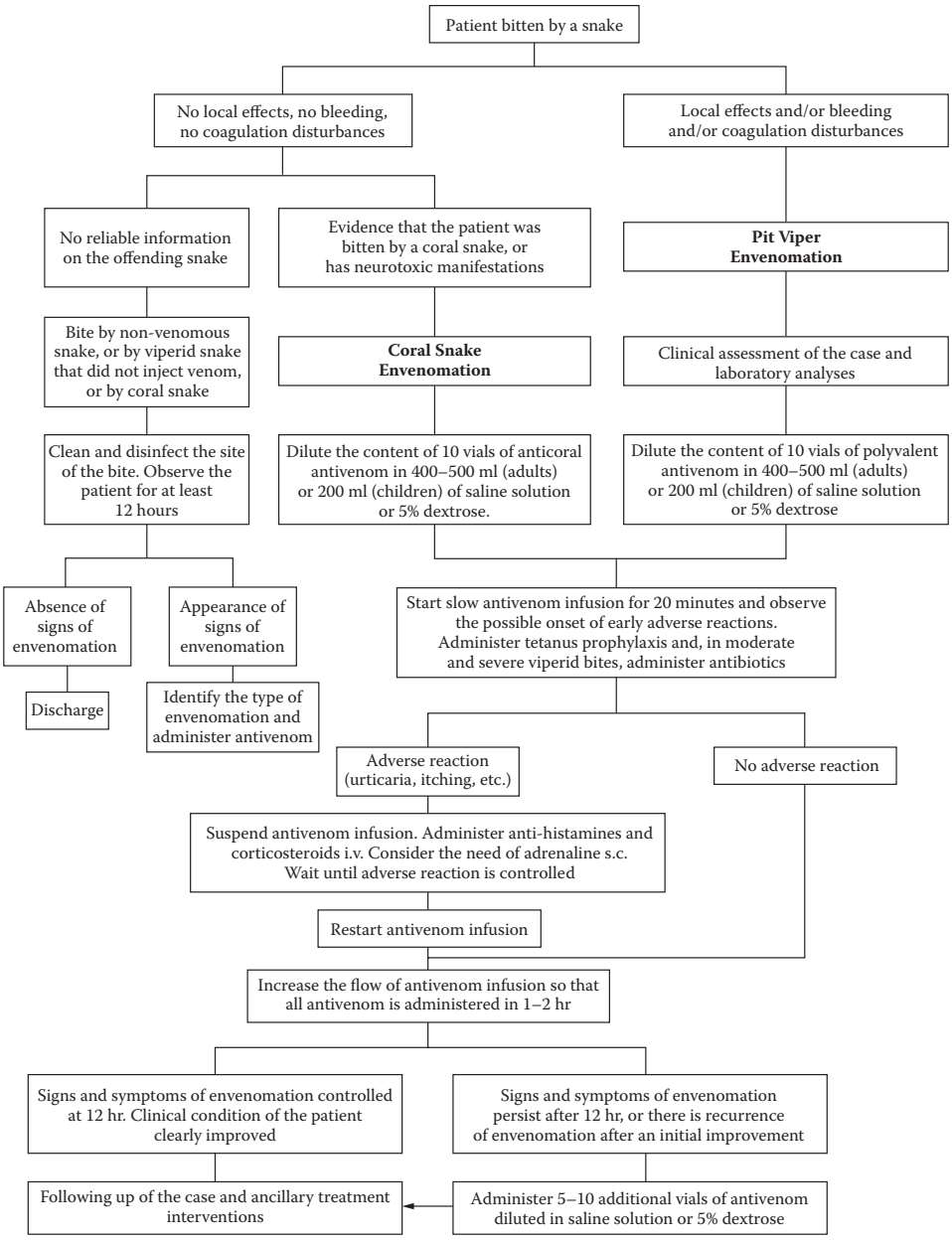

FIGURE 24.4 Algorithm used in Costa Rica for the diagnosis and antivenom therapy of snakebite patients. The recommended doses of antivenoms apply to the products manufactured by Instituto Clodomiro Picado (Universidad de Costa Rica). Only antivenom treatment is included; for details of other ancillary aspects of therapy, see text.

In some cases, the fluid therapy associated with antivenom infusion may correct the renal disturbances. Fluid therapy must be linked to the monitoring of central venous pressure, in order to detect fluid overload. If renal alterations persist, i.e., urine output does not improve, the administration of diuretic drugs such as furosemide or mannitol is required. When these interventions are ineffective, liquid ingestion must be carefully controlled and the patient has to be transferred to a specialized hospital unit for dialysis. In the case of pigmented urine, reflecting hemoglobinuria or myoglobinuria, the intravenous infusion of sodium bicarbonate is indicated.

e. Management of Respiratory Paralysis in Elapid Envenomations

In the event that a patient bitten by a coral snake, or a sea snake, has already developed respiratory alterations, endotracheal intubation and mechanical ventilatory support must be initiated. In addition, anticoral antivenom should be administered, since there is evidence that antivenom is able to reverse the binding of α-neurotoxins of *M. nigrocinctus* venom from the motor end plate (Alape-Girón et al., 1996b). There is no experimental or clinical evidence that anticholinesterase treatment is effective in envenomations by Central American coral snakes.

f. Management of Serum Sickness

The incidence of serum sickness in people treated with antivenom in Central America is unknown, since this phenomenon develops 7 to 14 days after antivenom administration, when patients have left the hospital. Serum sickness is characterized by urticaria, itching, fever, arthralgia, myalgia, and lymphadenopathy, and should be treated with corticosteroids (prednisolone) and antihistamines (chlorpheniramine) (Warrell, 1999).

g. Rehabilitation

A number of patients suffering viperid snakebite envenomations develop permanent sequelae secondary to tissue loss and dysfunction. Physiotherapy must be performed, and patients should also receive psychological support, since snakebites are often followed by episodes of posttraumatic stress (based on our unpublished observations in Costa Rica).

VI. CONCLUDING REMARKS: REDUCING THE IMPACT OF SNAKEBITE ENVENOMATIONS IN CENTRAL AMERICA DEMANDS INTERVENTIONS AT VARIOUS LEVELS

Despite considerable advances in confronting the problem of snakebite envenomations in Central America, much remains to be done. Regional efforts, promoted by national and regional health authorities, should be aimed at obtaining a more accurate epidemiological assessment on the incidence of snakebite envenomations in the region. Efforts should also be directed at improving the distribution of antivenoms to health centers in all areas of the region. The significant advances already made by governments in the acquisition of antivenoms should be complemented by innovative and effective strategies directed toward the deployment of these antivenoms to the health posts where they are most needed. It is furthermore necessary to develop regional guidelines for snakebite envenomation treatment. The experience gained in Costa Rica, where such guidelines have been developed and are widely distributed, could be extended to other countries in the region. In parallel with this, a permanent program of seminars and workshops for health workers should be promoted, in order to bring physicians, nurses, and other health staff members the basic aspects of snakebite envenomation therapy. The development of initiatives to identify and help people suffering from sequelae after snakebite envenomation, either physical or psychological, is a largely forgotten issue that needs to be addressed in Central America. Finally, public campaigns aimed at improving the prevention of snakebites should be promoted throughout the region, involving governmental agencies, local community organizations, and public-private partnerships of diverse sorts. All these concerted actions will result in the amelioration of human suffering caused by snakebite envenomation in the region.

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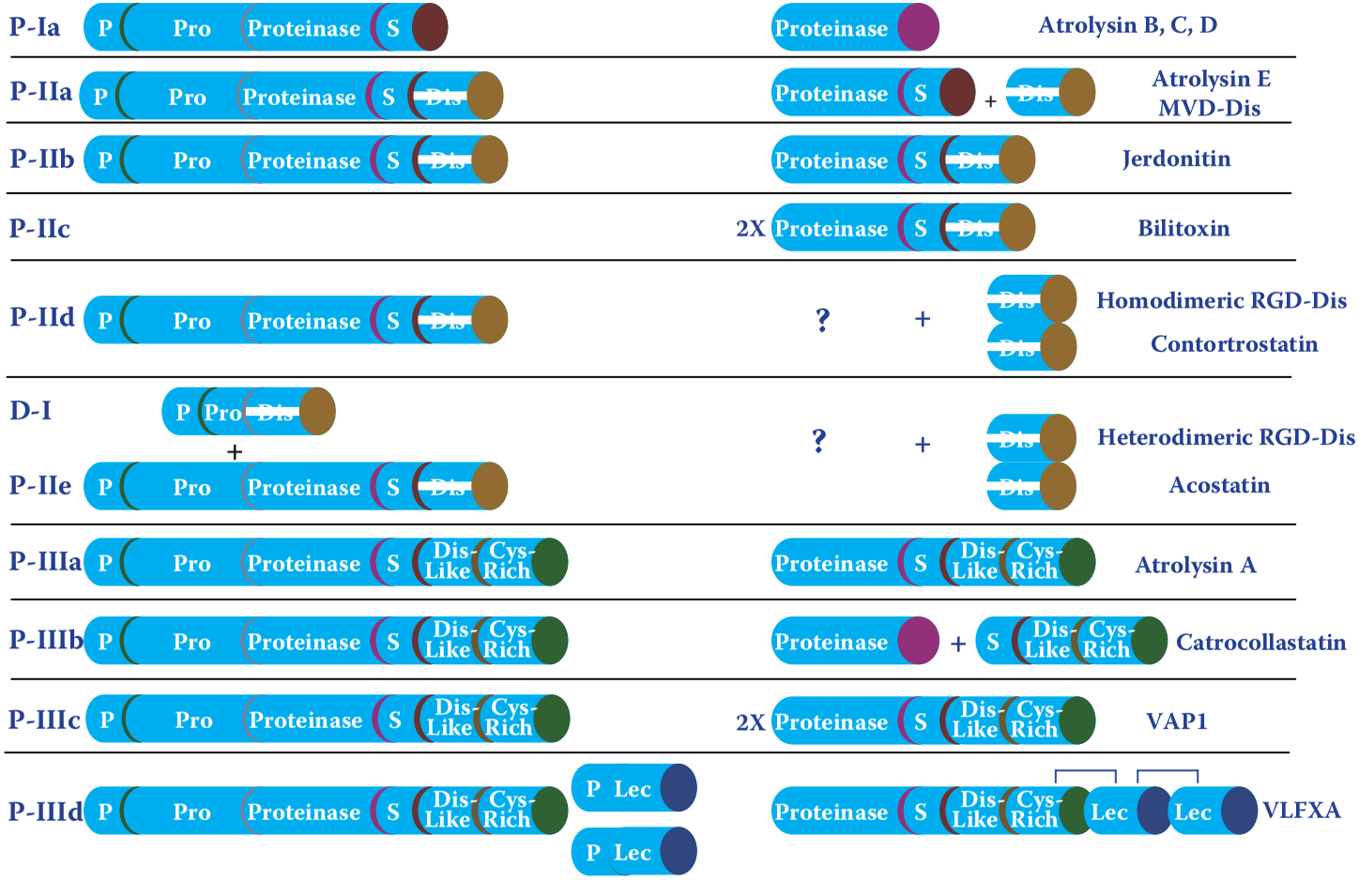
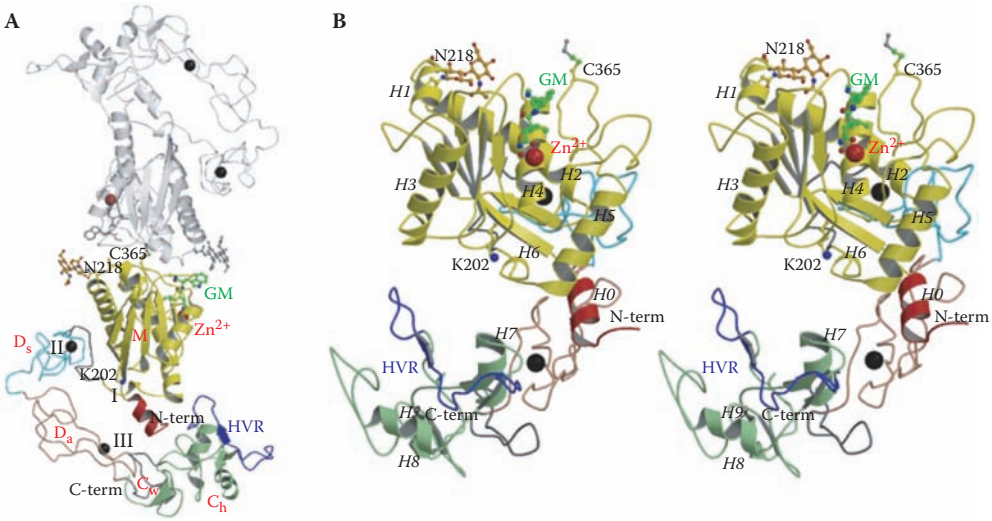
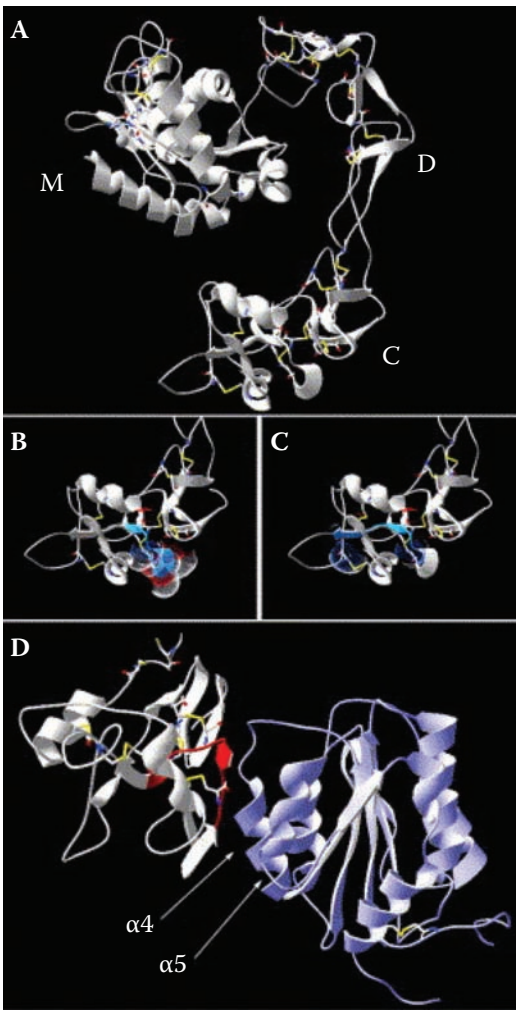
COLOR FIGURE 1.1 Representative examples of venomous squamate reptiles. (A) Gila monster (*Heloderma suspectum*), a member of the family Helodermatidae. (B) Small-scaled burrowing asp (*Atractaspis microlepidota*), family Atractaspididae. (Photograph by Kristen Wiley, courtesy Kentucky Reptile Zoo.) (C) Mangrove catsnake (*Boiga dendrophila*), family Colubridae. (D) Monacled cobra (*Naja kaouthia*), family Elapidae. (E) Northern blacktail rattlesnake (*Crotalus molossus molossus*), family Viperidae.



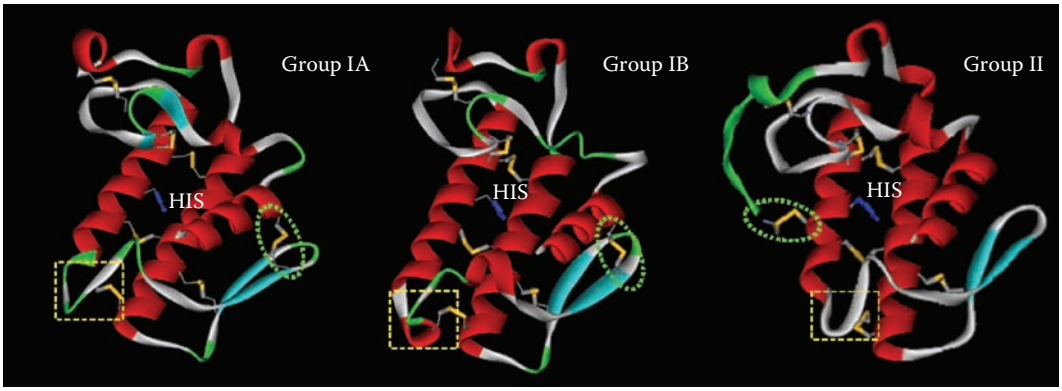
COLOR FIGURE 4.1 Schematic of SVMP classes. (Reprinted from Fox and Serrano, 2008, with permission from Wiley.)



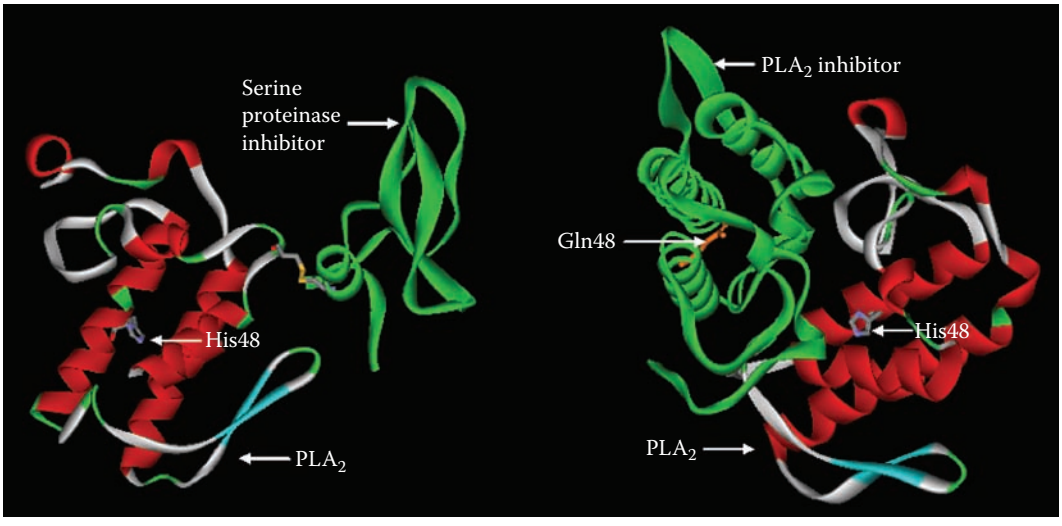
COLOR FIGURE 4.3 Metalloproteinase-disintegrin-cysteine-rich (MDC) architecture. (A) VAP1 dimer viewed from the non-crystallographic twofold symmetry (NCS) axis. The N-terminal helix (H0-helix), metalloproteinase domain (M-domain), linker, "shoulder" (D_{s-}), "arm" (D_{a-}), "wrist" (C_{w-}), and "hand" (C_{h-}) domains and hypervariable regions (HVRs) belonging to the one monomer are shown in red, yellow, gray, cyan, pink, gray, green, and blue, respectively. The disulfide-linked counterpart is shown in gray. Zinc and calcium ions are represented as red and black spheres, respectively. The NAG (*N*-acetyl-glucosamine, in orange) moieties linked to Asn218, the calcium-mimetic Lys202, and the bound inhibitor GM6001 (GM, in green) are in ball-stick representations. (B) Stereo view of VAP1 monomer from the direction nearly perpendicular to (A). The helix numbers are labeled. (Reprinted from Takeda et al., 2006, with permission from Macmillan Publishers Ltd.)



COLOR FIGURE 4.4 Structural analysis of the P-III SVMP jararhagin model. (A) View of jararhagin. Metalloproteinase (M), disintegrin (D), and cysteine-rich (C) domains are indicated. Zinc ion is represented as a gray sphere. (B and C) Peptides 365PCAPEDVKCG374 (Jar6) and 372KCGRLYCK379 (Jar7) are represented in blue. Solvent accessibility of the residues in each peptide is represented in dotted spheres and colored by atoms (carbon in white, oxygen in red, and nitrogen in blue). (D) Docked structure of jararhagin cysteine-rich domain (gray) and vWF A1 domain (light blue). The peptide Jar6 region is colored in red. vWF A1 a4 and a5 helices are indicated. Disulfide bonds are represented by yellow sticks. (Reprinted from Pinto et al., 2007a, with permission from Elsevier.)

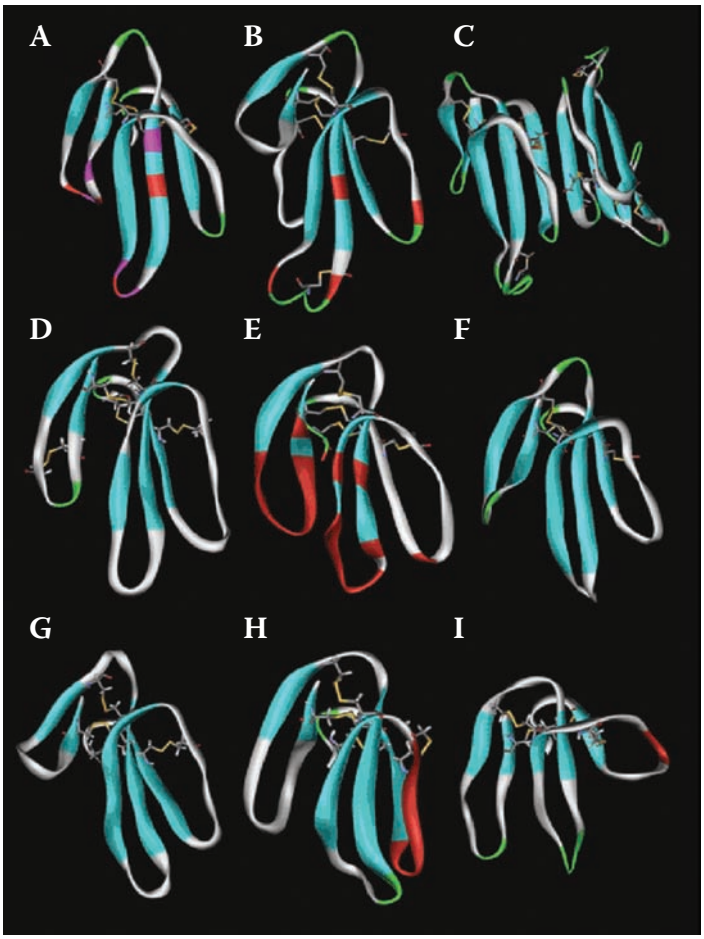


COLOR FIGURE 8.2 Three-dimensional structures of snake venom PLA_2 enzymes generated and modified using ViewerLite software. PLA_2 molecules of Group IA from *Naja naja* venom (Segelke et al., 1998), Group IB PLA_2 from *Ophiophagus hannah* venom (Xu et al., 2003), and Group II PLA_2 from *Deinagkistrodon* (*Agkistrodon*) *acutus* venom (Holland et al., 1990) are shown in the figure. Structurally these PLA_2 molecules have a common scaffold comprised of two major α-helices and β-wings, though they differ in their primary amino acid sequence. The elapid loops (between the first major α-helix and β a-wing) in Groups IA and IB are shown in violet. The pancreatic loop present in Group IB is shown in green, which is absent in Group IA. The C-terminal extension in Group II is shown in green. The disulfide bridge between the cysteine residues and the active site histidine residue are also shown in the figure. The unique disulfide bridges in Groups IA and IB (Cys11–Cys77) and Group II (Cys50–Cys134) are encircled and the loops are shown in a box.

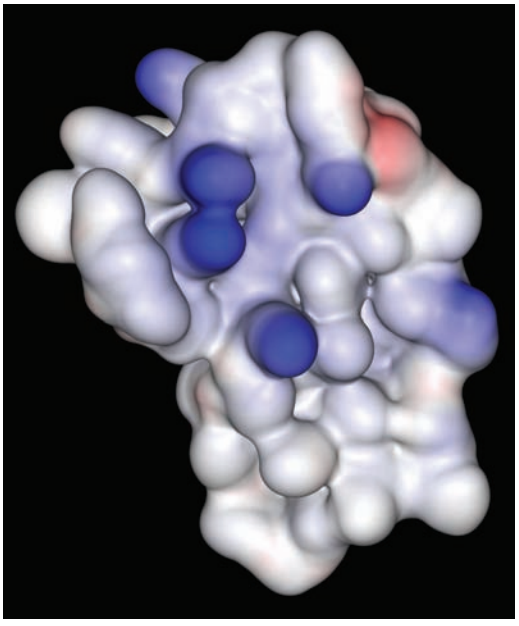


β-Bungarotoxin **Vipoxin**

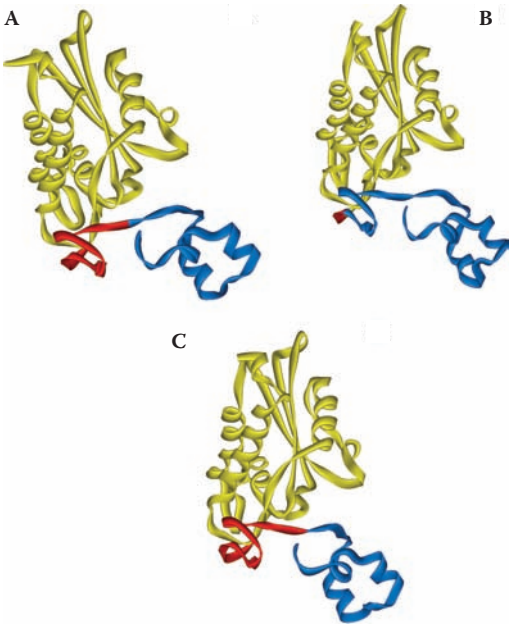
COLOR FIGURE 8.5 Crystal structures of β-bungarotoxin and vipoxin complexes. In β-bungarotoxin, the serine-type proteinase inhibitor is linked to the PLA_2 molecule by a disulfide (covalent) bond between Cys15 of the PLA_2 molecule and Cys55 of the serine-type proteinase inhibitor (Kwong et al., 1995). In vipoxin, two PLA_2 molecules interact noncovalently to form the complex. In one of the molecules the His48 is replaced with Gln48, which acts as an inhibitor to the PLA_2 enzyme (Perbandt et al., 1997). The critical disulfide bond in β-bungarotoxin and active site residues of vipoxin are shown.



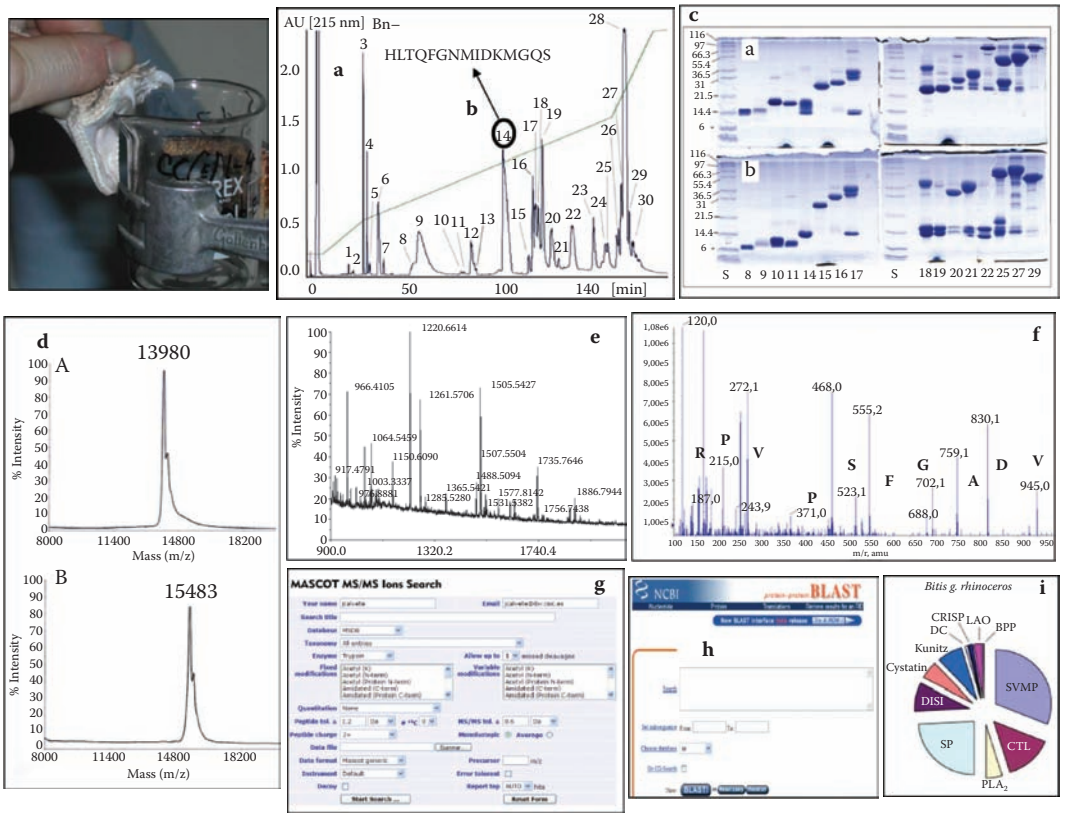
COLOR FIGURE 13.1 Similar structures, diverse functions. Three-dimensional structures of 3FTXs from representative snake venoms are shown. (A) Erabutoxin (3EBX). (B) α-cobratoxin (2CTX). (C) Dimer observed in κ-bungarotoxin (1KBA). (D) Candoxin (1JGK). (E) Fasciculin (1FSS). (F) Muscarinic toxin 2 (1FF4). (G) Cardiotoxin (2CRT). (H) FS_2 (1TFS). (I) Dendroaspin (1DRS). The cysteine residues are shown in stick representation, and disulfide bonds are shown in yellow. Important functional residues, in toxins with known structure-function relationships, are shown in red.



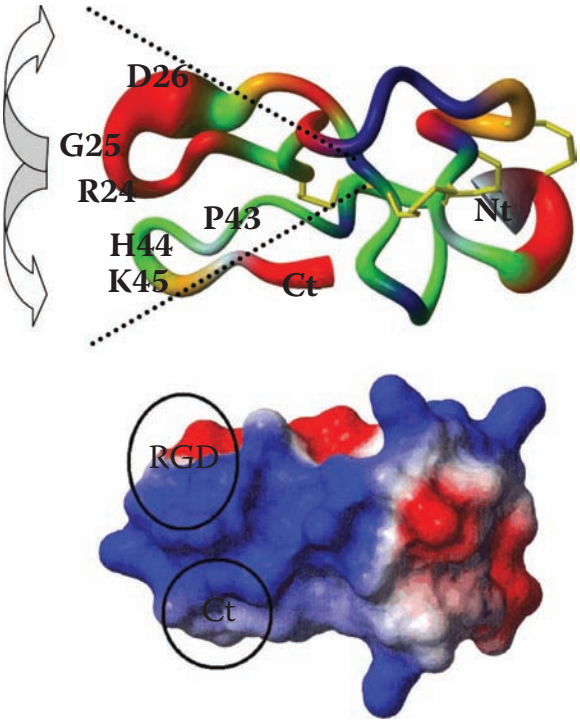
COLOR FIGURE 13.3 Surface charge view of cardiotoxin structure. Positive, negative, and hydrophobic residues are in blue, red, and white, respectively.



COLOR FIGURE 16.1 Snake venom CRISP structures generated by SWISS-MODEL. (A) Stecrisp (GenBank/EBI accession number 1RC9A). (B) Triflin (1WVRA). (C) Natrin (1XTAA). Ribbon color is used to differentiate structural domains as described by Guo et al. (2005). Yellow, N-terminal PR-1 domain; red, bridge region; blue, C-terminal cysteine-rich domain. Note the high degree of structural homology in all three molecules.

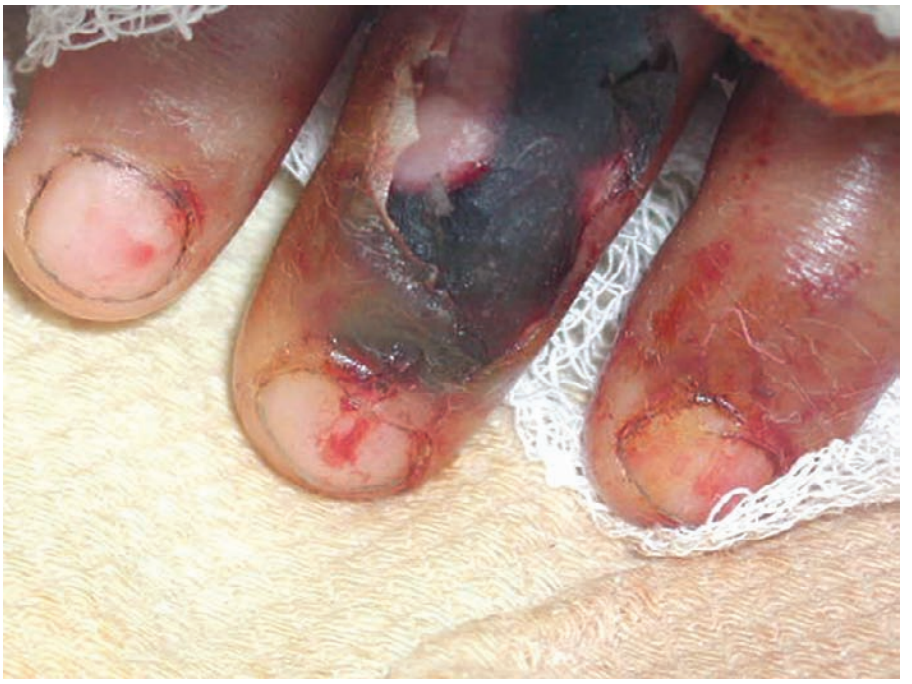


COLOR FIGURE 17.1 Snake venomics. Schematic representation of the steps typically followed in a snake venomics project. (a) Reverse-phase chromatographic separation of the venom proteins. (b) N-terminal sequencing of the isolated protein fractions. (c) SDS-PAGE of the RP-HPLC isolated proteins run under nonreduced (upper panel) and reduced (lower panels) conditions. (d) Determination of the molecular masses of the proteins isolated in (a), and quantitation of their sulfydryl group and disulfide bond contents. (e) MALDI-TOF mass fingerprints of in-gel digested protein bands excised from SDS-polyacrylamide gels run as in (c). (f) Amino acid sequence determination by nanospray-ionization CID-MS/MS of doubly and triply charged tryptic peptide ions obtained as in (e). (g) Database searches using MS/MS spectra in MASCOT. (h) MS/MS-derived amino acid sequence through Basic Local Alignment Search Tool (BLAST). (i) Summary of the relative amounts of toxin families using a pie chart representation (see also Table 17.1).

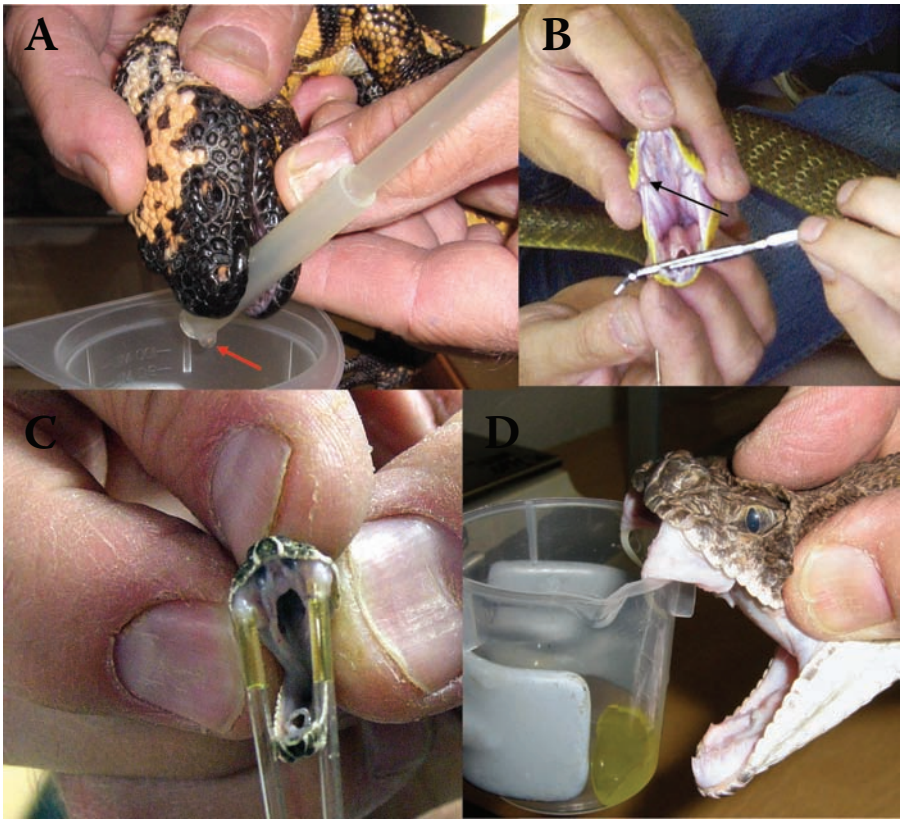


COLOR FIGURE 17.8 NMR structure of the short disintegrin echistain. The upper panel shows a "sausage" model of the solution structure of echistatin (PDB code 1RO3) highlighting the topology of the active tripeptide and the C-terminal region, which form a conformational epitope engaged in extensive interactions with the target integrin receptor, and display concerted lateral movements. The lower panel shows the surface electrostatic potential of echistatin. The molecule is depicted in the same orientation as the sausage model. The positively and negatively charged areas are colored blue and red, respectively. The integrin recognition motifs (RGD) and the C-terminal tail (Ct) are labeled and circled.

COLOR FIGURE 24.1 *Bothrops asper*, locally known as *terciopelo*, *barba amarilla*, or *equis*, is the most dangerous venomous snake in Central America, being responsible for a large number of cases every year. Adult specimen collected in Costa Rica. (Photo courtesy of Dr. Mahmood Sasa and published in Gutiérrez et al., *PLoS Medicine* 3 (2006): e150.)



COLOR FIGURE 24.3 Local necrosis in a finger of a child after a bite by *Bothrops asper* in Costa Rica. (Photo courtesy of Dr. María Luisa Avila-Agüero, Hospital Nacional de Niños, Costa Rica.)



Venom extraction from representative reptiles. (A) Gila monster, *Heloderma suspectum*; the head is held inverted over a beaker, and venom (clear, at arrowhead) slowly drips into the container. (B) Brown Treesnake, *Boiga irregularis*; capillaries placed over the rear maxillary fangs (arrowhead) collect venom. (C) Prairie rattlesnake, *Crotalus viridis viridis*; for neonate and small snakes, capillary tubes are placed over fangs to collect the small volume expressed. (D) Western diamondback rattlesnake, *Crotalus atrox*; adult snakes are extracted directly into small beakers. (A, C, D Photographs by C. Rex.)

The **Handbook of Venoms and Toxins of Reptiles** offers "one-stop shopping" to all biologists, biochemists, toxicologists, physicians, clinicians and epidemiologists, and informed laypersons interested in the biology of venomous reptiles, the biochemistry and molecular biology of venoms, and the effects and treatment of human envenomation. This book examines the topic generally, provides an overview of the current taxonomy of these reptiles, explains the similarities and differences in the venom delivery apparatus in different groups of reptiles, reviews state-of-the-art knowledge about specific venom components and their action, and summarizes effects of envenomation and treatment in humans on different continents.

Features

- Presents an overview of the biological relationships among venomous animals
- Provides a summary of the main structural features of the venom glands
- Supplies a detailed understanding of the structure/function relationships at the molecular level
- Includes a complete synopsis of assay procedures used to detect a specific class of venom enzymes

Produced by leading toxinologists, biologists, biochemists, and physicians from 12 countries, the book provides a broad, international perspective that bridges divergent areas. A synthesis of current knowledge about venoms and venomous reptiles, it contains a wealth of illustrations, including an 8-page color insert, that presents a view of reptile toxinology from the whole animal to the glands producing venoms to the molecular models and the mechanisms of actions of the toxins themselves.

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