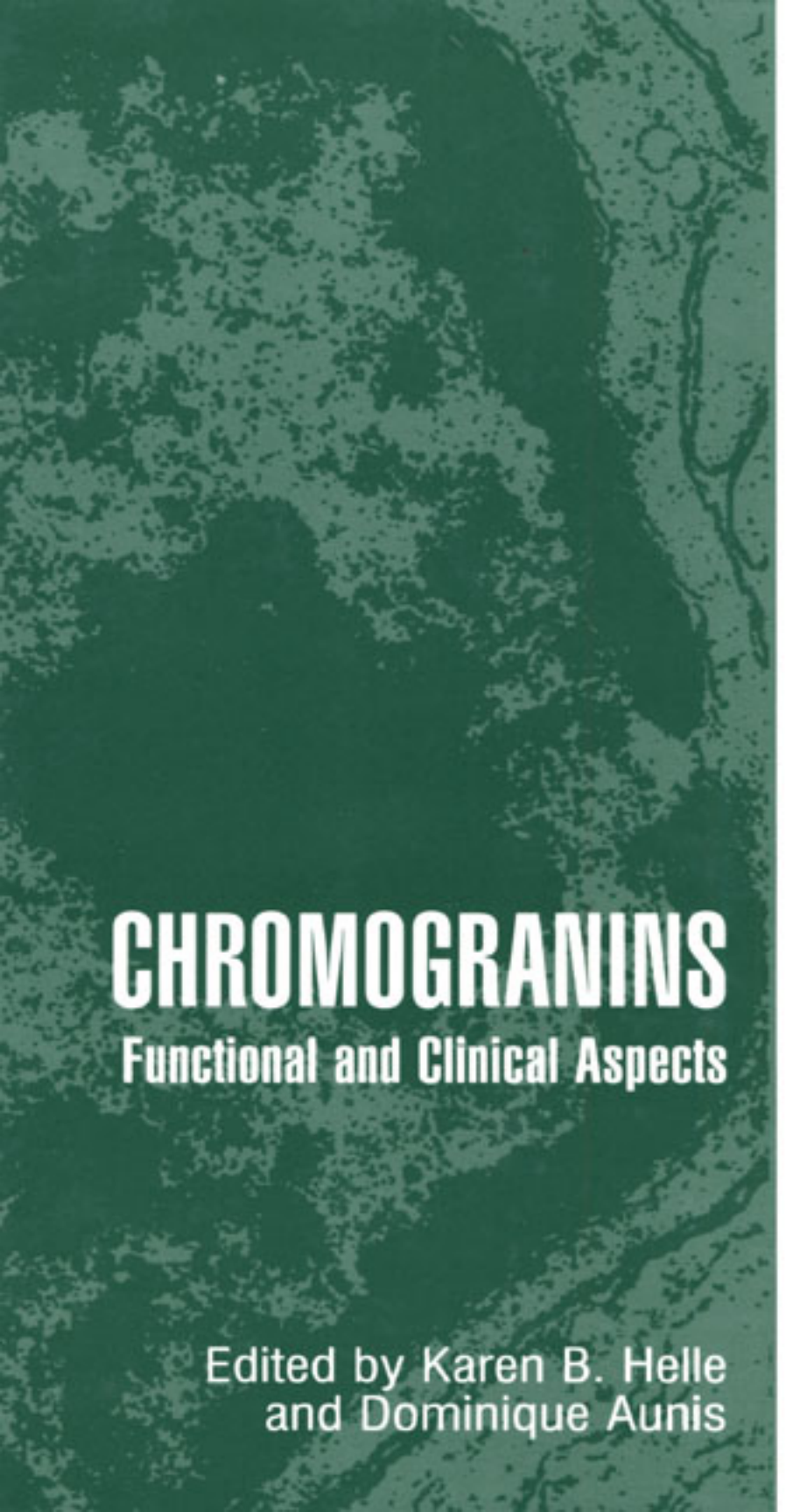

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 482

CHROMOGRANINS

Functional and Clinical Aspects

Edited by Karen B. Helle and Dominique Aunis

CHROMOGRANINS

FUNCTIONAL AND CLINICAL ASPECTS

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CHROMOGRANINS
FUNCTIONAL AND CLINICAL ASPECTS

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This book is dedicated to the memory of Dr. Hugh Blaschko, F.R.S. (1900–1993), in deep admiration for his integrity and high standards as a scientist and human being and in recognition of his initiation and long standing interest in chromograninresearch.

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PREFACE

Chromogranin research has finally come of age, about four decades after the first observations of their acidic nature. The accumulated literature embodies a wide range of aspects of this unique family of proteins and is not readily gathered in a comprehensive way. This volume, being the first publication to be solely devoted to the chromogranins, aims to meet the demand for an up-to-date overview of their biological activities and potential for clinical applications.

The series of contributions presented at the 10th International Symposium on Chromaffin Cell Biology in Bergen, Norway, August 22– 27, 1999, and the following workshop, August 28, 1999, form the core of the present volume, providing a "state of the art" with respect to functional implications of the chromogranins and their close relatives within the "granin" family.

In view of the rather long time lapse from the first discovery of these acidic proteins to the ongoing unmasking of their biological activities, this volume opens with a historical perspective, emphasising the main lines of progress and providing an introduction to the chapters focused on the intracellular functions of the chromogranins. The current aspects of chromogranin research is presented in a chapter which also brings the reader up to date on structural properties of relevance for the following chapters on tissue processing and the wide range of biological activities of the chromogranin-derived peptides. Clinical applications of the chromogranins as markers for neuroendocrine and nonendocrine tumours have multiplied in the last decade. This clinical information may, together with the overviews on the physiological aspects of the chromogranins, provide new ideas of the pathophysiological implications of these proteins and their regulatory peptides.

The specialised contributions have been grouped according to topics, opening with aspects related to intragranular functions. Thereafter, focus is shifted to the regulation of expression, transcription and secretion, followed by chapters on tissue-specific processing, transport, distribution, and on functional aspects of the biologically active sequences. The chapters dealing with clinical aspects open with an assessment of the quantitative assays for circulating chromogranins and their derived peptides, followed by applications in tumour diagnosis and analyses of physiopathological conditions.

More than 2,300 articles with chromogranin as a keyword have been entered into the Medline database since 1967. These articles represent an exponential growth in the research interest in the chromogranin field, with 833 articles published from 1996 to 1999. Despite this large body of information, much work remains before we may fully understand their physiological relevance.

The chromogranins still represent a fascinating challenge for basic and clinical work. Hence, the ultimate goal for this first book on the chromogranins is to inspire basic and clinical scientists to progress with new approaches to elucidate the physiological and physiopathological roles for the chromogranins and their derived peptides.

Karen B. Helle
Dominique Aunis

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Thanks are also due to the Nansen Foundation and Associated Funds, the Norwegian Academy for Science and the Humanities, Oslo, Norway, for support of the publishing costs, and to Institut National de la Santé et de la Recherche Médicale (INSERM), France, and the Norwegian Research Council Section for Medicine and Health for support of the editorial work. Finally, the editors are indebted to Mme Martine Rivet, Unité Biologie de la Communication Cellulaire, INSERM U-338, Centre de Neurochimie, Strasbourg, France, for excellent assistance in the editorial process.

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PART 1

INTRODUCTION

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1

THE CHROMOGRANINS

Historical Perspectives

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1. INTRODUCTION

The immunological approach to chromaffin granule research in the mid 1960s led to the discovery of co-secretion of a granule-specific, water-soluble protein and the catecholamine hormones (Banks and Helle 1965, see review by Helle 1990). This was the first experimental evidence of exocytosis, i.e. release of low molecular weight amine hormones in parallel with a large molecular weight, co-stored protein.

When the release of this protein from the stimulated calf adrenal gland *in vivo* was established, the term CHROMOGRANIN A (CGA), was introduced for the predominant satellite protein (Blaschko *et al* 1967, see review by Winkler and Fischer-Colbrie 1992). Since then a whole family of uniquely acidic proteins have been associated with storage and release of a wide range of hormones. Today this family, often collectively called *"granins"*, comprises 5 members, CGA, chromogranin B (CGB), secretogranin II (SGII), 7B2 and NESP 55 (see review by Huttner *et al* 1991).

These historical perspectives are focused on the highlights in chromogranin research, with emphasis on properties of relevance for their dual functions, as proteins for hormone storage and granulogenesis, and as prohormones for regulatory peptides. A major aim is to bring forth the early literature and ideas related to functions that has yet to be fully understood.

Chromogranins: Functional and Clinical Aspects, edited by Helle and Aunis
Kluwer Academic / Plenum Publishers, New York, 2000

2. CHROMOGRANINS AS SATELLITE PROTEINS IN HORMONE-STORING ORGANELLES

The first characterisation of the acidic nature of the water soluble protein in the bovine adrenomedullary granules was made by Hillarp in 1959 and in the following decade the main component was purified and characterised by its uniquely acidic amino acid composition (Helle 1966a, Smith and Kirshner 1967, Smith and Winkler 1967) and also detected in the splenic nerve of the ox (Banks *et al* 1969) and sheep (Geffen *et al* 1969a). When chromogranins were shown to be markers for neurotransmitter release from this sympathetic nerve in several species (DePotter *et al* 1969, Geffen *et al* 1969b), completely new ideas of neuronal function were formulated, such as exocytotic release of neurotransmitters (Stjärne *et al* 1970), coexistence of neuronal polypeptides and biogenic amines (Schultzberg *et al* 1978) and regulated biosynthesis and storage of biogenic amines and polypeptides (Sietzen *et al* 1987).

The unique acidity of CGA was reflected in an extremely low isoelectric point and a "random coil"-like configuration (Smith and Winkler 1967). In the early studies molecular weights in the range of 74,000 - 81,000 were assigned to CGA. However, by cDNA methodology the true molecular weight of the nascent CGA was less, i.e. 48,000 (Benedum *et al* 1986, Iacangelo *et al* 1986). Hence, in addition to the unique acidity, also the post-translational modifications, e.g. phosphorylation, sulfation and O-glycosylation (Smith and Winkler 1967, Settleman *et al* 1985b, Rosa *et al* 1985), contribute to the anomalous behaviour of the granins in gel electrophoresis and gel permeation chromatography. Furthermore, the post-translational modifications have a role in regulation of proteolytic processing (Metz-Boutigue *et al* 1998).

2.1 The Primary Structures

The most striking breakthrough in chromogranin research since the 1960ies was the elucidation of the complete cDNA coded sequences for CGA (Iacangelo *et al* 1986, Benedum *et al* 1986) and CGB (Benedum *et al* 1987). As a consequence of the new insight into the primary structures and genome organizations, the prohormone concept for the chromogranins was formulated (Eiden 1987), supported by the sequence homology between the middle region of CGA and the inhibitory peptide, pancreastatin, shown to suppress glucose induced insulin release from the porcine pancreas (Tatemoto *et al* 1986).

The other immunologically distinct group of acidic proteins, the CGBs (Fischer-Colbrie and Frischenschlager 1985, Settleman *et al* 1985b), were

also named secretogranin I (Rosa *et al* 1985). The human CGB, with its nascent molecular weight of 76,000, shares some features with CGA, such as an overall high content of acidic amino acids and proline and a disulphide-bridged loop in the N-terminal domains (Benedum *et al* 1987). CGA and CGB share also random coil properties, reflected in the heat and acid stability of these proteins (Rosa *et al* 1985) and in a relatively low affinity, high capacity binding of Ca^{2+} (Reiffen and Gratzl 1986a, Gorr *et al* 1989). By consensus, the name CGB was preferred for this granin, as a reflection of the many shared properties with CGA, in contrast to secretogranin II (Eiden *et al* 1987).

3. CHROMOGRANINS AS MARKERS OF THE DIFFUSE NEUROENDOCRINE SYSTEM

The findings that one or more of the chromogranins are conspicuous constituents of secretory granules within the diffuse neuoendocrine system was another significant leap forward (O'Connor 1983, Fischer-Colbrie *et al* 1985). Chromogranins occur in the parathyroid (Cohn *et al* 1981, 1982), in thyroid C-cells (O'Connor *et al* 1983), in the endocrine pancreas (Ehrhart *et al* 1986, Yoshi *et al* 1987) and in the adenohypophysis (O'Connor 1983, Serck-Hanssen and O'Connor, 1984) and also in non-adrenergic neurons in the central nervous system (Somogyi *et al* 1984). In addition, a wide range of peptidergic cells in the mucosal layer of the gastrointestinal tract also contain immunoreactive CGA (Grube *et al* 1987, Gauweiler *et al* 1988, Qian *et al* 1988), notably the rat enterochromaffin-like (Håkanson *et al* 1995) and the human enterochromaffin cells (Cubeddu *et al* 1996). An implication of the chromogranins in the immune system was also an early hypothesis (Angeletti and Hickey, 1985).

As reviewed by Winkler & Fischer-Colbrie (1992), CGA and CGB are expressed in a wide variety of neuroendocrine cell types, each of which co-express a unique and cell-specific polypeptide hormone or neurotransmitter. A single mammalian CGA gene appears to exist (Wu *et al* 1991, Iacangelo *et al* 1991). Despite their different chromosomal locations (Mahata *et al* 1996), CGA on the human chromosome 14 (Murray *et al* 1987) and CGB on the mouse chromosome 2 (Pohl *et al* 1990), the structure and exonic organisation of the CGA and CGB genes are similar in the regions corresponding to the N- and C-termini of the proteins.

In bovine chromaffin cells CGA and CGB together make up about 50% of the total soluble protein, but their biosynthesis is not closely linked to the secretory activity (Galindo *et al* 1990). It appears that CGA is synthesized in

excess in a "constitutive" manner and that decreased degradation of CGA rather than increased gene transcription may regulate CGA levels in the secretory cells. Much less is known about regulation of CGB synthesis.

4. CORE FUNCTIONS

A regulatory role of the chromogranins at the level of the secretory granules was an early assumption in view of their co-storage with amines, nucleotides, calcium and other peptide hormones. All of the aggregating and membrane-binding properties of the chromogranins so far accumulated, point to significant roles for the intact granins in granulogenesis, core formation and condensation. A role for the processed products in the osmotic regulation of the core, remains to be elucidated.

4.1 Models of Amine Storage

The early studies concentrated on the mature storage organelles and their capacities for stable binding of the high concentrations of costored catecholamines (CA), ATP and Ca^{2+}-ions in the adrenomedullary chromaffin granules (Blaschko *et al* 1956, Hillarp, 1959, 1960, Borowitz 1965). At first, most of the attention was directed to the apparent stoichiometry of opposite charges indicated by the molar ratio of CAATP of 4: 1 (Berneis *et al* 1969, Kopell and Westhead 1982). However, stable interactions between the small molecules could not be verified by NMR studies (Sharp and Richards 1977, Sharp and Sen 1979).

The NMR studies also made it evident that there was no specific interaction between CGA and the catecholamines (Sen and Sharp 1981). As an alternative, electrostatic interactions were proposed to account for the osmotically stable complex (Sen and Sharp 1982), between CA and the chromogranins (Uvnäs and Åborg 1977; 1980) and between ATP and the protein backbone within the storage complex (Sharp and Richards 1977, Sharp and Sen 1979). These early models based on nucleotide interaction with CGA have been substantiated by more recent findings (Yoo *et al* 1990).

A general role for the aggregating chromogranins as osmotic stabilisers, independent of their interaction with Ca^{2+}, was postulated by Helle *et al* (1985), on basis of concentration-dependent reductions in colloid osmotic pressures near the intragranular pH. On basis of Donnan forces arising from the condensed protein matrix in the mature granules (–180 mg/ml, Phillips *et al* 1977), an amine storage capacity could be calculated. Theoretically, the condensed matrix could accomodate 600 mM CA at a molar ratio of

CA:ATP of 4.6: 1, however, without anticipating any complex between CA and ATP or with these ions and CGA. Accordingly, at the intragranular pH of 5.7 this "Donnan model" predicts that the CA concentration would be 330 mM within the aggregated core which would be surrounded by an aquous phase of 216 mM CA consistent with a "two pool" model as first proposed by Uvnäs & Åborg (1970). The osmotic pressure exerted by this core was calculated to 254 mOsmol across the interphase, i.e. nearly isotonic, as required for osmotic stability. By this model (Helle *et al* 1985; 1990, see review by Helle *et al* 1993), the granins would function as osmotic stabilisers due to their acidic nature, and their capacity for cation storage would reflect the total negative charge within the condensed core, consisting of the co-stored proteins, nucleotides and peptides.

This "two pool" model for CA storage has gained support from the detailed quantitation of amine release from single chromaffin granules by amperometry (Wightman *et al* 1995). At pH 5.5 the core was stable, and the pre-spike "foot" was assumed to originate from a limited pool of free CA within the vesicle while the main spike reflected the more slowly dissolving core (Jankowski *et al* 1993, see Borges *et al,* this volume).

4.2 Calcium Storage

The importance of Ca^{2+} in the storage complex has been in the focus ever since this field was opened by Douglas & Rubin (1961). The EDTA-resistant calcium pool in mature chromaffin granules from perfused and stimulated adrenal glands is high, 288 nmol Ca^{2+}/mg protein (Serck-Hanssen and Christiansen 1973), and this calcium is stored at fixed ratios of 30 mol CA:4 mol ATP: 1 mol Ca^{2+} (Bolstad *et al* 1980).

Several values for Ca^{2+} binding to the purified CGA have since been reported, indicating at least two classes, one with a maximal capacity of 152 nmol Ca^{2+}/mg protein and moderate affinity (Kd 54 μM, Reiffen and Gratzl 1986b), and another, with 10 times higher capacity and 20 times lower affinity (Yoo and Albanesi 1991). Moderate affinity calcium binding was also expressed in the recombinant bovine CGA, concomitant with pH and concentration-dependent aggregation and a secondary structure consisting of about 50% random coil and 30% β-turns, with lesser amounts of calculated a helix and β-sheet (Angeletti *et al* 1992).

Thus, the accumulated evidence point to several classes of electrostatic Ca^{2+}-binding sites being associated with aggregation of CGA and CGB (Gorr *et al* 1989, Gerdes *et al* 1989). Ca^{2+} binding to these proteins was postulated to occur in the early stage of core formation when the uptake of

proportional amounts of CA and Ca^{2+} is limited to the stimulation period or shortly thereafter (Borowitz 1969). However, the ability to accumulate calcium in response to stimulation was not restricted to granules low in CA, suggesting a role as a calcium "pump" also for the mature granules (Serck-Hanssen and Christiansen, 1973).

Aggregation of chromogranins by different components of the core has been demonstrated. For instance, at 0.03 mg protein/ml Ca^{2+} was an efficient aggregator (Gorr *et al* 1989), while at 1 mg proteidml noradrenaline rather than Ca^{2+} was effective (Videen *et al* 1992). In our studies ATP and phosphate anions, but not Ca^{2+}, were important for aggregation at 1 – 12 mg protein/ml (Helle *et al* 1985, Helle *et al* 1990), indicating concentration-dependent phases in core formation. The final stage in core condensation, leading to a protein concentration of 180 mg/ml in the intact chromaffin granule, has yet to be elucidated.

4.3 Co-stored Peptides and Proteins

In the 1970ies a new family of matrix components, including opioid peptides and enkephalin-like material, were discovered in mammalian adrenal medullae (Schultzberg *et al* 1978, Viveros *et al* 1979). Since then, the analgesic enkephalins and also an antibacterial peptide have been identified as products of the adrenomedullary enkephalins (see review by Dillen *et al* 1993) and shown to be secreted along with the chromogranins (Metz-Boutigue *et al* 1993, see this volume).

A soluble fraction of the noradrenaline producing enzyme, dopamine β-hydroxylase (DBH) is also a characteristic core component (Viveros *et al* 1968, Geffen *et al* 1969). Moreover, the co-release of both chromogranins and DBH fiom neuronally stimulated cat adrenal medulla in an all-or-none fashion was an early finding (Kirshner and Viveros 1970).

Endogenous inhibitors of DBH (Belpaire and Laduron 1970) have been associated with the matrix. Apart from chelators of the enzyme bound copper, trypsin-resistant peptides have been suspected as inhibitors of the soluble DBH (Aunis *et al* 1978). However, neither the role for the low extracellular DBH activity nor the mechanisms leading to inactivation of the enzyme have been conclusively established (see Terland and Flatmark, this volume).

DBH-containing complexes of CGA were also early findings (Helle 1971, Helle *et al* 1978). The relationship between the membrane bound and soluble enzyme remained controversial (see review by Winkler 1976) until both hydrophobic and hydrophilic forms of the enzyme were demonstrated, indicating a precursor function for the hydrophobic, membrane form by

limited proteolysis (Bjerrum *et al* 1979), not only in the mature granules but also in the early, immature stages (Helle *et al* 1982).

The intact chromogranins have been assigned important roles in sorting of proteins destined for the regulated secretory pathway (Rosa *et al* 1989, Tooze and Huttner 1990, Parmer *et al* 1993a). In the trans-Golgi network (TGN) a dense-cored aggregate is assumed to form before being membrane bound and budding into a nascent secretory granule. In this process the aggregative properties of the chromogranins and their derived peptides are crucial. It is now evident that, in addition to acidification and Ca^{2+} accumulation as accumulating factors (Gorr *et al* 1989, Gerdes *et al* 1989, Yoo and Lewis, 1992 Angeletti *et al* 1992), these proteins engage in a range of protein-protein interactions, such as self aggregation (Helle *et al* 1985; 1990, Corti *et al* 1997, Thiele and Huttner 1998), complexing with DBH (Helle *et al* 1978) and t-plasminogen activator (Parmer et al 1997), and binding to membrane constituents (Helle and Serck-Hanssen 1969, Helle 1973, Settleman *et al* 1985a, Pimlikar and Huttner 1992, see Gerdes *et al,* this volume).

5. TISSUE-SPECIFIC PROCESSING

The evidence for a prohormone role of the chromogranins has grown exponentially since the primary sequences were deduced and the gene structures were unravelled in the late 1980ies. Characteristically, CGA, CGB and SgII contain numerous sites for potential proteolytic processing, and a large body of research has been devoted to studies of the proteolytic enzymes involved (see reviews by Dillen *et al* 1993, Parmer *et al* 1993b). In addition, the chromogranins have also been shown to be a target for extracellular proteases such as plasmin and to be co-released with the tissue-bound plasmin activator, t-PA, from the adrenal medulla (Parmer *et al* 1994a; 1994b).

Processing of the chromogranins seems to occur at some, but not all of the pairs of basic amino acids, and, in addition, at some single basic amino acids, preferentially at the C- and N-termini of the molecules (Wohlfarter et al 1988, Metz-Boutigue *et al* 1993, Kirchmair *et al* 1993, Strub *et al* 1995). The extent of processing is highly tissue-dependent, being least complete in the adrenal medulla and highest in the endocrine pancreas (Watkinson *et al* 1991, Gill *et al* 1991, Curry *et al* 1991).

Numerous peptide fragments have been described so far, some of them with biological activity. From CGA these are pancreastatin (Tatemoto *et al* 1986, Lewis *et al* 1988), the vasostatins I and II (Drees *et al* 1991, Aardal *et al* 1993, Helle *et al* 1993, Russel *et al* 1994), rat β-granin (Hutton *et al*

1987), parastatin (Fasciotto *et al* 1993), catestatin (Mahata *et al* 1997) and chromacin (Strub *et al* 1997). From CGB they are the N-terminal CGB_{1-40} and the C-terminal secretolytin ($CGB_{614-626}$) (Strub *et al* 1995).From secretogranin II four peptides are known to date, including the 33 amino acid long neuropeptide secretoneurin (Vaudry and Conlon 1991, Kirchmair *et al* 1993).

6. BIOLOGICAL ACTIVITIES

Most of the biological activities so far reported are inhibitory, and each peptide sequence appears to exhibit several activities, depending on the target system. Autocrine inhibition of secretion is a characteristic of pancreastatin in the endocrine pancreas (Tatemoto *et al* 1986), of vasostatins and parastatin in the parathyroid (Fasciotto *et al* 1993) and of catestatin in the adrenal medulla (Mahata *et al* 1997). Paracrine/endocrine inhibitions are also evident; by vasostatins acting directly on the smooth muscle component in blood vessels (Aardal *et al* 1993) and by catestatin indirectly via release of histamine *in vivo* (Kennedy *et al* 1998). In the parathyroid gland cells CGB_{1-40} may serve as an endocrine inhibitor on the same target system as for the autocrine inhibition by CGA_{1-40} (Angeletti *et al* 1996). Vasostatins may also induce fibroblast adhesion (Gasparri *et al* 1997) and microglial activation (see Ciesielski-Treska and Aunis, this volume).

Like-wise, secretoneurin exhibits several target-specific activities, indicating paracrine/endocrine enhancement of dopamine release and as well as cellular chemotaxis (Saria *et al* 1993, Reinish *et al* 1993). Finally, several of the CGA and CGB-derived peptides are inhibitory to microbial and fungal growth (Metz-Boutigue *et al* 1998), implicating the chromogranins in early defense reactions.

The previous postulates of the chromogranins being prohormones for a multitude of regulatory peptides (see reviews by Helle *et al* 1993, Helle and Angeletti 1994, 1998, Iacangelo and Eiden, 1995), lend considerable strength from the more recent findings, as detailed in the present contributions.

7. THE CIRCULATING CHROMOGRANINS

The release of immunoreactive CGA into the human circulation (O'Connor 1983, O'Connor and Bernstein 1984) provided the first indications of a circulating pool of CGA. Since then, a range of

radioimmunoassays have been developed for neuroendocrine tumour diagnosis and prognostic purposes (Hsiao *et al* 1990, Syversen *et al* 1994, Stridsberg *et al* 1995). The circulating CGA also serves as an indicator of sympathoadrenal activity (Cryer *et al* 1991) and of release from the enterochromaffin cells ofthe gastrointestinal tract (Cubeddu *et al* 1995).

In healthy humans both CGA and CGB circulate in the low nanomolar range, while the levels are several folds higher in patients with various forms of neuroendocrine tumours (Aardal *et al* 1996), Thus, in patients suffering from pheochromocytoma and other neuroendocrine tumours, the circulating levels of CGA (Hsiao *et al* 1990) as well as of CGB and SgII (Stridsberg *et al* 1995), are several folds higher than normal, and the levels of CGA and CGB may be taken as reliable indictors of tumour mass (Stridsberg and Husebye 1997). Also in the cerebrospinal fluid the chromogranins and their processed products have been identified as conspicuous constituents and may find use as diagnostic indicators of brain function in a number of neurological diseases (O'Connor *et al* 1993, Kirchmair *et al* 1994).

For assessment of physiological and pathophysiological aspects of the circulating chromogranins and their derived peptides, reliable quantitative data oftheir concentrations in the extracellular fluids are essential. However, the marked species differences in primary sequence and immunoreactive epitopes of CGA have limited the use of the human RIAs in cross-species analyses of circulating CGA (O'Connor *et al* 1989). Moreover, radioimmunoassays (RIAs) based on antibodies directed to the intact prohormones may not reliably quantify the processed peptides (see Stridsberg, this volume). A wide range of site-specific antisera are now available, and a number of RIAs have been developed, some specific for the free peptides while others may detect the specific sequence within the intact prohormone

8. CONCLUSIONS

The goal has been to draw the main lines ofprogress in the chromogranin field, from the early discoveries towards our current understanding of the functional implications. It is evident from the accumulated literature that an unforseen diversity of cellular processes involve chromogranins. A multitude ofactivites have been assigned to these proteins, some to the intact molecules while most to their processed products. Functional patterns are now emerging, revealing important functions for the intact molecules in granule biogenesis and in hormone co-storage with calcium and nucleotides within the neuroendocrine cells. Perhaps most astonishing is the plethora of extracellular effects by granin-derived peptides, implicating the chromogranins as

prohormones for regulatory peptides for a range of homeostatic mechanisms, as further detailed in the many contributions compiled in this volume.

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2

CHROMOGRANINS: CURRENT CONCEPTS

Structural and Functional Aspects

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1. INTRODUCTION

The secretory granules of adrenal medulla chromaffin cells synthesize, and store catecholamines. Upon cell stimulation that occurs during stress, adrenaline and noradrenaline are liberated into circulation to activate target organs. Besides catecholamines, chromaffin granules contain a large variety of molecules, essentially peptides and proteins which are co-secreted with the catecholamines.

Amongst the water-soluble proteins, chromogranins/secretogranins represent the major components. The first member of this family, chromogranin A (CGA) was identified more than 30 years ago as a component exocytotically co-released with catecholamines from adrenal glands. Since these first discoveries, several components have been characterized, sharing in common to be ubiquitously distributed in endocrine, neuroendocrine and nerve cells, to be stored in dense core secretory granules, to be acidic in nature and to most of them to be processed into low molecular weight peptides (see review by Simon and Aunis 1989). These protein components include chromogranin B/secretogranin I (CGB), chromogranin C/secretogranin II (SgII) and some others.

The present chapter deals first with the structural properties of chromogranins. From the structure of chromogranins/secretogranins, the dictinction between chromogranins and secretogranins requires clarification. Recent reports on post-translational modifications bring some light on the

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processing of chromogranins. Finally, the function of chromogranins as precursors to putative biologically active peptides is now well accepted ; it will only be shortly discussed since several chapters in this volume cover this aspect.

2. STRUCTURAL PROPERTIES OF CHROMOGRANINS

2.1 The Primary Structures

In 1986, the cloning of bovine CGA was reported by two independent groups (Benedum *et al* 1986, Iacangelo *et al* 1986). There is a unique gene for CGA protein (15 kbp including upstream regulating regions), and the cDNA clone hybridizes to a unique mRNA of 2100 bases. The open reading frame codes for a protein of 449 amino acids (Fig. 1) and the first 18 amino acids represent the signal sequence responsible for the passage of the nascent protein to and through the membrane of the rough endoplasmic reticulum. CGA is encoded by 8 exons : exon I and a short part of exon II contain the signal peptide, the rest of exon II starts the N-ending domain of CGA, exon III codes for the disulfide bonded S-S loop and a part of vasostatin I (up to residue 44), exon IV codes for the C-terminal domain of vasostatin I, exon V extends to the end of vasostatin 11, while exons VI and VII encodes for long internal regions of the protein and a minor part of exon VIII codes for the C-terminal end of CGA from amino acid 404 to amino acid 431. Dibasic sites are located at positions corresponding to exon ends, particularly in the N-terminal region of CGA implying structural relationship with the functional activity of these domains.

Since the first sequencing of bovine CGA, the structure of the protein from a variety of species has been reported. The position of splicing sites and the size of the 7 introns indicate a strong phylogenic conservation. A striking observation is that whereas the sequence homology in domains corresponding to exons VI to VII is particularly low (14-30% homology between frog and human, see Turquier *et al*, this volume), the N-terminal(1-76) and C-terminal ends are well conserved. In Fig. 2, the sequences of CGA_{1-76} from species reported so far have been aligned. It is clear that this part of CGA molecule represents a highly conserved segment, inasmuch occasional changes appearing conservative for most of them. This high conservation is indicative of important biological activity for this domain.

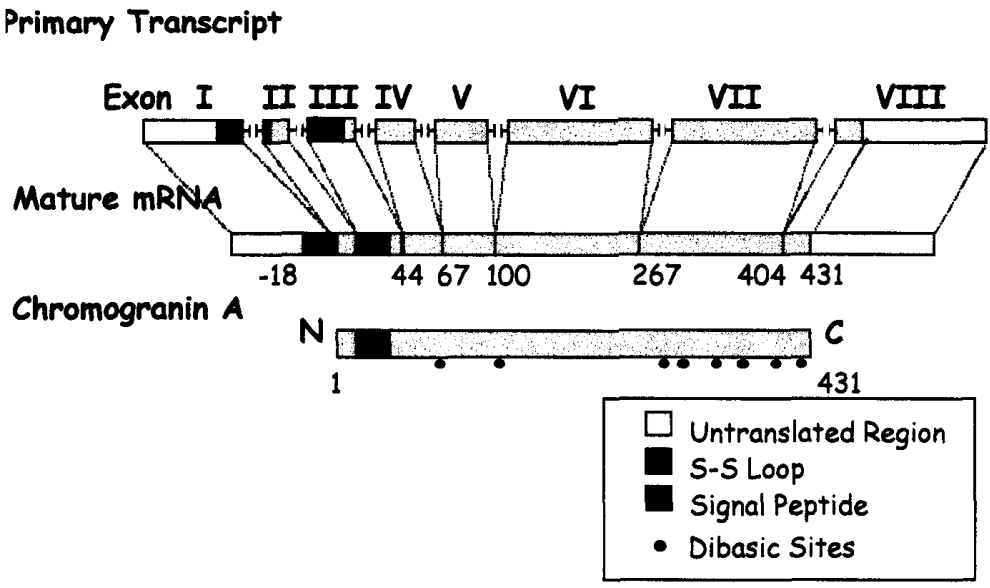

Figure 1. Structure of the primary transcript of bovine chromoganin A, of the mRNA and the protein. Eight exons code for the unique mRNA and a single protein chain.

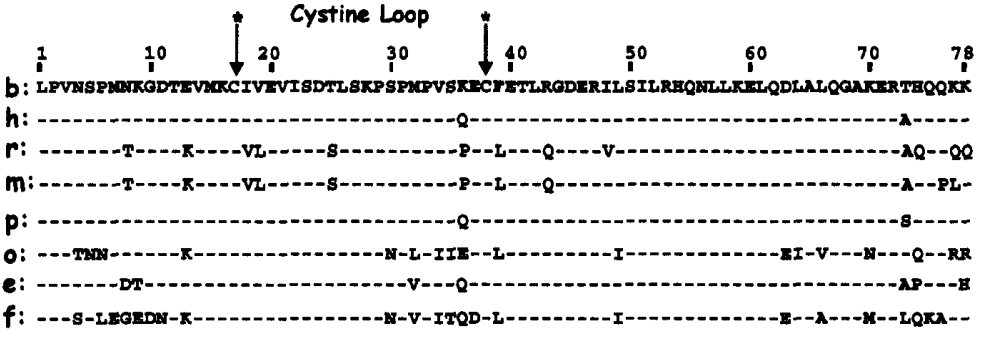

Figure 2. Structure of the 1-76 domain of bovine (b), human (h), rat (r), mouse (m), pig (p), ostrich (o), equine (e), and frog (f) CGA. This domain corresponds to vasostatin I, a peptide that is found as such in adrenal glands and in chromaffin granules after processing (Helle et al 1993, Lugardon et al 2000). Note that most of the changes are highly conservative : homology between i.e. frog and human vasostatin-I reaches 83%. The cystine loop present in all CGAs studied so far results from the formation of a disulfide bond between cysteine residues 17 and 38. Sequences of CGAs from bovine, human, rat, mouse, and pig have been collected in Simon and Aunis (1989), from ostrich in Lazure et al (l990), from equine in Sato et al (this volume), and from frog in Turquier et al (this volume).

The 3-D structure of CGA has not been extensively studied since it was demonstrated tha CGA adopts a random coil structure. CGA sequence includes ca. 10% proline residues inducing many turns and concomittant flexibility property. CGA 3-D structure is governed by medium ionic composition (Yoo and Albanesi 1990, Angeletti *et al* 1992) and in addition, depending on pH, monomeric, dimeric (100 kDa) and tetrameric (192 kDa) forms are detectable under equilibrium (Yoo and Lewis 1992).

The sequence of CGB has also been described (Benedum *et al* 1987) and the structural organisation of the gene has similar features to the CGA gene (Gerdes and Glombik, this volume) : 5 exons encode for 5 structural domains in CGB molecule. CGB N-terminal domain encoded by exons 2 and 3 shares strong homology with CGA N-terminal domain encoded by exons 2 and 3 : this is illustrated in Figure 3. Homology is also encountered for the C-terminal ends of CGA and CGB ; the structural relationship on both N- and C-terminal ends between the two proteins and the overall similarity in the organisation of their gene might be interpreted as CGB resulting from CGA gene duplication that appeared during evolution.

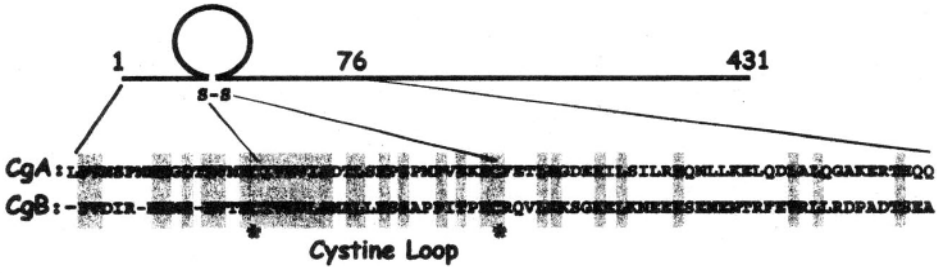

Figure 3. Sequence comparison of CGA and CGB N-terminal 1-76 domains. Note the homology of sequence particularly in the first 1-44 amino acids (corresponding to exons I and II in CGA gene). The disulfide bonded cystine loops are present in both proteins sharing the strongest homology.

The chromogranin/secretogranin family comprises other members. Chromogranin C/secretogranin II has been discovered on early 80's (Rosa and Zanini 1981) and its sequence (Gerdes *et al* 1989) appears to be rather divergent to that of CGA and CGB. The only similarity is the acidic nature as a consequence of the high content in acidic amino acids. The same observation applies to other acidic soluble proteins found so far in large

dense core secretory granules which strongly supports the suggestion that the true chromogranins are CGA and CGB : the name chromogranin has to be strictly reserved to these two proteins.

2.2 Post-translational Modifications

2.2.1 Bovine Chromogranin A

The bovine adrenal medullary CGA purified from chromaffin granules displays an apparent molecular mass of 70 kDa as estimated by SDS-polyacrylamide gel electrophoresis. The difference between its apparent molecular mass and the expected theoretical value deduced from amino acid composition (48 kDa) results in part from post-translational modifications, i.e. glycosylation and phosphorylation, in addition to the high content in acidic residues (21%). CGA has early been described as a *O*-glycoprotein containing 5.4% carbohydrate (mass/mass), consisting of di- and trisaccharides (Kiang *et al* 1982) and as a phosphoprotein with a ratio of 5 phosphorylated residues per protein molecule (Settleman *et al* 1985). It is only recently that post-translational modification sites were fully located and structurally identified using mild proteolysis, peptide separation, microsequencing and matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (Strub *et al* 1997).

Seven post-translational modification sites have been detected : two linked *O*-glycosylated moieties, each consisting of the trisaccharide NeuAcα 2-3Galβ l-3GalNAcα1, are located in the middle part of the protein on Ser_{186} and on Thr_{231} (Metz-Boutigue *et al* 1993). The glycan moiety on Ser_{186} is included in the antibacterial peptide named chromacin (Strub *et al* 1996). Four phosphorylation sites are present on serine residues at positions Ser_{81}, Ser_{307}, Ser_{372} and Ser_{376} and one additional phosphorylation site is located on residue Tyr_{173} (Fig. 4). It is interesting to note that no modification occur in the N-terminal domain of CGA. This was recently confirmed by studying the structure of CGA_{1-76} peptide purified from bovine chromaffin granules with the use of potent MALDI-TOF mass spectrometry (Lugardon *et al* 2000).

More recently, using a different mass spectrometry fragmentation approach, two *O*-glycosylation sites and five phosphorylation sites were also characterized (Bauer *et al* 1999). The glycosylation on Thr_{231} residue was confirmed, whereas glycosylated Ser_{167} was identified instead of Ser_{186}. Phosphorylation of serine residues in positions 81, 372 and 376 was confirmed with additional but partial phosphorylation on Ser_{124} and Ser_{297}.

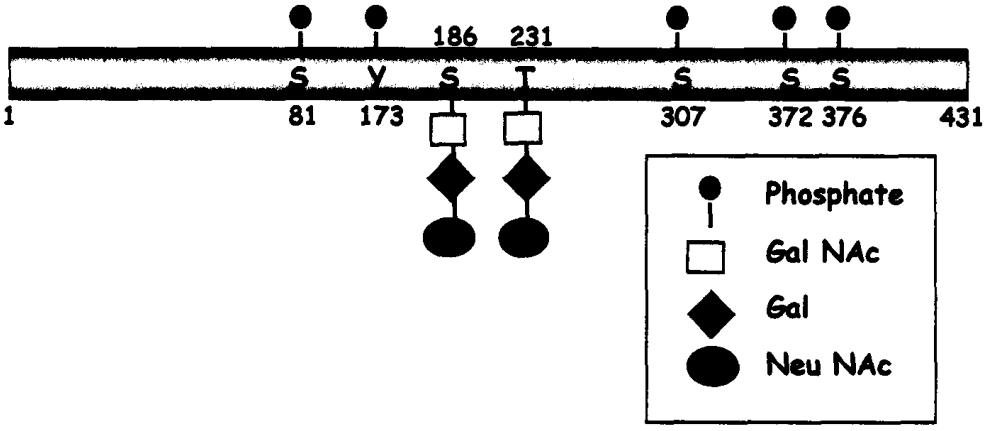

Figure 4. Post-translational modifications of bovine chromaffin granule CGA. Modifcations occur on serine (S), threonine (T), and tyrosine (Y) residues. Two O-glycosylated moieties with similar structures were found on Ser186 and T231. Five phosphorylated sites were identified (Strub et al 1997).

The location of the two glycan moieties in the central domain of CGA protects this part of the protein from proteolytic attack that occurs primarily and recurrently on N- and C-terminal ends of CGA (Metz-Boutigue *et al* 1993).

The sites bearing post-translational modifications on bovine CGA are highly conserved when analysing human, pig, rat, mouse CGA with bovine protein (Strub *et al* 1996), apart residue Tyr_{173} that is present only on the bovine and human CGA. Concerning enzyme mechanisms involved in phosphorylation of CGA, few studies have been reported yet. Sequence analysis reveals that some sites might be phosporylated by cyclic GMP-dependent kinase (Ser_{81}), cyclic AMP-dependent kinase (Ser_{307}, Ser_{372} , Ser_{376}) and/or protein kinase C (Ser_{81}, Ser_{372}, Ser_{376}). There is only one recent study that showed that CGA phosphorylation in cultured chromaffin cells might be directly linked with the secretory activity of cells (Yanagihara *et al* 1996).

2.2.2 Human Chromogranin A

Since 1989, it is well known that chromogranins can serve as immunocytochemical markers for neuroendocrine tissues and as a diagnostic tool for neuroendocrine tumors provoking a large interest in clinical studies

(Huttner *et al* 1991, Degorce *et al* 1999). However although the sequences of both human CGA and CGB have been reported nothing is known concerning their structure due to difficulty in purifying these proteins. It has been found that long fragments of CGA in rather large quantity are excreted into the urine of patients with carcinoid tumors (Stridsberg *et al* 1993). In a recent study we took advantage of this finding and used this fluid as the source for purifying the major truncated fragment CGA_{79-439}. We could obtain sufficient material to allow for the characterization of post-translational modifications (Gadroy *et al* 1998). Using mild proteolysis, HPLC, microsequencing and mass spectrometry analysis, six post-translational modifications were identified. Three *O*-linked glycosylation sites were located in the core of the protein at position Thr_{163}, Thr_{165} and Thr_{223} and three phosphorylation were located in the middle and C-terminal domain on residues Ser_{200}, Ser_{252} and Ser_{315}. The glycan moieties are variable from one site to the other composed of di-, tri- and tetrasaccharides. The phosphorylation sites are divergent from what was expected from conservative sequences provided from studies on bovine CGA. This results from the carcinoid nature of the protein used in this study ; this is supported by the observation that the structure of glycan moieties on human protein from carcinoid tumors GalNAcα-1 and Galβ1-3GalNAcα-1 correspond to antigens T and Tn, present on glycoproteins from tumoral origin. These glycan moieties on CGA from tumoral source are likely to exert specific protection from degradation and thus induce the genesis of fragments that do not occur in normal tissues. Therefore the post-translational modifications of human CGA from normal tissues await for complete determination albeit they are likely to be close to the ones reported for the bovine protein.

2.2.3 Chromogranin B and Secretogranins

Although the sequence of CGB and most if not all secretogranins are known, structural analysis have not been carried out so far. CGB is a glycoprotein containing phosphate and sulphate but the location of the sites bearing post-translational modifications and the complete nature of them await for their determination. In our laboratory we are currently studying the modifications on bovine CGB ; the first results show that they are complex and located through along the molecule with numerous phosphorylation sites (Metz-Boutigue and Aunis, unpublished data).

3. FUNCTIONS

Since their discovery, one major question has been the biological function of the chromogranins and secretogranins. Answers are not simple, and it is still an open questions although some clues has emerged on the last years, as explained below and in the chapters of this volume.

3.1 Sorting Mechanisms

Chromogranins are stored at rather high concentration in large dense core secretory granules of most endocrine and neuroendocrine cells and of many nerve cells in periphery and brain (Simon and Aunis 1989, Winkler and Fischer-Colbrie 1992). It was early thought that CGA could represent an element important in the binding of catecholamines in chromaffin granules thus lowering intragranular osmotic pressure that reaches several hundred mOsmol taking into account all individually stored components (see Helle, this volume). CGA has been shown to bind large amount of free Ca^{2+} (18 mol/mol CGA) but with weak affinity (Kd = 54 µM) (Reiffen and Gratzl 1986) and also to interact with ATP and catecholamines. Interactions between ATP, Ca^{2+} and catecholamines have been demonstrated to occur in vitro in reconstituted medium (Kopell and Weshead 1982); they were also observed in chromaffin granules by nuclear magnetic resonance although they seem to involve CGA and other proteins (Daniels *et al* 1978).

The presence of high CGA level in secretory granules was also interpreted as a specific mechanism in Golgi apparatus to concentrate chromogranins in secretory granules of the regulated pathway; this observation directed further research to this mechanism. Gerdes and Glombik (this volume) show that the disulfide bonded cystine loop in the N-terminal domain CGB corresponding to exon 3 plays a crucial role in the sorting of this protein. As CGA possesses an analogous disulfide bonded cystine loop in its exon 3, it is assumed that this domain in CGA plays a similar role as for CGB sorting although this point needs to be clarified.

Other domains of CGA (in exons 4-7) and CGB (in exon 4) have been found to be involved in multimeric interaction of these proteins suggesting a role in the pH/Ca^{2+}-dependent aggregation of these two proteins. At this stage numerous questions remain unanswered, concerning i) the specificity of the loop in sorting mechanism with regard to other proteins that do not possess this structure and that are still sorting and concentrated in secretory granules, ii) the apparent absence of structural organisation of CGA within secretory granules as deduced from biophysical technique analyses, iii) the requirement of membrane components as binding sites to CGs since up to now no specific attachment sites present in all secretory granules of the regulated pathway have been clearly identified.

3.2 Chromogranin/Secretogranin Processing

The early use of antibodies directed against CGA, CGB or SgII revealed many low molecular weight immunoreactive components. As shown on Figure 5, native CGA with the (apparent) molecular mass of 70 kDa had to be considered as the precursor to components resulting from proteolytic processing. The same observation was also made to other granule components.

A variety of proteolytic enzymes have been identified in large dense core secretory granules and they are responsible for the processing of granule proteins (see Laslop *et al,* this volume). Both CGA and CGB sequences are characterized by the presence of numerous dibasic sites representing putative site of cleavage to trypsin-like proteolytic enzymes although some of them are not necessarily used as such (Metz-Boutigue *et al* 1993). Most of these dibasic sites are very well conserved across species studied so far which should be of significance with regard to the biological function of generated peptides (Metz-Boutigue *et al* 1993, 1995).

A thorough study on CGA and CGB processing has been performed showing that in chromaffin granules these two proteins are attacked on both their N- and C-terminal sides giving rise to multiple low molecular chromogranin-derived peptides (Metz-Boutigue *et al* 1993, Strub *et al* 1995, Dillen *et al* 1993).

The processing of chromogranins is however rather complex because using immunocytochemistry generation of peptides appeared to be cell and tissue specific (Cetin *et al* 1993, Kimura *et al* 1995) giving rise to a multitude of peptides that are released upon cell stimulation and recovered as circulating in blood and lymph or present in fluids as synovial (Metz-Boutigue and Aunis, unpublished), salivary (Kanno *et al* this volume) and cerebrospinal (Aunis and Metz-Boutigue, unpublished) fluids.

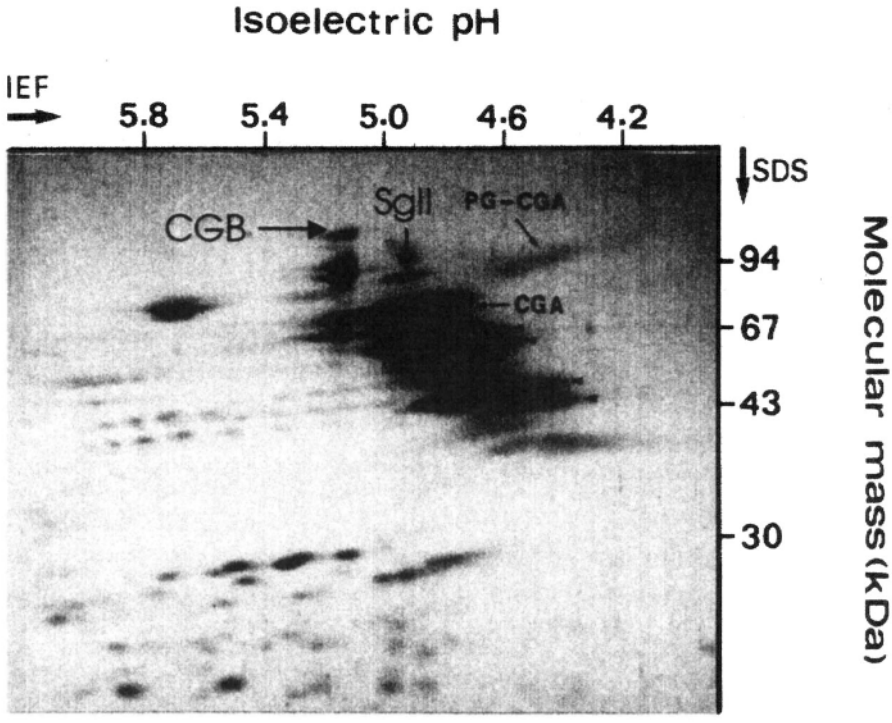

Figure 5. Separation by 2-D polyacrylamide gel electrophoresis of soluble proteins present in bovine adrenal medulla chromaffin granules. CGA precursor is indicated together with CGA low molecular weight derived peptides (small arrowheads). Long arrow points to CGB precursor (apparent molecular mass of 100 kDa), while the short arrow points to SgII precursor (apparent molecular mass of 90 kDa). PG-CGA indicates the polyglycopeptide recognized with antiserum directed against CGA but the structure (and the function) of which remaining to be determined yet.

3.3 Chromogranin-Derived Peptides

The discovery that pancreastatin, a novel peptide purified from pancreas able to inhibit insulin secretion from pancreatic ß-cells evoked with glucose was a CGA-derived peptide (Tatemoto *et al* 1986) paved the way to the suggestion that CGA might be the precuror to biologically active peptides and as a result should be considered as a prohormone protein (Eiden 1987, Huttner and Benedum 1987, Simon and Aunis 1989, Helle and Angeletti

1994). Since then, the proteolytic processing of chromogranins became a topic of growing interest ; chromogranin-derived peptides have been purified from tissues and characterized and for some of them biological activities have been attributed.

As early as 1988, it was established that CGA is the precursor to fragments able to inhibit the secretory activity on chromaffin cells (Simon *et al* 1988). It was only recently that this fragment has been identified as catestatin, a novel $CGA_{344-364}$ peptide characterized as a non-competitive nicotinic cholinergic agonist (Mahata *et al* this volume). This 21-amino acid peptide also displays inhibitory activity on nicotine desensitization at rather low concentration. No specific receptor has been identified yet. This peptide may be active on the regulation of chromaffin cell secretory activity as an autocrine regulating factor, however it remains to establish whether catestatin is generated in and released from secretory granules.

Pancreastin ($CGA_{248-293}$) has been extensively studied (Sanchez-Margalet, this volume). In addition to its activity on pancreatic ß-cells this long 49 amino acid peptide is able to modulate secretion in various glands and regulate glucose, lipid and protein metabolism in liver and adipose tissues. However, pancreastatin is present in a poorly conserved domain in CGA. This region shows a large nucleotide deletion in the frog gene coding for the region (Turquier *et al* this volume) ; homology stands as low as 45% and 55% when mouse is compared to bovine and rat to human respectively. Because of the low conservation of this region, the validity of its biological function is still an open question. Since pancreastatin also modulates glycogen metabolism in hepatocytes via a phospholipase C-linked G-protein signalling pathway (Sanchez-Margalet *et al* 1996), a specific receptor may exist, but still awaits complete identification.

Another extensively studied peptide is vasostatin, located in the N-terminal part of CGA (Helle, Brekke *et al* this volume). This CGA_{1-76} peptide has been named vasostatin-I because of its vasoconstriction inhibitory activity on isolated human blood vessels (Aardal *et al* 1993). This CGA region is the most conserved across species displaying 71% homology between frog and human (Turquier et al 1999). Some variation occurs concerning the rat peptide because CGA_{1-129} (known as B-granin, see Hutton *et al* 1987) is generated in place of CGA_{1-76} due to the absence of cleavage sites in position 76. CGA_{1-113} peptide named vasostatin-II exists also in adrenal medulla (Metz-Boutigue *et al* 1993) and it exerts activity on blood vessels. Smooth muscle cells display saturable binding sites to CGA_{1-76} and CGA_{1-40} fragments with a unique affinity constant close to 50 nM, a value that has to be compared to the concentration of circulating CGA-derived peptides in mammalian, to 1-7 nM (Stridsberg *et al* 2000). The role of vasostatin in the stress response could represent an important biological

function in addition to the role of catecholamines in regulating blood circulation demand during emergency and recovery. The identity of the vasostatin-binding receptor present on smooth muscle cells has yet to be identified and might represent a challenging step due to the low number of copies expressed on cell surface.

A member of the secretogranin family, SgII has been shown to be the precursor to a 33-amino acid peptide named secretoneurin that has been isolated from frog brain (Vaudry and Conlon 1991) and is also present in mammals (Kirchmair *et al* 1993). This peptide has chemotaxis property on immune cells (Reinisch *et al* 1993) and induces dopamine release (Agneter *et al* 1995, Saria *et al* 1993, Fischer-Colbrie *et al* this volume).

In addition to these CGA-derived fragments, some others have been identified but less studied. This is the case of parastatin ($CGA_{347-419}$) that causes inhibition of parathyroid cell secretion (Fasciotto *et al* 1993). Many CGA- and CGB-derived fragments with various length have been detected in secretory granules from various endocrine tissues including brain and shown to be released but no specific biological activity has been attributed to them yet.

4. ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF CG-DERIVED PEPTIDES

Our discovery of antibacterial activity in CGA was truly serendipitous. A few years ago when setting up a new radioimmunoassay for human CGA as diagnostic kit in cancer (Degorce *et al* 1999, Degorce this volume), we made a plasmid construction to express the human protein in bacteria (Taupenot *et al* 1995). To our great surprise we observed that the overnight expression of human CGA resulted in the complete disappearance of the host bacteria. This killing effect was not due to the plasmid itself and transfection process but was the result of CGA accumulation in bacteria. We concluded that there should be antibacterial activity associated with CGA. This initial puzzling observation led us to systematically examine the presence of antibacterial activity on each fraction isolated from HPLC column used to separate water-soluble peptidic material present in bovine chromaffin granules.

We were able to detect a large number of peptides active against the growth of Gram+ and Gram- bacteria (Metz-Boutigue *et al* 1998). The first peptide we identified was secretolytin a peptide corresponding to the C-terminal sequence of CGB (614-626) that was active in the μM range against Gram+ bacteria. A larger CGB-derived fragment (564-626) named

chrombacin was also active completely inhibiting the growth of *M. luteus* and *E. coli* at 2 μM. Secretolytin was found to possess structural homology with cecropins, a family of peptide isolated from insects and possessing potent antibacterial activity.

The second granule component we identified was enkelytin, a peptide located in a very conserved region of proenkephalin A able to act against pathogenic strain as *S. aureus* at 100 nM concentration. This peptide is post-translationally phosphorylated on two serine and full activity requires these modifications (Goumon *et al* 1998). The synthetic bisphophorylated peptide was shown to be fully active too (Goumon *et al* 1998). NMR conformational studies indicated strong structural requirements for activity (Kieffer *et al* 1998). Neither secretolytin nor enkelytin display any lytic activity on mammalian cells tested so far including erythrocytes. These two peptides are released from secretory granules upon chromaffin cell stimulation.

More recently, one peptide naturally generated from CGA processing in chromaffin granule matrix was identified as possessing antibacterial activity working at μM concentration ; this peptide was found to correspond to CGA_{1-76} vasostatin-I (Lugardon *et al* 2000). In addition to its antibacterial activity vasostatin-I displays antifungal activity since it is able to kill a large variety of filamentous fungi and yeast cells in the μM range (Metz-Boutigue *et al*, this volume). The antifungal spectrum displayed by vasostatin-I is quite large, also shared by the recombinant human peptide and synthetic rat vasostatin-I peptide. This antifungal activity of vasostatin-I complements its antibacterial activity providing highly benefitial survival strategy if one considers that these two activities represent functional biological activity. To a further characterization of vasostatin-I immunological detection of the peptide was carried out in secretions from human polymorphonuclear neutrophils (PMN) : the peptide was detected suggesting its presence in infectious fluids (Lugardon *et al* 2000).

The presence of enkelytin has also been shown in various infectious fluids and in PMN secretions (Goumon *et al* 1998). Cooperative action of vasostatin I and enkelytin with other antibacterial peptides such as defensins (Goumon *et al* 1998) is likely to take place in these fluids although it remains to be shown. Immunoblots of PMN secretory material with anti-vasostatin-I antibody revealed a CGA pattern similar to that reported for chromaffin granules. Therefore the presence of CGA and CGA-derived peptides in immunocytes is a new finding that opens new direction in neuroimmune field. Interestingly recent investigation in our laboratory has shown that microglial cells isolated from newborn rat brain and maintained in primary culture from quiescent state become activated when nM concentration of CGA is added to culture medium (Taupenot *et al* 1996). The activation of microglial cells was due to the N-terminal CGA_{1-76}

fragment vasostatin-I (Ciesielski-Treska *et al* 1998) since this fragment induced activation too. Activation of microglial cells result in the release of factors in the extracellular fluid that are toxic to cultured rat brain neurons. Interestingly when cultured medium conditioned with CGA-activated microglial cells was added to cultured neurons these cells started on irreversible apoptosis or necrosis. This observation suggested that in brain nervous cells and immune cells are cross-talking via CGA-derived peptides. Activation of microglial cells has been shown to occur in neuropathogenic syndroms as Alzheimer and Parkinson diseases and it has been proposed that CGA-derived peptides that are abundantly present in senile plaques or Lewis bodies may play some role yet to be determined (Ciesielski-Treska and Aunis this volume).

At this point, in mammals, vasostatin-I which is the first antifungal CGA-derived peptide isolated so far from adrenal chromaffin cells, represent a new weapon in innate immunity. We propose a role for this peptide during stress situations, protecting animal organisms from bacterial and fungal invasions. All these peptides thus represent a shield protecting animals from infections until acquired immunity becomes activated.

5. CONCLUSION

Although a large amount of work has been carried out since the discovery of the first chromogranin more than thirty years ago, many questions have yet to be answered. The structure, expression and localization of the chromogranin/secretogranin proteins are now well established. However, the crucial questions concern the biological function of these proteins and/or the peptides derived from them.

As an example vasostatin-I represent a multifacet component since a variety of different properties seem to be associated to this peptide. Therefore, the true challenge for the next years is to define the specific receptors on target cells and to understand how the peptide penetrates and kills bacteria and fungi. What could be the membrane interacting domains involved in sorting and aggregation, is also an important question to address, for the vasostatins and all granin-derived peptides that are generated in endocrine cells and neurons.

New vistas concern the role of CG-derived peptides in the neuroimmune field. The recent findings converge to point out major functions, opening the field and serving as challenges for future investigations.

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PART 2

INTRACELLULAR FUNCTIONS

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SIGNAL-MEDIATED SORTING OF CHROMOGRANINS TO SECRETORY GRANULES

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1. INTRODUCTION

The proteins chromogranin A (CGA) and B (CGB) are constitutents of neuropeptide- and hormone-containing secretory granules. As members of the granin protein family (Huttner *et al.,* 1991) they represent widespread marker proteins for these organelles. CGA was the first discovered member of this family (Helle, 1966, Blaschko *et al* 1967, Smith and Winkler 1967), followed by secretogranin II (SgII) (Rosa and Zanini 1981, Zanini and Rosa 1981, Lee and Huttner 1983), a more distantly related protein, and CGB (Lee and Huttner 1983, Falkensammer *et al* 1985). In addition, other members of this family have been described (Huttner *et al* 1991).

Because of their abundance, widespread distribution in neuroendocrine tissues, and specific localization in secretory granules (Wiedenmann and Huttner 1989, Winkler and Fischer-Colbrie 1992, Rosa and Gerdes 1994) the granins have been used as prime candidates to study sorting to these organelles.

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Secretory granules are the storage organelles of the regulated secretory pathway of neuroendocrine cells. The other pathway of protein secretion, common to all cells, is termed the constitutive pathway. Proteins of both pathways are synthesized in the rough endoplasmic reticulum (ER) and take the same intracellular route to the Golgi complex. In the most distal part of the Golgi complex, the trans-Golgi network (TGN), their transport routes diverge (Burgess and Kelly 1987, Tooze and Huttner 1990). Constitutively secreted proteins enter constitutive secretory vesicles (CVs) which fuse in an unstimulated manner with the plasma membrane. Regulated secretory proteins such as the granins, prohormones, and neuropeptides are sorted away from constitutive secretory proteins into immature secretory granules (ISGs) (Tooze and Huttner 1990), a short lived vesicular intermediate (Tooze *et al* 1991). This intermediate undergoes a complex maturation process including fusion of ISGs and budding of ISG-derived vesicles to remove cargo not destined for the mature secretory granule, the final product of this process (Arvan and Castle, 1998, Tooze, 1998). Regulated secretory proteins including the granins are stored in these organelles and undergo exocytosis upon an extracellular stimulus. During the last two decades extensive studies have been undertaken to identify sorting signals reponsible for directing regulated secretory proteins specifically to secretory granules. To date the granins belong to the best studied proteins in this regard and helped to elucidate sorting mechanisms and signals for the regulated pathway of protein secretion.

2. STRUCTURAL FEATURES OF CHROMOGRANIN A AND B

Besides their widespread distribution in neuroendocrine tissues CGA and CGB have one major advantage for analyzing sorting to SGs. Their structural organisation provides first clues in the search of putative sorting domains. CGB consists of 5 structural domains each encoded by a separate exon (Fig 1A) and CGA has a very similar domain structure. In particular two domains of CGA and CGB have attracted attention due to their high sequence conservation across species and between CGA and CGB (Benedum *et al* 1987, Simon and Aunis, 1989). One is located near their N-terminus, encoded by exon 3 (Pohl *et al* 1990) (Fig. IB), and the other is at their very C-terminus, encoded by exon 8 in the case of CGA (Wu *et al* 1991, Iacangelo *et al* 1991, Mouland *et al* 1994) and by exon 5 for CGB (Pohl *et al* 1990). Importantly, the domain encoded by exon 3 forms a disulfide-bonded loop structure consisting of 20 amino acids flanked by two

cysteins (Benedum *et al* 1987). The remaining parts of CGA and CGB encoded by exons 4-7 and exon 4 respectively, are much less conserved across species and between the two proteins. Nontheless they share the common feature of negative charge due to their high abundance in acidic amino acids. These domains representing more than 90% of the respective protein have been proposed to mediate the Ca^{2+}/pH-dependent aggregation (Gerdes *et al* 1989). The structural organisation provides the first clue in the search of putative sorting domains. Interestingly the more distantly related SgII which does not contain the disulfide-bonded loop structure and is encoded by one exon only (Schimmel *et al* 1992), is also highly abundant in acidic amino acids.

3. AGGREGATION – THE SOLE SORTING MECHANISM?

The phenomenon of aggregated regulated secretory proteins initially discovered by Pallade and colleagues using electron microscopy (Siekevitz and Palade 1966) was later found to be a general principle in cells bearing a regulated pathway of protein secretion (Tooze *et al* 1993, see references therein). This led to the proposal of the first function of aggregation which is the condensation of cargo for efficient storage in secretory granules. The most detailed studies on the aggregation of regulated secretory proteins were performed with the granins in biochemical approaches (Gerdes *et al* 1989, Gorr *et al* 1989, Yo and Albanesi 1990a, Chanat and Huttner 1991). Their aggregation is mediated by millimolar concentrations of calcium and a mildly acidic pH (Gerdes *et al* 1989) conditions mimicking the milieu of the TGN (Chanat and Huttner 1991). Importantly constitutive secretory markers were excluded from these aggregates (Gerdes *et al* 1989, Gorr *et al* 1989, Chanat and Huttner 1991). This observation led to the discovery of the second function of aggregation which is the segregation of regulated from constitutive secretory proteins. In consequence, an aggregation-sorting model emerged questioning the existence of specific sorting signals different from amino acid sequences mediating aggregation. However, given the necessity that during the biogenesis of ISGs at the TGN the formed aggregates have to be enwrapped by an ISG specific membrane, a specific cargo-membrane interaction has to occur. This interaction is thought to be facilitated by specific sorting signals recognizing ISG-specific membrane components in the TGN.

CGB is a paradigm for the discovery of sorting signals because both aggregative properties and a specific sorting signal have been found. Given

the domain structure of CGB we here summerize the sorting data available for the different domains and in a separate chapter, draw a comparison to the findings made for CGA.

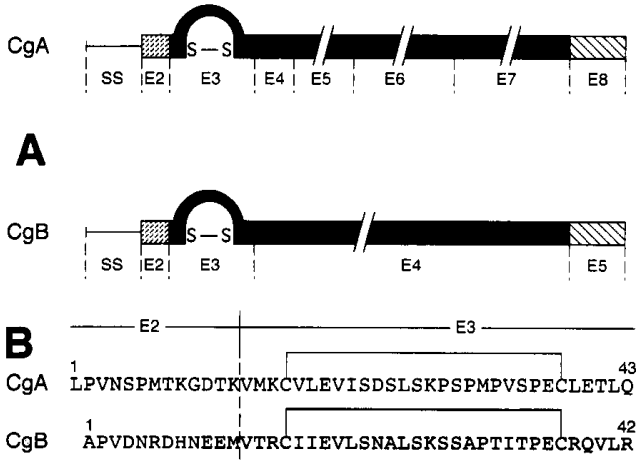

Figure 1. (A) Schematic representation of rat CGA and rat CGB. The protein domains of the mature proteins encoded by separate exons (E2-E8) are indicated. Note that exon 1 and partly exon 2 encode for the signal sequence (SS). Corresponding domains in CGA and CGB are depicted in the same shading. Black arc, disulfide-bonded loop, grey bar, highly acidic domain. (B) Alignment of the N-terminal amino acid sequences of rat CGA and rat CGB. E2, E3, protein domains encoded by exon 2 and 3 respectively; bracket, disulfide bond; grey box, specific sorting signal identified in rat CGB; numbers refer to the positions of amino acid residues in the mature proteins after cleavage of the signal peptide.

4. THE DISULFIDE-BONDED LOOP–A SPECIFIC SORTING SIGNAL

4.1 Essential Role

A first hint for the existence of a sorting signal in CGB was provided by the treatment of pheochromocytoma (PC 12) cells with dithiothreitol (DTT) leading to the reductive cleavage of disulfide bonds. Under these conditions missorting of CGB to the constitutive pathway of protein secretion was observed (Chanat *et al.,* 1993) but its aggregative properties were not affected (Chanat *et al* 1994). Based on this finding a more specific investigation on the role of the disulfide-bonded loop in the sorting of CGB was started. To investigate whether the amino acids forming the disulfide-bonded loop (Fig. 1B) are necessary for the sorting of CGB to SGs, a deletion mutant of human CGB was generated lacking amino acids 16-37 of mature hCGB including the two flanking cysteines. Ectopic expression of this deletion mutant by transfection of PC12 cells led to its sorting to SGs

whereas expression in PC12 cells by a recombinant vaccinia virus led to its missorting to the constitutive pathway of protein secretion. The main difference between the two expression systems is the presence of endogenous CGB in the transfection system as compared to its absence in the viral system due to host cell protein synthesis shut-off. Thus, the disulfide-bonded loop is essential for the sorting of CGB but coaggregation of loopless CGB with endogenous CGB containing the loop leads to sorting of these mixed aggregates in the transfection system.

The second conclusion obtained from this study is that regulated secretory proteins lacking a specific sorting signal but competent for aggregation, can be sorted via hitchhiking with proteins bearing a specific sorting signal. Direct proof for a coaggregative sorting was shown by viral coexpression of loopless CGB together with its full-length counterpart leading to sorting to SGs of the deletion mutant. In line with previous findings (Natori and Huttner 1995, Natori and Huttner 1996), this experiment shows that two classes of regulated secretory proteins may exist. One consists of proteins having both a specific sorting signal and the competence for aggregation. Proteins of the other class lack a specific sorting signal but are competent for aggregation. Their proper sorting relies on the former class. Thus, the chromogranins could play an important role as sorting helpers facilitating the sorting of other regulated secretory proteins as observed for a cleavage product of proopiomelanocortin (POMC) (Natori and Huttner 1996).

4.2 Sufficient Role

To test for a sufficient role of the loop in sorting, fusion proteins were generated containing the loop motif and al-antitrypsin (AT), a reporter protein secreted by the constitutive pathway. Sorting of AT alone with AT containing one loop, and AT with two loops, one at each terminus, were compared. Sorting of AT with one loop was significant but of low efficiency. Interestingly, AT with two loops was sorted very efficiently to SGs. By all criteria applied it was shown that amino acids 10-42 of rat CGB which form the disulfide-bonded loop motif are sufficient to direct a constitutive reporter protein to SGs (Glombik *et al* 1999). The observed missorting of the fusion proteins upon reductive cleavage of the disulfide-bond with DTT demonstrates that not only the amino acid sequence of the loop but also its three-dimensional conformation stabilized by the disulfide bond is essential for its function in sorting (Glombik *et al.,* 1999). Importantly, the lack of Ca^{2+}/pH-mediated aggregative properties implies that the sorting mechanism of the loop is different from milieu-dependent aggregation.

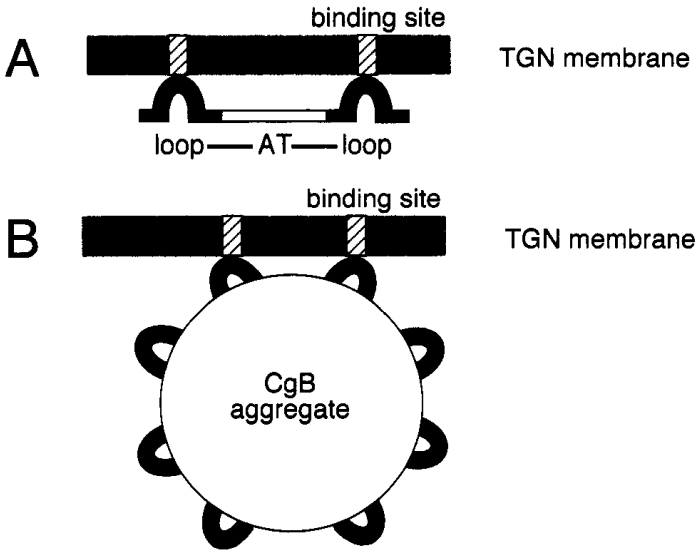

Figure 2. Sorting model based on membrane interaction of the disulfide-bonded loop in the TGN. (A) Fusion proteins consisting of AT and the disulfide-bonded loop do not undergo Ca^{2+}/pH-mediated aggregation in the TGN. Single molecules are the sorting units. Only if more than one loop is fused to AT efficient loopmediated membrane-binding in the TGN occurs. (B) CGB undergoes Ca^{2+}/pH-mediated aggregation in the TGN. The formed CGB aggregates are the sorting units to ISGs which have exposed multiple loops at their surface. More than one loop interacts with the membrane in the TGN leading to high binding efficiency.

The finding of membrane-bound full-length CGB (Pimplikar and Huttner 1992) pointed to an important role of this feature in the sorting of CGB. In line with the concept that regulated secretory cargo interacts with components of the TGN membrane giving rise to ISGs, membrane binding of the loop was detected in the TGN and at later stages of the regulated secretory pathway. Strikingly, as was observed for sorting, membrane binding was much more efficient when two loops were present on the constitutive reporter AT which does not undergo Ca^{2+}/pH-mediated aggregation (Glombik *et al* 1999). Thus, the disulfide-bonded loop of CGB represents a specific sorting motif which provides a link between cargo in the TGN and enwrapping membrane during ISG biogenesis. The synergistic effect of loop duplication on sorting and membrane binding efficiency led to the following hypothesis: Under physiological conditions the sorting units are not single CGB molecules but aggregates which presumably have exposed multiple loops on their surface. All of these loops may interact with TGN membrane components resulting in a high affinity binding (Fig. 2).

This suggests that in addition to concentration of regulated secretory proteins and segregation from constitutive secretory proteins, aggregation has a third function. This function is the multimerization of sorting signals of regulated secretory proteins (Fig. 2). Similar mechanisms for increasing the binding

affinity to a sorting receptor are known for the sorting of lysosomal hydrolases to lysosomes by the mannose-6-phosphate receptor (Tong *et al* 1989) and for the apical delivery of N-glycosylated proteins (Scheiffele *et al* 1995).

5. THE EXON 4- AND EXON 5-DOMAIN

The role of the domains encoded by the exons 4 and 5 was also assessed by the expression of AT fusion proteins. A fusion protein consisting of the exon 4 domain and AT was sorted with high efficiency to SGs in transfected PC12 cells (Glombik *et al* submitted). On the other hand a vaccinia virus expressed deletion mutant of CGB consisting only of the exon 4 and 5 domains was not sorted to secretory granules because of the absence of endogenous full-length CGB (Kroemer *et al* 1998). In biochemical experiments with perforated TGN vesicles the presence of the exon 4 domain was found to be necessary for the Ca^{2+}/pH mediated aggregation of CGB (Glombik *et al* submitted). Taken together these data are in line with the view that sorting of the fusion protein is accomplished by coaggregation with endogenous CGB. In regard to the hypothesis that aggregation has a role to increase the sorting efficiency of CGB by the multimerization of its specific sorting signal, this role can be assigned to the aggregative exon 4 domain. Indeed, expression of a fusion protein corresponding in its structure to a CGB in which the exon 4 domain was replaced by the non-aggregative reporter AT revealed a low sorting efficiency (Glombik *et al* submitted).

To investigate the role of the exon 5 domain of CGB in sorting to the regulated pathway, a fusion protein consisting of this domain and AT was expressed in transfected PC12 cells. The fusion protein was not found to be sorted to SGs (Glombik *et al* submitted). This finding is remarkable in light of the high sequence conservation of the exon 5 domain and suggests a so far unknown important function for this domain. Whether the role in oligomerization described for the corresponding C-terminus of CGA (Yoo and Lewis 1993, Thiele and Huttner 1998) is responsible for the high sequence conservation needs to be clarified.

6. IMPLICATIONS ON THE SORTING OF CGA

Two types of sorting information have been found in CGB. One is the specific sorting signal located in the exon 3 domain which exerts its function by the interaction with the TGN membrane. The other is located on the large exon 4 domain mediating Ca^{2+}/pH dependent aggregation. Only both types together lead to the high sorting efficiency of CGB under physiological conditions (Kroemer *et al* 1998).

Due to the fact that the disulfide-bonded loop is highly conserved between CGA and CGB (Fig 1B) it is very likely, that this structure also represents a sorting signal in the case of CGA. Although the influence of the CGA loop on sorting was not addressed so far, the second feature of a specific sorting signal, i. e. membrane binding, was analyzed in much detail by employing peptides (Yoo 1993) and deletion mutants (Yoo and Kang 1997). Interestingly, a peptide comprising the amino acid sequence of the CGA loop but lacking the two flanking cysteines bound to granule membranes (Yoo 1993). This suggests that the stabilization of the three-dimensional structure of the loop motif is not essential for its membrane binding. Furthermore, CGA interacts with the luminal loop of the inositol 1,4,5-trisphosphate receptor (Yoo and Lewis 1994) which has been proposed to exist in SG membranes (Blonde1 *et al* 1995, Gerasimenko *et al* 1996, Yoo and Albanesi 1990b). However, other studies reported that the Ca^{2+} stores of SGs are inositol 1,4,5-trisphosphate insensitive doubting the presence of inositol 1,4,5-trisphosphate receptors in secretory granules (Prentki *et al* 1984, Zacchetti *et al* 1991).

Interestingly the loop of CGA was found to mediate dimerization of CGA (Corti *et al* 1997, Thiele and Huttner 1998). It was proposed that this oligomerization is a prerequisite for the Ca^{2+}/pHdependent aggregation (Thiele and Huttner 1998) which is well characterized for CGA (Gorr *et al* 1989, Colomer *et al* 1996, Yoo and Albanesi 1990a). In analogy to exon 4 domain of CGB, the aggregative properties of CGA are most likely localized on the domain encoded by exons 4-7 (Fig. 1A). It can be surmized that the role of aggregation in sorting of CGA is similar to the one described for CGB.

7. SORTING MODEL

Based on the data available for CGB concerning its features aggregation, sorting signal and membrane association, we propose a sorting model that dissects the continuous sorting process of secretory cargo into three

mechanistic steps. Because the three features at least in part have been reported for many other regulated secretory proteins (Glombik and Gerdes *Biochimie* 2000, in press ; see references therein) this sorting model may be of general significance.

Before their arrival in the TGN, regulated and constitutive secretory proteins presumably exist as a mixture of non aggregated molecules. In the TGN regulated secretory proteins undergo a low pH/divalent cation-induced aggregation from which constitutive proteins are excluded (step 1, Fig. 3). This leads to segregation of the former from the latter class of proteins. Aggregate formation results in multimerization of specific sorting signals on the surface of the aggregates. This has two important functions in the sorting process which are tightly linked to each other. One is the higher binding efficiency to low affinity sorting receptors in the membrane (step 2, Fig. 3), the other is the recruitment and clustering of aggregate-enwrapping ISG-specific membrane by receptor binding (step 3, Fig. 3). It follows that the selective aggregation of regulated secretory proteins is the crucial step for secretory granule biogenesis. Notably, due to coaggregation even the regulated secretory proteins lacking a membrane-interacting specific sorting signal can be sorted according to this model.

8. PERSPECTIVES

The other classical granin, secretogranin II, lacks sequence homology to the conserved disulfide-bonded loop (Gerdes *et al* 1989). The fact, that secretogranin II is sorted to SGs in the vaccinia expression system (Samenfeld and Gerdes, unpublished observation) shows that it possesses in addition to its aggregative properties a specific sorting signal. Future work will show whether, although different in amino acid composition, both signals have a common structural motif.

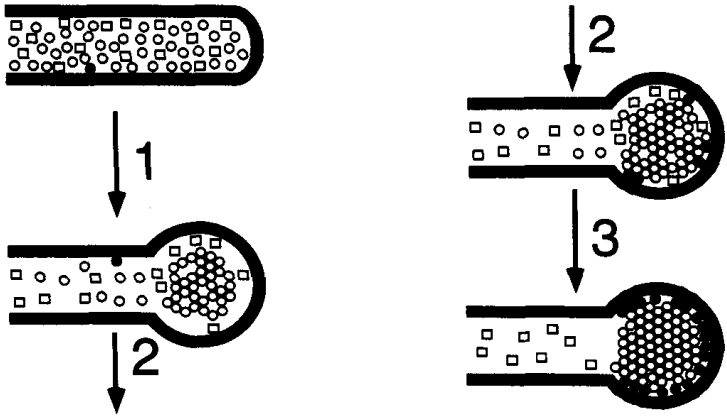

Figure 3. Sorting model. Upon entry in the TGN tubule, constitutive (squares) and regulated secretory proteins (circles) are mixed homogenously. Due to their nonaggregated state, only few molecules (filled circles) interact with low-affinity sorting receptors (black rectangle). In a first step regulated secretory proteins selectively aggregate in the lumen of the TGN (1). Aggregates as multivalent ligands efficiently bind (2) and thus cluster sorting receptors (3). Constitutive secretory proteins are excluded from the aggregates. Open squares, constitutive secretory proteins; open circles, regulated secretory proteins not interacting with a receptor; filled circles, membrane-bound regulated secretory proteins; black rectangles, sorting receptors.

Interestingly, for POMC a N-terminal disulfide-bonded loop structure not homologous to the loop of CGB was reported to contain sorting information (Cool *et al* 1995, Cool and Peng Loh, 1994). However it remained unclear at which level, i. e. TGN or ISGs, in the secretory pathway the identified sequence acted. Also the aggregative properties of this sequence were not analyzed. Nevertheless, it was reported by Loh and colleagues that the identified sequence binds to carboxypeptidas E (CPE, Cool *et al* 1997). The proposed concept that CPE is the sorting receptor of POMC, proinsulin and proenkephalin, which acts at the level of the TGN and directs the respective proteins to ISGs (Cool *et al.,* 1997) is highly debated (Irminger *et al* 1997, Thiele *et al* 1997). Irrespective of the exact role of CPE in the sorting of these three prohormones two lines of evidence exist which exclude CPE as a binding partner for chromogranins. First, in Neuro2A cells expressing CPE-antisense RNA, CGA was not missorted to CVs, whereas proinsulin and proenkephalin exhibited an increased basal release (Normant and Loh 1998). Secondly, despite the absence of CPE in exocrine tissue, CGB expressed in transgenic mice is sorted into amylase containing exocrine granules (Natori *et al* 1998). Thus, the binding site for the disulfide-bonded loop of CGB remains to be identified.

NOTES

Parts of this article have been published elsewhere.

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4

THE CONDENSED MATRIX OF MATURE CHROMAFFIN GRANULES

The Soluble Form of Dopamine β-Hydroxylase Is Catalytically Inactive

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1. INTRODUCTION

The activity of dopamine β-hydroxylase (dopamine β-monooxygenase, DBH, EC 1.14.17.1) in mature secretory granules of the bovine adrenal medulla has been reported to increase several fold on disruption of their limiting membranes (Belpaire and Laduron, 1968). This increase in DBH activity, known as DBH-latency, showed that DBH was on the lumenal side of the organelle and suggested that the vesicular uptake of one or more of the substrates could contribute to the regulation of noradrenaline (NA) biosynthesis (Belpaire and Laduron, 1968). In this context it is interesting to note that Sole et al. (1982) suggested that the vesicular dopamine (DA) uptake in cardiac sympathetic fibers limited myocardial NA biosynthesis in the cardiomyopathic hamster. Hence, in certain pathological conditions NA biosynthesis may in fact be limited by the transport of DA into the nerve vesicles. Levine et al. (1988) showed, on the other hand, that the uptake of DA in intact chromaffin granules was higher than the intravesicular NA biosynthesis, both at 0.1 and 10 mM external DA. These findings strongly suggested that in the mature chromaffin granules the supply of DA does not limit DBH activity.

As first reported by Levin and Kaufman (1961) DBH requires an external

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electron donor like ascorbate for the hydroxylation of DA to NA. Ascorbate is present in mature chromaffin granules at an estimated concentration of ~10 mM (Terland and Flatmark, 1975). The level of internal ascorbate (in chromaffin granule ghosts) has, however, been shown not to be rate-limiting for the DBH catalysed hydroxylation of DA if the external concentration is at least 2 mM (Herman et al., 1988; Dhariwal et al., 1989; Huyghe and Klinman, 1991; Wimalasena and Wimalasena, 1995). Thus, it is highly unlikely that the lack of substrates (ascorbate or DA) can explain the DBH-latency.

But how is it possible, then, to explain the large increase in DBH activity as measured *in vitro,* from 3 nmol NA/min/mg protein in intact chromaffin granules to 360 nmol/min/mg protein, i.e. a 120-fold increase in response to granule membrane solubilisation (Levine et al., 1988)? Part of the explanation may be that Levine et al. (1988) assayed the DBH activity of the lysed granules in the presence of acetate and fumarate, both known to increase the DBH activity (Craine et al., 1973). Hence, in their assay system, part of the latency obviously reflects an "anion-activation" of the enzyme (Craine et al., 1973) giving artificially high catalytic activities in detergent lysed granules compared to the DBH activity in intact chromaffin granules. A second contribution may be related to the observations by Foldes et al. (1 972) presenting evidence for an endogenous inhibitor of DBH activity in chromaffin granules. The putative inhibitor was stable on boiling and was not neutralised by Cu^{2+} or by *p*-hydroxymercuribenzoate (Foldes et al., 1972). Later, Levine et al. (1988) also presented evidence for one or more endogenous inhibitors of DBH, but no such inhibitor has so far been isolated or identified in chromaffin granules.

The aim of the present work is to study the effect of the matrix proteins (chromogranins) on the activity of DBH, and possibly extrapolate the results *in vitro* to the conditions in mature chromaffin granules *in vivo*. It is, however, important to note that the conversion of DA to NA is part of a complex physiological regulation of catecholamine biosynthesis that involves a large number of enzymes and takes place in different subcelluar compartments, as recently reviewed by Flatmark (1 999).

2. THE LATENCY OF DBH ACTIVITY IN CHROMAFFIN GRANULES DEPENDS ON THE CONCENTRATION OF GRANULE PROTEIN

The specific DBH activity in intact chromaffin granules (for experimental details, see appendix) was 12.8 ± 3.7 nmol octopamine/min/mg

protein (n = 6) with a linear relationship between the activity and the granule concentration (Fig. 1). As expected from the literature (Belpaire and Laduron, 1968; Levine et al., 1988) the DBH activity increased on solubilisation of the organelle by the non-ionic detergent Brij-58 (Fig. 1). Furthermore, it is seen that the latency of DBH, i.e. the ratio of the activity measured in the presence and the absence of the detergent, was strongly dependent on the concentration of granule protein (Fig. 2). At 0.025 mg protein/ml the DBH activity increased 6-fold (Fig. 2). At a granule concentration of 2 mg protein/ml, on the other hand, no increase in DBH activity was observed as a result of membrane solubilisation (Fig. 2).

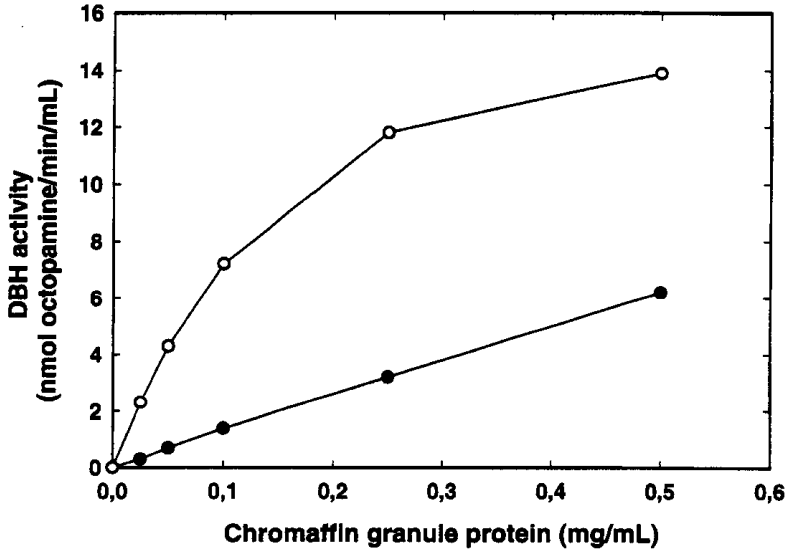

Figure 1. The DBH activity in intact chromaffin granules (•) or Brij-58 solubilised granules (o) as a function of granule protein concentration.

If the DBH latency in intact chromaffin granules was due to limited transport of the substrates across the granule membrane, one would expect a similar latency in chromaffin granule ghosts. But the latency in the ghosts was 1.8-fold at maximum (data not shown). Using 8 mM dopamine instead of 8 mM tyramine as the substrate, the latency-factor was only 1.2 (n = 5, data not shown). Hence, we did not find a statistically significant latency in the ghosts.

The specific DBH activity in chromaffin granule ghosts was 150 ± 36 nmol octopamine/min/mg protein (n = 8). Provided that DBH accounts for 20 % of the total membrane protein as found by Winkler and Westhead (1980) or 8 % as found by B. Almås (personal communication), the homospecific DBH activity in chromaffin granule ghosts was in the range 750 - 1875 nmol octopamine/min/mg DBH.

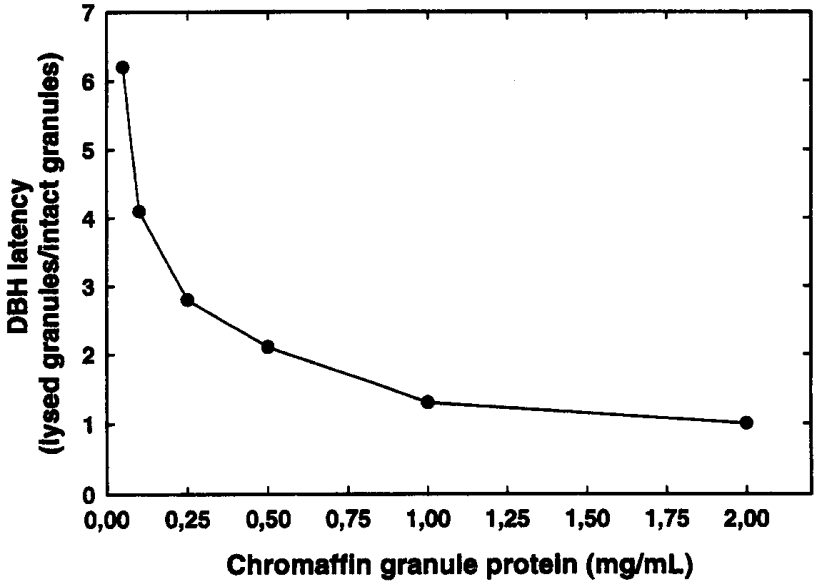

Figure 2. The latency of DBH (i.e. the ratio DBH activity in solubilised granules/DBH activity in intact granules) as a function of granule protein concentration. For the two lowest concentrations, seven different granule preparations were examined. For the other concentrations, two or three different granule preparations were examined.

It should be noted, however, that the ghosts contain a variable amount of the soluble form of DBH, as shown by Skotland and Flatmark (1979) using charge shift crossed immunoelectrophoresis. Hence, the homospecific DBH activity in the ghosts is an overestimation due to the fact that the ghosts contain residual (hydrophilic) matrix DBH (sDBH) in addition to the main, membrane-bound (amphiphilic) form of the enzyme (mDBH).

3. THE INHIBITORY EFFECT OF MATRIX PROTEINS ON sDBH ACTIVITY

The homospecific activity of sDBH in the dialysed matrix fraction decreased with increasing protein concentration, indicating that the activity of the sDBH was inhibited by a component in the matrix itself (Fig. 3). The activity of highly purified sDBH (two different preparations) was also markedly reduced from 800 ± 80 (n = 2) (control value) to 200 ± 40 nmol octopamine/min/mg sDBH when assayed in five different matrix preparations (0.5 mg matrix protein/ml). Heat treatment (95° for 10 min) of

the matrix proteins completely inactivated the endogenous sDBH activity and the heat-treated matrix inhibited the purified sDBH by 95 %.

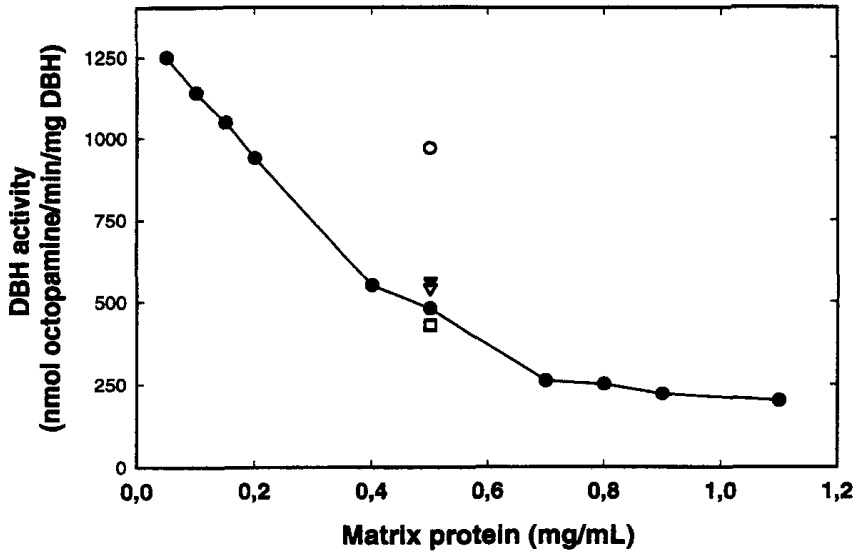

Figure 3. The homospecific activity of sDBH in a dialysed matrix fraction of chromaffin granules as a function of matrix protein concentration assuming that sDBH accounts for 4 % of the matrix proteins (Winkler and Westhead, 1980). The homospecific activity of four other matrix preparations are given at 0.5 mg protein/mL.

We also removed (precipitated) part of the chromogranins (mainly chromogranin A) from the matrix by adding 35 mM Ca^{2+} at pH 5.5 (Yoo and Albanesi, 1990). The resulting supernatant as well as the dissolved precipitated matrix proteins (chromogranins) did still inhibit purified sDBH. 20 mM Ca^{2+}, which is estimated to be present in the matrix of the intact organelles (Phillips et al. 1977) and promotes aggregation of matrix proteins if pH < 6.5 (Gerdes et al., 1989; Gorr et al., 1989), had no effect on the matrix-dependent inhibition of sDBH activity (data not shown).We also found that 20 mM NEM increased the sDBH activity in the dialysed matrix phase by 82 % ($P < 0.01$, $n = 4$) and increased the activity of the purified DBH by 70 % in accordance with the finding of Ljones et al. (1976). Finally, 10 µM Cu^{2+} activated the sDBH in the matrix by 60 %, but inhibited isolated, purified sDBH by 77 % (data not shown).

4. THE INHIBITION OF sDBH BY MATRIX PROTEINS IS ELIMINATED BY HIGH SALT CONCENTRATION

As expected from the literature (Craine et al., 1973) the addition of KCI to the assay medium markedly increased the sDBH activity (Fig. 4) as measured by a direct spectrophotometric method using 8 mM DA both as the substrate and electron donor (i.e. no ascorbate present). In addition we also found that KCI had a pronounced effect on the relationship between sDBH activity and the concentration of matrix proteins. In contrast to the non-linear relation between sDBH activity and the matrix protein concentration at zero KCI (as also found by the standard DBH assay, Fig. 3), an almost linear dose-response relationship was observed in the presence of 600 mM KCI (Fig. 4). It should be noted that very little effect was observed at around 60 mM, the estimated concentration for the total content of Na^+ and K^+ in isolated chromaffin granules (Haigh et al., 1989).

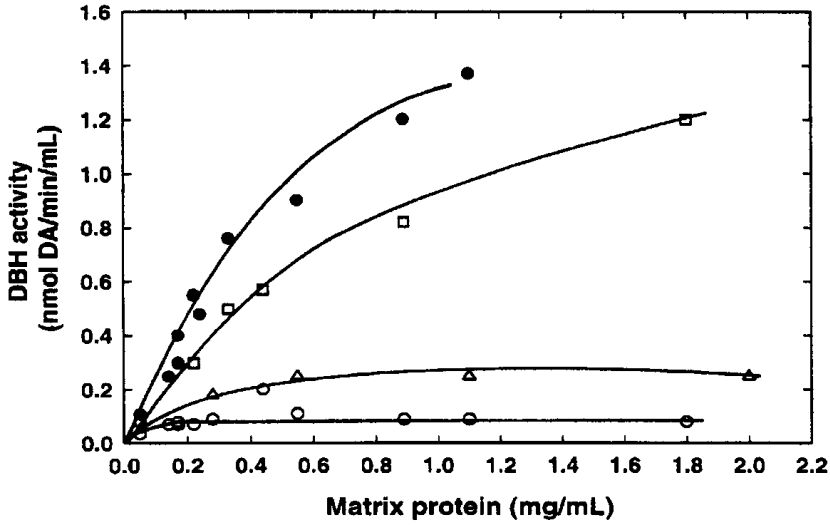

Figure 4. The effect of KCl on the sDBH activity in a dialysed matrix preparation as assayed by a direct spectrophotometric method (Levin and Kaufman, 1961; Terland et al., 1977). (O, no KCI; Δ 80 mM KCl; □, 320 mM KCl; ●, 600 mM KCl).

5. CONCLUSIONS

The important finding in the present study is not the latency per se (Fig. 1) but that the latency was highly dependent on the granule protein concentration and essentially absent at a granule concentration of 2 mg

protein/mL (Fig. 2). The lack of latency at high granule protein concentration most likely reflects the decrease in homospecific DBH activity by increasing matrix protein concentration as shown in Fig. 3. As the matrix-dependent inhibition is present in an extensively dialysed matrix fraction containing molecules ≥ 30 kDa (defined by the preparation procedure), even after heat-treatment, we conclude that the heat-stable acidic matrix glycoproteins (chromogranins) are responsible for the inhibition. A matrix fraction largely free of chromogranin A (precipitated by 35 mM Ca^{2+} at pH 5.5) as well as a preparation of redissolved precipitated matrix proteins (enriched in chromogranin A) also inhibited sDBH. This indicates that the matrix-dependent inhibition of sDBH results from the combined effect of several proteins identified in the matrix (Fischer-Colbrie et al., 1987). As the matrix-dependent inhibition of sDBH was largely overcome by high salt concentration (Fig. 4), we suspect that electrostatic interactions between sDBH and the chromogranins (Hogue-Angeletti 1977; Helle et al., 1978) inhibit the sDBH by an unknown mechanism. It should be noted that the mechanism behind the anion-activation of purified sBDH is also unknown (Craine et al., 1973).

5.1 Only mDBH Is Active in Mature Chromaffin Granules

Extrapolating to the *in vivo* situation, it should be noted that the matrix protein concentration is at least two orders of magnitude higher (180 mg/mL, see references in Njus et al., 1986) than used in our assays. Furthermore, the dense cores exist as a viscous, but fluid state at a hyperosmolar concentration of granule solutes with a restricted mobility of the intragranular solutes (Daniels et al., 1978; Sen and Sharp, 1982). We therefore conclude that sDBH is essentially catalytically inactive, *whereas only the membrane-bound amphiphilic form of DBH (mDBH) is active in the intact, mature chromaffin granules.* If mDBH accounts for 8 % of the membrane proteins [B. Almås, personal communication], the homospecific activity of mDBH in intact chromaffin granules is 800 nmol octopamine/min/mg mDBH. This figure compares well with the homospecific activity in the diluted matrix (1250 nmol/min/mg sDBH, Fig. 3), in the ghosts (750 – 1875 nmol/min/mg DBH, which most likely is a slight overestimation due to the presence of some sDBH as explained above) and for the purified enzyme (0.4 - 1.8 µmol/min/mg sDBH). Thus, our data support the conclusion that *mDBH has full enzymatic activity in the isolated mature, intact chromaffin granules.*

5.2 Is There a Role for Matrix DBH?

Chromaffin granules are specialised secretory organelles used for biosynthesis, storage and regulated release of catecholamines. They form by budding from the *trans*-Golgi network which involves a highly regulated sorting mechanism. Studies carried out in the neuroendocrine PC12 cell line have shown that the nascent granule is an immature secretory granule (ISG), defined as such because it has a distinct composition compared to the mature secretory granule (MSG) (Tooze, 1998).

The half-life of ISGs is about 45 min in PC12 cells (Tooze et al., 1991) whereas the MSGs are stable for several days in primary cultures of bovine adrenal chromaffin cells (Corcoran and Kirshner, 1990). Thus, the MSGs account for the major part of chromaffin granule population in these cells. The *in vitro* experiments in the present study strongly suggest that sDBH is essentially catalytically inactive in the condensed matrix of the MSGs also *in vivo* (schematically presented in Fig. 5).

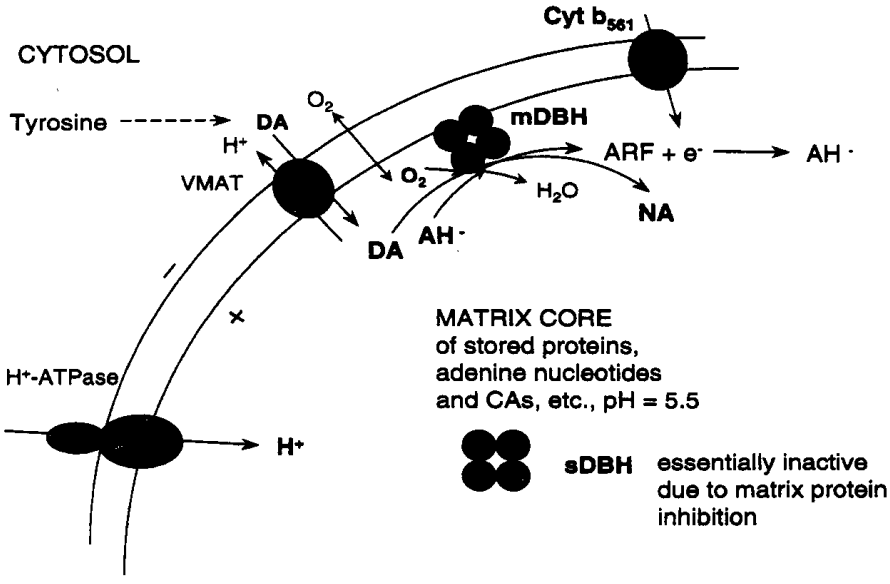

Figure 5. Schematic presentation of the conversion of DA to NA in mature chromaffin granules. As can be deduced from the observation in Fig. 3, sDBH has essentially no enzymatic activity in the condensed matrix. Hence, mDBH is solely responsible for the conversion of DA to NA in the mature chromaffin granules. *In vivo*, vesicular DA uptake is via VMAT (vesicular monoamine transporter), driven by the transmembrane proton electrochemical gradient generated by the V-type H^+-ATPase. Ascorbate free radical (AFR) generated by DBH is reduced (one-electron reduction) by cytochrome b_{561} (cyt b_{561}) to regenerate ascorbate (AH^-).

Chromogranins A and B have been proposed to play essential roles in the selective aggregation and sorting of matrix proteins to ISGs (Yoo, 1996). Furthermore, DBH and chromogranin A are incorporated simultaneously during granule biogenesis (Corcoran and Kirshner, 1990). Thus, sDBH may be inactive also in the matrix of ISGs. An important finding by Stjärne (1971) is that the newly formed NA (from radio-labelled tyrosine) is preferentially released from the isolated cat adrenal gland in response to acetylcholine or carbachol stimulation. These findings clearly demonstrate that this NA biosynthesis takes place in a release-ready pool of mature chromaffin granules, in which mDBH is active, located close to (docked with) the plasma membrane.

It is interesting to note that the net myocardial release of NA (unstimulated, non-regulated) is 20 times higher in chronic heart failure as compared to the non-failing heart (Swedberg et al., 1984). This high NA release takes place in spite of a subnormal (20 % of normal) NA content (Chidsey et al., 1966), a reduced number of sympathetic fibers (Oki et al., 1994) and a reduced (total, measured *in vitro*) DBH activity (Siltanen et al., 1982) in the failing human heart. Hence, in chronic heart failure DBH seems to be activated compared to normal conditions. The role of the two forms of DBH should be further investigated in chronic heart failure.

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APPENDIX

Isolation of Intact Chromaffin Granules and Chromaffin Granule Ghosts. Intact chromaffin granules were obtained by centrifugation in an isosmotic density gradient of Percoll-sucrose (Terland et al., 1979). Chromaffin granules were also isolated by a discontinuous sucrose density-gradient, lysed (hypotonic) and centrifuged in a final discontinuous density-gradient to yield chromaffin granule ghosts essentially free from mitochondrial and microsomal contamination (Terland and Flatmark, 1980).

Preparation of the Matrix Phase. The highly diluted matrix phase obtained as a supernatant after the initial hypotonic lysis of chromaffin granules was dialysed (Spectra/Por, cut-off 12-15 kDa, 4°) against five successive changes of 200-fold 7.5 mM MES buffer, pH 6.5 (6 h each) in order to remove the low molecular mass solutes (catecholamines, nucleotides, etc). Finally, the dialysed matrix was concentrated by ultrafiltration (Sartorius Membranfilter GmbH, Gottingen, Germany) with a cut-off at 30 kDa to a final concentration of 5 - 10 mg protein/ml. A heat-stable protein fraction was prepared from this concentrated matrix by heating at 95° for 10 min, followed by centrifugation (38 000 g, 15 min) to remove precipitated proteins (< 5 % of the proteins were removed). At pH 6.5 the addition of 35 mM Ca^{2}+ did not initiate any turbidity, i.e. no sign of chromogranin aggregation, whereas at pH 5.5 it resulted in a marked aggregation of chromogranins as expected from the literature (Yoo and Albanesi, 1990; Yoo, 1996). Approximately 60 % of the matrix protein were removed by this procedure. The precipitated proteins were collected by centrifugation, and dissolved in 50 mM PIPES buffer, pH 6.5.

Assay of DBH Activity. DBH activity was assayed at 25° in an isotonic medium containing 0.25 M sucrose (intact chromaffin granules only, otherwise sucrose was omitted), 50 mM PIPES buffer, pH 6.5,8 mM tyramine, 8 mM ascorbate and catalase (12 000 units/ml). After preincubation at 25° for 5 min, the reaction was started by adding tyramine and stopped after 10 min by an equal volume of ice-cold ethanol/acetic acid (pH = 4). The octopamine formed was assayed by HPLC (Flatmark et al., 1978). No KCI, fumarate or other anions were added in order to activate DBH, except where indicated (see Fig. 4). Tyramine was selected as the substrate to be hydroxylated because it has a non-saturable uptake in intact secretory granules

(Knoth et al., 1984), which is independent of MgATP if [tyramine] > 2.5 mM (Flatmark et al., 1982). By using tyramine at a high concentration instead of DA at a low concentration, we also avoided the problems related to the DBH-dependent oxidation of DA generating an inhibitor (DA-semiquinone free radical) of the vacuolar H^+-ATPase in the granule membranes (Terland et al., 1997). At low DA concentrations (< 100 µM), vesicular DA uptake depends on the transmembrane proton electrochemical gradient. If the proton pump is inhibited by a DBH-dependent DA product (Terland et al., 1997), DA may inhibit its own vesicular uptake. This may explain the low vesicular DA uptake in the absence of ascorbate found by Knoth et al. (1984) corresponding to 0.04 nmol/min/mg protein. In the presence of 2 mM external ascorbate, on the other hand, Ahn and Klinman (1987) obtained a DA (100 µM, external) uptake corresponding to 18 nmol DA/min/mg protein. A direct spectrophotometric assay of DBH in the matrix phase (Terland et al., 1977) was used in some experiments (Fig. 4). In this assay 8 mM DA was used both as the substrate to be hydroxylated and as the electron donor. The DA-semiquinone free radical generated in the reaction dismutates and finally generates dopaminochrome, which was followed spectrophotometrically at 485 nm. Chromaffin granules and chromaffin granule ghosts were solubilised by the non-ionic detergent Brij 58 (0.01%, w/v) which does not inhibit the DBH activity (Terland et al., 1977). Protein was assayed by the method of Bradford (1976) using bovine serum albumin as the standard. The soluble form of DBH (sDBH) was isolated from pooled matrix fractions as described by Ljones et al. (1976) and had a specific activity in the range of 0.4 - 1.8 µmol octopamine/min/mg DBH (n = 5; standard tyramine/ascorbate assay, no anion activation).

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5

FUNCTIONAL ROLE OF CHROMOGRANINS

The Intragranular Matrix in the Last Phase of Exocytosis

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1. INTRODUCTION

Chromaffin granules store catecholamines (CA) at very high concentrations, 0.5 to 1 M (Jankowski et al 1993b, Albillos et al 1997). Other soluble components, such as ATP, Ca^{2+}, ascorbic acid, opiates, dopamine-&hydroxylase and chromogranins (CGs), which are also concentrated within the granules can thus create a high intragranular osmotic pressure gradient compared with the cytoplasm (Winkler and Westhead 1980, Helle *et al* 1995). Complexation of intragranular substances, by Donnan forces, is the mechanism which has been proposed for the reduction of osmotic forces, thereby preventing granule lysis. The present view is that a major part of the intragranular CA is complexed, although a small fraction of free CA exists; and it is believed that both the free and bound CA are in equilibrium. It has been proposed that both chromogranin A (CGA) and ATP promote the passive aggregation of soluble substances forming the so-called intragranular matrix (Helle 1990). Several laboratory manoeuvres have been developed to study the nature of this "passive" interaction between intragranular components.

Recently, we have proposed that the interaction between CA-CGs is more complex than a simple and passive process of aggregation of

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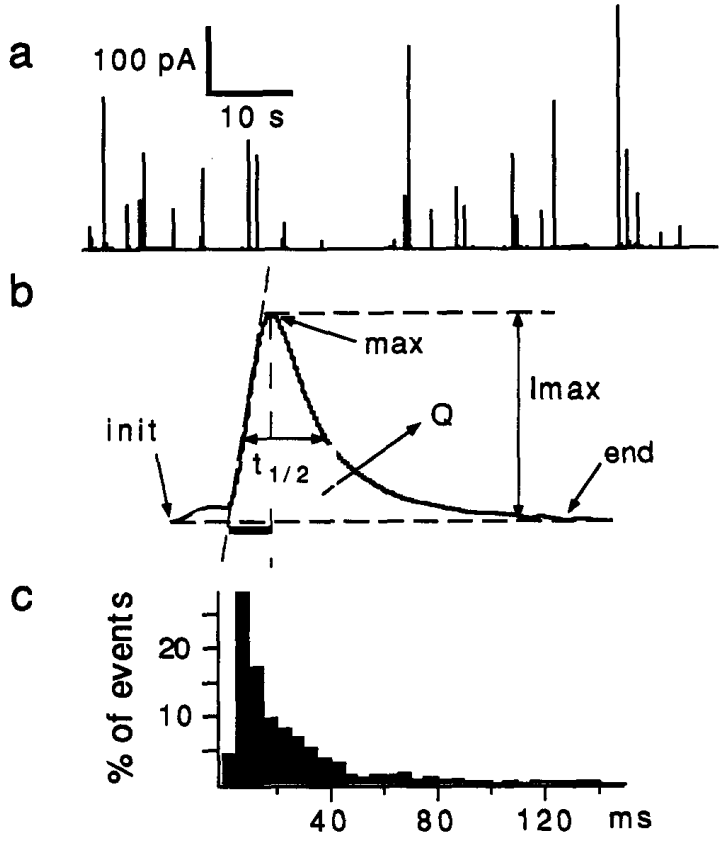

Figure 1. a. Amperometrical recording of secretory spikes in response to 5 mM BaCl2 for 5 s. Each transient corresponds to a single exocytotic event, observe the different spike heights. Vertical bar calibrates oxidation current, horizontal bar indicates time. b. Spikes parameters. Several measurements can be automatically done on the spikes obtained. Once the beginning and the end points are found, the maximum amplitude (Imax) can be calculated, and is expressed in PA. Spike width at halfheight t1/2, is expressed in ms. Granule net charge (Q) is obtained by integration of the area under the curve, which indicates the amount of oxidizing substances released and is expressed in pC. Some times, the ascending slope (dashed line) and the time to peak (thick horizontal line) are determined as well. c. Because large differences between individual spikes are currently observed in the amperometric recordings, histograms are frequently used to estimate the effects of a drug treatment, figure represents a normalised histogram of t1/2. On histograms, wider spikes are located on the right side of the graph.

intragranular substances. The activation of certain secondary messenger pathways could modulate this CA-CGs interaction thereby producing a slowing-down or acceleration of the exocytotic process. Considering the prevailing similarities between chromaffin granules and the dense core vesicles of sympathetic synapses, the apparent physiological relevance of this modulation arises from the fact that it can contribute to the modification of the synaptic performance at sympathetic junctions.

Although the term "exocytosis" is generally used as a synonym for secretion, in this chapter we will restrict it to describe the fusion event. Therefore, the term "exocytotic kinetics" will not consider granular movement towards the cell membrane or the secretory machinery, instead it will describe the time-course of CA extrusion from single vesicles which are amperometrically detected as secretory spikes.

In this chapter, we summarise several laboratory findings that suggest the active participation of CGs in the exocytotic process.

2. AMPEROMETRIC TECHNIQUES FOR THE STUDY OF EXOCYTOSIS

Amperometric techniques have largely contributed to our knowledge of exocytosis. Released CA are oxidized at the tip of a carbon fibre microelectrode, producing two electrons per molecule and rendering typical oxidizing current waves called secretory spikes. Spikes offer a direct 'observation' of the time-course kinetics of single secretory events and have been successfully used to characterise the late phase of exocytosis (Chow *et al* 1992). In addition, these provide valuable information about the previous storage conditions within the secretory vesicles.

Guidelines for electrode construction and instrumentation used for amperometry or voltammetry can be found elsewhere (Kawagoe *et al* 1993, Cahill *et al*1996). Changes in spike shape result from either variations in the CA granule content and/or in the exocytotic kinetics. In addition, changes in the fusion pore dynamics, water movement towards vesicle core or alterations in the association between CGs and CA will be reflected in the spike shape.

A typical trace of secretory events is shown in figure la. As it can be seen, there are large variations observed between individual spikes obtained from the same cell. For this reason, the analysis of exocytosis has to be based on the kinetic parameters measured from these spikes (Fig. lb).

In view of the variability observed in these parameters, a statistical analysis of an elevated number of spikes is obligatory to establish whether a particular cell treatment actually promotes real changes on exocytotic

kinetics. Histograms are the usual form of representation of data from spike population (Fig. 1c).

3. INTRAGRANULAR MATRIX DELAYS THE EXOCYTOTIC KINETICS

The main argument in favor of the role of intragranular matrix in CA complexation comes from the observation of secretory spikes. The time-course of a spike does not correspond to that expected for the free diffusion of CA towards electrode surface, instead it is considerably delayed. When a carbon fibre electrode is gently touching the cell surface, the distance between the cell and carbon surface is restricted to just the intervening water layer which allows the electrical connection between the carbon fibre and the reference electrode. We have estimated this gap as being about 10 to 20 nm, ten-fold smaller than the granule diameter. Simulations done using the diffusion constant for CA produce spikes of t1/2 in the µs range whereas in real spikes this typically ranges from 5 to 30 ms (Fig. 2c).

Several mathematical approaches have been used in an attempt to understand what are the subjacent mechanisms involved in the genesis of secretory spikes. Two kinds of spike models have been developed, macroscopic and microscopic. The macroscopic simulation consists of the use of mathematical equations to describe spikes, whereas microscopic simulations use single particle movements (random walk, Montecarlo simulations) to simulate the movement of a single molecule of catecholamine from the cell surface to the electrode surface.

By means of adjustments made in the variables, one can apparently reproduce a typical spike. However, computer simulation based only on free CA diffusion in water at a rate of 6×10^{-6} $cm^2 \cdot s^{-1}$ (Gerhardt and Adams 1982), failed to reproduce the time-course of real spikes.

In addition, the kinetics of secretion, expressed as acceleration or deceleration simulated by adjusted mathematical models, are quite different from real spikes. The same arguments can be used for spikes generated by the convolution of mathematical functions; again, these traces seem to reproduce the profile of typical spikes but in turn they fail to follow the phase plane which accurately describes their kinetic properties.

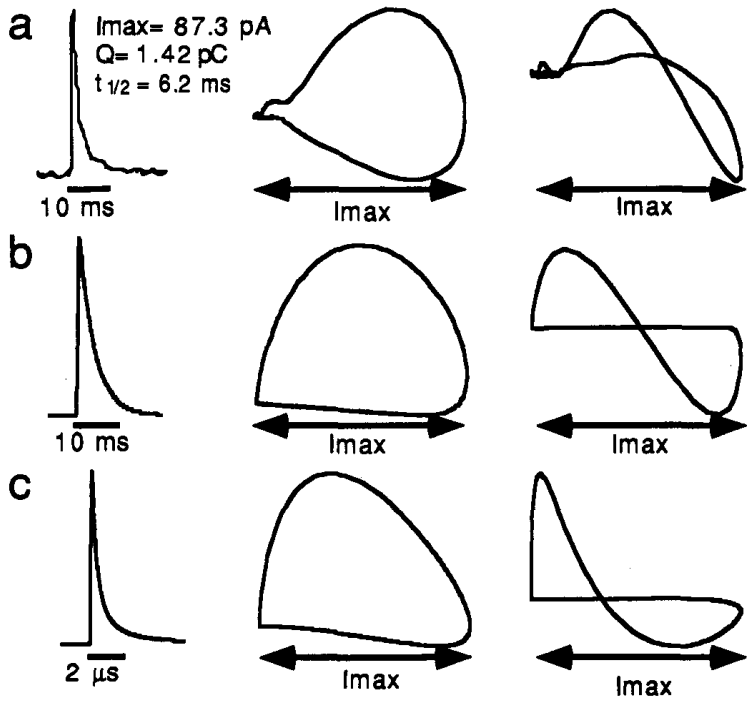

Figure 2. Kinetics analysis reveals important differences between real and model spikes. Traces on the left represent spikes (Imax vs. time), middle traces show first derivative vs. Imax, right traces show second derivative vs. Imax. Note the differences in the maximum speed, acceleration and deceleration. a. Real spike with the kinetic characteristics expressed in numbers. b. Exponentially modified gaussian (Jankowski et al 1993a). c. Random-walk simulations for an electrode placed at 20 nm from cell surface, note the difference in the time bar of this random-walk generated spike.

In figure 2, a secretory spike from a bovine chromaffin cell (a) is compared with a mathematically generated spike by the convolution of a gaussian and a decreasing exponential functions (b). In panel c, a random walk simulation obtained by Montecarlo methods is shown. In spite of an apparently similar profile, both computer-generated waves exhibit drastic differences in their time course compared with the original, which are evident in the first and the second derivatives. From these curves one can conclude that i) something is delaying the free diffusion of CA and ii) that exocytotic kinetics are more complex than the simple dissociation of CA from a gel. Diffusion of CA towards the electrode tip should hence be considered to be acornbination of two factors: the diffusion of CA within a

matrix gel and the extrusion of gel resulting from expansion caused by water uptake.

4. THE PROTEIC BASIS OF FUNCTIONAL INTRAGRANULAR MATRIX

A well-known phenomenon is that exocytosis is drastically accelerated when temperature is raised from 23°C to 37°C (Bittner and Holz 1992, Pihel *et al* 1996). Although fusion pore expansion can also be affected by temperature, an accurate analysis of secretory spikes indicate that the main alteration corresponds to the dissociation of intragranular matrix.

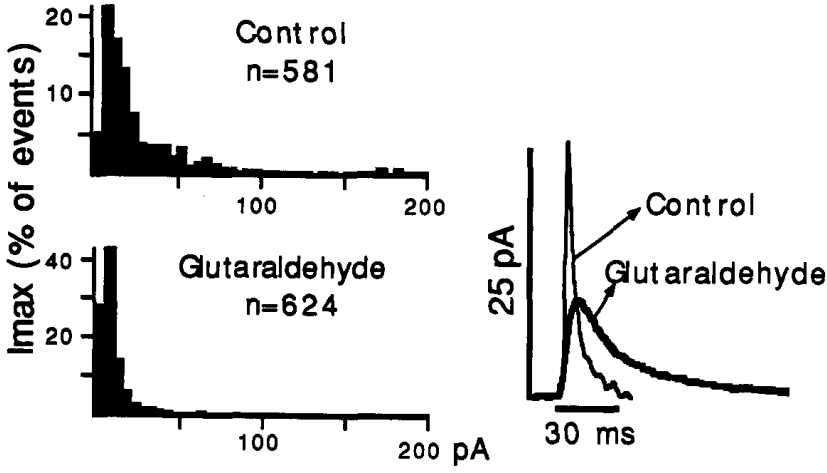

Figure 3. Effects of cross-linking agents. The experiments were done under hypertonic conditions. Cells were bathed at 750 mOsm and stimulated with Ba^{2+} 5 mM for 5 s. Under these conditions no secretory spikes were observed. However, spikes could be observed by puffing isotonic saline with no further increase in intracellular free Ba^{2+}, indicating that granules were prefusioned before isotonic pulse. Low concentrations of glutaraldehyde (0.1%) produced drastic effects on spikes shape. Histograms show Imax distribution in the absence (upper histogram) and in the presence of glutaraldehyde (lower histogram). On the right, representative traces with mean spike characteristics are shown. Control spike is indicated by the thin line, while spike obtained with glutaraldehyde 0.1 % is indicated by thick line. Note the smaller size of spikes when compared with control spikes obtained under isotonic conditions.

Other evidences come from the comparison of chromaffin- with mast cells-granules. This latter model has been extensively used for the study of intragranular matrix because a naturally occurring mutant -the beige mouse- has giant granules that can be directly observed by light microscopy (Zimmerberg *et al* 1987). Mast cell granules store histamine and serotonin and the matrix is composed mainly of heparan sulfates. In the beige mice mast cells, the concomitant use of amperometric, capacitance and image measurements has served to produce an accurate sequential picture of exocytosis.

Chromaffin granule matrix share many physico-chemical properties with those of the mast cell granule matrix. For instance, they behave similarly in response to temperature or to incubation with mono- or divalent cations (Pihel *et al* 1996). These changes are compatible with the shrinking or expanding dynamics observed in the beige mice mast cell granule matrix (Nanavatti and Fernández 1993), suggesting that nature has been provided with a widely distributed mechanism for the intragranular complexation and subsequent release of CA in which the intragranular matrix plays a critical role.

Other experimental evidence for the protein basis of the functional matrix comes from the observed effects of cross linker agents. Cell secretion evoked by external Ba^{2+} application is able to maintain secretion for minutes upon incubation with glutaraldehyde or formaldehyde. However, in spite of secretion being maintained, the time-course of single exocytotic event were profoundly affected (Fig. 3a). It can be argued that these cross linker agents alter the exocytotic kinetics by acting on the fusion-pore proteins. However, very low concentrations of glutaraldehyde persist in producing these effects, even in pre-fusioned granules obtained under hypertonic conditions (Borges et al 1997). Since it is unlikely that CA or ATP are affected by glutaraldehyde, these experiments suggest that the functional granule matrix is proteic.

Chromogranin A is the most likely candidate to account for the time-course profile of the spikes. This assumption arises not only from the fact that CGA is the most abundantly found protein within the granule, but also from the fact that its acidic characteristics maintain its stability at the intragranular pH of 5.5. The intragranular pH rises rapidly to 7.4 during exocytosis, facilitating CGNCA dissociation (see below).

5. PHYSIOLOGICAL MODULATION OF EXOCYTOTIC KINETICS

The drawings in figure 4 illustrate the sequence of events accompanying an exocytotic event. These recordings correspond to a 'patch-amperometry', as otherwise single granule fusion cannot be resolved by capacitance. The granule has to be attached to the cell membrane (docking and priming steps) before the fusion pore formation, these steps being "invisible" for either capacitance or amperometric measurements. Fusion pore produces a "jump" in the capacitance which is frequently accompanied by a pre-spike phenomenon, the so-called "foot", this reflecting the leakage of CA through the fusion pore. Subsequently, granule swelling occurs followed by pore dilatation and full fusion resulting in the amperometric spike.

The sequence of events presented here is related to the full fusion. However, it has been demonstrated that partial "kiss and run" fusion, resulting from the opening of a fusion pore, can account for the total emptying of granule content (Albillos *et al* 1997).

Exocytotic kinetics may be modulated, at least, at three different steps: i) fusion pore expansion, ii) Cl-/water influx and iii) CA/CGs dissociation.

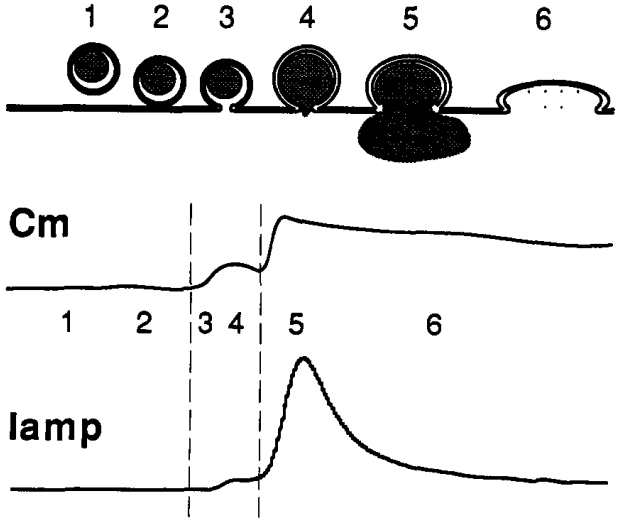

Figure 4. Relationship between the exocytotic steps, capacitance- (Cm) and amperometric-recordings (Iamp). Granule approaching and docking (1, 2) are "invisible" for both techniques. Pore formation (3) and granule swelling (4) are observed as a jump in the imaginary component of Cm and a 'foot' on amperometric records. Fusion pore expansion and granule emptying is observed in the Cm recording as a big jump and as a secretory spike in the Iamp traces (5,6). Note that the Cm recording precedes Iamp traces.

Fusion Pore. Although proteins involved in fusion pore formation and dilatation are far from being characterised and the lipids implicated in membrane fusion are unlikely to be modulated, there are a few contributions in the literature which indicate that this process can be affected by second messengers. Hartmann and Lindau (1995) described increases in the dilatation speed of fusion pore when intracellular Ca^{2+} concentrations rose to a micromolar range. In addition, Alés *et al* (1999) have recently shown that the number of partial exocytosis are increased when extracellular Ca^{2+} concentration rises.

Cl-/Water. The role of C1- channels has been recently implicated in the granule matrix expansion and extrusion. It has been shown that a net water flow escapes from the granules upon fusion and that this flow can be blocked by C1- channel antagonists (Terakawa *et al* 1999). However, at present, it is not clear what is the C1- source and what are the signals that trigger C1- channel opening.

Chromogranin/CA Dissociation. Although attempts to find specific binding sites on CGA for CA or ATP have failed, the consensus is that such interaction is rather unspecific and based on charge attraction. In this context, it has been shown that alterations in extracellular pH to 5.5 slow the granule emptying during exocytosis (Jankowski *et al* 1993b). In addition, changes in the external ionic composition modifies the time course of spikes, in a similar way that is observed in mast cells, suggesting that there is participation of electrostatic forces in the complexation mechanisms (Jankowski *et al* 1993a, Pihel *et al* 1996).

Providing that the granule emptying speed will produce changes in the CA concentrations that reach the postsynaptic cell in a sympathetic synapse, an attractive possibility is that synaptic performance could be modulated by modifying the speed of granule emptying. This mechanism would allow a neuron to increase or decrease the concentration of the neurotransmitter released, utilising the same amount of substance content in a secretory vesicle. Although this mechanism seems to be irrelevant in chromaffin cells where CA are quickly diluted into the blood stream, it can operate in dense core vesicles of sympathetic varicosities, which are closely related to chromaffin granules, thereby modifying the sympathetic synaptic performance.

As it was mentioned above, a series of different physical treatments of chromaffin and mast cells can result in alterations in the secretory spikes. However, these manoeuvres are unlikely to occur under physiological conditions.

Recently, we have found that PKG/NO system can modify dramatically the spike shape. These changes were evident within few seconds and were

fully reverted upon NO withdrawal with NO-scavengers. These effects were evident at NO concentrations considered to be within the physiological range and occurred within a few seconds. There are evidences indicating that this effect is mainly mediated through an action on the CA/CGA complex.

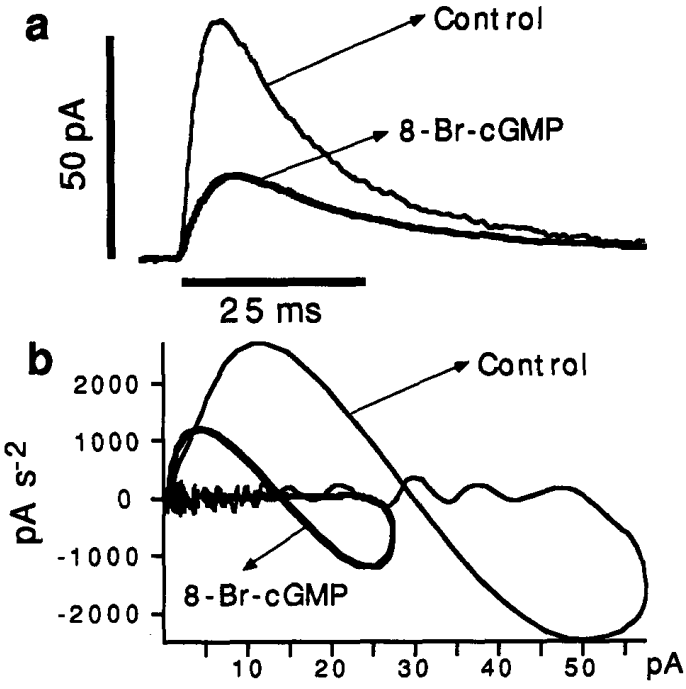

Figure 5. Effects of the stimulation of PKG on secretory spike shape. Cells were stimulated with 5 mM $BaCl_2$ for 5 s. Cells were incubated in the absence (thin trace) or in the presence of 8-Br-cGMP 10 µM for 15 min (thick trace). On the right panel, the second derivatives are plotted against Imax. There is no delay in the maximum acceleration of CA output location.

Figure 5 shows the effect of 8-Br-cGMP on spike shape. These changes were mimicked by NO and NO donors suggesting that there is involvement of the guanylate cyclase pathway. Although PKG can act on several target sites like fusion pore expansion or vesicle swelling, there are some evidences indicating the active role of the intragranular matrix. Part of this evidence has come from the analysis of the pre-spike features (feet). Fusion pore

expansion can be indirectly monitored by foot duration. We could not find any difference in feet duration between control and NO-treated cells.

Even more convincing evidence comes from experiments carried out under hypertonic conditions. We have shown that full granule fusion can be blocked by increasing the tonicity of the extracellular media (>700 mOsm, Borges *et al* 1997). However, the secretory machinery remains operative and a high number of granules are opened to the external medium. Full exocytosis can be assessed by a brief pulse of isotonic saline. The isotonic pulse does not change the Ca^{2+} uptake and spike production ceases immediately after the interruption of the isotonic pulse. The effect of PKG activation is still evident on these pre-fusioned granules, indicating that its target site of action is a step beyond fusion pore formation and expansion. Nitric oxide prevents the CA leakage from granules already fused. In figure 5b, the effect of NO on CA exocytotic kinetics is shown. This constitutes the first evidence indicating that the speed of exocytosis can be modulated by a second messenger. Since then, an increasing number of drugs and endogenous substances have been revealed to be putative modulators of exocytotic speed, several of them seeming to act on the intragranular matrix.

The major question opened by these results is what are the transduction mechanisms used to produce conformational changes on the affinity of CA for the granule matrix. Chromogranin A is the main candidate to be targeted for this process, considering that it is the most abundant protein found within the granule. However, no clear mechanisms have been described to explain how PKG activation can account for conformational changes on CGA. There are few granule membrane receptors described so far which are capable to interact with CGA. There have been some experimental evidences implicating IP3 receptors (Yoo and Albanesi 199 1, Yoo 1996). Recently, this association has been described as well for CGB (See the chapter by Yoo in this book). The major criticism against the role of IP_3 receptor comes from the fact that, even if there exist functional receptors on the granule membrane, they are unlikely to account for large conformational changes in an abundantly found protein like CGA. Nitric oxide itself could diffuse to the granules and exert its action directly on the granule matrix. However, there is not a soluble guanylate cyclase described so far inside granules and this cannot explain the effects of 8-Br-cGMP.

The characterisation of transduction routes that can account for the rapid changes induced by drugs or endogenous substances will require further investigations. The relevance of this novel route is that it can promote changes in the affinity of CGA for CA that can be responsible for changes in synaptic performance of sympathetic synapses. This is the first evidence which indicates that exocytotic kinetics can be modulated by drugs or endogenous substances.

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6

INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR AND CHROMOGRANINS A AND B IN SECRETORY GRANULES

Co-localization and Functional Coupling

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1. INTRODUCTION

The secretory granule of adrenal medullary chromaffin cells has been shown to release Ca^{2+} in response to IP3 (Yoo and Albanesi 1990a), and this observation has also been extended to the secretory granule of insulin-secreting pancreatic b-cells (Gerasimenko *et al* 1996), demonstrating the IP_3-sensitive intracellular Ca^{2+} store role of secretory granules. Recently, direct participation of secretory granule calcium in the control of cytoplasmic Ca^{2+} concentration has also been demonstrated in the secretory granules of goblet cells (Nguyen *et al* 1998) in that uptake of Ca^{2+} by secretory granule was temporally and spatially matched by simultaneous reduction of Ca^{2+} concentration in the surrounding cytoplasm whereas IP_3-mediated release of Ca^{2+} by the secretory granules resulted in the simultaneous increase of cytoplasmic Ca^{2+} concentration in the immediate vicinity of the secretory granules. The IP_3-sensitive Ca^{2+} store role of secretory granules of bovine adrenal medullary chromaffin cells was attributed to the presence of high-capacity, low-affinity Ca^{2+} storage protein CGA, binding 30-50 mol of Ca^{2+}/mol, inside the secretory granule (Yoo and Albanesi 1990a, 1991).

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2. COLOCALIZATION OF IP3RS AND CHROMOGRANINS AAND B

2.1 Immunogold Cytochemistry

In immunogold cytochemical study, IP_3R type 1 (IP_3R-1)-specific antibody (Yoo 1994) was shown to interact specifically with antigens in the secretory granules of bovine adrenal medullary chromaffin cells (Fig. 1), more than half of the immunogold-labeled IP3R-l localizing in the periphery of the granules. In line with the known existence of IP_3Rs in the endoplasmic reticulum (Mignery *et al* 1989, Matter *et al* 1993, Khan *et al* 1992), Fig. 1 shows the presence of IP3R in the endoplasmic reticulum, but not in the mitochondria.

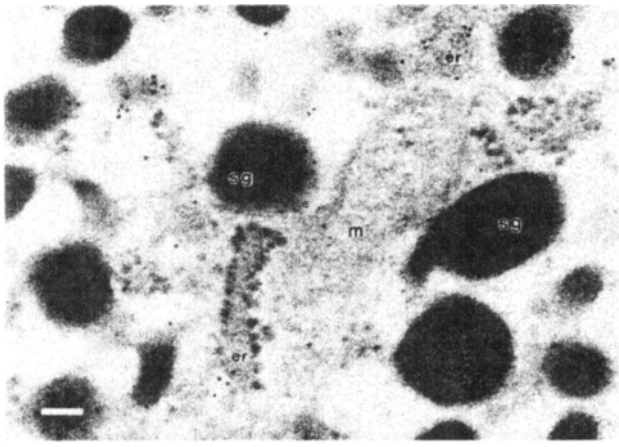

Figure 1, Immunogold Electron Microscopy Showing Localization of IP3R and CGA. More than half of the gold labeled IP3R-1 (10 nm) are localized near the membrane region of the secretory granules (sg). The gold particles are also shown in the endoplasmic reticulum (er) but not in the mitochondria (m). Bar = 100 nm.

Moreover, two other IP3R type-specific antibodies, i.e. type 2, and 3 antibodies, appeared to react only with the antigenic sites on the periphery of the secretory granules (S.H.Yoo, M.K.Kang, and H.S.Kweon, unpublished results), suggesting the localization of all three types of IP3R in the secretory granules. Therefore, the immunogold cytochemical results appear to unequivocally demonstrate the existence of the IP3Rs in the secretory granules.

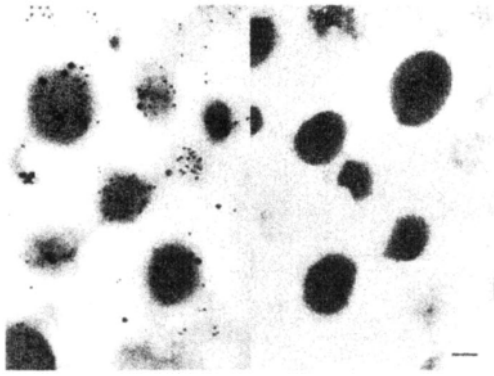

Figure 2. Immunogold Double Labeling of IP3R and CGA. Isolated secretory granules of bovine adrenal medullary chromaffin cells were double-immunolabeled for IP_3R (20 nm gold) and CGA (10 nm) with affinity-purified anti-rabbit IP_3R antibody (5) and monoclonal anti-mouse CGA antibody, respectively. The IP3R immunoreactivity is localized primarily in the periphery of the secretory granules. (Right), Control in the absence of the primary antibodies. Bar = 100 nm.

Further, coexistence of IP3R and CGA in the secretory granules was shown by double immunolabeling of the isolated secretory granules with CGA and IP_3R antibodies (Fig. 2). The IP3R-gold particles (20 nm) were primarily localized in the periphery of the secretory granules whereas the CGA-gold particles (10 nm) were distributed rather evenly over the granules, clearly demonstrating the colocalization of IP_3R and CGA in the secretory granules.

2.2 Direct Interaction between IP_3R and Chromogranins A and B

Although CGA has been shown to interact with several integral membrane proteins of secretory granules of bovine chromaffin cells, including the IP_3R (Yoo 1994), it was not clear whether purified intact IP_3R and chromogranins A and B can interact directly and what effect this interaction will exert on the IP_3-mediated Ca^{2+} release activity of P_3R. We have hence used purified bovine IP3R to test its interaction with CGA and CGB (Fig. 3). The GST-CGA and -CGB fusion proteins interacted with the purified IP_3R at the intravesicular pH 5.5 (Fig. 3).

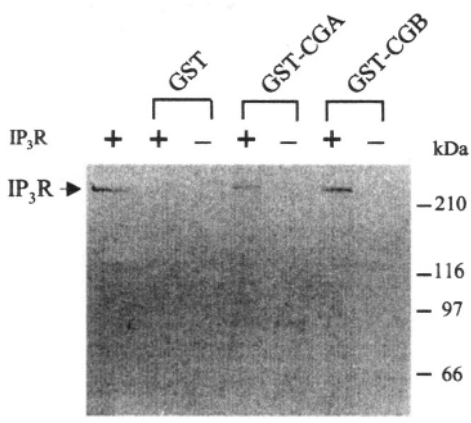

Figure 3. Purified Bovine IP_3R and Its Interaction with CGA and CGB. Purified IP_3R (0.5-0.7 μg) was reacted with GST-CGA and -CGB fusion proteins at pH 5.5, and the bound IP_3R was shown by Coomassie blue staining.

It is known that IP3R monomers interact with each other to form either homotetrameric or heterotetrameric IP_3R (Supattapone *et al* 1988, Maeda *et al* 1990, Chadwick *et al* 1990, Monkawa *et al* 1995, Jos eph *et al* 1995), forming a Ca^{2+} channel. Interestingly, CGA is also known to form a dimer at the near physiological pH 7.5 and a tetramer at the intravesicular pH 5.5 (Yoo and Lewis 1992, Thiele and Huttner 1998). Moreover, chromogranins A and B together form a CGA-CGB heterodimer at pH 7.5 but a CGA2CGB2 heterotetramer at pH 5.5 (Yoo and Lewis 1996). In addition, we have shown previously that tetrameric CGA interacts with four molecules of intraluminal loop peptide of IP3R (Yoo and Lewis 1995), thus implying an interaction between tetrameric chromogranin and tetrameric IP_3R. In this regard, direct interaction between the IP3R and Ca^{2+} storage proteins CGA and CGB sheds new light on the physiological significance of this coupling between tetrameric chromogranin and tetrameric IP3R in the secretory granules.

2.3 Cotransfection and Coimmunoprecipitation of IP_3Rs and Chromogranins A and B

To determine whether the IP3R and CGA or CGB are physically linked in the cells, the IP3R and CGA or CGB were cotransfected into COS-7 cells and coimmunoprecipitation was carried out (Fig. 4). Immuno-precipitation of the cell extracts, which had been cotransfected with hemagglutinin A

(HA)-tagged IP3R-1 (S.H.Yoo *et al* submitted, GenBank accession number AF157625) and CGA (Kang and Yoo 1997), by monoclonal HA antibody, followed by immunoblot analysis with CGA and HA antibodies (Fig. 4a), indicated coprecipitation of CGA and IP3R-1. Moreover, immunoprecipitation of the same cell extracts with CGA antibody, followed by immunoblot analysis with IP3R-1 and CGA antibodies (Fig. 4b), also indicated coprecipitation of CGA and IP3R-1, thus clearly demonstrating the complex formation between the IP3R and CGA in the cells.

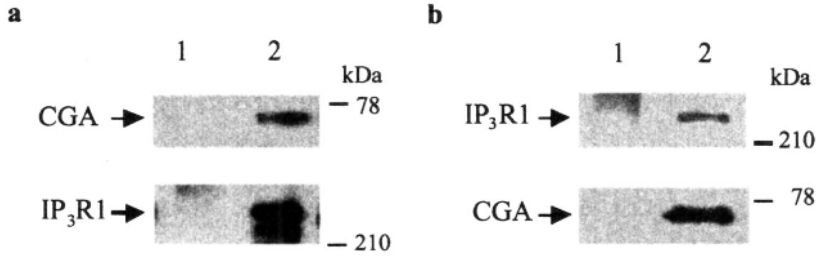

Figure 4. Co-immunoprecipitation of IP3R-I with CGA or CGB. a, COS-7 cells which were cotransfected with HA-tagged IP_3R-1 and CGA were immunoprecipitated with monoclonal HA antibody (mHA Ab), and were immunoblotted with monoclonal CGA antibody (mCGA Ab) (upper panel) and mHA antibody (lower panel), respectively. b, Same extracts were immunoprecipitated with CGA antibody and immunoblotted with HA (upper panel) and CGA (lower panel) antibodies. Preimmune IgG was used as control antibody (lane 1).

Likewise, immunoprecipitation of the cell extracts, which had been cotransfected with type 2-specific IP3R (IP3R-2) and CGB (Yoo and Kang 1997), with HA and CGB antibodies followed by immunoblot analysis with HA and CGB antibodies, also indicated the presence of IP_3R-2-CGB complex (Fig. 5, a and b). In a similar study, cotransfection of type 3-specific IP_3R (IP3R-3) and CGA into COS-7 cells and immunoprecipitation of the cell extracts also indicated coimmunoprecipitation of IP_3R-3 and CGA (Fig. 5, c and d), further indicating the existence of IP_3R-3-CGA complex in the cell.

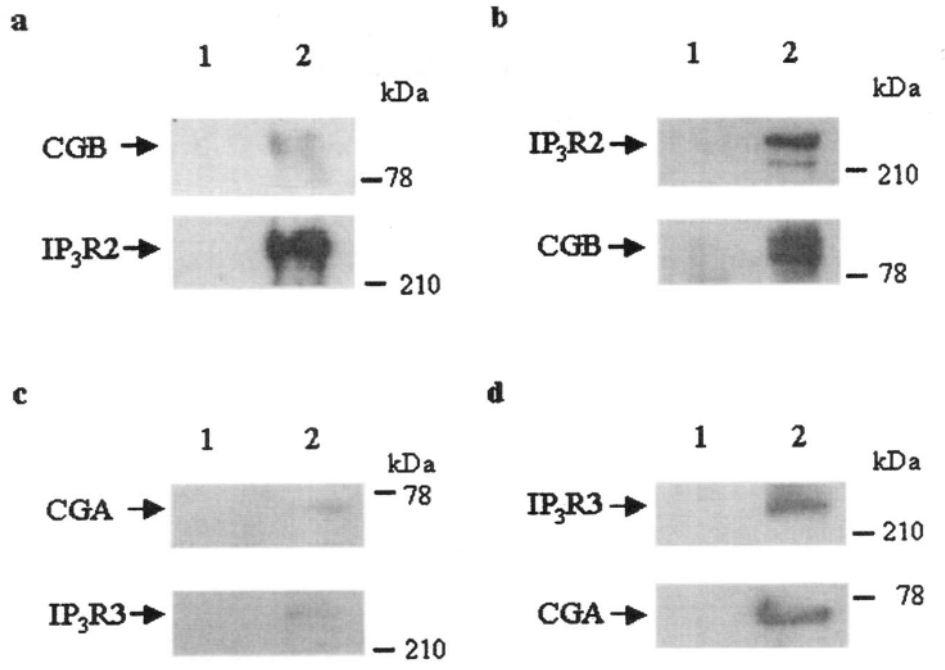

Figure 5. Co-immunoprecipitation of IP3R-2 and -3 with CGA and CGB. a, COS-7 cells which were cotransfected with HA-tagged IP_3R-2 and CGB were immunoprecipitated with monoclonal HA antibody (mHA Ab), and were immunoblotted with monoclonal CGB antibody (mCGB Ab) (upper panel) and mHA antibody (lower panel), respectively. b, Same extracts were immunoprecipitated with CGB antibody and immunoblotted with HA (upper panel) and CGB (lower panel) antibodies. Preimmune IgG was used as control antibody (lane 1). c and d, COS-7 cells which were cotransfected with HA-tagged IP3R-3 and CGA were immunoprecipitated with mHA (c) and CGA (d) antibodies, respectively, and each was immunoblotted with HA and CGA antibodies.

3. FUNCTIONAL COUPLING BETWEEN IP_3R AND CHROMOGRANIN A

3.1 IP_3-Induced Ca^{2+} Release from the IP_3R-Reconstituted Liposomes

To determine what effect CGA can exert on the Ca^{2+} release property of IP3R, IP3-induced Ca^{2+} release was determined using IP3R reconstituted liposomes in the presence and absence of CGA (Fig. 6). IP_3 was able to release Ca^{2+} from the IP3R-reconstituted liposome, but the amount was greatly reduced in the presence of IP3R Ab (Fig. 6a), suggesting IP_3-induced Ca^{2+} release through the reconstituted IP3R/Ca^{2+} channel.

Given the acidic intravesicular pH of 5.5 and the abundance of CGA in the secretroy granules, CGA was encapsulated into the proteoliposomes at

pH 5.5. The maintenance of pH gradient in and out of the proteolipsomes was confirmed by measuring the fluorescence of encapsulated oxonol V, which increases upon exposure to higher pH (not shown). From these CGA-encapsulated proteoliposomes at the intraliposomal pH 5.5, the initial 0.5-1 .0 μM IP_3 caused 2-3 times more Ca^{2+} release than those obtained in the absence of CGA (Fig. 6b).

This difference in the amount of Ca^{2+} released in response to the same amount of IP_3 gradually decreased as more Ca^{2+} is released as a function of increasing IP_3 concentrations, reaching a maximal level after addition of 4 μM IP_3. Further addition of IP_3 caused little or no increase of the emission fluorescence (not shown).

The total amount of Ca^{2+} releasable by IP3 was estimated to be about 55-60% of the total encapsulated Ca^{2+} considering the final fluorescence increase caused by Triton X-100 treatment.

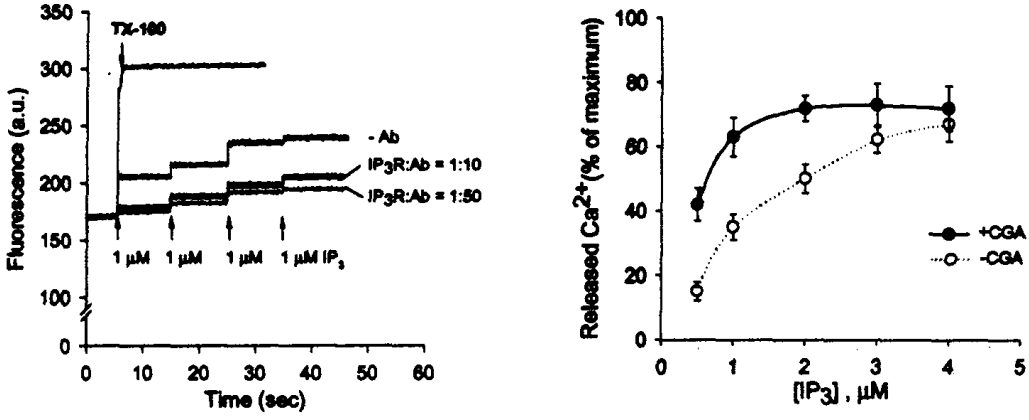

Figure 6. Effects of CGA on IP_3-induced Ca^{2+} Release from the IP3R-reconstituted Liposomes. a, IP_3-induced Ca^{2+} efflux through the proteoliposomes (300 μM Ca^{2+} inside) was determined by the fluorescence change of indo-I at 393 nm. Affinity purified anti-peptide IP_3R antibody was added at the IP_3R to anti-IP_3R IgG ratio of 1 : 10 and 1 : 50 (w/w). b, Ca^{2+} releases were measured after one-time dose of indicated concentrations of IP_3 to the proteoliposome solution (0.3 μM of IP_3R) containing 1 μM of indo-I at 35 ^{0}C. IP_3-induced fluorescent changes were compared to that obtained by 1% Triton X-100 (this value was set as 100%). Data shown are mean ± s.e. (n=3).

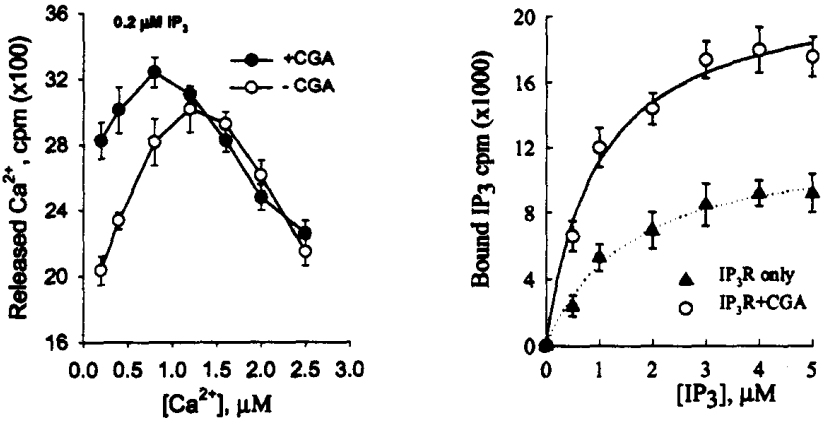

Figure 7. Effects of Ca^{2+} and the IP_3 Binding on the IP_3-induced Ca^{2+} Release. a, 0.2 μM IP_3 was added to the proteoliposomes containing ~300 μM Ca^{2+} including ~50,000cpm of $^{45}Ca^{2+}$ in the presence of increasing Ca^{2+} Concentrations, and the released Ca^{2+} was measured. Data, mean ± s.e. (n=3). b, The proteoliposomes were incubated with various concentrations of [^{3}H]IP_3. After incubation for 10 min at 35 °C, 0.1 volume of each sample was filtered through a concentrator (microcon from Amicon) with a molecular weight cutoff of 100,000, and the radioactivity of each filtrate was determined. Data, mean ± s.e. (n=3).

Our attempt to do similar experiments with CGB was not successful due to an extremely high aggregation property of CGB at the intravesicular pH 5.5.

In view of the potential inhibitory effect of increasing Ca^{2+} concentrations on the IP_3-induced Ca^{2+} release activity of IP3R-1 (Miyakawa *et al* 1999), we examined the Ca^{2+} release activity of the proteoliposome in response to a fixed amount of IP_3 in the presence of increasing concentrations of Ca^{2+} (Fig. 7a). In the Ca^{2+} concentration range of 0.1-1.0 μM, 0.2 μM IP_3 induced far greater Ca^{2+} release in the presence of CGA. But in the higher Ca^{2+} concentration range the difference in the amount of released Ca^{2+} disappeared, indicating the inhibitory effect of Ca^{2+} on the Ca^{2+} release property of IP_3R-1. These results clearly show that same amount of IP_3 releases far greater amount of Ca^{2+} from the proteoliposome in the presence of CGA when there is no inhibitory amount of Ca^{2+} in the liposome solution. The increased release of Ca^{2+} in the presence of CGA appeared to be due to the increased binding of IP_3 to the proteoliposome in the presence of CGA (Fig. 7b).

3.2 Structural and Motional Changes of IP3R by Interaction with Chromogranin A

Anisotropy measurement (Fig. 8a) indicated that the anisotropy values are significantly higher in the presence of CGA compared to those in the absence of CGA in the tested temperature range of 20-40 °C, suggesting that the molecular motion of IP_3R or its thermal flexibility was significantly decreased by its interaction with CGA. This indicates that CGA caused conformation changes of the IP_3R to a more rigid (or stabilized) structure that is conducive to IP_3 binding and better suited to Ca^{2+} release as shown in Figures 6-7. The CGA-induced conformational change of the IP_3R to a more rigid (or stabilized) structure is also confirmed by fluorescence resonance energy transfer study (Fig. 8b), in which higher resonance energy transfer from CPM-labeled to F-mal-labeled IP_3R was observed in the presence of CGA. This result also suggests conformational changes of IP_3R to a more stabilized state due to the presence of CGA in the proteoliposomes. Therefore, it appears that chromogranins play pivotal roles in controlling the Ca^{2+} release property of the IP_3R in neuroendocrine cells not only by binding and freeing of the intravesicular Ca^{2+} but also by modulating the channel activity of the IP_3Rs.

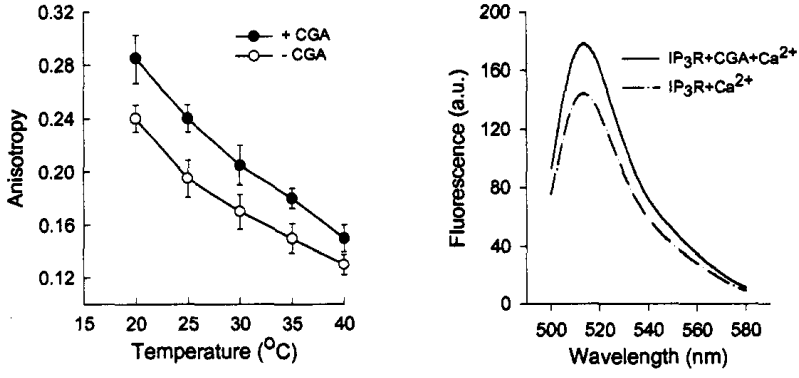

Figure 8. Anisotropy and Resonance Energy Transfer Studies of IP_3R in the Presence of CGA. a, Fluorescence emission anisotropy of IAEDANS (5-(((2 iodoacetyl) amino) ethyl) amino) naphthalene-1-sulfonic acid) (Molecular Probes) labeled to Cys residues of IP_3R shows that when CGA was present the anisotropy values were higher than those without CGA within all the temperature range tested. The anisotropy values also decreased with increasing temperatures regardless of the presence of CGA. b, The fluorescence resonance energy transfer from CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin) to F-mal (Fluorescein-5-maleimide) was measured after reconstituting the CPM- and F-mal-labeled IP_3R (1 : 1 mixture) into the liposomes. The emission fluorescence of F-mal was measured at 384 nm the excitation wavelength of CPM at 35 °C.

4. CONCLUSIONS

It has been shown previously that CGA in different conformations exhibits different Ca^{2+}-binding capacity and affinity (Yoo and Albanesi 1990b, 1991). In light of these observations, it is natural to think that the conformational changes of the IP_3R that occur as a result of IP_3 binding (Mignery and Südhof 1990) will be transmitted instantly to the coupled CGA and CGB in the secretory granules, causing their conformational changes. It may then be assumed that the resulting conformational changes in CGA and CGB will concomitantly result in lowering the affinity of chromogranins for Ca^{2+}, thus freeing some Ca^{2+} from the proteins and making them available for release through the IP_3R channel to the cytoplasm.

In view of the fact that the secretory granules contain up to 40 mM Ca^{2+} and most (>99.9%) of it stay bound to chromogranins in the secretory granule (Bulenda and Gratzl 1985), the coupling of calcium storage proteins to the IP_3R/Ca^{2+} channel appears to reflect the efficient and intricate structural organization of an intracellular Ca^{2+} store whose Ca^{2+} storage/release function should be strictly and subtly controlled. Given the physiological needs of cells to tightly control the cellular Ca^{2+}, the physical coupling of Ca^{2+} storage proteins to Ca^{2+} channels seemed to be a natural consequence. Despite the seemingly intricate structural organization inside the IP_3-sensitive Ca^{2+} stores, the amounts of Ca^{2+} released in response to a fixed amount of IP_3 vary widely in the cells (Muallem *et al* 1989, Meyer and Stryer 1990, Bootman *et al* 1992), probably due to multiple reasons, in the case of secretory granules, the amount of Ca^{2+} to be released in response to IP_3 will be determined by a combination of several factors, i.e. the IP_3 concentration introduced, the nature of tetrameric IP_3R channels formed, the types of chromogranins coupled to the IP_3R channel, and the Ca^{2+} charge states of coupled chromogranins.

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PART 3

TRANSCRIPTION, EXPRESSION, AND SECRETION

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7

REGULATION OF CHROMOGRANIN A TRANSCRIPTION AND CATECHOLAMINE SECRETION BY THE NEUROPEPTIDE PACAP

Stimulation and Desensitization

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1. INTRODUCTION

Pituitary adenylyl cyclase-activating polypeptide (PACAP) is a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) regulatory peptide family and occurs in both 27- (PACAP27) and 38- (PACAP38) amino acid forms (Arimura and Shioda 1995, Miyata *et al* 1992). PACAP and VIP peptides activate at least three distinct receptors that belong to the larger family of the seven transmembrane-spanning guanine-nucleotide-binding G protein-coupled receptors (GPCRs). Three classes of PACAP/VIP receptors (PAC1, VPAC1, VPAC2) have been identified and cloned (Pisegna and Wank 1993, Spengler *et al* 1993). PACl receptors, which have high selectivity for PACAP but low affinity for VIP, exist as six splice-variant forms (Pantaloni et al 1996, Spengler *et al* 1993). PACl can couple to activation of both the adenylyl cyclase (AC) and the phospholipase C-β/phosphoinositide (PLC-β/PI) pathways, through G_s and $G_{q/11}$ classes of Gα protein subunits (Hezareh *et al* 1996 Spengler *et al* 1993). Chromogranin A (CGA) is a regulated secretory protein which is a widespread constituent of

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neuroendocrine and neuronal secretory granules. CGA represents the major soluble protein of the secretory granule core of chromaffin cells (Iacangelo and Eiden 1995, O'Connor 1983). Its intracellular functions include binding of Ca^{2+}, ATP and catecholamines within the hormone storage vesicle (Videen *et al* 1992, Yoo and Albanesi 1991), and a trafficking role in guiding other secretory proteins into dense core vesicles within the regulated secretory pathway (Parmer *et al* 1993). CGA is also a pro-hormone giving rise by proteolytic processing to several bioactive peptides such as the catecholamine release-inhibitor *catestatin* (Mahata *et al* 1997).

The adrenal gland is innervated by a dense network of PACAP-immunoreactive fibers, and abundant PACAP binding sites occur on the surface of chromaffin cells (Moller and Sundler 1996). Over the past few years, several reports have provided evidence that in addition to the classic preganglionic neurotransmitter acetylcholine (ACh), the non-cholinergic transmitter PACAP is likely to play an important role in governing both secretion and synthesis of catecholamines in chromaffin cells (Isobe *et al* 1993, Marley *et al* 1996, Przywara *et al* 1996). However, recent studies have suggested a distinct role for PACAP in addition to that of ACh (Babinski *et al* 1996, Przywara *et al* 1996). In contrast to nicotine, whose secretory response in chromaffin cells shows rapid desensitization (Boksa and Livett 1984), chronic exposure of chromaffin cells to PACAP can evoke long-lasting Ca^{2+} influx and catecholamine secretion (Babinski *et al* 1996, Tanaka *et al* 1996), without apparent desensitization of the secretory response. We previously reported that secretory stimulation of PC 12 pheochromocytoma cells by nicotine stimulates transcriptional activation of the CGA gene, involving discrete CGA promoter domains (Tang *et al* 1996). In the present article, we explored the signal transduction pathways whereby PACAP modulates catecholamine secretion versus the expression of the CGA gene in PC 12 cells. We also questioned whether desensitization of both responses evoked by PACAP might occur.

2. ACTIVATION OF CGA GENE TRANSCRIPTION AND CATECHOLAMINE SECRETION BY PACAP

An important prerequisite for the significance of any study of transcription is that expression of the chromosomal gene be activated by the stimulus under investigation. Steady state analysis of CGA mRNA levels

indicated that PACAP augmented CGA mRNA by ~4- to 5-fold in PC12 cells (Fig 1A).

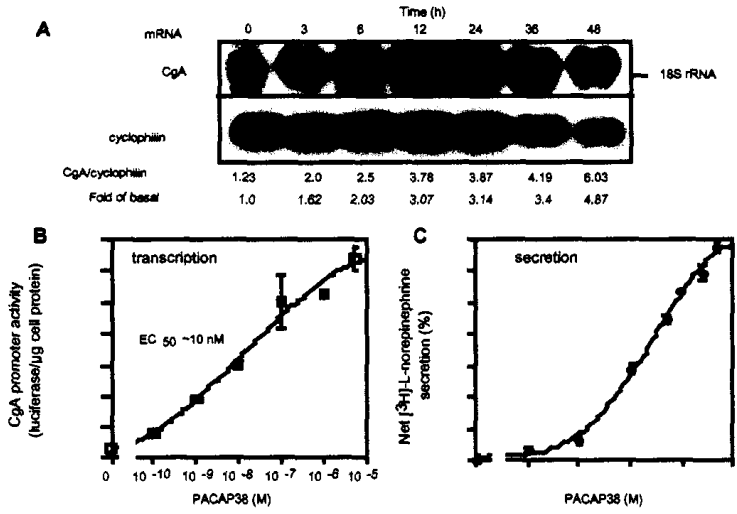

Figure 1. (A) Northern blot analysis of the time-dependent effect of PACAP on CGA mRNA. PC12 cells were treated with 250 nM PACAP38 for the indicated times. The cyclophilin probe recognizes a constitutive or housekeeping mRNA, at the same time point. (B) Acute stimulation of catecholamine secretion by PACAP. [^{3}H]-L-norepinephrine prelabeled PC 12 cells were treated for 20 min with indicated concentration of PACAP. (C) Dose-dependent effect of PACAP on CGA transcription. PC12 cells transfected with pXP1200 were treated for 20 h with indicated concentration of PACAP38. Reproduced in part from Taupenot *et al* (1998).

Furthermore, PACAP *trans*-activated a transfected 1200 bp mouse CGA promoter/luciferase reporter construct in PC 12 cells, in a concentration-dependent manner (Fig 1B). The promoter activity was increased significantly at 20 hours of exposure to a concentration of PACAP as low as 0.1 nM, with an EC50~10 nM. PACAP also triggered catecholamine secretion from PC 12 cells in a concentration-dependent manner. Increased norepinephrine release became significant at $\geq$ 1 nM peptide and reached a maximum at 1 µM peptide, with an EC50~23 nM (Fig IC).

3. PACAP SIGNALING TO CGA TRANSCRIPTION

Cyclic AMP response element (CRE) domains have been shown to mediate PACAP activation of gene expression in several neuroendocrine cell types, such as POMC expression in AtT20 corticotropes (Boutillier *et al* 1994). The CGA promoter's CRE domain is conserved across species

(Mouland *et al* 1994, Wu *et al* 1991) and responds to cAMP (Wu *et al* 1995). We therefore evaluated the role of this CRE in response to PACAP, a CAMP-generating stimulus. A series of CGA promoter 5' deletion mutant/reporter constructs revealed several regulatory domains in cis responding to PACAP stimulation (Fig 2A). Promoter domains whose deletion decreased the PACAP response were both distal (-1200 to -821 bp) and proximal (-77 to -61 bp). While deletion of a domain from -181 to -77 bp increased PACAP response, deletion of the region -77 to -61 bp caused a dramatic decrement (77%) in the PACAP response. This domain contains the mouse CGA promoter's functional CRE: [-71 bp]5'-TGACGTAA-3'[-64 bp] (Wu *et al* 1995). Co-transfection of PC12 cells with a dominant negative mutant of the CRE-binding protein CREB (KCREB), (Walton *et al* 1992) together with either a 77 bp or 1200 bp CGA promoter/luciferase reporter also blunted the PACAP response (Fig 2B). Deletion or point mutations of the CGA CRE element markedly diminished PACAP effects, and transfer of the CGA CRE box to a neutral, heterologous thymidine kinase promoter also conferred activation by PACAP (Taupenot *et al* 1998). These findings suggest that the CGA CRE domain is entirely necessary and, to some extent, sufficient to account for the PACAP response.

In chromaffin cells, PACAP may stimulate both AC/cAMP and PLC-β/PI/Ca^{2+}pathways (Deutsch and Sun 1992). Expression of the protein kinase A (PKA) inhibitor PKI or an inactive catalytic subunit of PKA (ΔPKAβ) strongly inhibited PACAP38-induced CGA promoter *trans*-activation (Fig 2C and 2D), suggesting that upregulation of the CGA gene by PACAP is dependent upon mobilization of the AC/cAMP/PKA pathway. Taken together, these findings suggest that the CGA CRE domain is entirely necessary and, to some extent, sufficient to account for the PACAP response. Thus, PACAP signals to CGA transcription through the proximal CRE domain in *cis*, and through cAMP, PKA and CREB in *trans.*

4. DESENSITIZATION OF PACAP-INDUCED CGA TRANSACTIVATION AND CATECHOLAMINE SECRETION

Sustained exposure of GPCRs to their cognate agonists generally results in a desensitization of the receptor responsiveness. Rapid desensitization results from uncoupling of the heterotimeric G protein from its receptor, as a consequence of receptor phosphorylation (Freedman and Lefkowitz 1996). We recently reported that PACAP-induced catecholamine secretion and

CGA transcription are both mediated by the PACl receptor isoform (Taupenot *et al* 1998). Indeed, Tanaka et al. reports that the "hop" splicing variant of PACl is present in chromaffin cells (Tanaka *et al* 1998). To date, homologous desensitization of the PACl receptor in chromaffin cells remains uncharacterized.

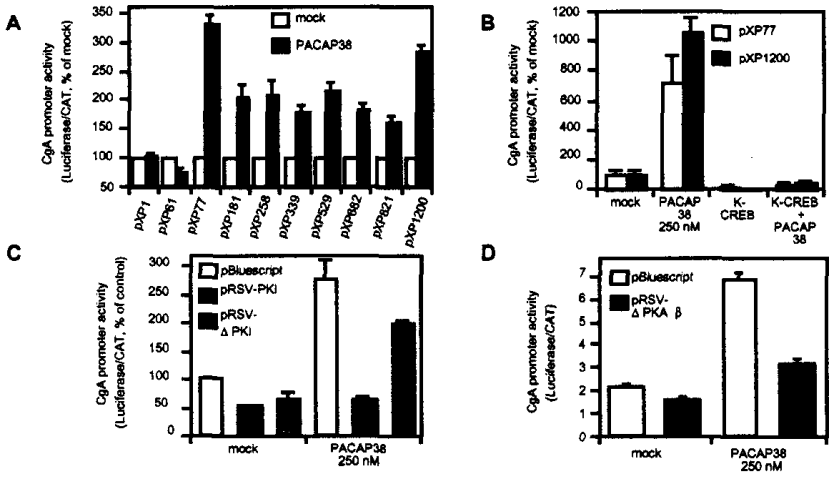

Figure 2. (A) Response of 5' deletions of the CGA promoter to stimulation by PACAP38. Promoter deletion positions are numbered by reference to bp upstream of the mouse CGA transcription initiation site. (B) Effect of expression of the dominant negative mutant KCREB on 77 or 1200 bp CGA promoter *trans*-activation. In all 3 experiments, cells were exposed for 20 h with or without 250 nM PACAP. Effect of expression of the PKA inhibitor, PKI (C) or the PKA inactive catalytic subunit (D) on the response of CGA promoter to PACAP38. PC12 cells were co-transfected with pXP1200 along with either βRSV-PKI (active PKI), pRSV-ΔPKI (inactive mutant PKI) or pRSV-ΔPKAβ (inactive b catalytic subunit). Cells were incubated for 20 h with or without 250 nM PACAP38. pBluescnpt was co-transfected for DNA mass balance. Reproduced in part from Taupenot *et al* (1 998).

Prolonged exposure of chromaffin cells to PACAP can evoke long-lasting Ca^{2+} influx (Tanaka *et al* 1996) and catecholamine secretion with no apparent desensitization (Babinski *et al* 1996). We show here that prior exposure of cells to PACAP for 10 min, followed by washing (twice for 5 min), inhibited the subsequent stimulatory effect of PACAP on catecholamine secretion (Fig 3A). Desensitization of the secretory response (IC_{50}~1.3 nM) is obtained with 18-fold less PACAP than that required to induce secretion (EC_{50}~23 nM), suggesting that the signaling pathway underlying PACAP-induced secretory desensitization might be distinct from that mobilized during secretion. Homologous desensitization of the secretory response did not result from depletion of the intracellular pool of releasable secretory granules since PACAP pre-incubation did not affect catecholamine

secretion induced by direct membrane depolarization with KC1 (data not shown). Surprisingly, desensitization of the secretory response occurred only when pre-incubation of the cells with PACAP was followed by repeated washing before rechallenge with the polypeptide. After washing, at least partial dissociation of PACAP from its binding site might be a necessary step to achieve full desensitization/downregulation of PAC1, perhaps as a consequence of incompletely characterized changes in receptor conformation.

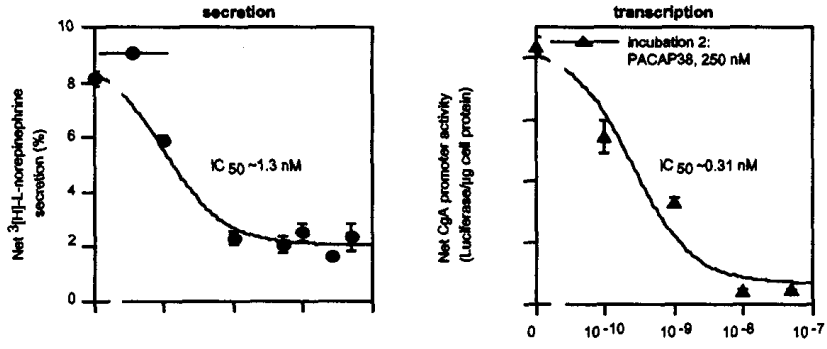

Figure 3. PACAP-induced catecholamine secretion and CGA *trans*-activation are subject to desensitization in PC12 cells. (A) Desensitization of subsequent catecholamine release by prior exposure to PACAP. [^{3}H]-L-norepinephrine prelabeled cells were preincubated for 10 min with indicated concentration of PACAP38 (*incubation* 1). Cells were washed twice with secretion medium (2 x 5 min) and subsequently exposed for 20 min to 250 nM PACAP38 (*incubation* 2). (B) Dose-dependent effect of PACAP preincubation on CGA transcription desensitization. 30 min after transfection of cells with pXP1200, cells were exposed to the indicated concentrations of PACAP38 for an additional 150 min (*incubation* 1). Following two washes with 2 ml of medium, cells were incubated for 4 hours (*incubation* 2) in mock buffer or in 250 nM PACAP38. Reproduced in part from Taupenot *et al* (1999).

As revealed by a Ca^{2+} uptake experiment, desensitization of the secretory response results in a diminution of extracellular Ca^{2+} influx (Taupenot et al 1999) which' is consistent with earlier studies by us and others which indicate that Ca^{2+} influx through L-type channels is an essential requirement for PACAP secretory action (Tanaka *et al* 1996, Taupenot *et al* 1998, Taupenot *et al* 1999).

We then questioned whether transcriptional activation of CGA by PACAP could undergo homologous desensitization similar to that observed for secretion. Pre-exposure of cells to PACAP, followed by washing, markedly impaired the subsequent stimulatory effect of PACAP on transcriptional activation of the CGA promoter (Fig 3B). Desensitization of PACAP-induced transcription became significant when cells were pre-

exposed to 0.1 nM PACAP, and complete desensitization of transcription was reached following a 10-50 nM PACAP pre-incubation (IC_{50}~0.31 nM; Fig 3B).

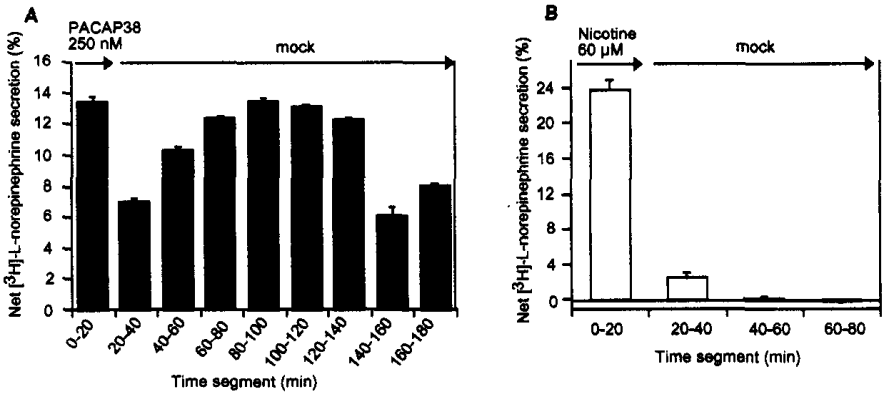

Figure 4. [^{3}H]-L-norepinephrine prelabeled PC12 cells were exposed for 20 min to mock, 250 nM PACAP38 (A) or to 60 µM nicotine (B). After a 20 rnin incubation and at the following indicated time segments, extracellular media were collected and replaced by secretion buffer alone (mock). Then at the final 20 min time segment, cells were lysed and the intracellular [^{3}H]-L-norepinephrine content was determined. Catecholamine secretion in any given 20 rnin time segment was evaluated by counting the amount of [^{3}H]-L-norepinephrine released, and dividing by the sum of the amount released during that 20 min plus the amount remaining in the cells at the end of that time period. For any given time segment, the intracellular [3H]-L-norepinephrine content at the end of that period represents the radioactive signal in the final cell lysate summed with the radioactive signal measured in the extracellular media of all further time segments until the end of the experiment (the point of cell lysis). Reproduced from Taupenot *et al* (1999).

The IC_{50} for this inhibition (~0.31 nM) was far (32-fold) lower than the dose required for PACAP to trigger transcription (EC_{50}~10 nM). Moreover, the inhibitory effect of PACAP (10 nM) 3 hour preincubation on CGA promoter expression was prolonged, and could still be detected after 24 hours of subsequent high dose PACAP (data not shown). Muller *et al.* (1998) recently reported that pretreatment of mouse central catecholaminergic CATH.a cells with PACAP enhanced subsequent PACAP-induced CREB-mediated transcription, a finding in sharp contrast with our current results, considering that the CGA promoter CRE domain is sufficient to account for PACAP stimulation (Fig 2 and Taupenot *et al* 1998). The discrepancy between our findings and those of Muller *et al.* may reflect differential cAMP responses in PC12 versus mouse CATH.a cells. In PC12 cells PACAP signals to CGA transcription through the CRE in *cis* and through cAMP, PKA and CREB in *trans* (Fig 2). However, in CATH.a cells

both CREB protein and its mRNA are downregulated by forskolin-induced increases in cAMP (Widnell *et al* 1996).

5. PRESENCE OF A NONDESENSITIZING PHASE IN PACAP-EVOKED CATECHOLAMINE SECRETION

Acute (≤20 min) exposure of PC12 cells to PACAP elicited long-lasting catecholamine release for up to 3 hours (Fig 4A). Thereafter, even replacement of the PACAP-containing medium by PACAP-free medium did not prevent this sustained PACAP-evoked catecholamine secretion. When cumulative catecholamine release was measured for 3 hours after an acute exposure (20 min) of cells to PACAP, a remarkable 76.9±0.22 % of total cell content of catecholamines had been secreted. In sharp contrast, acute treatment of PC 12 cells with nicotine induced massive but time-limited secretory response. Catecholamine secretion returned to a basal value within 40-60 min (Fig 4B). Since the secretory response to PACAP is subject to homologous desensitization after even a 10 min pre-incubation (Fig 3A), the long-lasting (20-1 80 min) PACAP secretory response (Fig 4A) is an initially unexpected characteristic of the PC12 cell secretory response to PACAP. In chromaffin cells, the signaling pathway underlying the action of PACAP on catecholamine secretion remains unclear. Some authors suggest that cAMP mediates the stimulatory effect of PACAP (Perrin *et al* 1995, Przywara *et al* 1996) whereas others report that voltage-operated Ca^{2+} channels (VOCCs) play a key role in secretion (Isobe *et al* 1993, O'Farrell and Marley 1997).

Our data reveals that PACAP exhibits dual secretory effects on PC12 cells, which can be discriminated by their sensitivity to the to the selective L-type voltage-operated Ca^{2+} channel (VOCC) antagonist nifedipine: the initial phase of the secretory response may be defined as a dihydropyridine (DHP)-sensitive response, whereas the long-term response is DHP-insensitive (Fig 5A). The potential contributions of DHP-insensitive VOCCs to the prolonged secretory effect of PACAP was further examined by testing the effect of a combination of the N-type VOCC blocker ω-conotoxin GVIA with the N- and P/Q-type antagonist ω-conotoxin MVIIC or the effects the T- and L-type VOCC inhibitor bepridil or the T-type inhibitor flunarizine. Extended catecholamine secretion was unaltered by the combination of the two ω-conopeptides or by the two chemical inhibitors (data not shown).

Whereas PACAP-induced transcriptional activation of CGA is a Ca^{2+}-independent process (Taupenot *et al* 1998), extracellular Ca^{2+} entry through cell surface Ca^{2+} channels appears to be a key event in PACAP's effect on

both initial and prolonged catecholamine release. Calcium uptake and [^{3}H]-L-norepinephrine release experiments revealed that initial secretion evoked

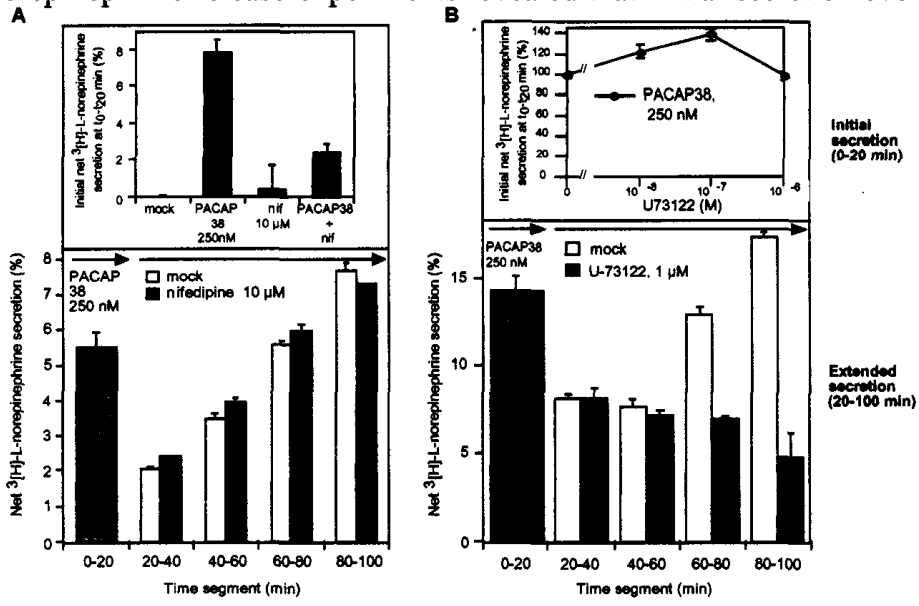

Figure 5. (A&B), *upper panels.* Effect of nifedipine or U-73122 on catecholamine secretion over the initial (20 min) secretion. [^{3}H]-L-norepinephrine-prelabeled PC12 cells were incubated in indicated concentration of U-73 122. (A&B), lowerpanels. Effect of nifedipine or U-73 122 on extended catecholamine secretion. [^{3}H]-L-norepinephrine-prelabeled PC 12 cells were exposed to mock or 250 nM PACAP38. After a 20 min incubation and at the indicated later time segments, extracellular media were collected and exchanged for mock medium or secretion medium plus 10 µM nifedipine or 1 µM U-73 122. Reproduced from Taupenot *et al* (1999).

by PACAP requires a massive influx of external Ca^{2+} likely through L-type VOCC (Fig 5A and Taupenot *et al* 1999). In contrast, lower magnitude Ca^{2+} entry through Ca^{2+} channels sensitive to broad spectrum blockers (Zn^{2+}, Ni^{2+}, Cd^{2+}) but insensitive to DHP is required to maintain long-lasting effect of PACAP on catecholamine release (data not shown ; Taupenot *et al*1999).

6. ROLE OF PLC-β IN PACAP-INDUCED CATECHOLAMINE SECRETION

PAC1 not only stimulates the effector AC, but also transduces its signal through the PLC-β/PI pathway in PC12 and other cell types (Spengler *et al* 1993). Monomeric a subunits of the G_q family of G proteins as well as βγ heterodimeric subunits of the $G_{i/o}$, class mediate activation of PLC-β

(Gudermann *et al* 1997). These two families of G proteins can be distinguished by their sensitivity to pertussis toxin (PTX): $G_{i/o}$ family are sensitive to ADP-ribosylation by PTX whereas the Gq family is PTX-insensitive. We found that neither initial (20 min) nor extended (20<t<100 min) secretion after PACAP was sensitive to PTX, effectively ruling out Gi or G_o isoforms (data not shown). Continuous exposure of cells to U-73 122, a selective antagonist of PLCs, impaired the prolonged secretory response triggered by PACAP without affecting the initial phase of the secretory response (Fig 5B). Hence, and in contrast to initial secretion, inhibition of sustained catecholamine release by U73 122 suggests involvement of a $G_{q/11}$/PLC-β/PI signaling pathway.

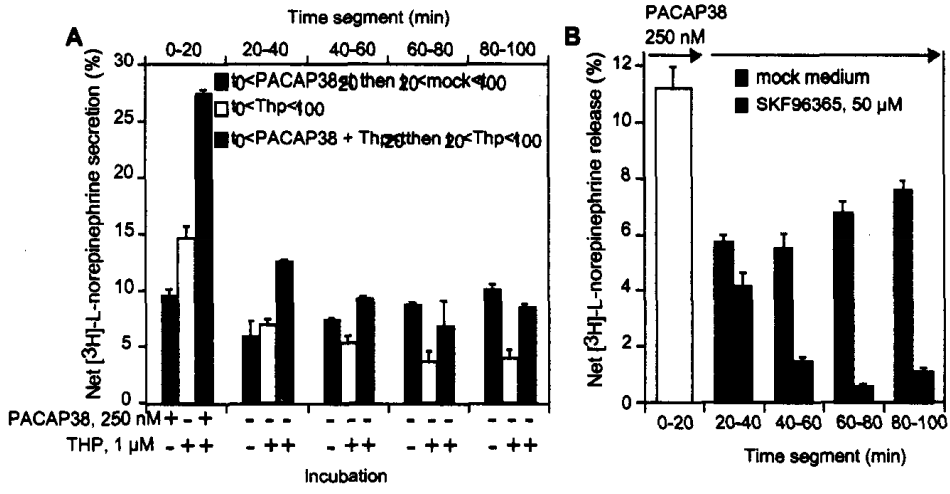

Figure 6. Effect of thapsigargin and SKF96365 on PACAP-induced norepinephrine release. [^{3}H]-L-norepinephrine prelabeled PC12 cells were exposed to mock (A, B), 250 nM PACAP38 (A,B), 1 µM thapsigargin (ThP; A) or 250 nM PACAP38 plus 1 µM ThP (A). After a 20 min incubation and at the following indicated time segments, extracellular media were collected and changed for mock (A, B), 1 µM ThP (A) or 50 µM SKF96365. Reproduced from Taupenot *et al* (1 999).

7. ROLE OF STORE-OPERATED CALCIUM CHANNELS IN PACAP TRIGGERED CATECHOLAMINE SECRETION

Store-operated Ca^{2+} entry (also referred as capacitative Ca^{2+} entry) from the extracellular space is a widespread mechanism in non-excitable cells where depletion of intracellular Ca^{2+} stores signals the opening of store-operated Ca^{2+} channels (SOCCs) in the plasma membrane (Favre *et al* 1996).

In excitable cells the role of capacitative Ca^{2+} entry is less clear, since these cells possess other Ca^{2+} entry pathways such as VOCCs. In PC12 cells, the SOCC pathway may be initiated by several perturbations of intracellular Ca^{2+} stores, including activation of ryanodine receptors (RyRs), inositol 1,4,5 trisphosphate (P_3) receptors, or inhibition of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) by thapsigargin (Bennett *et al* 1998). However, the contribution of cell surface SOCCs to neurotransmitter release from chromaffin cells (e.g. catecholamine) remains unsettled (Koizumi and Inoue 1998, Powis *et al* 1996). SERCA inhibition by agents such as thapsigargin characteristically triggers sustained, capacitative Ca^{2+} entry (Favre *et al* 1996). As shown in Fig 6A, depletion of intracellular Ca^{2+} stores of PC12 cells by continuous exposure to thapsigargin stimulated norepinephrine release, which showed only little desensitization for a period of up to 100 min. Co-application of thapsigargin together with PACAP caused additive stimulation of secretion within the initial (0-20 min) phase. Thereafter, by 40-100 further minutes in PACAP-free secretion medium, the secretory response in cells treated with initial (20 min) PACAP plus sustained (0-100 min) thapsigargin was no greater than in cells treated with initial PACAP alone (Fig 6A).

Taken together, these data suggest that PACAP and thapsigargin use distinct Ca^{2+} entry pathways to elicit the initial phase ($\leq$20 min) of catecholamine release, while the less than additive effect of combined PACAP and thapsigargin during the prolonged phase of secretion suggests that the two compounds may share the same signaling pathway, i.e. Ca^{2+} release from IP3-sensitive intracellular stores, followed by activation of capacitative Ca^{2+} entry. To further probe a possible contribution of SOCCs to long-lasting catecholamine secretion evoked by PACAP, we tested the effect of SKF96365, a SOCC/non-VOCC blocker (Favre *et al* 1996). SKF96365 markedly inhibited extended norepinephrine release for up to 100 min (Fig 6B), supporting the involvement of a store-operated Ca^{2+} entry mechanism in PACAP-induced extended secretion.

8. CONCLUSIONS

We propose a model depicting putative signal transduction pathways underlying PACAP-induced CGA transcription and catecholamine secretion in PC12 cells (Fig 7). PACAP mediates both secretion and transcription through the PAC 1 receptor, but with quite different post-receptor signaling pathways. PACAP signals to CGA transcription through a Ca^{2+}-independent

pathway involving the CGA promoter CRE domain in cis and PKA and the transcription factor CREB in *trans*. PACAP-evoked secretion and transcription are subject to homologous desensitization in PC 12 cells; however, PACAP also provokes long-lasting secretion, even under dose and time circumstances where acute, DHP-sensitive secretion has been desensitized. While initial secretion is mediated by an L-type VOCC, extended secretion may involve a SOCC activated through a $G_{q/11}$/PLC-β/PI signaling pathway. Further characterization of PACAP signaling pathways will require definitive identification of the SOCC channel involved in the sustained catecholamine release.

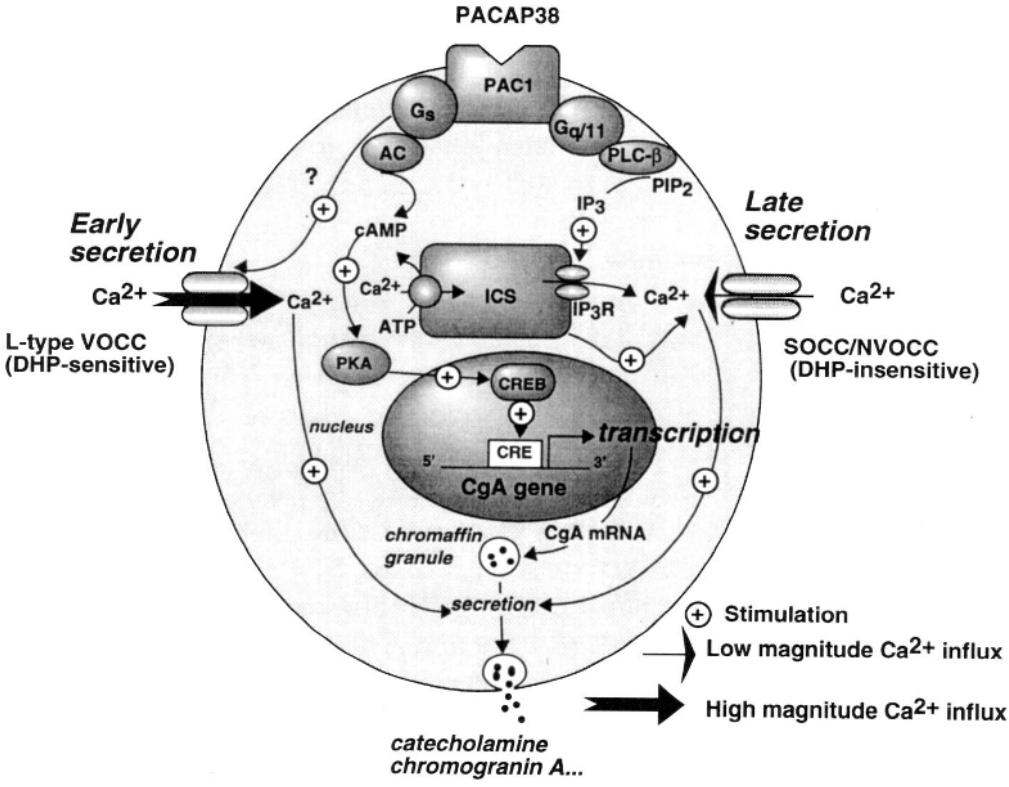

Figure 7. Proposed signal transduction pathways underlying PACAP-induced catecholamine release and chromogranin A transcription in PC12 cells. CRE: cAMP response element (here: [-7 1 bp]5'-TGACGTAA-3'[-64 bp]). CREB: homodimeric CRE-binding/*trans*-activating protein. AC: adenylyl cyclase. G_S: stimulatory heterotrimeric G protein. Gq/1 1 heterotrimeric G protein of the $G_{q/11}$ family. ICS: intracellular Ca2+ store. PIP_2: phosphatidylinositol-4-bisphosphate. IP3: Ins(1,4,5)P3. IP3: receptor for Ins(1,4,5)P_3. NVOCC: non voltage-operated Ca^{2+} channel. SOCC: store-operated Ca2+ channel. DHP: dihydropyridine Ca^{2+} channel antagonist. (+): stimulation or activation. Reproduced from Taupenot *et al* (1 999).

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8

NEUROENDOCRINE CELL-SPECIFIC EXPRESSION AND REGULATION OF THE HUMAN SECRETOGRANIN II GENE

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1. INTRODUCTION

Chromogranins are secretory products of endocrine and neuronal cells. Owing to their selective and abundant expression in secretory cells, chromogranins are widely used as markers for the diagnosis of endocrine and neuroendocrine neoplasms (Rosa and Gerdes 1994). However, the molecular mechanisms that govern their restricted expression are not fully understood. In addition, it is well known that the expression level of a particular chromogranin depends on the cell type. For instance, secretogranin II (SgII) is highly expressed in gonadotrope cells compared to other pituitary cells (Rosa *et al* 1985), or in glucagon-producing alpha cells compared to the other pancreatic endocrine cell types (Schmid *et al* 1994), suggesting that cell-specific determinants may play critical roles in specifying the expression of chromogranins.

The effects of a variety of treatments on SgII gene expression have been studied in different models *in vivo* or *in vitro*. For example, in the rat brain, SgII mRNA levels are increased in the paraventricular and supraoptic nuclei of the hypothalamus following osmotic stimulation (Mahata *et al* 1992a), or in hippocampal neurons after kainic acid injection (Mahata *et al* 1992b). In

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the adrenal gland, reserpine-induced splanchnic nerve stimulation also leads to an increase in SgII mRNA levels (Hofle *et al* 1991).

These regulatory effects probably involve various signal transduction pathways that ultimately modulate the activity of the SgII gene promoter. The signalling pathways that regulate SgII gene expression have been investigated in cultured cells under treatment by different stimulators. Thus, cell-depolarization by potassium which triggers calcium influx, or activation of protein kinases A and C by forskolin or phorbol ester treatment, respectively, have been shown to increase SgII mRNA levels in bovine chromaffin cells (Fischer-Colbrie *et al* 1990), in cultured cortical neurons (Scammell *et al* 1995) or in neuroblastoma cells (Weiler *et al* 1990). Nerve growth factor (Laslop and Tschernitz 1992), nicotine (Wolkersdorfer *et al* 1996) or histamine (Bauer *et al* 1993) are first messengers which stimulate these different transduction pathways in order to regulate SgII gene expression in neuroendocrine cells.

Cloning of the SgII gene and functional characterization of its promoter will now allow to understand the molecular mechanisms underlying the restricted and cell-specific expression of the SgII gene in neuroendocrine cells and its regulation by a variety of neuronal and humoral factors.

2. STRUCTURE OF THE HUMAN SGII GENE

The gene encoding the human SgII protein has been isolated and its structure characterized. The organization of this gene is relatively simple since there are only two exons separated by a 3-kb intron, and the entire coding region for the SgII protein is contained within the second exon (Fig 1).

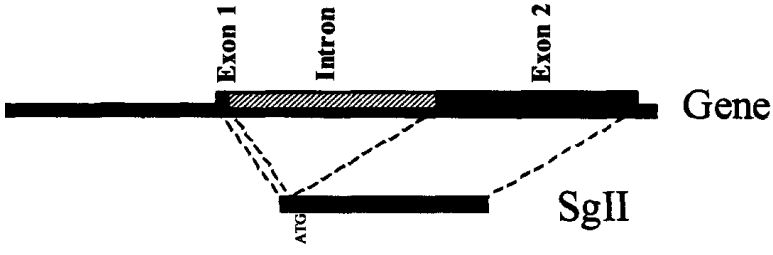

Figure 1. Schematic representation of the organization of the human SgII gene. The gene contains two exons separated by the single intron. *ATG* refers to the initiating methionine codon of SgII sequence.

Sequence analysis of the human SgII gene promoter revealed the occurrence of a typical TATA box present 23 nucleotides upstrean of the transcription initiation site that was mapped on this gene by RNAse protection assay (Desmoucelles *et al* 1999). Within the promoter, several potential transcriptional regulatory sites have been found including a consensus cyclic AMP-responsive element (CRE), activator protein-1 (AP-1)-binding sites, several enhancer box (E box)-binding sites as well as an Oct-1 recognition site (Table 1).

Table 1. Potential transcriptional cis-active elements in the human SgII gene promoter

Cis-element	Sequence	Position	Consensus
CAMP-responsive element (CRE)	TGACGTCA	-62, -69	TGACGTCA
Activator protein-1 (AP-1)	GGAGTCA	-43,-49	TGA(C/G)TCA
	TGACACA	-306,-312	
	TGAGTCA	-1126,-1132	
Enhancer box (E box)	CAGGTG	-200,-205	CANNTG
	CAAATG	-820, -825	
	CACATG	-1094, -1099	
	CAATTG	-1128, -1133	
	CAGATG	-1319, -1324	
	CAAATG	-1554, -1559	
Oct-1	TTATTTGCAT	-1247,-1256	$((A/T)_4TNCAT)$

These potential regulatory elements have been also described in several other genes expressed in neuronal, endocrine and neuroendocrine cells. Oct-1 -binding sites which are targets of POU-homeodomain transcription factors (Wegner *et al* 1993) were characterized in the promoters of two neuropeptide genes, the vasoactive intestinal polypeptide, VIP (Hahm and Eiden 1998) and the gonadotropin-releasing hormone (Clark and Mellon 1995) genes. Homeodomain proteins contribute to the basal expression of these two genes in neuronal cells. The expression and regulation of the genes encoding growth hormone and prolactin are also under the control of a POU-domain protein, the transcription factor Pit-1 (Bradford *et al* 1997).

E box elements are recognition sites for helix-loop-helix proteins, and have been shown to be implicated in the transcriptional activity of neuropeptide or peptide hormone genes such as the proopiomelanocortin gene in the pituitary (Therrien and Drouin 1993) or the secretin gene in enteroendocrine cells (Mutoh *et al* 1997). Because the SgII gene is widely expressed in neuronal and endocrine cells, the existence of several potential helix-loop-helix and homeodomain protein-recognition sites in its promoter, along with other sites which recognize more ubiquitous transcription factors

(Table 1), could represent the basis for its widespread and yet cell-specific expression.

3. BASAL EXPRESSION OF THE SGII GENE IN NEUROENDOCRINE CELLS

It is well established that the expression level of members of the chromogranin family of proteins is highly cell-dependent (Fischer-Colbrie *et al* 1990, Helman *et al* 1988, Mouland *et al* 1994). For instance, basal levels of SgII mRNA are higher in the corticotrope cell line AtT-20 than in the neuroblastoma cell line SH-SY5Y, and no expression occurs in a non-neuronal, non-endocrine cell line, the epithelial kidney cell line LLC-PK1 (Fig 2).

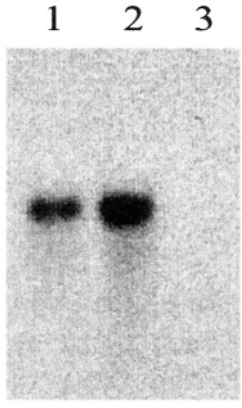

Figure 2. Neuroendocrine cell-specific expression of SgII mRNA. Expression is detected in the neuroblastoma SH-SY5Y (1) and the corticotrope AtT-20 (2) cell lines, but not in the renal epithelial cell line LLC-PKl (3).

Recently, several studies dealing with the neuroendocrine cell-specific expression of the SgII gene have been reported (Desmoucelles *et al* 1999, Jones *et al* 1996, Mahata *et al* 1999). Transfection of different cell lines with chimeric reporter constructs driven by the SgII gene promoter revealed that the CRE site plays a major role in the activity of the promoter. Deletion of this element almost abolished the activity of the human SgII promoter (Fig 3). Similar results have been reported for the mouse SgII gene (Mahata *et al* 1999) and for the rat (Wu *et al* 1995) or human (Canaff *et al* 1998) chromogranin A (CGA) genes. A CRE has been also characterized in the chromogranin B (CGB) gene (Jungling *et al* 1994), suggesting that the

presence of a CRE may represent the common denominator for the expression of chromogranin genes in neuroendocrine cells.

However, the CRE may not be sufficient to direct neuroendocrine cell-specific expression of chromogranins given the ubiquitous character of the CRE-binding proteins (CREB/CREM/ATF family members) whose expression occurs in neuroendocrine as well as non-neuroendocrine cells (Meyer and Habener 1993). Nevertheless, it is known that a CRE can bind to different combinations of basic-leucine-zipper proteins (Chatton *et al* 1994, Hai and Curran 1991) and it is therefore possible that the CRE site binds specific complexes that only occur in neuroendocrine cells and not in other cells.

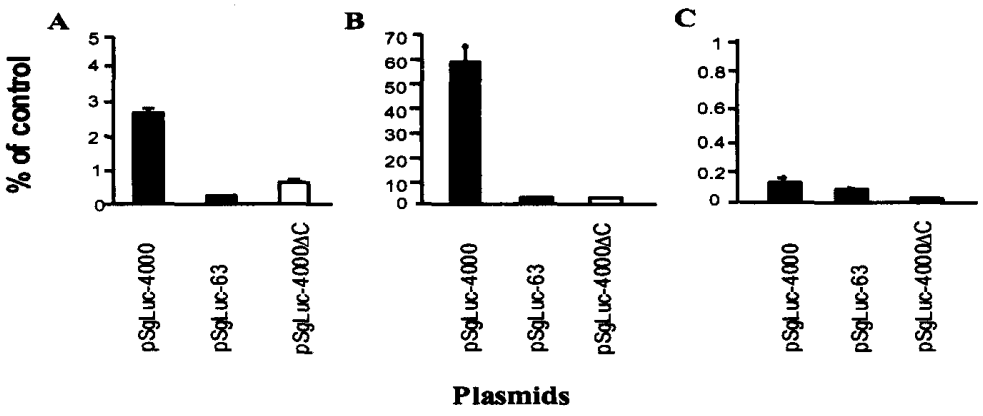

Figure 3. Cell-specific expression of the human SgII gene. Constructs with 4 kb, 63 bp, or 4 kb with deleted CRE of the SgII promoter linked to luciferase were transfected in SH-SY5Y (A), AtT-20 (B) and LLC-PK1 (C) and their activity was measured and expressed as the percentage of a SV40-luciferase construct activity.

Alternatively, CRE-binding proteins may interact with tissue-specific coactivators belonging to other transcription factor families, which bridge the activated CRE-binding proteins to the basal transcriptional machinery (Goodman and Mandel 1998). It has recently been shown that CREM, a CRE-binding protein modulator and a CREB family member, specifically interacts with a transcription factor called ACT (activator of CREM in testis) in a tissue-specific manner to activate the basal transcriptional machinery (Fimia *et al* 1999). This coactivator is a member of a large transcription factor family whose members exhibit tissue-specific expression. Such proteins may represent candidates that impart neuroendocrine cell-specificity to chromogranin genes through the CRE.

Finally, the CRE and the CRE-binding proteins may represent only a part of the mechanism allowing neuroendocrine cells to express chromogranin genes. Thus, it has been shown for the SgII (Desmoucelles *et al* 1999, Jones

et al 1996) as well as for the CGA (Canaff *et al* 1998, Nolan *et al* 1995, Wu *et al* 1995) genes that other cis-regulatory elements and consequently their cognate transcription factors may be important for the fine-tuning of the cell-specific expression of these genes. Although little is known on these elements or the proteins that interact with them, it is possible that the synergistic action of several elements may be responsible for the cell-specificity. There are several examples in the literature showing that a CRE and other cis-active elements may cooperate to impart the cell-specific expression of certain genes. The transcriptional activity of the somatostatin and VIP genes for example requires the cooperative effect of CRE-binding proteins and homeodomain transcription factors (Hahm and Eiden 1998, Schwartz and Vallejo 1998).

To add to the complexity of the mechanisms that regulate chromogranin gene expression, it has been recently shown that a sequence homologous to a serum response element is important for the neuroendocrine cell-specific transcriptional activity of the mouse SgII gene promoter (Mahata *et al* 1999). Inspection of the human promoter sequence showed that this element is not conserved, indicating that species-specific cis-elements and cognate transcription factors may also be involved in the regulation of chromogranin gene expression.

4. REGULATION OF SGII GENE TRANSCRIPTION BY SECOND-MESSENGER PATHWAYS

The control of the expression of the chromogranin genes has been extensively studied, and the data indicate that these genes may be differentially regulated in a gene- and cell-dependent manner. For example, SgII gene expression is stimulated by histamine in bovine chromaffin cells whereas CGA or CGB gene expression is not altered by this neurotransmitter (Bauer *et al* 1993), suggesting that gene-specific mechanisms are involved.

On the other hand, several examples of differential regulation of a given chromogranin gene depending on the cell type have been reported (Iacangelo *et al* 199 1). We found that SgII gene expression is up-regulated by forskolin and the phorbol ester TPA in the neuroblastoma cell line SH-SY5Y but not in the pituitary corticotrope cell line AtT-20 (Fig 4A, B). Transfection of reporter gene constructs driven by the SgII promoter in these two cell lines revealed that protein kinases stimulated the transcriptional activity of the SgII gene through the CRE in the neuroblastoma cell line (Fig 4C). In contrast, no effect was observed in the corticotrope cell line, in very much the same way as the endogenous gene (Fig 4D).

Thus, it appears that the SgII CRE mediates responsiveness to second-messenger stimulation, most likely by binding activated CREB transcription factor family members. However, additional factors are probably necessary to impart the cell-specific regulation. It has been shown that stimulation of SgII gene expression in pituitary GH cells is not observed unless protein synthesis is inhibited by cycloheximide treatment and that the stimulatory effect observed in the presence of forskolin and cycloheximide is mediated by the SgII CRE (Jones and Scammell 1998). This observation indicates that inhibitory factors may prevent activation of SgII gene transcription through the CRE. The authors could not find any difference in the CRE-bound proteins from nuclear extracts of forskolin- *vs* cycloheximide/forskolin-treated cells using gel-shifting assay. This result indicates that protein-protein interactions rather than protein-DNA interactions are probably responsible for the regulation of the SgII gene in GH cells.

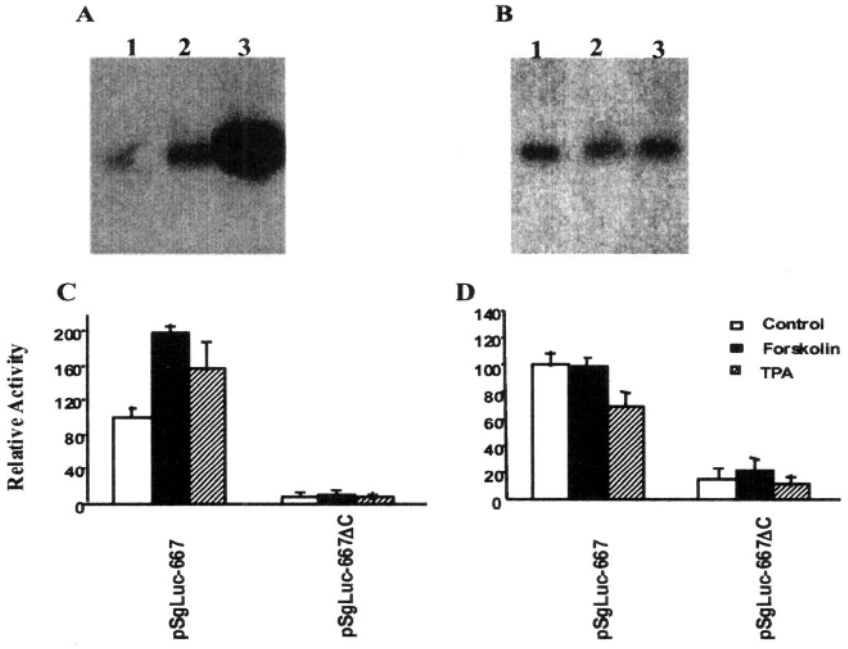

Figure 4. Regulation of SgII mRNA levels and SgII promoter activity by second-messenger pathways. SgII mRNA levels were analyzed in SH-SY5Y (A) and AtT-20 (B) cell lines that were treated either with the vehicle only (1), with forskolin (2) or with TPA (3). Regulation of the activity of the SgII promoter with or without the CRE in SH-SY5Y (C) or AtT-20 cells (D).

This is a good example of how the SgII gene could be regulated in a cell-specific manner, although other mechanisms may also occur. Synergistic interaction between CRE-binding proteins and cell-specific transcription factors is another possible mechanism that may occur to confer cell-specific regulation to the SgII gene. Such a mechanism has been described for the effect of cAMP on dopamine beta-hydroxylase gene transcription which requires the homeodomain protein Arix in addition to CREB (Swanson *et al* 1997).

CREB has been shown to be implicated in cAMP regulation of the mouse SgII gene transcription in HIT cells and SN56 cells (Cibelli *et al* 1996), or the rat CGA gene in PC12 cells (Taupenot *et al* 1998). However, it is not known yet whether CREB is sufficient to elicit the response of chromogranin genes to cAMP stimulation or whether other factors whose expression may be cell-specific, are also involved. In addition, protein kinase C stimulation of SgII gene transcription also appears to involve the CRE, but the transcription factors involved remain unkown.

5. CONCLUSION

The elucidation of the structure of the SgII gene in several mammalian species has led to the appreciation of the major role played by the consensus CRE found within the proximal promoter, in both the basal expression and the regulation of this gene by second messengers. The second-messenger signalling pathways are probably activated *in vivo* to mediate the action of different neurotransmitters and hormones that regulate SgII gene expression in neurons and endocrine cells. At present, the mechanisms that govern chromogranin gene expression are only beginning to be understood. Thus, although all the chromogranin genes characterized so far bear a CRE in their promoters, this cis-active element and cognate binding proteins are probably not sufficient to impart the gene- and cell-specific expression and regulation of this family of secretory proteins. Future studies using site-directed mutagenesis of the putative regulatory elements present in the promoters of these genes, such as those described above for the SgII gene (Table 1), and functional characterization of the resulting constructs for their basal and stimulated expression in different model systems, will undoubtedly shed light on the mechanisms of chromogranin gene transcriptional activity in the neuro-endocrine system. An example of the contribution of other cis-active elements in addition to the CRE in chromogranin gene regulation has been reported recently for the gastrin effect on CGA gene transcription in enterochromaffin-like cells. The action of the gastric peptide is exerted

through increased binding of both the Sp1/Egr-1 transcription factor and CREB to their target sites in the CGA promoter (Hocker *et al* 1998).

Identification of the transcriptional cis-active sites within the promoters of the different chromogranins will allow to understand their differential expression in normal and tumoral neuroendocrine cells.

ACKNOWLEDGMENTS

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9

CHARACTERIZATION OF CHROMOGRANINS IN THE FROG *Rana ridibunda*

Structure, Expression, and Functional Implications

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1. INTRODUCTION

Secretogranin II (SgII) and chromogranin A (CGA) are members of the chromogranin family of secretory polypeptides that are present in large dense-core vesicles of endocrine and neuronal cells. The physiological function of chromogranins is not completely understood, but the widespread and abundant expression of these proteins has led to their use as markers for endocrine neoplasm diagnosis. Chromogranins are characterized by a high proportion of acidic amino acids that represent 20-30% of the total amino acid content, the presence in their sequences of multiple pairs of basic amino acids which are potential cleavage signals for prohormone convertases, and their localization in secretory vesicles from which they are released upon stimulation (Simon and Aunis 1989).

The structural characteristics of chromogranins have oriented the investigations on their function mainly into two directions. First, because chromogranins are components of the intragranular complex and because of their tendency to aggregate in high calcium and acidic pH conditions which are the hallmark of the intragranular milieu, it has been suggested that these proteins may participate in the intragranular events leading to secretory granule formation (Iacangelo and Eiden 1995). Second, based on the fact that chromogranins are sites of endoproteolytic processing which gives rise

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to smaller peptides that are released from endocrine and neuronal cells, it is thought that the released peptides may represent biologically active molecules (Winkler and Fischer-Colbrie 1992).

The structure of chromogranins has been elucidated only in mammals where a high degree of sequence identity has been found. Since evolutionary pressure should have acted to conserve the biologically active regions, characterization of chromogranins in nonmammalian vertebrates should provide crucial information on the functional determinants of these proteins. Amphibians, which are the first vertebrates that have evolved to a terrestrial life, are a group of extraordinary interest in which to investigate the neurochemical facets of evolution. We have thus decided to isolate the cDNAs encoding CGA and SgII in the frog *Rana ridibunda*, a species which is widely used for studying neuroendocrine regulatory mechanisms.

2. CHARACTERIZATION OF FROG SGII AND CGA

In order to clone the cDNAs encoding chromogranins in the frog, we took advantage of highly conserved sequences of chromogranin-derived peptides that have been previously characterized. In particular, the 33-amino acid peptide secretoneurin (SN), which is a SgII-processing product that has been isolated from the frog brain (Vaudry and Conlon 1991) and which exerts some biological activities (Reinisch *et al* 1993, Saria *et al* 1993). On the other hand, within mammalian CGA, the sequence of the C-terminal region has been found to be more conserved than other parts of the protein, and its processing has been described (Winkler and Fischer-Colbrie 1992).

2.1 Sequence Analysis of Frog SgII

Using PCR and library screening, the cDNA encoding frog SgII has been isolated and characterized. Frog SgII contains 574 amino acids, 20% of which are aspartic or glutamic acid residues. The protein encompasses 11 pairs of basic amino acids. Comparison of frog and human SgII revealed an overall amino acid sequence identity of 46%, with the highest sequence identity occurring mainly in the regions encoding SN, its C-terminal flanking sequence and the C-terminal part of the protein (Anouar *et al* 1996). Interestingly, the sequences of these conserved regions are delimited by preserved dibasic amino acids which represent sites of cleavage by prohormone convertases (Fig 1).

Cloning of SgII cDNA from Xenopus (Holthuis and Martens 1996) also revealed the high conservation of the SN sequence and its downstream flanking peptide, whereas the cloning of SgII cDNA from goldfish showed

that only the sequence of SN has been highly conserved in fish (Blazquez *et al* 1998).

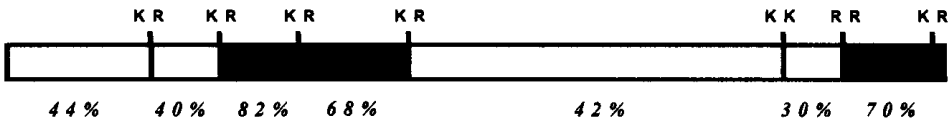

Figure 1. Schematic representation of SgII showing the amino acid sequence conservation between the frog and human proteins. Conserved pairs of basic amino acids and the percentages of amino acid identity are indicated. The shaded zones correspond to the most conserved regions within SgII.

Along with the facts that SN has been isolated from the frog brain and has been shown to exert biological activities in mammals such as a chemotactic activity during neurogenic inflammation and wound healing, these data indicate that SN should be considered as an authentic biologically active peptide. In addition, recent studies have shown the existence of SN-specific binding sites on human monocytes (Kong *et al*1998, Schneitler *et al*1998).

2.2 Sequence Analysis of Frog CGA

The cDNA encoding frog CGA has been isolated from a pituitary cDNA library. Frog CGA is a 399-amino acid protein including a signal peptide of 18 amino acids. Like its mammalian homologues, frog CGA is highly acidic, 28% of its amino acids being either glutamic acid (19%) or aspartic acid (9%) residues. The amino acid sequence of frog CGA comprises 11 pairs of basic residues (Turquier *et al* 1999).

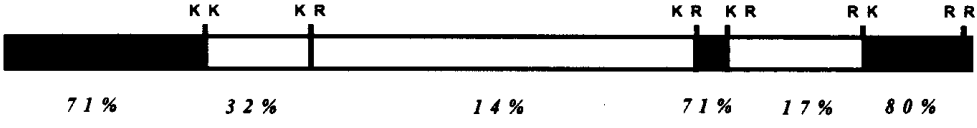

Figure 2. Schematic representation of CGA showing the amino acid sequence conservation between the frog and human proteins. Conserved pairs of basic amino acids and the percentages of amino acid identity are indicated. The shaded zones correspond to the most conserved regions within CGA.

Alignment of the frog and mammalian CGA amino acid sequences revealed an overall identity of 40-44%. The sequence identity is confined

mainly to three regions (70-80%) of the protein that are flanked by conserved pairs of basic amino acid residues. The other regions of the molecule exhibit only 14-30% identity (Fig 2). This finding suggests that processing of CGA through cleavage of dibasic sites could give rise to three peptides which may be of relevance for the physiological function of CGA.

In fact, two of the three conserved peptides have been previously isolated from mammalian tissues. The N-terminal peptide has been identified in bovine adrenal medulla (Aardal and Helle 1992) and rat insulinoma cells (Hutton *et al* 1987). This peptide has been named vasostatin in consideration of its vasoconstriction inhibitory effect on human blood vessels (Aardal et al 1993). The second highly conserved segment of CGA is a tetradecapeptide, named WE-14, which has been previously purified from a human ileal tumor (Curry *et al* 1992) and from pheochromocytoma tissue (Conlon *et al* 1992). The frog peptide is one amino acid shorter than its mammalian homologues. The fact that the sequence of such a discrete region of CGA has been conserved from amphibians to mammals is suggestive of an important function. The third conserved region is the C-terminal part of CGA which may give rise to a 35-amino acid peptide that has not been described to date and may represent a novel processing product of CGA. Characterization of this peptide in CGA-producing tissues would enable to demonstrate that all conserved regions of CGA exist as mature peptides *in vivo*, and therefore their consideration as the functional determinants of CGA could be further supported.

Besides, several CGA-derived peptides have been described for which biological activities have been attributed. These peptides have been obtained by purification from biological samples, by *in vitro* proteolysis of purified CGA or by direct design and synthesis of CGA-derived sequences. Pancreastatin (Pst) is a 49-amino acid peptide that has been initially isolated from the porcine pancreas and shown to inhibit insulin (Tatemoto *et al* 1986) or parathyroid hormone secretion (Fasciotto *et al* 1989). Molecular cloning of CGA (Benedum *et al* 1986, Iacangelo *et al* 1988) has subsequently revealed that Pst is in fact a product of CGA endoproteolytic processing. This observation provided the first evidence for the occurrence of a CGA-derived peptide that exerts a biological activity (Eiden 1987, Huttner and Benedum 1987), establishing thus a precedent for chromogranins as precursors to hormones. Cloning of frog CGA made it possible to seek the sequence conservation of Pst during evolution. Unexpectedly, it was found that the sequence of Pst is poorly conserved and that there is a large nucleotide deletion in the region of frog CGA which would correspond to the region encoding Pst in mammalian CGA. This finding does not support a role for Pst as an authentic regulatory peptide. Nevertheless, two hypotheses could be put forward to explain why this peptide is poorly conserved in frog

despite the fact that it has been characterized in mammals. First, Pst could be encoded by a separate gene in submammalian species, as suggested by an immunohistochemical study on Pst and CGA distribution in lower vertebrates (Reinecke and Maake 1993). Second, Pst may not exist in lower vertebrates but could have emerged during evolution of the CGA sequence. Pst exhibits a limited conservation even between mammalian species, indicating that the sequence of this peptide evolved rapidly during phylogeny. This is reminiscent of the peptide growth hormone-releasing hormone (GHRH) whose sequence conservation between mammalian species is rather modest (approximately 60% sequence identity) compared to the majority of neuropeptides, and which has not been identified with certainty in lower vertebrates. However, it is thought that the GHRH peptide of submammalian vertebrates could be located on the precursor of the neuropeptide pituitary adenylate cyclase-activating polypeptide (Parker *et al* 1997). Molecular cloning approaches could be helpful to identify a Pst-like gene in nonmammalian species. In the meantime, cloning of CGA from other species will undoubtedly shed light on the evolution of the Pst sequence.

More recently, other peptides that could derive from CGA have been described. These include chromacin, a peptide that has been shown to possess antibacterial activity (Strub *et al* 1996), and catestatin which inhibits nicotine-stimulated catecholamine release from adrenal medullary chromaffin cells (Mahata *et al* 1997). Catestatin has been found to act as a noncompetitive antagonist at the cholinergic nicotinic receptor. We found only a modest conservation of the sequences of chromacin and catestatin between frog and mammals. In addition, it is not known at present whether these peptides are generated *in vivo*. If this were the case, then their lack of conservation across vertebrate species would suggest that, as hypothesized above for Pst, these peptides may have emerged late during evolution.

3. EXPRESSION AND REGULATION OF FROG SGII AND CGA GENES

The distribution of chromogranins in mammals has been extensively studied. By contrast, little is known about the tissue-distribution and gene expression of chromogranins in nonmammalian species. We have used Northern blot and *in situ* hybridization techniques to determine the Occurrence of SgII and CGA mRNA in the central nervous system and in peripheral tissues of frog.

3.1 Tissue Distribution of Frog SgII mRNA

Northern blot analysis showed that SgII mRNA is highly expressed in the brain, the pituitary or the spinal cord and to a lesser extent in the adrenal (Fig 3A). Other tissues including liver, kidney or testis did not exhibit any hybridization signal (Fig 3A), indicating that in amphibians as in mammals, expression of the SgII gene is mainly confined to endocrine and neuronal cells. Within the frog brain, SgII mRNA is widely distributed with high concentrations occurring in the pallium, the thalamic or hypothalamic nuclei (Fig 3B). Overall, these data show that the distribution of SgII in nonmammalian species is similar to that previously described in mammals.

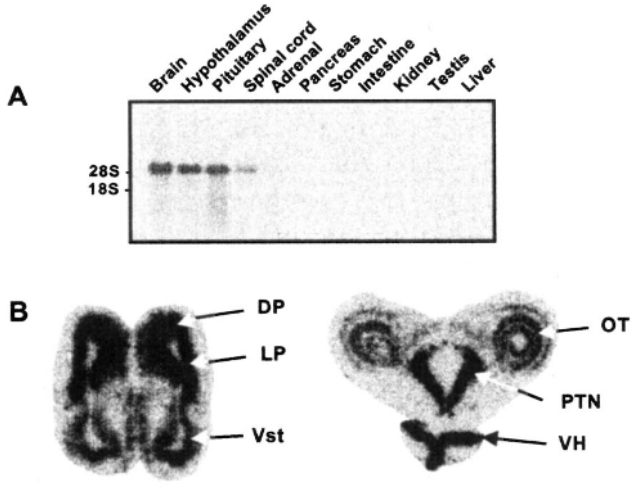

Figure 3. Tissue-distribution of SgII mRNA in the frog *Rana ridibunda*. A: Northern blot analysis of SgII mRNA in various frog tissues. B: *In situ* hybridization analysis of SgII mRNA in the frog brain. Frontal sections at the level of the telencephalon and the diencephalon are shown. DP, dorsal pallium; LP, lateral pallium; OT, optic tectum; PTN, posterior thalamic nucleus; VH, ventral hypothalamic nucleus; Vst, ventral striatum.

3.2 Tissue Distribution of Frog CGA mRNA

Like SgII mRNA, frog CGA mRNA is expressed in neuronal and endocrine tissues. However, it was found that CGA mRNA is intensely expressed in the pituitary, particularly in the distal lobe as revealed by in situ hybridization (not shown), compared to other tissues such as the brain, the spinal cord and the adrenal gland (Fig 4A). In mammals, expression of the CGA gene in the anterior pituitary is tightly regulated by estrogens and glucocorticoids (Anouar *et al* 1991, Fischer-Colbrie *et al* 1989).

Collectively, these observations suggest that CGA may play an important function in the secretory activity of adenohypophysial cells.

In the frog brain, CGA mRNA exhibited a widespread distribution with high levels in thalamic and hypothalamic areas (Fig 4B). In the spinal cord, both the dorsal and ventral horns were strongly labeled (not shown).

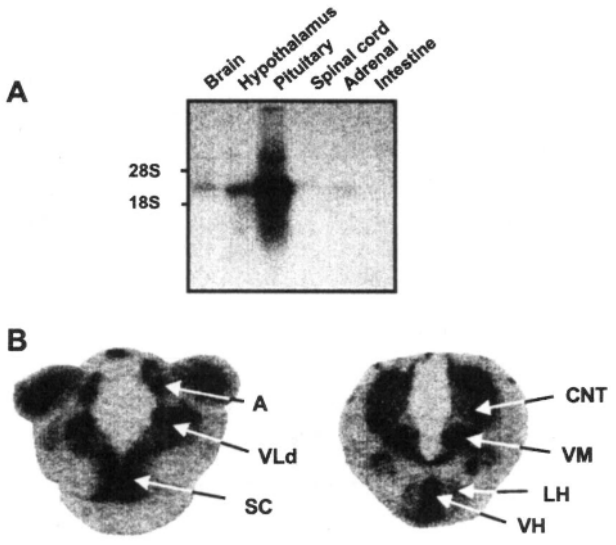

Figure 4. Tissue-distribution of CGA mRNA in the frog *Rana ridibunda*. A: Northern blot analysis of CGA mRNA in various frog tissues. B: *In situ* hybridization analysis of CGA mRNA in the frog brain. Frontal sections at the level of the diencephalon are shown. A, anterior thalamic nucleus; CNT, central thalamic nucleus; LH, lateral hypothalamic nucleus; SC, suprachiasmatic nucleus; VH, ventral hypothalamic nucleus; VM, ventromedial thalamic nucleus; VLd, dorsal part of the ventrolateral thalamic nucleus.

3.3 Regulation of Chromogranin Gene Expression in the Pars Intermedia of the Frog Pituitary

In amphibians, the melanotrope cells of the neurointermediate lobe of the pituitary produce melanotropic peptides that induce skin darkening. This neuroendocrine process enables amphibia to adapt to the color of their environment. The expression of the proopiomelanocortin (POMC) gene, which encodes the precursor of melanotropic peptides, is highly dependent on the color of background. The POMC gene is actively transcribed when animals are adapted to a dark environment and, inversely, its activity is repressed when animals are adapted to a white background (Ayoubi *et al* 1992). Since CGA mRNA was detected in the neurointermediate lobe of the frog pituitary by *in situ* hybridization histochemistry (data not shown), we

sought to determine whether its levels could be regulated during the background adaptation process. We found that CGA mRNA levels are regulated in concert with those of POMC during background color adaptation, *i.e.* CGA gene expression is also increased in black- *vs* white-adapted animals (Fig 5).

It is interesting to note that SgII mRNA levels are also regulated during background adaptation of *Xenopus* (Holthuis and Martens 1996), suggesting that several members of the chromogranin family may participate in the physiological processes that control this neuroendocrine regulatory mechanism. Chromogranins may be involved in the intracellular trafficking events that occur in melanotrope cells to allow the storage and release of melanotropic peptides. On the other hand, since it has been recently shown that SgII is highly processed in the pituitary of *Xenopus* (Van Horssen and Martens 1999), chromogranins may also act as precursors to hormones that could participate in the regulatory mechanisms occurring during adaptation.

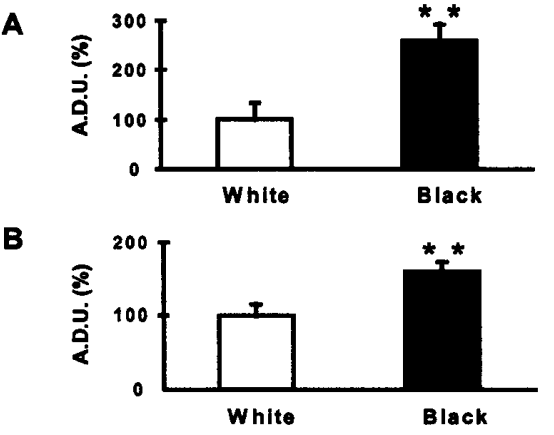

Figure 5. Coordinate regulation of CGA (A) and POMC (B) mRNA levels in the frog pars intermedia. Values are expressed as percentages of arbitrary densitometric units (A.D.U.) in black- vs white-adapted animals. **: $p<0.01$ (Student's t test).

4. CHARACTERIZATION OF CHROMOGRANIN-DERIVED PEPTIDES

The cloning of frog SgII and CGA revealed the high sequence conservation of discrete regions that sometimes correspond to previously isolated peptides such as SN, WE-14 or vasostatin. Other conserved regions had not been described before as mature peptides. Since these regions are flanked by potential cleavage sites, the generation of the free peptides is very likely. The characterization of these novel peptide species is required to understand the processing and possibly the function(s) of chromogranins.

We decided to identify these peptides in endocrine and neuroendocrine tissues by using antibodies that were raised against the human peptide sequences. Among these sequences, a peptide of 66 amino acids which could derive from SgII and which exhibits 68% identity between frog and human species (Fig 1) has been studied first. This peptide has been named EM66 with respect to its N- and C-terminal residues in the human sequence, which are a glutamic acid and a methionine, respectively. Immunohistochemical analysis using EM66 antibodies revealed intense labeling of adrenochromaffin cells in the adult and fetal human adrenal gland (Anouar *et al* 1998). Analysis of adrenal extracts by high performance liquid chromatography followed by EM66 radioimmunoassay of the eluted fractions showed the existence of a large EM66-immunoreactive material exhibiting the same retention time as the EM66 peptide, in both adult and fetal adrenals (Fig 6).

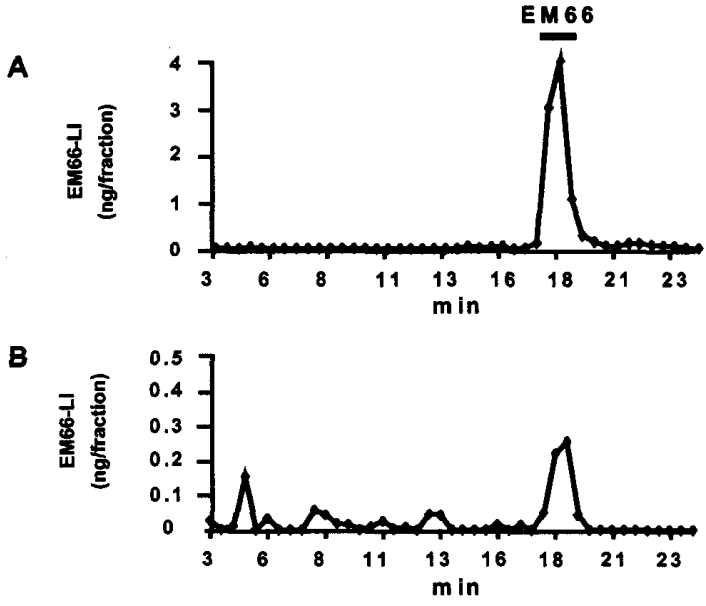

Figure 6. High performance liquid chromatography analysis of EM66-like immunoreactivity in adult (A) and fetal (B) human adrenal gland extracts. The eluted functions were radioimmunoassayed for EM66. The bar above the peaks indicates the elution position of recombinant EM66 peptide.

Taken together, these data demonstrate the occurrence of EM66 as a mature peptide in the human adrenal gland. In addition, EM66 is present in the fetal human adrenal gland, indicating that SgII is processed during the early stages of the adrenal gland development.

Recently, we have been able to show a cell- and tissue-specific distribution of EM66 peptide in the rat. Further studies are in progress to correlate the occurrence of EM66 with a potential role in the neuroendocrine system. By characterizing the occurrence and the distribution of the diverse peptides generated from chromogranins, whose sequences have been selectively preserved in phylogenetically distant species, we believe that further insights could be obtained to elucidate the function of chromogranins.

5. CONCLUSION

Up to now, investigations on the function of chromogranins have been conducted mainly in mammalian species. Although much has been learned from these studies on the structure, properties and distribution of chromogranins, their physiological function is still a matter of debate. Studies in distant vertebrate species will bring novel information that may help to solve the enigma of chromogranin function. From the cloning of the frog SgII and CGA proteins, we could learn that evolutionary pressure has acted to conserve the sequence of specific amino acid stretches within these proteins. The fact that some of these conserved regions have been characterized as free peptides such as SN or vasostatin for which biological activities have been described, strongly suggests that one of the functions of chromogranins is to serve as precursors to biologically active peptides. This notion still needs further support, for example by characterization of the receptors for these peptides. On the other hand, chromogranins exhibit a high content of acidic amino acids in all species studied to date, suggesting that this property is probably important for their function. It is conceivable that before chromogranins are released from the cells to act as neuropeptides or hormones, they may participate in the intracellular events leading to secretory granule formation as has been previously suggested (Huttner and Natori 1995). Their highly acidic nature could be the basis for such a complementary role.

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10

EXPRESSION OF EQUINE CHROMOGRANIN A

cDNA Sequence and Distribution of mRNA in Endocrine and Exocrine Tissues

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1. INTRODUCTION

Chromogranin A (CGA) is a member of a family of highly acidic proteins, chromogranins, which are co-stored with catecoholamines in the adrenal medulla, and co-released with adrenaline and noradrenaline (NAd) in response to splanchnic stimulation (Banks and Helle 1965; Blaschko et al. 1967). CGA is now known to be stored in secretory granules in a wide variety of neurons and paranurons secreting catecholamines and peptide hormones (Cohen et al. 1984; Dillen et al. 1993; O'Connor 1983; Winkler and Fischer-Colbrie 1992).

Chromogranins are widely distributed in paraneurons and neurons (Cohen et al. 1984; Dillen et al. 1993; O'Connor 1983; Winkler and Fischer-Colbrie 1992). The different members of the family [chromogranin A (CGA), chromogranin B / secretogranin I (CGB / SgI), chromogranin C / secretogranin II (CGC / SgII), secretogranin III, secretogranin IV or HISL-19 antigen and secretogranin V or 7B2] are now considered as precursor proteins that are exclusively located (Aunis et al. 1998). We now report the

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amino acid sequence of equine CGA, which contains possible precursor proteins flanked by appropriate signals for secretory granule processing.

2. cDNA SEQUENCE OF EQUINE CGA

The total RNAs of an adult thoroughbred horse for RT-PCR and northern blot analysis were extracted by guanidinium/cesium-chloride ultracentrifugation, and they were preserved in a deep freezer before using them.

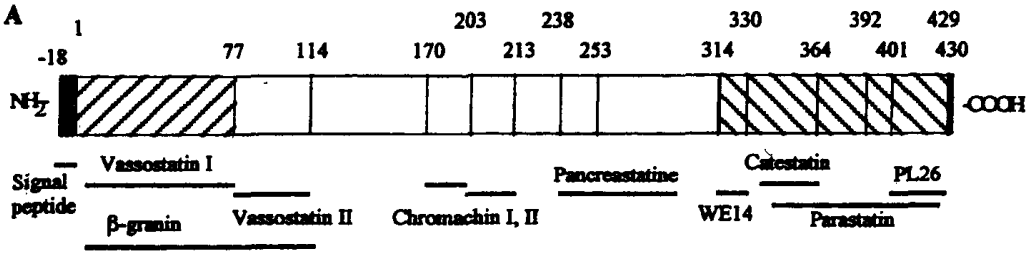
To amplify the complete cDNA sequence of equine CGA, RT-PCR and 5'/3' RACE (rapid amplification of cDNA ends) methods (Frohman 1994) were performed for total RNA of an adrenal medulla. The PCR products were subcloned into pGEM-T Easy vector (Promega) and the cDNA sequences were determined by an A.L.F. automated sequencer (Pharmacia Biotech). The entire cDNA sequence and the suspected amino acids sequence were analysed and compared with the registered data of the GenBank database by personal computer based sequence analysing software (DNASIS-Mac; Hitachi Software).

The region-specific anti-equine CGA (335-365) serum (RY472: Yanaihara Institute Inc., Fujinomiya Japan) was developed with due consideration for the regional analogy with the 344-374 sequence of human CGA (Konecki et al. 1987; Mouland et al. 1994). We purposely selected equine CGA (335-365) for the synthetic immunogen to produce region-specific CGA antibodies since the region-specific anti-human CGA (344-374) serum was shown to be sensitive and useful anti-serum for the measurement of CGA (Nishikawa et al. 1998; Yanaihara et al. 1999).

The resected tissues (adrenal gland, pituitary gland, pancreas and submandibular gland) were fixed with bouin buffer, routinely processed and embedded in paraffin. The sections were incubated with a rabbit polyclonal antiserum against equine CGA (335-365) diluted 3000-fold volume overnight at room temperature. Then immunostaining was carried out according to the avidin-biotin peroxidase complex (ABC) technique with an ABC kit (Histofine kit; nichirei, Tokyo, Japan). The antigen-antibody reaction was visualized by incubation in 0.05M Tris-HCI buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine (DAB) and 0.002% H_2O_2.

3. THE DISTINCTIVE HOMOLOGY OF CGA SEQUENCES

We could obtain the complete cDNA sequence for equine CGA by RT-PCR and 5'/3'RACE methods. The nucleotide sequence of equine CGA reported in this study was deposited in GenBank database with the accession number AB025570. The total 1842bp cDNA sequence contained a 1347 bp of open reading frame encoding 448 amino acids of the deduced equine CGA. The suspected amino acids sequence of equine CGA was compared with the registered data of other animals on GenBank database by the personal computer based sequence analyzing software (DNASIS-Mac; Hitachi Software). The homology percentages of the amino acids sequences of bovine (Iacangelo et al. 1986), porcine (Iacangelo et al. 1988b), human (Konecki et al. 1987), mouse (Wu et al. 1991), rat (Iacangelo et al. 1988a) and frog (Turquier et al. 1999) to equine CGA are shown in Fig. 1.



B

Equine	1- 77	78-313	314-430	(Amino acid No.)
Human	94.8	52.5	90.6	
Porcine	93.5	49.1	81.4	
Bovine	92.2	38.9	90.6	
Mouse	81.8	26.6	80.5	
Rat	83.1	27.9	83.3	
Frog	66.2	6.2	39.0	(%)

Figure 1. A; Schematic representation of equine CGA with identified degradation products. The numbers indicate basic amino acids that is putative processing site of equine CGA. B; The percentages of identity between the amino acid sequences of equine CGA and reported other animals CGA are indicated.

The sequences at N- and C- terminal regions were well conserved in equine CGA. These N- and C-terminal regions contain several proteolytic sites for processing putative biologically active peptides. It is known that the first 18 amino acids residues constitute the signal peptide and the 19-113 amino acid residues of bovine CGA constitute the vasostatin/beta-granin which are known as antibacterial peptides or multiple important roles in the process of regulated hormone secretion (Iacangelo and Eiden 1995; Metz-Boutigue et al. 1998; Winkler and Fischer-Colbrie 1992).

The C-terminal 347-419 amino acids residues of porcine CGA constitute the parastatin which is known as peptides that strongly inhibit glucose induced insulin release. (Tatemoto et al. 1986). The 344-364 amino acids residues of bovine CGA constitute the catestatin which is known as peptides that inhibit catecholamine release (Mahata et al. 1997). Similar biologically active peptides may also be expected in the corresponding regions of the well-conserved N- and C- termini of the equine CGA molecule.

4. IMMUNOHISTOCHEMISTRY OF CGA

Chromogranin A-like immunoreactivity (CGA-LI) was detected in cells of adrenal gland, pituitary gland, pancreas, submandibular gland, thyroid gland, parathyroid gland, and endocrine cells of the gastric body and intestine. Among these cells, endocrine cells in the adrenal medulla, the anterior pituitary and the pancreatic islet were intensely immunoreactive with this antiserum (Fig.2), and weak but substantial immunoreactivity was found in cells of the duct of submandibular gland.

The immunoreactive CGA-LI in the cells of the duct of submandibular gland may correspond to CGA-LI stored in the cells, since we have shown that CGA-LI was detected in the cells of convoluted granular tubules (CGT) in the rat, that highly concentrated CGA (~1mM) released by compound exocytosis into saliva in response to 1μM noradrenaline or to 100 μM accetylcholine (Kanno et al., 1999).

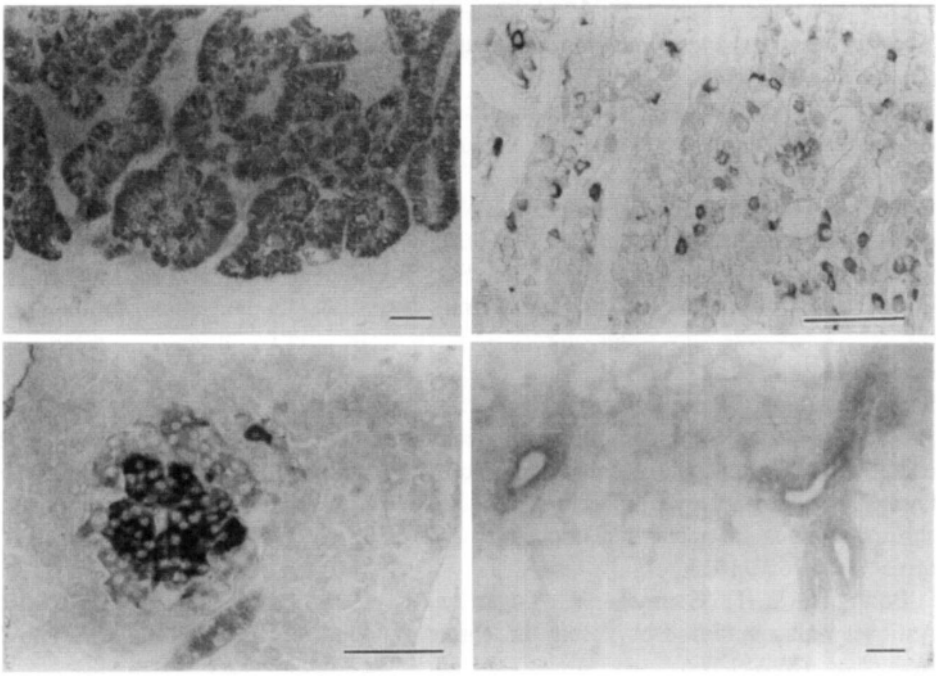


Figure 2. CGA-like immunoreactivity in the equine tissues. A; The adrenal medulla was intensely immunoreactive with the antiserum against equine CGA(335-365). B; In the anterior pituitary, scattered endocrine cells were strongly reactive. C; CGA-like immunoreactivity was localized in the pancreatic islet, especially the central region, in which glucagon cells. D; Weak but substantial immunoreactivity was also detected in the ducts of submandibular gland. The bars indicate 5 μm.

5. CONCLUSION

In conclusion, we could confirm the expression of CGA in the equine submandibular gland. We consider that the salivary level as well as the plasma level of the CGA-LI can be a quantitative index for monitoring the activity of the sympathetic nervous system in physical and psychological stress of race horses.

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11

SALIVARY SECRETION OF CHROMOGRANIN A

Control by Autonomic Nervous System

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1. INTRODUCTION

Most of the secretory proteins of mammalian salivary glands are mainly synthesized and stored in secretory granules in acinar cells, and are extruded by exocytosis into saliva via b-adrenergic receptor activation. In the rat and mouse, however, substantial amounts of a number of different polypeptides and proteins with potent biological effects are known to be synthesized in the specialized segment of the duct system (the granular convoluted tubule, GCT) of the submandibular glands (Gresik *et al* 1996, Sato and Miyoshi 1998). These biologically potent polypeptides and proteins include nerve growth factor, epidermal growth factor (EGF), transforming growth factor-a, hepatocyte growth factor, erythroid differentiation factor, insulin-like growth factor, and kallikrein-like proteinases (Gresik *et al* 1996). In mouse submandibular gland, four members of the tissue kallikrein family are predominantly expressed , they are mK1, mK9, mK13, and mK22, all of which exhibit extensive homology in their amino acid sequences. The

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function of these enzymes has been understood to be EGF binding for mK9, b-NGF endopeptidase activity for mK22, protein-converting enzyme activity for mK13, and true tissue kallkrein activity for mK1 (Hosoi *et al* 1994). mK13, as well as mK9 and mK22, processes prorenin to give renin and /or arginyl renin (Hosoi *et al* 1998). Among these biologically potent proteins, secretion of true tissue kallikrein (rK1) from the granular cells in rat submandibular gland has most extensively been studied (Anderson *et al* 1995, Proctor *et al* 1997). These peptides and proteins are extruded by exocytosis of secretory granules in the cells of GCT via probably an a-adrenergic receptor activation (Abe and Dawes 1978).

A novel member of the biologically potent proteins secreted by the cells in GCT is chromogranin A (CGA) (Yanaihara, *et al* 1998, Kanno *et al* 1998, 1999).

2. SECRETION OF CGA IN SALIVARY GLAND

CGA is a member of a family of chromogranins (CGs), which are co-stored and co-released with adrenaline and noradrenaline (NAd) in the adrenal medulla in response to stimulation of the splanchnic nerve, a presynaptic nerve of sympathetic nervous system (Bank and Helle 1965, Blaschko et al 1967). CGs are now known to be stored in a wide variety of neurons and paraneurons secreting peptide hormones (Cohn *et al* 1984, O'Connor 1983, Winkler and Fisher-Colbrie 1992, Dillen *et al* 1993, Stridsberg 1995) and are recently considered to be precursors of multifunctional hormones (Helle and Angeletti 1998, Aunis et al 1998). Immunoreactive CGs are found in a wide variety of neurons and paraneurons as well as in serum, liquor and urine (Winkler and Fisher-Colbrie 1992). Yanaihara et al (1998) estimated CGA-like immunoreactivity (CGA-LI) in saliva by region-specific radioimmunoassay for CGA, and gave a view that the salivary CGA-LI may be a sensitive biochemical index of psychological stress response. This view was supported by the result that salivary CGA-LI elevated promptly prior to an elevation of salivary cortisol level in psychosomatic stresses, oral presentation and car driving in male volunteers (Nakane *et al* 1998). The source of salivary CGA-LI may not be the adrenergic neurons and paraneurons, but be the exocrine cells in the salivary glands. The exocrine secretion of CGA-LI has been substantiated by the following results : i) CGA-LI was identified in the secretory granules in cells of GCT in the submandibular gland of rat (Kanno *et al* 1998), ii) Highly concentrated CGA-LI was extruded from the cells of GCT into saliva when the autonomic nerves innervating the submandibular gland was electrically stimulated (Kanno *et al* 1998) in anaesthetized rats, iii) When

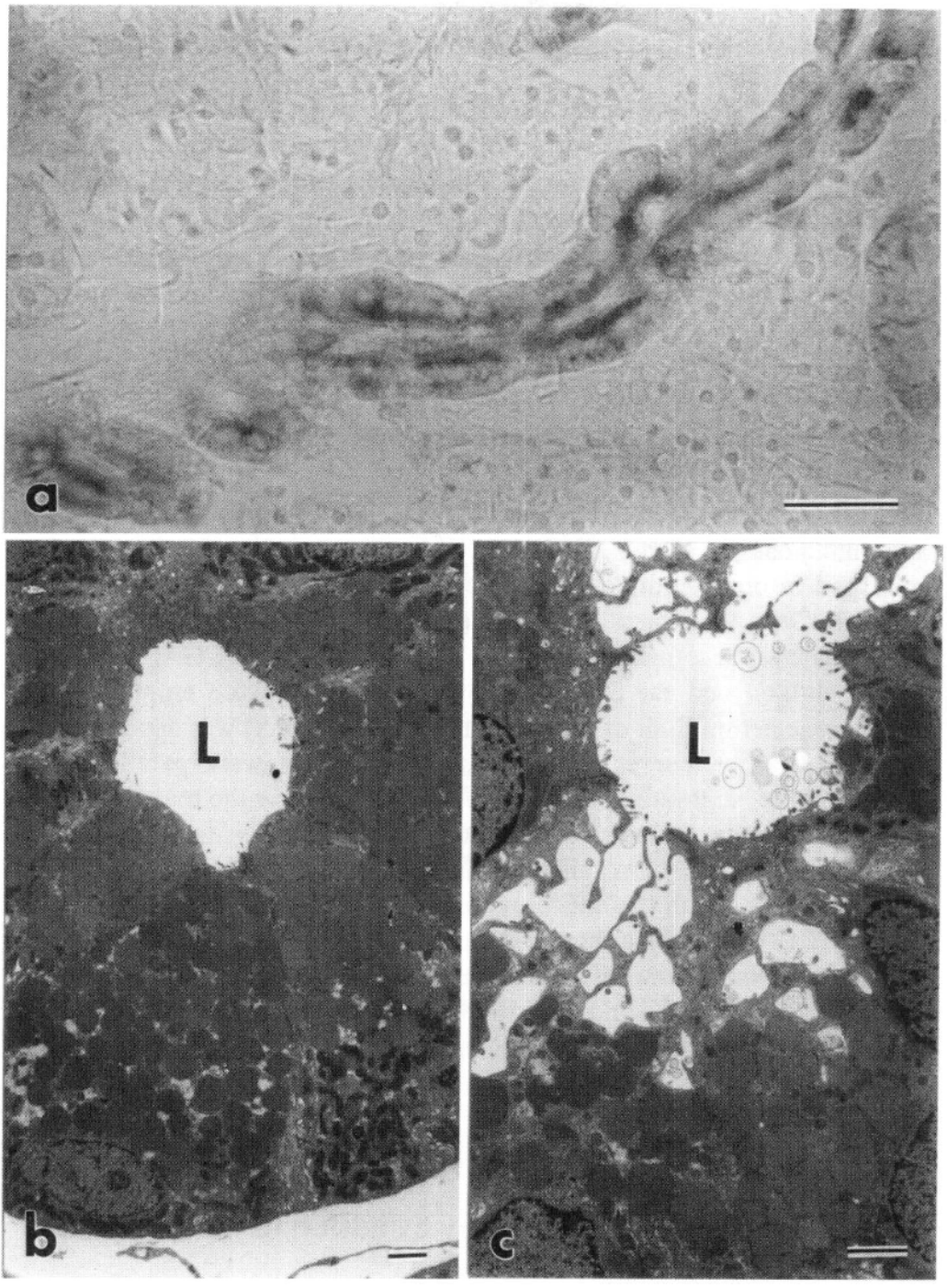

Figure 1. Intense immunoreactivity (IR) with CGA antiserum is found in GCT (a). The IR is shown to be localized at the apical regions of GCT cells of the rat submandibular gland. Transparent electron micrographs showing the ultrastructure of the GCT cells in the control group 0). Stimulation with 1 μM NAd induced degranulation by compound exocytosis (c). L : lumen. Scale bars : 50 μm in (a) and 2 pm in (b) and (c).

the isolated gland was humorally stimulated with 1 μM NAd or with 100 μM acetylcholine (ACh) (Kanno *et al* 1999). The mode ofNAd-induced extrusion of secretory granules of GCT cells is compound exocytosis (Kanno, *et al*

1999). The stimulation with phenylephrine (< 1 μM) induced a dose-dependent increase in salivary CGA-LI secretion (Asada, N., Kanno, T., Nagasawa, S., and Yanaihara, N., unpublished observations). These results support a novel view that CGs may not only be precursors of peptidergic neurotransmitters and hormones in neurons and paraneurons but also be secretory proteins synthesized and extruded via probably an a-adrenergic receptor activation from the GCT cells.

2.1 Region-Specific Immunoassay of CGA-LI

CGs have been measured in plasma, urine, and saliva by a region-specific radioimmunoassay (RIA) for rat CGA using anti-rat CGA(359-389) developed in our institute (Nishikawa *et al* 1998). Anti-rat CGA(359-389) serum (RY76 , Yanaihara Institute Inc., Fujinomiya, Japan) generated in a rabbit was used at a final dilution of 1 : 8,400 and 125I-rat CGA(359-389) as labeled antigen and rat CGA(359-389) as standard were employed. The minimum detection limit of the radioimmunoassay (RIA) was approximately 1.80 fM. Dilution curves of rat plasma and saliva were parallel to the standard curve, suggesting that the RIA can be used for the measurement of rat CGA-LI levels. CGA-LI was imunohistochemically ascertained in the cells of GCT in rat submandibular gland with this anti-rat CGA(359-389) serum as well as with the antiserum against CGA(1-28), pancreastatin (33-51), and CGA(94-139) although strength of reactivity became weaker in order of mention. Neural elements and acinar cells reacted little, if any, to any of these antisera (Kanno *et al* 1998). Figure 1 a shows the localization of CGA-LI in rat submandibular gland. The cells in GCT reacted to the antiserum against CGA(359-389). These results indicate that CGs may be contained in cells of GCT.

2.2 Extrusion of CGA-LI by Stimulation with Nad and ACh

When the isolated submandibular grand of rat was stimulated with 1 μM NAd, electron micrographs showed marked change in the ultrastructure of the cells in GCT (Kanno *et al* 1999). Cytoplasmic granules adjacent to the apical cytoplasmic membrane were opened to the lumen and vigorously fused to granules localized in the deeper region of cells, displaying compound exocytosis, and the apical half of the cells was occupied by round

or irregular-shaped empty spaces. The compound exocytosis in response to stimulation with 1 μM NAd was shown in Figure 1c. These results show that the contents of secretory granules in the cells are released mainly by exocytosis into the saliva.

2.3 Biologically Potent Peptides Contained in Human Salivary Gland

In the rat and mouse, substantial amounts of a number of different polypeptides and proteins with potent biological effects are known to be synthesized in the GCT cells, which are identified only in the rodent submandibular gland. Question now raised is the distribution of the biologically potent polypeptides and proteins including CGs in salivary glands in mammals other than the rodent. The subcellular distribution of EGF was recently demonstrated by means of a post-embedding immunogold cytochemical method in human parotid and submandibular gland (Lantini and Cossu 1998). Acinar secretory granules were reactive for EGF and a great number of cytoplasmic vesicles in acinar and ductal cells were reactive for EGF. These results suggest that EGF enters saliva and is then released not only through granule exocytosis by acinar cells, but also via other routs. The vesicular system revealed in both acinar and ductal cells could play a fundamental role in driving some products both towards the lumen and towards baso-lateral cell surfaces and intracellular spaces.

Garrett *et al* (1998) also showed the different mode of secretion of kallikreins from the GCT cells in rat submandibular glands. Kallikreins in sympathetic saliva arise from exocytosis of prepachaged granules in GCT, whereas the kallikreins in parasympathetic saliva must come from a non-granular pool, and are likely to be secreted through a constitutive vesucular route. Constitutively secreted true tissue kallikrein (rK1) has been found to have a different molecular form from that in secretory granules. The rat submandibular glands also contribute to the kallikreins normally circulating in the blood. Serum levels of kallikrein are independent of the amounts scereted into the saliva, so are likely to have arisen from constitutive secretion via the basal sides of the cells.

It seems possible that the constitutive pathway may also be responsible for the secretion of newly synthesized CGA-LI from the basolateral portion of cell membrane to blood stream via interstitual fluid (endocrine secretion), whereas the regulated pathway is activated by an a-adrenergic mechanism and may be responsible for the exocrine secretion by exocytosis of matured granules containing CGA-LI into the salivary duct at the apical portion of cell membrane.

2.4 Regulated and Constitutive Secretion of Biologically Potent Peptides in Other Glands

As it has been mentioned above, in addition to various growth factors, tissue kallikrein activity processing prorenin to renin and /or arginyl renin is also synthesized and stored in the GCT of the duct in the submandibular glands of the rodents (Penschow and Coghlan 1993). In some strains of male mice, renin was detected in the submandibular and sublingual salivary glands, but not in the parotid glands, and was restricted to the striated or granular portions of excretory ducts (Kon 1999). In the juxtaglomerular cells in the mouse kidney, inactive prorenin was shown to release by a constitutive pathway, while active renin was released by a regulated pathway by exocytosis (Berka *et al* 1992). The constitutive (nonstimulable) pathway may be responsible for the secretion of newly synthesized renin and that it may not be stimulated by a D-adrenergic mechanism (Kawamura *et al* 1988). The renin secretion by both pathways is toward the intercellular spaces, blood or lymphatic circulation, and, by definition, is endocrine or paracrine in nature (Kon *et al* 1995). In the coagulating gland of mice, large amounts of mRNA and renin are detected, and dual modes of exocrine secretion of renin are proposed , a usual exocrine secretion and a lysosomedependent exocrine secretion. In both modes, renin is extruded toward the seminiferous lumen.

2.5 CGA as an Index of the Autonomic Nervous Activity Consisting of Initial Psychosomatic Phase in Stress Responses

The principal conclusion in our recent studies is that, in response to activation of the autonomic nervous system or to the chemical transmitters of the system, the exocrine cells composing the GCT segment of duct secrete highly concentrated CGA-LI into the saliva in the rat submandibular gland. Support for this conclusion comes from the following results. First, under pentobarbitone anaesthesia, electrical stimulation of the sympathetic and parasympathetic nerves innervating the submandibular gland caused prompt elevation of CGA-LI level in the saliva from non-collectable level to 63 1 nM in contrast to small rise in plasma CGA-LI level from resting 0.97 nM to

1.23 nM. The intramural injection of ACh (total 0.2 ml of 0.1 mM solution) into the submandibular gland induced a distinct increase in salivary flow, which contained 5,964 nM CGA-LI (Kanno *et al* 1998). Second, vascular perfision of isolated submandibular gland of rat with a solution containing 1 μM NAd caused copious salivary flow containing concentrations of CGA-LI as high as 0.9 mM. Stimulation with ACh at high concentration, 100 μM, increased the CGA-LI secretion (0.6 mM) (Kanno *et al* 1999). Third, CGA-LI was immunohistochemically ascertained in the exocrine cells of GCT but not in neural elements nor in acinar cells in rat submandibular gland (Kanno *et al* 1998) (Fig. la). Electron microscopic examination showed vigorous compound exocytosis of secretory granules in the cells of GCT when the submandibular gland was stimulated with 1 μM NAd (Kanno *et al* 1999) (Fig. 1c).

These results indicate that activated level of the autonomic nervous system can be monitored by the level of CGA-LI in saliva secreted from the rat submandibular gland, and provide an experimental basis for the view that the salivary CGA-LI secretion may be a sensitive and quantitative index of the activity of the sympathetic nervous system innervating the gland. Since the initial phase of stress responses, the flight/fright response, is known to be regulated by the sympaththetic-adrenomedullary system in the brain and at the periphery, the magnitude of the initial phase can be expressed by the level of salivary CGA-LI.

3. CONCLUSION

We have provided novel evidence that CGA-LI is stored in the cells of granular convoluted tubule, and is secreted into saliva by stimulation with NAd in the isolated and perfused rat submandibular gland. The cells are also known to secrete various growth factors, which are released into blood stream. The cells may release CGA-LI into blood stream (endocrine secretion) in addition to salivary release (exocrine secretion). The endocrine-exocrine secretion of the cells resembles the secretion of renin in the cells of juxtaglomerular apparatus and coagulating gland.

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PART 4

TISSUE-SPECIFIC PROCESSING, TRANSPORT, AND DISTRIBUTION

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12

PROTEOLYTIC PROCESSING OF CHROMOGRANINS

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1. INTRODUCTION

The group of chromogranins comprises chromogranin A and B, secretogranin II and 111, and the proteins VGF and NESP55. Based on several common features these components were consolidated in a family of related proteins (for reviews see Fischer-Colbrie *et al* 1995, Iacangelo and Eiden 1995, Rosa and Gerdes 1994, Wider and Fischer-Colbrie 1992). They are widely expressed in neuroendocrine tissues where they localize to the soluble content of large dense-core vesicles; they are exocytotically released from neurons and endocrine cells upon cell depolarization; their unique primary amino acid sequences are characterized by a high abundance of acidic residues and in addition they contain multiple pairs of consecutive basic residues as well as several monobasic residues forming classical cleavage sites for trypsin-like endopeptidases. The chromogranins share this latter attribute with other prohormones and proneuropeptides which are sequentially processed by endopeptidases and a carboxy- or aminopeptidase to liberate the active peptide hormones and neuropeptides (reviewed in Dillen *et al* 1993, Parmer *et al* 1993, Perone and Castro 1997, Seidah *et al* 1998). Therefore, even though the physiological function of the various chromogranins is still elusive, it is reasonable to assume that they represent the precursors for smaller peptides with functional significance.

Chromogranin: Functional and Clinical Aspects, edited by Helle and Aunis
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1.1 Chromogranin-derived Peptides

Up to now several fragments produced via the proteolytic cleavage of chromogranins have been characterized after isolation from tissue or after enzymatic digestion of purified chromogranin substrates. The first of these peptides reported was pancreastatin, which originates from the central region of the chromogranin A molecule and inhibits glucose-stimulated insulin release from pancreatic beta cells and secretion of parathyroid hormone from the parathyroid gland (Fasciotto *et al* 1989, Tatemoto et al 1986). A similar activity blocking parathyroid hormone release was attributed to a stretch located in the C-terminal portion of chromogranin A and called parastatin (Fasciotto *et al* 1993). Chromogranin A was further shown to give rise to the N-terminal fragments vasostatin I and II that can antagonize the endothelin-mediated contraction of smooth muscle in certain vascular regions (Aardal et al 1993) as well as hormone secretion from parathyroid cells (Drees *et al* 1991). Recently, a peptide named catestatin with antagonistic effects towards the nicotine-provoked secretion of catecholamines was described (Mahata et al 1997). Chromacin in glycosylated and phosphorylated form can exert antibacterial action (Strub *et al* 1996). Bacteriostatic activity was also ascribed to the peptide secretolytin encompassing 13 amino acids at the C-terminus of chromogranin B (Strub *et al* 1995). Secretoneurin, a peptide produced from secretogranin II (Kirchmair *et al* 1993, Vaudry and Conlon 1991), induces chemotaxis (Reinisch et al 1993) and dopamine release (Agneter *et al* 1995, Saria *et al* 1993). Besides, a multitude of chromogranin-derived fragments have been reported, to name only a few, e.g. GE-25 (Kirchmair *et al* 1995), WE-14 (Conlon *et al* 1992, Curry *et al* 1992), PE-11 (Kroesen *et al* 1996), BAM-1745 (Flanagan *et al* 1990), the 21 kDa N-terminal part of secretogranin II (Lame et ul 1991, Van Horssen and Martens 1999), the peptide EM-66 located adjacent to secretoneurin on the precursor secretogranin II (Anouar *et al* 1998) and the C-terminal octapeptide of NESP55 (Ischia *et al* 1997) that was originally purified by Sigafoos and co-workers (1993).

The majority of the above mentioned processing products are flanked by basic amino acid pairs and thus the prohormone convertases are the most likely candidate enzymes for the liberation of these peptides. Figure 1 schematically illustrates the amino acid sequences of chromogranins where the dibasic cleavage sites, as represented by lysine and arginine residues, are indicated. The human chromogranin A molecule contains 10 such basic pairs, 19 pairs or triplets are found in mouse chromogranin B, 9 pairs in rat secretogranin II and 6 in bovine NESP55. The work in our laboratory has focussed on the peptides whose sequences are high-lighted in this figure: a peptide of 25 amino acids derived from chromogranin A and named GE-25 according to the first and last amino acid in the single letter code, the undecapeptide PE-11 from chromogranin B, secretoneurin from secretogranin II and the octapeptide GAIPIRRH from NESP55.

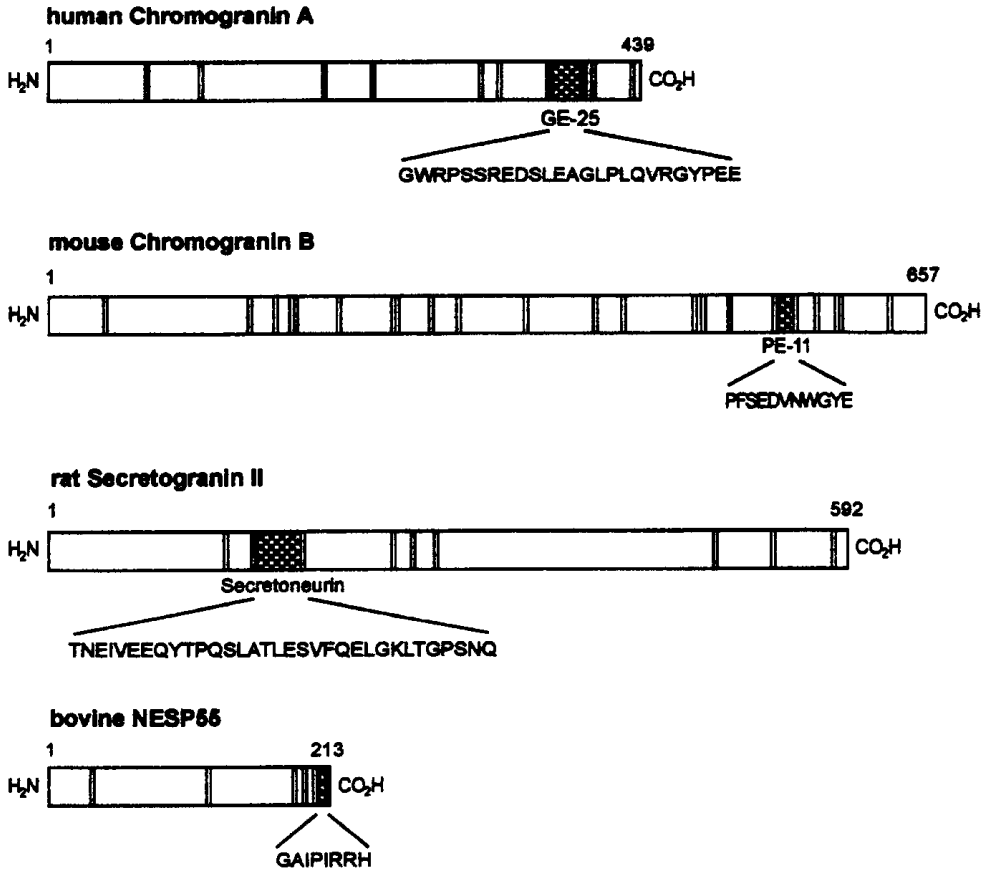

Figure 1. Schematic drawing of chromogranin A, chromogranin B, secretogranin 11, and NESP55 from various mammalian species. The vertical bars mark pairs of basic amino acids that consist of lysine and/or arginine residues. The total numbers of amino acids composing the primary sequences are given above the C-terminus. Below each molecule the sequences of the peptides, which we have investigated in detail, are indicated.

1.2 NESP55 as a Novel Chromogranin Member

The neuroendocrine secretory protein of M_r 55,000 (NESP55, Ischia *et al* 1997) is the newest member of the chromogranin family. This recently cloned protein is heat-stable, acidic from its primary amino acid sequence and posttranslationally further acidified by addition of keratansulfate glycosaminoglycan chains (Weiss *et al* 2000). It is present in the large secretory vesicles of endocrine cells and neurons, like the other chromogranins, but is less widely distributed than these proteins. Interestingly, the C-terminal octapeptide GAIPIRRH has been isolated

previously from bovine chromaffin granules in a search for novel small peptides released from the adrenal gland (Sigafoos *et al* 1993). In the adrenal medulla, where the highest levels (2355 finol) of GAIPIRRH immunoreactivity were detected, NESP55 is preferentially localized to the adrenergic cell population (Bauer *et al* 1999b). Within the brain high amounts of NESP55 mRNA were detected in several regions of the hypothalamus, the midbrain and noradrenergic and serotonergic neurons of the brain stem (see Table 1, Bauer *et al* 1999a).

Table 1. NESP55 mRNA in different areas of the rat brain, as determined by in situ hybridization

Brain area	Intensity of labelling
Cortical areas	
Hippocampus	
Hypothalamus	++
Midbrain	+++
Brain stem	+++

NESP55 gained some interest when a genomic imprinting of this gene was discovered (Hayward *et al* 1998, Peters *et al* 1999). These studies demonstrated that NESP55 is expressed exclusively from the maternal allele. Genomic imprinting is a genetic mechanism that determines expression or repression of genes according to their parental origin and has been shown for about 20 genes. Currently, the mechanisms leading to this imprinting are investigated in detail.

2. IN VITRO PROCESSING OF CHROMOGRANINS IN CULTURED CELLS

Several studies on the proteolytic processing of chromogranins in vitro were published recently, using mainly two different experimental approaches: in cells expressing endogenous prohormone convertases the synthesis of these proteins is - at least partially - knocked out by antisense mRNA. Alternatively, overexpression of specific convertases can be achieved by means of transfection or infection techniques. The kexin/subtilisin-like prohormone convertases represent a growing family of related endopeptidases and up to now include seven different members, i.e. PCI/PC3, PC2, furin, PACE4, PC4, PC5 (-A and -B) and PC7 (for a review see Seidah et al 1998). Because of the cellular and subcellular co-localization of PC 1 and PC2 with chromogranins mainly these two members were investigated.

Changes in the proteolytic processing of chromogranin A were analyzed using both knockout and overexpression of the prohormone convertases PC1, PC2 and furin. Concerning PC1, recent data demonstrated that after PC1 knockout via expression of antisense mRNA larger chromogranin A fragments accumulated and smaller fragments declined, as shown by imunoblotting (Eskeland *et al* 1996), and that this convertase is also essential for the generation of the chromogranin Aderived peptide pancreastatin in the human pancreatic carcinoid cell line BON (Udupi *et al* 1999). For PC2, stable transfection of SH-SYSY human neuroblastoma cells (expressing no endogenous PC1 or PC2) revealed a significant change in the pattern of chromogranin A processing including the generation of the peptide GE-25 (Doblinger and Laslop, to be published). Similarly, in PC2 overexpression experiments in AtT-20 cells (already expressing some endogenous PC2) an enhanced proteolysis of chromogranin A was observed (Eskeland et al 1996). The knockout of furin in these cells led to a shift in chromogranin Aderived breakdown products towards larger molecular forms, but the effect was not comparable in its efficacy to PC1 repression (Eskeland *et al* 1996).

To study the proteolytic processing of chromogranin B various prohormone convertases were overexpressed together with the substrate chromogranin B by use of a vaccinia virus infection system. The most active enzyme was PC1, which generated considerable amounts of smaller cleavage products including the free peptide PE-11. Some processing and limited production of PE-11 was seen by PC2 and PACE4, whereas furin produced mainly larger fragments. In contrast, PCS-A and PCS-B showed no significant effects. In PC12 cells stably transfected with PC1 or PC2 and expressing endogenous chromogranin B, however, the free peptide PE-11 was formed not only by PC1, but very efficiently also by PC2 (Laslop *et al* 1998).

In the case of secretogranin II, co-infections employing vaccinia viruses revealed a high proteolytic activity of PC1 with substantial production of secretoneurin (Hoflehner *et al* 1995). In analogy to the infection experiments for chromogranin B mentioned above PC2 displayed only a weak capacity to process secretogranin II and no free secretoneurin peptide could be detected (Hoflehner *et al* 1995). On the other hand, when we transfected PC12 cells stably with PC1 or PC2, we observed an extensive cleavage of the intact precursor secretogranin II by both enzymes and PC2 seemed to be especially capable of generating smaller fragments including secretoneurin (see Figure 2, Laslop *et al* 1998). Similarly, Dittié and Tooze (1995) described the appearance of several secretogranin 11-derived processing products in PC12 cells stably transfected with PC2.

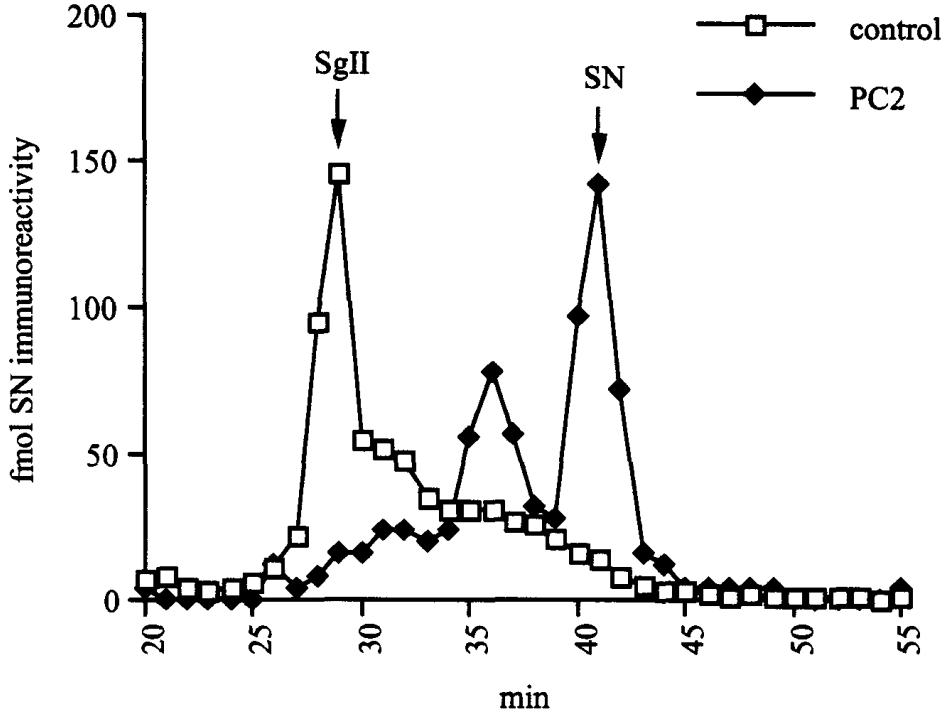

Figure 2. Proteolytic processing of secretogranin II in stably transfected PC12 cells. Heat-stable extracts from mock-transfected PC12 cells (control) and PC12 cells stably transfected with PC2 were size-fractionated over gel filtration chromatography followed by radioimmunoassay of the individual column fractions for secretoneurin. The elution times of intact secretogranin II (SgII) and free secretoneurin (SN) are indicated.

For NESP55 only limited data about its proteolytic processing are available. In experiments using the Semliki Forest virus PC1 exhibited a profound proteolytic conversion of the co-infected substrate NESP55 in BHK cells, whereas PC2 again was less effective in this system (Doblinger, Lundstrom and Laslop, unpublished observation). Nevertheless, both PC1 and PC2 liberated the C-terminal octapeptide GAIPIRRH from NESP55, which is also found in its free form in several tissues in vivo (see below).

Resuming these results, it can be concluded that chromogranins are suitable substrates for prohormone convertases, in particular for PC1 and PC2. With respect to PC1, the data obtained in several experimental systems were in good agreement and demonstrated a powerful processing activity of this enzyme towards the different chromogranins. On the other hand, the remarkable proteolytic action of PC2, as observed in transfected cells

expressing endogenous chromogranins, could not be reproduced in experiments using the virus infection technique where cells were co-infected with a prohormone convertase and a chromogranin substrate. An explanation for this phenomenon might rely in the unique properties of PC2. As the only member of the subtilisin/kexin-like convertase family PC2 needs for its intracellular activation a chaperone-like helper protein, i.e. the neuroendocrine protein 7B2 (Muller and Lindberg 1999, Seidah *et al* 1998). It is likely that in case of infection models the endogenous production of 7B2 in infected cells is down-regulated due to the massive overexpression of virus products, and therefore only a limited activation of PC2 might occur when this experimental approach is used.

3. IN VIVO PROCESSING OF CHROMOGFUNINS IN DIFFERENT TISSUES

A number of studies were performed in our laboratory and in collaboration with other groups to analyze the proteolytic conversion of chromogranins in various neuronal and endocrine tissues. With radioimmunoassays specific for the peptides quoted above the existence of these peptides in vivo could be demonstrated after chromatographic size fractionation of tissue extracts. In general, the highest degrees of processing were found in the nervous system, e.g. in brain, in the posterior pituitary, in the splenic or sciatic nerves and in the vas deferens. This applied especially to secretogranin II and chromogranin B, where hardly any forms of the intact molecules could be measured and short fragments including the free peptides secretoneurin and PE-11, respectively, were the most abundant molecular species (Kroesen *et al* 1996, Leitner *et al* 1996 1999, Li *et al* 1999 1998, Liang *et al* 1999, Lovisetti-Scamihorn *et al* 1999b). Also in the case of chromogranin A a substantial maturation of the large precursor to intermediate-sized fragments and to GE-25 occurred in neuronal tissues (Kirchmair *et al* 1995, Leitner *et al* 1999, Lovisetti-Scamihorn *et al* 1999b). Concerning NESP55, the results obtained from bovine brain differed when compared with the other chromogranins in as much as NESP55 was not significantly processed within the hypothalamus and caudate nucleus (Lovisetti-Scamihorn *et al* 1999a). However, in the posterior pituitary as well as in peripheral nerve terminals shorter molecular forms of NESP55 including the free octapeptide GAIPIRRH dominated (Lovisetti-Scamihorn *et al* 1999a, b).

A considerable processing of the chromogranins was also found in various regions of the gastrointestinal tract and in the pancreas, but only limited proteolytic conversion appeared in the anterior pituitary (Kirchmair

et al 1995, Leitner *et al* 1996, Lovisetti-Scamihom *et al* 1999a, Watkinson *et al* 1991). For chromogranin A and secretogranin II the degree of processing was lowest in the adrenal medulla (Kirchmair *et al* 1995, Leitner *et al* 1996, Watkinson *et al* 1991). We have speculated that this observation could possibly be explained by an inhibition of prohormone convertases in the presence of very high levels of catecholamines, as typical for the adrenal medulla (Wolkersdorfer *et al* 1996). Indeed, high concentrations of catecholamines inhibited the enzymatic activity of PC1 and furin in vitro and lowering of the catecholamine content in cultured chromaffin cells by means of reserpine or alpha-methyl-p-tyrosine enhanced the processing of chromogranins (Watkinson and Robinson 1992, Wolkersdorfer *et al* 1996).

Again, for NESP55 a somewhat different picture emerged in the adrenal medulla. This new chromogranin is cleaved more efficiently in the adrenal gland, producing not only intermediate-sized molecules but also substantial levels of the C-terminal octapeptide (Ischia *et al* 1997). Thus, a distinct regulatory mechanism may exist for the processing of NESP55 that determines its proteolytic maturation and the release of putatively functional peptide products from the adrenal medulla and other neuroendocrine cells.

4. CONCLUSION

A number of peptides derived from the large chromogranin precursor proteins have been identified in neuroendocrine tissues and several of them have been attributed with functional data. In addition, various fragments were shown to be liberated by the action of prohormone convertases. This is in accordance with the concept that these peptides represent the mediators of at least part of the yet unidentified physiological functions of the chromogranins.

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13

ENDOTHELIAL HANDLING OF CHROMOGRANIN A

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1. INTRODUCTION

The chromogranins (CGs) are expressed in CNS, sympathetic neurons and numerous neuroendocrine cells (see review by Winkler and Fischer-Colbrie 1992). In recent years knowledge about the biological activities of these acidic proteins and peptides derived by N- and C-terminal processing have accumulated, and in most target tissues studied the CG-derived peptides appear to have inhibitory effects.

Recent data indicate that blood vessels are likely targets for the vasostatins, causing suppression of the tone development in the smooth muscle layers (Aardal and Helle 1992, Aardal *et al* 1993, Brekke et al, this volume). A question of interest is whether the circulating pool of CGs may serve as the main source of vasostatins in regulation of vascular tone. To reach the muscle layer the CGs have to cross the vessel wall. The endothelium, lining the vessels, forms a barrier to transvascular exchange of proteins due to endothelial cell tight junctions. Several different cellular structures have been suggested, however, to be involved in transendothelial transport. Thus shuttling plasmalemma1 vesicles, trans-endothelial channels, non-specific adsorptive endocytosis or receptor-mediated transcytosis appear

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to mediate exchange, depending on the transport being specific or non-specific (see Simionescu and Simionescu 1991).

The properties of the endothelial cells may vary according to the position along the vascular tree. Although the aorta is not generally regarded as an important site for exchange of macromolecules, isolated aorta endothelial cells in culture have been widely applied in model studies of endothelial transport (King and Johnson 1985, Milton and Knutson 1990, Schnitzer and Oh 1994, Taylor *et al* 1993). The goal of the present work was to investigate interactions of CGA with aortic endothelial cells, and for this purpose cultured bovine aorta endothelial cells (BAEC) were chosen to comply with the species origin of CGA to be studied.

2. EFFECT OF CGA ON MEMBRANE POTENTIAL IN BAEC

Changes in the membrane potential (Em) of endothelial cells modulate calcium-influx and calcium-dependent endothelial functions (He and Curry 1995). Chemical stimulation of the endothelial layer may cause the release of vasodilatory or vasostimulatory compounds for the underlying smooth muscle layer (Furchgott 1983, Vanhoutte *et al* 1986). To establish if the dilatory activity of the vasostatins (see Helle, this volume) may be secondary to direct effects of the peptides on the endothelium, the influence of CGA on the Em of BAEC was first investigated.

We have previously shown that the lipophilic fluorescent dye Rhodamine 6G can be used as a non-invasive probe to determine changes in the membrane potential in cultured BAEC in suspension (Mandala *et al* 1999). When BAEC were incubated in presence of 1 nM –10μM of intact CGA and vasostatins for 20 min at room temperature, some small but insignificant fluctuations in Em were observed, as illustrated in Fig.1, A and C. The shorter N-terminal CGA-derived peptide CGA1-40 (1 μM) appeared, on the other hand, to have a slight and slowly developing depolarising effect (Fig. 1 B). From these results it seems reasonable to conclude that in micro- molar concentrations intact CGA and peptides derived from the N-terminal domain have no significant modulatory effects on the membrane potential of BAEC. Other CGA derived peptides are currently under investigation for membrane potential effects in this model.

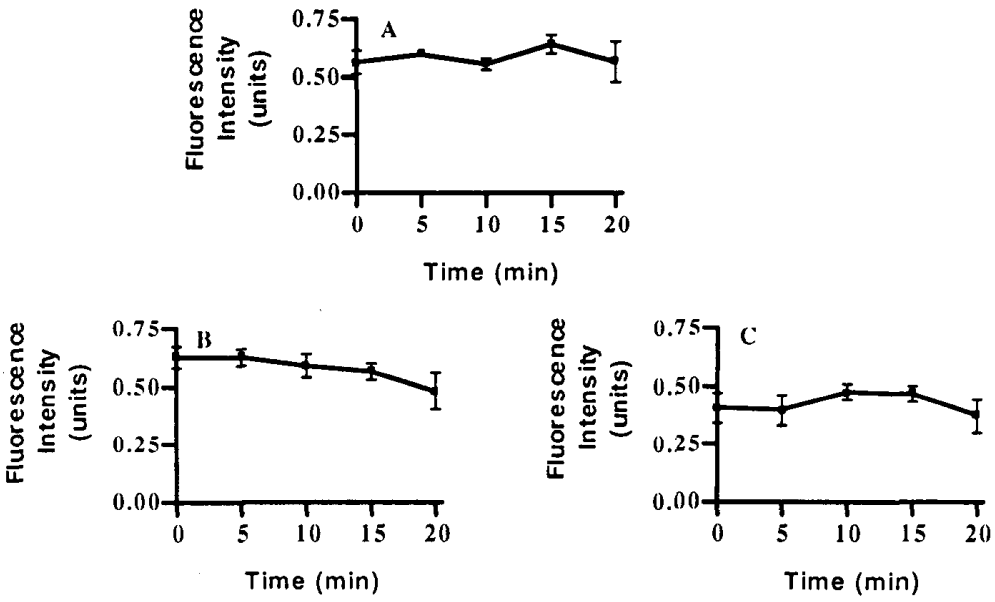

Figure 1. Effect of (A) CGA, (B) CGA1-40 and (C) vasostatin I (CGA1-76) on fluorescence intensity as a measure of Em changes in BAEC. BAEC (5 x 10^5 cells/ml) were pre-incubated in presence of 1 µM CGA and CGA peptides for 20 min at room temperature in Krebs-Ringer phosphate buffer containing Rh 6G (0.7µM). Fluorescence at time zero in absence of peptides represents the control. Bars indicate SEM, n = 3.

3. AFFINITY BINDING AND LIGAND-RECEPTOR CROSS-LINKING STUDIES

To examine if CGA, nevertheless, binds specifically to BAEC, cells were incubated for 3 h at ~12°C with 125I-labelled CGA (~20,000 cpm/150 µl) in presence of increasing concentrations of unlabelled CGA. Under these conditions maximal binding was obtained at 10^{-9} M CGA (~15% of added tracer) but ~50 % CGA was still bound in the presence of 6.6 x 10^{-6} M unlabelled CGA (Fig. 2, left panel). When binding was examined after incubation overnight at 4°C only 3% of added tracer was associated with the cells. The displacement curve was, however, similar to that obtained at 12°C with ~50 % and ~10 % of ^{125}I-CGA still bound to the cells in the presence of 6.6 x 10^{-6} and 6.6 x 10^{-5} M unlabelled CGA, respectively (Fig. 2, left panel). The differences in total binding of ^{125}I-CGA at the two temperatures thus indicated some internalisation of CGA during the relatively short incubation

at the higher temperature. No difference in binding was observed when 0.1 or 1% bovine serum albumin was present in the binding buffer.

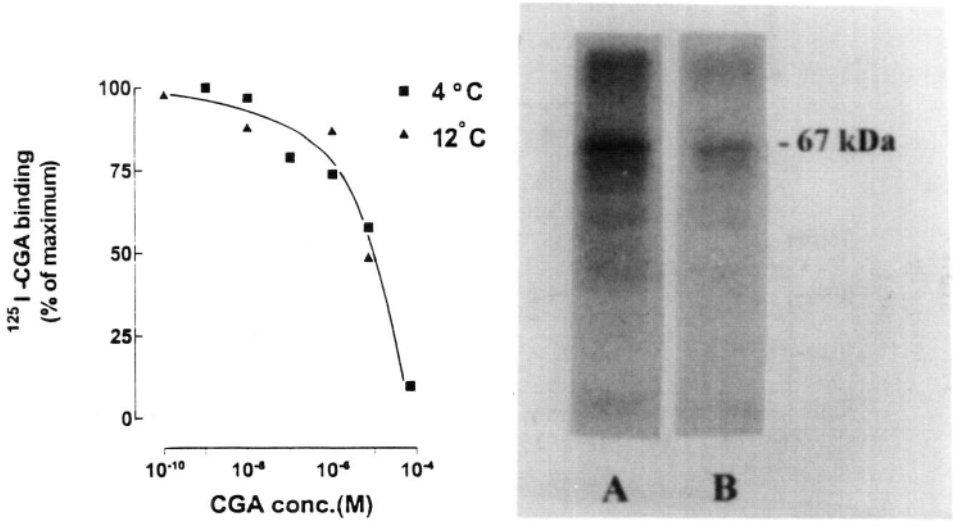

Figure 2. Affinity binding of ^{125} I-labelled CGA to bovine aorta endothelial cells. Cells were incubated for 3 h at 12°C or overnight at 4°C with tracer amount of ^{125} I-CGA in presence of increasing concentrations of unlabelled CGA (left panel). Results, means of 2 determinations, are expressed in % of maximal binding obtained at 10^{-9} M CGA (left panel). For cross-linking of ^{125}I-CGA to BAEC (right panel), cells were incubated with 10^{-10} M ^{125}I-CGA for 3 ½ h at 12ºC in absence (lane A) or presence (lane B) of 6.6 x 10^{-6} M unlabelled CGA. Cells were cross-linked with 0.5 mM DSS and examined by SDS gel under reducing conditions.

To identify a possible specific receptor for CGA, affinity cross-linking was performed. Cells were incubating in presence of 0.5 μCi/ml (2.5 x 10^{-10} M) ^{125}I-CGA in the absence or presence of 6.6 x 10^{-6} M unlabelled CGA for 3 ½ h at 12°C followed by cross-linking with disuccinimidyl suberate (DSS) and gel electrophoresis under reducing conditions. Surprisingly, the autoradiograph (Fig. 2, right panel) revealed a pattern of broadly distributed bands, in contrast to the normally expected distinct band for a specific binding site. The similarity of banding with and without competition with unlabelled CGA (Fig. 2, lane A and B) strongly suggests that the labelled bands reflected internalised CGA rather than a specific binding site of ~67 kDa. No specific binding site was disclosed when ^{125}I-CGA was cross-linked to isolated cell membranes (data not shown), an observation supporting the

conclusion that ^{125}I-CGA cross-linked to intact cells actually represented molecules trapped inside the cells.

Hence, the results show that CGA binds to endothelial cells with low affinity but high capacity. A specific high affinity binding site for CGA was not identified, and in this respect BAEC differ from the calf aortic smooth muscle cells, which express a binding site of ~74 kDa (Angeletti *et al* 1994). The ability of excess CGA to compete for binding of labelled CGA while high concentrations of the negatively charged serum albumin molecule of similar molecular weight had no effect, indicated nevertheless some specificity of the binding.

4. BINDING AND INTERNALIZATION STUDIES

The time course of binding was studied in more detail by incubating cells with tracer amounts of labelled CGA in presence of 10^{-8} M unlabelled CGA. To evaluate the amount ^{125}I-CGA internalised, cells were stripped of surface-bound CGA by acid wash (Haigler et al., 1980). Both binding and internalisation were temperature dependent and increased by time, reaching maximum after approximately 2 h of incubation (Fig. 3, A and B).

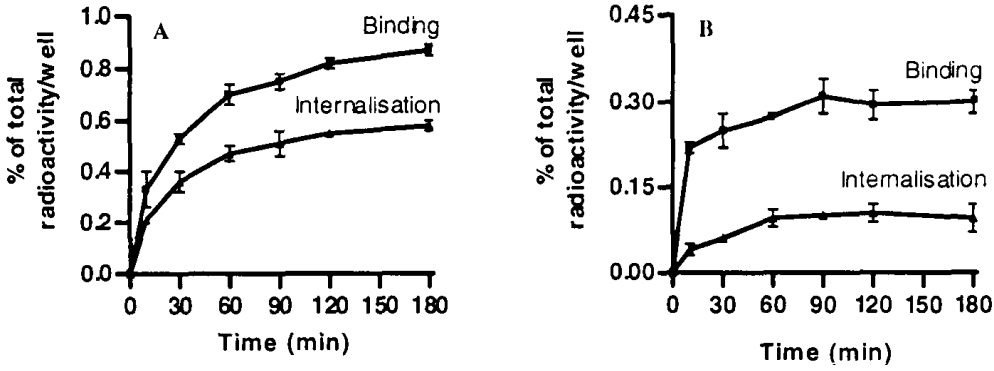

Figure 3. Binding and internalization of CGA. Confluent monolayers of BAEC were incubated with tracer amounts of ^{125}I-CGA and 10^{-8}M unlabelled CGA at 37°C (A) or at 4°C (B) for the time given. Cells were stripped of surface-bound CGA by acid-wash. Values are means ± SEM, n = 3. Note difference in scale on Y-axis in (A) and (B).

At 37°C internalised CGA accounted for nearly 70% of total radioactivity associated with the cells. Total binding expressed as % of total cpm added to

the wells, was relatively low, however, indicating a non-specific receptor-independent binding (see also Fig. 2).

It is known that pH may affect cell surface charge, and the effect of different pH in the medium on binding and internalisation was therefore examined. As shown in Fig. 4, left panel, both binding and internalisation were significantly higher at pH 6.8 than at pH 7.4, while a corresponding increase (0.6 units) to pH 8.0 caused no significant changes.

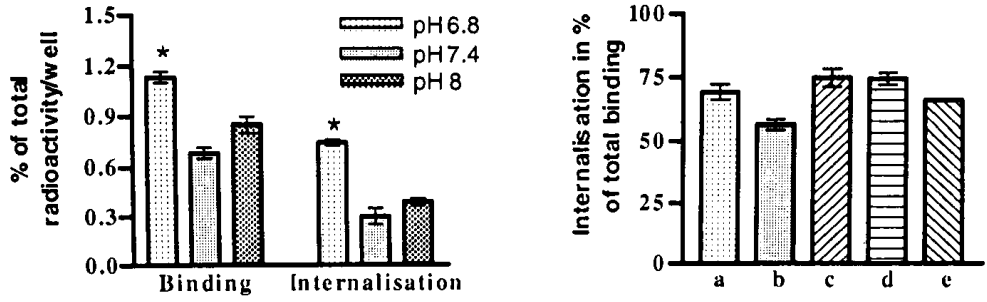

Figure 4. Effects of pH and perturbed vesicle transport on CGA binding and internalization. Cells were incubated for 1 h with 125I-CGA (left panel) at different pH or (right panel) under the following conditions), a) control, b) K^+-depleted cells, c) monensin, d) vinblastine and e) hyperosmolar 1 M sucrose (see Methods for details). Binding and internalization were measured as described in Fig. 3. Values are means ± SEM, n = 3.

To distinguish between possible uptake mechanisms, binding was studied under various conditions known to perturb specific and non-specific membrane transport (Fig. 4, right panel). Monensin, affecting endosomal pH, and hyperosmolar conditions (1 M sucrose) had no effect on either binding (data not shown) or internalisation (Fig. 4, right panel, c and d). Vinblastine, causing disruption of microtubuli, enhanced binding relative to controls (data not shown) but fractional internalisation (internalised CGA in % of total cell-associated CGA) was the same as in controls. The only condition under which binding and internalisation were significantly affected, was in K^+-depleted cells, which exhibited lowered binding (data not shown) as well as internalisation of CGA (Fig. 4, right panel, b).

These results show that both binding and internalisation of CGA are temperaturedependent with internalised CGA accounting for a relatively high fraction of total cell-associated CGA. The lack of a specific binding site, discussed under section 2, therefore suggests an uptake by non-specific fluid phase endocytosis. The effect of pH on binding and internalisation

indicates, however, that electrostatic forces may be involved in CGA binding to the cell surface or to a restricted fraction of plasmalemma1 vesicles (see Simionescu and Simionescu 1991). It has been suggested that the pulmonary endothelium due to negative charge restricts trans-capillary movement of anionic molecules (Swanson and Kern 1994). It seems unlikely that lowering pH to 6.8 significantly reduces the total negative charge of the acidic CGA. On the other hand, if a negative charge also applies to BAEC, it is more likely to expect that a decline in pH to 6.8 may reduce the negative surface charge on the cells, thus facilitating a non-specific adsorptive endocytosis of CGA. Intracellular K^+ depletion, shown to disrupt coated pit-dependent endocytosis (Larkin *et al* 1983) had a small but significant inhibitory effect on binding and internalisation. Hyperosmolar conditions, also known to inhibit coated pit-dependent endocytosis (Oka *et al* 1989), had on the other hand no effect. Hence, whether CGA is endocytosed by fluid phase or coated-pit dependent endocytosis can at the present not be decided.

5. TRANSCELLULAR TRANSPORT

Endothelial transcellular transport of ^{125}I-CGA over time was monitored using monolayers of BAEC seeded in transwell inserts. Endothelial cells, like epithelial cells, form tight junctions. In contrast to the epithelial tight junctions, the endothelial junctions do not form a barrier to paracellular electrolyte movement (Milton and Knutson 1990). Measurements of electrical resistance as a test of an intact monolayer is therefore not applicable to BAEC. The cells were therefore examined by light microscopy and used when intact monolayers had formed. ^{125}I-CGA was added to the inserts together with ^{3}H-inulin as a marker of non-specific paracellular transport. The cells were incubated at 37°C. At given time intervals aliquots were collected from the insert and the well in the culture dish, i.e. at the luminal and abluminal side of the cells, respectively. The clearance of the two substances across the cell layer and the gelatine-coated membrane alone is illustrated in Fig. 5, A and B. The clearance of inulin followed a linear time course both in absence and presence of cells in the inserts, with permeability coefficients (PC) estimated to 2.0×10^{-5} cm/s and 1.3×10^{-5} cm/s, respectively. The clearance of CGA followed a more complex time course under both conditions. The flux (µl/min) of CGA was initially faster than that of inulin but slowed down on prolonged incubation beyond 30 min. This indicated the presence of smaller, labelled CGA-fragments or free iodine in the medium added to the inserts, and which readily diffused by the paracellular route. Trichloroacetic acid precipitation, often used to control the integrity of transported protein molecules (Bastian *et al* 1997), could not

be applied to quantify the acid-soluble CGA. A more reliable estimation of CGA transport across endothelial monolayers thus necessitates quantification by RIA combined with Western blots.

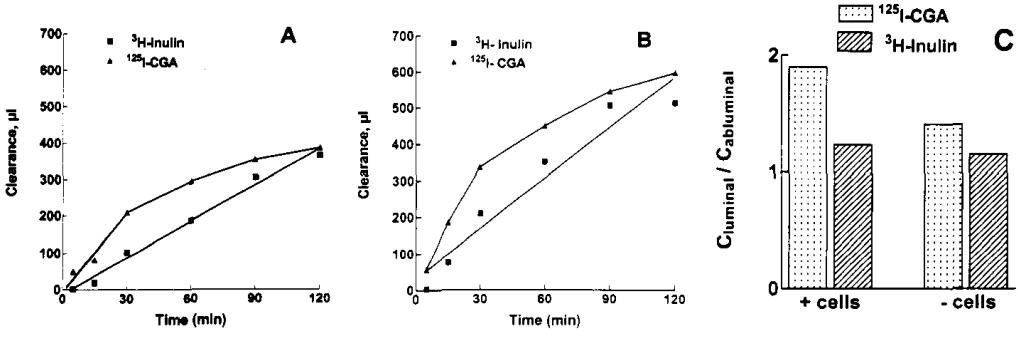

Figure 5. Luminal to abluminal clearance of ^{125}I-CGA across BAEC monolayers. 10^{-8} M ^{125}I-CGA and 3H-inulin were added to the luminal side of cells grown on gelatine-coated porous transwell membranes (A) or to coated membranes alone (B). Cells were incubated at 37°C and aliquots (50 µl) were collected from the luminal and abluminal side of the monolayer at the time given or after 23 h of incubation (C). Results are the means of 2 experiments, measured in duplicate.

Transfer of ^{125}1-CGA in the reverse direction from the abluminal to the luminal side, although at a somewhat slower rate, was demonstrated in one experiment (data not shown), indicating that release of internalised CGA may also occur from the endothelium to the circulation. According to the two-compartment model suggested by Hsiao *et al* (1990), the circulating CGA equilibrates between two pools, the extravascular pool being 24-fold higher in capacity than the intravascular pool. Considering the apparent ability of endothelial cells like BAEC to internalise and release CGA at the luminal side and the total mass of endothelial tissue, it seems likely that the endothelium may account for the extravascular pool, proposed by Hsiao and coworkers.

Transfer of CGA was also measured after incubating the cells for 23 h at 37°C. As shown in Fig.5 C, the concentration of ^{3}H-inulin at the two sides of the transwell membrane was almost equal in absence and in presence of cells. CGA, on the other hand, was distributed unequally over the

monolayer, the concentration on the luminal side of the cells being nearly double that on the abluminal side.

Hence, these first observations on transendothelial transport of CGA indicate that a monolayer of endothelial cells like BAEC imposes a restriction on the transfer of this macromolecule relative to the marker for paracellular transport. A paracellular transport of small CGA fragments can, on the other hand, not be excluded. The observed transport kinetics indicate that the CGA transfer is slow but may take place in both direction. A slow release of CGA from a large endothelium pool may contribute to the relatively constant level of CGA normally found in blood, increasing only under extreme stress or pathological conditions.

6. CONCLUSION

The present study is the first to address the question of interaction between CGA and endothelial cells. It is evident that neither intact CGA nor N-terminal peptides activate endothelial cells by affecting the membrane potential. Nevertheless, bovine aorta endothelial cells (BAEC) bind and intemalise intact CGA in a temperature-dependent manner, and the binding occurs by low affinity and high capacity. BAEC do not express specific, high affinity membrane-associated binding sites for CGA. Cell surface charge appears, on the other hand, to be important for the low affinity binding, seemingly followed by non-specific adsorptive endocytosis involving plasmalemma1 vesicles. The different kinetics for CGA and inulin migration across the BAEC monolayer indicate that CGA is slowly transported by a transcellular route, consistent with transcytosis involving shuttle of vesicles between the luminal and abluminal side of the endothelial cell. Intemalised CGA may account for a pool of trapped CGA in equilibrium with circulating CGA.

Taking into account the regional differences in endothelial properties and functions, the present approach to analyse CGA exchange in the macrovascular model should also be applied to endothelial cells derived from the microvasculature.

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APPENDIX

Experimental details

Materials. All media, foetal calf serum (FCS) and antibiotics were obtained ffom GIBCO BRL (Baisley, Scotland). Disuccinimidyl suberate (DSS) was purchased from Pierce (Rockford, Illinois., USA). Bovine chromogranin A, purified from the medulla as described elsewhere (Stridsberg et al. 2000), was iodinated by the chloramine T method (Hunter and Greenwood 1962). 3H-inulin (0.71 µCi/mmol) was purchased ffom Amersham Int. (Bucks, UK). Cell culture plastic ware ffom NUNC (Roskilde, Denmark) and the transwell inserts, pore size 0.4 µm and growth area 4.7 cm^2, from Costar (Cambridge, MA, USA).

Methods. Endothelial cells were obtained ffom bovine aortas by collagenase digestion as described by Rosenthal and Gotlieb (1 990). The cells were seeded in gelatin-coated dishes and kept in DMEM/Fl2 supplemented with 10% FCS and antibiotics. The cells, grown at 37ºC in 5 % C02, received fresh medium every 3 days. Confluence was reached within 6-7 days at which the monolayers exhibited the typical cobblestone pattern. All experiments were performed on confluent cells between 3rd and 15th passage and which had been preincubated for 4 h in serum-free DMEM/F 12 at 37ºC. CGA effects on the membrane potential were examined on cells in suspension as previously described in detail (Mandalà *et al* 1999), using the lipophilic fluorescent cationic dye Rhodamine 6G with high permeability in cells membranes as probe. Studies of CGA binding and intemalisation were performed using Hepes binding buffer, pH 7.4, supplemented with 1 % bovine serum albumin (Dahmer et al 1989). For affinity binding and crosslinking studies Krebs-Ringer phosphate buffer containing tracer amounts of 125I-labelled CGA (– 0.05 µCi/ml and 0.5 µCi/ml, respectively) and unlabelled CGA in concentrations as given was applied. Cross-linking was performed essentially as described (Serck-Hanssen and Sovik 1991) by addition of disuccinimidyl suberate after incubating cells with 1251-CGA at 12°C for 3 ½

h. SDS-gel electrophoresis was carried out under reducing conditions using in 7.5 % gels. The gels were dried and exposed to Kodak X-Omat films.

Transendothelial migration experiments were carried out on cells seeded in Costar transwell inserts, growth area 4.7 cm^2 and pore size 0.4 μm. DMEM, 2.6 ml, was added to the lower chamber and 1.5 ml, containing tracer amounts of labelled and 10-8 M unlabelled CGA, to the insert, i.e. at the luminal side of BAEC. Tritium-labelled inulin (~10-8 M) was included as a control for paracellular transport. 50 μl medium from both chambers were collected at given time points and counted. Transport is expressed as clearance; $[CGA]_{lower\ chamber}$ x Volume $_{lowerchamber}$ / $[CGA]_{upper\ chamber}$ The permeability constant, PC, was calculated from the clearance rate and surface area as described by Milton and Knutson (1 990).

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TISSUE PLASMINOGEN ACTIVATOR AND CHROMAFFIN CELL FUNCTION

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1. INTRODUCTION

Tissue plasminogen activator (t-PA) is a serine protease that plays the dominant role in dissolving fibrin clots within the vascular tree by activating plasminogen to plasmin, the primary fibrinolytic enzyme (Carmeliet et al 1994). t-PA is distributed widely throughout the neuroendocrine system (Kristensen *et al* 1985; Kristensen *et al* 1986; Bansal and MacGregor 1992; Desruisseau-Gonzalvez *et al* 1993; Sappino *et al* 1993; Friedman and Seeds 1994) in addition to its expression by endothelial cells (Todd 1959). t-PA has a signal peptide which allows its entry into the secretory pathway, that is, across the endoplasmic reticulum, followed by transport through the Golgi stacks. Within endocrine cells and neurones, secretory proteins are sorted at the trans-Golgi network into either the constitutive or regulated pathway (Kelly 1985). Proteins entering the regulated pathway are concentrated and stored in vesicles, and subsequently released following stimulation by a secretagogue. In contrast, in the constitutive secretory pathway, newly synthesised protein is not stored but is transported directly to the cell surface and secreted even without any extracellular signal (Kelly 1985).

Recently, we investigated the expression and trafficking of t-PA in chromaffin cells (Parmer et al 1997b). Our studies demonstrated that t-PA is synthesised by chromaffin cells, where it is targeted into the regulated

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pathway of secretion, that is, into catecholamine storage vesicles, and is co-released with catecholamines by exocytotic release from these organelles in response to sympathoadrenal stimulation. Thus, we have identified a potential new role for the chromaffin cell as a source of t-PA, and secretion of t-PA by the regulated pathway suggests a newly recognised mechanism to control the activity of t-PA by allowing its immediate introduction into the extracellular milieu in a rapid response to specific mediators.

In this review, we will summarise recent studies demonstrating that t-PA is targeted to the regulated secretory pathway in neuroendocrine cells, and studies which identify a specific subcellular granule, the catecholamine storage vesicle, in which t-PA is stored. Also, we will review the potential targeting of t-PA to the regulated secretory pathway in other cell types, and the possibility that inhibitors of t-PA may be targeted also to the regulated secretory pathway. In addition, we will comment on potential mechanisms by which t-PA is targeted to the regulated pathway of secretion, and discuss potential physiological implications of the trafficking of t-PA to regulated secretory vesicles.

2. TARGETING OF t-PA TO THE REGULATED SECRETORY PATHWAY IN CHROMAFFIN CELLS

t-PA expression, subcellular localisation, and secretagogue-mediated t-PA release, have been investigated in chromaffin cells from several sources. These have included rat PC-12 cells (Greene and Tischler 1976), primary cultures of bovine adrenal chromaffin cells, and human pheochromocytoma, a catecholamine-producing tumour of the adrenal medulla and, therefore, a source of human chromaffin cells. Synthesis and expression of t-PA was demonstrated in each of these chromaffin cell sources [PC12 cells (Leprince *et al* 1991; Pittman and DiBenedetto 1995; Gualandris *et al* 1996; Parmer *et al* 1997b) bovine adrenal chromaffin cells (Parmer *et al* 1997b) and human pheochromocytoma (Parmer *et al* 1997b)]. A variety of methods have been utilised, including Northern blotting with a specific t-PA cDNA probe, metabolic labelling and immunoprecipitation with t-PA specific antiserum, functional t-PA enzyme activity assays and specific t-PA immunoassays to confirm that the plasminogen activator activity expressed in chromaffin cells is authentic t-PA (Parmer *et al* 1997b).

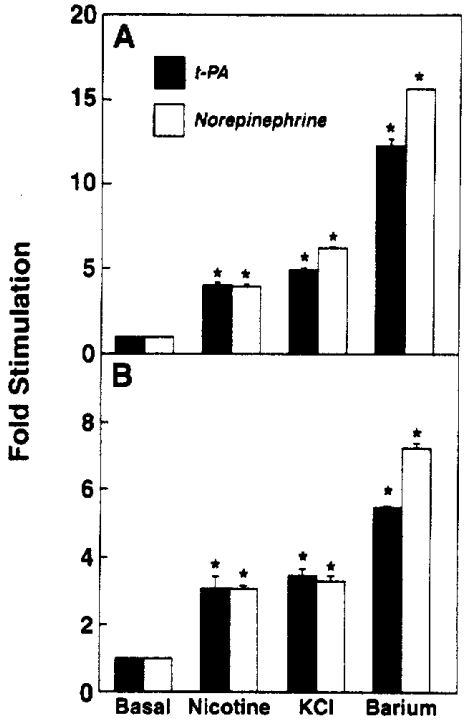

Figure 1. Co-release of t-PA with catecholamines from chromaffin cells. PC-12 cells (A) or bovine adrenal chromaffin cells (B) were labelled with [^{3}H]-norepinephrine and incubated at 37°C for 30 min in release buffer (10 mM HEPES, pH 7, 150 mM NaCI, 5 mM KCl, 2 mM $CaCI_2$) in either the presence or absence of 60 μM nicotine, 55 mM KCl or, 2 mM $BaCI_2$. After aspirating the release buffer, cells were harvested and lysed and the release of t-PA antigen (filled bars) and [^{3}H]-norepinephrine (open bars) were determined. Percent secretion was calculated as the amount in release buffer/total (amount in release buffer + amount in cell lysate), and the results expressed as fold stimulation compared with basal (unstimulated) values. Reprinted with permission from (Parmer *et al* 1997b).

To examine whether t-PA was targeted to the regulated secretory pathway, we evaluated the subcellular localisation of chromaffin cell t-PA using functional secretagogue release studies (Fig. 1). In these experiments, t-PA antigen and norepinephrine were measured in releasates from PC-12 cells (Fig. 1, Panel A) under basal conditions and after 30 minutes of exposure to several chromaffin cell secretagogues, including 60 μM nicotine, 55 mM KC1 and 2 mM $BaC1_2$. Significant increases in t-PA secretion were measured in response to all of these chromaffin cell secretagogues, approximately 4-fold for nicotine, 5-fold for KC1, and 12-fold for $BaC1_2$ (Parmer et al 1994a; Parmer *et al*1997b). Moreover, t-PA release occurred in

parallel with release of norepinephrine, consistent with release from the same subcellular pool, the catecholamine storage granule.

t-PA release from PC-12 cells has been observed also in response to membrane depolarization with tetraethylammonium chloride, and the release is dependent upon the influx of calcium ions (Gualandris *et al* 1996). This rapid release is consistent with release of presynthesized t-PA from a storage pool since no changes in gene expression or protein synthesis are required (Gualandris *et al* 1996).

Furthermore, when PC-12 cells are transfected with an expression vector driving t-PA expression, the expressed t-PA is also stored in a releasable pool as demonstrated by its release in response to carbachol (Harrison *et al* 1996). t-PA is also released from primary bovine adrenal chromaffin cells in response to the three chromaffin cell secretagogues (nicotine, KC1, and $BaC1_2$) (Fig. 1. Panel B) (Parmer *et al* 1994a; Parmer et al 1997b). The secretagogue-mediated t-PA release is in parallel with norepinephrine secretion, consistent with exocytotic release of t-PA from catecholamine storage vesicles in this additional chromaffin cell source.

We also evaluated the subcellular localisation of the chromaffin cell t-PA using subcellular fractionation (Parmer *et al* 1997b). PC-12 cells were labelled with ^{3}H-norepinephrine, homogenised and subjected to sucrose density fractionation (Fig. 2). ^{3}H-norepinephrine and t-PA antigen were colocalized to the same subcellular fraction with a major peak at 1.4 M sucrose (Parmer et al 1997b). This 1.4 M peak is consistent with the buoyant density which we and others have demonstrated previously for catecholamine storage vesicles isolated from PC-12 cells (Parmer *et al* 1993; Schubert and Klier 1977; Roda *et al* 1980).

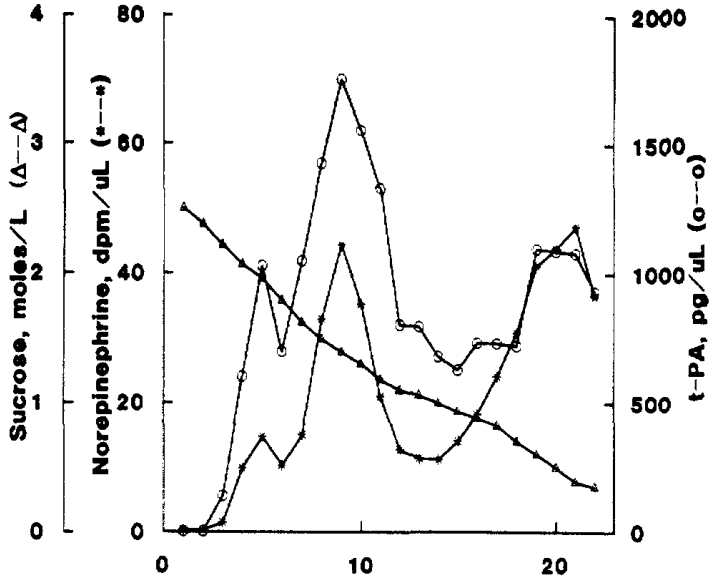

Figure 2. Co-localization of t-PA and norepinephrine in sucrose density gradient fractions. PC-12 cells were labelled with [^{3}H]-norepinephrine and fractionated on a continuous sucrose density gradient. Fractions were collected and assayed for [^{3}H]-norepinephrine by liquid scintillation counting, t-PA by enzyme-linked immunosorbent assay, and sucrose concentration by refractometry. Reprinted with permission from Parmer et al 1997b.

In another chromaffin cell source, catecholamine storage vesicle lysates isolated from human pheochromocytoma tumours were enriched ~30-foldin t-PA antigen, compared to tumour homogenate (Parmer *et al* 1997b). The enrichment in t-PA antigen paralleled the enrichment in catecholamines, suggesting that both are localised to the same subcellular fraction (Table).

Table 1. t-PA and catecholamines in human pheochromocytoma: Enrichment in chromaffin vesicle fractions.

Fraction	t-PA (ng/mg protein)	Catecholamines (μg/mg protein)
Tumor homogenates (*n*=9)	0.37±0. 12	14.80±5.04
Chromaffin vesicle lysates (*n*=9)	10.70±4.73*	220.58±73.46*

*P<0.05, compared with tumour homogenates. Reprinted with permission from Parmer et al 1997b.

3. POTENTIAL TARGETING OF t-PA TO THE REGULATED SECRETORY PATHWAY IN OTHER NEUROENDOCRINE CELL TYPES

Evidence is now emerging that other neuroendocrine cell types may target t-PA to specific subcellular granules. Specifically, t-PA and parathyroid hormone exhibit parallel calcium-regulated release from 24-hour cultures of human parathyroid cells, raising the possibility that t-PA and parathyroid hormone may follow the regulated secretory pathway in this neuroendocrine cell type (Bansal and MacGregor 1992). In addition, when t-PA was stably expressed in the murine pituitary derived AtT20 cell line, cAMP stimulated its secretion and, t-PA was colocalized with ACTH, a marker of the AtT20 storage granule as assessed using confocal microscopy (Santell *et al* 1999). Furthermore, PC-12 cells treated with nerve growth factor (NGF) differentiate into nondividing cells which exhibit characteristics of sympathetic neurones (Greene and Tischler 1976). t-PA is released by carbachol and KCI (Gualandris *et al* 1996; Harrison *et al* 1996) also in NGF-treated PC-12 cells. This implies that neuronal cells also may target t-PA to catecholamine storage vesicles and/or to other secretory vesicles.

4. CONSIDERATION OF t-PA INHIBITORS

The net result of t-PA release must be considered in the context of the presence of t-PA inhibitors in the cells or in the extracellular milieu. Free t-PA (~70 kDa) as well as complexes of t-PA with a size consistent with t-PA complexed to an inhibitor (~110 kDa) are detected in PC-12 cell lysates by immunoprecipitation (Fig.3) (Parmer *et al* 1997b).

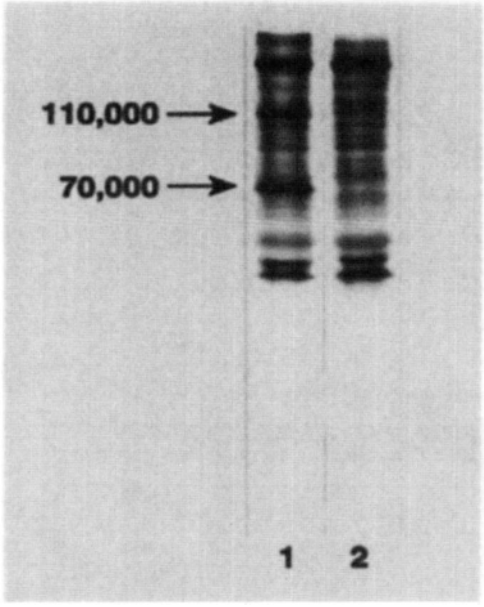

Figure 3. Synthesis of t-PA by PC-12 cells. PC-12 cells, grown to 50% confluency, were cultured in the presence of [35S]-methionine (50 µCi/6 cm plate) for 4 hr at 37°C. Cell lysates were immunoprecipitated with an IgG fraction of either rabbit anti human t-PA antiserum (lane 1) or normal rabbit serum (lane 2). Reprinted with permission from Parmer et al 1997b.

However, by fibrin zymography [a technique which simultaneously evaluates electrophoretic mobility and plasminogen activating activity (Loskutoff and Schleef 1988a)], free (uncomplexed t-PA) is the major species detected in release medium when PC-12 cells (Parmer *et al* 1994a; Parmer *et al* 1997b; Gualandris *et al* 1996) or bovine adrenal chromaffin cells (Parmer *et al* 1994a; Parmer *et al* 1997b) are stimulated by secretagogues (Fig.4).

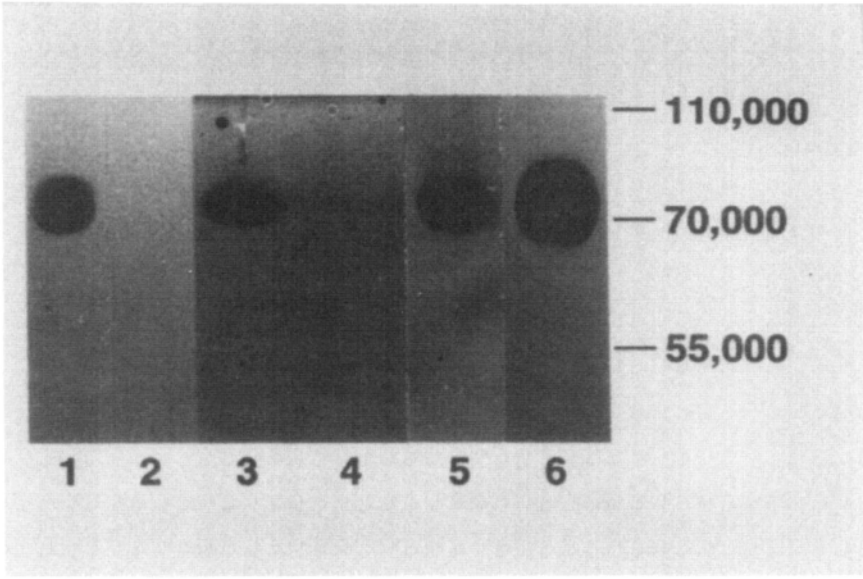

Figure 4. Fibrin zymography of catecholamine storage vesicle proteins. Samples were electrophoresed on 10% sodium dodecyl sulphate gels and subjected to fibrin zymography as described (Loskutoff and Schleef 1988b) for either 17 hr (lanes 1,2, and 6), 20 hr (lanes 3 and 4), or 3 hr (lane 5). Lanes are as follows: 22 µl of conditioned media fiom PC-12 cells stimulated for 30 min with either 2 mM BaCl2 (lane 1) or buffer (lane 2); 30 µl of conditioned media fiom bovine adrenal chromaffin cells stimulated for 30 min with either 2 mM BaCl2 (lane 3) or buffer (lane 4); human pheochromocytoma chromaffin vesicle lysate (lane 5); 2 ng of human recombinant single chain t-PA (lane 6). Reprinted with permission fiom Parmer *et aI* 1997b.

Thus, it is not known whether inhibitors are co-stored and co-secreted with t-PA. Interestingly, transfection of AtT-20 murine pituitary cells with a cDNA for PAI-1 results in PAI-1 expression and targeting of this inhibitor to the regulated secretory pathway (Gombau and Schleef 1994). Leprince and co-workers could not detect PAI-1 antigen using a panel of monoclonal and polyclonal anti-human PAI-1 antibodies in PC-12 samples in which t-PA inhibitory activity was present (Leprince *et al* 1991). This result could have been due to lack of cross reactivity between rat PAl-l and the anti-human PAI-1 antibody or to the possibility that the t-PA-inhibitor complex is formed with another inhibitor, PAI-2 or the newly recognised axonally secreted serine protease inhibitor, neuroserpin, which also inhibits t-PA activity (Hastings *et al* 1997). Further studies are needed to resolve whether t-PA inhibitors are targeted to the regulated pathway of secretion in the chromaffin cell and to identify these inhibitors.

5. POTENTIAL MECHANISMS FOR TARGETING OF t-PA TO THE REGULATED SECRETORY PATHWAY

Two potential mechanisms have been proposed for targeting of proteins into the regulated pathway and for sorting regulated secretory proteins away from constitutive proteins at the trans-Golgi cisternae: (1) receptor-dependent protein targeting and (2) selective protein aggregation and condensation. In the first mechanism, targeting to the regulated pathway is due to binding to specific receptors or to carrier proteins in the Golgi membrane (Chung et al 1989). In the second mechanism, sorting of regulated secretory products from the soluble (constitutive) proteins in the pathway is due to formation of molecular aggregates triggered by conditions in the trans-Golgi milieu (acidic pH and millimolar concentrations of calcium ions) (Huttner *et al* 1991; Chanat and Huttner 1991). Proteins of the chromogranin/secretogranin ("granin") family may play a crucial role in the targeting of proteins destined for the regulated secretory vesicle via the second mechanism by forming aggregated "granin" complexes to which other proteins destined for the regulated secretory vesicle bind and are separated from constitutively secreted proteins in the trans-Golgi network with subsequent movement into dense core regulated secretory vesicles. Selective aggregation of proteins of the "granin" family is induced by a low pH and high calcium environment, corresponding to that in the trans-Golgi network lumen (Chanat and Huttner 1991). In recent studies we found that t-PA binds specifically to chromogranin A (Parmer *et al* 1997a), the major secretory protein in catecholamine storage vesicles, as well as in secretory vesicles throughout the neuroendocrine system. Moreover, the interaction is enhanced under conditions of the trans Golgi network (low pH and high Ca^{+2}). Binding of t-PA to aggregated chromogranin A complexes may provide a mechanism for the targeting of t-PA to the regulated pathway of secretion in chromaffin cells and in other neuroendocrine tissues.

6. PHYSIOLOGICAL IMPLICATIONS OF REGULATED SECRETION OF t-PA

Under basal conditions, constitutive release from the endothelial cell is considered to be the major source of circulating t-PA (Todd 1959). However, t-PA is released also into the circulation in response to distinct stimuli (Emeis *et al* 1996). This response is rapid, suggesting secretion from

stored pools. The mechanisms by which this response occurs in vivo and the cellular sources are not known. The acute release of t-PA has been demonstrated in response to a variety of circumstances/conditions including anxiety (Macfarlane and Biggs 1946; Schneck and von Kaulla 1961), exercise (Biggs *et al* 1947), surgery (Macfarlane and Biggs 1946), caloric tests (Macfarlane and Biggs 1946; Schneck and von Kaulla 1961), electroconvulsive therapy (Fantl and Simon 1948), cerebral arteriography and pneumonencephalography (Macfarlane and Biggs 1946; Schneck and von Kaulla 1961). Notably, these types of stimuli also activate the sympathoadrenal system causing exocytotic release of amines and proteins from catecholamine storage vesicles. Thus, the recent studies reviewed here, which demonstrate regulated secretion of t-PA from catecholamine storage vesicles, suggest that these subcellular organelles may serve as a reservoir, and sympathoadrenal activation may be an important physiologic mechanism, for rapid release of t-PA.

Catecholamines (primarily epinephrine) stimulate t-PA release into the circulation and this was originally thought to be due to their action upon vascular endothelium (Biggs *et al* 1947). However, during exercise, only ~50% of the increase in plasma t-PA level, is attributable to this mechanism [exercise-induced increases in catecholamines causing endothelial cell t-PA release (Chandler *et al* 1992)]. Furthermore, the exercise-induced release of t-PA is not fully abolished by b-adrenergic receptor antagonists (Cohen *et al* 1968; Korsan-Bengsten and Conradson 1974; Britton et al 1976) implying that a portion of the t-PA is not released in response to direct action of the catecholamines on cellular receptors. Thus, release of catecholamine storage vesicle contents during sympathoadrenal activation may enhance the profibrinolytic capability of plasma by the direct release of t-PA and, secondarily, by release of catecholamines which may act upon endothelium to release endothelial cell t-PA.

The demonstration of concomitant release of t-PA and catecholamines from the same subcellular granule further predicts that t-PA antigen levels should be elevated in essential hypertension, a disease characterised by augmented sympathoadrenal activity. This prediction has been borne out in several studies [e.g. (Trifiletti *et al* 1995)]. However, PAI-1 levels can also be increased in hypertension (Trifiletti et al 1995). Thus, the net fibrinolytic activity in hypertension may even be reduced in the face of increased circulating t-PA antigen. Further studies are necessary to establish the extent of the role of the chromaffin cell and sympathetic neurones as a source of t-PA release in response to stress.

The presence of t-PA within catecholamine storage vesicles has important implications for neuroendocrine function. t-PA is synthesised by neurones in most areas of the brain (Sappino *et al* 1993) and may participate in neuronal

regeneration and migration (Pittman *et al* 1989; Krystosek and Seeds 1981; Moonen *et al* 1982; Seeds *et al* 1992; Qian *et al* 1993). t-PA plays a role in excitotoxin-induced injury to the hippocampus as shown in studies with t-PA deficient mice (Tsirka *et al* 1995). Also, t-PA deficient mice show differences in synaptic transmission and in long term potentiation (Huang *et al* 1996; Frey *et al* 1996). Infusion of KCI into brains of adult mice increases hippocampal t-PA activity (Gualandris *et al* 1996). These studies raise the interesting possibility that the release of stored t-PA from neurosecretory vesicles may contribute to the aforementioned functions of t-PA within the brain.

The widespread neuroendocrine localisation of t-PA also has suggested that t-PA may contribute to prohormone processing (Kristensen *et al* 1986). Our results suggest that chromaffin cell t-PA can participate in the plasmin-dependent processing of chromogranin A to bioactive peptides which modulate catecholamine release.

Thus, targeting of t-PA to specific subcellular granules has broad implications for both neuroendocrine and vascular function and represents a heretofore unrecognised interaction between the fibrinolytic and sympathoadrenal systems.

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15

CHROMOGRANIN A IMMUNOREACTIVITY IN NEUROENDOCRINE CELLS IN THE HUMAN GASTROINTESTINAL TRACT AND PANCREAS

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1. INTRODUCTION

Chromogranin A (CGA), an acidic glycoprotein with 439 amino acids and a molecular mass of 48 kD, was first isolated from chromaffin cells in the adrenal medulla (Banks and Helle 1965, Helle 1966, Blaschko *et al* 1967, Schneider *et al* 1967). Sixteen years later, immunocytochemical studies showed that CGA immunoreactivity was present in most neuroendocrine organs (Lloyd and Wilson 1983, O'Connor 1983, O'Connor *et al* 1983a, 1983b). Since then CGA has served as an important broad-spectrum histochemical marker for the identification of neuroendocrine cells and tumours (Wilson and Lloyd 1984, Facer *et al* 1985). CGA antibodies have been commercially available for the past two decades, and are widely used in routine pathology.

Evidence that CGA is a prohormone, i.e. a precursor to other biologically active peptides, was based on the biochemical findings of several molecular forms of CGA (Fischer-Colbrie 1987, Iacangelo *et al* 1988) and on the isolation and characterization of pancreastatin (Tatemoto *et al* 1986, Efendic *et al* 1987).

The amino acid sequence of human CGA contains 10 pairs of basic amino-acids, which are potential cleavage sites for specific endogenous proteases, giving rise to various smaller fragments that may be biologically

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active. These peptides are, following their localization from the N- to the C-terminal regions, vasostatins (amino acid sequences CGA 1-17/113 ; Helle *et al* 1990, Drees *et al* 1991), chromostatin (CGA 124-143 ; Galindo *et al* 1991), chromacins (CGA 173-194 ; Strub *et al* 1997), pancreastatin (CGA 243-294 ; Tatemoto *et al* 1986), WE-14 (CGA 316-329 ; Curry *et al* 1992), catestatin (CGA 344-364 ; Mahata *et al* 1997), parastatin (CGA 347-419 ; Fasciotto *et al* 1993) and GE-25 (CGA 367-391 ; Kirchmair *et al.* 1995).

In man, post-translational processing of CGA has been reported in adrenal chromaffin cells and pancreatic endocrine cells (Hutton *et al* 1987, Watkinson *et al* 1991, Wohlfarter *et al* 1988), as well as in a few neuroendocrine tumours. Chromatographic separations of tissue extracts have shown that the extent of CGA processing varies in different neuroendocrine tissues (Helle and Angeletti 1994, Iacangelo and Eiden 1995).

In man, the largest total amount of CGA is extracted from the adrenal medulla, followed by the pituitary gland, gastrointestinal (GI) tract and pancreas. Other endocrine organs contain very small amounts of CGA (Takiyyuddin *et al* 1990). Little is known about the differential CGA processing in the various parts of the GI tract and pancreas.

The aim of this chapter is to review the localization of CGA immunoreactivites in neuroendocrine cells in various parts of the human GI tract and in pancreas, with antibodies raised against different regions of the human CGA molecule ; this is a first step to increase our knowledge about the functional role of CGA and its fragments.

2. IMMUNOREACTIVITY WITH MONOCLONAL ANTIBODIES TO CGA

2.1 Distribution in the Human Gastrointestinal Tract and Pancreas

Several studies have been performed regarding the distribution of CGA-immunoreactive cells in the human GI tract and pancreas (Buffa *et al* 1989, Schmid *et al* 1989, Rindi *et al* 1986, Varndell *et al* 1985, Portela-Gomes *et al* 1997).

These studies have been performed by using a commercial monoclonal antibody to CGA (mabCGA; clone LK2H10) raised from a human pheochromocytoma (Lloyd and Wilson 1983). However, there are no written reports Stating where this mabCGA binds on the CGA molecule. Preincubation of this mabCGA antibody with antigens corresponding to

different parts of the CGA molecule has indicated that this commercial mabCGA most likely binds somewhere in the CGA sequence 250 to 284, i.e., in the N-terminal part of pancreastatin (Portela-Gomes and Stridsberg unpublished observations).

Immunoreactive cells to mabCGA were observed in all parts of the GI tract and in the pancreas, where they were seen at all levels of the mucosa, though mainly in the middle third portion. In the GI tract, immunoreactive cells to mabCGA were most numerous in the antrum, decreased in frequency from the duodenum to distal ileum, and increased in the colon. These immunoreactive cells were also present in the corpus and in the Brunner's glands. The intensity of the immunoreactivity to mabCGA was strong and even in all GI regions studied except the antrum, where it varied. In the endocrine pancreas there were also variations in the staining intensity in different cells (see below).

2.2 Co-localization of Immunoreactivity to mabCGA in Neuroendocrine Cells

The GI tract and pancreas contain several types of neuroendocrine cells, i.e., endocrine cells that share histochemical features with neurons. There are neuroendocrine cell types that are distributed throughout the whole GI tract, such as the enterochromaffin (EC ; serotonin-producing) cells and somatostatin cells, but most neuroendocrine cell types have a more local distribution. Thus, enterochromaffin-like (ECL) cells are limited to the corpus; gastrin cells to the antrum, but also to a much lesser extent to the duodenum; cholecystokinin (CCK), secretin and gastric inhibitory polypeptide (GIP) cells are concentrated in the duodenum; enteroglucagon cells in the duodenum, ileum and colon, and neurotensin cells mostly in the ileum. The endocrine pancreas contains four major cell types, A, B, D and PP cells, synthesizing glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. B cells also express islet amyloid polypeptide (IAPP or amylin ; Westermark *et al* 1986), and a small fraction of A cells, GIP (Alumets *et al* 1978).

A systematic study of the co-localization of the immunoreactivity with mabCGA in different neuroendocrine cell types has been performed in histologically normal mucosa from different parts of the human GI tract, by using double or triple immunofluorescence stainings (Portela-Gomes *et al* 1997). In this study, the majority of ECL cells in the gastric oxyntic mucosa was immunoreactive to mabCGA. The EC cells in the GI tract displayed mabCGA immunoreactivity, except in the duodenal villi, where a minority was immunoreactive. Most gastrin cells showed immunoreactivity to

mabCGA, but the staining intensity was mostly weak, sometimes hardly observed. The majority of duodenal CCK cells in the villi and in the crypts was immunoreactive to mabCGA, but only few in the Brunner's glands. Regarding the secretin cells, only less than 50% showed immunoreactivity to mabCGA, which is in some contrast with reports by Buffa *et al* (1989), who found reactivity to mabCGA in all these cells. The co-localization of immunoreactivities to enteroglucagon and mabCGA varied in the different intestinal regions. In the duodenal mucosa, the majority of enteroglucagon cells reacted to mabCGA, which is in agreement with the results reported by Varndell *et al* (1985) ; however, this cell type did not react to mabCGA in Brunner's glands. In the ileum and colon, where enteroglucagon to a large extent is co-localized with peptide tyrosine tyrosine (PYY), immunoreactivity to mabCGA appeared in only some of these cells. GIP cells, localized in the duodenum, and neurotensin cells, in the ileum, also displayed a varying content of immunoreactivity to mabCGA. Virtually all somatostatin cells in the GI tract were non-reactive to mabCGA. Wiedenmann *et al* (1988) reported similar results. The reason for this latter finding is unclear, but one explanation may be that the antigen epitope corresponding to mabCGA is present in too low concentration to be detected with the immunostaining method used. Other explanations may be that the antigen can be masked by granule-related proteins, or is proteolytic fragmented in such a way that it does not react with mabCGA, or that is non-existent.

In the pancreas, the staining results with mabCGA are contradictory, some authors report that only glucagon cells are stained (Lloyd and Wilson 1983), others show that also insulin cells are weakly stained (Grube *et al* 1986, Buffa *et al* 1989) ; these varying staining results may depend on differences in the sensitivity of the staining method used, or in tissue preparation masking the CGA epitope.

2.3 Intracellular Localization

In the GI tract, the immunoreactivity to mabCGA was mostly concentrated in the infranuclear (basal) region of the cells. This intracellular distribution was more marked in the antral EC cells, where the immunoreactivity was localized mainly adjacent to the basal cell membrane. In some gastrin cells and in the few somatostatin cells expressing CGA, the entire cytoplasm was stained. Thus, the immunoreactivity to mabCGA varied in the different regions of the GI tract, but also among the different cell types, as well as within the same cell type.

3. CGA IMMUNOREACTIVITY WITH ANTIBODIES RAISED TO DIFFERENT REGIONS OF THE CGA MOLECULE

3.1 Distribution

Immunocytochemical studies in the human GI tract and pancreas with region-specific antibodies are sparse. The presence of beta-granin (corresponding to human vasostatin) and WE- 14 (corresponding to human CGA 352-372) have been described in the human GI tract and pancreas (Hutton *et al* 1988, Gleeson *et al* 1996). Immunocytochemical studies in pancreas have also been performed with pancreastatin (porcine sequence) and chromostatin (bovine sequence) antibodies (Schmidt *et al* 1988, Cetin *et al* 1993). A systematic immunocytochemical investigation in different parts of the GI tract and in pancreas was performed (Portela-Gomes *et al* 1999) by using polyclonal antibodies (raised by M. Stridsberg) to the following 12 regions of the human CGA molecule: amino acid sequences CGA1-17, 17-38, 63-76, 100-113, 116-130, 176-195, 238-247, 284-301, 324-337, 361-372, 375-384 and 411-424.

Both the GI tract and pancreas contained immunoreactive cells with all the region-specific antibodies tested, but the distribution patterns varied. An intense to moderate immunoreactivity was found with the antibodies to the N-terminal and the mid portions of the CGA molecule, with some regional exceptions: the antibodies corresponding to amino acid sequences CGA 63-67 (C-terminal vasostatin-I), CGA 116-130 (chromostatin) and CGA 238-247, displayed a weak immunoreactivity in the corpus, while CGA 116-130 showed no immunoreactivity in the antrum and a weak reaction in the pancreas. The C-terminal part of CGA molecule showed a weaker immunoreactivity; the sequences CGA 361-372 (N-terminal parastatin, N-terminal GE-25) and CGA 375-384 (mid portion of parastatin, C-terminal GE-25) showed a weak immunoreactivity in the corpus and antrum, the latter antibody also in duodenum. The antibody to CGA 411-424 (C-terminal parastatin) was weakly immunoreactive in the antrum and colon.

The weak immunoreactivity to some fragments of CGA, particularly in the C-terminal portion of CGA, may indicate that they are present at very low concentration, or that the antibody-binding epitopes may be masked either by other granule proteins or by the histological processing. However, it is more likely that CGA is proteolitically processed in such a way not reacting with the antibodies used. This is in line with biochemical findings of a predominant processing of CGA in the C-terminal end (Metz-Boutigue *et al*1993, Eskeland *et al* 1996).

3.2 Co-localization

By using double immunofluorescence technique, the comparison of the immunostaining with the different CGA region-specific antibodies and mabCGA showed a similar staining pattern ; only occasionally were cells stained with only one of the two antibodies, but the fluorescence intensities could vary. This discrepancy in the staining intensity was markedly apparent in pancreas, where a stronger immunoreactivity was found in the glucagon rather than in the insulin cells with the antibodies to CGA 1-17 (N-terminal vasostatin), 176-195 (chromacin) and 284-301 (C-terminal pancreastatin) and, as earlier mentioned, also with mabCGA. All the other region-specific antibodies showed a more homogeneous staining in these two cell types. A similar immunostaining pattern to the former, in pancreatic islets, was reported by Schmidt *et al* (1988) who used porcine C-terminal pancreastatin antibodies. Weak immunostaining to CGA 116-130 (chromostatin) was found in few islet cells, contrary to the results reported by Cetin *et al* (1993) who found strong immunostaining by using antibodies to bovine CGA sequences. This discrepancy may be due to species differences of the peptide sequences used for antibody production.

A remarkably stronger staining intensity was obtained with the CGA116-195 (chromacin) antibody compared to all the other antibodies. This antibody stained a larger cytoplasmic area than mabCGA in most cells ; this finding was more apparent in the EC cells. Gastrin cells in the antrum and duodenum showed a larger cytoplasmic area immunostained with the CGA 238-247 antibody than with mabCGA. These differences in cytoplasmic distribution may indicate an intracellular specific processing of CGA. Somatostatin cells, either in the GI tract or in pancreas, were mostly unreactive with all CGA region-specific antibodies.

4. SUBCELLULAR LOCALIZATION

Few ultrastructural immunocytochemical studies have been carried out concerning the demonstration of CGA in human GI tract and pancreas.

Only A (glucagon) cells have been reported to show ultrastructural immunoreactivity to mabCGA (Varndell *et al* 1985, Lukinius et al 1992). Lukinius *et al* (1992) have observed that the immunoreactivity to mabCGA was mainly localized in the lucent halo of the secretory granules of the A cells of human pancreatic islets. By using a CGA 116-439 antibody, which includes sequences from chromostatin to C-terminal CGA, these latter authors have also shown immunolabelling in the electron-dense core of B (insulin) cell granules, in the lucent halo of A cell granules, and in D

(somatostatin) cell granules. The D cell immunolabelling was, however, weak. Probably these cells represent the minority somatostatin cell population mentioned above.

5. CONCLUSION

The human GI tract and pancreas contain numerous neuroendocrine cells of various types and most of them display CGA immunoreactivity. Little is known about the extent to which the CGA molecule is processed to smaller fragments in these cells. The present chapter analyses the CGA immunoreactivity in the various neuroendocrine cell types in the human GI tract and pancreas with different region-specific antibodies and a monoclonal CGA antibody widely used.

On the whole, immunoreactivity with the antibodies raised to the N-terminal and mid parts of the CGA molecule was stronger than that to the C-terminal part, indicating that the C-terminal part of the CGA molecule is more processed. These findings are in accordance with biochemical studies. In the different parts of the GI tract and pancreas, different degrees of immunoreactivity intensity could be observed with the various region-specific antibodies, suggesting a differential processing of chromogranin A in different parts of the GI tract.

Both GI and pancreatic somatostatin cells were, with few exceptions, unreactive with all antibodies. The intensity of the immunostaining in gastrin, EC, glucagon and insulin cells varied apparently with the region-specific antibodies, while the other cell types were stained more homogeneously, suggesting that there is a different processing of CGA in the neuroendocrine cell types.

The intracytoplasmic distribution of the CGA immunoreactivities with the different CGA region-specific antibodies varied to some extent ; the immunoreactivities were mainly localized to the infranuclear region (GI tract) or to the vascular pole (pancreatic islets). The whole cytoplasm was immunostained with the antibody to CGA 176-195 (chromacin) in most neuroendocrine cells, and with the antibody to CGA 238-247 in gastrin cells.

The observed variations in the immunoreactivity of the various CGA region-specific antibodies in the GI tract and pancreas point to the possibility that a different processing of CGA can occur and/or that there may be a masking of CGA epitopes.

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CHROMOGRANIN A AND ITS DERIVED PEPTIDES IN THE RAT AND PORCINE GASTRO-ENTERO-PANCREATIC SYSTEM

Expression, Localization, and Characterization

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1. INTRODUCTION

Numerous studies of normal and neoplastic tissues have demonstrated that chromogranin A (CGA) is expressed in the majority of neuroendocrine cell types (for review see Weidenmann and Huttner 1989). However, the reports on which of the different neuroendocrine cell types that exhibit CGA immunostaining has often been contradictory. The most probable explanation for these discrepancies is the different epitope specificity of the sera employed in each study. The generation of region- and site-specific antisera (Fig. 1) allowing the chemical and histochemical characterisation of CGA derived peptides has offered a more comprehensive understanding of the localization and posttranslational processing of CGA in the

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neuroendocrine cell populations (Curry *et al* 1991, Iacangelo *et al* 1995). This paper reviews the localization of CGA and of CGA-derived peptides in the gastro-entero-pancreatic system of rat and pig.

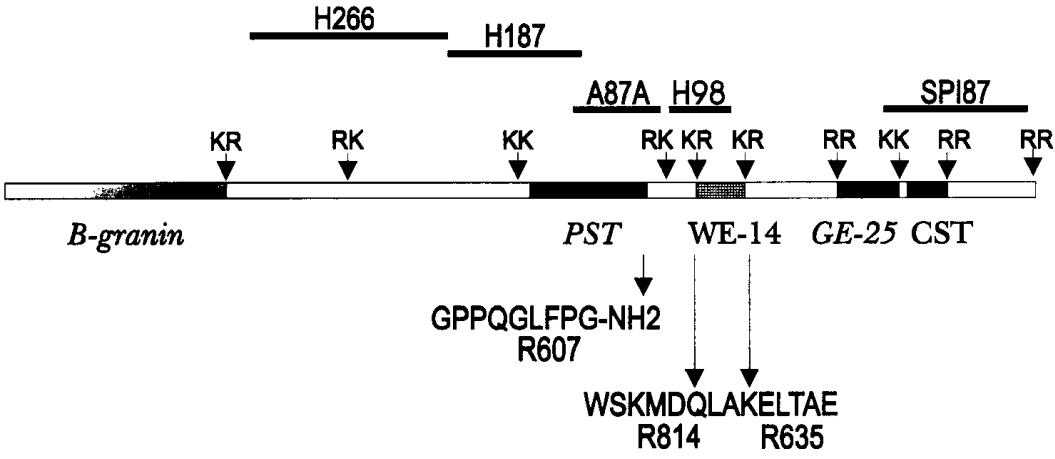

Figure 1. Diagrammatic representation of rat CGA indicating the sites of characterized peptides. The regional-specificity of rat CGA b-galactosidase fusion protein sera (—) and the amino acid sequence employed in the generation of site-specific sera are indicated (Curry *et al*1991, Iacangelo *et al* 1995).

2. ONTOGENY

Embryologically the gastro-entero-pancreatic (GEP) system is derived from the endoderm (Le Douarin 1982, Gittes and Rutter 1992). The gastrointestinal tract is formed as a simple multilayered tube of stratified epithelium that is substituted by a layer of columnar epithelial cells (Ponder *et al* 1985). Two ductal outgrowths of the foregut develop to form the dorsal and ventral lobes of the pancreas that subsequently fuse (Dubois *et al* 1989). Stem cells within these tissues give rise to the diverse population of

neuroendocrine cell types observed throughout the GEP system (Rutter 1980, Ponder *et al* 1989).

In the fetal rat pancreas CGA or CGA-derived products were observed at 13.5 days gestation (Jin *et al* 1992) and pancreastatin (PST)-like immunoreactivity (LI) (Barkatullah *et al* 1997) two days following the detection of insulin (Yoshinari and Daikoku 1982) and one day after the detection of glucagon cells (Jin *et al* 1992). WE-14 immunostaining was not detected until 15.5 days gestation (Barkatullah et al 1997). The prohormone convertases (PCs) PC3/1 and PC2 which cleave proinsulin, proglucagon and CGA (Arden et ai 1993), were detected in fetal mouse pancreatic tissue at 10 and 11 days gestation (Marcinkiewicz *et al* 1994) at a time when the fetal rat islet hormones were first detected. A large population of CGA and WE-14 immunopositive cells were observed in the primordial pig pancreas at 22 days gestation (Fig. 2), one day earlier than previously reported (Zabel *et al* 1994) and remained throughout gestation (Fig. 2). Characterization of the CGA- LI revealed a complex pattern of processing to generate PST-LI and WE-14-LI (Fig 3).

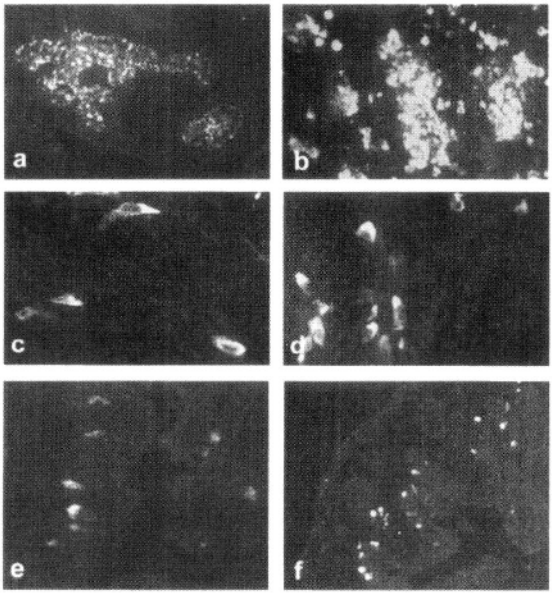

Figure 2. Porcine fetal GEP tissues displaying WE-14 immunoreactive endocrine cells in the: primordial pancreas at 22 (a, x150) and 76 days gestation (b, x200), gastric mucosa at 52 (c, x300) and 74 days gestation (d x300), and in the proximal intestine at 52 (e, x300) and 74 days gestation (f xl 80).

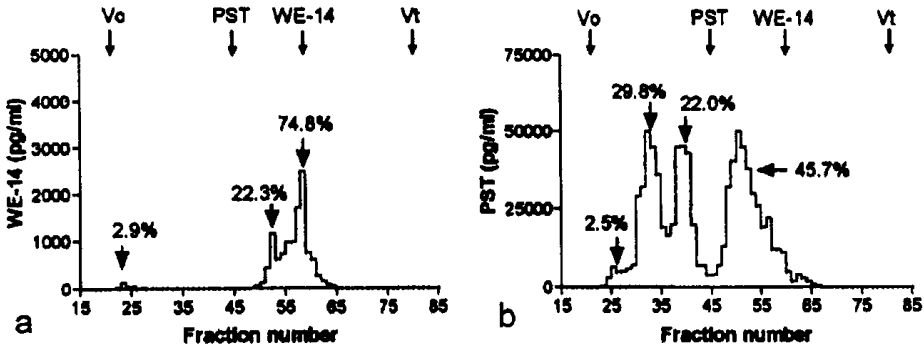

Figure 3. Chromatographic characterization of WE-LI and PST-LI in fetal pig pancreatic extracts at 68 days gestation.

PST-LI and WE-14 immunostaining was detected in the fetal rat stomach at 15.5 days gestation (Barkatullah *et al* 1997), 2.5 days before CGA was localized with histidine decarboxylase (HDC) in rat ECL cells (Sundler 1998). Intestinal cells in the fetal rat exhibited CGA immunostaining at 14.5 days gestation. PST-LI and WE-14 immunoreactivity was observed in endocrine cells, one and two days after CGA, respectively. The sequential expression of CGA and its derived peptides may reflect the timedependent expression of the PC's in endocrine cells in the developing fetal stomach. The density of CGA and WE-14 positive cells increased with the progressive maturation of the GEP system in both fetal rat (Barkatullah *et al* 1997) and pig tissues.

3. ISLETS OF LANGERHANS

Morphologically the pancreatic islets are composed of four major cell types that are characterized by their peptide products (Curry *et al* 1994): namely insulin, glucagon, somatostatin and PP cells. Perfusion experiments employing radiolabelled islets and rat insulinoma cells revealed the co-secretion of insulin and a 21 kDa protein (Hutton *et al* 1982), subsequently named β-granin (Hutton *et al* 1985). Perfusion of the porcine pancreas demonstrated the parallel secretion of insulin and PST-LI (Ostenson et al 1989). The endocrine cells of the pancreatic islets differ with respect to the CGA products they contain. Colocalisation studies revealed that WE-14 is expressed with insulin, glucagon and pancreatic polypeptide, but not with somatostatin, in porcine islets (Curry *et al* 1997). Light microscopy (Fig. 4) and ultrastructural analysis localized β-granin immunoreactivity to the B-cell

secretory vesicle (Hutton et al 1987) whereas all cell types seemed to contain PST-LI.

The isolation of porcine PST (Tatemoto et al 1986) and the simultaneous cloning of bovine CGA (Iacangelo *et al* 1986) led to speculation that CGA was the PST precursor. Chromatographic characterization of porcine pancreatic extracts detected three PST-like immunoreactants, the predominant molecular form coeluted with synthetic pig PST (Bretherton-Watt *et al* 1988). Analysis of rat pancreatic extracts indicates that in this tissue PST does not represent the predominant product (Curry *et al* 1990). Instead, extended forms of PST-LI predominates. Following the determination of rat CGA cDNA β-granin was shown to represent residues 1-128 and is flanked C-terminally by a pair of basic amino acids which represent the cleavage site (Hutton et al 1988). Rat CGA contains ten pairs of basic amino acids that represent putative cleavage sites (Fig. 1). The generation and application of region- and site-specific antisera revealed a differential pattern in the rat endocrine cell population. N- and C-terminally directed rat CGA sera exhibit a variable pattern and intensity of immunostaining in cells of the islet core and periphery (Fig. 4). Pulse-chase radiolabelling studies demonstrated that the β-granin precursor was synthesized as a 98 kDa proprotein that was processed to generate β-granin, a 45 kDa peptide representing the central domain of CGA and a 14 kDa peptide corresponding to the C-terminus. The generation of these peptide products was indicative of cleavage at Lys^{129}-Arg^{130} and Lys^{357}-Arg^{358}. The 45 kDa intermediate fragment was further processed to generate a 5 kDa PST-LI and a 3 kDa peptide corresponding to WE-14 (Arden *et al* 1994).

4. THE ENDOCRINE CELL POPULATION OF THE GASTROINTESTINAL TRACT

The mucosa of the stomach contains at least six endocrine cell populations that can be distinguished by their histochemical and ultrastructural features. ECL and A-like cells are unique to the oxyntic (acid-secreting) mucosa, gastrin and enterochromaffin (EC) cells are located in the pyloric mucosa, while D (somatostatin) and D_1/P cells are located in both the oxyntic and pyloric mucosa (Capella *et al* 1991, Sundler and Hakanson, 1991). The ECL cells contain histamine and histidine decarboxylase (HDC) and represent the predominant cell type in the oxyntic mucosa, constituting 65-75% of all endocrine cells in this location.

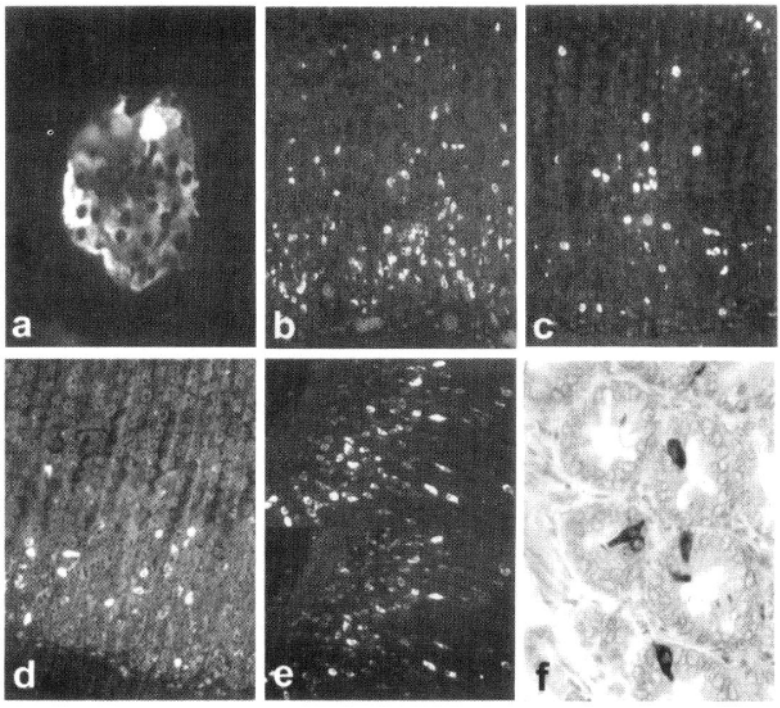

Figure 4. Adult GEP tissue displaying β-granin immunoreactivity in a pancreatic islet (a, x500), a large population of oxcyntic mucosal endocrine cells exhibiting H266 immunostaining (b, x220) with a subpopulation of cells exhibiting A87A (c, x220) and H98 immunoreactivity (d, x220). Pyloric mucosal endocrine cells simultaneously immunostained with B-granin [upper image] and SP187 [lower image] (e, x180). Flask-shaped endocrine cells in the duodenum exhibiting PST-LI immunostaining (f, x400).

Colocalization studies revealed the parallel expression of PST-LI and HDC immunostaining in the ECL cell population. WE-14 immunoreactivity was also evident in ECL cell population (Norlen *et al* 1997). Chromatographic characterization revealed a complex pattern of CGA proteolysis in oxyntic and pyloric extracts. Three PST-like immunoreactants were detected, the predominant large immunoreactant exhibited CGA-like mobility while the a PST-LI immunoreactant co-eluted with synthetic rat PST. A small PST-LI immunoreactant was also detected (Curry *et al* 1990, Norlén *et al* 1997). Analysis of rat plasma revealed that in the rat the ECL cell represents the major source of circulating PST-LI (Hakanson et al 1995, Kimura *et al* 1997, Norlén *et al* 1997). Fasting, feeding and gastrin and omeprazole administration modulated CGA expression in these cells (Dimaline et al 1993, Norlén *et al* 1997). Sustained hypergastrinemia caused an initial reduction in the cellular intensity of PST-LI and WE-14 immunostaining. Quantitative analysis of oxyntic mucosal extracts revealed a similar decrease in tissue concentration, while parallel chromatographic analysis revealed the selective depletion of small PST-like and WE-14-like

immunoreactants. This was explained by the preferential mobilization of mature secretory vesicles, containing highly processed and thus smaller CGA fragments, in response to hypergastrinemia (Norlén *et al* 1997). These studies have demonstrated modulation of CGA expression and proteolysis in response to physiological and pharmacological stimuli.

A diverse population of endocrine cell types are distributed in the musosa of the small intestine and colon. Detailed analysis of the human intestinal tract has revealed that different endocrine cell types exhibit distinct patterns of granin immunostaining (Portela-Gomes *et al* 1997). PST immunostaining has been detected in endocrine cells in porcine intestine (Bretherton-Watt *et al* 1988) and CGA, b-granin and PST-LI was detected rat intestine and colon (Curry *et al* 1990, 1991). Although the CGA-derived peptides seems to be differentially expressed, characterization of the proteolysis of CGA in rat intestinal and colonic extracts was similar to that observed in oxyntic extracts, thus demonstrating that CGA is most probably subject to similar posttranslational processing in gastric, intestinal and colonic endocrine cells (Curry *et al* 1990).

5. CONCLUSION

Collectively, these studies provide an insight into some of the dynamic factors that modulate the CGA expression, posttranslational processing and secretion during ontogeny and in the mature neuroendocrine cell population of the GEP system. The dynamic changes probably reflect the differential expression of proteolytic enzymes that take part in the processing of CGA. Understanding the mechanisms that control the expression and processing of CGA-derived peptides will help us elucidate their functional significance.

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PART5

FUNCTIONAL ASPECTS OF BIOLOGICALLY ACTIVE SEQUENCES

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REGULATION OF PARATHYROID SECRETION

Chromogranins, Chemokines, and Calcium

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1. INTRODUCTION

The parathyroid gland secretes parathyroid hormone (PTH), a primary regulator of serum calcium concentration. Changes in serum calcium concentration then regulate PTH secretion by negative feedback. This regulatory interplay can be modulated by biologically active peptides, including chromogranin A-derived peptides and interleukin-8. Fluctuations in intracellular calcium ion concentration appear not to play a role in the signalling mechanisms of these processes.

1.1 Characteristics of Parathyroid Secretion

The parathyroid gland is part of the complex of tissues and glands responsible for regulating calcium homeostasis and bone density. The parathyroid secretes parathyroid hormone, which causes calcium mobilization from bone, and decreases calcium clearance in kidney. Parathyroid differs from other endocrine cells in that secretion increases with diminishing levels of extracellular calcium, and decreases as the levels of extracellular calcium are raised. Secretion is a continuum, without a discrete switch. Thus, secretion of PTH from the parathyroid gland is not regulated by quantal stimuli, but by sensitive calcium receptors (Brown *et al* 1993). These G-protein coupled receptors continuously "monitor" the concentration

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of calcium in extracellular fluids, maintaining it in a narrow range around 1-1.2 mM for normal individuals. As the calcium concentration falls, PTH secretion increases, as it rises, PTH secretion is diminished. The calcium receptor is present in other tissues as well, for example, the thyroid gland, which also secretes calcitonin, with an effect opposite to that of PTH on calcium and bone metabolism. However, the signal transduction pathways in the thyroid C-cells are different, such that the thyroid secretes calcitonin in response to increased levels of extracellular calcium.

PTH production is regulated by 1,25-dihydroxy vitamin D_3, which decreases the amount of PTH mRNA, and consequently lowers the overall level of PTH secreted (Russell et al 1990, Cantley *et al* 1985). The vitamin D receptor is itself a transcription factor that regulates transcription of PTH and other mRNAs upon binding 1,25-dihydroxy vitamin D_3. As described below, N-terminal peptides from the prohormone chromogranin A, which is co-secreted from parathyroid with PTH, attenuate PTH secretion as well (Angeletti *et al* 1994, 1996). In parathyroid, 1,25-dihydroxy vitamin D_3 also regulates chromogranin A, increasing levels of mRNA and secretion, opposite to the effects on PTH (Cantley *et al* 1985). The chemokine interleukin-8 (IL-8) has also now been found to regulate both PTH secretion and PTH synthesis (Angeletti *et al* 1998). There is evidence of a regulatory interaction between IL-8 and the vitamin D receptor (Angeletti and Russell, unpublished observations). Other factors which potentially regulate PTH secretion include the complex of peptidergic nerve fibers which innervate both the parathyroid cells, and the blood vessels within the parathyroid.

2. CHROMOGRANIN PEPTIDES MODULATE PTH SECRETION

Peptides containing the amino acid sequence 1-76 of chromogranin A, originally described in the parathyroid gland as parathyroid secretory protein-1 (Cohn *et al* 1981), can inhibit PTH secretion as effectively as high physiologic concentrations of calcium. The biological mechanism is not yet entirely understood.

The peptide purified from the natural source is biologically active, and it is produced in by parathyroid, adrenal, and other tissues. Drees et al (1991) first found that a naturally derived amino terminal peptide of Mr 26,000 was an effective inhibitor of PTH secretion (Fasciott *et al* 1990, 1993). We have identified the naturally occurring amino terminal peptide CGA_{1-76}, also termed vasostatin (Aardal and Helle, 1992), which would be produced by proteolytic cleavage at the first set of dibasic residues, in parathyroid cell culture medium. Although we noted other amino terminal chromogranin A

fragments in the medium, the Mr 26,000 peptide noted by Drees *et al* (1991) was not observed. The cGA_{1-76} peptide and that generated by cleavage of chromogranin A at the second paired basic site have also been shown to be produced by adrenal chromaffin cells. Thus, inhibition of parathyroid secretion could occur by either an autocrine or an endocrine mechanism. Whether this peptide is produced by intracellular or extracellular processing is not known. However, prohormone convertases that could cleave this site are found in the parathyroid (Lame *et al* 1982, Angeletti and Zouheiry, unpublished observations).

Synthetic peptides corresponding to the natural sequence are biologically active (Russell *et al* 1994, Angeletti *et al* 1996). Not only is CGA_{1-76} functional, but CGA_{1-40} is active, showing that the first 40 amino acids are sufficient for biologic activity. In addition, the disulfide bond is required for activity, as demonstrated by the observations that the 22 amino acid loop sequence by itself retained partial activity, and that reduction and alkylation of CGA_{1-40} completely abolished its inhibitory effect. The ability of synthetic chromogranin A and chromogranin B amino terminal peptides to inhibit parathyroid secretion demonstrates that the unmodified primary structure is sufficient for biological activity if the disulfide bond is intact. Investigation of the structure-function relationships of these peptides was pursued using alanine residues to substitute for other classes of amino acids found within the sequence. It was found that there are specific structural requirements to the peptide's biological activity.

In contrast to the amino terminal peptides, peptides derived from other regions of chromogranin A and chromogranin B, which included $CGA_{403-428}$, CGB_{1-16}, $CGB_{316-326}$, and $CGB_{635-657}$, had no significant effect on PTH secretion. Other peptides, notably pancreastatin and parastatin, which are located at the carboxy terminus of chromogranin A, also have been shown to inhibit PTH secretion. Thus far, however, it has not been established that these peptides are processed or secreted from the parathyroid or other endocrine cells.

Neutralizing antibodies to chromogranin A stimulate parathyroid secretion. Within 1.5 hours after addition of CGA_{1-76} antisera to the incubation medium, the presence of CGA_{1-76} antisera at a dilution of 1:500, results in a 75% increase in PTH secretion as compared with low calcium alone. By 6 hours, the effect of the antisera on secretion diminished to 35% above controls, and showed essentially no effect by 24 hours. The decrease in effect was likely due to competition with intact chromogranin A, which the antiserum recognizes with equal affinity. Control antibodies to other chromogranin A peptides and to other proteins did not demonstrate this biological effect.

Chromogranin A peptides act via a G protein-coupled receptor (GPCR) mechanism. In addition to their effects on PTH secretion, radioactively labelled chromogranin A peptides bind specifically to a 78 kDa protein on the parathyroid cell surface. Although a much more detailed study would be required to definitively correlate protein binding with biologic activity, it has been found that only the biologically active peptides are effective as competitors. Even recombinant full length chromogranin A was not an effective competitor, which is consistent with other studies that have shown that proteolytic processing of chromogranin A is required for its biologic activities. The action of chromogranin A peptides on PTH secretion was blocked by pertussis toxin, suggesting that a GPCR mechanism is involved.

3. INTERLEUKIN-8 MODULATES PTH SECRETION

Interleukin-8 (IL-8) is a chemotactic cytokine, or chemokine, which is produced in response to inflammatory stimuli, and whose action aids in the migration of neutrophils and other white blood cells to the affected location in the body. It is known as a powerful chemotactic agent, which also activates cell adhesion molecules and stimulates angiogenesis. Both are different biological components of the inflammatory response. While attempting to clone a receptor for the N-terminal chromogranin A peptides, we found that there is a high level of expression of the IL-8 type B receptor (CXCR2) in the parathyroid gland, and that treatment of isolated bovine parathyroid cells with IL-8 increases the levels of PTH secretion and PTH mRNA (Angeletti *et al* 1998). Thus, the action of IL-8 on parathyroid could have consequences for bone formation.

This discovery raises many questions about IL-8 itself. Its functions define it as an episodic hormone whose actions will be limited in time and space, depending on the type and duration of infection or inflammation. It is not known if its expression is only limited to peripheral blood monocytes and endothelial cells, or if the expression of IL-8 and its congeners is more widespread.

Are its actions truly limited to inflammation events, or is IL-8 also a peptide hormone or growth factor of broader utility? The history of the past several decades of peptide hormone, growth factor and cytokine research has amply demonstrated that biologically active peptides first found in endocrine glands, neurons and the brain, or in the immune system will usually be found elsewhere, exhibiting effects that may be specific to an anatomical location or to the signal transduction system found within a particular cell type.

4. INTRACELLULAR CALCIUM LEVELS DO NOT REFLECT SECRETION STATUS

Although the parathyroid gland responds to stimulus with extracellular calcium (Cao) in a manner opposite to that of other endocrine cells, the intracellular apparatus and mechanisms for secretion of these cells have been thought to be very similar. Small, transient changes in intracellular calcium ion concentration (Cai) are an established part of the mechanism of secretion of most endocrine cells. Consistent with early reports by Nemeth and Scarpa (1986, 1987) using cuvette methods, our experiments with confocal imaging have found that changes in intracellular calcium levels do not correlate at all to the secretion status of the parathyroid cell (Russell *et al* 1999). In brief, increases in Cao > 0.25 mM cause a rapid and sustained increase in Cai, which does not diminish unless the Cao is reduced by addition of BAPTA, a chelating agent. Even if observations were continued for as long as 20 minutes, the internal Cai did not decrease to the starting levels. With many control experiments, we noted that this sustained rise in Cai appeared to be related to the magnitude of the change in Cao, not to the absolute value of Ca_o. Thus, the sustained increase in Cai could be observed both at 3 mM Cao, where the cells would not be secreting PTH, and at 0.5 mM Cao, where the cells would be actively secreting PTH. These sustained increases could be observed when the increments in Cao were as small as 0.25 mM. However, when the changes in Ca_o were reduced to 0.1 mM or less, changes in Cai, when they occured, were rapid and transient. In control experiments, we verified that the final levels of PTH secretion are the same whether the Ca_o was changed in large or small increments. The same cells which showed these subtle responses could be made to show the sustained response if the size of the calcium additions was then increased. Thus, it appears that changes in Cai are not coupled to PTH secretion. Addition of chromogranin A peptides and other biologically active peptides also did not alter levels of intracellular calcium in parathyroid cells. It is possible that the dramatic changes in Cai represent a pathological response, since physiological changes in Ca_o do not occur in such large increments.

5. CONCLUSION

Secretion of PTH from parathyroid glands can be regulated by extracellular calcium concentrations, l,25-dihydroxy vitamin D_3, chromogranin peptides, and interleukin-8. More detailed studies of the mechanism of secretion and the pathways of signal transduction must be pursued.

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VASOSTATINS

Vascular Targets

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1. INTRODUCTION

The N-terminal domain of chromogranin A was named vasostatin when it was first established that the natural cleavage products suppressed vasoconstriction in isolated human blood vessels (Aardal and Helle 1992, Aardal *et al* 1993). Two polypeptides, $CGA_{1\text{-}76}$ and $CGA_{1\text{-}113}$, arise from cleavage at the first and second pair of basic amino acid residues and have been named and numbered VS-I (VS-I) and vasostatin II (VS-11), respectively. The sequence of VS-I is highly conserved in man and the domestic mammals (Benedum *et al* 1986, Iacangelo *et al* 1986, Konecki *et al* 1987). In the rat the sequence homology to the bovine CGA is less. For instance, the rat CGA lacks a pair of basic amino acids at position 77-78 and contains an eicosaglutamine insert at position 75-92 (Iacangelo *et al* 1988). As a consequence, rat $CGA_{1\text{-}129}$, named b-granin (Hutton et al 1987), is distinct from that of VS-I and VS-II, not only in length but also in primary

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structure at the C-terminal side of the disulphide "loop". A vascular effect of b-granin has yet to be reported.

Proteolytic processing at the N-terminus of CGA results in a multitude of truncated forms (Metz-Boutigue *et al* 1993), and these shorter peptides may for practical reasons be included in the term vasostatins. Much less is known about the functional aspects of peptides derived from the N-terminus of CGB (Benedum *et al* 1987). The release of CGB_{1-40} from K^+-stimulated bovine chromaffin cells has been demonstrated (Strub *et al* 1995). Thus, the N-terminal products of CGA and CGB, the vasostatins and CGB1-40, may appear as secretory products of the diffuse neuroendocrine system and as such be considered as candidates for regulatory peptides for the vascular system (Helle and Angeletti, 1994, 1998). Apart from an autocrine function, as in the bovine parathyroid cells (Russel *et al* 1994, Angeletti *et al* 1996, 1997), the vasostatins may also have paracrine and endocrine effects, e.g. in blood vessels in close apposition to release from sympathetic nerves (Liang *et al* 1996), and in blood vessels exposed mainly to the blood borne prohormones (O'Connor and Bernstein, 1984, Cryer *et al* 1991).

1.1 Vascular Targets

The mechanisms involved in regulation of blood pressure and blood volume are complex. Although the level of activity and stress may vary, blood pressure remains remarkably constant. Research on the role of peptide hormones in blood volume and pressure homeostasis has led to recognition of a large number of vasoactive peptides, among them the vasostatins.

The blood vessel wall is not a homogenous system. The endothelial lining constitutes a metabolically active, semipermeable barrier which responds to physical and chemical stimuli, producing a range of potent, contracting and relaxing agents (Moncada and Vane 1979, Palmer *et al* 1987, Yanagisawa *et al* 1988).

Vascular smooth muscle shows marked regional selectivity for the vasodilators. Differences in endothelial reactivity as well as in smooth muscle properties make it necessary to distinguish between conduit vessels and resistance elements (Bevan and Bevan 1988, Mulvany and Aalkjær 1990, DeFily and Chilian, 1995). The macrovascular elements are in most cases passively dilated by pressure while the resistance vessels may respond actively to pressure, however, with species and organ specific variations.

1.2 Experimental Models

In the stressed organism, blood flow through vital organs must be maintained. Autoregulation of blood flow, e.g. in the heart, involves a multitude of region-specific regulatory mechanisms, some of which are understood while others remain to be resolved (DeFiley and Chilian 1995).

In vitro models are now commonly used to provide detailed information on functional aspects of specific regions with respect to inherent properties and receptor responses to neurotransmitters, hormones and metabolites. Two main approaches have been made, studying isometric tension in mechanically stretched ring preparations, or diameter changes in transmurally stretched, pressurised vessels, with or without luminal flow. The former is suitable for studies of conduit vessels, i.e. arteries and veins of 1-3 mm internal diameter, while the latter is preferred for small arteries and arterioles of 50 – 300 µm internal diameter (Halpern *et al* 1984, Mulvany and Aalkjær 1990).

1.3 A Working Hypothesis

We have postulated a role for the vasostatins as regulatory peptides in the protection against excessive responses to stress (Helle and Angeletti 1998). Included in this hypothesis is a role for the vasostatins in maintainance of blood flow by a reduction of the vascular tone. In the conduit vessels the tone may reflect mainly neurogenic and/or peptidergic vasoconstriction, while in the small arteries and arterioles myogenic and metabolic mechanisms may be dominating components for the tone (Bevan and Bevan 1988, Mulvany and Aalkjær 1990, De Filey and Chilian 1995). The suppressive effects of vasostatins on vascular tone might therefore be mediated by different mechansims in the micro- and macrovascular elements, reflecting differences in intrinsic mechanisms of relevance for the basal tone.

2. THE MACROVASCULAR MODELS

For experiments with the natural bovine vasostatins it was important to obtain vessels from a species with highest possible sequence homology compared to the bovine CGA (Benedum *et al* 1986, Konecki *et al* 1987). When human internal thoracic artery (hITA) and saphenous vein (hSV),

commonly used as grafts in coronary artery bypass operations, became available as discarded tissue from the clinic, these vessels were characterised for basic regulatory properties (Aardal *et al* 1991) before screening for vasoactivity in the N-terminal domains of the bovine CGA.

2.1 Vasostatin Responses in the Human Conduit Vessels

The source of natural vasostatins in these first experiments was the bovine preparation representing the N-terminal fragments of CGA VS-I and VS-II in a molar ratio of 2: 1. The human artery and vein, at the same preload (1 5mN) in the wire myograph, revealed different patterns of endothelium-dependent and independent constrictor responses to noradrenaline (NA)

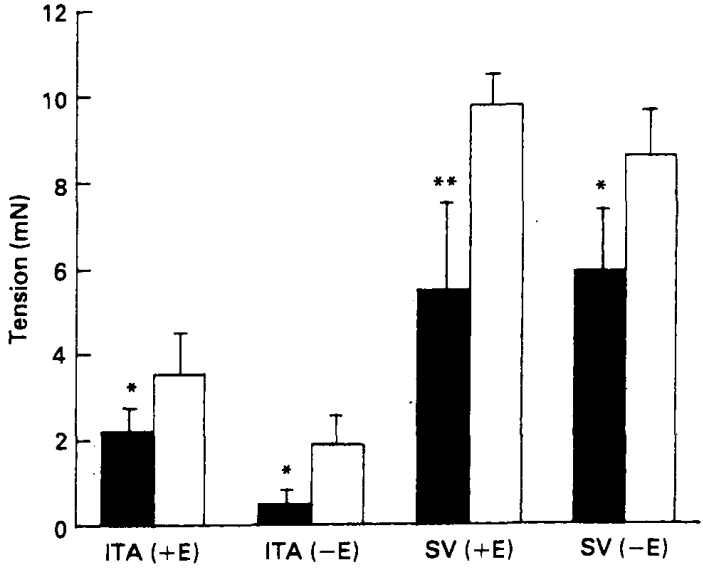

Figure 1. Role of endothelium for suppressive effects of bovine vasostatins on ET-1 evoked contractions in segments of human ITA and SV. The tension responses were those recorded 10 min after adding 35 nM ET-1 to matched pairs of segments of ITA (n=6) and SV (n=10) with (+E) or without (-E) intact endothelium. In the first cycle all segments were preincubated for 15 min with vasostatins (72nM) before adding ET-I (filled bars). The responses were recorded for another 45 min after which the medium was washed every 8 to 10 min. By this protocol the tension response after the first cycle had returned to baseline after 1 hr. Thereafter the segments were exposed to a second cycle of ET-1 (open columns), omitting preincubation in vasostatins. The results for both cycles are given as means ± SEM ***P<0.02, *P<0.05 for the difference between the first and the second cycle for each sets of segments by the Wilcoxon test for dependent variables. (Fig. 6, Aardal et al 1993)
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(Aardal *et al* 1991), ET-1 (Aardal *et al* 1993) and high K+ (Aardal and Helle 1992), consistent with previous literature on canine vessels (Miller *et al*

1989). The natural bovine vasostatins were without intrinsic effect on the mechanically stretched hITA and hSV. Vasostatin effects on established tone in the two models were therefore examined by several different approaches. Firstly, the vasostatins were found to be without effects on the sustained phase of the ET-contracted hITA and hSV. Secondly, the role of the vasostatins for the development of ET-1 constriction was examined in paired segments with and without an intact endothelium. In vessels exposed to two cycles of ET-1, the responses in the first cycle in presence of the natural vasostatins were significantly below the second cycle responses (Fig 1). These findings were the first to demonstrate an effect of the N-terminal peptides of CGA on vascular tension in conduit vessels. Moreover, the bovine peptides were active in human vessels, indicating species cross-over activity for the vasostatins.

Inhibitory effects on ET-1 constrictions were also observed with the synthetic VS-I (CGA_{1-76}) in the endothelium-denuded hSV (Angeletti *et al* 1994). By comparison, the suppressive effects of the synthetic VS-I (40 nM) was of the same order as observed with the natural vasostatins, containing 72 nM of VS-I and VS-II in a 2:1 ratio. These findings indicated that the suppressive activity was confined to the VS-I domain.

2.1.1 Role of Ca^{2+} for the Vasostatin-mediated Suppression of the ET-1 Responses in Endothelium-denuded hITA and hSV

In general the constriction by ET-I is initiated by a PLC-activated signalling leading to release of intracellular Ca^{2+} and a rise in tension while the sustained phase depends on influx of Ca^{2+} (Highsmith *et al* 1992). The slow reversal and subsequent desensitisation of the ET-1 response may reflect internalisation of the agonist-receptor complexes (Resink *et al* 1990). Arteries and veins may also differ in sensitivity to ET-1 (Miller *et al* 1989). Because of the difficulty in washing out the sustained response to ET-I in Ca^{2+}-containing solutions, the role of extracellular Ca^{2+} for the vasostatin effects was therefore investigated by a protocol by which each segment was exposed to only one cycle of ET-1 (Aardal and Helle 1992). In the matched pairs one segment was incubated with and the other without vasostatin for the last 15 min of a 30 min period in Ca^{2+} -free medium before adding ET- 1. Subsequently, the full ET-1 was unmasked by addition of extracellular Ca^{2+}. Constrictor responses to ET-1 were evident in the Ca^{2+} free medium in both vessels. In hITA it was the final, Ca^{2+}-dependent component of the ET-constriction that was changed by the vasostatins, from a sustained response without vasostatins to a reversible response in the vasostatin-treated vessels (Aardal and Helle 1992). In hSV the effects of the vasostatins were

different, revealing a significant reduction in response to ET-1 in absence of extracellular Ca^{2+}, but no additional effect on the final Ca^{2+}-dependent phase of the ET-1 constriction. These experiments led us to conclude that the vasostatins suppressed the tone developed by ET-1 in both vessels, however via different Ca^{2+}-dependent pathways. In hITA the sustained, Ca^{2+} dependent phase, was modulated, while in hSV it was the initial phase, independent of Ca^{2+}-influx, that was reduced in the presence of vasostatins.

2.1.2 Vasostatin Effects on Responses to Noradrenaline and High K+ in the Endothelium-denuded hSV

The vein was more reactive to high K^+ and noradrenaline (NA) than the artery at the same preload (Aardal *et al* 1991). The natural vasostatins and synthetic VS-I suppressed the constrictions evoked by high K^+ and NA by the same protocol as for ET-1 in vessels subjected to a 30 min period of Ca^{2+}-depletion before addition of the constrictor (Aardal and Helle 1992). Moreover, inhibitory effects by the natural and synthetic CGA_{1-40} were also evident on the responses to high K^+ and NA, but not by CGA_{1-40} on the ET-lresponse (Angeletti *et al* 1994). These venous suppressions were seemingly independent of influx of extracellular Ca^{2+} and led us to conclude that CGA1-40, containing the disulphide bridged "loop", was sufficient for full inhibitory effect on the transient contractions evoked by high K^+ and NA in the human vein. In contrast, the full-length VS-I appeared to be necessary for the sustained suppression by ET-1 in the same vessel. Finally, the inhibitory effects by the full-length VS-I on three different vasoconstrictors were taken as indications of a vasodilatory mechanism by VS-I distal to a direct binding to the respective agonist receptors.

2.2 Binding Protein for Vasostatins in Calf Aortic Smooth Muscle Cells

A calf aortic smooth muscle cell line was used to study binding of iodinated, tyrosinated cGA_{1-76} and CGA_{1-40} to intact cells (Angeletti *et al* 1994). Maximal binding was achieved by 1 hr incubation, and the specific binding of CGA_{1-40} was saturable, with a K_D of ~45 nM (Fig.2). The linear Scatchard plot indicated a single class of binding sites. Unlabelled VS-I (CGA1-76) as well as CGA_{1-40} could compete equally well for the binding site and the binding curves for iodinated VS-I and CGA_{1-40} showed similar characteristics. Moreover, ET-1 at concentrations from 10 – 50 nM could not displace the labelled CGA_{1-40} or CGA_{1-76}, making it highly unlikely that the

labelled peptides had bound to the agonist binding site on the vascular ET-1 (ET_A) receptor.

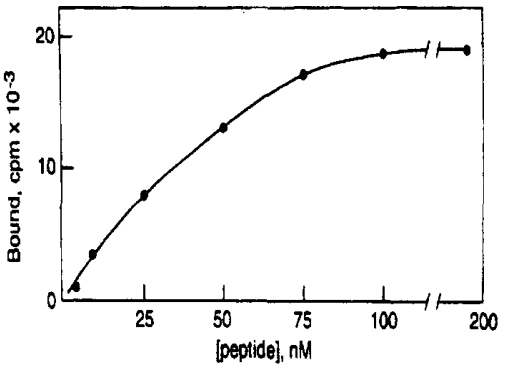

Figure 2. Specific binding of ^{125}I-CGA1-40 to a calf aortic smooth muscle cell line. (Fig 5, Angeletti *et al* 1994, by permission from Acta Physiol.Scand.)

In additional crosslinking experiments a single band of 78 kDa was detected in intact and solubilized cells. Assuming binding of one molecule of peptide per polypeptide chain, the binding polypeptide would have a molecular weight of 74 kDa. No specific membrane-associated binding could be detected, nor was any labelled polypeptide detected after crosslinking of the peptides to other non-relevant cell lines. This line of evidence was consistent with a specific vasostatin-binding membrane protein on the surface of smooth muscle cells of macrovascular origin.

2.3 Lack of Vasostatin Effects on the Membrane Potential in Bovine aorta Endothelial Cells

Cultured bovine aortic endothelial cells were used as models for examination of endothelial effects of intact CGA and vasostatins (Mandala et al, this volume). and bradykinin have previously demonstrated to hyperpolarise and depolarise, respectively, the plasma membranes of bovine aortic endothelial cells (Mandala *et al* 1999).

In contrast, the intact CGA as well as the natural VS-I[2], recombinant VS-I and VS-II[3] and the synthetic peptide cGA1-40[4] failed to modify the

[2] Gift from Dr. Karine Lugardon, INSERM Unite 338, Strasbourg, France.

membrane potential at concentration ranges from 1 nM to 10 μM (see Mandala *et al,* this volume).

3. THE MICROVASCULAR MODELS

Resistance calibre arteries from bovine microcirculations were chosen in order to avoid species problems between the agonists and putative receptors. The primary choice was coronary arteries, in view of the importance of autoregulated blood flow through the heart (DeFiley and Chilian 1995). However, no previous literature existed on the properties of bovine resistance arteries, apart from our study of the arterial input to the bovine adrenal gland (Kirkeleit *et al* 2000). For an assessment of vasostatin responses in coronary resistance arteries (bCoA), the functional properties of this model had first to be established. To this end the pressure myograph system was chosen, with automated video dimension analysis of the diameter changes in response to pressure changes (Halpern *et al* 1984). By this approach the vasostatin responses could be compared in the resistance arteries from microcirculations as functionally different as the heart and the adrenal gland.

3.1 Vasostatin Responses in Tonically Active Small Arteries

The pressurised bovine resistance arteries from the subepicardial coronary (bCoA) and the adrenal capsular-cortical regions (bAA) responded to the natural bovine VS-I with reduction in the calcium-dependent, basal tone (see Brekke et al, this volume). The dilator effect of the natural bovine VS-I was mimicked by the recombinant human VS-I and by CGA1-40 , consistent with a vasodilatory effect of VS-I located within the first 40 amino acid residues. Moreover, the effect of CGA1-40 was concentration-dependent between 0.1 nM and 1 μM with closely similar curves for bCoA and bAA, demonstrating for the first time that the vasostatins may serve as dilators of tonically active, microvascular elements.

Vasodilation in resistance arteries is commonly associated with membrane hyperpolarization involving a series of ion channels, among them the K channels (Nelson *et al* 1990, Nelson and Quayle 1995), located on both elements of the myoendothelial unit (Daut *et al* 1994). Both bovine

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[3] Gifts from Dr. Angelo Corti, DIBIT, San Raffaele Hospital Scientific Institute, Milan, Italy
[4]Gift from Dr. Mats Stridsberg, University Hospital, Uppsala, Sweden.

models expressed a Ca^{2+}-dependent basal tone that was autoregulated over the physiologically relevant range of blood pressures. Moreover, this tone was regulated by aminopyridine-sensitive mechanisms, suggestive of stretch-activated, voltage-activated K^+ channels under the in vitro pressurised conditions in both bCoA and bAA. Thus, the vasostatin-evoked dilation was an addition to that resulting from pressure-activated, aminopyridine-sensitive K channels.

The influence of other types of K channels on the basal tone and the vasostatin dilations were also examined. For instance, the vasostatin evoked dilations in both models persisted in addition to the dilation evoked by modest increases in extracellular K^+. Nevertheless, when examined in further detail in bCoA, the dilation by CGA_{1-40} was abolished by 30µM Ba^{2+}, not only at the basal tone (at 5.9mM K^+) but also at increased extracellular K^+ up to 16mM. (Brekke *et al,* this volume). Hence, Ba^{2+}, an efficient inhibitor of the K^+-evoked dilation, also appeared to be an efficient inhibitor of the CGA_{1-40} evoked dilation. implicating an ion sensitive mechanism. However, the dilator effect of CGA_{1-40} was abolished also by tetraethylammonium (TEA) and glibenclamide, i.e. established blockers of Ca^{2+}-activated and ATP-inhibited K channels, respectively (Nelson and Quayle 1995). Thus, these findings indicate that the membrane potential, seemingly a result of contributions from several of the K channel types in bCoA, is of importance for the vasostatin-evoked dilations

4. COMPARISON OF MACRO- AND MICROVASCULAR EFFECTS OF VASOSTATINS

The accumulated evidence thus indicates that resistance arteries as well as conduit vessels are likely targets for the vasostatins, causing suppression of tone development in the smooth muscle components. This conclusion lends support from the studies on endotheliumdenuded human conduit vessels, the high affinity binding sites for VS-I on the cultured calf aorta smooth muscle cells and the lack of membrane potential changes by vasostatins in the bovine aortic endothelial cells. An endothelium-independent vasodilation by the CGA_{1-40} sequence of VS-I was also evident in the pressurised bovine epicardial resistance vessels without luminal flow. An exception to this rule was the bovine adrenal capsular-cortical arteries. Here, endothelial vasodilators appeared to contribute to the basal tone (Kirkeleit *et al* 2000), and activation of endothelial ion channels by the vasostatins cannot be excluded at present. It should be kept in mind that these findings do not exclude a vasostatin-activated vasodilation via the

endothelium during luminal flow in either of the bovine resistance arteries. This aspect will be investigated in future studies.

There are major differences in autoregulatory and myogenic mechanisms and in endothelial and metabolic modulations across the various microvascular domains, between organs and within each organ, e.g. in the heart (DeFily and Chilian 1995). The vascular effects of the vasostatins, so far reported, therefore strictly apply to the regions and species from which they have been obtained. Nevertheless, these selected macro- and microvascular models differ in one important aspect, namely the nature of the vascular tone on which the vasostatins excert their dilator effects. The tone may provide the clue to the mechanisms involved. For instance, the basal tone in bCoA and bAA depended not only on influx of Ca^{2+} but also the membrane potential resulting from several activated K channel types, i e. intrisinc mechanisms. In hITA and hSV, tone depended on external activation. In these macrovascular models the vasostatins caused suppression of tone development only when present before the tonic agonist was added, in contrast to the tonically active resistance arteries in which the vasostatins have intrinsic activity. At present one can only speculate on a role for the resting membrane potential for manifestation of the vasostatin effects in the tonic resistance areteries.

5. VASOSTATINS AS REGULATORY PEPTIDES

Among the criteria set for a regulatory peptide (Iacangelo and Eiden 1995), there should be cross-species activity, as demonstrated with the natural bovine vasostatins in the human conduit vessels and with the recombinant human VS-I in bAA (Brekke *et al,* this volume). There should also be effects by synthetic homologues, as demonstrated with the synthetic VS-I in the endotheliumdenuded human vein and the bovine smooth muscle cell line. Moreover, in the two latter models, the effects of the natural and synthetic VS-I could be mimicked by a truncated sequence, CGA_{1-40}. Hence, the vasodilator activity is confined to the N-terminal40 amino acid residues which include the disulphide "loop", analogous to the inhibitory activity of CGA_{1-40} on secretion of parathyroid hormone from the bovine parathyroid cells (Angeletti *et al* 1996, Russel *et al* 1994).

For a regulatory peptide the concentration range for the effects should match the circulating levels in vivo. In man the plasma levels of the prohormone CGA run in the low nM range, with 20 fold or higher levels in cases of neoplasia (Stridsberg *et al* 1995, Aardal *et al* 1996). The concentrations of CGA in the bovine circulation range from 1-2 nM (Dillen *et al* 1989) to 7 nM (Stridsberg *et al* 2000). In the resistance vessels a significant vasodilation was apparent at the low nanomolar range of CGA_{1-40},

consistent with a functional relevance for the effect. Similarly, the high affinity binding demonstrated in the isolated calf aorta cell line indicated a significant binding in the same low nanomolar range. Thus, it seems likely that the dilatory responses, so far demonstrated for the selected microvascular and macrovascular models, may have functional relevance.

6. CONCLUSION

This overview discuss the existing evidence for a dilatory function for the vasostatins in the mammalian circulation. The intrinsinc vasodilation by the CGA1-40 sequence of VS-I now demonstrated in resistance arteries of the bovine heart and adrenal gland, strongly suggests a role for the vasostatins in regulation of blood flow in vital organs. Moreover, the close agreement between the in vitro effective concentrations and the circulating levels of the prohormone, strengthen our hypothesis of a physiological role for the vasostatins as dilators, notably under periods of metabolic and environmental stress.

The mechanisms involved in vasostatin-evoked vasodilation still remains to be clarified, and a wide range of questions must now be addressed; in particular the mechanisms behind the effects of vasostatins on macrovascular and microvascular smooth muscle cells, the properties of the vasostatin-binding membrane protein(s) and identification of the signalling pathways to which they are coupled.

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19

VASOSTATINS

Dilators of Bovine Resistance Arteries

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1. INTRODUCTION

The functional properties of blood vessels change along the vascular tree, with the small caliber arteries and arterioles representing the main sites for regulation of blood flow through an organ (DeFily and Chilian 1995). Vascular tone in resistance arteries is the sum of intrinsic and extrinsic regulatory factors. These are of both physical and chemical nature, involving luminal pressure, Ca^{2+}-homeostasis, endothelial factors, neurotransmitters and neuropeptides as well as a wide range of circulating hormones. In many cases the small arteries are tonically active and potassium channels serve as important regulators of this tone (Bevan and Bevan 1988; Mulvany and Aakjær 1990; Nelson *et al* 1990; Nelson and Quayle 1995; Quayle *et al* 1997; Standen and Quayle 1998). Opening or closing of specific K^+ channels may have a substantial effect on the membrane potential and so on the degree of vasoconstriction. In view of the previously described vasosuppressive effects of vasostatins on the tonic responses to several classes of constrictors in human conduit vessels (Aardal and Helle 1992; Aardal *et al* 1993; Angeletti *et al* 1994), it became of great interest to investigate whether vasostatins could be assigned dilator activity at the level of small resistance arteries.

The focus of the present report is on the natural bovine vasostatin I (bVS-I) and its synthetic derivative CGA1-40 and how these peptides may affect the vascular tone in resistance arteries from functionally different organs. In the heart and adrenal gland, blood vessels are assumed to be

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exposed to the prohonnone CGA and free VS-I, derived from local release and the circulating pools. Bovine capsular-cortical arteries from the adrenal gland (Kirkeleit *et al* 2000) and coronary resistance arteries were chosen as references for the vasostatin effect on small caliber arteries. For an interpretation of vasostatin modulation it was crucial to define the basic properties of our model vessels; whether they displayed a passive dilation in response to pressure, as in the porcine coronary resistance arteries (Nakayama *et al* 1988), or a myogenic, Ca^{2+}-dependent basal tone, as already established in the bovine adrenal resistance arteries (Kirkeleit *et al* 2000). Therefore, both types of vessels were compared by the same methodology and experimental designs, examining the modulatory effects of the vasostatins for concentration dependency and relationship to potassium channel activation.

2. BASIC PROPERTIES OF THE BOVINE MODELS

In the pressure myograph changes in the luminal diameter of a blood vessel can be studied with or without luminal flow (Halpern *et al* 1984). By this methodology the vascular tone may be defined as a function of transmural pressure. Also, potential tone-regulating factors may be examined, and so facilitate a better understanding of the physiological relevance of the observed phenomena.

The bovine coronary resistance arteries (bCoA) were subepicardial, 3rd - 4th order branches of the left anterior descending coronary artery (LAD), while bovine adrenal arteries (bAA) were taken from the ~ 400 parallel arteries entering the capsular-cortical region of the gland (Kirkeleit *et al* 2000). Both models had an internal diameter of ~ 250 µm and were not denuded of their endothelium prior to experimentation.

Like bAA, bCoA expressed a Ca^{2+}-dependent tone, consistent with myogenic activity (Fig. 1A). Moreover, this active tone was maintained at a constant level over a pressure range from 50 to 150 mm Hg in vitro, indicating autoregulation of the basal tone in both models. In this respect the active, autoregulated tone in bCoA was in marked contrast to the passive dilation by increased pressures in the porcine coronary arteries by a similar experimental design (Nakayama *et al* 1988).

Potential tone-regulating factors were compared in the two models at a fixed intraluminal pressure (75 mmHg), corresponding to the average in vivo pressure in the 250 µm sized resistance arteries in cattle. Two categories of inherent tone regulators were first examined. The inhibitor of nitric oxide

(NO) synthase, L-NAME, was without effect on the basal tone in both vessels. Indomethacin, an inhibitor of the cyclooxygenase enzyme, was inactive in bCoA (Fig. 1B) while enhancing the tone in bAA (Fig. IC). Thus, the transmural pressure appeared not to activate endothelial release of an indomethacin sensitive relaxing factor in bCoA, in contrast to bAA.

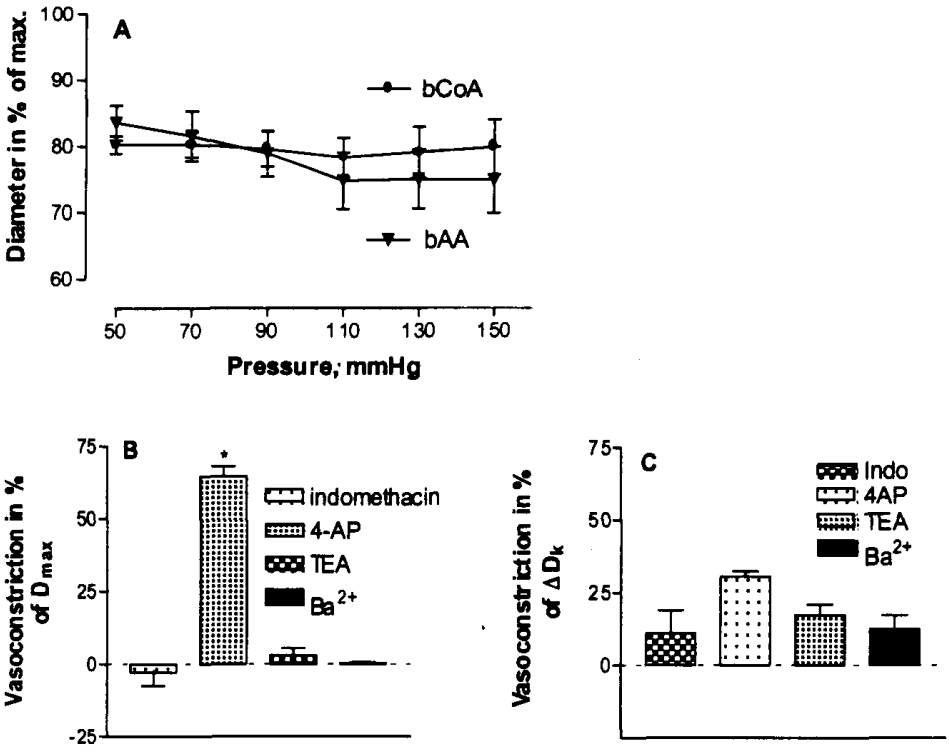

Figure 1. Myogenic tone, autoregulation and contribution of K^+ channels to the basal tone in bCoA and bAA by the pressure myograph system without intraluminal flow. In A) the pressure-diameter relationship, expressed in % of the diameter in the papavarine dilated vessels. In B) and C) the constrictor effects of indomethacin (100 µM), and antagonists of K^+ channel types (4-AP 1 mM, TEA, 0.1 mM, Ba2+ 30 µM) on the basal tone at 70-75 mmHg in B) bCoA and C) in bAA. The constrictor effects are expressed in % of the maximal constriction to 80 mM KCl. Each series consisted of n = 6 vessels. In B) * $p < 0.05$ for significance for difference from basal tone. In C) All responses were significantly different from the basal tone ($p < 0.05$).

Secondly, the contribution of K^+ channels to the basal tone was examined by means of established antagonists to the main channel categories (Standen and Quayle 1999). Aminopyridines caused marked vasoconstriction in both models (Fig. 1B and IC), implicating voltage dependent K^+ channels (K_V), presumably activated by the pressure induced stretch. Tetraetylammonium (TEA) and 30 µM Ba^{2+}, antagonists of Ca^{2+} activated K^+ - and inward

rectifier channels, respectively, were virtually without effect in bCoA (Fig. lB), while of some effect in bAA (Fig. IC).

These experiments revealed that the autoregulated basal tone in both models was the net result of oppositely directed forces, namely a myogenic contractile activity counteracted by pressure activated opening of K^+ channels, seemingly dominated by aminopyridine-sensitive types.

Modest increases in extracellular K^+ may arise in response to metabolic activity, being linked to enhanced blood flow in several vascular beds, such as the brain, heart and skeletal muscle (Haddy and Scott 1968).The same increase is also associated with vasodilatory responses in smaller coronary arteries and arterioles (Quayle *et al* 1997; Knot *et al* 1996). In the present study both models were therefore examined for responses to modest increases in extracellular K^+. Elevating extracellular K^+ from 6 to 16 mM caused vasodilation (Fig. 2A and 2B, control curves), which was completely abolished by 30 μM Ba^{2+}

These experiments demonstrate that in addition to the prominent influence of aminopyridine-sensitive ion channels on the basal tone, the bovine resistance arteries from the coronary and adrenal microcirculation are also dilated by increased extracellular K^+ presumably via the Ba^{2+} sensitive inward rectifier, KIR, channels.

3. DILATION IN RESPONSE TO VASOSTATINS

As a first approach the natural bVS-I was examined for effects on the basal tone and thereafter as a function of extracellular K' (Fig. 2A and 2B, upper curves). Consistent diameter increases were obtained at a concentration of 0.1 μM, i.e. of the same order as previously demonstrated to suppress the tonic vasoconstriction to ET-1 of the human conduit vessels (Aardal *et al* 1993). Similar vasodilations were observed with 100 nM of the recombinant human VS-I ($hCGA_{1-8}$)[1] and VS-II ($hCGA_{1-115}$)[1] when examined in bAA. Thus, a suppressive effect by vasostatins was not limited to the tonic vasoconstrictions by exogenous constrictors in human conduit vessels, but also applicable in the tonically active, bovine resistance arteries from two functionally very different organs such as the heart and adrenal gland.

[1] Gifts from Dr Angelo Corti, Osp. S. Raffaele DIR. Recerca, Milan, Italy.

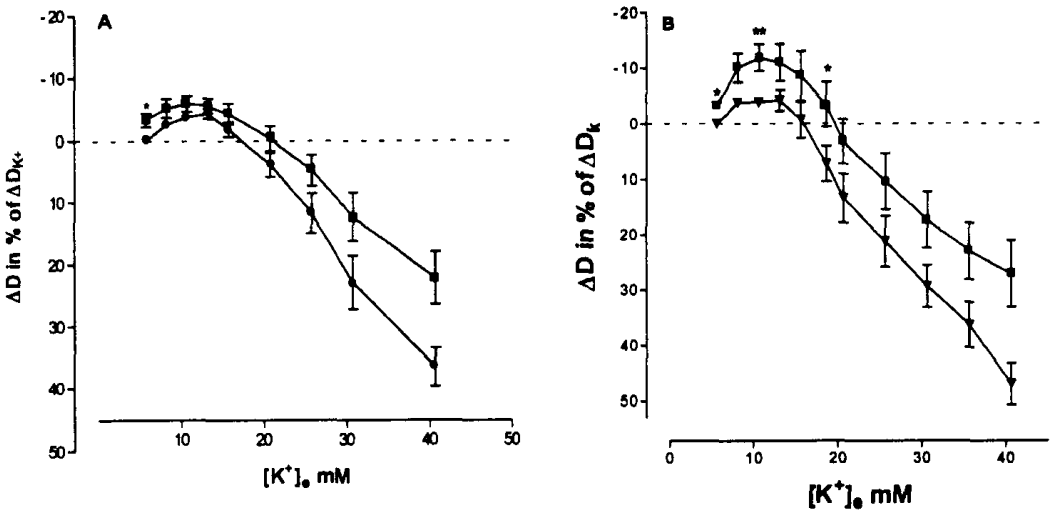

Figure 2. Vasodilation by extracellular K^+ (lower curves) and in presence of the natural bVS-I (1 00 nM, +, upper curves) in A) bCoA and B) bAA. The natural bVS-I was prepared as described by Lugardon *et al* 2000. Negative and positive values for the diameter changes (Δ D) on the Y-axis refer to dilation and constriction, respectively. Significantly different from control (baseline tone at 5.9 mM K^+) indicated by * $p < 0.05$; ** $p < 0.02$. n = 6 vessels in each group.

3.1 Vasostatin-evoked Dilation and Extracellular K^+

In bAA the intrinsic activity of natural bVS-I (0.1 μM) persisted and was further enhanced by a modest increase in extracellular K^+ ([K'],) (Fig. 2B). This vasodilation was maintained up to a $[K^+]_o$ of 19 mM (Kirkeleit *et al* 2000). In bCoA the significant effect of 1 μM CGA1-40 (Fig. 4) was also maintained in addition to the vasodilation caused by $[K^+]_o$ from 6 to 26 mM, while the additive effect of 0.1 μM at this $[K^+]_o$ range was not significant (Fig. 2A).

3.2 Concentration Dependent Vasodilation by CGA1-40

The synthetic peptide CGA_{1-40} mimicked the vasodilation expressed by the natural bVS-I in both models, and the effects were concentration dependent between 0.1 nM and 10 μM in bCoA and from 1 nM to 10 μM in bAA (Fig. 3).

The relative dilatory effects of CGA_{1-40} in the two arteries were closely similar, and the concentration range was in accordance with the effective concentrations of natural bVS-I and the recombinant human vasostatins.

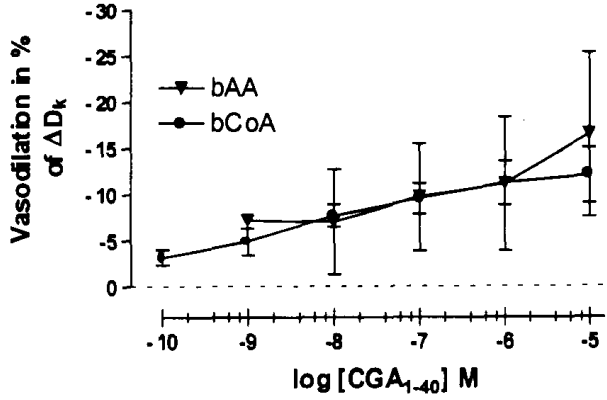

Figure 3. Concentration–responsecurves for synthetic CGA_{1-40} on basal tone in bCoA (n = 6) and bAA (n = 2). Negative values for the diameter changes (Δ D) on the Y-axis refer to vasodilation. The peptide was a gift from Dr. Mats Stridsberg, the University Hospital, Uppsala, Sweden.

Thus, in the bovine resistance arteries, analogous to that in the human saphenous vein, the dilatory effect of the vasostatins resided in the first 40 amino acids residues of CGA.

3.3 Inhibitors of CGA_{1-40} - evoked Dilation in bCoA

The significant dilatory effects of the vasostatins and the vasoconstrictor responses by K^+ channel antagonists, suggested a link between these phenomena. With the pressurized bCoA as a model, the response to 1 μM CGA_{1-40} alone was a significant vasodilation. As shown in fig. 4, this peptide effect could be completely abolished in presence of either 30 μM Ba^{2+}, TEA or glibenclamide, indicating that the membrane potential maintained by activated potassium channels might be a prerequisite for further dilation by the CGA_{1-40} domain of the vasostatins.

The peptide effect was also compared to that of CGB_{1-40}, a similar sized, "loop" containing chromogranin peptide, previously shown to inhibit bovine parathyroid secretion (Angeletti *et al* 1996). By itself CGB_{1-40} was without intrinsic effect on either bCoA or bAA in contrast to CGA_{1-40}, consistent with a functional specificity for the CGA_{1-40} structure. Interestingly, the dilatory response to CGA_{1-40} was slightly reduced by the presence of an

equimolar concentration CGB $_{1\text{-}40}$ suggestive of some competition at a binding site for $CGA_{1\text{-}40}$.

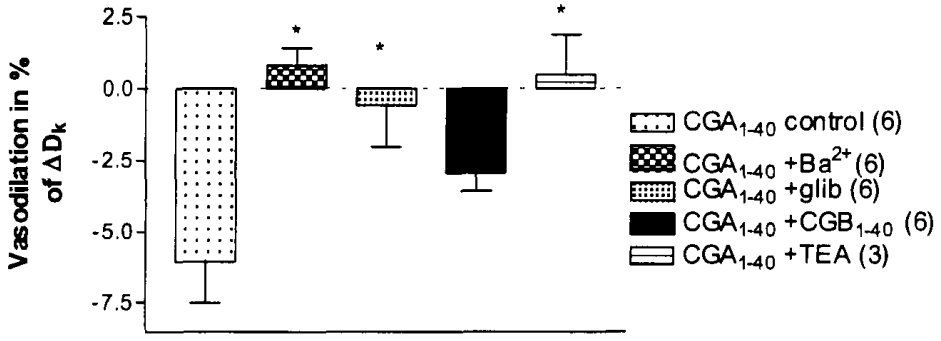

Figure 4. Effects of CGB1-40 and K^+ channel antagonists (0.1 mM TEA, 30 μM Ba^{2+} and 10 μM glibenclamide) on the vasodilation by CGA1-40 (1 μM) in bCoA. Negative and positive values for the diameter changes (Δ D) on the Y-axis refer to dilation and constriction, respectively. Number of vessels (n) in each series. *p < 0.05 for significant difference from CGAl-40 alone.

4. CONCLUSIONS

This study clearly shows that the natural bVS-I and its "loop" containing sequence $CGA_{1\text{-}40}$ possess a significant, concentration-dependent dilatory capacity in pressurized, autoregulating resistance sized vessels from the bovine heart and adrenal gland. These findings imply a role for vasostatins in blood flow regulation in both the coronary and adrenal microcirculation. The observed vasostatin evoked dilation can be blocked by a range of K^+ channels antagonists. It may therefore seem likely that the membrane potential per se is of importance for the vasostatin effect. Future studies with electrophysiological methods may clarify the nature of the membrane activated mechanisms behind the presently demonstrated regulatory function of the vasostatins.

ACKNOWLEDGMENTS

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20

PANCREASTATIN

Biological Effects and Mechanisms of Action

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1. INTRODUCTION

Evidence is accumulating for pancreastatin (PST) being a regulatory peptide. Although the physiological role of pancreastatin has yet to be fully established, a multitude of effects has been ascribed, implicating PST in modulation of endocrine and exocrine secretion, and in regulation of glucose, lipids and protein metabolism. Autocrine, paracrine and endocrine actions have been observed in a variety of systems, but the molecular mechanisms underlying most of them still await clarification.

It is well established that PST represents one of the main bioactive peptides derived from chromogranin-A (CGA), and should be taken into account in a discussion of the still elusive, functional role of CGA.

In the present chapter we will review the different actions ascribed to PST. We will describe in more detail the molecular mechanisms involved in the metabolic actions in liver and adipose tissue, with emphasis on our previous and current results regarding the pharmacology of PST receptor and signaling. Finally, we will propose a model of the putative function of PST as a stress peptide.

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1.1 Structure, Processing and Secretion

PST was first isolated from porcine pancreas (Tatemoto *et al* 1986) as a 49 amino acid peptide (5 kDa) with no apparent homology with any known family of gastrointestinal peptide hormones.

Species-specific differences in PST sequence were soon unraveled. Thus, rat and mouse share 88% of the sequence, whereas bovine and mouse share only 45%, and rat and human 55%. Human PST sequence has 71% homology with that of the porcine peptide (Hutton *et al* 1988, Konecki *et al* 1987).

Processing of CGA is species- and tissue-specific, being extensive in the pituitary and endocrine pancreas (Curry *et al* 1991, Watkinson 1991). Different molecular forms of PST may arise, depending on the extent of processing, sharing the biologically active C-terminal part, not only in the rat (Curry *et al* 1990, Håkanson *et al* 1995) but also in human tumors (Schmidt *et al* 1988, Sekiya et al 1988, Funakoshi *et al* 1989a, Tamamura *et al* 1990). In human plasma the major molecular forms are PST-52 and a larger, intermediate precursor of 15-20 kDa (Kitayama *et al* 1994).

Different phosphorylated forms of PST have been reported depending on the tissue localization (Watkinson *et al* 1993). A correlation is apparent between the phosphorylation level of CGA and its processing in different tissues. For instance, CGA and PST are highly phosphorylated in the pancreas, from which mature PST is secreted, whereas CGA is sparcely phosphorylated in the ileum, where processing into PST has not been detected.

Extracellular processing of CGA may be due either to the proteases co-secreted from the secretory cells or by exoproteases localized on the extracellular side of the cell plasma membrane (Leduc *et al* 1990). CGA is more fully processed in stomach, specially endocrine cells of the antrum (Håkanson *et al* 1995). The best characterized physiological system of PST secretion is the enterochromaffin-like (ECL) cells from the gastric antrum (Håkanson *et al* 1993). These cells respond to gastrin stimulation increasing both PST secretion and CGA mRNA levels (Håkanson *et al* 1995). In the rat in vivo, increasing gastrin levels either by infusion or indirectly, suppressing acid secretion, increases PST levels; whereas decreasing gastrin levels by fasting or antrectomy, is followed by a decrease in PST levels. Finally, patients with gastrinoma have increased plasma PST, either secreted by the tumor or released upon gastrin secretion levels (Syversen *et al* 1993).

Plasma porcine PST-like immunoreactivity levels have been shown to increase 50% (from 100 to 150 pM) in response to a meal. In the perfused porcine pancreas, PST-like immunoreactivity is released in parallel with insulin in response to insulinotropic stimuli (Östenson *et al* 1989a). Elevated

PST levels in plasma have been found in response to glucose loading in type 2 diabetes (Funakoshi *et al* 1990), in hypertension (Sanchez-Margalet *et al* 1999, and in pregnant women with gestational diabetes (Sánchez-Margalet *et al* 1998).

2 BIOLOGICAL EFFECTS

A multitude of biological effects have been ascribed to PST which may act as an autocrine, paracrine and endocrine peptide (Sánchez-Margalet et al 1996a), depending on the organ and target cells (Fig. 1). For instance, PST affects release of the co-released hormones in an autocrine manner, in the endocrine and exocrine pancreas and in the parathyroid. In the gastric mucosa, a paracrine effect may occur by the ECL cell release of PST, modulating gastric secretion. Modulation of glucose metabolism in the liver, affecting glycogen metabolism, implies, on the other hand, an endocrine effect, while PST effects on adipocytes may indicate both paracrine and endocrine modulation via neuronally released and/or circulating peptide and prohormone. Consistent with PST modulation of carbohydrate metabolism, administration of PST in the rat also decreases catecholamine levels in the rat under surgical stress (Sánchez-Margalet and Goberna 1993b).

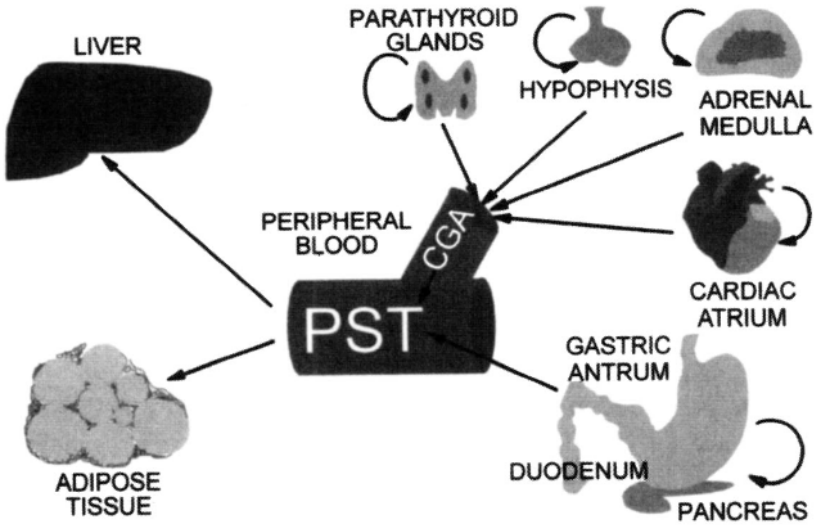

Figure 1. Target tissues of pancreastatin action.

A role for PST in modulation of brain centers for regulation of blood glucose is indicated from experiments by which intracranial administration of PST elevated blood glucose and free fatty acids in the rat, an action

opposing of that of insulin (Flood *et al* 1988). PST has also been located in senile plaques in Alzheimer disease (Sekiya *et al* 1994).

2.1 Effects on Glandular Secretions

The evidence for PST affecting both endocrine and exocrine secretion in the pancreas is accumulating and both actions appear to require the amidated C-terminal sequence.

2.1.1 The Endocrine Pancreas

The first described effect of PST was the inhibition of glucose-stimulated insulin secretion in the porcine pancreas, specially the first phase of insulin secretion (Tatemoto *et al* 1986, Efendic *et al* 1987) and since confirmed in vitro and in vivo in the rat (Funakoshi et al 1989). PST has also been shown to inhibit insulin secretion from the cell line RIN m5F, either stimulated by carbachol (Lorinet *et al* 1989), phorbol ester, or ionophore A23187 (Hertelendy *et al* 1996). These effects are pertussis toxin-sensitive, suggesting the invovlement of a G protein of the Gi family in the PST action, as discussed in detail below.

PST inhibits the stimulatory action of agents other than glucose, such as arginine, GIP, VIP, CCK and glucagon (Schmidt et al 1987, Peiró *et al* 1991, Sánchez-Margalet *et al* 1992a), and even IBMX and sulphonylurea (Silvestre *et al* 1988, Ostenson *et al* 1989b).

The effects of PST may be species dependent, as PST had no effect on glucose-induced insulin release in the dog in vivo (Ohneda *et al* 1989), only slightly increasing the insulin secretion stimulated by arginine or theophyllin, although PST inhibited the CCK-8-stimulated insulin release (Inui *et al* 1989). Also, in the isolated perfused pig pancreas, PST was devoid of effect on endocrine secretion (Holst *et al* 1990)

PST stimulates glucagon release in vivo in mice (Ahren *et al* 1988) and rats (Funakoshi *et al* 1989b) and in vitro in the pig (Efendic *et al* 1987). In dogs PST inhibits secretion of pancreatic polypeptide upon feeding (Gomez *et al* 1997). Taken together, these data suggest a role for PST as a regulatory peptide with autocrine and paracrine actions modifying the insulin/glucagon ratio with a final effect on glucose mobilization.

2.1.2 Exocrine Pancreas

PST also inhibits secretion from the exocrine pancreas in rats after physiological stimulation in vivo, central vagal nerve stimulation and stimulation with CCK-8 (Miyasaka *et al* 1989, 1990). The results from in vitro studies are contradictory, reporting on inhibitory and stimulatory effects of PST on CCK-8 evoked secretion (Ishizuka *et al* 1988b, Funakoshi *et al* 1989c), and PST also slightly stimulated amylase secretion from pieces of porcine pancreas (Adeghate *et al* 1996).

The mechanisms of PST action on exocrine pancreas seems to involve presynaptic modulation of acetylcholine release from vagal system (Herzig *et al* 1992), thus explaining the controversial effects observed in vitro. In fact, other inhibitors of exocrine secretion in vivo, such as somatostatin, do not inhibit it in vitro (Von Shonfeld *et al* 1989), implicating PST as a new mediator in the islet-acinar axis.

2.1.3 Gastric Secretory Cells

The ECL cells from the gastric antrum are an important source of PST (Håkanson *et al* 1993, 1995), implying a role in the paracrine regulation of gastric acid secretion. However, PST modulation of gastric secretion is still controversial. While PST inhibits gastric acid secretion from rabbit isolated parietal cells (Lewis *et al* 1988), PST in vivo seems to enhance gastric acid secretion in the conscious dog when stimulated by a peptone meal, phenylalanine or glucose (Hashimoto *et al* 1990).

The PST action in the gastric parietal cell seems to involve a pertussis toxin-sensitive G protein, mediating an inhibition on the intracellular cAMP levels (Lewis *et al* 1989).

2.1.4 Parathyroid Cells

An inhibitory effect of PST on parathormone secretion (PTH) was observed in porcine (Fasciotto *et al* 1989) and bovine (Drees *et al* 1992) parathyroid cells upon stimulation with either low calcium concentration or phorbol ester (non-physiologic), in accordance with increase in PTH secretion when incubating parathyroid cells with anti-PST antibodies (Fasciotto et al 1990). Moreover, PST inhibited also the transcription of the parathyroid hormone and CGA genes and decreased the stability of the respective mRNAs (Zhang *et al* 1994). However, the parathyroid gland, being abundant in CGA (Cohn *et al* 1982), does not yield much PST.

Accordingly, an autocrine role for PST on parathyroid secretion seems rather unlikely. On the other hand, PST is present in porcine calcitonin producing C cells (Bretherton-Watt *et al* 1988), and a species- specific role for PST in paracrine/endocrine regulation of the thyroid-parathyroid axis can at present not be excluded.

2.2 Effects on Hepatic Glycogen Metabolism

PST activates glycogenolysis in the rat in vivo and in vitro, implicating a hyperglycemic effect by direct stimulation on liver metabolism of glycogen (Sánchez *et al* 1990). This hypothesis was confirmed by our further studies in isolated rat hepatocytes, and the glycogenolytic effect was similar to that of glucagon in potence. The mechanisms of these effects will be discussed in detail below .

In addition, PST also inhibits insulin-stimulated glycogen synthesis, but unlike glucagon PST does not affect the rate of insulin-stimulated glycolysis (Sánchez-Margalet and Goberna 1994a). Thus, although the glycogenolytic effect of PST is comparable to that of glucagon, the latter produces a higher hyperglycemia. Presumably, PST enhances the hyperglycemic effect of glucagon (Sánchez-Margalet et al 1992b).

2.3 Effects on Adipocyte Metabolism

The adipocyte is a newly discovered cell target for PST (Sánchez-Margalet and González-Yanes 1998). In the isolated rat, PST dose dependently inhibits basal and insulin-stimulated glucose transport, lactate production and lipogenesis, impairing the main metabolic actions of insulin. These effects were observed in a wide range of insulin concentrations, leading to a shift to the right in the dose-response curve, with maximal effect and IC50 value at 10 nM and 1 nM, respectively. Moreover, PST has a dose dependent lipolytic effect in rat adipocytes (ED^{50} 0.1 nM), although it was completely inhibited by 10 nM insulin. In contrast, PST dose dependently stimulated protein synthesis and enhanced insulin-stimulated protein synthesis

2.4 Other Biological Activities

PST has inhibitory effect on cell growth in a variety of pancreatic cell lines (Smith and Kramer 1989). PST inhibits DNA synthesis in rat fetal islets (Sjoholm 1991) and inhibits basal and CCK-stimulated pancreas growth in mice (Smith *et al* 1991).

PST has been shown to have an in vitro effect by modulating the formation of insoluble fibres of amyloid polypeptide (Janciauskiene *et al* 1997). Actually, the deposit of amyloid fibres seems to be mediated by the inappropiate balance between the amyloid ,peptide, insulin, C-peptide and PST.

Another PST sensitive tissue seems to be the cardiac atrium. For instance, PST increased the secretion of sulphated proteoglycans by neonatal rat atrial cells (Gorr and Pence 1995). This effect of PST was found to be even higher than that of endothelin-1.

Recently, a role of PST as an immunomodulator has been proposed (Haberstock-Debic *et al* 1997), co-stimulating the mitogenic response of peripheral blood T lymphocytes to non-specific stimuli (lectins).

3. MECHANISMS OF ACTION

The molecular mechanisms underlying the PST effects in hepatocytes (Sánchez-Margalet et al 1996b) and adipocytes have been in focus for most of our work these last 10 years. So far, the accumulated data points to the presence of PST binding proteins i.e. receptors, in liver and adipose membranes, associated with modulation of intracellular calcium and involving some of the G proteins (Fig. 2).

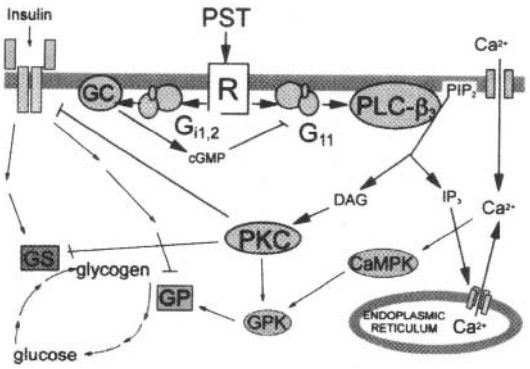

Figure 2. Model for the mechanism of pancreastatin action in the hepatocyte. Pancreastatin first binds to the receptor in the plasma membrane and activates the signaling. A Ga11 activates PLC-β3 increasing IP3 and DAG production, and this way, activating PKC and calcium dependent kinases to mediate the metabolic effect. PKC can phosphorylate the insulin receptor to inhibit its activity and further counterregulate insulin action.The activation of Gai1,2 seems to mediate the cGMP production to inhibit PLC activity to downregulate the pancreastatin signaling.

3.1 In Hepatocytes

The mechanism of PST action in rat hepatocytes has been intensively studied by our group (Sánchez-Margalet et al 1996b). The effect of PST on glycogen metabolism in rat hepatocytes is independent on cAMP, but both extracellular and intracellular calcium are needed for this PST action (Sánchez *et al* 1992). Moreover, we have shown that PST dosedependently increases intracellular calcium, involving both pertussis toxin-sensitive and -insensitive mechanisms (Sánchez-Margalet *et al* 1993). The mobilization of intracellular calcium is mediated by the production of inositol 1,4,5-triphosphate (IP3) through a pertussis toxin-insensitive mechanism, whereas the stimulation of calcium influx involves a pertussis toxin-sensitive mechanism. Thus, PST dosedependently stimulates phospholipase C (PLC) increasing the production of IP3 and diacylglycerol (DAG) in rat hepatocytes, leading to the activation of protein kinase C (PKC) (Sdnchez-Margalet *et al* 1994a).

PST also increases the production of cGMP involving a pertussis toxin-sensitive G protein (Sánchez-Margalet and Goberna 1994b). The physiological role of cGMP in liver metabolism has yet to be clarified. Some evidence point to cGMP as a mediator of PLC inhibition stimulated by PST, presumably serving as a negative feedback or down-regulation in the PST signaling.

Using different approaches (GTP-γ-S binding, GTP-azido photolabeling, GTPase activity), we have demonstrated that the pertussis toxin-insensitive stimulation of PLC by PST in rat liver membranes is mediated by the activation of a G protein of the $G\alpha_{q/11}$ family, whereas the PST pertussis toxin-insensitive G protein belongs to the $G\alpha_{i1,2}$ family (Santos-Alvarez *et al* 1998, Santos-Alvarez and Sánchez-Margalet 1999), suggesting that $G\alpha_{l1}$ rather than $G\alpha_{q}$ mediates the PST activation of PLC in rat liver membranes and that PLC-β3 is the specific PLC isoform activated by PST in rat liver membranes (Santos-Alvarez and Sánchez-Margalet 1998).

A cross-talk between the PST receptor and insulin signaling in HTC rat hepatoma cells is indicated (Sánchez-Margalet 1999) demonstrating that PST inhibits insulin receptor tyrosine phosphorylation and the tyrosine phosphorylation of its substrates. Thus, PST may impair insulin signaling (P13K and S6 kinase) in the early events of the insulin receptor signal transduction cascades. This effect of PST is mediated by the activation of classical isoforms of PKC, which phosphorylates the insulin receptor in ser/thr residues. The most recent findings indicate that PST stimulates MEK/MAPK activity through the activation of the $G\alpha_{q/11}$ protein (Sánchez-Margalet *et al* 2000).

3.2 In Adipocytes

Similar to the signaling of PST in rat hepatocytes, PST action in rat adipocytes is mediated by the activation of PLC through the stimulation of a G protein of the $G\alpha_{q/11}$ family (González-Yanes *et al* 1999). Another G protein of the $G\alpha_{i1,2}$ is also activated by PST but to a lesser extent. The specific isoform activated by PST in rat adipocyte membranes is also PLC-β3.

PST does not increase cAMP production in adipocyte membranes, but we do not know yet whether there is some indirect effect of PST on the cAMP levels (i.e. modulating phosphodiesterase activity) in the intact adipocyte to partially account for the previously observed lipolytic action (Sánchez-Margalet and González-Yanes 1998).

Recently, we have found that PST signaling in the rat adipocyte can also cross-talk with insulin receptor, inhibiting the signaling (tyrosine kinase, substrates phosphorylation, PI3K activity, GLUT4 traslocation) by the activation of classical PKC isoforms, and therefore preventing the glucose and lipid metabolism. On the other hand, PST stimulates MAPK activity, providing some molecular basis for the reported stimulatory effect on protein synthesis (González-Yanes and Sánchez-Margalet 2000).

4. THE PANCREASTATIN RECEPTOR

Despite the multitude of effects ascribed to PST, specific receptors have proved difficult to obtain from the secretory cells. In our approach we have taken rat liver membranes as a starting material, using radioiodinated rat PST to target high affinity binding proteins (Sánchez-Margalet *et al* 1994b). First, the ligand binding site was selective for the rat PST sequence, with low affinity for human PST in displacement experiments. The PST binding was highly sensitive to guanine nucleotides and could be adsorbed by lectins. Thus, the PST binding protein appeared to be a monomeric glycoprotein functionally coupled to some G protein. A single binding site, with a Bmax of 15 fmol/mg of protein and a K_d of 0.2 nM could be demonstrated, correlating well with the ED_{50} previously obtained for PST effects in rat hepatocytes (Sánchez-Margalet *et al* 1993, 1994a), and in accordance with PST levels in pig plasma (Bretherton-Watt *et al* 1988).

The PST binding protein could be solubilized from rat liver membranes in a functional state, still coupled to some G proteins (Sánchez-Margalet and Santos-Alvarez 1997). Binding data for the solubilized protein corresponded to that in membrane particles, i.e. with a Bmax of 14 fmol/mg of protein, a K_d of 0.3 nM, and a similar sensitivity to guanine nucleotides. Molecular

characterization by gel filtration and cross-linking with the radioligand revealed two components, a peak of 80 kDa corresponding to the protein band observed in the control soluble membranes, and another peak of 170 kDa consisting of the protein associated with a G protein of the $G\alpha_{q/11}$ family. Moreover, the PST binding activity could be precipitated with anti-$G\alpha_{q/11}$ antibodies, suggesting a physical association of the PST binding protein with the G protein (Santos-Alvarez *et al* 1998). Taken together, we assume that this PST binding glycoprotein serves as the PST receptor in the rat hepatocytes.

Finally, this PST receptor-$G\alpha_{q/11}$ protein complex have been purified by affinity chromatography in a two steps procedure (Santos-Alvarez and Sánchez-Margalet 2000): a first lectin-chromatography using wheat germ agglutinin immobilized to agarose, and a second affinity-ligand column with biotinylated pancreastatin immobilized to Streptavidin-Sepharose. This is an important step towards sequencing and cloning of this PST receptor.

5. PANCREASTATIN: A STRESS PEPTIDE?

In the light of the biological actions ascribed to PST, this peptide may have some physiological role locally modulating secretion in glands where this peptide is actually processed and secreted. Therefore, we postulate that PST serves as an autocrine and paracrine regulatory peptide of endocrine and exocrine secretion and in this respect plays a significant physiological role (Fig 3).

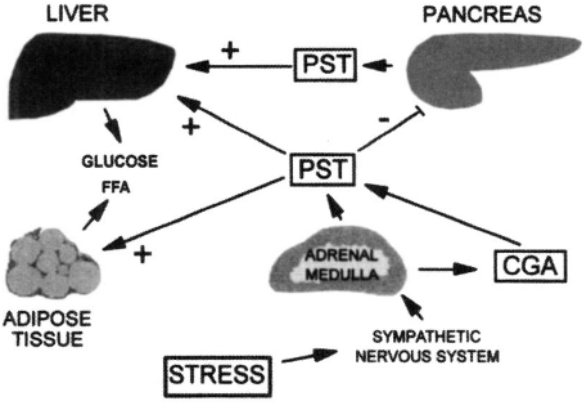

Figure 3. Physiological model of the putative role of pancreastatin in the response to stress. Pancreastatin levels are increased as a consequence of the release of chromogranin-A from sympathetic nervous system. Pancreastatin may provide energy supply by releasing glucose from the liver and free fatty acids (FFA) from adipose tissue. These effects are potentiated by decreasing the insulin/glucagon ratio secreted by pancreatic islets.

The fact that circulating PST levels may be increased in stressful conditions when CGA is co-secreted with catecholamines (Cryer *et al* 1991) lends further support to our hypothesis. We have characterized high affininity PST receptors and their signal transduction in liver and adipose tissue, which are the basis for the molecular mechanisms of the PST effects observed in glucose and lipid metabolism. In this context one would expect that endocrine actions of PST to take place in stressful conditions when circulating PST levels are high enough to interact with specific receptors in target cells. Further studies of the molecular mechanisms underlying these effects are in progress to substantiate this hypothesis.

The glycogenolytic, lipolytic and insulin-counterregulatory effects of PST could play a role in the physiology of the stress. Besides, PST can modify the insulin/glucagon ratio to further increase these effects. In this context, PST could play a role not only in the physiology of stress, but also in pathophysiological conditions such as insulin-resistant states.

Moreover, these metabolic actions of PST would be complementary to those described for other CGA derived peptides, such as vasostatin (modulating vasodilation and fighting infection) and catestatin (negative feed-back for catecholamine secretion) (see the corresponding chapters in this book for full information). Hence, this model may be helpful to understand the function of PST and in this respect also be useful to complete the picture of CGA function.

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21

THE NOVEL CATECHOLAMINE RELEASE-INHIBITORY PEPTIDE CATESTATIN (CHROMOGRANIN $A_{344-364}$)

Properties and Function

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1. INTRODUCTION

Chromogranin A (CGA), a highly acidic secretory protein, was initially discovered in catecholamine storage vesicles of the adrenal medulla (Blaschko *et al* 1967, Huttner *et al* 1991, Takiyyuddin *et al* 1990, Winkler and Fischer-Colbrie 1992). It belongs to the chromogranin/secretogranin protein family, which also includes chromogranin B and secretogranin II (Fischer-Colbrie *et al* 1995, Huttner *et al* 1991, Winkler and Fischer-Colbrie 1992). These proteins are ubiquitously distributed in endocrine, neuroendocrine, and neuronal cells. CGA is encoded by eight exons with a molecular mass of –48kDa. The primary structure of this protein reveals conserved pairs of 8-10 dibasic sites, which are potential sites of proteolytic cleavages for the generation of biologically active peptides. These peptides include pancreastatin (porcine $CGA2_{40-288}$), which impairs glucose tolerance by inhibiting glucose-stimulated insulin release from pancreatic islet beta-cells (Tatemoto *et al* 1986), and by triggering hepatic glycogenolysis (Sanchez-Margalet 1999), the vasodilator (vascular smooth muscle-relaxing) vasostatin (human CGA_{1-76}) (Aardal *et al* 1993), parastatin (porcine $CGA_{347-419}$) which inhibits PTH secretion by parathyroid chief cells (Fasciotto *et al*

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1993), and the novel fragment catestatin (bovine $CGA_{344-364}$) which inhibits catecholamine release, as a nicotinic cholinergic antagonist, in apparent negative feedback (autocrine/paracrine) fashion (Mahata *et al* 1998, Mahata *et al* 1997). Catestatin also inhibits desensitization of catecholamine release induced by nicotine (Mahata *et al* 1999). A hydrophobic moment plot shows that catestatin is the only segment of CGA predicted to contain amphiphilic β-strand (Tsigelny *et al* 1998) and molecular modeling of the catestatin region reveals a β-strand/loop/β-strand structure, especially in a hydrophobic environment (Tsigelny *et al* 1998). The present article will focus on the primary structure and function of catestatin.

2. EFFECT OF CATESTATIN ON CATECHOLAMINE SECRETION

2.1 Identification of the Catecholamine Release-Inhibitory Peptide Catestatin

In 1988, it was reported (Simon et al 1988) that catecholamine secretion from bovine chromaffin cells can be inhibited by peptides generated from CGA, but the identity of that peptide remained elusive. To identify the secretion-inhibitory domain within CGA, we synthesized 15 peptides (average length, 22 residues; range, 19-25 residues) spanning 336 amino acids: or 78% of the length of the bovine CGA 431 amino acid mature protein, and then tested their efficacies at 10 μM dose on nicotine-induced norepinephrine secretion from PC 12 cells according to the method described previously (Mahata *et al* 1996). The results identified bovine $CGA3_{44-364}$ as the active domain, which profoundly inhibited nicotine-induced catecholamine secretion. Since this domain inhibits catecholamine release we named it as “catestatin”. We then determined the potency of catestatin using logarithmically ascending doses (0.1 to 10 μM) of this peptide on nicotine-induced catecholamine secretion from PC12 cells (Fig. 1) and found IC50s from 200-400 nM (Mahata *et al* 1998, Mahata *et al* 1997). This inhibitory effect was also seen in bovine chromaffin cells and in neurite-bearing (post-nerve growth factor treatment) PC12 cells (Mahata *et al*1997).

2.2 Relative Potencies of Several Species' (Human, Bovine, or Rat) Forms of Catestatin, in Nicotine-induced Catecholamine Release

To identify catestatin effects across species we tested potencies of several species' (human, bovine, or rat) forms of catestatin on nicotine-induced catecholamine secretion from PC12 cells. The IC_{50} values for inhibition of nicotinic-stimulated secretion were: human catestatin, ~0.31 μM; bovine catestatin, ~0.3 μM; and rat catestatin, ~1.2 μM (Mahata *et al* 1997).

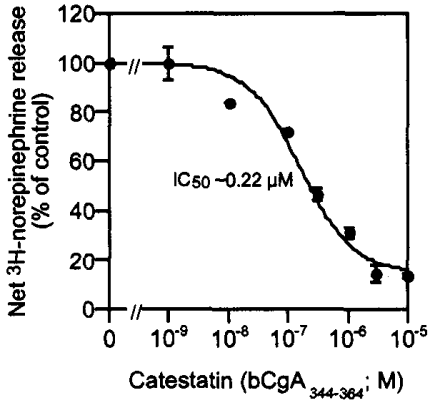

Figure 1. Potency of catecholamine secretion inhibition by catestatin. [^{3}H]L-norepinephrine-prelabeled cells were incubated with 60 μM nicotine either alone or in combination with ascending logarithmic doses of catestatin (0.01-10 μM) for 30 min. Control (100%) net norepinephrine release is that in the presence of nicotine stimulation alone, without catestatin. bCgA, bovine CGA.

2.3 Specificity of Catestatin Effect for Neuronal Nicotinic Receptors

To establish specificity we tested the effects of catestatin on catecholamine secretion evoked by secretagogues which bypass nicotinic receptors, such as the membrane depolarizing agent KCI (55 mM) which opens voltage-gated calcium channels; ATP (100 μM) which stimulates P_{2x} purinergic receptors; $BaCl_2$ (2 mM), an alkaline earth whose effects require participation of calcium channels; the peptidergic stimulus PACAP (pituitary adenylyl cylase-activating polypeptide: 250 nM) which acts through G-protein-coupled receptors; a calcium ionophore (A23 187, 1 μM); or

alkalinization of the chromaffin vesicle core (chloroquine, 1 mM). Catestatin failed to exert antisecretory activity on such non-nicotinic secretagogues, indicating its specificity for the nicotinic acetylcholine receptor (Mahata *et al* 1997).

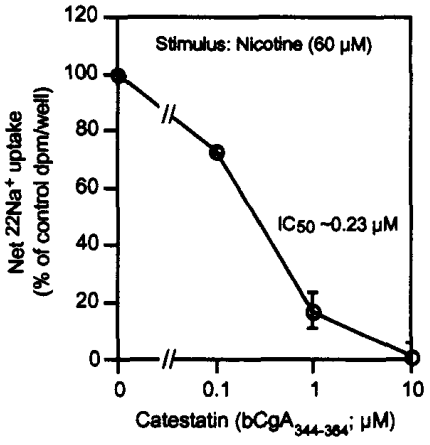

Figure 2. Catestatin effect on nicotine-induced uptake of $^{22}Na^{+}$ in PC12 cells. Cells were treated with $^{22}Na^{+}$ plus nicotine (60 µM), in the presence or absence of bovine catestatin (0.1-10 µM), for 5 min, followed by removal of the medium and cell lysis for measurement of $^{22}Na^{+}$ uptake. Control (100%) net $^{22}Na^{+}$ uptake is that in the presence of nicotine stimulation alone, without catestatin.

2.4 Catestatin's Effect on Nicotinic Cationic Signal Transduction

The physiological secretagogue acetylcholine or its analog nicotine cause influx of Na^{+} upon binding to the nicotinic acetylcholine receptor. Influx of Na^{+} depolarizes the cell membrane resulting in influx of Ca^{2+} which then induces catecholamine secretion. To identify at which step of nicotinic cationic signal transduction catestatin acts we studied influx of Na^{+} and Ca^{2+} as described previously (Mahata *et al* 1997) in the presence of catestatin, and found that catestatin blocked nicotine-induced uptake of both Na+ (Fig. 2) and Ca^{2+} influxes, meaning that catestatin acts at the very first step in nicotinic signal transduction. Interestingly, the blockade of Na^{+} uptake paralleled blockade of catecholamine secretion (Mahata *et al* 1997).

2.5 Catestatin's Site of Action

A competitive antagonist competes with agonist at the agonist-binding site to exert its inhibitory effect. Non-competitive antagonists, on the other hand, do not compete with agonist for the binding site and therefore cannot be reversed by an increase of agonist concentration. We treated PC12 cells with log10-ascending doses of nicotine (10-1000 μM) either alone or with ascending doses of catestatin (0.1-10 μM) for 30 min, after which cells were harvested for measurement of norepinephrine release. Even very high doses of nicotine (100-1000 μM) could not overcome catestatin inhibition of norepinephrine release (Fig. 3), thereby establishing catestatin as a non-competitive nicotinic antagonist (Mahata *et al* 1997).

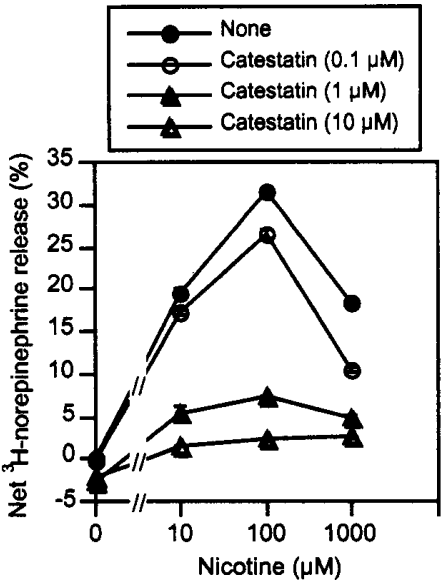

Figure 3. Noncompetitive nicotinic cholinergic inhibition by catestatin. [^{3}H]-L-norepinephrine preloaded cells were treated $\log_{10}$-ascending doses of nicotine (10- 1000 μM) either alone or in combination with $\log_{10}$-ascending doses of catestatin (0.1-10 μM) for 30 min before measurement of norepinephrine secretion.

3. CATESTATIN PROCESSING *IN VIVO*

3.1 Proteolytic Processing of the Catestatin Region from CGA

Several proteolytic cleavages have been reported in the catestatin region which in bovine CGA is bounded by furin recognition sites, RXXR, at its NH2 and COOH termini, and a dibasic site, RR, at its COOH terminus. We also found an extensive processing of this region after immunoblotting of bovine chromaffin granule soluble core proteins with an antibody directed against synthetic catestatin. An antiserum directed against synthetic catestatin was found to reverse the antisecretory activity of low molecular weight chromaffin vesicle peptides obtained by gel chromatography, indicating processing of CGA to catestatin inside chromaffin granules. This was further confirmed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) on chromaffin vesicle peptides, revealing a mass corresponding to a longer form of catestatin (bovine $CGA_{332-362}$). Other approaches utilizing HPLC separation, immunoblot detection, MALDI-MS and N-terminal sequencing revealed a peptide corresponding to bovine $CGA_{343-364}$. Further studies are in progress to address this issue.

4. EFFECT OF CATESTATIN ON DESENSITIZATION OF CATECHOLAMINE RELEASE

4.1 Catestatin's Effect on Desensitization of Catecholamine Release

Nicotinic receptors undergo desensitization upon prolonged or repeated exposure to agonist (Mahata *et al* 1999). Desensitization may be modulated by non-competitive nicotinic blockers (Ochoa *et al* 1989), calcium (Ochoa *et al* 1989), and peptides including substance P (Livett and Marley 1993) and calcitonin gene-related peptide (Ochoa *et al* 1989). Since catestatin acts as a non-competitive nicotinic cholinergic antagonist and has actions like substance P on catecholamine release, we tested the hypothesis that catestatin also modulates desensitization of catecholamine release induced

by nicotinic agonists. We found that catestatin inhibited desensitization of catecholamine release with an IC_{50} of ~0.31 μM (Fig. 4) (Mahata *et al* 1999). The details of the method have been described previously (Mahata *et al* 1999).

4.2 Relative Potencies of Several Species' (Human, Bovine, or Rat) Forms of Catestatin, in Desensitization of Catecholamine Release

We tested potencies of several species' (human, bovine, or rat) forms of catestatin on desensitization of catecholamine release induced by nicotine in PC12 cells. The IC_{50} values for inhibition of nicotinic desensitization of release were : human catestatin, ~0.22 μM ; bovine catestatin, ~0.31 μM ; rat catestatin, ~0.62 μM (Mahata *et al* 1999).

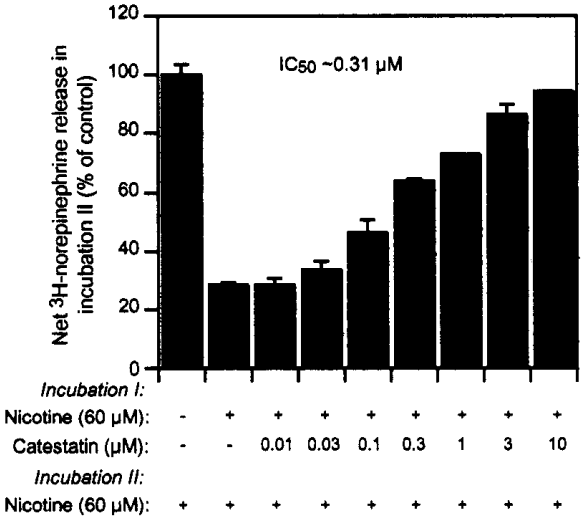

Figure 4. Inhibition of desensitization of catecholamine release by catestatin. [^{3}H]-L-norepinephrine pre-loaded cells were treated with nicotine (60 μM) either alone or in combination with loglo-ascending doses of catestatin (0.01-10 μM) for 10 min (incubation I), washed twice (6 min each), and then secretion was rechallenged with nicotine (60 μM) for 10 min (incubation 11) before measurement of norepinephrine secretion. Control cells received nicotine only in incubation II, and the control secretion is considered 100%.

4.3 Site of Action of Catestatin on Desensitization of Catecholamine Release

To test catestatin's mechanism of action on desensitization, we used logarithmically ascending doses (10-1000 μM) of nicotine to induce desensitization in the presence or absence of 10 μM dose of catestatin. We found that even very high doses of nicotine failed to overcome inhibition of desensitization caused by preexposure to 10 μM of catestatin, indicating an action of catestatin as non-competitive with respect to agonist (Fig. 5) (Mahata *et al* 1999).

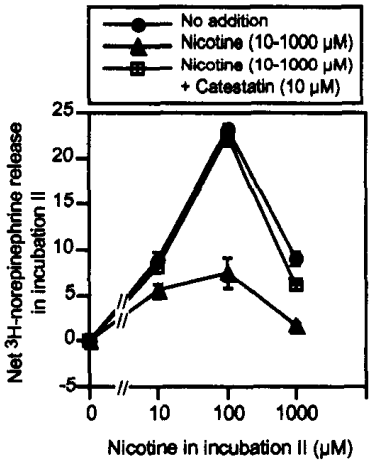

Figure 5. Noncompetitive inhibition of nicotinic desensitization by catestatin. [^{3}H]-L-norepinephrine preloaded cells were treated with log10-ascending doses of nicotine (10-1 000 μM) either alone or in combination with a desensitization-blocking dose (10 μM) of catestatin for 10 min (incubation I), washed twice (6 min each), and rechallenged with log10-ascending doses of nicotine (10-1000 μM) for 10 min (incubation II) before measurement of norepinephrine secretion. Control cells received nicotine only in incubation II (reproduced with permission from The American Society for Biochemistry & Molecular Biology).

4.4 Catestatin's Specificity on Desensitization of Catecholamine Release

Like its effect on secretion (Mahata *et al* 1997) catestatin failed to block desensitization induced by agents which bypass nicotinic cholinergic receptors, such as KCl (55 mM) and ATP (100 μM), indicating catestatin's

specificity for the nicotinic receptors in its action on desensitization (Mahata *et al* 1999).

4.5 Catestatin's Blockade of Desensitization at the Initial Step in Nicotinic Signal Transduction

Prior nicotinic desensitization caused 82% diminution of $^{22}Na+$ uptake. Catestatin dosedependently inhibited this decline in $^{22}Na^{+}$ uptake with an IC_{50} ~0.31 μM (Fig. 6). This IC_{50} is comparable to the peptide's IC_{50} ~0.31 μM on secretion (Mahata *et al* 1999).

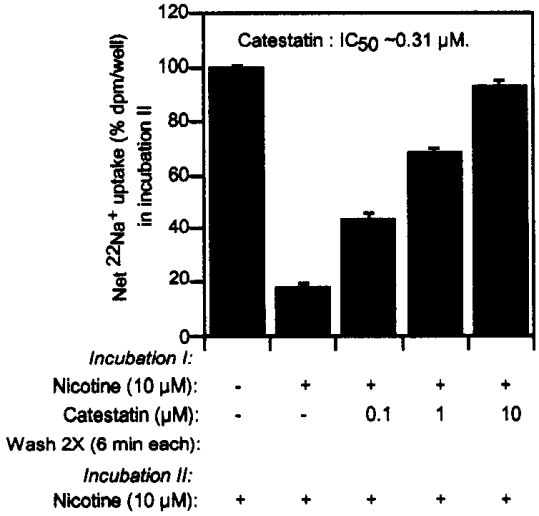

Figure 6. Catestatin blockade of desensitization of $^{22}Na^{+}$ uptake. PC12 cells were treated with nicotine (10 μM) either alone or in combination with bovine catestatin (10 μM) versus vehicle in incubation I, then washed twice (6 min each), and finally treated with nicotine (10 μM) for 10 min in incubation II in the presence of extracellular ^{22}Na (reproduced with permission from The American Society for Biochemistry & Molecular Biology).

4.6 Potency of Catestatin over Nonpeptide Nicotinic Antagonists on Desensitization of Catecholamine Release

We compared the potencies of catestatin to nonpeptide nicotinic antagonists on desensitization of catecholamine release. The IC_{50} values were : catestatin, ~0.24 μM ; trimethaphan, ~18.0 μM ; procaine, ~14.4 μM ;

phencyclidine, >10 μM ; cocaine, ~23.1 μM ; clonidine, ~4.91 μM ; or hexamethonium, >1000 μM. Thus catestatin is far more potent than non-peptide antagonists on blockade of desensitization of catecholamine release (Mahata *et al* 1999).

4.7 Lack of Cooperativity in Catestatin's Effect on Secretion or Desensitization

Our recent report (Mahata *et al*1999) showing Hill plots with slopes near unity (slope = 0.878 for secretion, slope = 0.958 for desensitization) suggests noncooperativity for catestatin's inhibitory effects on both catecholamine secretion and desensitization of catecholamine release.

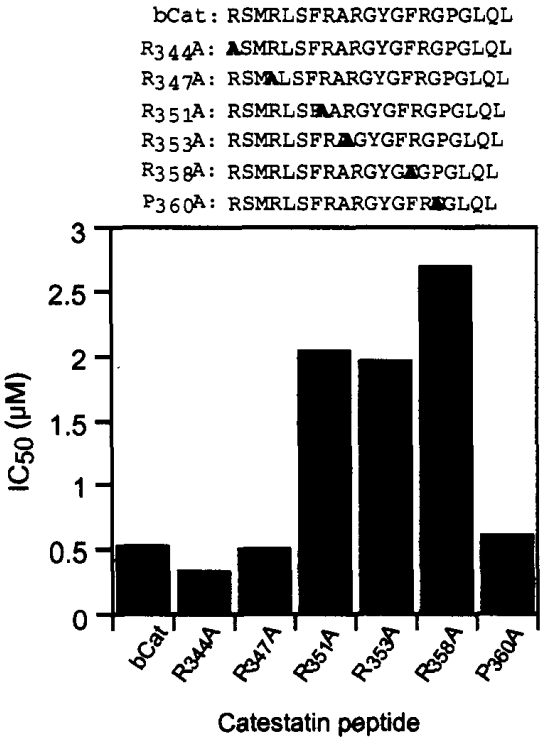

Figure 7. Effect of alanine substitution of arginine and proline residues in the catestatin sequence on catecholamine secretion. [^{3}H]-L-norepinephrine pre-loaded cells were treated with nicotine (60 μM) either alone or in combination with log10-ascending doses of catestatin (or point mutants of catestatin) for 30 min, followed by measurement of norepinephrine secretion. Mutant alanine residues are shown in bold. BCat: bovine catestatin.

5. IDENTIFICATION OF MINIMAL ACTIVE REGION IN CATESTATIN

To define the minimal active region within catestatin, we tested potencies of synthetic serial 3-residue deletion fragments to inhibit nicotinic-stimulated catecholamine secretion from PC 12 pheochromocytoma cells: truncations from the N-terminus began to lose potency after removal of even 3 amino acids, while C-terminal truncations of up to 6 residues actually enhanced potency, and bi-directional truncations progressively lost potency after removal of 3-6 residues. Thus, a completely active core sequence of catestatin was constituted by $CGA_{344\text{-}358}$ (unpublished observation). Substitution of arginines by alanine reveal that arginines at positions 351, 353 and 358 are important for the antisecretory activity of catestatin (Tsigelny *et al* 1998) (Fig. 7).

6. ROLE OF INDIVIDUAL AMINO ACIDS IN CATESTATIN'S EFFECT ON SECRETION AND DESENSITIZATION

To identify crucial residues within the active core mediating the effects of catestatin on catecholamine secretion and desensitization, we tested serial single amino acid truncations or single residue substitutions by alanine, and found important roles for Leu^{348}, Phe^{350}, Arg^{351}, and Arg^{358} on catecholamine secretion, and crucial roles for Met^{346}, Leu^{348}, Ser^{349}, and Arg^{358} on desensitization of catecholamine release (unpublished observation).

7. THE SEARCH FOR SYNTHETIC ANTAGONISTS OF CATESTATIN ACTION

For many peptide hormones and neurotransmitters, ligand/receptor interactions may be conceptually divided into those occurring at binding

domains versus activation domains, and selective interaction of a ligand with a binding domain may result in antagonist action. For example, pituitary adenylyl cyclase-activating polypeptide (PACAP) is inhibited by its fragment $PACAP_{6-27}$ (Robberecht *et al* 1992), while vasoactive intestinal polypeptide628 (VIP_{6-28}) (Fishbein *et al* 1994) blocks VIP responses. We explored this possibility for catestatin, by testing the ability of its inactive amino- and carboxy-terminal fragments to block the action of the full-length peptide, but the activity of the full-length peptide was unimpaired (Fig. 8).

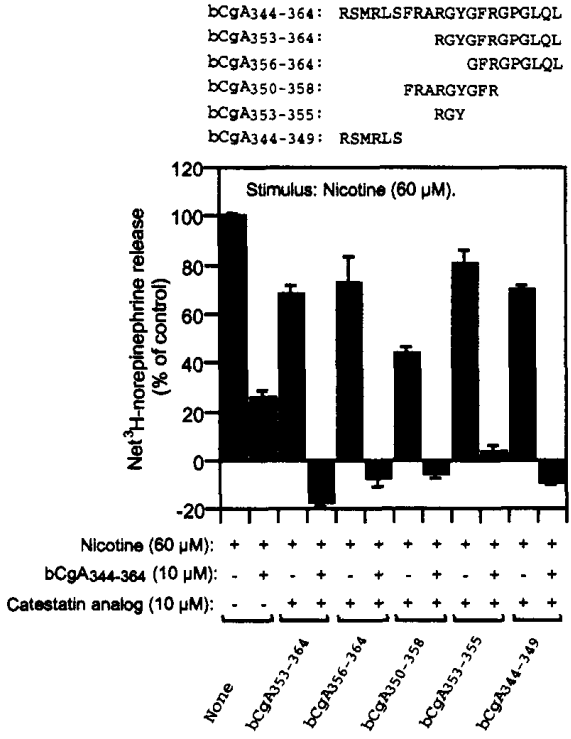

Figure 8. Role of discrete catestatin domains as potential antagonists of catestatin action. PC12 cells were loaded with [^{3}H]-L-norepinephrine and the effects of inactive catestatin analogs were examined by incubating cells with nicotine, in the presence or absence of full length catestatin (bovine $CGA_{344-364}$), either alone or in combination with inactive catestatin deletion fragments for 30 minutes, followed by measurement of norepinephrine secretion. Control (100%) net norepinephrine release was that achieved in the presence of nicotine stimulation alone, without catestatin.

8. CONCLUSION

The results presented above establish that catestatin, comprising a small 21-amino acid oligopeptide domain within CGA (bovine $CGA_{344\text{-}364}$), is a potent inhibitor of catecholamine secretion (IC_{50} ~0.2-0.4 μM) as well as nicotinic desensitization (IC_{50} ~0.3 1 μM) of catecholamine release. This peptide appears to be formed in vivo in chromaffin granules. The inhibition of secretion and desensitization is selective for the physiologic (nicotinic cholinergic agonist) stimulus to chromaffin cell secretion. At the nicotinic receptor, the inhibition of secretion and desensitization is non-competitive with agonist. Nicotinic cationic signal transduction for both secretion and desensitization is specifically impaired by catestatin. We conclude that catestatin may contribute to a novel, autocrine, homeostatic (negative-feedback) mechanism modulating catecholamine release from chromaffin cells (Fig. 9). Catestatin's inhibition of desensitization of catecholamine release might be advantageous to an organism during stress, when the peptide might act to sustain catecholamine release.

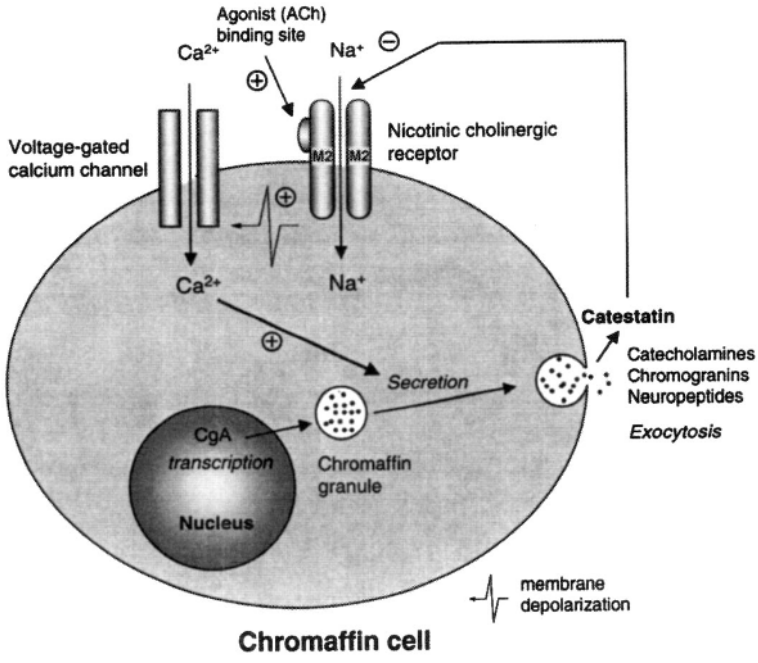

Figure 9. Model for the action of catestatin on catecholamine secretion from chromaffin cells. When acetylcholine (the physiological stimulus for catecholamine secretion) binds to the nicotinic cholinergic receptor it induces influx of sodium, which depolarizes the cell membrane, allowing cytosolic influx of calcium through voltage-gated calcium channels. After exocytotic release, catestatin inhibits nicotinic cationic signal transduction pathway, to modulate (in negative-feedback fashion) catecholamine secretion from chromaffin cells.

ACKNOWLEDGMENTS

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22

SECRETONEURIN - A NOVEL LINK BETWEEN THE NERVOUS AND THE IMMUNE SYSTEM

Conservation of the Sequence and Functional Aspects

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1. INTRODUCTION

Secretoneurin (SN) is a novel neuropeptide expressed in many neuronal and endocrine tissues (Fischer-Colbrie *et al*1995, Kirchmair *et al* 1993). It is derived from the 286-amino acid precursor, secretogranin II (preprosecretoneurin), which is encoded by a gene localised to chromosome 2q35-q36 (Mahata *et al* 1996). Secretogranin II comprises together with chromogranins A and B a class of acidic, heat-stable proteins (Iacangelo and Eiden 1995, Simon and Aunis 1989, Winkler and Fischer-Colbrie 1992), which are localised to the large-dense type of neuroendocrine secretory vesicles and typically processed within these vesicles to smaller sized fragments. Upon stimulation of cells the chromogranins are secreted en bloc with hormones, neurotransmitters and neuropeptides into the circulation or the synaptic cleft.

In the brain, SN is expressed in phylogenetically older parts, overlapping partly but not completely with established neurotransmitter and neuropeptide systems (Marksteiner *et al* 1993). In the peripheral nervous system SN is found in sympathetic and sensory fibres (Dun *et al* 1997, Kirchmair et al 1994, Li *et al* 1999, Schürmann *et al* 1995). SN has been detected in several endocrine tissues, including the adrenal medulla, endocrine pancreas, posterior pituitary and the endocrine cells of the gastrointestinal tract (Schurmann *et al* 1995). In fact, in the rat (Leitner *et al* 1996) the endocrine cells of the gastrointestinal tract represent the largest pool (55.3%) of total

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SN-immunoreactivity followed by the central nervous system (33%). These two tissues therefore are the main source of SN-immunoreactivity secreted into the serum and the cerebrospinal fluid, respectively (Ischia *et al* 2000, Miller *et al* 1996).

Tumours derived from endocrine tissues, including pheochromocytomas, neuroblastomas, ganglioneuromas, pituitary adenomas, C-cell carcinomas or pancreatic islet carcinomas synthesise significant amounts of SN. Antisera towards SN are useful histochemical markers for these tumours (Eder *et al* 1998, Prommegger *et al* 1998, Tötsch *et al* 1994). Furthermore, increased levels of SN have been reported in sera from patients suffering from oat cell carcinomas, pheochromocytomas, gastroenteropancreatic endocrine tumours and advanced prostate carcinomas (Ischia *et al* 2000).

Preprosecretoneurin (secretogranin 11) mRNA is up-regulated in cultured cells by cell depolarisation, cAMP, protein kinase C and nerve growth factor (Fischer-Colbrie *et al* 1990, Laslop and Tschernitz 1992, Scammell *et al* 1995) and in vivo in animal models for cortical spreading depression (Shen and Gundlach 1998), temporal lobe epilepsy (Mahata *et al* 1992), ischemia, and after treatment with antipsychotic drugs (Kroesen *et al*1995).

2. CONSERVATION AND STRUCTURE OF SECRETONEURIN

SN displays a remarkable degree of sequence conservation during evolution. It was detected in endocrine tissues and brain nuclei of many mammalian species by Western blotting, immunocytochemistry and RIA.

SN was originally purified by reversed phase HPLC from the frog hypothalamus (Vaudry and Conlon 1991) and is generated in vivo in the brain of birds, reptiles, amphibian and fish (Leitner *et al* 1998). The primary amino acid sequence of SN from several species obtained by cDNA cloning of its precursor or amplification of the corresponding mRNA by RT-PCR is given in Table 1.

Among mammalian species SN is highly conserved to more than 90% (Leitner *et al* 1998). Amphibian SN and goldfish SN are 76% and 61% identical, respectively (Anouar *et al* 1996, Holthuis and Martens 1996). Goldfish SN is C-terminally extended by one amino acid and thus comprises 34 amino acids (Blázquez *et al* 1998). Two stretches in the middle of SN including the sequences QYTP and LATLE/QSVFQ/EEL are a 100% conserved, whereas the C-terminal end of SN varies considerably between species. It is interesting to note that this highly conserved sequence of SN displays, as detected by NMR, a pronounced secondary structure, and is part of one contiguous a-helix (Oulyadi *et al* 1997).

Table 1. Phylogenetic conservation of secretoneurin

Species	Sequence	Sim.	Idt.
Human	TNEIVEEQYTPQSLATLESVFQELGKLTGPNNQ	100%	100%
Hamster	----------	100%	100%
Porcine	----------	100%	100%
Rat	----------S--	97%	97%
Mouse	----------S--	97%	97%
Bovine	----------N----------S-	94%	94%
Chicken	----------MA--S-H	94%	88%
Guinea-pig	----I------LN----------R--S-	88%	85%
Rana	------G----------Q----------K-QA-N	88%	82%
Xenopus	-S----G-----N----Q----------K-QG-H	85%	76%
Goldfish	---NA-------K----Q---E--SGIAAS-ANS	70%	61%

The amino acid sequence of SN from various species is given in the single letter code, only amino acids differing from human SN are shown. Sim., similarity; Idt., identity.

SN is flanked on both ends within its precursor secretogranin II (preprosecretoneurin) by two consecutive basic amino acids. These two pairs of basic amino acids, typically Lys-Arg, are highly conserved during evolution (Fig. 1). At these sites SN is generated in vivo by prohormone convertases PC1 and PC2 (Dittié and Tooze 1995, Hoflehner *et al* 1995). These two endopeptidases, which belong to the subtilisin-like proteases, are localised to the large dense core vesicles of neuroendocrine tissues and typically generate neuropeptides and hormones from their precursors (Seidah *et al* 1993).

Let us now compare the phylogenetic conservation of secretogranin II (preprosecretoneurin) with that of SN. Mammalian secretogranin II is well conserved between species throughout the entire molecule (Fig. 1). In amphibian secretogranin II, the two well-conserved regions correspond to SN plus the adjacent EM66 peptide. Goldfish secretogranin II in general, shares a poor homology with the mammalian homologues. Interestingly, the only part of secretogranin II, which is significantly conserved, corresponds to SN (Fig. 1). This exclusive conservation of the SN sequence within secretogranin II further corroborates that SN indeed represents the functional peptide within secretogranin 11.

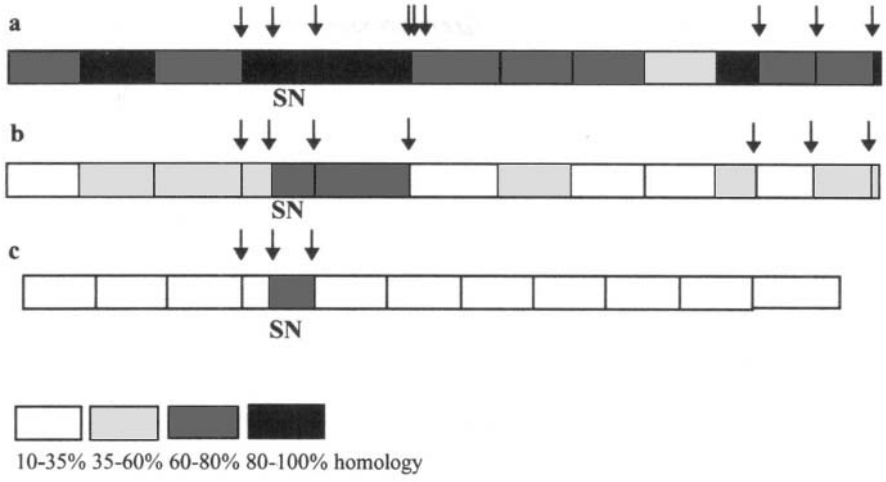

Figure 1. Conservation of secretogranin II (preprosecretoneurin) during evolution . The degree of homology of a) human vs. mammalian (bovine, rat, mouse), b) human vs. amphibian (Rana, Xenopus) and c) human vs. fish (goldfish) secretogranin II is given. Arrows indicate conserved pairs of consecutive basic amino acids. Note that the SN peptide represents the only part of secretogranin II significantly conserved across species.

3. FUNCTIONAL ASPECTS

Neurogenic inflammation is triggered by the activation of neuropeptide receptors on different cell types. Inflammatory cells are attracted when tissue sustains an immunological, chemical or mechanical injury. Leukocytes and mesenchymal cells are activated and migrate into the site of inflammation, release mediators, proliferate, and synthesise and remodel matrix. Any of these cellular responses is partly mediated by the local release of neuropeptides from sensory nerve endings.

Experiments by Storch *et al* (1996) suggested a potential role for SN in inflammatory reactions for the first time. The local presence of SN within the central nervous system of the rat was shown to influence the topographical distribution of inflammatory cell infiltrates in acute T cell mediated encephalomyelitis. Clustering of macrophages, but not of T-lymphocytes, was observed at sites of SN immunoreactivity in all stages of

experimental autoimmune encephalomyelitis in the rat, suggesting a proinflammatory role for SN at inflammatory sites *in vivo*.

Initially, SN was shown to induce dopamine release from rat striatal slices in a dose and calcium dependent manner (Saria *et al* 1993). Subsequently, a potent chemotactic activity of SN towards monocytes was characterised (Reinisch *et al* 1993). Table 2 presents an overview of established biological effects of SN.

Table 2. Biological effects of secretoneurin

	Reference
Dopamine release from rat striatal slices *in vitro*	(Saria *et al* 1993)
Neurotransmitter release (dopamine, dynorphin) from basal ganglia by in vivo microdialysis	(Agneter et al1995, You *et al*1996)
Monocyte chemotaxis	(Kong *et al* 1998, Reinisch *et al* 1993, Schratzberger *et al* 1997)
Eosinophil chemotaxis	(Dunzendorfer *et al* 1998)
Fibroblast chemotaxis	(Kähler *et al* 1996)
Deactivation of chemotaxis of neutrophils	(Schratzberger *et al*, 1996a)
Stimulation of the proliferation and migration of vascular smooth muscle cells	(Kähler *et al* 1997b)
Stimulation of the migration and inhibition of proliferation of endothelial cells	(Kähler *et al* 1997a)
Stimulation of the transendothelial migration of monocytes	(Kähler *et al*1999)
Activation of endothelial cells for neutrophil adherence	(Gruber *et al* 1997)
Gonadotropin II release from goldfish pituitary	(Blázquez *et al*1998)
Survival of cerebellar granule cells	(Fujita *et al* 1999)
Inhibition of serotonin and melatonin release from pinealocytes	(Simonneaux *et al* 1997)

3.1 Leukocytes

3.1.1 Monocytes

SN stimulates the directed, selective migration of human monocytes in vitro and in vivo at nanomolar concentrations (Reinisch *et al* 1993). Likewise, a significant migratory response of the monocytic cell line U937 towards a soluble gradient of SN and its C-terminal fragment was seen in both, the classical Boyden chamber chemotaxis assay and a transendothelial migration model (Kähler *et al* 1999). For human monocytes and U937 cells the maximal migratory response was comparable to the effect of the well-characterised monocyte chemoattractant N-formyl-Met-Leu-Phe (fMLP). A polyclonal SN antiserum specifically blocked the effect in both cell types.

Transmigration of U937 cells across endothelial monolayers in response to SN was shown to require the CD11/CD18 integrin complex, like other chemoattractants (Kähler *et al* 1999).

Specific binding sites for SN were identified on the human monocytes and the two monocytic cell lines MonoMac-6 and U937 (Kong *et al* 1998, Schneitler *et al* 1998). Scatchard analysis revealed a single class of binding sites with a K_d value of 7.3 nM and a B_{max} of 322 fmol/mg protein for MonoMac-6 cells (Schneitler *et al* 1998). This K_d in the low nanomolar range is well compatible with that of other monocyte chemoattractants, such as monocyte chemotactic protein-1 (MCP-1) and N-formyl-Met-Leu-Phe (fMLP). Competition studies demonstrated that a peptide comprising the 15 C-terminal amino acids of SN was able to displace iodinated SN, while shorter C-terminal fragments were ineffective. Other neuropeptides such as calcitonin-gene related peptide, substance P or galanin, as well as the chemokine receptor ligand RANTES or the monocyte chemoattractants MCP-1 and fMLP did not displace SN. Therefore SN mediates its effects via a specific receptor unique for this neuropeptide.

The signalling pathway downstream of the SN receptor has been characterised in human monocytes to some extent. Pre-incubation with pertussis toxin and cholera toxin selectively inhibited monocyte chemotaxis towards SN (Kong *et al* 1998, Schratzberger *et al* 1996b). Staurosporine, an inhibitor of protein kinase C, significantly suppressed directed monocyte locomotion elicited by SN, suggesting that activation of protein kinase C is involved in the signalling process (Schratzberger *et al* 1996b). Tyrphostin-23, an inhibitor of tyrosine kinase, did not affect monocyte SN chemotaxis (Schratzberger *et al* 1996b). SN slightly elevated intracellular Ca^{2+} levels in one study (Schratzberger *et al* 1996b), however, another report failed to demonstrate a rise (Kong *et al* 1998). Incubation of monocytes with the cAMP analogue 8-Br-CAMP inhibited SN induced migration (Kong *et al* 1998). Thus, a classical g-protein receptor coupling to protein kinase A and C transduction pathways, e.g. via G_i, mediates SN receptor signalling. The detailed characterisation of the signalling mechanisms has to await the cloning of the SN receptor.

3.1.2 Eosinophils

In addition to monocytes, for which a chemotactic activity of SN has now been well established, human eosinophils are also attracted. SN is a potent eosinophil chemoattractant in vitro (Dunzendorfer *et al* 1998), equally potent as interleukin-8. The chemotactic activity of SN was established by checkerboard analysis, inhibition of the effect by specific polyclonal antibodies and receptor desensitisation experiments.

In human eosinophils staurosporine or tyrphostin-23 did not alter SN induced migration, whereas pre-incubation with wortmannin or 3-isobutyl-1-methylxanthine completely blocked SN-induced eosinophil locomotion (Dunzendorfer *et al* 1998). Therefore, SN mediated eosinophil chemotaxis involves the activation of wortmannin-sensitive pathways, like phospholipase D or phosphatidyinositol-3-kinase and is inhibited by increases in cAMP.

3.1.3 Neutrophils

Human neutrophils are a common target of neuropeptides in vitro and in vivo. However, SN did not induce directed migration of human neutrophils in vitro at biological relevant concentrations (Schratzberger *et al* 1996a). Furthermore, no effect on neutrophil priming, respiratory burst activity and adherence to endothelial cell monolayers were found. However, pre-treatment of human neutrophils with SN resulted in an increase in spontaneous migration, and a significant antagonism of fMLP-induced neutrophil locomotion (Schratzberger *et al* 1996a).

3.1.4 Lymphocytes

Lymphocytes play a key role in human inflammatory responses. SN was ineffective in eliciting significant lymphocyte migration in vitro for both the T- and the B-subset of these cells (Schratzberger *et al* 1997). Also, SN did not affect lymphocyte proliferation (Schratzberger *et al* 1997).

3.2 Endothelial Cells

In *in vitro* experiments SN was shown to inhibit proliferation of human umbilical vein endothelial cells, bovine aortic endothelial cells and rat pulmonary endothelial cells, which were stimulated with an endothelial growth factor like fibroblast-growth factor (Kähler *et al* 1997a). In contrast to its inhibitory potency on cell proliferation, SN stimulated endothelial migration *in vitro* (Kähler *et al* 1997a). It also enhanced adhesion of human neutrophils to SN-activated endothelial cells in an in vitro testing system (Gruber *et al* 1997). In these experiments SN stimulated neutrophil adherence in a protein kinase C dependent manner.

3.3 Mesenchymal Cells

3.3.1 Fibroblasts

In human skin fibroblasts induction of locomotion to a soluble gradient of SN was found (Kähler *et al* 1996). The maximal dose response was comparable to that of human leukocytes. In contrast to other neuropeptides, like substance P, SN failed to induce fibroblast proliferation *in vitro* (Kähler *et al* 1996).

3.3.2 Smooth Muscle Cells

SN induced a directed, selective migration of cultured rat aortic smooth muscle cells *in vitro*. It also stimulated cell proliferation and DNA synthesis *in vitro.* The specificity of the SN induced responses was confirmed by incubation with SN antibodies and trypsinisation of SN before treatment (Kähler *et al* 1997b).

4. CONCLUSION

SN represents a novel neuropeptide, which is found in high concentrations in several regions of the brain, endocrine cells of the gastrointestinal tract, as well as in peripheral sympathetic and sensory nerves. Several biological effects including monocyte chemotaxis and release of dopamine fiom the rat striatum have been established.

SN is co-localised with substance P and calcitonin-gene related peptide in sensory nerve endings (Kirchmair *et al* 1994). In response to chemical, mechanical or immunological injury these peptides are released fiom sensory nerve endings resulting in an inflammatory response called neurogenic inflammation. Leukocytes, endothelial and mesenchymal cells are the major cellular compartments involved in inflammatory responses. SN was shown to affect these cells *in vitro* and *in vivo* and thus provides a novel link between the nervous and immune system. Consequently, an implication of SN in inflammatory diseases like asthma, bowel disease in the gastrointestinal tract or rheumatoid arthritis can be postulated.

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23

CHROMOGRANIN A INDUCES A NEUROTOXIC PHENOTYPE IN BRAIN MICROGLIAL CELLS

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1. INTRODUCTION

The central nervous system (CNS) is made up of several cell types : neurons, microglial cells and macroglial cells, including astrocytes and oligodendroctytes. There is an increasing evidence of closely coupled multidirectional interactions between neuronal and glial signaling systems that are implicated in brain development and pathology (Fig 1). Microglial cells are the resident macrophages of the nervous system where they play an important role in supporting neuronal survival and differentiation by releasing neurotrophins and stimulating their production in astrocytes (Banati and Graeber 1994, Giulian *et al* 1995, Kaul *et al* 1999). The major role of microglia is their participation in immunological processes. Microglia express class I and II major histocompatibility complex molecules, secrete and respond to cytokines and function as scavanger cells due to their phagocytic properties. Although the activation of microglia represents a beneficial physiological response in host defense, sustained activation of microglial cells may however be harmful and lead to the extensive damage of neighbouring cells particularly of neurons known to be extremely sensitive to extracellular factors (Giulian *et al* 1993, Chao *et al* 1995). Thus, accumulating evidence has implicated the inflammatory process initiated by microglia in the pathogenesis of chronic neurodegenerative disorders, i.e.

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Alzheimer's disease, Parkinson's disease and multiple sclerosis (McGeer *et al* 1993, Giulian *et al* 1995, Dickson 1997).

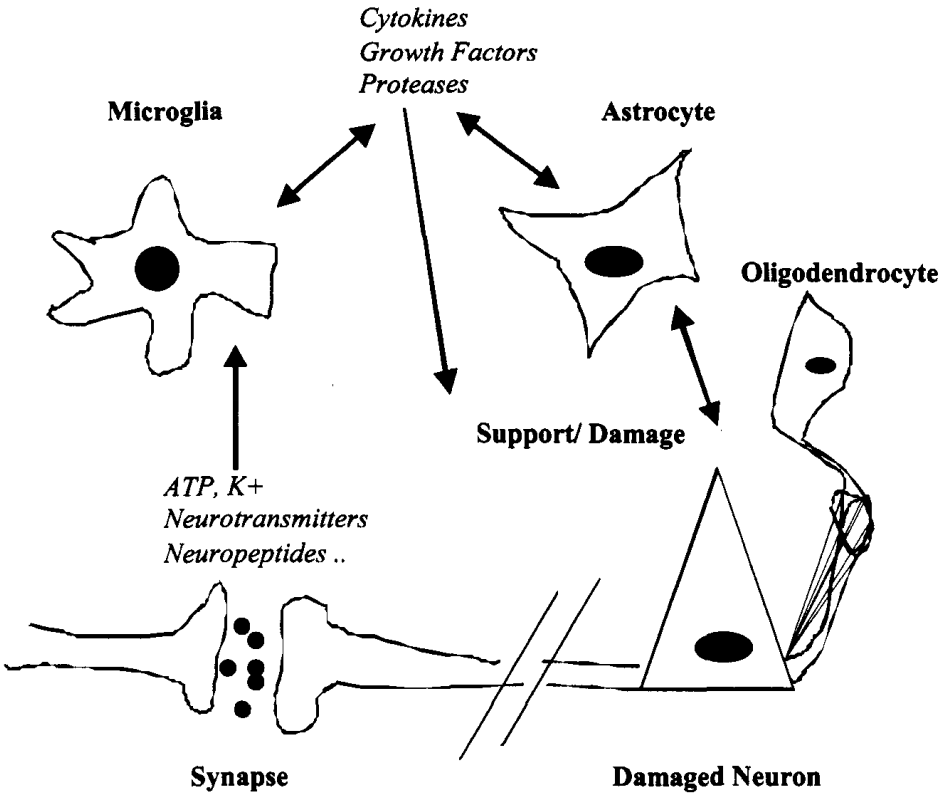

Figure 1. Scheme for multiple mechanisms in the activation of microglial cells.

The brain-derived factors which control microglial activation are still under investigation. Besides D-amyloid protein, various proteins and inflammatory cytokines have been described in the extracellular deposits found in neurodegenerative disease (Dickson 1997, Ferrer *et al* 1998) and the possible combination of these molecules could cause gradual chronic and sustained immune responses in microglia.

Chromogranin A (CGA), a polypeptidic chain of 43 1-445 amino acid residues corresponding to a 48-52 kDa glycoprotein (Simon and Aunis 1989, Winkler and Fischer-Colbrie 1992, Strub *et al* 1996) has been found in senile

and pre-amyloid plaques (Muñoz *et al* 1990, Muñoz 1991, Dickson 1997). CGA has also been detected in large dystrophic neurites containing the amyloid precursor protein (Yasuhara *et al* 1994, Ferrer *et al* 1998). As a possible indication of its potential role in generation of inflammatory response in microglia, the N-terminal domain of CGA has been recently shown to modulate adhesion and spreading of cultured fibroblasts (Gasparri *et al* 1997). Together these findings prompted us to examine whether CGA may modulate the functional activity of brain microglial cells in culture. We have shown previously that microglial cells isolated from rodent brain express in vitro Fc and complement type 3 (CR3) receptors, and are activated by exposure to lipopolysaccharide (LPS) (Ciesielski-Treska *et al* 1991, Bader *et al* 1994).

2. EFFECT OF CGA ON MICROGLIAL CELLS

As a first step in order to examine the possibility that CGA could represent an endogenous factor capable of triggering the immune response in microglia, we have compared the effects of CGA and LPS, a well-known activator of microglia, on the morphology of cultured microglial cells and on the activity of nitric oxide synthase (Taupenot *et al* 1996). Both CGA purified from bovine chromaffin granules (bCGA) as well as recombinant human CGA (rec.hCGA) induced in microglia a flattened amoeboid shape associated with the translocation of cytoplasmic actin to the subplasmalemmal space. The CGA-evoked changes in the organization of actin were different from that induced with LPS suggesting that CGA may activate a specific, different signaling pathway. Furthermore, CGA like LPS elicited a rapid and large calcium transients within min after application preceeding the reorganization of actin and changes in cell morphology that were observed 10-15 min later.

Both CGA anf LPS triggered the accumulation of nitrite (NO) in the culture medium by a mechanism that was shown to be dependent on protein synthesis. The effect of CGA was dose-dependent and the production of NO was inhibited with competitive inhibitors of inducible nitric oxide synthase (iNOS). The CGA-induced stimulation of iNOS required tyrosine kinase activity since genistein completely blocked the accumulation of NO in the medium. All these modifications occurred when CGA was added to culture medium in the nM range.

Destruction of neurons by apoptosis and necrosis is the underlying mechanism in a variety of irreversible neurodegenerative diseases and thus represents an area of intense interest (Pettmann and Henderson 1999). Therefore we have investigated whether CGA might provoke directly or

indirectly neuronal cell injury (Ciesielski-Treska *et al* 1998). For that purpose, the effects of CGA on neuronal survival and proliferation were studied by analysing the organisation of neurofilaments and the uptake of GABA in neurons purified from rat brain cerebral hemispheres and maintained either in culture alone or in co-culture with rat brain microglial cells (in a 1/1 ratio). We have observed that CGA was unable to exert a direct toxic effect on neurons but provoked a dramatic neuronal injury with massive degeneration when neurons were cocultured in the presence of microglial cells. The development of neuronal cell injury required several days of incubation with CGA and this rather slow timecourse of the CGA-evoked neurotoxic response is consistent with the fact that CGA does not interfere directly with neurons but is likely to induce an immune-mediated pathway through the activated phenotype of microglia.

The incubation of neurons with culture medium conditioned by CGA-activated microglia provoked a similar neuronal injury indicating that toxic factors are secreted by CGA-activated microglia and that these factors are active despite the spatial segregation of microglia and neurons. Although the exposure of microglial cells to CGA induced a marked accumulation of pro-inflammatory cytokine TNF-α in the conditioned medium, a direct correlation between the neuronal damage and the levels TNF-α when applied to neurons in culture could not been established. Furthermore, the inhibition of iNOS with specific inhibitors could not protect neurons indicating that the predominant neurotoxicity is not dependent on NO nor on TNF-α production by and release from microglia. In contrast, in preliminary experiments we have found that signaling cascade initiated in microglia with CGA triggered secretion of low molecular weight, diffusible neurotoxic factors that are partially heat-stable. The identity of these factors remain to be established.

To identify the possible domains within CGA protein that could be responsible for the secretion of neurotoxins by microglia we have focused our attention to vasostatin I (Aardal *et al* 1993), the most conserved domain across species from frog to human. A recombinant fragment of the human protein corresponding to residues 1-78 (Gasparri *et al* 1997) was tested. For comparison, we also examined the effects of synthetic peptides corresponding to residues 173-194 and 614-626 in CGA and CGB sequence respectively. In neuronal/microglial cell co-cultures, only the N-terminal 1-78 fragment.of CGA abolished GABA uptake in a dosedependent manner, thus revealing neuronal cell injury ; these data suggest that the microglia-activating domain is localized in the N-terminal region of the protein.

3. CONCLUSION

Neurodegenerative diseases are characterized by a progressive loss of neurons but the underlying mechanisms are not well understood. Several lines of evidence indicate that neurons and microglial cells cooperate in the initiation and development of toxic signaling pathways. Both cell-surface interactions and released molecules seem to be involved in the process of neuronal degeneration. Our results indicate that CGA, which is up-regulated in many neuropathologies, represents an endogeneous inflammatory molecule responsible for the induction of signalling cascade leading to secretion of neurotoxic factors from microglia (Fig 2).

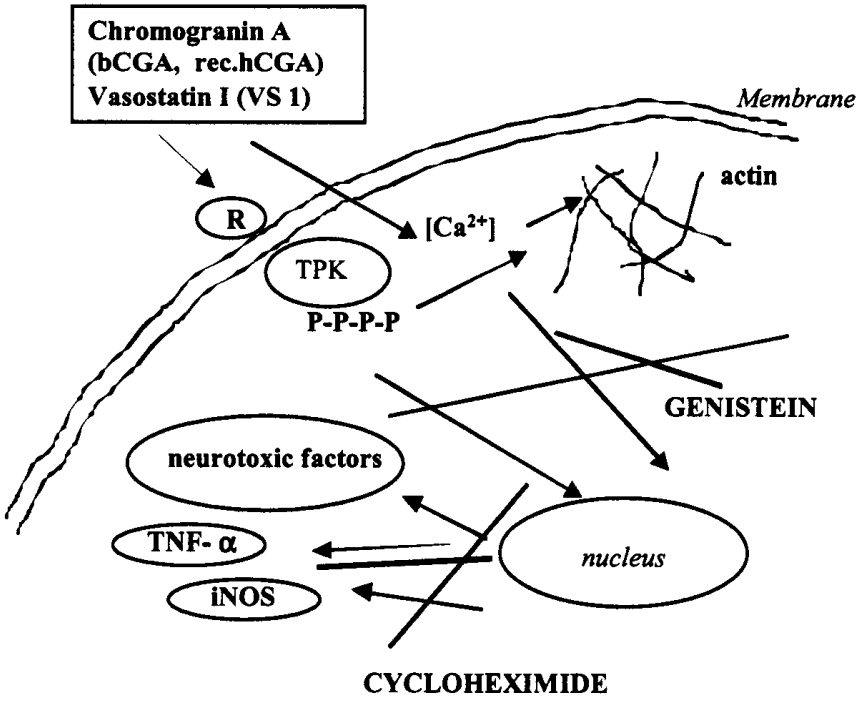

Figure 2. Schematic illustration of the effect of CGA and human recombinant vasostatin-1 peptide on microglial cells in culture (TPK, tyrosine protein kinase).

In line with our original findings, Kingham *et al* (1999) recently reported that microglia exposed to CGA release glutamate and induce apoptosis in

cerebellar granular neurons in culture. The demonstration that N-terminal fragment of CGA stimulates the secretion of neurotoxins from microglia to a similar extent as total CGA suggests that the active domain is present in this region of the CGA molecule.

The importance of the N-terminal region in microglial cell activation is suggested by the fact that it is the only region which exhibits a high degree of conservation between species, reflected in the 97-99 % homology between porcine, bovine, human and rat (Simon and Aunis 1989). It is flanked by a major proteolytic cleavage site and is processed from CGA within secretory granules (Metz-Boutigue *et al* 1993, Strub *et al* 1996). Several biological activities have been attributed to the N-terminus of CGA, including the autocrine modulation of parathyroid cell secretion (Drees *et al* 1991), a vasoinhibitory activity (Aardal *et al* 1993) and a potent antibacterial activity (Lugardon *et al* 2000, Metz-Boutigue *et al* this volume). Perhaps more relevant to the present study is the capacity of the N-terminal domain of CGA to modulate adhesion and spreading of fibroblasts (Gasparri et al 1997). This observation correlates well with preliminary studies in our laboratory that indicated that CGA at nanomolar concentration can stimulate the migration of rodent microglia and human monocytes (unpublished data). In the central nervous system, CGA is present in neurons and astrocytes either as an intact molecule or as smaller fragments (Muñoz 199 1, Mahata *et al* 1991, EI Majdoubi *et al* 1996). However, the presence of CGA and the degree of processing vary considerably between different brain regions and the functional significance of CGA-derived peptides in the brain remains to be investigated. The occurrence of N-terminal fragments of CGA in neurodegenerative brain lesions will be an interesting challenge for future investigations.

Together these results support the idea that CGA accumulating in senile and pre-amyloid plaques and in dystrophic neurites represents one of the putative neurotoxic signaling molecules in neurodegenerative diseases.

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24

ANTIBACTERIAL AND ANTIFUNGAL PEPTIDES DERIVED FROM CHROMOGRANINS AND PRO-ENKEPHALIN-A

From structural to biological aspects

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1. INTRODUCTION

Secretory granules from adrenal medullary chromaffin cells contain a complex mixture of molecules including catecholamines, low-molecular mass constituents and numerous water-soluble peptides and proteins ; all these components are released into the circulation in response to splanchnic nerve stimulation. Large amounts of chromogranins (CG) and proenkephalin-A (PEA) are present in the adrenal medullary chromaffin granules (Simon and Aunis 1989, Dillen *et al* 1993), and we have used these organelles as a model to study their processing (Metz-Boutigue *et al* 1993, Strub *et al* 1995). During the course of our studies we found that antimicrobial peptides are present in the intragranular matrix and are released in the extracellular medium on chromaffin cell stimulation (Strub *et al* 1995, Strub *et al* 1996a, b, Goumon et al 1996, 1998, Metz-Boutigue *et al* 1998). These peptides, derived from CGs and PEA, are active against Gram-positive and Gram-negative bacteria and are also able to kill fungi. More recently, we have identified these peptides in biological fluids implicated in defence mechanisms and in secretions of polymorphonuclear neutrophils (Goumon *et al* 1998, Lugardon *et al* 2000).

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2. NATURAL ANTIMICROBIAL PEPTIDES

From the perspective of a microbe, the tissues of living multicellular organisms are rich sources of nutrients. Thus, to avoid being parasitized and digested, all living multicellular organisms have developed a rapid and effective response to challenge pathogens. Among the different mechanisms which have evolved to act efficiently, the production of a large variety of natural antimicrobial peptides is attracting increasing attention. The importance of these molecules is clearly established in the immune defence of invertebrates (Boman 1995, Hoffmann *et al* 1996), while in vertebrates they act as a first line of defence against pathogens and in the control of natural flora (Boman 1995, Zasloff 1992). These antimicrobial peptides are located at sites exposed to microbial invasion such as the epithelia of amphibian (Zasloff 1992), mammals (Bevins 1994) and insects (Hoffmann 1995, Hoffmann *et al* 1996). They are present in the hemolymph of insects and stored in the secretory granules of immune cells in mammals and birds (Gennaro *et al* 1991, Lehrer *et al* 1993, Evans *et al* 1994, Harwig *et al* 1994). In addition, human intestinal Paneth cells, which are secretory epithelial cells located at the bottom of the crypts in the small intestine, express defensins (Ouellette and Selsted 1996), also produced in various epithelia such as psoriatic skin (Harder *et al* 1997) and trachea (Diamond *et al* 1996, Russell *et al* 1996). Surveys of antimicrobial peptides have revealed links between the antimicrobial peptides in vertebrates and those in invertebrates (Charlet *et al* 1996, Lee *et al* 1997, Zhao *et al* 1997). Recently, new data have highlighted similarities between pathogen recognition, signalling pathways and effector mechanisms of "innate immunity" in Drosophila and mammals (Hoffmann *et al* 1999). Adaptive immune responses are not the only mechanisms used by vertebrates to fight against microorganisms. Vertebrates have also a highly diverse range of responses essential to health and independent on lymphocyte receptors. Since these host defences are shared by all individual and are present at birth, they are grouped under the heading "innate immunity". Thus, it is apparent that innate immunity is an evolutionary ancient defence mechanism.

As the need for new antimicrobial agents in the recent years is rapidly rising due the fast growing number of antibiotical-resistant bacteria, the interest in antibacterial peptides is expanding. Their therapeutic potential in immunodeficient patients, in chemotherapy, in organ grafting, and against antibiotic-resistant bacterial infections, are currently under intensive elucidation.

In the present review we will focuse on three antimicrobial peptides, predominantly present in chromaffin granules, named vasostatin-I, corresponding to the N-terminal domain of bovine chromogranin A (CGA_{1-}

$_{76}$), secretolytin corresponding to the C-terminal domain of chromogranin B ($CGB_{614-626}$) and enkelytin which is the bisphosphorylated C-terminal peptide of proenkephalin-A (PEA209-237) (Fig. 1). We will summarize our present knowledge concerning the structural and biological characterization of these antimicrobial peptides and discuss their involvement in the innate immunity.

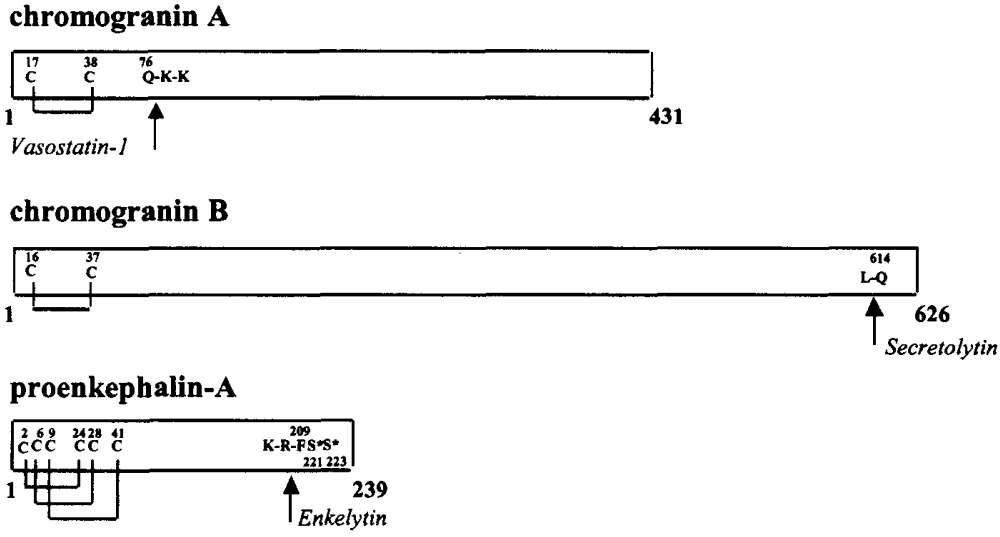

Figure 1. Antibacterial and antifungal bovine chromogranin-A, chromogranin-B, and proenkephalin-A-derived peptides : vasostatin-I, secretolytin, and enkelytin. Arrows show the proteolytic sites. The disulfide bridges and the phosphorylated serine residues are indicated (S*I).

3. ANTIMICROBIAL PEPTIDES DERIVED FROM CHROMOGRANINS

3.1 N-terminal Chromogranin A-Derived Peptide (1-76)

3.1.1 Vasostatin-I : A Multifunctional Peptide

Chromogranin A (CGA), the major member (40% of total soluble bovine chromaffin granule proteins) of CGs, has been extensively studied since its

discovery. A range of biological activities are now attributed to peptides located along the sequence of CGA. Amongst these peptides, we found vasostatins, the collective term refering to the vascular inhibitory effects of N-terminal fragments CGA_{1-76} (vasostatin-I) and CGA_{1-113} (vasostatin-II) (Aardal and Helle 1992, Aardal *et al* 1993, Brekke *et al* this volume).

The N-terminal natural bovine CGA_{1-76} corresponds to a very conserved domain, showing high yield of identity with human (Konecki et al 1987), pig (Iacangelo *et al* 1988a), rat (Iacangelo *et al* 1988b), equine CGA (Sato et al 1999) and frog CGA (Turquier et al 1999) respectively. In all species examined so far, the disulfide bridge C_{17}-C_{38} is conserved and the sequence SILRHQNLLKELQ (CGA_{50-62}) is strictly unchanged.

Vasostatins are predominantly generated in the matrix of chromaffin granules and co-released with catecholamines in the extracellular medium upon chromaffin cell stimulation (Metz-Boutigue et al 1993) and also secreted upon stimulation of the isolated retrogradely perfused bovine adrenal gland (Helle et al 1993). In addition, they have been immunodetected in the large dense core vesicles travelling down sympathetic axons and are released from nerve terminals in response to stimulation (Liang et al 1995). The vasoinhibitory effect has been reproduced with recombinant and synthetic human vasostatin fragments (Taupenot et al 1995). In addition, vasostatin-I exhibits other biological activities, such as the autocrine inhibition of parathyroid hormone secretion (Russell et al 1994), regulation of cell adhesion (Gasparri et al 1997) and neurotoxic effects in neuronal/microglial cell co-cultures (Ciesielski-Treska et al 1998). It has been demonstrated that the disulfide-bonded loop included in vasostatin-I plays a crucial role for oligomerization of CGA (Thiele and Huttner 1998). Furthermore, intragranular vasostatin-I may interact with components on the secretory vesicle membrane in a pH-dependent manner (Yoo 1993). All these properties indicate that vasostatins are likely to play important biological functions, yet to be more extensively elucidated.

3.1.2 Antimicrobial Activities of Vasostatin-I

Antibacterial activity against Micrococcus luteus (Gram-positive bacteria) was detected in several fractions after separation by HPLC of the soluble material present in chromaffin granules. One of the fractions was highly active (Lugardon et al 2000) and automatic Edman degradation revealed unique sequence corresponding to the N-terminal end of bovine CGA. MALDI-TOF mass spectrometry analysis indicated that this CGA-fragment corresponded to the peptide CGA_{1-76}, known as vasostatin-I, including the intact disulfide cystine loop C_{17} -C_{38} (Fig. 1).

Natural bovine vasostatin-I is selectively active against M. luteus and Bacillus megaterium, completely inhibiting completely their growth at 2 µM and 0.2 µM respectively. In contrast, no activity is detectable at 10 µM against other Gram-positive bacteria such as Bacillus cereus, Bacillus subtilis, Streptococcus pyogenes, Mycobacterium fortuitum, Staphylococcus aureus, Listeria monocytogenes and Gram-negative bacteria as Escherichia coli D31, 022, Enterobacter cloacae, Salmonella typhimurium, Klebsiella pneumoniae and Pseudomonas aeruginosa.

To complete the spectrum of activity of vasostatin-I, its ability to affect the growth of filamentous fungi (Table I) and yeast cells was examined. Surprisingly, this peptide in the concentration range of 1-10 µM is strongly active against a variety of filamentous fungi including Neurospora crassa, Aspergillus fumigatus, Alternaria brassicola, Nectria haematococca, Fusarium culmorum, Fusarium oxyporum, while being inactive against Trichophyton mentagrophytes (Table I).

Table I. Antifungal activity of bovine vasostatin-I, human recombinant VS-I, synthetic rat CGA7-57 and related peptides. MIC100 in µM is the minimal concentration completely inhibiting fiiamentous fungi in the standardized conditions, as described in Material and Methods. (-), no detectable activity.

	MIC100 (µM)						
Filamentous fungi	**vasostatin-1**	**VS-1**	**rat CGA7-57**	**CGA synthetic peptides**			
				47-60	41-60	41-70	47-70
Neurospora crassa	3	10	10	7	100	7	5
Aspergillus fumigatus	5	-	-	50	-	-	30
Alternaria brassicola	3	1	20	10	-	7	10
Nectria haematococca	1	3	10	7	30	7	7
Fusarium culmorum	1	5	20	20	-	10	10
Fusarium oxyporum	10	-	-	50	-	100	30
Trichophyton mentagrophytes	-	-	-	50	-	-	30

In addition, vasostatin-I is active against the yeast forms of Saccharomyces cerevisiae and Candida albicans at the concentration of 10 µM. After removal of vasostatin-I-containing medium and substitution with fresh vasostatin-I-free medium, fungi were unable to restart their growth, suggesting that this peptide possesses fungicidal activity. The specificity towards bacteria and fungi is remarkable since vasostatin-I is inactive against bovine erythrocytes. Its antibacterial and antifungal properties are

lost after treatment with proteolytic enzymes, indicating that this activity is bonafide associated to peptidic material. We can mention that the intactness of the disulfide bridge is not essential for the expression of the antifungal property, whereas it is important for the antibacterial activity

To complete these studies, the recombinant vasostatin-I (VS-I) corresponding to the sequence of human CGA_{1-78}, bearing the tripeptide SerThrAla on the N-terminal end (Corti et al 1997) is active against the growth of *M. luteus* and *B. megaterium* at a concentration of 30 µM and inhibits the growth of several fungi such as *N. crassa, A. brassicola, N. haematococca* and *F. culmorum* at concentration of 1-10 µM (Table I). However, VS-1 is inactive against the growth of yeast cells. Alkylation of VS- 1 impairs its antibacterial activity without affecting its antifungal activity whereas treatment with chemical oxidants do not modify its antifungal activity.

The antimicrobial property of the synthetic peptide corresponding to rat CGA_{7-57} was also examined. This peptide is inactive against the growth of Gram-positive bacteria, *M. luteus* and *B. megaterium* at a concentration of 30 µM, revealing that it does not possess the structural features necessary for antibacterial activity. In contrast, it was fully active against *N. crassa, A. brussicola, N. haematococca* and *F. culmorum* (Table I), but inactive against the growth of yeast cells. The antifungal activity of the synthetic peptide does not require intact disulfide cystine bridge.

In conclusion, natural vasostatin-I is a secretory peptide with potent antibacterial and antifungal activity. Whereas the disulfide bridge in vasostatin-I is crucial for its antibacterial activity, it does not seem to be required for its antifungal activity. Based on these findings we have proceeded to identify the active antifungal sequence.

3.1.3 Identification of Antifungal rhVS-1-derived Peptides

Digestion of human recombinant VS-1 (rec.hVS-1) with endoproteinase Glu-C generated short fragments that were separated by HPLC. In one major peak, a peptide corresponding to $CGPL_{47-60}$ was active against *N. crassa* with a MIC100 of 7 µM. In order to characterize the activity spectrum of this C-terminal domain of rhVS-1, different synthetic peptides were prepared. As illustrated in Fig. 2, synthetic CGA_{47-70} peptide affects *Fusarium culmorum* growth inducing its complete disappearance at 10 µM.

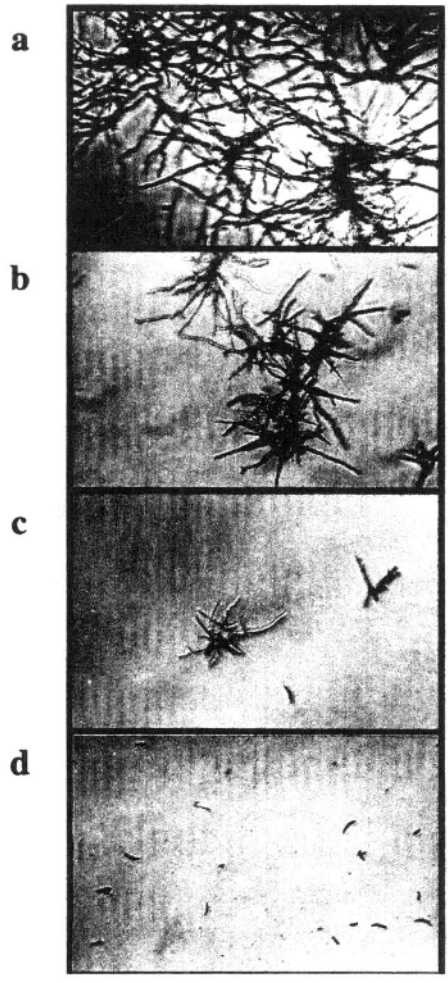

Figure 2. Inhibition of fungal growth by synthetic peptide CGA_{47-70}. Phase-contrast photomicrographs were taken after 48 h incubation of *Fusarium culmorum* spores in suspension in Potato Dextrose Broth medium in the absence (a) and in the presence of 5 µM (b), 7 µM (c), 10 µM (d), of CGA_{47-70}. Magnification : x 40.

In order to investigate the structural features involved in the antifungal activity of the highly conserved C-terminal moiety of VS-1, we tested the activity of the synthetic peptides CGA_{41-60}, CGA_{41-70} and CGA_{47-70} to establish the role of the tripeptide $R_{43}G_{44}D_{45}$ and the minimal length of the peptide chain (Table I) : (i) CGA_{41-60} displays a rather weak activity suggesting that the tripeptide $R_{43}G_{44}D_{45}$ may confer some hindrance for the antifungal activity, (ii) CGA_{41-70} is able to kill several fungi suggesting the importance of the C-terminal end in this fragment that somehow counteracts the inhibitory effect of the tripeptide $R_{43}G_{44}D_{45}$, (iii) fragments CGA_{41-60} and CGA_{47-70} are active showing a relationship between their activities and the peptide chain length, (iv) in contrast with natural vasostatin-I (CGA_{1-76}), all the C-terminal synthetic peptides are inactive against yeast cells up to 100 µM.

3.1.4 Characterization of CGA-derived Fragments in Polymorphonuclear Neutrophil (PMN) Secretions

To further study CG-derived peptides in defence mechanisms, we looked for their presence in inflammatory fluids and in secretions of immune cells. The monoclonal anti-CGA 5A8 antibody directed against the epitope CGA_{47-68} epitope (Corti *et al* 1997) was used on material secreted from human PMNs. Several N-terminal CGA-derived fragments were immunodetected with surprisingly a pattern similar to that found for chromaffin granule soluble material : a 70 kDa protein corresponding to native CGA and low-molecular weight components identified as processed CGA_{1-394}, CGA_{1-272}, CGA_{1-209}, CGA_{1-115} and CGA_{1-78} fragments on the basis of the human CGA sequence (Konecki *et al* 1987) and the cleavage sites (Metz-Boutigue et al 1993). The fuzzyness of some bands results from the presence of post-translational modifications (see Aunis and Metz-Boutigue this volume) and from an endogenous cleavage point located at the N-terminal end at position V_3-N_4 (Metz-Boutigue *et al* 1993). In conclusion, CGA-derived fragments are present in and secreted by PMNs and thus may locally be recovered in specific infectious fluids where they exert their antibacterial and antifungal activity.

3.2 Secretolytin, the C-terminal Antibacterial Peptide Derived from Chromogranin B (CGB614-426)

A few years ago, we detected a major peptide released from stimulated chromaffin cells (Strub *et al* 1995). This peptide was the CGB614-626 fragment

located at the C-terminal end (Fig. l). Its sequence is strongly homologous to the N-terminal lytic domain of insect cecropin and, as predicted from this structural similarity, it displays potent antimicrobial activity. This peptide, named secretolytin fully inhibits the growth of *M. luteus* at 2 μM concentration.

Furthermore, we have characterized the structural features of secretolytin necessary for the antibacterial activity. Using synthetic related peptides prepared taking into consideration modifications related to the length of secretolytin and the distribution of polar and hydrophobic residues, we were able to show that the antibacterial activity is directly related to the alpha-helical structure (Strub *et al* 1996a). On a more physiological aspects, we found that the amount of secretolytin in released material is quite variable from one preparation to another, suggesting that physiological (seasonal, alimentary) or pathological conditions leading to the processing of CGB have yet to be elucidated.

An extension of the cecropin-like family has recently been reported suggesting the high conservation of these antimicrobial molecules along the evolution. The human stomach is commonly colonized by the bacterium *Helicobacter pylori* which is a predisposing factor for gastrointestinal illness, such as gastritis and peptic ulcers. On the basis that *H. pylori* may have beneficial effects on infected carriers who are exposed to other gastrointestinal pathogens, it has thus been established that antibacterial activity can be detected in crude lysate from *H. pylori* (Pütsep *et al* 1999). This antibacterial activity is protease sensitive, suggesting that *H. pylori* like some other bacteria could produce one or more antimicrobial peptides, and it was suggested that this antibacterial property may be displayed by a cecropin-like peptide. A consensus motif for N-terminal residues from insect cecropins was used to search into database. The N-terminal sequence of RpL1 from *H. pylori* was listed out. This finding suggests that cecropins have probably evolved from an early rpll gene in a prokaryota that passed from being an intracellular parasite, ending up as an organelle. When the rpll gene moved from the organelle to the host nucleus, a duplicated sequence could have begun to evolve towards a specialized antimicrobial peptide.

4. ENKELYTIN , THE ANTIMICROBIAL PEPTIDE DERIVED FROM PROENKEPHALIN-A

4.1 Processing of Proenkephalin-A

The processing of proenkephalin-A (PEA) has been extensively studied in adrenal medulla chromaffin cells (Dillen *et al* 1993, Rostovstev *et al* 1994) as well as in stably transfected mouse anterior pituitary tumor (AtT-20) cells (Mathis and Lindberg 1992). PEA maturation by processing proceeds through an orderly series of steps. Several opioid peptides including M-enkephalin and L-enkephalin in the ratio 4: 1 , two C-terminally extended variants, the heptapeptide (M-enkephalin-Arg-Phe) and the octapeptide (M-enkephalin-Arg-Gly-Leu) are liberated by cleavage of the precursor at pairs of basic residues (Kojima *et al* 1982). Similarly to other precursors, bovine PEA maturation appears to first begin with the removal of the C-terminal peptide B (Stem *et al* 1981, Liston *et al* 1984, Mathis and Lindberg 1992), corresponding to bovine $PEA_{209-239.}$

4.2 Enkelytin, $PEA_{209-237}$, peptide

4.2.1 Relation of the Antimicrobial Activity with Conformational Features

Different PEA-derived peptides (PEAPs) have been isolated from the complex mixture of intragranular matrix and amongst them the natural antibacterial peptide, enkelytin has been identified as the bisphosphorylated form of $PEA_{209-237}$ (Fig. 1 ; Goumon *et al* 1996). Enkelytin is active on *M luteus* and *B. megaterium* killing bacteria in the 0.2 - 0.4 μM range. Using microsequencing and MALDI-TOF spectrometry, the antibacterial activity has been correlated with the presence of phosphate groups on Ser_{221} and Ser_{223} residues (Goumon *et al* 1996). Enkelytin is active in the micromolar range against several Gram-positive bacteria including the pathogenic strain *Staphylococcus aureus*, but it is unable to inhibit Gram-negative bacteria and fungi growth.

In order to correlate the antibacterial activity of enkelytin with structural features of the peptide, natural and synthetic enkelytinderived peptides were tested (Goumon *et al* 1998). Natural enkelytin and $PEA_{209-239}$ fragment known as peptide B completely inhibit the growth of Gram-positive bacteria, but were inactive to *E. coli* in the concentration range 0.2 - 3 μM (Goumon *et al* 1998). Synthetic enkelytin displays rather low activity because only 10% of the synthetic peptide adopts the right conformation that

bears the antibacterial activity, suggesting important conformational differences amongst different synthetic isoforms. Three-dimensional ^{1}HNMR analyses of $PEA_{209-237}$ show that residue Pro_{227} is responsible for conformational cis-trans isomerization changes (Kieffer *et al* 1998). In contrast with the natural and synthetic bisphosphorylated peptide, the non-modified synthetic peptide displays a low antibacterial activity indicating that the two phosphorylated serine residues play an important conformational role. The synthetic $PEA_{209-237}$ peptide with three glutamic residues in place of the three putative phosphorylated serine residues Ser_{215}, Ser_{221} and Ser_{223} inhibits by 20% *M. luteus* growth at 100 µM, suggesting the importance of negative charges in the expression of the antibacterial activity of enkelytin.

In order to correlate the antibacterial activity with the length of the sequence, N- and C-terminal enkelytin-derived peptides were tested ($PEA_{209-220}$ and $PEA_{224-237}$). These two peptides are poorly active (20-25% inhibition at 500 µM). In addition, shorter C-terminal fragments ($PEA_{230-237}$ and $PEA_{233-237}$) are completely inactive at the same concentration. To complete these data, these peptides have neither of detectable hemolytic activity in the concentration range from 0.2 to 500 µM. In conclusion, the antibacterial activity of enkelytin is directly related to three parameters : i) the length of the peptide chain, ii) the natural conformational constraints induced by the three proline residues $Pr0_{212}$, Pro_{214}, Pro_{227}, and iii) the phosphorylation state of Ser_{221} and Ser_{223}.

Circular dichroïsm spectra (CD) recorded with increasing percentage of trifluoroethanol (TFE) shows that synthetic $PEA_{209-237}$ adopts progressively an helical structure, as the percentage of TFE increases. The presence of helical structures was confirmed from ^{1}HNMR spectra of synthetic $PEA_{209-237}$ in domains extending from Ser_{215} to Gly_{219} and from Glu_{228} to Phe_{236}. ^{1}HNMR analysis indicates that the 3-D structure of $PEA_{209-237}$ adopts an L-shape (Goumon *et al* 1998, Kieffer *et al* 1998). When the two serine residues Ser_{221} and Ser_{223} of enkelytin are phosphorylated, the negatively charged phosphate groups probably induce conformational changes by electrostatic interactions, thus opening the angle formed by the two helical domains.

4.2.2 Characterization of Enkelytin-derived Fragments in PMN Secretions

To further characterize the biological function of enkelytin, we looked for its presence in biological fluids involved in defence mechanisms. Using the combination of immunoblotting, microsequencing and MALDI-TOF mass spectrometry analysis, antibacterial C-terminal PEA-derived fragments were identified in numerous bovine and rabbit infectious fluids and also in

secretory materials collected from human PMNs (Goumon *et al* 1998). Enkelytin was detected and associated with other antibacterial peptides including defensins and bactenicins in these infectious fluids (Goumon *et al* 1998).

5. CONCLUSIONS

In 1995, we reported for the first time the presence of antimicrobial peptides in the matrix of secretory granules from adrenal medulla. These new peptides, which inhibit bacteria and fungi growth, are processed from the N-terminal (vasostatin-I) and the C-terminal (secretolytin, enkelytin) ends of precursors CGA, CGB and PEA as a result of different proteolytic enzymes also present in secretory granules. These peptides are released from stimulated chromaffin cells, but also as a novel product from stimulated polymorphonuclear neutrophils. Interestingly, in these secretory fluids the concentration of the antimicrobial peptides fall in the micromolar range (Goumon *et al* 1998), i.e. close to the concentration necessary for the antimicrobial activity.

Some of these peptides share structural features with other antimicrobial peptides. For instance, secretolytin has a consensus sequence with the cecropin lytic moiety while the active C-terminal fragment of vasostatin-I (CGA_{47-66}) has some homology with the C-terminal fragment of neuropeptide Y (Metz-Boutigue and Aunis unpublished) that displays both antibacterial and antifungal activities (Vouldoukis *et al* 1996). Moreover, the antibacterial activity of some CG- and PEA-derived fragments depends on post-translational modifications such as O-glycosylation (prochromacin) (Strub *et al* 1996b) and phosphorylation (enkelytin) (Goumon *et al* 1996, 1998) These modifications occur at an early stage within the pro-secretory granules and may represent regulatory steps related to still unknown physiological or pathological conditions.

Antibacterial peptides have to be positively charged in order to bind to bacterial surfaces. Secretolytin and vasostatin-I are positively charged with respective net charges of +3 and +1, but curiously enkelytin and peptide B possess net charges of -7 and -8 respectively. As for most of all antibacterial peptides described so far, the mechanism by which enkelytin and peptide B inhibit bacteria growth is completely unknown. In addition, the presence of vasostatin-I and enkelytin in infectious fluids together with other antimicrobial peptides supports their potential role in host defence mechanism. Defensins and bactenecins probably are assumed to form molecular heterocomplexes with a consequent synergistic activity (Goumon *et al* 1998).

As previously reported, vasostatin-I and enkelytin correspond to the most highly conserved domains in their precursors. Thus, the antimicrobial activities displayed by these peptides are likely to have occurred early in evolution. Interestingly, CGs appear to be phylogenetically old early during since chromogranin-like immunoreactivity is detectable in unicellular and muticellular organisms alike, from Paramecium, through coelenterates, bivalved molluscs, lower vertebrates to birds and mammals (see reviews in Simon and Aunis 1989, Helle 1990). Furthermore, CGs and PEA are widely distributed not only in endocrine, neuroendocrine and nerve cells but also in immune cells. Their liberation from these cells indicates that these peptides are likely to play a role in inflammatory processes. Therefore we suggest that in stress situations, these peptides might act by rising an immediate protective barrier against infection. Hence, the antibacterial and antifungal activities might provide a highly beneficial strategy against pathogenic invasion.

PEA has been reported to be significantly expressed in the immune system, providing a basis for neuroimmune interactions (Zurawski *et al* 1986, Linner *et al* 1991, Rosen *et al* 1989, Martin *et al* 1987). The involvment of opioids in the regulation of interactive events between the nervous and immune systems appears to have a long evolutionary history. The relationship between the immune and nervous systems also exists in invertebrates (Stefano *et al* 1990) and the corelease of enkelytin and M-enkephalin represent an unified neuroimmune protective response to stress situations that may be accompanied with infectious diseases. Furthermore, recent evidence obtained in vitro has shown that vasostatin-I activates microglial cells in primary culture (Taupenot *et al* 1996) resulting in the liberation of neurotoxic factors (Ciesielski-Treska *et al* 1998, Ciesielski-Treska and Aunis, this volume).

To conclude, the identification of different classes of antibacterial peptides within CGA, CGB and PEA active in a diverse range of organisms, including prokaryotes, bivalves, frogs, mammals suggests that they play an important role in innate immunity for the host defence. Thus, the characterization of new non-toxic antimicrobial peptides derived from endogenous precursors is a topic of growing interest in relation to their therapeutic use.

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PART 6

CLINICAL ASPECTS OF THE CIRCULATING PROHORMONES

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25

MEASUREMENTS OF CHROMOGRANINS AND CHROMOGRANIN-RELATED PEPTIDES BY IMMUNOLOGICAL METHODS

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1. INTRODUCTION

Antibodies against chromogranins have been used for immunocytochemical staining of tissue specimens, both normal tissue and tumour tissue. Also Western blots and dot blot assays have been employed in estimations of tissue concentrations of chromogranins. However, these methods are semi-quantitative and not sensitive enough to allow accurate estimations of circulating levels.

The first radioimmunoassay for measurements of chromogranins was introduced in 1986 (O'Connor and Deftos 1986). Since then other assays for measurements of intact human chromogranin A (CGA) have been reported. Also two assays developed against bovine CGA, but with sufficient cross-reactivity to allow measurements of human CGA, have been reported, see table 1. At present, two commercial assays for CGA are available (DAKO and CIS-BIO). Assays for measurements of defined regions of CGA have also been established. These include methods specific for pancreastatin from different species and other regions of CGA, see table 2. For chromogranin B (CGB) only region-specific assays have been presented, see table 3. Region specific assays for measurements of secretogranin II (SgII) have been presented as assays for secretoneurin and other regions, see table 4.

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Table 1. Assays designed to measure intact chromogranin A

Antibodies raised against	Amino acid sequence covered	Reference	Comments
Purified protein from pheochromocytoma	Human CGA1-439	(O'Connor and Deftos 1986), (O'Connor et al 1989)	Competitive RIA
Purified protein from adrenal medulla	Bovine CGA 1-431	(Dillen et al 1989)	EIA, two monoclonal antibodies. Cross reaction to human CGA
Purified protein from adrenal medulla	Bovine CGA 1-431	(Kawakubo. et al 1989)	EIA, two polyclonal antibodies
Purified protein from pheochromocytoma	Human CGA1-439	(Eriksson et al 1990)	Competitive RIA. Possibly cross reaction to and detection of CGB
Purified protein	Human CGA 1-439	(Bender et al 1992)	LIA, one monoclonal antibody and one polyclonal antibody
Purified protein from pheochromocytoma	Human CGA1-439	(Syversen et al 1992)	Competitive RIA
Purified protein from pheochromocytoma	Human CGAl-439	(Nobels et al 1993)	Competitive RIA
Purified protein from urine from patients with carcinoid tumours	Human CGA116-439	(Stridsberg et al 1993), (Stridsberg et al 1995)	Competitive RIA
Purified protein from pheochromocytoma	Human CGA 1 16-439	(Corti et al 1996)	EIA, two monoclonal antibodies recognising CGA 68-90
Recombinant protein	Human CGA 1-439	(Degorce et al 1999), Commercial kit, CIS-BIO	RIA, two monoclonal antibodies recognising CGA 145-245
Purified protein from urine from a patient with a carcinoid tumour	A 23 kDa C-terminal fragment of human CGA	Commercial kit, DAKO	EIA, two polyclonal antibodies

2. CLINICAL SIGNIFICANCE OF CHROMOGRANINS

Already at the first presentation of radioimmunoassay for measurements of CGA the clinical use was outlined (O'Connor and Deftos 1986). Tumours of neuroendocrine origin usually present with increased plasma levels of CGA. The neuroendocrine tumours are derived from the neuroendocrine cells and typical neuroendocrine tumours are carcinoid tumours,

pheochromocytomas, neuroblastomas, small cell lung cancers, hyperparathyroid adenomas, pituitary tumours, prostate cancers and pancreatic islet tumours and including the MEN1 and MEN2 syndromes. This also includes the different pancreatic islet cell syndromes, namely the insulinomas, the glucagonomas, the somatostatinomas, the Zollinger-Ellison syndrome, the Verner-Morrison syndrome, the PPomas and the non-functioning neuroendocrine tumours. The most useful assays for tumour detection have been those that measure the whole molecule. Assays measuring specific defined parts of the molecule usually have lower sensitivity in detecting patients with neuroendocrine tumours (Stridsberg et al 1995). The clinical use of chromogranins is described more extensively in the next chapter.

Table 2. Assays designed to measure specific regions ofthe chromogranin A molecule

Antibodies raised against	Amino acid sequence covered	Reference	Comments
Synthetic peptide, Human pancreastatin 24-52	Human CGA 273-301	(Tateishi et al 1989)	Competitive RIA. Cross reaction to porcine Ps, not to bovine Ps
Synthetic peptide, Porcine pancreastatin 39-49	Porcine CGA278-288	(McGrath-Linden et al 1991)	Competitive RIA. Cross reaction to bovine Ps and, human Ps
Synthetic peptide, Porcine pancreastatin 33-49	Porcine CGA272-288	(Iguchi et al 1992)	Competitive RIA. Cross reaction to human Ps
Synthetic peptide, Human pancreastatin 35-52	Human CGA 283-301	(Stridsberg et al 1995)	Competitive RIA
Synthetic peptide, Bovine GE-25	Bovine CGA367-391	(Kirchmair et al 1995)	Competitive RIA
Synthetic peptides	Rat CGA 1-28 Rat CGA 94-130 Rat CGA 296-314 Rat CGA 359-389	(Nishikawa et al 1998a)	Competitive RIA:s. Partial cross reaction of CGA 359-389 to human CGA
Synthetic peptide	Human CGA 344-374	(Nagasawa et al 1998)	Competitive EIA
Synthetic peptide	Human CGA 344-374	(Nishikawa et al 1998b)	Competitive RIA
Synthetic peptides	Human CGA 1-9 Human CGA 116-124 Human CGA 210-222 Human CGA 340-348 Human CGA 432-439	(Jensen et al 1999)	Competitive RIA:s

3. CIRCULATING CHROMOGRANINS

Different neuroendocrine tissue contains different amounts of chromogranins (Takiyyuddin et al 1990). Calculated from the tissue wet weight the adrenal medulla is the organ that contain the highest concentration of CGA, 175 μg/g, while the pituitary contained only 43 μg/g wet weight. In pancreas the concentration of CGA was about 9 μ g/g and in the stomach and the small intestine the CGA concentrations were about 4 pglg wet weight (Takiyyuddin et al 1990). However, in these latter organs the number of neuroendocrine cells compared to other cells are rather low which can explain the apparently low concentrations of CGA per wet weight. In fact, the total number of neuroendocrine cells in the stomach and the small intestine may very well exceed that of the adrenal medulla. It is therefore plausible that in normal, healthy individuals, the circulating pool of chromogranins is made up of the chromogranin released from the different neuroendocrine cells in the body. These neuroendocrine cells are likely to contribute to the circulating amounts of chromogranins in comparison to the number of cells and the hormonal release activity of the cells. Thus, it has been shown that suppression of hormonal activity with somatostatin decreased the plasma levels of CGA by 48% and that selective disruption of sympathetic outflow decreased the plasma CGA by 25% (Takiyyuddin et al 1991). For endocrine tumours, it has been shown that the plasma concentration of CGA is proportional to the endocrine cell (tumour) mass (Hsiao. et al 1990b, Wassberg et al 1996, Janson et al 1997).

Table 3. Assays designed to measure chromogranin B

Antibodies raised against	Amino acid sequence covered	Reference	comments
Synthetic peptide, GAWK 20-38	Human CGB 439-457	(Iguchi et al 1988)	Competitive RIA
Synthetic peptide	Human CGB 312-331	(Stridsberg et al 1995)	Competitive RIA
Synthetic peptide, PEl 1	Rat CGB 552-574	(Marksteiner et al 1999)	Competitive RIA

The circulating half-life of CGA has been examined after surgical resection of pheochromocytomas (Hsiao et al 199Ob). The plasma concentration fitted best to a two-dimensional model with an initial rapid half-life of 16 minutes, followed by a longer half-life of 520 minutes. Similar experiments have been performed at the Uppsala University Hospital in Sweden. These data show a functional half-life of 150 minutes for CGA after surgical resection of pheochromocytomas (unpublished data).

4. MEASUREMENTS OF CHROMOGRANINS

Today measurements of CGA are routine procedures in the management of neuroendocrine tumours. Most CGA methods are classical competitive assays that make use of one polyclonal antibody. However, some methods are non-competitive assays that use two antibodies, which bind to different parts of the CGA molecule. This approach may be less useful since it requires binding to a larger part of the CGA molecule. As described in other chapters, chromogranins are often cleaved into smaller fragments and consequently these kits may give false low values in some patients. The ideal assay would be one that measure both intact and fragments of CGA.

Table 4. Assays designed to measure secretogranin II

Antibodies raised against	Amino acid sequence covered	Reference	Comments
Synthetic peptide, SgII	Human SgII 133-151	(Kirchmair et al 1993)	Competitive
Synthetic peptide, secretoneurin	Human SgII 154-186		RIA:s
Synthetic peptide	Human SgII 223-249	(Stridsberg et al 1995)	Competitive RIA

In most neuroendocrine tumours CGA is more abundant than CGB and CGA is thus usually a better circulating tumour marker than CGB. However, in some tumour patients one can find increased circulating CGB while CGA is normal. It can therefore be suggested that both CGA and CGB should be analysed to increase the sensitivity when chromogranins are used as tumour markers.

The most common non-tumour associated increase of CGA is decreased renal function (Hsiao et al 1990a). Other important non-tumour associated increases of CGA are type A gastritis and treatments with proton pump inhibitors (Sanduleanu et al 1999). However, none of these parameters affect the measurements of CGB (Stridsberg and Husebye 1997).

Concentrations of CGA, measured in the assays using purified chromogranin as standard, are mostly presented in ng/mL (= µg/L). Usually the circulating levels of CGA in humans are measured to about 50-100 µg/L. The measured concentration in a given sample is dependent on what epitopes the antibodies recognise and how they can bind to these epitopes. Different pairs of antibodies can give different measured concentrations, as described for development of non-competitive methods (Corti et al 1996, Degorce et al 1999). One explanation of this behaviour is that some antibody-binding epitopes on CGA may be masked by the 3-dimensional folding of the molecule. This has been shown earlier when an antibody specific for pancreastatin did not bind to intact CGA, but showed a partial binding to partly processed CGA (Stridsberg et al 1993). When the

standardpreparations are more defined, i.e. when synthesised peptides are used, concentrations are usually given in pmol/L. Since plasma chromogranins are circulating both in the intact form and as fragments, the molar presentation may be a more accurate way to express concentrations of chromogranins.

5. FUTURE PERSPECTIVES

Today measurements of chromogranins are important in the management of patients with neuroendocrine tumours. Some specialised laboratories can offer CGA analyses and at present also two commercial assays are available. However, laboratories that can offer measurements of CGB are rare. It has been shown that CGA is a better tumour marker than neuron specific enolase (NSE) and the a-subunit of glycoprotein hormones for patients with gastrinomas, pheochromocytomas, carcinoid tumours, non-functioning pancreatic endocrine tumours and medullary carcinomas (Nobels et al 1997). Likely, CGA was found to be a better tumour marker than NSE and urinary 5-HIAA for patients with histopathologically assessed neuroendocrine tumours including foregut, midgut and hindgut carcinoid tumours and pancreatic endocrine tumours (Bajetta et al 1999). It is likely that the clinical use of chromogranins will increase and CGA will probably defend its position as the first choice of tumour marker for neuroendocrine tumours.

Different neuroendocrine cells can process chromogranins differently and for example it has been shown that immunocytochemical staining for the N-terminal part of CGA (vasostatin I) can distinguish between ileal and lung carcinoids (Cunningham et al 1999). Thus, it is likely that region-specific assays, detecting specific processed forms of chromogranins present in the circulation, can improve the diagnostic value of chromogranins. It is not unlikely that the increased turnover of chromogranins in neuroendocrine tumours may result in a decreased processing, i.e. a proportional increased release of intact chromogranin compared to processed chromogranin. If this impaired processing is more pronounced in more malignant tumours it is possible that the quotient between intact CGA and a defined split product can serve as an indicator of malignancy. It is also known that there is a difference in the expression of CGA relative to CGB in different neuroendocrine tissue. Thus, it is possible that also the proportion between CGA and CGB may turn out to have some diagnostic value.

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26

CHROMOGRANINS AS DIAGNOSTIC AND PROGNOSTIC MARKERS IN NEUROENDOCRINE TUMOURS

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1. INTRODUCTION

The physiological function of chromogranin A (CGA) is gradually elucidated, although many questions still remain. Its ubiquitous presence in neuroendocrine tissues and its co-secretion with peptide hormones and amines makes it a suitable tissue and serum marker for neoplasms of neuroendocrine origin (O'Connor *et al* 1983, Eriksson *et al* 1990, Deftos 1991, Stridsberg et al 1995). Proteolytic processing of CGA occurs during its presence in the dense core vesicles (Iacangelo and Eiden 1995). Depending on the type and amount of proteases present (proconvertases), the type and amount of the proteolytic cleavage product may vary in different neuroendocrine tissues (Iacangelo and Eiden 1995, Deftos *et al* 1990). The present data support that peptides derived from intragranular processing of CGA are co-released with resident peptide hormones and amines and exert an immediate autocrine and paracrine modulatory effect on the secretory activity on neuroendocrine cells. A classical endocrine effect on distant tissues might also be possible as these fragments of CGA are also released into the circulation (Deftos 1991).

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2. IMMUNOHISTOCHEMICAL DETECTION OF CHROMOGRANINS

CGA is found throughout the neuronal and neuroendocrine system. The endocrine cells are expressing the protein in the endocrine cells of the anterior pituitary, parafollicular C-cells of the thyroid, chief cells of the parathyroids, islet cells of the pancreas and chromaffin cells of the adrenal medulla (Larsson *et al* 1992). CGA is also present in the wide spread neuroendocrine system of the bronchial and gastrointestinal tract and of the skin (Merkel cells), see review (Winkler and Fischer-Colbrie 1992). CGA positive cells have also been found in the prostate (Deftos *et al* 1996). Cells that are not of neuroendocrine origin, such as follicular cells of the thyroid gland and steroid hormone secreting cells of the adrenal cortex and the gonads, lack CGA.

Table 1. Neuroendocrine tumors which usually show positive immunostaining for chromogranin A

Locations of tumours	Syndromes
Anterior pituitary tumours	ACTH (Cushing)
	LH/FSH
	GH (Acromegaly)
	TSH
	Prolactinoma (CGB positive!)
	Null cell adenoma
Parathyroid tumours	
Medullary thyroid carcinoma	
Merkel cell tumour	
Neuroendocrine GEP-tumours	Carcinoids (foregut, midgut, hindgut)
	ECL-oma
	Gastrinoma
	Glucagonoma
	Insulinoma (CGB positive!)
	Somatostatinoma
	VIP-oma
	Nonfunctioning tumours
Bronchial carcinoids	Ectopic ACTH producing tumours
Endocrine pancreatic tumours	
Ganglioneuroma, neuroblastoma	
Pheocromocytoma	
Small cell lung cancer	
Prostate cancer	

Immunohistochemical techniques to detect the presence of CGA in tumour tissues are widely used in clinical practice. A list of tumours, for which CGA can serve as a tissue marker, is presented in Table 1. In addition to typical neuroendocrine neoplasm, tumours derived from the nervous system, such as ganglioneuroblastoma, ganglioneuroma and neuroblastoma

also show imunostaining for CGA (Wiedenmann and Huttner 1989, Winkler and Fischer-Colbrie 1992). Also tumours of the prostate demonstrate positive staining for CGA (Deftos et al 1996). Even those neuroendocrine tumours that have lost their ability to produce peptide hormones (e.g. null cell pitutuitary adenomas) can show CGA positive immunostaining. CGA immunostaining is also positive in neuroendocrine cell hyperplasia, such as gastric enterochromaffin like cells (ECL-cells; chronic atrophic gastritis), pulmonary neuroendocrine cell hyperplasia and prostate hyperplasia (Gould et al 1988, Larsson *et al* 1992, Belaiche *et al* 1993).

The prolactin producing cells in the pituitary lack CGA expression but show positive staining with chromogranin B (CGB) antibodies. The same is also true for the p-cells of the pancreas, which sometimes are negative for CGA. It should also be pointed out that several non-endocrine tumours might show positive immunoreaction for CGA in a limited number of cells, for example in colorectal cancer and exocrine pancreatic cancer.

3. PLASMA MEASUREMENTS OF CHROMOGRANINS

Chromogranins are co-released with the peptide hormones present in secretory granules, which opens the possibility to use circulating CGA concentrations as markers of the secretion from neuroendocrine neoplasms. CGA has been used for clinical purposes for about 15 years and data are reported from several research centres, but CGB measurements are reported from only one research centre (Stridsberg *et al* 1995). Since CGA is stored in a majority of different neuroendocrine tumours, the release to the circulation can be used as a ”general” marker for various neuroendocrine tumours. CGA might also be an excellent marker for the so called ”non-functioning” neuroendocrine tumours, which lack other suitable tumour markers. It may also be of value to analyse CGA when existing tumour markers are either unstable or rapidly fluctuating, such as serotonin and cathecolamines in plasma. CGA can be used for differentiation between endocrine tumours of neural crest or other origin, such as ectopic source of Cushing´s syndrome.

Several radioimmunoassay procedures have been developed during recent years and also commercially assays are available (see chapter X). Some of these assays are not measuring intact CGA but more splice products such as pancreastatin. Published results of circulating levels of CGA from different centers might vary depending on what assay that has been used as well as the patient material. CGA as such is a very stable molecule, no special precautions are needed to handle or store the serum or plasma. Mast assays are sufficiently sensitive to measure CGA in normal subjects. Circulating

levels are slightly higher in postmenopausal women but without any clear diurnal variation. Elevated levels can be encountered in patients with renal or hepatic failure. CGA is co-released with cathecolamines and one might expect a slight elevation of its serum levels in circumstances of stress (Hsiao 1990). However, even intense sympatico-adrenal stimulation, such as during excessive medical stress, insulin induced hypoglycemia, cardiac arrest or during strenous exercise, CGA levels are only slightly increased (Cryer *et al* 1991, Elias *et al* 1992). Patients with inflammatory conditions of the bowel, such as ulcerative colitis and Crohn's disease might also present slightly elevated levels (Granberg *et al* 1999).

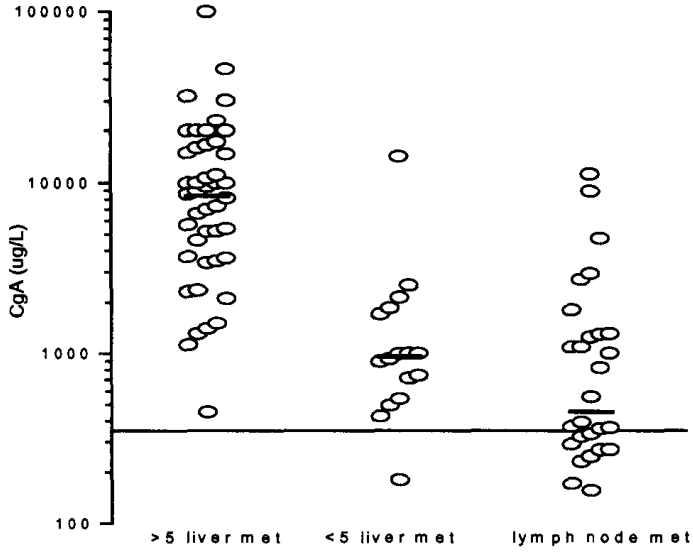

Figure 1. Chromogranin A levels in carcinoid tumour patients with respect to tumour mass. Plasma levels were significantly higher in patients with more than five liver metastases compared to less than five liver metastases. Logarithmic scale on the Y-axis. The horizontal line indicate the upper reference limit of plasma CGA.

The plasma levels of CGA are elevated in patients with various types of peptide producing neuroendocrine turnours. There is a clear correlation between tumour burden and serum CGA concentration (Hsiao. *et al* 1990, Wassberg *et al* 1996, Janson *et al* 1997, Granberg *et al* 1999), see also figure 1. The highest levels are recorded in patients with metastatic carcinoid tumours, particularly midgut carcinoids (Stridsberg *et al* 1995, Janson *et al* 1997, Nobels *et al* 1997). Plasma levels of more than 1000 times the upper reference limit have been reported. In malignant endocrine pancreatic tumours plasma CGA are varying from slightly elevated to extremely high levels. CGB is usually a better marker for insulin producing tumours than CGA. In a recent study CGA was measured in patients with multiple

endocrine neoplasia type 1 (MEN-1). There was a clear correlation between tumour mass and circulating levels. MEN-1 patients without known pancreatic involvement showed elevated CGA levels in 45%. Furthermore, 60% of patients with biochemically unequivocal tumours of the pancreas and 100% of patients with radiological visible tumours had increased levels of CGA (Granberg *et al* 1999). In an earlier study, involving 44 patients with malignant carcinoid tumours, 17 patients with sporadic endocrine pancreatic tumours and 11 patients with endocrine pancreatic tumours and MEN-I, plasma levels of CGA was increased in 99%, whereas CGB was elevated in 88% and pancreastatin in 46% of the patients (Stridsberg *et al* 1995), see figure 2. When using a specific pancreastatin assay and comparing it with a specific for CGA assay, it is quite clear that measurement of pancreastatin is inferior to measurements of the whole molecule (Stridsberg *et al* 1995). A possible explanation for this difference might be that some tumours are lacking necessary enzymes for splicing off pancreastatin from the "mother" molecule.

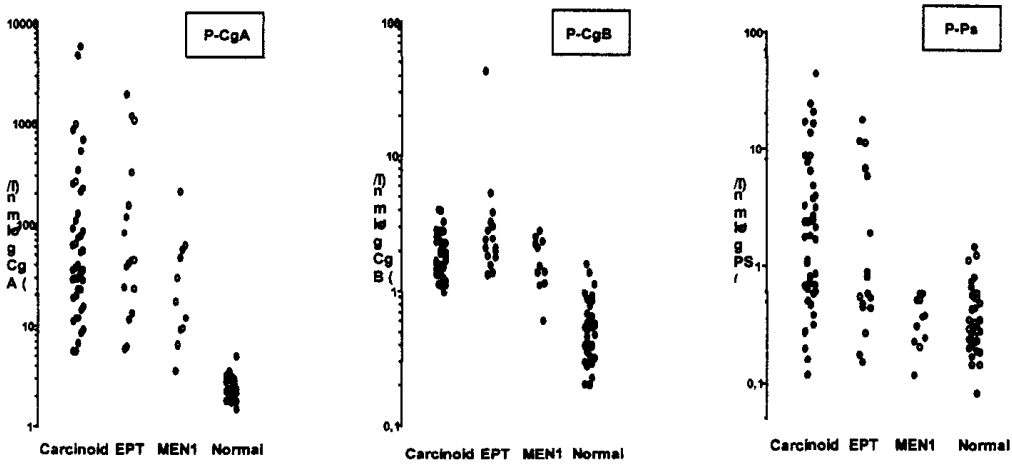

Figure 2. Plasma levels of CGA, CGB, and pancreastatin in patients with neuroendocrine tumours.

In another study, CGA measurement was superior to measurements of urinary 5-HIAA, neuron specific enolase (NSE) and carcino-embryonic antigen (CEA) as tumour marker in neuroendocrine tumours (Bajetta *et al* 1999). The specificity was 86% and sensitivity 68% for CGA in a total of 127 patients with neuroendocrine tumours. Patients with gastrin producing tumours (gastrinomas) very often show increased levels of CGA. However, several studies have indicated that plasma CGA is released from the enterochromaffin like cells (ECL cells) rather than by co-release from the gastrinoma itself (Stabile *et al* 1990, D'Adda *et al* 1990, Syversen *et al* 1993). Hypergastrinemia induces hyperplasia of the ECL cells of the

stomach. Patients treated with proton pump inhibitors show elevated CGA levels (Sanduleanu *et al* 1999). CGA can serve as a useful serum marker for patients with pheochromocytomas, which usually show markedly elevated CGA concentrations (Hsiao 1990, Stridsberg and Husebye 1997). No substantial elevation of CGA levels is seen in patients with other causes of hypertension. Drugs used in the diagnosis or treatment of pheochromocytomas such as clonidin, metoprolol, phentolamine and tyramine seem to have little effect on plasma CGA concentration (Takiyyuddin *et al* 1990, Hsiao *et al* 1990).

CGA is a very useful marker for carcinoids of the foregut origin such as bronchial carcinoids with or without ectopic adrenocorticotrophin hormone (ACTH) secretion. As mentioned above, CGA concentration in classical midgut carcinoids are significantly elevated. Many patients with the carcinoid syndrome show 100-1000 fold increase. Such levels are rarely found in foregut carcinoids or hindgut carcinoids. There is no significant correlation between peptide hormone levels or amine levels and CGA concentrations in tumour patients. Treatment with somatostatin analogues and alpha interferon significantly reduces the CGA levels in carcinoid tumour patients, which by that reduce the correlation between tumour mass and CGA levels. However, CGA is very useful to indicate tumour progression during treatment because at tumour progression the levels start to increase earlier than changes of the tumour size on CT-scan or MRI. Increases of CGA were associated with progressive disease in 83% of the cases indicating a value of this marker in the follow-up of the patients (Bajetta *et al* 1999).

CGA might be a very usuful marker to localize the tumour responsible for the Cushing syndrome in a patient. CGA levels are usually normal in patients with pituitary cortico-trophic adenomas as well as in adrenal cortisol producing tumours, but are significantly increased in ectopic ACTH producing tumours of the pancreas and/or the lung (Nobels *et al* 1994). About one third of patients with small cell lung cancer present increased levels of CGA indicating the neuroendocrine nature of these tumours (Nobels *et al* 1997).

Elevated CGA leves are not entirely specific for a neuroendocrine tumour. Slightly elevated levels have been identified in patients with non-endocrine tumours (Kadmon *et al* 1991, Syversen *et al* 1995). Neuroendocrine cells are multifocally located in small nests or scattered within these tumours. Obviously these cells might produce peptides that affect the growth of the tumour cells. Prostatic adenocarcinomas co-express CGA and prostate-specific antigen (PSA) suggesting a common malignant precursor cell (Deftos *et al* 1996). Limited data suggested that prostatic adenocarcinoma containing neuroendocrine cells are more resistant to hormonal treatment. Similarly patients with colorectal adenocarcinoma containing numerous neuroendocrine cells seems to have a worse prognosis (Syversen *et al* 1995, Hamada *et al* 1992). On the other hand, patients with

pancreatic adenocarcinoma (Pour *et al* 1993), or with non-small cell cancer whose cancer contains many neuroendocrine cells, seems to have a better prognosis (Sundaresan *et al* 1991). Patients with neuroendocrine tumours such as carcinoid tumours, an analysis of prognostic factors in 301 patients showed that plasma CGA is an independent predictor of bad prognosis in midgut carcinoid patients together with advanced age (Janson *et al* 1997). The various observations that increased levels of CGA correlates with bad prognosis in different tumour diseases might indicate a role of CGA as a stimulator of tumour growth, either the molecule itself or any of the splice products. Therefore ongoing studies analysing splice products of CGA in carcinoid patients might reveal a correlation between some of the fragments and prognosis in those patients.

4. FUTURE PROSPECTS

It is quite obvious that CGA is currently the most suitable marker both for immunohistochemical measurements and as a serum marker in patients with neuroendocrine tumours. It will also be of interest to analyse the different fragments from CGA and CGB molecules to see possible correlation with tumour biology, location and molecular genetics. It has been suggested that CGA might be a molecule for in vivo imaging techniques of neuroendocrine tumours and development of monoclonal antibodies against CGA or Cg-B has been reported (Colombo *et al* 1996). Preliminary data has been reported showing some interesting results but so far any clinically working scintigraphy method has not emerged.

To learn more about the chromogranins as markers of neuroendocrine tumours one has to get more information about the physiology of these molecules and also their regulation of secretion. Treatment using antisense technique against the CGA molecule could be of benefit for patients with neuroendocrine tumours.

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27

ASSESSMENT OF CHROMOGRANIN A USING TWO-SITE IMMUNOASSAY

Selection of a Monoclonal Antibody Pair Unaffected by Human Chromogranin A Processing

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1. INTRODUCTION

For many years, chromogranin A (CGA) immunohistochemistry has been an uncontested criterion of neuroendocrine differentiation. It has also been shown that plasmatic CGA level determination was an important element in the diagnosis of pheochromocytoma and of numerous endocrine and neuroendocrine tumours. In prostatic cancer, a high level of CGA has been associated with a poor prognosis. Most of these studies have referred to the assessment of intact and fragmented CGA. As the processing of the protein targets both C and N-terminal ends in a recurrent manner, we have generated 24 monoclonal antibodies against native and recombinant human CGA and developed a two-site radioimmunoassay by selecting monoclonal antibodies that recognise the central domain of CGA. Using this assay, we have also demonstrated that most circulating CGA forms can be detected by this antibody combination. On the contrary, those which associate a median-type antibody with another against more C-terminally located epitopes were shown to be substantially affected by CGA proteolysis.

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2. CLINICAL VALUE OF CHROMOGRANIN A

2.1 CGA Immunohistochemistry

The literature concerning the immunohistochemical detection of CGA is extremely abundant, reflecting its value as a marker for neuroendocrine differentiation. The use of monoclonal and polyclonal antibodies has confirmed the presence of different-sized neuroendocrine cell contingents in many normal tissues such as thyroid, parathyroid, pituitary, adrenals (the main synthesis organ), gastrointestinal tract (another important site for synthesis, given the large representation of neuroendocrine cells), pancreas, lung or breast (reviewed in Wilson and Lloyd 1984). Because of the dispersion of neuroendocrine cells throughout the human body, the expression of granins, and more particularly CGA, is not limited to the corresponding endocrine tumours, but can also be associated with pathologies or organs which do not usually constitute a CGA source. For instance, CGA immunoreactive cells have been detected in breast (Weiler *et al* 1988), prostate (Deftos *et al* 1996), stomach (Blumenfeld *et al* 1996) or colon (Pagani *et al* 1996), in all of which the occurrence of mixed neoplasia is not uncommon. In this context however, the value of CGA detection remains subject to debate. This expression is frequently associated with that of other granins, hormones or neuropeptides (calcitonin, gastrin, serotonin...). In the gastrointestinal tract, Portela-Gomes *et al* (1997) showed that CGA could be detected in most cell types (except somatostatin secreting cells). CGA expression is also much more ubiquitous than that of chromogranin B or secretogranin 11.

In the central nervous system, the occurrence of CGA has been demonstrated by many authors in the senile plaques in brain of patients with Alzheimer's disease and in the Lewy's bodies in brain of patients with Parkinson's disease (Muñoz *et al* 1991, Nishimura *et al* 1994). In another study, the CGA/synaptophysin ratio determined by immunoblot on cerebral extracts was shown to be substantially more elevated in the case of Alzheimer's disease. On the other hand, CGA levels detected in CSF did not differ from normal samples (O'Connor and Bernstein 1984) nor could any correlation between CSF and seric levels be established (Blennow *et al* 1995). Nevertheless, the same work seemed to prove that CGA concentrations were significantly lower in type I Alzheimer's disease (pure) than in type II (senile dementia).

Finally, interest in CGA expression in prostate has motivated an increasing number of studies over the past few years (reviewed in Di Sant'Agnese *et al* 1992). Many neuroendocrine products originate from normal and pathological prostate : calcitonin, calcitonin gene-related peptide

(CGRP), parathyroid hormone related peptide (PTHrp), neurone specific enolase (NSE) and CGA among others (Deftos and Abrahamsson 1998). It was shown that CGA positive cells did not express androgen receptor and were thereby forming a hormone-resistant contingent (Bonkhoff 1998). The detection of this cell population on its own is not indicative of proliferation, even though CGA immunohistochemistry often depends on sampling quality and yields focal or scattered positivity in many cases (Abrahamsson *et al* 1998). Nevertheless, it is most likely that these neuroendocrine contingents exert endocrine and paracrine stimulation on contiguous cells by secreting bioactive peptides including those coming from CGA processing.

2.2 CGA Assessment in Plasma

The clinical value of CGA assessment in plasma or serum had first been demonstrated for pheochromocytoma (O'Connor and Deftos 1998) and rapidly extended to other endocrine tumours with particularly significant elevations for intestinal carcinoids and pancreatic endocrine tumours (O'Connor *et al* 1993, Nobels *et al* 1997). Unlike other biological markers such as plasmatic catecholamines, CGA levels in blood remain unaffected by stress or by any medication used for pheochromocytoma treatment (Hsiao *et al* 1990). Moreover, similar CGA levels were found for patients suffering from essential hypertension and normal individuals, which reinforced the interest in this marker for pheochromocytoma diagnosis. On the other hand, frequent elevations have been noted in case of impaired renal function and this possibility should be investigated as part of the overall patient assessment (Hsiao *et al* 1990).

In a recent study, Nobels *et al* (1997) have shown that plasmatic CGA concentration was a general indicator of neuroendocrine differentiation, but could not challenge the results from more specific secretions such as NSE in small cell lung carcinoma. It was also clear from the same study that there was a correlation between CGA levels and tumour burden. Several authors have also observed a similar correlation among a population of patients suffering from neuroblastoma at different stages (Hsiao *et al* 199 1, Wassberg *et al* 1996). Other authors have more recently demonstrated that plasmatic CGA elevation in rats grafted with the SH-SY5Y neuroblastoma cell line was associated with tumour extension, unlike NSE or urinary homovanillic acid levels.

As mentioned earlier, the occurrence of neuroendocrine cell contingents in adenocarcinoma is not negligible, as numerous studies of prostatic carcinoma have shown. In a restricted series of patients with stage D2 prostatic adenocarcinoma, Kadmon *et al* (1991) observed a significant increase of CGA levels in 50 % of the cases. These results were confirmed by others (Cussenot *et al* 1996, Deftos 1998), where admittedly limited

populations presented CGA elevations, mostly associated with advanced stages of the disease. Pathological CGA levels could also be correlated to a shorter survival, even for earlier stages (Deftos *et al* 1996).

3. METHODOLOGICAL ASPECTS OF CGA IMMUNOASSAY

Similarly to the work published by O'Connor *et al* (1984, 1989), most CGA immunoassays have been developed on the basis of a competitive assay using iodinated CGA, following purification of the protein from human chromaffin granules. It appears, therefore, that the majority of the clinical results obtained refer to the assessment of intact CGA and most probably to all or part of the fragments generated after CGA proteolytic degradation.

More recently, a few studies were carried out with an immunoenzymometric assay using a polyclonal antibody raised against the 23 kDa C–terminal fragment of human CGA. In most cases, the same antibody was used on the solid phase (catcher antibody) as well as conjugated to the enzyme (Boomsa *et al* 1995). In spite of a significant correlation, the comparison of this system with a radioimmunoassay using the same antibody gave rise to a certain number of inconsistent results (Syversen *et al* 1994). Given the importance of CGA processing, particularly at the C-terminal domain, it may be speculated that a more or less selective recognition of the different fragments occurs.

The question of the clinical value of the assessment of a possibly more specific fragment coming from the proteolytic breakdown of CGA could be raised. Nevertheless, the comparison with the pancreastatin assay (249-301 degradation fragment) has shown that the results were indisputably in favour of total CGA assessment (Stridsberg *et al* 1995).

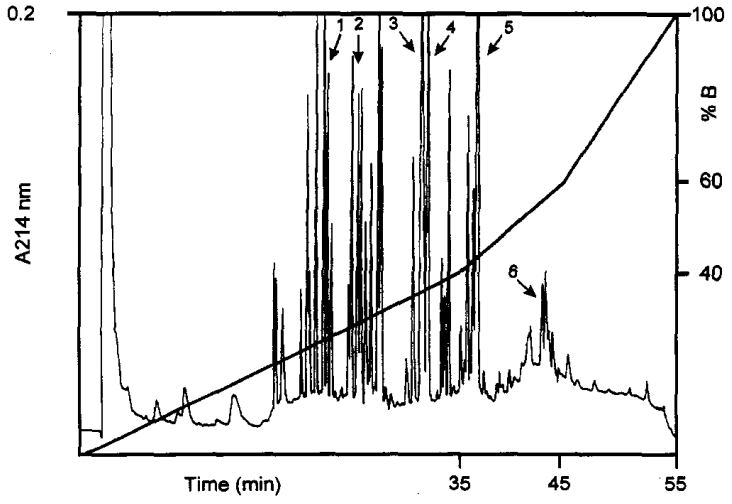
4. DEVELOPMENT OF AN IMMUNORADIOMETRIC ASSAY FOR HUMAN CGA

According to the data previously published, the clinical value of CGA assessment relies on the detection of both intact CGA and its main degradation fragments. At the same time, CGA proteolysis was shown to be a recurrent event that concerned simultaneously both N and C-terminal ends (Metz-Boutigue *et al* 1993). The aim of this study was therefore to obtain

antibodies directed against the median part of the protein, putatively less affected by degradation, in order to trap total CGA.

We therefore generated a first series of mouse monoclonal antibodies (Mabs) after immunisation with human CGA purified from pheochromocytoma (Degorce *et al* 1999). In a second step, we used a preparation of recombinant human CGA (rhCGA) produced in E. coli to immunise a new set of mice (Taupenot et al 1995). Hybridoma screening was performed against iodinated rhCGA, and a total of 24 clones were selected for further investigation.

A)



B)

Fraction	Antibody	N-terminal sequence	Fragment	Mass (Da)
1	CGS17	RLEGQEE	339-355	2053.9
2	CGS30	AEGNNQA	145-197	5635.8
3	CGS20	EIRKGESRS	246-303	6224.8
4	CGS06	GLSAEPG	198-245	5265.4
5	CGS21	LXFRARA	356-400	5145.8
6	CGS32	GXTEVMKXIV	10-400	43532
-	CGS04	LEGQEE	340-394	n.d.
-	CGS10	GYPEEK	395-439	n.d.

Figure 1. HPLC profile of proteolysed rhCGA (A) and characteristics of the immunoreactive fragments. Mabs CGS04 and CGS10 were mapped after separation of peptides by 2-D electrophoresis followed by westem-blot and N-terminal sequencing. Mass spectroscopy was not carried out in these cases.

Epitope mapping was carried out on Biacore®, again using rhCGA. Antibodies could be classified into 8 distinct groups (Degorce *et al* 1999), and one antibody representative of each group was further addressed on the CGA sequence. For this second cartography, we monitored the proteolysis of rhCGA by endolysine C or trypsin. All generated fragments were then isolated by HPLC and tested against the 8 representative Mabs. All peptides recognised were next submitted to N-terminal sequencing by Edman degradation followed by mass spectroscopy, which allowed the precise deduction of the detected sequences.

As shown in Figure 1, epitopic groups mainly span the two C-terminal thirds of human CGA, with the exception of CGS32 which most likely recognises a conformational epitope.

Following the above assumptions, we developed a radioimmunoassay (RIA) with one of the Mabs against the median part of the protein, CGS06. The antibody, immobilised on a solid phase, bound radiolabelled rhCGA. This binding was displaced by increasing CGA amounts contained in plasma or sera. We performed a clinical evaluation of this assay with 46 plasma from healthy subjects and 21 1 others from patients with neuroendocrine or non neuroendocrine cancers (Figure 2). Wilcoxon-Mann-Whitney analysis showed statistically significant differences when each population was compared to the normal one ($p < 0.0001$ in all cases), except that of small cell lung cancers which was too small to be analysed. Lower differences were observed when the comparison was made versus the population of other cancers, but the presence of mixed tumours could not be totally excluded among the 32 cases. These results were in accordance with previously published data (Hsiao *et al* 1990, O'Connor *et al* 1989, Stridsberg *et al* 1985).

All antibodies were then tested in pairs (one solid phase, one tracer) and the performance of each combination was evaluated through the assessment of three pools i) of normal plasma, ii) of plasma coming from patients with pheochromocytoma, iii) of plasma from patients with intestinal carcinoids. The three samples were assayed in the reference RIA and were found to be at 40, 675 and 1600 ng/ml respectively. Our results showed that only the combinations involving median-type antibodies (i.e. antibodies against the median part of the protein : CGS30, CGS06, CGS20) could discriminate among the pools, according to the RIA results. On the other hand, all combinations with C-terminal type antibodies showed either a lack of sensitivity, or were not able to discriminate the pheochromocytoma pool from the normal one (with, for some of them, a pathological/normal ratio lower than 1), or the pheochromocytoma from the carcinoid.

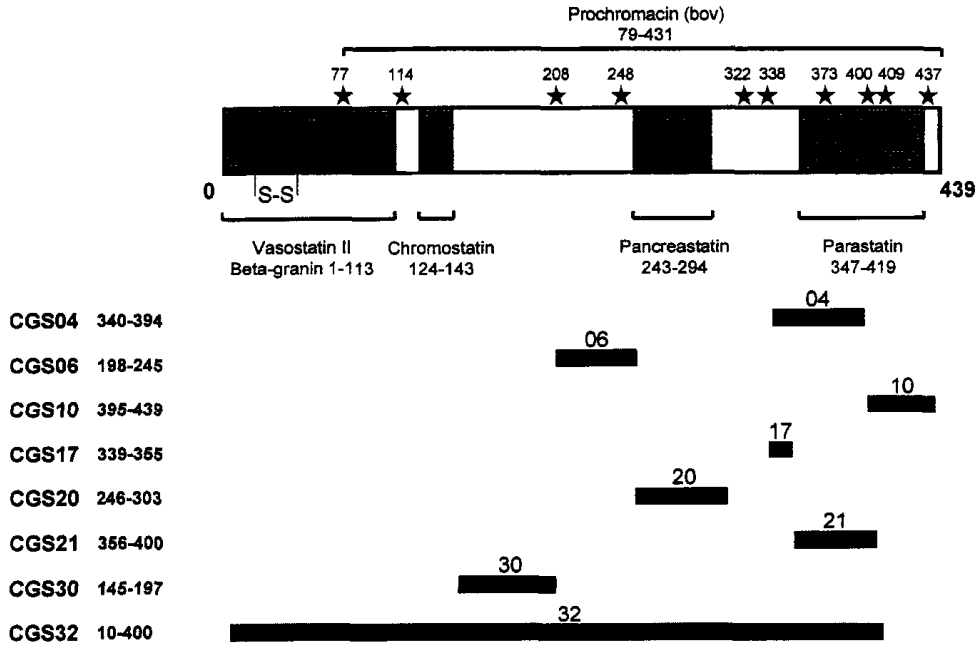


Figure 2. Human CGA sequence and position of the different epitopes. Stars and corresponding numbers indicate dibasic cleavage sites.

Finally, a series of 20 normal plasma and 39 plasma from neuroendocrine tumours were tested in three immunoradiometric assays associating the median-type Mab CGS06 with other antibodies spanning the C-terminal half of CGA, and compared to the data obtained with the reference RIA (Fig 4). When CGS29, a Mab directed against the C-terminal end, was used, CGA was undetectable in most of the samples. This confirms the extensive proteolysis of this region which encompasses 4 putative cleavage sites, An intermediate situation was observed with CGS04 whose epitope is obviously less affected by the degradation. In this case however, CGA concentration was significantly lowered in 8 samples out of 39. The CGS06/CGS30*, which recognises respectively the 198-245 and 145-197 domains, was correlated to the RIA. In its final configuration, the CGA RIACT assay was performed in two steps (over night + 1 hour) at room temperature. Assay optimisation allowed improvement of between and within run precision (lower than 5 and 8 % respectively). The detection limit was found to be 1 ng/ml and the hook effect was over 210,000 ng/ml.

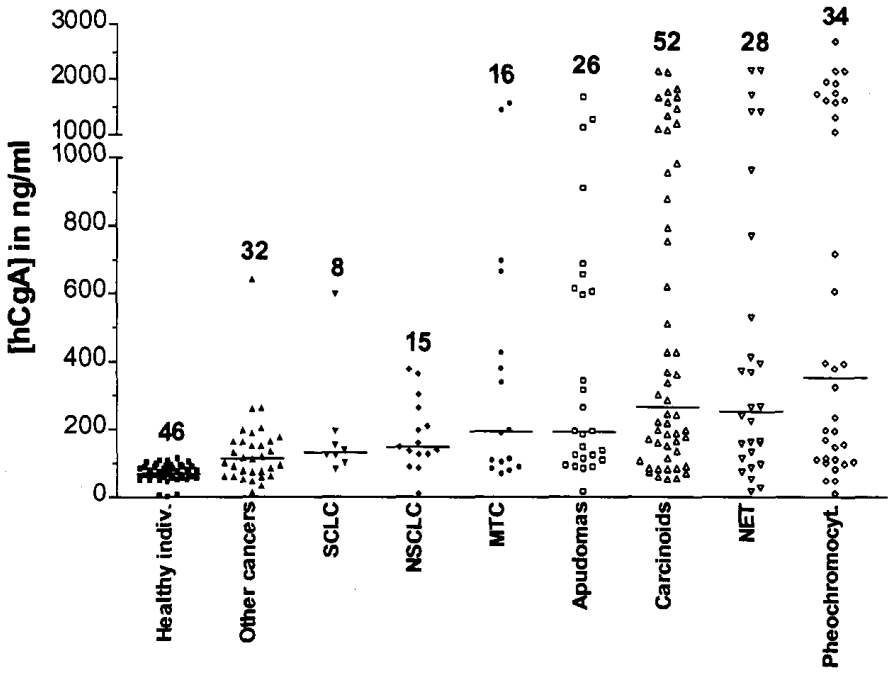


Figure 3. Distribution of the different populations assayed with the reference RIA. Population sizes are shown above each plotted series. Line segments represent median values. SCLC : small cell lung cancer. NSCLC : non small cell lung cancer. MTC : medullary thyroid carcinoma. NET : neuroendocrine tumours.

5. CONCLUSION

Chromogranin A is a multi-facet protein, playing an essential pro-hormonal role through its proteolysis, and the anti-bacterial properties of some of the degradation fragments (Lugardon *et al* 2000) open indisputable perspectives concerning the diversity of its functions. CGA has long been used as a generic marker for the determination of neuroendocrine differentiation, and numerous studies have more recently demonstrated its clinical value in serum. Because of the complexity of its proteolysis and post-translational maturation (Simon and Aunis, 1989, Gadroy *et al* 1998), CGA assessment needs to take into account both intact and major fragmented forms of the protein. Throughout the development of this assay, it was shown that only an appropriate positioning of antibodies on the median domain of the protein could enable a reliable determination of total

circulating CGA, since this region is seldom affected by proteolysis. Using the CGA RlACT assay, Baudin *et al.* (1998) have shown in a comparative

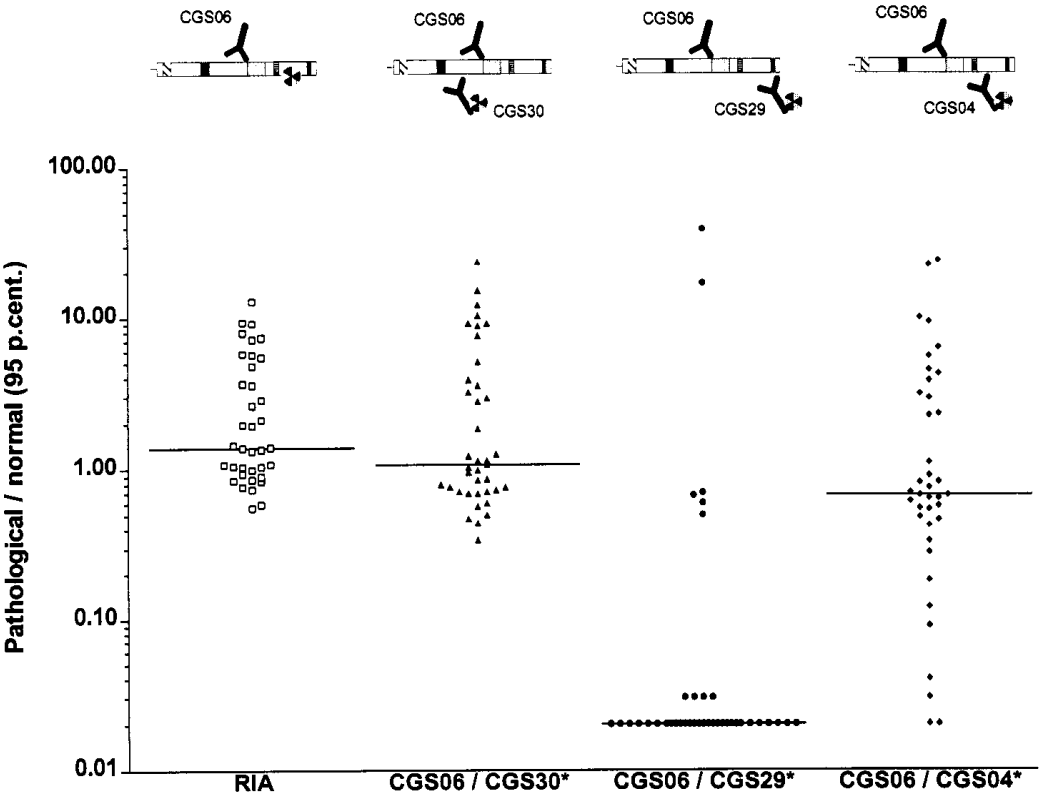


Figure 4. Comparison of three different immunoradiometric assays versus the reference RIA. Data are expressed as the concentration ratio of pathological plasma / 95 percentile value of the normal population (n = 20). Line segments indicate the median value for each group.

study with NSE that CGA concentration in patients suffering from neuroendocrine tumours was particularly well correlated with tumour extension and was therefore a potential marker for disease follow-up. In a recent study on prostatic pathologies, Ravery *et al* have also underlined the predictive value of CGA immunodetection in tissues (Ravey *et al* 1996). Similarly, elevation of CGA levels were shown to depend on tumour stage and significantly higher figures were obtained for patients with cancer compared to those suffering from prostatic hyperplasia.

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CHROMOGRANIN A AND TUMOR NECROSIS FACTOR-α (TNF) IN CHRONIC HEART FAILURE

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1. INTRODUCTION

Chronic heart failure (CHF) is a clinical syndrome related to the inability of the heart to pump blood at a rate commensurate with the metabolic demand of tissues due to abnormalities in the cardiac function.

Several hormonal and neuroendocrine changes have been described in CHF (Nicholls *et al* 1996). For instance, catecholamines, vasopressin, endothelins and components of the renin-angiotensin-aldosterone system are activated to induce vasoconstriction and sodium-water retenction. Other hormons are also secreted to counterbalance these effects, such as atrial natriuretic peptides and brain natriuretic peptides. Since some of these hormons are co-stored with chromogranin A (CGA) within the secretory granules of endocrine cells (Winkler and Fischer-Colbrie 1992), we have recently measured the levels of CGA in the serum of CHF patients and normal subjects. We have found that circulating CGA increases in patients, depending on the severity of the disease, and that the increase is an independent prognostic indicator of mortality (Ceconi *et al* 2000).

Several lines of evidence suggest that CHF is also characterised by elevated levels of molecules usually associated with an inflammatory response. Tumor Necrosis Factor α(TNF), soluble TNF receptors (sTNF-Rs), soluble CD14, intercellular adhesion molecule 1, and interleukin-6 are increased in CHF and have pathophysiological and prognostic implications

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(Anker *et al* 1997, Ferrari *et al* 1995b, Kapadia *et al* 1998, Levine *et al* 1990, MacGowan *et al* 1997, Torre-Amione *et al* 1999). In particular, the negative inotropic effects of TNF, together with its known activity in producing anorexia and cachexia, left ventricular dysfunction and remodelling, pulmonary edema, and cardiomiopathy (Eichenholz *et al* 1992, Hegewisch *et al* 1990, Lo *et al* 1992, Mann 1996, Yokoyama *et al* 1993), suggest that this cytokine plays a primary pathophysiologic role in the development of heart failure.

While TNF may contribute directly to the syndrome of heart failure, through effects on the periphery or on cardiac cells (Torre-Amione *et al* 1999), the biological meaning of the raised levels of sTNF-Rs in this disorder and the mechanism of their release are more obscure.

To investigate the relationships between neuroendocrine activation and activation of the TNF/sTNF-Rs system we have measured CGA, TNF and sTNF-Rs in the serum of CHF patients. We have found that circulating CGA is increased and strongly correlates with sTNF-Rs, and to a lower extent with TNF, hinting at a functional integration of the mechanisms that cause CGA secretion and TNF receptor shedding in these patients.

2. CIRCULATING CGA CORRELATES WITH TNF AND sTNF-Rs IN CHF PATIENTS

One-hundred-sixty consecutive elective patients with CHF (NYHA I, n = 9 ; NYHA 11, n = 63 ; NYHA 111, n = 57 ; NYHA IV, n = 31) and 103 healthy subjects were studied. Patients hospitalised to be submitted to a possible cardiac transplantation were also enrolled. Patients with significant concomitant diseases such as infection, renal failure, pulmonary disease, thyroid disease, malignancy or collagen vascular disease were excluded. The aetiology of CHF was coronary artery disease (61.9%), dilated cardiomyopathy (25.6%), valvular heart disease (6.3%), hypertensive heart disease (0.6 %), while the remaining part (5.6%) was uncertain. The clinical characteristics of these patients, as well as their therapeutic regimens (including the use of ACE-inhibitors and b-blockers) are in keeping with the CHF population followed by Hospital Cardiological Units in Italy. None of the patients were treated with intravenous inotropic agents or with mechanical-support assistance.

Serum CGA was measured in 162 patients and 103 healthy subjects using a sandwich ELISA based on the anti-CGA mAb B4E11 and an anti-CGA rabbit polyclonal antiserum (detection limit : 10 ng/ml). MAb B4Ell is a mouse IgGl which recognises an epitope overlapping with residues 68-70 of

human CGA (Corti *et al* 1996). Intra and inter-assay coefficients of variation (CV,%) were 5.2 ± 1.09 % and 28.5 ± 15.2 % (n = 10), respectively ; mean analytical recovery of internal CGA standards added to samples containing various amounts of CGA was 105.9 % (range 98-118, n = 10). A good parallelism of response with recombinant CGA assays was obtained in five serum samples at various dilutions. The specificity for CGA was further confirmed by the following observations : a) spiking of five serum samples with 5 µg/ml of CGA(68-91) peptide, containing the B4Ell epitope, efficiently inhibited the ELISA signal ; b) no signal was obtained when mAb B4E11 was omitted or when an irrelevant IgGl was used in the solid-phase.

TNF and sTNF-Rs antigens were measured in the serum of 129 patients (NYHA I, n = 8 ; NYHA II, n = 48 ; NYHA III, n=42 ; NYHA IV, n= 31) as described (Ferrari *et al* 1995a).

The circulating levels of CGA in normal subjects (median 71.6 ng/ml, interquartiles 39-116.3) were lower than in CHF patients. Class IV patients showed the highest serum levels of CGA (median 545 ng/ml (inter quartiles 231-1068), being statistically different from class III (median 279 ng/ml, inter quartiles 203-516) ($p < 0.001$). Serum levels of CGA in class II (median 146.9 ng/ml, inter quartiles 108.3-265.5) were significantly higher ($p < 0.05$) than in class I (median 109.7 ng/ml, inter quartiles 97-137) and lower ($p < 0.05$) than in class 111 (Ceconi *et al* 2000).

Antigenic TNF was modestly increased in patients depending on the severity of CHF (mean ± SD (pg/ml) : normal subjects, 23.1 ± 4.65 ; class I, 29.89 ± 7.13 ; class II, 33.5 ± 15.2 ; class III, 38.3 ± 10.2 ; class IV, 44.6 ± 15.31). Although a low cytolytic activity was detectable in some patients, the mean activity was not significantly different from that of the normal subjects, suggesting that inactive forms were present, presumably related to complexes with soluble receptors and/or monomeric subunits (Corti *et al* 1994) .

STNF-RI was also increased depending on the severity of the disease (mean ± SD (ng/ml) : normal subjects, 0.96 ± 0.18 ; class I, 1.37 ± 0.55 ; class II, 1.57 ± 0.63 ; class III, 1.96 ± 0.81 ; class IV, 3.5 ± 2.31).

A similar increase was observed also with sTNF-RII (mean ± SD (ng/ml): normal subjects, 2.28 ± 0.42 ; class I, 3.49 ± 1.23 ; class II, 3.98 ± 1.69 ; class III, 4.38 ± 1.64 ; class IV, 6.6 ± 4.61).

CGA levels correlates closely with sTNF-RI ($r = 0.566$, $p < 0.001$), sTNF-RII ($r = 0.776$, $p < 0.001$) and weakly with TNF antigen ($r = 0.39$, $p < 0.001$).

Since CGA is likely secreted by neuroendocrine cells in these patients these correlations suggest that the neuroendocrine mechanisms that lead to CGA release and those that trigger activation of the TNF system are functionally integrated in CHF.

3. WHICH IS THE SITE OF PRODUCTION OF CGA AND TNF/sTNF-Rs IN CHF?

The tissue origin of circulating CGA and TNF/sTNF-Rs in CHF is unknown. The levels of CGA in CHF patiens are relatively high. In some patients we have detected up to 50-100 nM, thus levels comparable to those found in patients with neuroendocrine tumors. While in tumor patients the source of abnormally high levels of CGA is often the tumor itself, speculation on the tissue origin of CGA is more difficult.

CGA was originally discovered as an abundant soluble protein of adrenal medullary chromaffin granules and was found later to be a member of a family of regulated secretory proteins present in the electrondense granules of many other neuroendocrine tissues and of the nervous system (Qian *et al* 1988, Rosa and Gerdes 1994, Serck-Hanssen and O'Connor 1984, Simon and Aunis 1989, Winkler and Fischer-Colbrie 1992). For instance, CGA is a component of dense-core synaptic granules in many areas of the central and peripheral nervous systems (Adam *et al* 1993, Muñoz *et al* 1990) and is co-stored with various hormones in the secretory vesicles of neuroendocrine cells of the gastrointestinal tract (Qian *et al* 1988), hypophysis (Serck-Hanssen and O'Connor 1984) , thyroid (O'Connor *et al* 1984), parathyroids (Cohn *et al* 1982) and pancreas (Ehrhart *et al* 1988). CGA is released in the extracellular environment together with the co-resident hormones and reaches the blood stream via the capillaries or the lymph vessels (Winkler and Fischer-Colbrie 1992).

Although the atrial myocardium also stores CGA, we found no or little correlation with atrial natriuretic peptide. This argues against the hypothesis that atrial myocytes are the main source of CGA. Other CGA-rich organs are likely to contribute to the circulating levels. Of note, no correlation was observed between CGA and single hormones, such as noradrenaline, adrenaline, aldosterone, and plasma renin activity, suggesting that more than one organ or tissue contributes to the circulating CGA.

TNF, like CGA, can also be produced by many cell types, including macrophages, endothelial cells, lymphocytes, fibroblasts, muscle cells, and cardiac myocytes (Fiers 1995, Tone-Amione *et al* 1996). The origin of circulating soluble receptors in CHF is even more obscure, as besides the heart, a large number of different cell types bear TNF receptors and can potentially shed soluble receptors (Fiers 1995, Torre-Amione *et al* 1995, Torre-Amione *et al* 1996, Vandenabeele *et al* 1995). Thus, although several lines of evidence suggest that the heart itself could be a source of TNF and sTNF-Rs, speculation on the cellular origin of these circulating molecules in CHF is very difficult.

The trigger of TNF production is also unclear. Recently, it has been

observed that sCD14 is also increased. On this basis it has been proposed that activation of the TNF system could be a consequence of an increased blood pressure in the mesenteric artery, that in turn causes an increased permeability of the bowel, bacterial translocation and monocyte/macrophage activation by endotoxin via CD14, marked by sCD14 (Anker *et al* 1997). However, activation of the TNF system by endotoxin still remain speculative as other stimuli could cause sCD14 shedding.

Considering the strong neuroendocrine activation in this patients and the notion that several hormones and neurotransmitters can affect the physiology of immune cells it is possible that the neurohormonal mechanism that leads to the increased production of CGA is also at the base of the raised TNF/TNF-Rs levels in CHF. Interestingly, a previous study showed that 10 nM CGA can trigger TNF production by rat microglial cells in vitro (Ciesielski-Treska *et al* 1998). We have therefore hypothesized that an increased production of CGA, or a fragment of it, may contribute to a local or a systemic production of TNF by inflammatory cells. However, when we challenged monocytes/ macrophages from normal subjects with recombinant CGA no TNF/sTNF-Rs release was observed in vitro, apparently arguing against this hypothesis. Still we cannot exclude that other cells are targets of CGA in CHF patients or that combination with other factors are necessary for this effect.

Alternatively, both circulating CGA and TNF/sTNF-Rs may simply represent an epiphenomenon of a pathophysiological mechanism activated in CHF.

4. DOES CGA AND TNF PLAY A PHYSIOPATHOGENETIC ROLE IN CHF?

Although the role of CGA in the maturation of secretory granules is more delineated the function of this protein once it is secreted is more obscure. It has been proposed that CGA represents a precursor of biologically active peptides with endocrine, paracrine, and autocrine functions (Deftos 1991). For instance, CGA residues 248-293 were found to be homologous to pancreastatin, a pancreatic peptide that inhibits insulin secretion (Tatemots *et al* 1986), whereas catestatin, a peptide corresponding to residues 344-364 of bovine CGA, inhibits secretion of catecholamines from chromaffin cells and noradrenergic neurites (Mahata *et al* 1997). Retrogradely perfused and nerve-stimulated bovine adrenal medullae release the CGA fragments corresponding to aminoacids 1-76 and 1-113. These fragments have been named vasostatin I and II (VS-1 and VS-2), respectively, because of their inhibitory effects on vascular tension (Aardal and Helle 1992, Aardal *et al* 1993, Helle *et al* 1993). We do not know whether vasostatins and other peptides are released in

circulation in CHF patients since our assay is unable to distinguish the intact CGA molecule from fragments. Even in the case vasostatin fragments are released one should wonder whether their levels are sufficiently high to contribute to the mechanisms that counterbalance the vasoconstrictive drive in these patients.

Another question that is raised by the results of this study is whether the correlations between TNF/sTNF-Rs and CGA have a functional significance. It has been proposed that overexpression of TNF in the heart contributes to important changes in cardiac structure and left ventricular remodelling, possibly by affecting the adhesion of cardiac myocytes to extracellular matrix proteins (Mann *et al* 1995), or by affecting the extracellular matrix tunover (Bozkurt *et al* 1998). Indeed, recent data have shown that pathophysiologically relevant concentrations generated with osmotic infusion pumps in rats promote left ventricular disfunction and remodelling, in part by causing degradation of the fibrillar collagen matrix (Bozkurt *et al* 1998). Marked changes in the structure and shape of the heart of transgenic mice overexpressing TNF within the heart were observed also by other groups (Bryant *et al* 1998, Kubota *et al* 1997). Thus TNF has been proposed as a candidate, along with norepinephrine, endothelin and angiotensin 11, for the mediation of progression of myocardial remodelling and dysfunction (Bristow 1998).

Recently, we found that CGA fragments can modulate the adhesion of fibroblasts to extracellular matrix proteins (Gasparri *et al* 1997). CGA N-terminal fragments corresponding to residues 1-78, 1-115 and 7-57 can induced adhesion and spreading of fibroblasts on plates coated with collagen I or IV, laminin, FCS but not on BSA. The long incubation time required for optimal adhesion (4 h) suggest that the adhesive activity is likely indirect and requires other proteins made by the cells. Interestingly, a recent work by Soriano and coll. (Soriano *et al* 1999) have shown that 5 nM CGA can increase deposition of basement membrane components, such as collagen type IV, laminin and perlecan by mammary epithelial cells and alter ductal morphogenesis *in vitro.* These author proposed that CGA may play a role to modulate basement membrane remodelling, even in tissue distant from the site of release.

Given the importance of cardiac fibroblasts in extracellular matrix production and collagen turnover in myocardial remodelling and repair (Weber 1997), and considering that factors that affects fibroblast adhesion can affect also their physiology, an interesting possibility is that excessive secretion of TNF and CGA contribute in regulating cardiac remodelling.

Although no definite answer can be given to most of the above questions, the results suggest that a regulatory link exists, direct or indirect, between cells that release CGA and TNF/sTNF-Rs in CHF that may deserve further investigations.

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CHROMOGRANIN A (CGA) AND THE ENTEROCHROMAFFIN-LIKE (ECL) CELL

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1. INTRODUCTION

Chromogranins are acidic proteins confined to secretory granules in neuroendocrine cells (Huttner *et al* 1991). They seem to have an important role in granule formation (Parmer *et al* 1993) and to be precursors for biological active peptides (Tatemoto *et al* 1986). Since chromogranins are localized to neuroendocrine cells (Schmid *et al* 1989), they may be used as markers for hyperplasia and neoplasia originating from these cells. Moreover, chromogranins are released together with different active hormones/mediators and may be detected in blood by sensitive immunoassay methods (O'Connor *et al* 1989, Syversen *et al*1994).

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2. CHROMOGRANIN A IN PLASMA

The concentration of chromogranins in blood reflects the mass of neuroendocrine cells (Borch *et al* 1997). Therefore, determination of chromogranins and particularly one of them, chromogranin A (CGA) in blood has become the initial examination when suspecting a neuroendocrine tumor. Neuroendocrine cells are spread among other cells in virtually every epithelial surface of the body. They can give rise to tumors where the tumor cells have maintained their capability to produce active hormones/mediators and give rise to syndromes secondary to hormonal over-production. Some of the neoplastic neuroendocrine cells do not produce active hormones/mediators, but nevertheless release CGA which may be used in the diagnosis of such tumors (O'Connor and Deftos, 1986). Furthermore, it has become apparent that the neuroendocrine cell gives rise not only to the relatively benign so-called carcinoid tumors, but also highly malignant ones like the oat-cell carcinoma of the lung (Bensch *et al* 1968), and neuroendocrine carcinoma of the stomach (Rindi *et al* 1999) to which an important proportion of gastric cancers previously classified as adenocarcinomas of the diffuse type belongs (Waldum *et al* 1991,1998; Qvigstad *et al* 2000). It is also reason to postulate that the neuroendocrine cells play an important role in the carcinogenesis of other organs than those two foregut derived organs, the lungs and the stomach. In fact, neuroendocrine differentiated tumor cells are found in many tumors (Syversen *et al* 1995), and their significance has yet not been fully assessed. The embryological origin of the neuroendocrine cells (Andrew *et al* 1998, Waldum *et al* 1999) has great implications for the significance of neuroendocrine tumor cells in tumors classified as adenocarcinomas.

3. THE ENTEROCHROMAFFIN-LIKE (ECL) CELL

The neuroendocrine cells in the stomach are among the most abundant of the body, and among these the enterochromaffin-like (ECL) cell is the most prevalent constitutes about 65 % of such cells in the rat, and 35 % in man (Sundler and Håkanson, 1991). Gastrectomy in the rat markedly reduces the concentration of pancreastatin, a split product of CGA (Håkanson *et al* 1995), indicating that the ECL cell releases a disproportionate large quantity of CGA to the blood. The release of CGA to the blood does not depend upon the co-release of hormones/mediators as depletion of histamine in the ECL cell did not reduce the pancreastatin release from the rat isolated stomach (Chen *et al* 1996).

In man the ECL cell constitutes about 35 % of neuroendocrine cells in the oxyntic mucosa of the stomach (Sundler and Håkanson, 1991). The ECL cell is able to self-replicate (Tielemans *et al* 1990), and its growth (Tielemans *et al* 1990) as well as function (Sandvik *et al* 1987) is regulated by gastrin by interaction with the gastrin CCK-B receptor (Brenna and Waldum, 1992). Hypergastrinemia induces ECL cell hyperplasia in rodents (Havu, 1986, Mattson *et al* 1991) as well as man (Hodges *et al* 1981, Maton *et al* 1990). It has been claimed that gastritis itself could provoke the growth of the ECL cell (Lamberts *et al* 1993). However, ECL cell density is similar in patients with the same level of hypergastrinemia, whether induced by atrophic gastritis (Sjsblom *et al* 1989) or gastrinoma (Maton *et al* 1990), strongly suggesting that gastrin is the important factor. The vagal nerves also have an effect on ECL density (Axelson *et al*1988) probably mediated by pituitary adenylate cyclase activating polypeptide (PACAP), which like gastrin, stimulates the release of histamine from the ECL cell (Lindstrsm *et al* 1997). Even relatively short-lived and moderate rise in gastrin induces an increase in CGA as seen in patients treated for three months with a proton pump inhibitor inducing a slight hypergastrinemia and secondary ECL cell hyperplasia (Waldum *et al* 1996). Patients with hypergastrinemia due to reduced gastric acidity secondary to gastritis may have a pronounced increase in blood CGA values (Borch *et al* 1997). In patients with gastrinoma the gastrinoma tumor cell has been claimed to be the main source of the increased CGA (Goebel *et al* 1999), but ECL cell hyperplasia is probably the most important factor for the CGA elevation in patients with gastrinoma (Syversen *et al* 1993). Thus, the antral gastrin (G) cell does produce much more chromogranin B than CGA (Portela-Gomes *et al* 1997), and it is probable that this is also the case in gastrinoma tumor cells. Moreover, blood CGA increases with blood gastrin up to a concentration at which gastrin exerts maximal trophic effect on the ECL cell (Goebel *et al*1999), indicating that the ECL cell is a main source for the CGA elevation in gastrinoma patients.

4. ECL CELL HYPERPLASIA

If hypergastrinemia persists for a long time, ECL cell hyperplasia may develop into ECL cell neoplasia in the rat (Havu 1986) and man (Wilander, 1980). With time relatively benign ECL cell carcinoids may be transformed into highly malignant tumors (Qvigstad *et al* 1999). Animals or patients with ECLomas also have increased blood CGA, but hitherto no study has been done following blood CGA in patients with hypergastrinemia to see whether CGA rises when ECL cell carcinoids develop. It should also be taken into

consideration that the ECL cell carcinoids secondary to hypergastrinemia, may develop into more malignant ECL cell carcinomas with loss of differentiation and expression of CGA (Qvigstad *et al* 1999). In fact, an important proportion of human gastric carcinomas is derived from ECL cells (Waldum *et al* 1991,1998; Qvigstad *et al* 2000), which has been confirmed by immunohistochemistry together with the tyramide signal amplification method (Adams, 1992), showing that most of the tumor cells were CGA immunoreactive (Qvigstad *et al* 2000). However, only a small proportion of those ECL cell derived carcinomas had elevated blood CGA values (Waldum *et al* 1998).

5. CONCLUSIONS

To conclude, the ECL cell is important for the blood CGA concentration, and since gastrin is the principle regulator of the ECL cell mass, it is important to assess blood gastrin in patients with CGA elevation (Kleveland *et al* 2000) , particularly since atrophic gastritis with reduced gastric acidity is such a common disease (Siurala *et al* 1968). Moreover, the fact that drug induced reduction of gastric acidity results in hypergastrinemia and thus ECL cell mass elevation (Waldum *et al* 1996), CGA determination in blood may be used to control treatment with inhibitors of gastric acid secretion. When determining CGA in blood it is necessary to know whether the patients are taking inhibitors of gastric acid secretion as well as serum creatinine since reduction of renal function greatly affects blood CGA values (Hsiao *et al* 1990). Furthermore, serum gastrin should be determined in patients with elevated CGA values to detect patients having an increased ECL cell mass secondary to atrophic gastritis, before starting a search for a neuroendocrine tumor in patients with elevated CGA.

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CHROMOGRANINS IN NON-ENDOCRINE TUMOURS

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1. INTRODUCTION

Neuroendocrine cells contain many large dense core granules filled with peptide hormones, prohormone convertases (PC1/3, PC2), amidating enzymes, biogenic amines, and chromogranins. Chromogranin A (CGA) and chromogranin B (CGB) are members of the granin family. CGA and CGB have been isolated from bovine adrenal medulla and their distribution is well known: these two proteins are almost ubiquitously present in the cells of neuroendocrine organs and neuroendocrine tumours. Both CGA and CGB are considered as useful markers for neuroendocrine tumours (Kimura *et al* 1988, 1997a,b) as well as prohormone convertases (PC1/3, PC2) and amidating enzymes (Kimura *et al* 2000). On the other hand, neuroendocrine cells are occasionally present in greater or lesser degree in non-endocrine tissues; along this line they have especially been investigated in the gastrointestinal tract (GI-tract). Neuroendocrine cells in the GI-tract produce many hormones that display paracrine or autocrine functions involved in cell growth and homeostasis. The role of chromogranins in normal tissues, however, is still not elucidated. The presence of neuroendocrine cells in non-endocrine tumours from the breast, prostate, GI-tract and lung has previously been investigated and discussed with regard to their clinical significance.

The familial breast and ovarian cancer suppressor gene BRCAl encodes for an 1863 amino acids RING-finger protein (Miki *et al* 1994). Jensen *et al*

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(1996) reported a perfect granin consensus sequence at BRCAl amino acids 1214-1223 in a functional domain (Table 1); they also showed that the biochemical properties, expression and cellular localisation of BRCA 1 protein share many biochemical features with granins (Jensen *et al* 1996). Mucinous carcinoma of the breast that is a slowly progressive tumour contains many neuroendocrine cells. The majority of neuroendocrine tumours are also slowly progressive tumours. An intrinsic inhibitory function of somatostatin through somatostatin receptors on cell surface in these tumours (Kimura *et al* 1999a), may be one of the reasons for the slow progression. It would therefore be most interesting to see if a relationship exists between the presence of chromogranins and tumour progression, and if the sequence homology of the granins and BRCAl could be a clue to reveal the mechanisms involved in tumour growth and progression.

Table 1. Granin and BRCA sequences (Jensen *et al* 1996)

	Species	Amino acid
Consensus		ENLSXXDXEL
BRCAl	Human	ENLSSEDEEL
BRCA2	Human	ESNSIADEEL
CGA	Human	ESLSAIEAEL
CGB	Human	ENLAAMDLEL

2. CHROMOGRANINS IN NON-ENDOCRINE TUMOURS

There have been many previous investigations on the presence of neuroendocrine cells in non-endocrine tumours. The functions of neuroendocrine cells in such tumours are not elucidated yet. In addition to hormones produced by neuroendocrine cells, CGA and CGB may have some roles on tumour growth or tumour progression. The chromogranins are ubiquitous proteins that are co-stored and co-secreted with many peptide hormones, all appearing to be powerful inhibitors of endocrine secretions (Koeslag *et al* 1999). Recent biological studies have revealed that many bioactive peptides are processed from chromogranins including CGA and CGB (Metz-Boutigue *et al* 1993, Laslop *et al* 1998). No chromogranin-derived peptides directly mediating tumour growth or suppression has been

characterised so far, however, the possibility of discovering such an active peptide cannot be excluded.

3. GRANINS AND BRCAl IN BREAST CARCINOMA

Chromogranins share homology with a short sequence in BRCAl gene product (Jensen *et al* 1996). Tissue localisation of BRCAl in the breast is constantly observed in all ductal and stromal cells, and is very similar to that of mdm2 and p27 (both are p53 regulator) (Kimura *et al* in preparation). Loss of BRCAl immunoreactivity has been reported in both familial breast carcinomas and sporadic breast carcinomas. BRCA 1 was reduced or absent in the majority of familial and early onset breast tumours (Jarvis *et al* 1998). Scully *et al* (1996) stated that RING finger sequence of the BRCAl protein is consistent with a C-terminal globular domain analogue to a p53-binding domain. If chromogranis have similar function in tumour suppression as BRCAl through p53 function, it is reasonable to assume that tumours containing many neuroendocrine cells should progress slowly and be stable. In breast tissue, Bussolati *et al* (l985) found scattered neuroendocrine cells positive to Grimelius argyrophilic staining or to chromogranin immunostaining in normal breast tissue. These cells were dispersed or more often focally localised in some lobular ductules or intralobular and interlobular ducts. CGA has been detected in human colostrum and milk using biochemical techniques (M.H. Metz-Boutigue & D. Aunis personal communication) and immunohistochemistry (Kimura *et al* 1999b). CGA was frequently detected in non-invasive carcinomas, however, the number of cells immunoreactive to CGA remarkably decreased in the invasive carcinomas (Kimura *et al* 1999b). A comparative study showed that the tissue distribution of CGA and BRCAl are very different; however a statistically significant correlation between the expression of CGA and that of BRCAl was found in breast carcinoma devoid of lymph node metastasis (Kimura et al 1999b). This latter study thus suggests that CGA and CGB may play some role on homeostasis in cell growth as well as BRCAl and the disappearance of chromogranins breaks such homeostasis and induces tumour invasiveness and growth.

4. CGA IN OTHER CARCINOMAS

In addition to breast carcinoma, other carcinomas also have scattered neuroendocrine cells. The clinical significance of neuroendocrine

differentiation in these carcinomas has been debated. Expression of neuroendocrine proteins CGA and CGB has been reported to be a negative prognostic factor in prostatic carcinoma (Abrahamsson *et al* 1989, Angelsen *et al* 1997). However, in carcinomas of other organs, such as pulmonary carcinoma (Kiriakogiani-Psaropoulou *et al* 1994, Sundaresan *et al* 1991) or colonic carcinoma (Lloyd *et al* 1998, Foley *et al* 1998, Saclarides *et al* 1 994), no significant correlation between the presence of neuroendocrine cells in tumour tissue and patients' survival has been found. These studies were carried out only in advanced cancers, but not in cancers at early stages. Based on our study on breast cancers, we strongly suggest that the loss of neuroendocrine cells and as a consequence of chromogranins in early carcinoma may be one of the important triggers for promoting advanced carcinoma. Further studies on chromogranins with regard to tumour suppression and promotion are necessary to bring a definite conclusion on this interesting and important topic.

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PART 7

CONCLUDING REMARKS

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31

CHROMOGRANIN A IN HUMAN DISEASE

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1. INTRODUCTION

The chromogranins or secretogranins are a family of acidic soluble proteins initially described in the core of the adrenal medullary chromaffin granules (Helle 1966), but subsequently identified in secretory granules throughout the neuroendocrine system, including both peptide- and amine-secreting endocrine secretory cells (Cohn *et al* 1982, O'Connor 1983, O'Connor *et al* 1983a, O'Connor *et al* 1983b) and a variety of neurons, both central and peripheral (O'Connor and Frigon 1994, O'Connor *et al* 1991).

Here we consider the diagnostic value of chromogranin A (CGA) in human disease, including pheochromocytoma as well as other neuroendocrine neoplasia, and consider its emerging usefulness in discriminating the neuroendocrine character of a variety of other neoplasia.

2. MEASUREMENTS OF CGA

The first human CGA measurements were derived from a radioimmunoassay (O'Connor and Bernstein 1984) based on CGA purified

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from human pheochromocytoma (O'Connor *et al* 1984). It was necessary to develop a species-specific human CGA immunoassay, since there is poor interspecies immunologic crossreactivity among CGAs (O'Connor and Bernstein 1984, O'Connor and Frigon 1984) perhaps because the immunodominant epitopes of CGA may lie in regions of the mid-molecule whose amino acid sequence is not well conserved across species (Gill *et al* 1992). Later human CGA radioimmunoassays contained modifications to decrease throughput time (O'Connor *et al* 1989). As detected by such human CGA radioimmunoassays, human CGA immunoreactivity circulates in the rage of ~1-3 nM.

The human CGA radioimmunoassay was quickly applied to plasma from patients with pheochromocytoma, 80-90% of whom had marked plasma concentration elevations (O'Connor and Bernstein 1984).

3. PHEOCHROMOCYTOMA

Pheochromocytoma remains a challenging diagnosis (O'Connor 1996) for which measurement of CGA may be a valuable adjunct. In patients with sporadic (non-familial) pheochromocytoma, elevation of CGA was 83% sensitive and 96% specific for the diagnosis (Hsiao *et al* 1991), and CGA concentration correlated with tumor mass. Plasma CGA was little (if at all) influenced by pharmacologic diagnostic tests for pheochromocytoma (alpha-adrenergic blockade with phenoxybenzamine or phentolamine, alpha-2-agonist treatment with clonidine, beta-adrenergic blockade with metoprolol, or non-exocytotic catecholamine release with tyramine).

In familial pheochromocytoma (Hsiao *et al* 1990b), CGA measurement was a useful (sensitive and specific) test for pheochromocytoma among members of an extended kindred at risk for pheochromocytoma because of autosomal dominant Von Hippel-Lindau disease. After resection of pheochromocytoma, plasma CGA falls bi-exponentially with an initial component half-life of ~16 minutes, and a subsequent slower decline with an ~8.7 hour half-life.

In a subject with factitious (feigned) pheochromocytoma, normal plasma CGA was an important clue to the factitious illness, despite episodic elevation of blood pressure and plasma catecholamines (Kailasam *et al* 1995). In malignant pheochromocytoma, plasma CGA is far higher than in benign pheochromocytoma, and there is less overlap in CGA than in plasma epinephrine or norepinephrine values; thus, CGA measurement may be of value in this diagnostic distinction (Rao *et al* 2000).

4. OTHER NEUROENDOCRINE NEOPLASIA

Shortly after the observation that CGA was found widely distributed in peptide- and amine-containing secretory granules throughout the neuroendocrine system, a human CGA radioimmunoassay was applied to plasma of patients with neuroendocrine neoplasia (O'Connor and Deftos 1986). Once again, this assay was based on CGA isolated from chromaffin granules of human pheochromocytoma. Plasma CGA was elevated in patients with not only pheochromocytoma, but also parathyroid adenoma or hyperplasia, medullary thyroid (C-cell) carcinoma, C-cell hyperplasia, small-cell lung carcinoma, pancreatic islet cell tumor, aortic body tumor, and carcinoid tumor; indeed, the very highest plasma CGA concentrations (up to >100,000 ng/ml, or >1000 times normal) were observed in patients with metastatic carcinoid tumors. By contrast, patients with non-peptide-producing endocrine neoplasia (such as papillary/follicular thyroid carcinoma, or adrenal cortical carcinoma) or non-endocrine neoplasia (osteosarcoma, or pancreatic adenocarcinoma) had normal plasma CGA. Thus, measurement of plasma CGA emerged as a probe of the neuroendocrine character of human neoplasms. In several neoplasms with possible or disputed neuroendocrine character (renal cell carcinoma, malignant melanoma, choriocarcinoma, or thymoma) plasma CGA was normal (O'Connor *et al* 1989).

In children with neuroblastoma (Hsiao *et al* 1990c) measurement of plasma CGA provided a sensitive (91%) and specific (100%) approach to the diagnosis. CGA rose as a function of disease stage or tumor burden ($r=0.76$, $p<0.01$), and the level of CGA elevation predicted patient survival.

In patients with gastrinoma (Zollinger-Ellison syndrome), CGA is also elevated (Stabile *et al* 1990), though the source of the plasma CGA excess may be the enteroendocrine cells in the stomach rather then the pancreatic gastrinoma itself, since gastrectomy alone markedly reduces plasma CGA in these patients. This is perhaps not surprising, since elevated circulating gastrin in these patients may act as a trophic factor to stimulate enteroendocrine cell proliferation in the stomach (Simonsson *et al* 1988). When this disorder is associated with parathyroid adenoma in patients with Multiple Endocrine Neoplasia Type 1 (MEN-1), the elevation in serum calcium (or perhaps elevated parathyroid hormone itself) might also stimulate CGA release from islet cells or enteroendocrine cells (Nanes *et al* 1989).

5. NEOPLASIA OF MIXED CELL TYPE: PROSTATE AND BREAST

Measurement of circulating CGA has proven to be useful in evaluating the neuroendocrine character of neoplasia (O'Connor and Deftos 1986, O'Connor *et al* 1989). Intriguingly, malignant melanoma, renal cell carcinoma, thymoma, and choriocarcinoma, which might be suspected to have neuroendocrine character, do not seem to produce CGA.

Some common neoplasia, such as carcinomas of the prostate and breast, may be heterogeneous in histologic cell type. Some cells in prostate (Deftos and Abrahamsson 1998) or breast (Sapino *et al* 1998) carcinoma immunostain for CGA, suggesting neuroendocrine character, and the chromogranin expression pattern may correspond to tumor prognosis (Cox *et al* 1999). Plasma CGA is elevated in a subset of prostate cancer patients, in whom it may predict hormone responsiveness (Wu *et al* 1998).

In patients with a variety of non-neuroendocrine neoplasia undergoing antineoplastic chemotherapy, especially with the drug cisplatin, both plasma CGA and urinary 5-hydroxyindole acetic acid (5HIAA, a serotonin metabolite) rise in parallel shortly after drug treatment, coinciding with chemotherapy-associated emesis (Cubeddu *et al* 1995a, 1995b). The co-release of serotonin and CGA likely represents exocytosis from gut enteroendocrine cells; the released serotonin may then act in the central nervous system to trigger emesis.

6. ARTERIAL HYPERTENSION (HIGH BLOOD PRESSURE)

6.1 Human Hypertension

Human "essential" (idiopathic) hypertension is a common though etiologically complex trait with substantial heritable determination, and evidence of an etiologic role for increased sympathoadrenal activity (O'Connor *et al* 2000).

In patients with essential hypertension, plasma CGA is typically elevated by ~50-100% as compared to age-matched normotensive controls (Takiyyuddin *et al* 1995), consistent with other lines of evidence for increased sympathoadrenal activity in this disorder and suggesting exocytosis as the mechanism of the excess catecholamine release. Furthermore, when adrenal medullary stores of CGA are released by the stimulus of insulin-evoked hypoglycemia, patients with essential

hypertension display an increase in such releasable stores of CGA ; thus, both storage and release of CGA are augmented in essential hypertension.

Twin (monozygotic and dizygotic) studies in humans indicate substantial heritability for plasma CGA (Takiyyuddin *et al* 1995). However, the still-normotensive adult offspring of patients with essential hypertension (as compared to hypertension family history negative control subjects) do not have elevated plasma CGA, nor are their releasable adrenal medullary stores augmented. Hence, elevation of exocytotic sympathoadrenal activity may not be an especially early feature in the course of development of essential hypertension.

Plasma CGA typically does not decline with effective antihypertensive therapy (Takiyyuddin *et al* 1995, Wu *et al* 1994), suggesting that the CGA elevation is not simply secondary to blood pressure elevation. Lack of elevation of CGA in several forms of secondary hypertension (Takiyyuddin *et al* 1990) is also consistent with this viewpoint.

During antihypertensive treatment with agents which diminish exocytotic catecholamine release (Kailasam *et al* 1995), both CGA and catecholamines may decline in plasma.

In human essential hypertension, plasma pancreastatin immunoreactivity is also elevated (Sanchez-Margelet *et al* 1995a, Sanchez-Margelet *et al* 1995b). This observation is of potential pathophysiologic importance, given the multiple dysglycemic actions of pancreastatin (Sanchez-Margelet *et al* 2000, see chapter 20, this volume) and the state of insulin resistance typically seen in patients with essential hypertension (Reaven *et al* 1996).

6.2 Experimental (Rodent) Hypertension

In a rodent model of human hypertension, the spontaneously (or genetically) hypertensive rat (or SHR), both plasma and adrenal CGA concentrations are increased, suggesting a parallel with human hypertension (O'Connor *et al* 2000), and the increase is seen at the earliest ages studied, even before the development of frank hypertension. Although adrenal CGA gene expression is increased in the SHR, the CGA locus on rat chromosome 6 (Simon-Chazottes *et al* 1993, Mahata *et al* 1996) does not co-segregate with blood pressure in the F2 generation of an intercross between the SHR strain and a normotensive control strain, the Wistar Kyoto (WKY) rat (O'Connor *et al* 2000). Thus, the CGA gene itself is not clearly implicated in rodent genetic hypertension, despite the biological roles of the CGA fragments catestatin (Mahata *et al* 1997) and vasostatin (Angeletti *et al* 1994, Brekke *et al* 2000) in controlling catecholamine release and vascular smooth muscle contraction, respectively.

In a mouse genetic hypertension model (O'Connor *et al* 2000) adrenal CGA expression was actually diminished, in contrast to results in the rat; thus, elevation of CGA is not a completely consistent feature of genetic hypertension.

Adrenal CGA expression is increased in a rodent model of secondary (acquired) hypertension: unilateral renal artery stenosis (2-kidney-1-clip hypertension) in the rat (Takiyyuddin *et al* 1993); thus, pressor conditions other than genetic hypertension can also augment CGA biosynthesis in vivo.

7. NEUROLOGIC DISEASE AND CEREBROSPINAL FLUID (CSF)

Both CGA (O'Connor *et al* 1993a) and its interior fragment GE25 (Kirchmair *et al* 1994) are found by radioimmunoassay of human CSF.

CGA immunoreactivity is present in CSF at about twice its concentration in blood plasma, despite the far lower total protein concentration in CSF; thus, CSF CGA seems to derive from the central nervous system, rather than by diffusion from the plasma into CSF (O'Connor *et al* 1993a). However, CSF CGA did not correlate with concentrations of norepinephrine or norepinephrine metabolites in CSF, and selective disruption of central noradrenergic neuronal activity by the alpha-2-adrenergic agonist clonidine decreased CSF norepinephrine though not CSF CGA; nor did effective antihypertensive treatment with the beta-adrenergic antagonist propranolol change CSF CGA. Thus, CSF CGA concentration is not closely coupled to central noradrenergic neuronal activity.

CGA concentration was lower in ventricular than in lumbar CSF, and was diminished in patients with Parkinson disease (O'Connor *et al* 1993a), though the diagnostic specificity of this finding is uncertain. In patients with Parkinson disease, adrenal-tocaudate autografts do not result in an increase in CSF CGA (Shults *et al* 1991, O'Connor *et al* 1993a).

Despite evidence that CGA may be a major constituent of senile cerebral plaques in Alzheimer disease (Munoz 1991), CSF CGA is not systematically different from control values in patients with Alzheimer disease (O'Connor *et al* 1993b, Blennow *et al* 1995).

CSF CGA values are not systematically different from the normal range in patients with schizophrenia (Van Kammen *et al* 1992).

8. RENAL FAILURE AND URINARY EXCRETION OF CGA

8.1 Plasma CGA

In patients with diminished renal function, plasma CGA is elevated (Hsiao *et al* 1990a); indeed, plasma CGA increases as a function of decline in glomerular filtration rate ($r=0.82$, $n=125$, $p<0.0l$), and the CGA values in patients with end stage renal disease (on dialysis treatment) are as high as those seen in patients with neuroendocrine neoplasia (O'Connor and Deftos 1986).

Gel filtration coupled to radioimmunoassay, or region-specific immunoprecipitations, suggested that the elevated CGA immunoreactivity seen in renal failure resulted from accumulation of immunoreactive mid-molecule fragments of the intact, parent molecule (Hsiao *et al* 1990a). In renal failure, plasma CGA did not closely parallel the plasma concentrations of either catecholamines or parathyroid hormone (Hsiao *et al* 1990a).

Thus, renal function (or at least serum creatinine concentration) must be evaluated when interpreting plasma CGA results. In patients with known renal failure, suspected pheochromocytoma can be better evaluated with plasma or urinary catecholamine or catecholamine metabolite assays (Takiyyuddin *et al* 1990, Hsiao *et al* 1991, Hsiao *et al* 1990a, O'Connor 1996).

8.2 Renal excretion of CGA

Weiler *et al* (1991) suggested that CGA immunoreactivity may be filtered at the renal glomerulus, and degraded by renal proximal tubular endocytosis. In healthy controls, we found that CGA immunoreactivity is not detectable in urine, but in patients with renal failure and substantial proteinuria, CGA immunoreactivity is readily detected at concentrations near those found in plasma (Hsiao *et al* 1990a). In a patient with carcinoid tumor, renal excretion of a CGA fragment was of sufficient magnitude to enable its purification, and the establishment of immunoassays (Stridsberg *et al* 1993).

8.3 Other Organ Failure (liver, heart)

In hepatic failure (O'Connor *et al* 1989), CGA was also moderately (up to ~4-fold) elevated, suggesting that the liver, too, may provide a route of CGA removal from plasma. Alternatively, neuroendocrine activation in the setting of hepatic failure might also be responsible for the elevation in CGA.

In congestive heart failure (Corti, *et al* 2000, see chapter 28) plasma CGA rose as a function of disease severity, and in parallel with other features of neuroendocrine activation in this syndrome. Patients with class IV (the most severe) heart failure had CGA elevations up to ~7.6-fold of normal.

9. CONCLUSIONS

CGA can be reliably measured by immunological methods, in such human biological fluids as plasma (or serum), cerebrospinal fluid, or urine. In plasma or serum, CGA has proven to be a useful clinical tool in evaluating suspected or actual neuroendocrine neoplasia, from both a diagnostic and a prognostic standpoint. CGA measurement also yields insight into the pathogenesis of such human disease states as essential (genetic) hypertension, in which elevated CGA suggests enhanced exocytotic sympathoadrenal activity as an etiologic culprit in the syndrome. Analysis of CGA secretion may assist in profiling the neuroendocrine character of such neoplasia as prostate carcinoma, as well as in prediction of response to treatment. Finally, plasma CGA is influenced by organ failure (kidney, liver, and heart); evaluation of the functional status of such organs is necessary in order to interpret the meaning of elevations in CGA.

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A PHYSIOLOGICAL ROLE FOR THE GRANINS AS PROHORMONES FOR HOMEOSTATICALLY IMPORTANT REGULATORY PEPTIDES ?

A working Hypothesis for Future Research

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1. INTRODUCTION

The concept of stress (Selye 1936) describes a state of threatened homeostasis. The main purpose of the stress response is to regain equilibrium via a chain of reactions co-ordinated by the brain and effected via the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis. As amply documented in the chapters of this volume, the chromogranins and secretogranin II are co-released with the respective hormones from the sympathoadrenomedullary division and other elements of the diffuse neuroendocrine system. Accordingly, there is an overflow to the circulation of both catecholamines and chromogranins in similar, low nanomolar ranges, reflecting the intensity of activation. However, while the catecholamines have long since been recognised as primary stress hormones, the implications of the co-released chromogranins in the stress response have proved difficult to envisage.

In view of the wealth of information accumulated in this volume, the chromogranins now deserve attention as potential stress signals. The main emphasis of this concluding chapter is to discuss a unifying concept for the chromogranins and their derived peptides as physiologically important regulators, based on the current evidence of their biological activities.

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2. HOMEOSTATIC MECHANISMS

The term homeostasis describes the various physiological arrangements which serve to restore the normal state of the internal environment once it has been disturbed (Cannon 1932). Many of the regulatory mechanisms that act to maintain the constancy of the internal environment operate on the principle of negative feedback, leading to compensatory changes until equilibrium is restored.

Apart from the major homeostatic mechanisms maintaining tonicity, volume and specific ionic composition of the extracellular fluids, there are also important mechanisms maintaining the levels of glucose, fatty acids and other nonionized substances of importance in the regulation of energy balance. Moreover, when the sympathoadrenal division discharges as a unit in emergency situations, also the perfusion of vital organs such as the brain, heart and working muscles is favoured. While the role of the catecholamines in these situations are undisputable, functional implications of the co-released chromogranins have yet to be established.

Another important homeostatic mechanism is the regulation of plasma calcium via the negative feedback on the parathyroid and calcitonin-secreting C-cells of the thyroid. The fact that this counter-regulatory pair of hormones is co-secreted with chromogranin A (CGA) has been used in a more general model of integral controllers in "zero steady-state error" homeostasis (ZSSE), in which the rising levels of co-secreted CGA will eventually inhibit secretion by both types of endocrine cells (Koeslag *et al* 1999).

Other important environmental threats to the organism are tissue damage and the invasion of foreign organisms, such as bacteria and fungi, through open wounds in the skin and other epithelial linings. Tissue damage is immediately sensed and transmitted via sensory nerves to the central and autonomic divisions of the nervous system, activating adequate counteracting strategies. In the first phase of microbial invasions, before antibody production becomes operable, the organism have to depend on the already synthesised agents with antimicrobial potencies. In the more recent years chromogranins and secretogranin II have been implicated in various aspects of the immune system (see Chapters 22 - 24).

Neuroendocrine tumours impose another threat, being accompanied by conspicuous discharge of chromogranins into the circulation (see Chapters 26 - 29). It is not yet established how this pathological discharge affect the homeostatic capacity of the patient. Of particular interest would be to establish whether prohormone processing proceeds in a manner that is beneficial to the host or to the tumour in the different stages of tumour development (see Chapter 31).

3. SOURCES OF THE PROHORMONES AND THEIR ACTIVE PEPTIDES

3.1 Chromogranin A, Vasostatins, Chromacin, Pancreastatin, and Catestatin

CGA is widely distributed in the diffuse neuroendocrine system, being co-released with a range of neurotransmitters, amine and peptide hormones. Evidence is also accumulating for CGA being released from exocrine glands of the gastrointestinal tract (see Chapters 11, 16 and 20). Processing of this prohormone varies between tissues and species, indicating cell- and tissue-specific cleavage products for autocrine, paracrine and endocrine modulations (Table 1). Three of the peptide products are implicated in autocrine inhibition of hormonal release, i.e. the vasostatins in the parathyroid release of PTH (see Chapter 17), pancreastatin in the pancreatic release of insulin and glucagon (see Chapter 20), and catestatin in the adrenal medullary release of catecholamines (see Chapter 21). Chromacin, on the other hand, accounts for an antibacterial domain which beside vasostatin-1, makes CGA also a multifactorial source of antimicrobial peptides (see Chapter 24).

The stimuli for release of the chromogranins derive from a wide range of environmental and intrinsic paths, ultimately raising the concentrations of the unprocessed and processed prohormones in the extracellular space, i.e. in the interstitium and the circulation. Taken together, the release from gland cells and nerve terminals would contribute to paracrine effects while the endocrine effects would depend on the subsequent overflow to the circulation. Release from immunocytes like polymorphonuclear neutrophils may also contribute to paracrine modulations and endocrine effects (see Chapter 24). Besides the parathyroid cells and the C-cells of the thyroid (Koeslag et al 1999), the most likely targets for the vasostatins as paracrine and endocrine signals seem to be the vascular system (see Chapter 13, 18 and 19), and for pancreastatin, the liver and adipocytes besides the pancreatic a and b cells (see Chapter 20). Nothing is at present known about the interstitial concentrations of chromogranins and their derived peptides while the reported plasma levels are in the sub-nanomolar to the low nanomolar range. Further information on the quantitative aspects will be essential for evaluation of the postulated physiological relevance of the biologically active chromogranin-derived peptides.

Table 1. Chromogranin and Secretogranin Derived Peptides with Biological Activities

Prohormone and Peptides	Effects	Species	Chapter
Chromogranin A			
CGA_{1-40}	Inhibit PTH secretion	Bovine	17
CGA_{1-76}(VS-I)	"		
CGA_{1-113} (VS-II)			
CGA_{1-40}	Inhibit vasoconstriction	Human	18
CGA_{1-76}(VS-I)	"	Bovine	19
CGA_{1-76}(VS-I)	Stimulate fibroblast adhesion	Human	28
CGA_{1-76}(VS-I)	Inhibit bacterial and fungal growth		24
CGA_{1-76}(VS-I)	Stimulate microglia	Rat	23
CGA_{79-431} (prochromacin)	Inhibit bacterial and		24
$CGA_{,73-194}$ (chrornacin)	fungal growth		
$CGA_{240-288}$(pancreastatin)	Inhibits glucose-stimulated insulin release	Porcine Rat	20 20
$CGA_{240-288}$(pancreastatin)	Stimulates glucagon release	Mice, Rat, Porcine	20 20 20
$CGA2_{40-288}$(pancreastatin)	Activates liver glycogenolysis and adipocyte lipolysis	Rat	20
	Inhibits adipocyte lipogenesis	Rat	20
$CGA_{240-288}$(pancreastatin)	Inhibits PTH secretion	Bovine	a)
$CGA_{344-364}$(catestatin)	Inhibits catecholamine release	Bovine	21
$CGA_{344-364}$(catestatin)	Stimulate histamine release	Rat	b)
$CGA_{347-419}$(parastatin)	Inhibit PTH secretion,	Bovine	c)

Prohormone and Peptides	Effects	Species	Chapter
Chromogranin B			
CGB_{1-40}	Inhibits PTH secretion	Bovine	17
$CGB_{564-626}$ (chrombacin)	Inhibit bacterial growth		24
$CGB_{614-626}$ (secretolytin)	Inhibit bacterial growth		24
Secretogranin II			
$SgII_{154-186}$ (secretoneurin)	Stimulated dopamine release from striatal slices and basal ganglia	Rat	22
$SgII_{154-186}$ (secretoneurin)	Inhibits release from pinealocytes	Human	22
$SgII_{154-186}$ (secretoneurin)	Stimulate chemotaxis (eosinophils, fibroblasts)	Human	22
$SgII_{154-186}$ (secretoneurin)	Deactivate chemotaxis of neutrophiles	Human	22
$SgII_{154-186}$ (secretoneurin)	Inhibits proliferation while stimulating migration of endothelial and vascular smooth muscle cells, and	Human Bovine Rat	22
	transendothelial migration of monocytes	Human	22
$SgII_{154-186}$ (secretoneurin)	Activates endothelial cells for neutrophil adherence	Human	22
$SgII_{154-186}$ (secretoneurin)	Stimulate survival of cerebellar granule cells	Mouse	22
$sgII_{154-186}$ (secretoneurin)	Stimulate release of pituitary gonadotropin II	Gold-Fish	d)

[a,b,c,d)] For full references see relevant chapters (n) for [a)] Fasciotto *et al* 1989 (20), [b)] Kennedy *et al* 1998 (1), [c)] Fasciotto *et al* 1993 (17), [d)] Blázquez *et al* 1998 (22).

3.2 Chromogranin B, CGB_{1-40}, and Secretolytin

Chromogranin B (CGB), commonly found in co-storage with CGA in species and tissue-specific ratios, is most frequently processed to a larger extent than CGA (see Chapters 2, 12 and 24). However, biologically active peptides have so far been located only at the N- and C-terminal domains, with effects limited to an endocrine inhibition of hormone release from the parathyroid by CGB_{1-40} and an antibacterial effect of secretolytin, the C-terminal peptide $CGB_{614-626}$ (Table 1).

3.3 Secretogranin II and Secretoneurin

Secretogranin II and its derived peptide, secretoneurin ($SgII_{154-186}$), are found in high concentrations in several regions of the brain, in endocrine cells of the gastrointestinal tract as well as in peripheral sympathetic and sensory nerves (see Table 1 and Chapter 22). Secretoneurin is co-localised with substance P and calcitonin-gene related peptide in sensory nerve endings and co-released with these peptides in response to chemical, mechanical or immunological injury. These sensory peptides are implicated in neurogenic inflammation which in the case of secretoneurin involves mobilisation of leucocytes and endothelial and mesenchymal cells as cellular targets. Accordingly, secretoneurin is implicated as another link between the nervous and immune system.

4. THE CHROMOGRANIN- AND SECRETOGRANIN II- DERIVED PEPTIDES AS HOMEOSTATIC REGULATORS ?

It seems evident from the accumulated literature that three of the CGA-derived peptides, vasostatin-I, pancreastatin and catestatin are involved in autocrine, negative feedback regulation of hormone secretion in selected glands. The vasostatins are effective inhibitors of PTH release at low calcium levels while pancreastatin inhibits insulin release at high glucose levels and catestatin inhibits the adrenomedullary catecholamine hormone release at sympathoadrenal activation. These properties by three different CGA sequences imply a role for CGA as a multifactoral prohormone for important homeostatic mechanisms, i.e. the regulation of plasma calcium, glucose and catecholamines. With respect to calcium and glucose homeostasis, both being under control of counter-regulatory pairs of hormones, CGA has been proposed as an inhibitor in a robust "zero steady-state error" type of homeostasis (Koeslag *et al* 1999), implicating

vasostatins and pancreastatin as the respective regulatory peptides in these examples of ZSSE.

Moreover, these two CGA sequences exhibit a plethora of effects beyond this type of homeostatic control. The vasostatins, the most highly conserved domain of the prohormone, appear to be the most functionally versatile of the CGA peptides, counteracting also vasoconstrictor mechanisms in micro- and macrovascular elements, presumably contributing to enhanced blood flow to vital organs. Lastly, vasostatins appear to be of importance for the immune response in the brain where a transition of the microglial cells from a quiescent to an activated, macrophage-like phenotype may be activated by vasostatin-I (see Chapter 23). Fibroblast adhesion stimulated by vasostatin-I may also be part of the myocardial response to chronic heart failure (see Chapter 28). The signalling pathways are not known, nor if other CGA- or CGB-derived peptides act on other immunocytes.

Pancreastatin, accounting for a domain with relatively low sequence homology between species, nevertheless emerges as an endocrine principle for activation of liver glycogenolysis and adipose tissue lipolysis (see Table 1 and Chapter 20). In these endocrine aspects pancreastatin seemingly acts in synergy with the sympathoadrenal system. Hence, the pancreastatin sequence of CGA may now be regarded as a regulatory peptide for maintenance of plasma glucose. Based on the various activities of the vasostatins, pancreastatin and catestatin, we propose, as a working hypothesis that these CGA-derived peptides have functional relevance as homeostatic regulators.

Secretoneurin is so far the only peptide with biological activity derived from secretogranin II (See Table 1 and Chapter 22). Besides stimulating the brain release of dopamine, an important inhibitory neurotransmitter in motor control, secretoneurin seems to be implicated in neuroimmune regulation, by modulating chemotaxis and proliferation of a wide range of cells involved in tissue repair.

Some of the granin-derived peptides thus appear to be involved in cross-talk between the endocrine, the nervous and the immune systems. These properties add new perspectives to the roles played by the chromogranin-derived peptides and secretoneurin in regulation of cells within each system.

5. A ROLE FOR THE ANTIMICROBIAL PEPTIDES IN INNATE IMMUNITY

Each organism possesses a variety of weapons for its protection against pathogenic invasions. In the first line of defence we find a range of agents that are distinct from the adaptive immunity system. These agents are

present at birth and account for the "innate immunity" which is an ancient evolutionary defence system.

Serendipitously, several chromogranin-derived peptides have been found to display antimicrobial and antifungal activity, the most potent being vasostatin-I (see Chapter 2 and 24). This peptide is able to kill Gram+ bacteria as well as a large variety of fungi and yeast without sharing structural homology with other known classes of antibacterial peptides. In addition to their location in endocrine tissues, the chromogranins and their derived peptide are also present in and released from a range of immune cells, appearing in inflammatory fluids (Metz-Boutigue and Aunis, unpublished observations).

6. CONCLUSION

A multitude of regulatory peptides derive from the "granin" family of prohormones. At present CGA stands out as the most versatile prohormone, giving rise to three different peptide principles, the vasostatins, pancreastatin and catestatin. CGB, being more extensively processed than CGA in most systems and species, may in the future appear to have specific regulatory functions. On the other hand, secretogranin II serves as prohormone for only one, conspicously active principle, nevertheless engaged in a wide range of modulatory activities related to tissue repair.

This first volume devoted to the chromogranins and secretogranin II as prohormones for regulatory peptides, provides ample evidence of their widely different effects and target tissues. The majority of properties so far assigned to these proteins and peptides fits into patterns of protective strategies which may not be called upon unless the organism is exposed to various stressful situations requiring immediate protection, e.g. via short-term adaptive mechanisms for homeostatic control and via the innate immunity system. It seems evident from the overviews collected in this volume, that we are now in the beginning of an understanding of how some of these homeostatic control mechanisms may involve the chromogranins and their derived peptides.

Furthermore, the clinical applications of chromogranins as markers for neuroendocrine tumours, chronic heart failure, and brain disorders like Parkinson's and Alzheimer's, also open for new insight into the pathophysiological implications of the high plasma levels of these prohormones and their regulatory peptide principles.

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