MOLECULAR BIOLOGY INTELLIGENCE UNIT

Gerald Zamponi

# Voltage-Gated Calcium Channels

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MOLECULAR BIOLOGY
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# Voltage-Gated Calcium Channels

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# PREFACE

Voltage-gated calcium channels are essential mediators of a range of physiological functions, including the communication between nerve cells, the regulation of heart beat, muscle contraction, and secretion of hormones such as insulin. Consequently, these channels are critical pharmacological targets in the treatment of a variety of disorders, such as epilepsy, hypertension, and pain. Voltage-gated calcium channels have therefore been subject to intense study by numerous investigators over the past few decades, and an immense body of work has accumulated. In this book, we provide the first comprehensive overview of our current state of knowledge concerning this exciting field of research. Leading off with a general review of calcium signaling and techniques to measure calcium channel activity, the book delves into a provocative overview of the history of the calcium channel field, contributed by one of the key pioneers in the field, Dr. Richard Tsien. This is followed by an in depth review of the biochemical and molecular biological characterization of calcium channel genes by Drs. Catterall and Snutch whose research has resulted in major advances in the calcium channel field. A number of chapters are dedicated towards various aspects of calcium channel structure and function, including channel gating, permeation, modulation and interactions with members of the exocytotic machinery—contributed by both established leaders and rising stars in the field. The next series of chapters is concerned with pharmacological and physiological aspects of voltage-gated calcium channels including genetic diseases linked to calcium channel genes. The book concludes with an overview of the effects of targeted calcium channel gene disruption in mice.

Over the past two decades, considerable progress has been made in terms of understanding the molecular physiology of voltage-gated calcium channels, yet, the work is far from complete. Identification of novel small organic calcium channel inhibitors remains a key priority towards treating diseases linked to these channels, and only recently has the first crystal structure of a calcium channel subunit been solved. Over the next decade, one may expect that current knowledge about the molecular structure of calcium channels will be used to understand, in detail, the function of these channels in their native cellular environment and in human physiology, and my fellow contributors and I look forward to being part of this effort.

*Gerald W. Zamponi, Ph.D.*

# CHAPTER 1

# $Ca^{2+}$ Chemistry, Storage and Transport in Biologic Systems:
## An Overview

**Tashi G. Kinjo and Paul P.M. Schnetkamp**

Calcium ions play a critical role in most if not all cellular processes. It has even been demonstrated that $Ca^{2+}$ currents in root tips, in combination with gravity, are responsible for their downward growth.[1] Most of these effects are mediated by both temporally and spatially tightly controlled changes in cytosolic free $Ca^{2+}$ brought about by activation of $Ca^{2+}$ influx pathways in the cell membrane or by activation of intracellular $Ca^{2+}$ release channels, and countered by transporters acting as $Ca^{2+}$ pumps. Voltage-gated $Ca^{2+}$ channels are a preeminent class of plasma membrane proteins providing regulated $Ca^{2+}$ influx, and the remainder of the volume is dedicated to their many different facets. In this introductory chapter, we will briefly review other aspects of cellular $Ca^{2+}$ homeostasis. We will review some of the chemical properties of $Ca^{2+}$ that are important to its role in biology, we will review intracellular $Ca^{2+}$ stores, and we will review other $Ca^{2+}$ handling proteins. Due to space limitations we will not be able to refer to the original literature in most cases, but have to limit ourselves to a relatively small number of recent reviews that could provide the reader further guidance into the many exciting and sometimes controversial topics of current research in $Ca^{2+}$ transport and storage in cells.

## Why $Ca^{2+}$: Unique Chemical and Physical Features

The chemical features and physical characteristics of the doubly charged $Ca^{2+}$ contribute favorably to its role in biology. This has been extensively reviewed by Williams[2,3] and a brief synopsis is provided here.

$Ca^{2+}$ displays a highly ionic character in its bonding. The covalent contribution in bond formation increases as one moves across the series from $Ca^{2+}$ to $Zn^{2+}$. The highly ionic character of $Ca^{2+}$ in bond formation restricts its interactions to liganding oxygen donating groups such as carbonyl, carboxyl, alcohols and ethers. Divalent cations that display highly covalent bond formations, such as $Cd^{2+}$, interact with stronger donating groups such as nitrogen and sulfur donors, and are thus removed from competing with $Ca^{2+}$ for binding.

$Ca^{2+}$ is a rather large ion with a crystal ionic radius of 0.99 Å. This is larger than the majority of divalent cations including those in the third row of the periodic table from $Mn^{2+}$(radius of 0.80Å) across to $Zn^{2+}$ (radius of 0.74Å). $Cd^{2+}$ is very close in size with a radius of 0.97Å. Relative to the other ions in the second period, $Ca^{2+}$ is larger than $Mg^{2+}$ (radius of 0.66Å), however, smaller than $Sr^{2+}$ (01.12Å) and $Ba^{2+}$ (1.34Å).

In terms of the binding capability, ionic radius is an important determinant as to the strength and type of ligand that will bind to a particular ion. If this alone was the determining factor for the binding of potential ligands, then it would follow that smaller divalent cations would exhibit the strongest ligand binding due to a stronger electrostatic field. This, however, is not the

*Voltage-Gated Calcium Channels*, edited by Gerald Zamponi.
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case. Smaller ions necessitate closer association of donor groups in three-dimensional space. This restricts the binding of ligands to smaller ions due to steric hindrance within the liganding molecule. However, if a divalent cation is too large, the reduction in electrostatic field causes a decrease in binding. $Ca^{2+}$ provides the best balance between the two opposing forces; steric hindrance and electrostatic field intensity.

Ionic radius also determines the solubility of a particular ion with other ions. $Mg^{2+}$ can pack tightly into the small holes created in the lattice of smaller anions such as hydroxide and fluoride ions. $Ca^{2+}$, however, is too large to fit into the holes in the lattice structure created by $OH^-$ and $F^-$ ions. Consequently, $Mg^{2+}$ rather than $Ca^{2+}$, tends to precipitate out with small anions. $Ca^{2+}$ precipitates out with larger anions such as phosphates and carbonates or polyanions such as nucleic acids and acidic proteins.

## $Ca^{2+}$ Evolution: A Rejection from Cytoplasm

An interesting theory on the evolution of the role of $Ca^{2+}$ in biology has been presented by Williams,[2,3] based on the ability for $Ca^{2+}$ to precipitate organic anions. From life's very beginnings, it was essential that $Ca^{2+}$ be separated from organic anions to avoid precipitation. Thus, $Ca^{2+}$ had to be rejected from the cytoplasm of the earliest cells. This initial simple rejection of $Ca^{2+}$ is still observed for all organisms, ranging from prokaryotes to multicellular eukaryotes, where intracellular free $Ca^{2+}$ concentrations are maintained on the order of $10^{-7}$M. In the earliest forms of life, namely prokaryotes, $Ca^{2+}$ was simply rejected to intracellular levels on the order of $10^{-5}$M. Little function for intracellular $Ca^{2+}$ is found in anaerobic bacteria to this day.

With the development of eukaryotes came the compartmentalization of the cytoplasm. It was then necessary for eukaryotic cells to develop intracellular signaling in order to coordinate function and placement of these organelles. The $Ca^{2+}$ features outlined above makes $Ca^{2+}$ an excellent second messenger, fast kinetics, and easy liganding ability. Therefore, eukaryotic cells started to use $Ca^{2+}$, and the previously set up $Ca^{2+}$ electrochemical gradient from prokaryotes, for coordination of events within the cell. It is not until the development of unicellular eukaryotic cells, such as yeast, that we observe intracellular $Ca^{2+}$ binding proteins. Emergence for the use of $Ca^{2+}$ precipitates as external support and protective structures begin in eukaryotes with calcium carbonate shells. Multicellular eukaryotes then develop organized internal calcium phosphate precipitates in the form of bone, which act not only to provide structural support, but act as a $Ca^{2+}$ reservoir for maintenance of circulating blood $Ca^{2+}$ levels. The progression of the extensive use of $Ca^{2+}$ is then based on its initial rejection from prokaryotic cells, creating a gradient for $Ca^{2+}$ entry. This entry, in combination with the unique binding characteristics of $Ca^{2+}$, allowed eukaryotic cells to use this divalent cation as an essential element for the many processes in which it is used today.

## $Ca^{2+}$ Signaling and Storage: The Endoplasmic Recticulum

The endoplasmic recticulum is generally the predominant intracellular $Ca^{2+}$ store. The ER is critical not only for $Ca^{2+}$ storage, but release of $Ca^{2+}$ from the ER is responsible for rapid transmission of $Ca^{2+}$ signals from the periphery to the center of the cell and for local $Ca^{2+}$ signaling.[4] Interestingly, changes in ER $Ca^{2+}$ handling is thought to be involved in neural ageing[5] and improper ER $Ca^{2+}$ control has pathogenic implications.[6] Resting ER free $Ca^{2+}$ concentration is thought to be on the order of several hundred μM,[7] in addition, lumenal ER $Ca^{2+}$ is stored by high capacity-low affinity $Ca^{2+}$ buffering proteins including calsequestrin and calreticulin.[8]

This resting level of lumenal ER free $Ca^{2+}$ is the result of a balance between $Ca^{2+}$ uptake via sarcoplasmic-endoplasmic type ATPases (SERCAs)[9] and release. $Ca^{2+}$ is released from the ER in either a stimulated or passive manner.

Stimulated release occurs through the activation of two receptor types; ryanodine receptors(RyR) and inositol 1,4,5-triphosphate($IP_3$) receptors discussed in more detail later.[8] In addition, other receptors may exist that release $Ca^{2+}$ via cyclic-ADP-ribose and nicotinic

acid adenine dinucleotide phosphate (NAADP). Suprastimulation with high levels of $Ca^{2+}$ releasing agonists reduce ER free $Ca^{2+}$ to only the tens of μM range.[8] Inactivation of $Ca^{2+}$ release channels by high cytosolic $Ca^{2+}$ may contribute to this incomplete emptying. Interestingly, sustained submaximal levels of cytosolic $IP_3$ causes only partial transient release of calcium from the ER. Whereas additional levels of $IP_3$ induce more pulses of $Ca^{2+}$ release. This is known as the quantal $Ca^{2+}$ release phenonmenon.[11] The exact mechanism of this phenomenon, however, remains elusive.

Passive release is less well-defined and has been shown in many cell types to be remarkably dynamic as inhibition of the SERCA pump by thapsigargin often results in a rise in cytosolic free $Ca^{2+}$ within a few seconds. This passive leak has been shown to be insensitive to inhibitors of both $IP_3$ and RyRs, but responsive to ATP levels.[10] Recent evidence has implicated a protein involved in apoptosis, Bcl2, as having a possible role in passive $Ca^{2+}$ leak from the ER.[8]

## The Role of $Ca^{2+}$ within the ER

$Ca^{2+}$ is fundamental for proper function of the ER. Indeed, evidence has accumulated that indicates that even ER structure and integrity are dependent upon lumenal free $Ca^{2+}$. Reports have indicated that loss of ER lumenal $Ca^{2+}$ results in ER fragmentation.[12] Additionally, it has been shown that high levels of cytoplasmic $Ca^{2+}$ can result in the loss of continuity of the ER membrane.[13] Prevention of ER fragmentation has been observed upon protein kinase C(PKC) activation, which could possibly provide the ER with a protective mechanism upon store depletion with $IP_3$/DAG producing agonists.[13,14] However, another group has since then observed that the protective effects of PKC activation occur only when fragmentation is caused by an increase in cytoplasmic $Ca^{2+}$ and not when caused by ER $Ca^{2+}$ depletion.[15]

In addition to $Ca^{2+}$ handling, the ER is the site of protein processing, storage and transport. Interestingly, many $Ca^{2+}$ binding proteins found within the ER also act as chaperone proteins. The two roles served by these proteins, chaperone and $Ca^{2+}$ buffer, are not separate, but are intimately associated. Maturation and proper folding of proteins is affected by depletion of ER $Ca^{2+}$.[8] The formation of chaperone complexes in the ER depends on $Ca^{2+}$ concentration.[16] Additionally, depletion of ER $Ca^{2+}$ increases protein degradation.[17]

The ER alone is not responsible for correct trafficking and maturation of proteins. The ER is in constant communication with the Golgi network with proteins and vesicles being transported between the two. $Ca^{2+}$ has been found to play an important role in this communication. Transport between the ER and Golgi has been shown to require certain levels of cytosolic free $Ca^{2+}$ and may be dependent upon a local $Ca^{2+}$ gradient in the cytoplasm separating these two organelles. In addition, transport of vesicles between the ER and Golgi depends on the presence of internal $Ca^{2+}$, within both the ER and Golgi, as transport did not occur following ER and Golgi emptying with $Ca^{2+}$ ionophores and the SERCA pump inhibitor thapsigargin.[8]

$Ca^{2+}$ also acts to control $Ca^{2+}$ management within the ER as a feedback mechanism. There are several examples of this phenomenon, however, the most extensively studied is capacitative $Ca^{2+}$ entry (CCE) through store-operated $Ca^{2+}$ channels (SOCs). It has been found that depletion of lumenal ER free $Ca^{2+}$ can stimulate the entry of extracellular $Ca^{2+}$ into the cell in order to refill the ER(reviewed in ref. 18). CCE is discussed in later text. Passive $Ca^{2+}$ leak out of the ER is also dependent on ER lumenal $Ca^{2+}$. Observation have indicated that low levels of ER $Ca^{2+}$ (100 μM) greatly reduced this passive leak, additionally, ER $Ca^{2+}$ affects $IP_3R$, SERCA activity and even expression of the ER $Ca^{2+}$ binding protein calreticulin.[8]

## ER Heterogeneity: $Ca^{2+}$ Tunneling Versus $Ca^{2+}$ Compartmentalization

Petersen provides an excellent discussion on the topic of ER calcium distribution.[11] An outline of this discussion is provided here. Classically, the endoplasmic recticulum has been thought of as one continuous membrane bound enclosure with equal protein expression and equilibrated ion concentrations throughout. ER protein expression, however, is not uniform but heterogeneous with specific localization of proteins into domains. Different SERCA

subtypes and $Ca^{2+}$ release channels are found in different parts of the ER. This differential ER protein distribution is separate from, but related to the topic of ER calcium distribution. Recently, this topic has been the subject of debate. There have been many reports that $IP_3$ induced and Ry/caffeine induced $Ca^{2+}$ release occur through separate calcium stores. Imaging experiments have indicated that $IP_3$ induced $Ca^{2+}$ release is spatially distinct, and can be independently emptied, from release induced by Ry/caffeine.[4] Currently, however, the classical view of an equilibrated ER $Ca^{2+}$ distribution is accepted based on the results of diffusion using uncaged $Ca^{2+}$ within the ER.

## $Ca^{2+}$ Signaling and Storage: Mitochondria

Since the 1960s, it has been known that mitochondria are capable of accumulating $Ca^{2+}$. The importance of this uptake, however, was thought to be minimal as physiologic increases in cytosolic $Ca^{2+}$ was seen as being insufficient to activate mitochondrial $Ca^{2+}$ uptake mechanisms. Interest in mitochondrial $Ca^{2+}$ uptake was then sparked by experiments using the $Ca^{2+}$-indicating protein aequorin, targeted to the mitochondrial matrix. These experiments indicated that mitochondrial matrix $Ca^{2+}$ levels transiently increased following treatment with $IP_3$ liberating agonists.[19] Mitochondria exhibit two mechanisms of $Ca^{2+}$ uptake. Primarily, $Ca^{2+}$ uptake occurs via a $Ca^{2+}$ uniporter. The molecular characterization of this uniporter, however, remains elusive. Evidence has indicated that this uniporter functions like a channel with an increase in open probability associated with increased local $Ca^{2+}$ concentrations(reviewed in 20). $Ca^{2+}$ uptake through this uniporter is known as the Ruthenium Red (RuR)-sensitive pathway. The driving force for $Ca^{2+}$ entry through the uniporter is provided by the mitochondrial membrane potential, reported to be -150 to -200mV with respect to the cytosol.[21] The uniporter has a rather high $Ca^{2+}$ dissociation constant ($K_d$>25 μM),[19] much greater than physiologic changes in net cytosolic $Ca^{2+}$. Mitochondria, however, are subject to much higher cytoplasmic concentrations of $Ca^{2+}$ in the form of microdomains that are released upon $IP_3$ receptor opening in the ER. In fact, the ER and mitochondria have been found to be very closely associated in space(<100nm), and in $Ca^{2+}$ communication(see next section).[22] A second form of mitochondrial $Ca^{2+}$ uptake has also been reported and is known as the rapid uptake pathway.[23] Little is known about this mechanism of uptake. Mitochondrial $Ca^{2+}$ uptake is balanced by $Ca^{2+}$ extrusion through the mitochondrial $Na^+Ca^{2+}$ exchanger, which exhibits different characteristics than the plasma membrane exchanger.[21]

## Mitochondrial $Ca^{2+}$: Uptake Function and Effects

The main role of mitochondria has long been known to be oxidative phosphorylation.[24] Not surprisingly then, mitochondrial $Ca^{2+}$ levels influence this process. The citric acid cycle is rate limited by levels of dehydrogenases. Expression of these enzymes is upregulated, with associated increase in ATP production, in response to accumulation of mitochondrial $Ca^{2+}$.[25]

A more unexpected role of $Ca^{2+}$ uptake by mitochondria involves the fine tuning and shaping of intracellular $Ca^{2+}$ signaling. A conventional view of $Ca^{2+}$ signaling within a cell consists of a wave of $Ca^{2+}$ propagating across the cytoplasm of the cell. This propagation is thought to occur by CICR through $IP_3$ receptors in the ER. The wave starts with a local increase in cytoplasmic $Ca^{2+}$. This sensitizes $IP_3$ receptors and enables their activation at lower levels of $IP_3$. The $IP_3$ receptor activates and releases $Ca^{2+}$, which acts to sensitize the $IP_3$ receptors on adjacent ER membrane. In this manner the wave of $Ca^{2+}$ is released into the cytoplasm across the cell. The action of mitochondria in shaping of these signals is to restrict the rate of wave propagation and dampen its amplitude as it moves across the cell. Inhibition of mitochondrial $Ca^{2+}$ uptake results in a constant rate of wave propagation and an increase in the rate of rise and amplitude of the signal.[22] In pancreatic acinar cells, mitochondria found along the center line separating the apical and basal portions, has been shown to restrict the spread of $Ca^{2+}$ waves from one end to the other.[26]

Under certain conditions, (oxidative stress, adenine nucleotide depletion and increased inorganic phosphate levels) an increase in intra mitochondrial $Ca^{2+}$ concentration will trigger the formation of a pore complex known as the permeability transition pore (mPTP). This complex is made up by several proteins located at the close contact points between the inner and outer mitochondrial membranes. The function of the mPTP, however, remains to be evaluated. Several theories have been put forward including $Ca^{2+}$ signal modulation, apoptosis initiation, or $Ca^{2+}$ release.[22]

## $Ca^{2+}$ Buffering: Cytosolic and Lumenal

Protein bound calcium accounts for between 95% and 99% of the total $Ca^{2+}$ load of a cell.[27] The proteins that are involved in this binding are collectively known as $Ca^{2+}$ buffering proteins and belong to the EF-hand family of $Ca^{2+}$ binding proteins.[28] $Ca^{2+}$ buffering proteins serve several functions within cells. Besides their obvious function to buffer cytoplasmic/lumenal free $Ca^{2+}$ levels, they can act as $Ca^{2+}$ delivery proteins, signaling proteins, and chaperones. A soluble cytosolic protein that demonstrates two of these functions is parvalbumin(PV). This high affinity $Ca^{2+}$ binding protein is found in high concentration in the sarcoplasm of vertebrate fast contracting muscles, where it is involved in muscle relaxation. The process of muscle relaxation occurs when $Ca^{2+}$ dissociates from troponin C, moves towards, and is transported into the sarcoplasmic recticulum (SR). The sarcoplasm/cytoplasm of cells is not a free space in which $Ca^{2+}$ can diffuse, but is instead filled with negatively charged proteins capable of binding to $Ca^{2+}$. Therefore, $Ca^{2+}$ diffusion occurs rather slowly in these areas. In order to facilitate $Ca^{2+}$ translocation, $Ca^{2+}$ is taken up by PV, which moves freely to other locations in the cell. It is generally thought that after muscle contraction, $Ca^{2+}$ is taken up by PV, which then relocates to deliver the $Ca^{2+}$ to the SERCA pump of the SR. There has also been evidence for a direct interaction between PV and the SR through the binding of PV to an SR protein. This binding occurs in a $Ca^{2+}$-dependent manner.[29]

$Ca^{2+}$ buffering in intracellular organelles involves high binding capacity, low affinity ($K_d$.1mM) $Ca^{2+}$ binding proteins including; calnexin, calreticulin, calsequestrin and endoplasmin.[8] ER $Ca^{2+}$ buffering in muscle cells is primarily a function of calsequestrin.[30] The ER of other cell types employs calreticulin, which acts not only in $Ca^{2+}$ buffering, but as a molecular chaperone protein.[31] Lumenal ER $Ca^{2+}$ binding capacity has been reported to be quite low compared to that of the cytosol. In mouse pancreatic acinar cells, cytosolic $Ca^{2+}$ binding capacity was observed to be two orders of magnitude greater than that of the ER.[32] This high cytosolic binding capacity has been reported for some cell types, while found to be lower in others.[11] A complete picture of ER lumenal binding capacity and mobilities of the $Ca^{2+}$ buffering proteins involved, in both the ER and the cytosol, needs further investigation.

## Channels that Lead to an Increase in Cytosolic $Ca^{2+}$

Temporal and spatial control of increases in cytosolic free $Ca^{2+}$ is of paramount importance for proper functioning of most cells, and aberrant $Ca^{2+}$ homeostasis can rapidly result in cell death. Therefore, it is not that surprising that members of several different gene families of $Ca^{2+}$ permeable channels may contribute to $Ca^{2+}$ influx into the cytosol. The first members of most of these gene families were discovered in the eighties and nineties, quickly followed by cloning of related gene products; by now with the human and mouse genomic sequencing projects nearing completion, a complete membership list is probably available for most gene families. Many of these channels contributing to $Ca^{2+}$ influx into the cytosol can be found coexpressed in most cell types, especially in cells of excitable tissues. To reflect the diversity of specific cellular functions, most of the above gene families contain many different but related members, and this complexity is further increased by the presence of different splice variants. Although the occurrence of different splice variants is common, only in very few cases has a physiological significance been established with certainty.

## Intracellular $Ca^{2+}$ Release Channels

Two families of intracellular $Ca^{2+}$ release channels have been found to date, the $IP_3$ receptor[33] and the Ryanodine receptor (RyR),[34] each family consisting of three distinct mammalian genes. The $IP_3$ and Ryanodine receptors are among the largest ion channel proteins and form functional tetramers with a MW of about 1.2 and 2 million daltons, respectively.

### *Ryanodine Receptors*[34]

Three isoforms of mammalian RyR have been cloned: RyR1 is expressed in skeletal muscle (5040 residues), RyR2 is expressed in cardiac muscle (4945 residues), and RyR3 is expressed in skeletal muscle (4871 residues) as well, but in lower amounts than RyR1. RyR1 and RyR2 are critical to proper functioning of the host tissue as knocking out these genes in mice is lethal; in contrast, the RyR3 knockout is relatively normal. The ryanodine receptor functions as an intracellular $Ca^{2+}$ release channel responding to a depolarization of the plasmalemma, although the mechanism is quite different for the RyR1 and RyR2 isoforms, respectively. In skeletal muscle, membrane depolarization is directly mediated via protein-protein interactions involving the plasmalemma dihydropyridine receptor (i.e., voltage-gated calcium channel) and the RyR1 located in the sarcoplasmic reticulum. In heart muscle, membrane depolarization results in $Ca^{2+}$ influx via voltage-gated calcium channels that is used as a trigger for further $Ca^{2+}$ influx by opening RyR2 in the sarcoplasmic reticulum. The conductance properties of RyR channels are regulated by cytosolic $Ca^{2+}$, $Mg^{2+}$, ATP and phosphorylation. Channel opening is promoted by low cytosolic $Ca^{2+}$ (1-10 μM) and inhibited by high $Ca^{2+}$ (>1 mM); channel opening also appears regulated by luminal $Ca^{2+}$, but this is less clearly defined. RyR1 is more sensitive to regulation by $Mg^{2+}$ and free ATP compared with the other two isoforms: free $Mg^{2+}$ is a potent inhibitor, while ATP activates RyR. In addition to cytosolic $Ca^{2+}$, $Ca^{2+}$ release from RyR can also be stimulated by cyclic-ADP-ribose, by caffeine, and by high concentrations of ryanodine. Ryanodine receptors have also been shown to associate with several other intracellular proteins including calmodulin, calsequestrin (the major soluble $Ca^{2+}$ binding protein in the SR), and immunophilin FK-506 binding proteins. Of all of the effectors of RyR function, $Ca^{2+}$ is clearly the most significant.

### *$IP_3$ Receptors*[33]

Three isoforms of mammalian $IP_3$ receptors have been cloned containing around 2700 amino acids each. Isoforms show about 72% homology amongst each other, and, similar to the ryanodine receptor, functional channels are thought to be tetramers of identical subunits. For each subunit, three main domains can be distinguished: the N-terminal ligand binding domain (~500 residues), followed by a long regulatory domain (~1600 residues) containing among other things two consensus ATP-binding sites and the binding domains for calmodulin and FK-506 binding protein, and the C-terminal ion channel domain (~450 residues) that anchors the $IP_3$ receptor to the membrane. The C-terminal ion channel domain shows homology to the ion channel domain of the ryanodine receptor. $IP_3$ opens channels in a cooperative fashion suggesting that each subunit requires $IP_3$ to bind for channels to open; half of the channels are opened at <1 μM $IP_3$. As with ryanodine receptors, cytosolic $Ca^{2+}$ exerts biphasic effects with potentiation of $IP_3$ effects at low $Ca^{2+}$ concentration and inhibition of channel activity at high $Ca^{2+}$ concentration. $IP_3$ receptors are also regulated by ATP in a biphasic manner with low ATP concentrations increasing channel activity and high ATP levels leading to a decrease in channel activity. $IP_3$ receptors show tight interactions with two cytosolic proteins, calmodulin and immunophilin FK-506 binding protein, proteins that also have been shown to interact with ryanodine receptors. Both calmodulin and FK-506 binding protein appear to have inhibitory effects on channel activity. The $IP_3$ receptor has also been suggested to directly interact with store-operated $Ca^{2+}$ channels as will be discussed below.

As the three different $IP_3$ receptor subtypes are expressed to various degrees in different cell types, and can often be found in the same cell type, it has been of interest to characterize functional differences that might suggest distinct roles for different subtypes. Differences in

$Ca^{2+}$ sensitivity to open channels have been observed for the type-1 and type-3 receptors, respectively, which have led to the suggestion that the type-3 receptor is ideally suited for providing the first burst of $Ca^{2+}$ at very low agonist concentrations, while the type-1 receptor may be better suited for regenerating $Ca^{2+}$ waves.[35] Although oscillatory changes in $Ca^{2+}$ were long known in such excitable tissues as neurons or cardiomyocytes, the advent of intracellular $Ca^{2+}$-indicating dyes revealed oscillatory $Ca^{2+}$ waves in many nonexcitable cells. Differential regulation of $IP_3$ receptors by $IP_3$ and $Ca^{2+}$ are likely to play key roles in generating and maintaining $Ca^{2+}$ waves.[36]

## Plasma Membrane $Ca^{2+}$ Channels

In addition to the voltage-gated $Ca^{2+}$ channels that form the main focus of this volume, three main groups of other plasma membrane proteins allow $Ca^{2+}$ entry into the cell:

1. Most ligand-gated cation channels lead to membrane depolarization by providing relatively nonselective passage of both $Na^+$ and $K^+$. However, many of these channels including the neuronal acteylcholine receptors, ionotropic glutamate receptors (in particular the NMDA and AMPA subtypes), and cyclic nucleotide gated channels have significant permeabilities for $Ca^{2+}$ as well, and $Ca^{2+}$ influx via such channels has significant physiological effects. We will discuss this in more detail for the cyclic nucleotide-gated channels.
2. Extracellular ATP acts on cell surface receptors of the P2X and P2Y type. The P2Y type are G-protein coupled receptors and agonist binding often leads to a rise in intracellular $Ca^{2+}$ via the $IP_3$ pathway. In contrast, P2X receptors are cation channels that lead directly to an increase in intracellular $Ca^{2+}$.
3. Store-operated $Ca^{2+}$ entry (SOCE), also referred to as capacitive $Ca^{2+}$ entry, is a ubiquitous mechanism that opens a $Ca^{2+}$ entry pathway in the plasma membrane after intracellular $Ca^{2+}$ release has depleted intracellular $Ca^{2+}$ stores. Members of the TRP gene family are considered to be the most likely candidate for SOCE.

## Cyclic Nucleotide-Gated Channels

Cyclic nucleotide-gated channels were first described in vertebrate rod photoreceptors. They are found in the outer segment organelles of both rod and cone photoreceptors where the cGMP-gated channels carry the light-sensitive current. Cyclic nucleotide-gated channels have now been found in many different tissues, although their function has only been clearly delineated in sensory cells.[37] In retinal photoreceptors, 10-20% of the inward current is carried by $Ca^{2+}$ under physiological conditions, leading to high sustained $Ca^{2+}$ concentrations of about 500nM in darkness. Modulation of the cytosolic $Ca^{2+}$ concentration by light-dependent changes in $Ca^{2+}$ influx feeds into a powerful negative feedback loop, which involves guanylyl cyclase and mediates the process of light adaptation. Cyclic nucleotide-gated channels are heterotetramers consisting of CNGA and CNGB subunits.

## *P2X* Receptors

*P2X* gene products are found in many tissues including motor neurons, sensory neurons, airway epithelia, bone, kidney and homopoietic tissue.[38] They form fast ATP-gated nonselective cation channels, some of which have a significant $Ca^{2+}$ permeability. Seven genes coding for P2X receptor subunits have been identified. The P2X subunit proteins range from 384 to 595 residues, and contain 2 transmembrane spanning helices, separated by a large (~280 residues) extracellular domain. The extracellular domain contains ten conserved cysteine residues and fourteen conserved glycine residues, and two critical disulfide bonds contributing to the ATP binding pocket. Functional P2X receptors are thought to be homo- or heteromultimers, perhaps containing three or six subunits. Two main groups of P2X receptors can be distinguished, those that quickly desensitize (<300 ms) and those that do not or very slowly desensitize. Homomeric P2X7 proteins have the additional property of being permeable to small molecules with a MW of less than 900 kDa.

## SOCE and the TRP Gene Family

Agonist-induced rise in intracellular free $Ca^{2+}$ displays in most cases an initial transient phase due to $Ca^{2+}$ release from intracellular stores, followed by a more sustained phase that requires the presence of extracellular $Ca^{2+}$. The latter was initially named capacitive $Ca^{2+}$ entry, and is now often referred to as store-operated $Ca^{2+}$ entry or SOCE. Unlike voltage-gated channels, SOCE often shows a high selectivity for $Ca^{2+}$ over $Ba^{2+}$ and $Sr^{2+}$. SOCE has been most convincingly demonstrated with the use of fluorescent intracellular $Ca^{2+}$-indicating dyes. Characterization of $Ca^{2+}$ currents induced by store depletion has proven much more difficult, most likely due to the small currents involved and due to low single channel conductance. Such currents have now been described in a limited number of cell types and are referred to as $I_{CRAC}$ currents. Although the molecular entities underlying SOCE have not been resolved unambiguously, mounting evidence suggests that members of the TRP family are involved.[39] TRP stands for transient receptor potential referring to a protein in *Drosophila* photoreceptors that carries a light-activated inward $Ca^{2+}$ current in the fly visual excitation process. The mammalian TRP gene family contains seven members, most containing about 860 residues. TRP topology is thought to consist of 6 transmembrane spanning helices with a poreforming reentrant loop located between the fifth and sixth helical segments, analogous to the topology of members of the superfamily of voltage-gated channels. The N-terminus is located in the cytoplasm and contains in most cases ankyrin-like repeats as well as a coiled-coiled domain, which may represent sites for interaction with other proteins. The C-terminus is quite variable in length and appears to be more specific for each individual TRP member. Heterologous expression of different members of the TRP gene family has become an important tool to investigate the molecular mechanism of SOCE as will be discussed below.

## Mechanisms of Store-Operated $Ca^{2+}$ Entry

One of the most scrutinized concepts in the study of $Ca^{2+}$ homeostasis of the past decade is the mechanism by which store depletion leads to $Ca^{2+}$ entry via the plasma membrane. Although much progress has been made, consensus on unambiguous identification of such a mechanism has remained elusive. Four different mechanisms have been proposed:[40-42]

1. A diffusible $Ca^{2+}$ Influx Factor (CIF) was the first mechanism considered and several partially purified fractions have been reported to activate $Ca^{2+}$ influx in some cell systems. However, no clear identification has yet been made.
2. The $Ca^{2+}$ regulation mechanism proposes that $Ca^{2+}$ in a restricted space close to the release channel keeps it inhibited; emptying of $Ca^{2+}$ stores removes the source of the inhibitory $Ca^{2+}$ and opens the release channel.
3. The exocytosis model proposes that emptying $Ca^{2+}$ stores promotes fusion of vesicles containing the $Ca^{2+}$ release channels with the plasma membrane, thus introducing functional channels to the plasma membrane.
4. Conformational coupling between the $IP_3$ receptor in the ER with release channels in the plasma membrane.

The latter model has received a lot of attention recently, particularly in cellular systems after transfection with TRP3. Such experiments provide strong evidence for conformational coupling between TRP3 and $IP_3$ receptors. In contrast, elimination of all three $IP_3$ receptor subtypes from a preB-lymphocyte cell line eliminated agonist-induced $Ca^{2+}$ release, but normal SOCE was observed after store depletion with thapsigargin. As suggested by Putney et al,[41] this may indicate that multiple mechanisms exist that couple store depletion to $Ca^{2+}$ entry, similar to the different mechanisms by which ryanodine receptors are activated in skeletal muscle compared with heart muscle.

## Mechanisms to Lower Cytosolic $Ca^{2+}$

$Ca^{2+}$ extrusion from the cytoplasm is an energy-requiring process as $Ca^{2+}$ needs to be moved against a large electrochemical gradient. Two classes of proteins contribute to $Ca^{2+}$ extrusion

from the cytoplasm, those that move $Ca^{2+}$ at the expense of ATP hydrolysis and those that utilize the energy provided by the inward $Na^+$ gradient to couple $Ca^{2+}$ extrusion to $Na^+$ influx. Two gene families of ATP-dependent $Ca^{2+}$ pumps have been identified: the PMCA gene family of pumps found in the plasma membrane and the SERCA gene family of pumps found in the endo- or sarcoplasmic reticulum. Likewise, two gene families of $Na^+/Ca^{2+}$ exchangers have been described: the NCX gene family operates at a stoichiometry of $3Na^+/1Ca^{2+}$, while the NCKX gene family operates at a stoichiometry of $4Na^+/1Ca^{2+}+1K^+$. Thus, members of the NCKX gene family use both the inward Na+ gradient and the outward $K^+$ gradient for $Ca^{2+}$ extrusion.

## PMCA Gene Family

Four members of the mammalian PMCA gene family have been identified, each consisting of about 1200 residues.[43] PMCA belongs to the superfamily of P-type ATPases with particularly high expression levels in brain. PMCA operates as an electrogenic $Ca^{2+}/H^+$ exchanger with a 1:1 stoichiometry and a submicromolar affinity for $Ca^{2+}$. PMCA is thought to be regulated by calmodulin, acidic phospholipids, by neuroactive steroids, and by phosphorylation.

## SERCA Gene Family

Three members of the mammalian SERCA gene family have been identified. SERCA1 represents the $Ca^{2+}$ pump in the SR, expressed mainly in fast-twitched skeletal muscle, and serves the distinction of being the first $Ca^{2+}$ transport protein for which the crystal structure has been obtained.[44,45] Like PMCA, SERCA $Ca^{2+}$ pumps belong to the P-type ATPases and also consist of a single polypeptide unlike most other P-type ATPases which are heterodimers. SERCA pumps contain ten membrane spanning alpha helices, and three distinct cytosolic domains, the activation domain, the phosphorylation domain and the nucleotide binding domain. SERCA pumps are regulated by a small protein called phospholamban; binding of phospholamban leads to a reduction in the apparent affinity for $Ca^{2+}$.

## NCX Gene Family

Three members of the mammalian NCX gene family have been identified.[46] NCX proteins contain about 940 residues and are thought to consist of sets of 5 and 4 transmembrane spanning helices separated by a large hydrophilic loop located in the cytosol. The second set of TM helices is also thought to contain a reentrant loop structure. NCX1 is the dominantly expressed isoform which plays a critical role in $Ca^{2+}$ homeostasis in heart muscle. Several splice variants have been identified. NCX is a bidirectional $Ca^{2+}$ transporter and the direction of $Ca^{2+}$ flux is dependent on the electrochemical $Na^+$ gradient. This exchanger is regulated by secondary interactions with both its substrates, named $Na^+$-dependent inactivation and secondary activation by cytosolic $Ca^{2+}$. In addition, NCX is activated by phosphatidylinositol-4,5-bisphosphate. NCX1 was the first transporter shown to be regulated by phosphatidylinositol-4,5-bisphosphate; regulation by phosphatidylinositol-4,5-bisphosphate has now been shown for several other channels and transporters including the rod cGMP-gated channel, the PMCA $Ca^{2+}$ pump and many others.[47] Very recently, voltage-gated $Ca^{2+}$ channels were shown to be regulated by phosphatidylinositol-4,5-bisphosphate as well.[33]

## NCKX Gene Family

Four members of the mammalian NCKX gene family have been identified, which range in size from about 660 residues to 1210 residues.[37,46,48] Like NCX, NCKX mediates bidirectional $Ca^{2+}$ fluxes, dependent in this case on both the transmembrane $Na^+$ and $K^+$ gradients. Although some NCKX isoforms appear to enjoy widespread tissue distribution, very little is known about NCKX physiology outside of the vertebrate retina. In both rod and cone photoreceptors, the NCKX1 and NCKX2 isoforms, respectively, extrude $Ca^{2+}$ that enters the outer segments of rod and cones via the cGMP-gated channels in darkness. Under bright illumination,

NCKX quickly lowers cytosolic $Ca^{2+}$ and initiates a negative feedback loop that mediates the process of light adaptation. In bovine rod photoreceptors, a rod NCKX1 dimer has been shown to form a complex with the rod cGMP-gated channel heteromultimer.[37,49]

In summary, calcium ions are of fundamental importance for cell function, and consequently, calcium concentrations are spatially and temporally controlled by a plethora of calcium handling proteins. The remainder of this book will focus in detail on one family of calcium permeant proteins—voltage-gated calcium channels.

## Notes

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# CHAPTER 2

# Monitoring Intracellular $Ca^{2+}$ in Brain Slices with Fluorescent Indicators

**Sean J. Mulligan and Brian A. MacVicar**

## Introduction

Imaging fluorescent chemical indicators specific for calcium ($Ca^{2+}$) has provided important insights into our current understanding of the many $Ca^{2+}$ regulated cellular processes in the brain such as neurotransmitter release and synaptic plasticity. In this chapter we discuss the use of fluorescent $Ca^{2+}$ indicators for the measurement of intracellular concentrations ($[Ca^{2+}]_i$) in brain slices. Single-wavelength intensity-modulating indicators are contrasted with dual-wavelength ratiometric indicators and high versus low-affinity indicators are described. The advantages and disadvantages of using a particular indicator form (free acid, AM-ester or dextran conjugate) for reliable $Ca^{2+}$ imaging are outlined. Finally, we review calibration methods to estimate intracellular $[Ca^{2+}]$ from both nonratiometric and ratiometric indicators. This chapter should provide a guide to how and when to use various $Ca^{2+}$ sensitive fluorescent indicators to map the spatio-temporal dynamics of intracellular $[Ca^{2+}]$ in brain slices.

## $Ca^{2+}$ Sensitive Fluorescent Chemical Indicators

Our current understanding of the numerous $Ca^{2+}$ regulated physiological cellular phenomena in the brain has been greatly facilitated by the use of the $Ca^{2+}$ sensitive fluorescent chemical probes developed by Tsien and colleagues.[1-10] The most widely used fluorescent indicators for intracellular measurement of free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) are based on the $Ca^{2+}$ chelator 1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) (Fig. 1). BAPTA has high selectivity for $Ca^{2+}$ ($K_d \approx 100nM$ at pH 7.0) over competing concentrations of $Mg^{2+}$ and an extremely fast on rate ($10^8$-$10^9 M^{-1}S^{-1}$) for $Ca^{2+}$ binding. The main $Ca^{2+}$ sensitive fluorescent indicators are obtained by coupling different fluorophores with varying spectral properties to the $Ca^{2+}$ sensor BAPTA. The binding of $Ca^{2+}$ to these $Ca^{2+}$ sensitive indicators alters the excitation or emission spectra such that the fluorescence of the indicator that binds the $Ca^{2+}$ can be easily distinguished from the fluorescence of the indicator that remains $Ca^{2+}$ free. The most useful fluorescent probes are those with large molar extinction coefficients and quantum yields that exhibit strong and stable fluorescence well above any background tissue autofluorescence. The wide range of $Ca^{2+}$ sensitive indicators now available (www.probes.com) can be divided into several operational classes based on a number of criteria, the advantages and disadvantages of which should be considered when selecting a probe for a particular experiment; 1) single-wavelength intensity-modulating probes vs. dual-wavelength ratiometric probes, 2) $Ca^{2+}$ binding affinity, and 3) indicator form (salt, AM ester or dextran conjugate).

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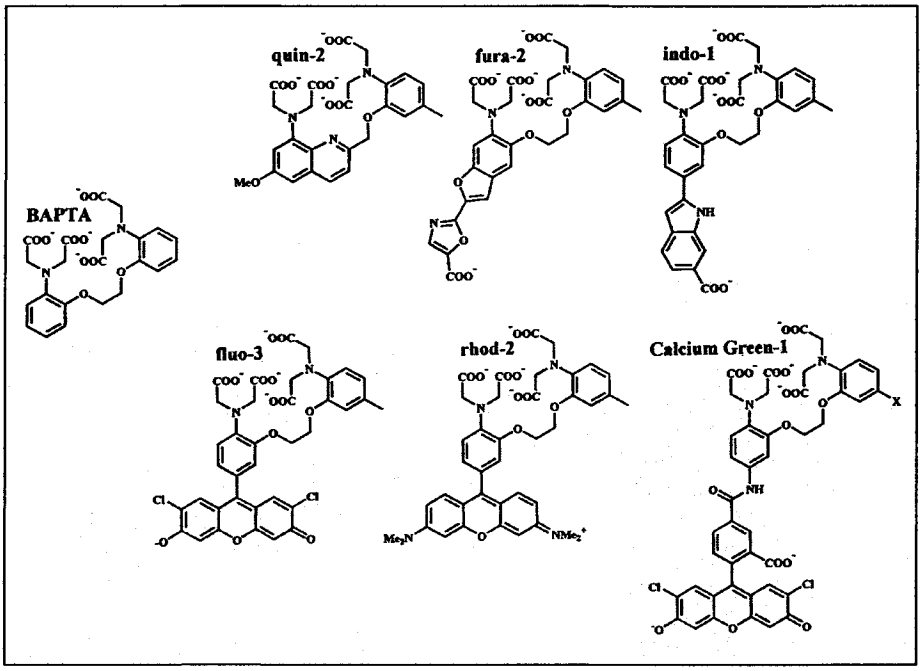



Figure 1. Chemical structures of the $Ca^{2+}$ chelator BAPTA and the widely used BAPTA based fluorescent indicators quin-2, fura-2, indo-1, fluo-3, rhod-2, and Calcium Green-1. Adapted with permission from Tsien RY. Monitoring Cell Calcium. In: Carafoli E, Klee C, eds. Calcium as a Cellular Regulator. New York: Oxford University Press, 1999:28-54.

## Single-Wavelength Probes

Increases in $[Ca^{2+}]_i$ result in dramatic increases in the quantum yield of single wavelength probes and hence the intensity of fluorescence emission, while the spectral maxima remain unchanged (i.e., no spectral shift). Fluo-3, rhod-2, and Calcium Green-1 and Calcium Green-2 are examples of commonly used single wavelength $Ca^{2+}$ sensitive indicators that undergo an ~100-fold increase in fluorescence emission upon binding $Ca^{2+}$[11] (Fig. 2). The high quantum yields characteristic of these single wavelength indicators, serves two important functions when attempting to image $[Ca^{2+}]_i$ in the fine structures associated with neurons and glial cells in brain slices. First, because all $Ca^{2+}$ indicators bind $Ca^{2+}$, they act as additional buffers in the cell and can significantly alter $Ca^{2+}$ dynamics.[12] In an attempt to measure undistorted changes in $[Ca^{2+}]_i$ it is necessary to balance the relatively low indicator concentrations needed to best report unaltered dynamics of free $[Ca^{2+}]_i$ with the need for sufficient fluorescence intensity that is gained with higher indicator concentrations. The high quantum yield of these indicators permits the use of lower dye concentrations to best report $[Ca^{2+}]_i$ dynamics while at the same time still achieving the necessary signal-to-noise for quantitative measurement. Secondly, laser intensity can be attenuated. The benefit of this is several-fold. First, fluorophores in the excited state are more prone to chemical reactions with other molecules and other fluorophores that can result in irreversible destruction or photobleaching, the final product being nonfluorescent.[13] With laser attenuation, the lifetime of the fluorophore is prolonged. Secondly, fluorophores may not only undergo complete destruction and become nonfluorescent from photodamage, but may remain fluorescent, however unable to report $Ca^{2+}$ changes. For example, basal fluorescence in the apical dendrites of neocortical neurons has been shown to increase linearly with

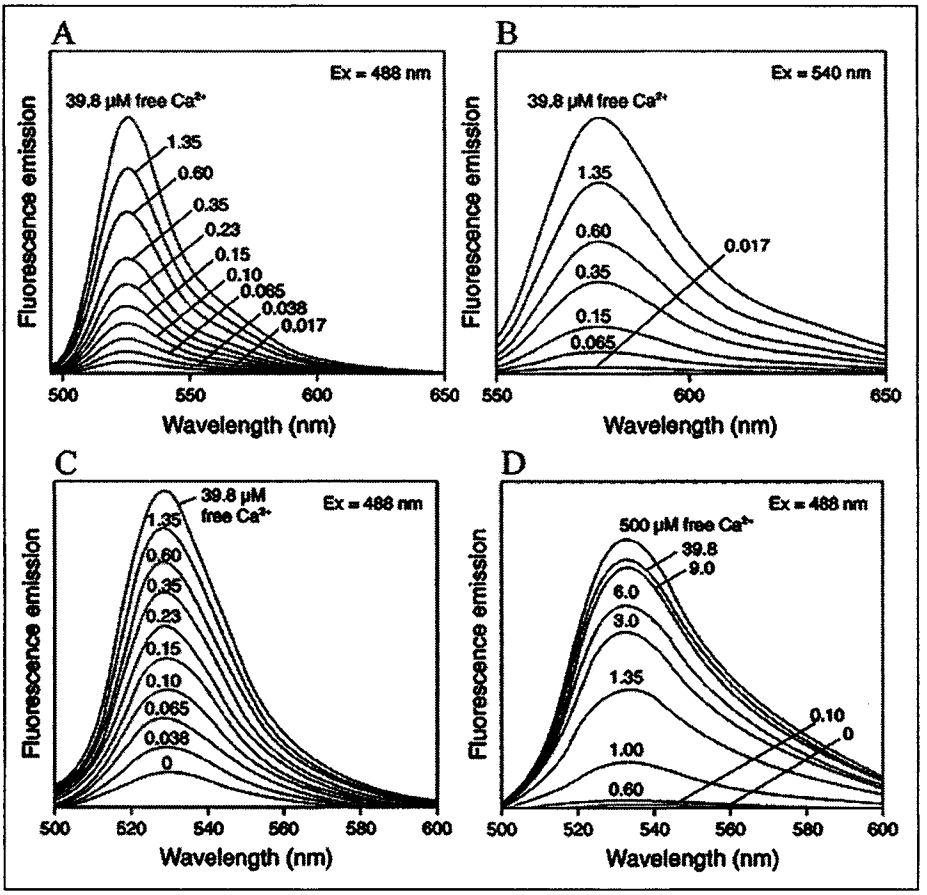



Figure 2. The $Ca^{2+}$-dependent fluorescence emission spectra of the single wavelength $Ca^{2+}$ sensitive indicators fluo-3 (A), rhod-2(B), Calcium Green-1(C), and Calcium Green-2(D). These indicators are excited by visible wavelengths of light and undergo dramatic increases in the intensity of fluorescence emission upon binding $Ca^{2+}$ with no spectral shift. Adapted with permission from Molecular Probes.[11]

cumulative photodamage.[14] The increased basal fluorescence is thought to result from irreversible photo-induced changes in the fluorophores that bind them to the cellular matrix or membranes. The bound fluorophore is molecularly altered and no longer able to report changes but remains fluorescent. Further, laser attenuation increases cell viability by decreasing phototoxic effects to the cell, independent of photodamage to the fluorophore.

An important characteristic of single wavelength $Ca^{2+}$ sensitive indicators is that they are excited by visible wavelengths of light. This makes this class of indicators well-matched for laser scanning microscopy because they are compatible with the standard fluorescein and rhodamine optical filter configurations common to most imaging system set-ups and they do not require the specialized chromatically corrected optical components necessary for use with UV excitation.[15] Most laser-based systems are supplied with visible excitation lasers that are ideal for efficient excitation. The most common being the 488nm argon-ion laser that is optimal for excitation of the popular indicators fluo-3, $Ca^{2+}$ Green-1, $Ca^{2+}$ Green-2 and Oregon Green 488 BAPTA, and the 543 nm helium/neon-ion laser that is well suited to the excitation maxima of the common red-shifted wavelength probes rhod-2 and $Ca^{2+}$ orange. In addition, visible wavelength light is

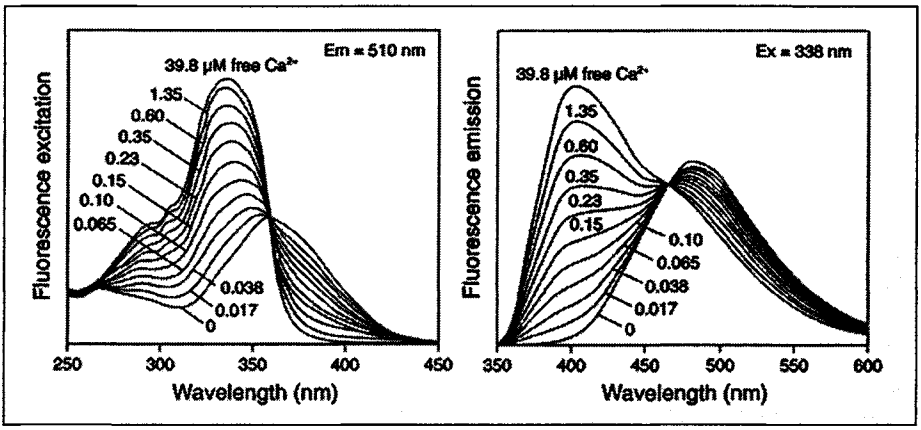



Figure 3. The $Ca^{2+}$-dependent fluorescence excitation spectra of fura-2 (left) and the emission spectra of indo-1 (right). Both of these common dual-wavelength ratiometric indicators are excited by UV wavelength light and show fluorescence intensity changes and spectral shifts in their excitation spectra (fura-2) or emission spectra (indo-1) with changing $[Ca^{2+}]$. Adapted with permission from Molecular Probes.[11]

less cytotoxic than that of UV excitation[16] and minimizes background scattering and the contribution of tissue autofluorescence, because in mammalian cells, intrinsic fluorescence is dominated by proteins and reduced nucleotides that exhibit strong fluorescence after ultraviolet (UV) excitation (for ex; NADH, NADPH; absorption maximum, 340nm; emission maximum, 460nm).[13] Finally, visible wavelength excited indicators are compatible with photolabile 'caged' $Ca^{2+}$ chelators used for rapidly changing $[Ca^{2+}]_i$ that require UV (~350nm) excitation.[15,17,18]

## Dual-Wavelength Ratiometric Probes

Like the single-wavelength probes, the ratiometric probes also show fluorescence intensity changes with changing $[Ca^{2+}]$. Unlike the single-wavelength probes however, the spectral maxima of the $Ca^{2+}$ -free and the $Ca^{2+}$ -bound forms of the ratiometric probes are at different wavelength locations (i.e.; they exhibit a spectral shift). The two most commonly used ratiometric indicators are fura-2 and indo-1.[1] Both these indicators are excited by UV wavelength light with resultant emission in the visible spectrum. They display $Ca^{2+}$ dependent spectral shifts in either their excitation spectra in the case of fura-2, or emission spectra in the case of indo-1 (Fig. 3). The $Ca^{2+}$ -free form of fura-2 displays high fluorescence emission intensity (collected at ~510 nm) when excited by 380nm wavelength light and low emission intensity upon binding $Ca^{2+}$. Reciprocal changes occur in fluorescence emission intensity when fura-2 is excited at 340nm wavelength light (i.e.; fluorescence emission intensity is low in the $Ca^{2+}$ -free form and highest upon binding $Ca^{2+}$). In contrast, indo-1 is excited at a single wavelength of light (~340 nm) and the fluorescence intensity monitored at two emission wavelengths. The emission spectral maxima of indo-1 shifts from ~480 nm in the $Ca^{2+}$-free form to ~400 nm when the indicator is saturated with $Ca^{2+}$.

Because this class of indicators exhibit $Ca^{2+}$ dependent spectral shifts, fluorescence intensity measurements can be made at two different wavelengths of the spectrum and a ratio calculated to determine $[Ca^{2+}]_i$ (see below). The ratiometric measurements are independent of the fluorescence intensity changes that arise from confounding factors unrelated to changes in $[Ca^{2+}]_i$ such as variations in indicator concentration, cell thickness, focal plane, cell volume or movement, and photobleaching effects to the probe. This fact makes the ratiometric indicators powerful tools for monitoring and quantifying Intracellular $[Ca^{2+}]$. Ratiometric imaging of $[Ca^{2+}]_i$ in brain slices using fura-2 has greatly increased our understanding of the spatio-temporal dynamics of $[Ca^{2+}]_i$ in fine neuronal structures such as dendrites[19-24] and presynaptic terminals.[25-30]

***Table 1. Properties of commonly used high- and low-affinity $Ca^{2+}$ indicators***

| Indicator | $Ca^{2+}$ Free Excitation | $Ca^{2+}$ Free Emisson | $Ca^{2+}$ Bound Excitation | $Ca^{2+}$ Bound Emisson | $K_D$ μM |
|---|---|---|---|---|---|
| **High-Affinity Indicators** | | | | | |
| Fluo-3 | 503 | | 506 | 526 | 0.39 |
| $Ca^{2+}$ Green-1 | 506 | 531 | 506 | 531 | 0.19 |
| $Ca^{2+}$ Green-2 | 506 | 536 | 503 | 536 | 0.55 |
| Oregon Green 488 BAPTA-1 | 494 | 523 | 494 | 523 | 0.17 |
| Rhod-2 | 548 | | 552 | 578 | 0.57 |
| $Ca^{2+}$ Orange | 549 | 575 | 549 | 576 | 0.185 |
| Fura-2 | 363 | 512 | 335 | 505 | 0.145 |
| Indo-1 | 346 | 475 | 330 | 401 | 0.23 |
| **Low-Affinity Indicators** | | | | | |
| Mag-Fura-2 | 369 | 511 | 329 | 508 | 25 |
| BTC | 464 | 533 | 401 | 529 | 7 |
| Mag Fura-5 | 369 | 505 | 330 | 500 | 28 |
| Magnesium Green | 506 | 531 | 506 | 531 | 6 |
| Ca Green-5N | 506 | 532 | 506 | 532 | 14 |

## $Ca^{2+}$ Binding Affinity

Fluorescent $Ca^{2+}$ indicators bind $Ca^{2+}$ in a reversible manner and as a result act as $Ca^{2+}$ buffers once introduced into cells. These exogenous buffers can significantly alter $Ca^{2+}$ dynamics.[12,27,28,31-34] In order to reduce the buffering effects on $Ca^{2+}$ signals, an important criterion for selecting an indicator is matching the $Ca^{2+}$ binding affinity of the indicator to the range of interest over which $[Ca^{2+}]_i$ changes occur during a particular experiment. $Ca^{2+}$ concentrations in astrocytes have been shown to be elevated to 3.5 μM with dopamine application[35] and estimates of $Ca^{2+}$ concentrations exceeding 200 μM have been reported at the inner membrane surface next to voltage-gated $Ca^{2+}$ channel clusters ($Ca^{2+}$ microdomains) in the presynaptic terminals of neurons during stimulation.[36] The common $Ca^{2+}$ indicators mentioned above belong to the high-affinity class of fluorescent $Ca^{2+}$ indicators, having $K_D$'s in the nanamolar range (Table 1). These high-affinity indicators greatly underestimate the increases in $[Ca^{2+}]_i$ during 'physiological' stimulation typically seen in neurons and astrocytes because $Ca^{2+}$ concentrations above 1 μM produce almost complete binding saturation.[11] For example, in the apical dendrites of neocortical layer V and hippocampal CA1 pyramidal neurons in rat brain slices, the amplitude of a single action potential evoked fluorescence transient was reduced by a factor of about three with just 125μM Fura-2.[12] The situation is equally dramatic at the presynaptic terminal. During trains of action potential evoked $[Ca^{2+}]_i$ increases, the fluorescence changes per spike decreased markedly during the train using high-affinity indicators such as Fura-2 and Calcium-Green-2[27,28,33] (Fig. 4 top). The $[Ca^{2+}]_i$ increases during the train saturate the responses of the indicators and thus the fluorescence transient is not linearly related to the changes in $[Ca^{2+}]_i$ and accurate quantification is not possible (given that an equal amount of $Ca^{2+}$ enters the presynaptic terminal with each stimulus in the train (300 nM;).[27]

Low-affinity $Ca^{2+}$ indicators have been developed that have greatly improved our ability to more accurately follow and quantify the activity evoked $[Ca^{2+}]_i$ changes in neurons and astrocytes in brain slice preparations. Mag-fura-2,[37] mag fura-5,[38] BTC,[39] Magnesium Green,[11] and Calcium-Greeen-5N[40] are some of the most widely used low-affinity $Ca^{2+}$ indicators that can detect $[Ca^{2+}]_i$ levels in the micromolar range (Table 1). The spectral shifts of the Fura-2

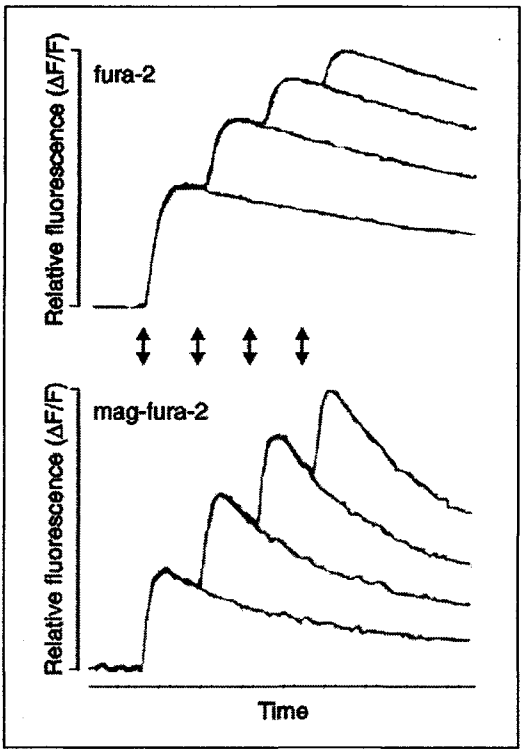



Figure 4. High-affinity $Ca^{2+}$ indicators distort rapid $Ca^{2+}$ changes. During trains of action potential evoked $[Ca^{2+}]_i$ increases (20Hz stimulus pulses indicated by double-arrows), the fluorescence changes per spike decrease markedly due to saturation of the high-affinity indicator Fura-2. The $[Ca^{2+}]_i$ increases do not saturate the response when the low-affinity indicator mag-fura-2 is used. Each stimulus in the train produces approximately the same change in fluorescence and thus the fluorescence transient is linearly related to the changes in $[Ca^{2+}]_i$. In addition, the decay time course of the $Ca^{2+}$ transients using the low-affinity indicator mag-fura-2 is undistorted. Reprinted with permission from Molecular Probes[11] and Regehr WG, Atluri PP, Calcium transients in cerebellar granule cell presynaptic terminals. Biophys J 1995; 68(5):2156-70.

analogs mag-fura-2 and mag-fura-5 are very similar to those of fura-2 but occur at higher $Ca^{2+}$ concentrations ($K_D$'s $\approx$ 25 μM and 28 μM respectively). BTC exhibits a shift in excitation maximum from ~480 nm to ~400 nm upon binding $Ca^{2+}$ which makes it the only low-affinity ratiometric indicator ($K_d \approx 7$ μM) with visible excitation wavelengths. BTC thus permits ratiometric measurements of $[Ca^{2+}]_i$ while retaining the advantages associated with using longer wavelength excitation (outlined above). The spectral properties of the single-wavelength low-affinity Calcium-Green analoges Calcium Green-5N and Magnesium Green are the same as Calcium Green-1 but have $K_D$'s $\approx$ 14 μM and 6 μM respectively.

In addition to underestimating increases in $[Ca^{2+}]_i$, high-affinity $Ca^{2+}$ indicators significantly distort the kinetics of rapid $Ca^{2+}$ changes associated with the physiological processes that operate on millisecond time scales in neurons and astrocytes. Both the high- and low-affinity classes of fluorescent indicators have $Ca^{2+}$ binding on-rates in the range of $10^8$-$10^9 M^{-1}s^{-1}$. They differ significantly however with respect to their off-rates. Low-affinity indicators have much faster $Ca^{2+}$ dissociation rates that make them much more suitable for tracking the kinetics of rapid $Ca^{2+}$ fluxes (the backward rate constants (k-) for Fura-2 and Mag-Fura2 are ~$100s^{-1}$ and $26{,}000s^{-1}$ respectively). For example, the decay time constants of fluorescent transients evoked

by single back-propagating action potentials in the apical dendrites of neocortical and hippocampal CA1 neurons increased dramatically with increasing concentrations of the high-affinity indicator Fura-2.[12,41] Fura-2 concentrations of 20, 80 125, and 250 μM corresponded to decay time constants of 78, 117, 265 and 703 msec respectively.[12] In contrast, in a study with parallel experimental procedures, high concentrations (up to 2mM) of the low-affinity indicators Magnesium Green and Oregon green BAPTA-5N did not distort the decay time course of action potential evoked $[Ca^{2+}]_i$ transients, but in fact, were faster than the predicted 'physiological' $[Ca^{2+}]_i$ transient (estimated from linear regression extrapolation to zero fura-2, during Fura-2 concentration dependence of decay time constant experiments).[24] At the presynaptic terminal the properties of the fluorescent transients also depend on the affinity of the indicator used. As discussed above, during trains of action potential evoked $[Ca^{2+}]_i$ increases, the fluorescence changes per spike decrease markedly during the trains using high-affinity indicators such as fura-2 and Calcium-Green-2 (Fig. 4 top). However, the $[Ca^{2+}]_i$ increases during trains did not saturate the response when low-affinity indicators were used. Each stimulus in the train produced approximately the same change in fluorescence and thus the fluorescence transient is linearly related to the changes in $[Ca^{2+}]_i$ and accurate quantification is possible.[27,28,33] (Fig. 4 bottom) The slow $Ca^{2+}$ binding off-rates of the high-affinity indicators make them unable to reliably track the decay time course of the $Ca^{2+}$ transients in presynaptic terminals[27,28,33,42] (Fig. 4 for qualitative decay comparison). At climbing fiber presynaptic terminals, single action potential evoked Calcium Green fluorescent transients decayed with a half decay time of 168 msec compared to only 33 msec with the novel low-affinity indicator Fluo-4 Dextran.[43]

## $Ca^{2+}$ Indicator Form

### *Salts (Free Acids)*

The common salt (or free acid) form of the $Ca^{2+}$ indicators are negatively charged polycarboxylate anions that are membrane impermeant and so must be introduced into cells by microinjection. Because this form of the indicator is negatively charged, cells may be filled by iontophoretic injection using high resistance sharp electrodes (50-150 MΩ) that contain millimolar indicator concentrations.[22,44,45] Cells are rapidly filled with indicator using this technique while the intracellular environment is not dialyzed with pipette solutions (hyperpolarizing at -0.2-0.5nA for 2-5 minutes loads an entire neocortical pyramidal cell with indicator).[46] Patch-clamping neurons and astrocytes in brain slices in the whole-cell configuration[47] with low resistance pipettes (3-10 MΩ) has become the preferred method for loading impermeant indicators into cells and simultaneously making electrophysiological measurements. Micromolar concentrations of indicator (50-500 μM) are loaded by the rapid diffusional exchange that takes place between the low resistance pipette tip and the intracellular cytoplasm.[48] This allows for defined intracellular indicator concentrations to be determined, which is a critical parameter in indicator calibrations (see below). The great advantage of using the free acid indicator form (in addition to avoiding the problems associated with using the AM-ester form -see below) is the remarkable fluorescence signal-to-noise achieved. Images from the finest neuronal structures are possible, including individual dendritic spines, axons and presynaptic terminals, and the $Ca^{2+}$ transients in these structures can be reliably quantified (Fig. 5A,B).[29,30,49-53]

## Dextran Conjugates

Fluorescent $Ca^{2+}$ indicators may be conjugated to dextrans, which are 3000 to 500,000 MW biologically inert hydrophilic polysaccharides. As with the salt forms, dextran conjugates are not membrane permeable and so must gain intracellular access by whole-cell patch pipette. Iontophoretic injection is not suitable because there is a reduction in the negative charge per unit mass. While the spectral characteristics of the dextran conjugates are similar to the free acid forms, the $Ca^{2+}$ binding affinity is slightly reduced. For example, the $K_D$ of the salt form of Fura-2 is 145 nM compared to 200-400 nM for Fura-2 dextran.[11] Molecular probes (Eugene,

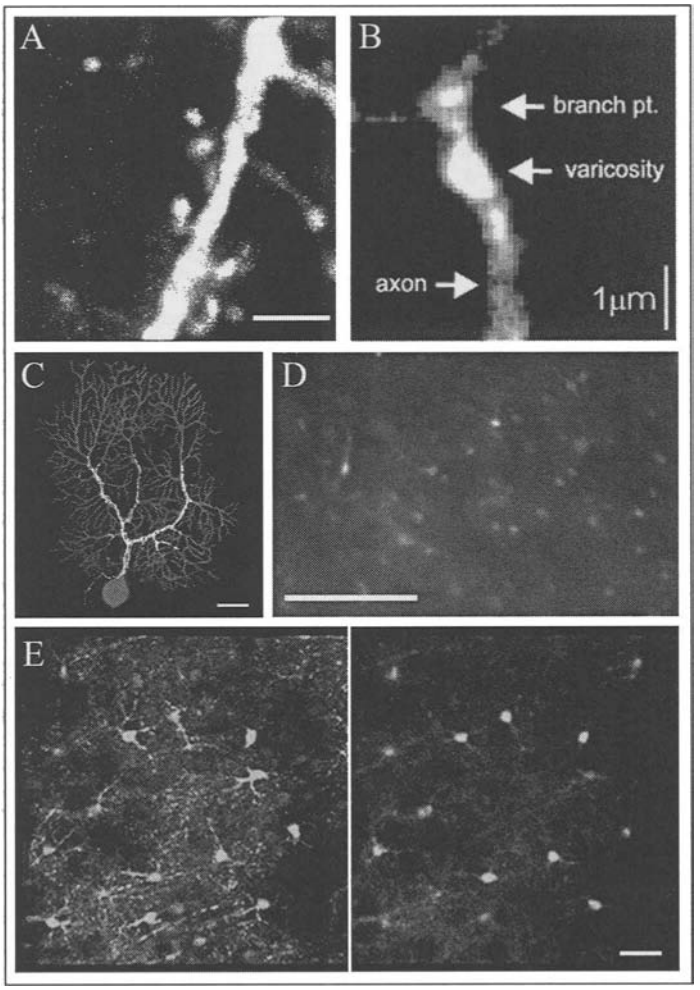



Figure 5. Neurons and astrocytes in the brain slice preparation loaded with the free acid, dextran conjugate, or AM-ester forms of $Ca^{2+}$ sensitive indicators. A) Two-photon image of a pyramidal neuron dendrite with spines from mouse visual cortex filled with 200 μM of the free acid form of Calcium Green-1 by whole cell patch pipette. (scale=5 μm) Reprinted with permission from Holthoff K, Tsay D, Yuste R. Calcium dynamics of spines depend on their dendritic location. Neuron. 2002 Jan 31;33(3):425-37. B) Two-photon image of an axonal arbor of a layer 2/3 neocortical pyramidal neuron filled with 100 μM of the free acid form of Oregon BAPTA-1 via patch pipette. (scale=1 μm) Reprinted with permission from Cox CL, Denk W, Tank DW, Svoboda K. Action potentials reliably invade axonal arbors of rat neocortical neurons. Proc Natl Acad Sci U S A 2000; 97(17):9724-8. C) An overlay of two confocal image stacks in a sagital cerebellar slice showing a Calcium Green Dextran-labeled climbing fiber incoming axon (white) innervating the thick proximal dendrites of a Purkinje neuron (grey) labeled with the fluorescent dye Alexa Fluor 568 hydrazide (Molecular Probes). (scale=20 μm) Reprinted with permission from Kreitzer AC, Gee KR, Archer EA, Regehr WG. Monitoring presynaptic calcium dynamics in projection fibers by in vivo loading of a novel calcium indicator. Neuron. 2000; 27(1):25-32. D) Dozens of neurons in layer 2/3 somatosensory cortex are labeled with the AM-ester form of Fura-2. The brain slice was taken from an 18 day old rat. (scale=100 μm) Reprinted with permission from Smetters D, Majewska A, Yuste R. Detecting action potentials in neuronal populations with calcium imaging. Methods. 1999;18(2):215-21. E) Two-photon image stack f GFP labeled astrocytes (left) loaded with $Ca^{2+}$ Orange-AM (right). (scale=20 μm). Mulligan and MacVicor unpublished observations.

OR) offers a 70,00MW dextran simultaneously conjugated to Calcium Green-1 and the $Ca^{2+}$-insensitive dye Texas Red that provides ratiometric $Ca^{2+}$ measurements with visible wavelength excitation. Dextran-conjugated $Ca^{2+}$ indicators retain all the advantages the salt form indicators have over AM-ester forms, and in addition, are much less likely to bind to cellular proteins and are better retained in cells and less resistant to extrusion.[11] The fact that they do not leak out of cells makes them ideally suited for long-term $Ca^{2+}$ measurements and excellent retrograde and anterograde tracers.[54,55] Krietzer et al (2000)[43] have loaded dextrans in to the cellular nuclei of the inferior olive in vivo, and monitored $Ca^{2+}$ dynamics days later in presynaptic terminals at the cerebellar climbing fiber to Purkinje cell synapse (Fig. 5C).

## AM-Esters

Loading neurons and astrocytes in brain slices can be achieved without the use of micropipettes for indicator injection and disruption to cell membranes. The negative charged carboxylate groups of the salt and dextran-conjugated forms of $Ca^{2+}$ indicators that render them hydrophilic and cell impermeant, may be derivatized as acetoxymethyl (AM) –esters.[5] The AM-ester form of the $Ca^{2+}$ indicator is lipophilic, membrane-permeant and insensitive to ions. Incubation of brain slices in a solution of the AM-ester form results in cellular loading by diffusion through the cell membrane. Once inside the cells, the acetyl-ester linkages are then hydrolyzed by intracellular esterases to release the active $Ca^{2+}$ sensitive indicator, which is now membrane-impermeant and trapped in the cytoplasm.

The advantage of using the AM-ester form of indicator, is that it facilitates the bulk loading of many cells or many specific cellular structures, while avoiding delicate intracellular microelectrode techniques required for loading the salt and dextran-conjugate forms. This method then, provides a way of monitoring intracellular $Ca^{2+}$ dynamics in many neurons (or neuronal processes) and astrocytes in a brain slice. For example, Yuste and colleagues have been able to bulk load AM-ester forms of $Ca^{2+}$ indicators into populations of neurons in brain slices and image somatic $Ca^{2+}$ transients (Fig. 5D).[56-58] By stimulating one excitatory neuron while imaging hundreds of others they are able to identify connected neurons and begin to explore cortical microcircuitry. Regehr and Tank (1991)[59] developed a method for selectively filling presynaptic terminals in adult brain slices by localized perfusion with the AM-ester form of Fura-2. They and others have used this method to image presynaptic $Ca^{2+}$ transients and have greatly contributed to our current understanding of the role of $Ca^{2+}$ in synaptic transmission at central synapses.[26-28,42,60-67] Astrocytes in brain slices are readily loaded with AM-esters (Fig. 5E). Results from studies monitoring $Ca^{2+}$ transients in AM-ester loaded astrocytes has forced us to expand our current view of the functional roles astrocytes play in theø central nervous system. For example, it is now believed that astrocytes are involved in activity-dependent modulation of synaptic transmission and that spontaneous intrinsic $Ca^{2+}$ oscillations in astrocytes can propagate and act as a primary source for generating neuronal activity.[68,69]

There are a number of pitfalls of using AM-ester $Ca^{2+}$ indicators. First and foremost there is a dramatic reduction in the cellular fluorescence signal-to-noise over background compared with the intracellular injected forms due to nonspecific loading and indicator that remains extracellular but fluorescent. Another major problem is incomplete hydrolysis of the esters. The fully esterfied form of Fura-2 for example contains five AM groups linked to the carboxylate groups.[11] If there is not complete hydrolysis of all five groups a new form that is $Ca^{2+}$ insensitive yet highly fluorescent remains.[70] Incomplete hydrolysis can also result in sequestering or compartmentalization inside the cell.[71,72] If the cell has low esterase activity and slow de-esterification results, the indicator is able to move freely from the cytoplasm and into intracellular organelles such as mitochondria, sarcoplamic reticulum, lysosomes and the nucleus where it then becomes cleaved and remains trapped there but unable to report cytoplasmic $Ca^{2+}$ changes.[73] Extrusion across the plasma membrane by the multidrug resistance transporter is also a problem that is specific to the AM form but not the free acid or dextran forms.[74] The above problems make it virtually impossible to perform quantitative indicator calibrations in

vivo (see below). A final complication of the AM-ester form is that the successful loading of neurons is highly age dependent. Indeed, few labs have been able to load any neurons in adult brain slices (but esee ref. 56).

## Quantitative Measurement of $[Ca^{2+}]_i$

### *Ratiometric Calibration*

The best quantitative descriptions of $[Ca^{2+}]_i$ can be achieved when using dual-wavelength ratiometric dyes, such as fura-2, because fluorescence measurements can be made at two different wavelengths and a ratio calculated to determine $[Ca^{2+}]_i$ independent of the fluorescence intensity changes that arise from confounding factors unrelated to changes in $[Ca^{2+}]_i$, such as variations in indicator concentration, cell thickness, focal plane, cell volume or movement, photobleaching effects to the probe, and instrument sensitivity. The relationship between $[Ca^{2+}]_i$ and the experimentally measured fluorescence is described in the calibration equation given by Grynkiewicz et al (1985);

$$[Ca^{2+}]_i = K_D\ (R\text{-}R_{min}/R_{max}\text{-}R)\ (S_{f2}/S_{b2})$$

For Fura-2, $K_D$ is the dissociation constant of Fura-2, R is the background corrected experimentally measured ratio of fluorescence intensity produced by 340 nm and 380 nm excitation, $R_{min}$ is the ratio at 0 $[Ca^{2+}]_i$, $R_{max}$ is the ratio at a saturating $[Ca^{2+}]$, and $S_{f2}/S_{b2}$ is the ratio of the fluorescence intensities in 0 $[Ca^{2+}]$ and saturating $[Ca^{2+}]$. To determine $R_{min}$, $R_{max}$, and $S_{f2}/S_{b2}$, in vitro or in vivo calibrations must be performed and requires that the $K_D$ for the indicator be known. These calibrations entail using a set of buffer solutions of known $[Ca^{2+}]$, and are available in 'Kit' form from Molecular Probes. When doing calibrations, it is again important to consider the $Ca^{2+}$ binding affinity of the indicator. Kits for high-affinity $Ca^{2+}$ indicators contain 10 mM $K_2$EGTA and 10 mM CaEGTA buffered solutions that provide a range of "zero" free $Ca^{2+}$ to 40 μM free $Ca^{2+}$. Solutions for low-Affinity $Ca^{2+}$ Indicator calibration provide a range from 1 μM to 1 mM free $Ca^{2+}$. The choice of whether to do an in vitro or in vivo calibration will depend on the indicator loading technique used during the experiment, i.e.; bulk loading with AM-esters or whole-cell patch pipette.

AM ester-loading indicators require calibrations to be performed in vitro, under conditions that closely mimic experimental conditions. Incomplete hydrolysis of the AM-ester, indicator compartmentalization, and ineffective access of ionophores (used to equilibrate $[Ca^{2+}]_i$ with buffers of known $Ca^{2+}$ concentrations) into regions within brain slices, as well as indicator leakage from the cells when permeabilized all make in vivo calibration impractical.[26,11] In vitro calibrations suffer however, from the fact that the spectral properties of the indicator are altered by the pH, ionic strength, intracellular binding and uptake and viscosity of the cytoplasmic environment and thus do not represent in vivo conditions.[72,75] In an attempt to correct viscosity, a viscosity correction (0.7-0.85) may be applied.[76] In vivo calibrations are possible when indicator loading with whole-cell patch pipettes because the pipette has direct access to the intracellular environment and thus circumvents the problems associated with using ionophores for the equilibration of internal and external $[Ca^{2+}]$.[48] During these calibrations, standard intracellular patch solutions are loaded to different known $[Ca^{2+}]$ levels, the fluorescence ratios experimentally measured and the parameters $R_{min}$, $R_{max}$, and $S_{f2}/S_{b2}$ determined.

### *Single-Wavelength Calibration*

Single-wavelength indicators are fluorescence intensity modulating probes and so do not allow for wavelength ratioing. As an alternative, when imaging $[Ca^{2+}]_i$ in neurons and astrocytes in brain slices it is typical to report $[Ca^{2+}]_i$ changes in normalized terms of relative florescence changes over the baseline fluorescence level, commonly known as $\Delta F/F$.[51,50,69,77-80]

Defined as;

$$\Delta F/F = ((F_1 - B_1) - (F_0 - B_0))/(F_0 - B_0)$$

where $F_1$ and $F_0$ are fluorescence in the imaged region of interest at any given time point and at the beginning of the experiment respectively, and $B_1$ and $B_0$ are the background fluorescence at any given time point and at the beginning of the experiment respectively. Background values are taken from an adjacent area distant to any indicator-containing structures. Although reporting $[Ca^{2+}]_i$ changes in terms of $\Delta F/F$ does not allow for quantitative molar measurement of $[Ca^{2+}]_i$, it is independent of indicator concentration, optical path and imaging sensitivity and is useful for reporting relative changes, induced for example by drug application. To accurately assess relative $[Ca^{2+}]_i$ changes however, it is necessary to show that the fluorescent transients are linearly related to the changes in $[Ca^{2+}]_i$. This condition is best met by using low-affinity indicators, as saturation of high-affinity indicators occurs during most physiological increases in $[Ca^{2+}]_i$ at typical indicator concentrations used and results in nonlinear changes.[12,27,28,51,56,81,82]

In theory, fluorescence signals from single wavelength indicators can be converted to $[Ca^{2+}]_i$ by the equation given by Grynkiewicz et al (1985);

$$[Ca^{2+}]_i = K_D (F-F_{min}/F_{max}-F)$$

where $K_D$ is the dissociation constant of the indicator, F is the background subtracted experimentally measured fluorescence intensity, $F_{min}$ is the fluorescence intensity at 0 $[Ca^{2+}]_i$, and $F_{max}$ is the fluorescence intensity at a saturating a $[Ca^{2+}]$. Analogous to the situation using ratiometric indicators, the indicator $K_D$ must be known and $F_{min}$ and $F_{max}$ determined from calibrations with solutions of known $[Ca^{2+}]$. $F_{min}$ and $F_{max}$ can only be reliably established in an in vitro setting at the same indicator concentration, optical pathlength and without any photobleaching or change in instrument sensitivity because the great variability in these requirements makes performing in vivo calibrations not practical. Quantitative measurements of intracellular $Ca^{2+}$ concentrations may be determined from single wavelength indicators in vivo however, if coloaded with another indicator of a differing wavelength. For example, because the single-wavelength indicators Fluo-3 and Fura Red exhibit reciprocal shifts in fluorescence intensity upon binding $Ca^{2+}$, ratiometric measurements of $[Ca^{2+}]_i$ are possible.[83,84] Simply coloading single-wavelength $Ca^{2+}$ indicators with $Ca^{2+}$-insensitive indicators that are excited at the same wavelengths, but detected at much longer wavelengths can also be done to make ratiometric measurements possible.

Changes in calcium concentration ($\Delta[Ca^{2+}]_i$) can be quantified by two methods with single-wavelength indicators, one method for use with low-affinity $Ca^{2+}$ indicators and the other for use with high-affinity indicators. The method appropriate for use with low-affinity indicators is based on the relative florescence changes over baseline fluorescence levels ($\Delta F/F$). Fluorescence intensity changes can be converted to changes in $[Ca^{2+}]_i$ using the following formula;[85]

$$(\Delta[Ca^{2+}]_i = (K_D / (\Delta F/F)_{max}) (\Delta F/F) (\Delta F/F << (\Delta F/F)_{max})$$

where, the parameters are the same as above and $(\Delta F/F)_{max}$ is the maximal change in fluorescence upon indicator saturation, which can be estimated in neurons using strong stimulation. The formula is only applicable if the indicator is far from saturation during evoked changes in $[Ca^{2+}]_i$ , a condition met with low-affinity $Ca^{2+}$ indicators. The method appropriate for use with high-affinity indicators is derived from the nonlinear dependence of fluorescence on $[Ca^{2+}]_i$ caused by high-affinity indicator saturation. Two closely timed stimulation pulses produces fluorescent transients (for example, action potential evoked $Ca^{2+}$ influx into dendritic spines[77] or presynaptic terminals,[55,85] the second of which is smaller than the first when loaded with high-affinity $Ca^{2+}$ indicators but not low-affinity indicators.[27,33,55,77,85] This indicates that partial saturation of the high-affinity indicators is responsible for the $\Delta F/F$ decrements and enable quantification of changes in intracellular $Ca^{2+}$ influx ($\Delta[Ca^{2+}]_i$) per action potential using the following formula;

$$\Delta[Ca^{2+}]_i = ([Ca^{2+}]_0 + K_D) (1 - \alpha) / 2\alpha$$

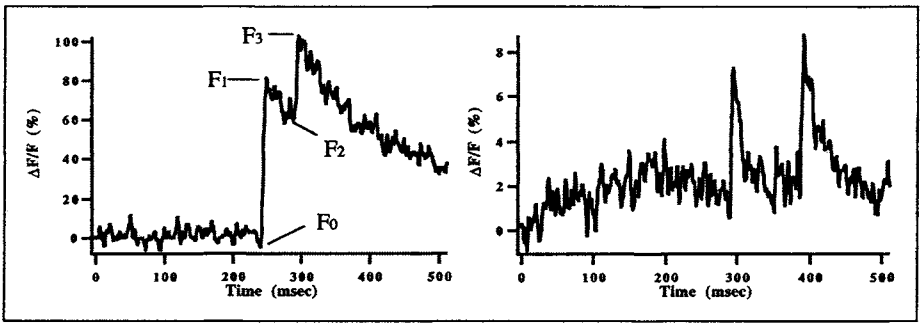



Figure 6. Quantification of changes in intracellular $Ca^{2+}$ influx per action potential is possible using high-affinity indicators. Two closely timed stimulation pulses produces fluorescent transients in a CA1 neuron, the second of which is smaller than the first when loaded with the high-affinity $Ca^{2+}$ indicator Calcium Green-1 (500 μM)(left), but not with the low-affinity indicator Magnesium Green (500 μM)(right). See accompanying text for explanation of symbols. Reprinted with permission from Yuste R, Majewska A, Cash SS, Denk W. Mechanisms of calcium influx into hippocampal spines: heterogeneity among spines, coincidence detection by NMDA receptors, and optical quantal analysis. J Neurosci 1999; 19(6):1976-87.

where $\alpha = (F_3 - F_2) / (F_1 - F_0)$ and $[Ca^{2+}]_0$ is the estimated resting $Ca^{2+}$ concentration, $K_D$ is the dissociation constant of the indicator, $F_0$ is the indicator fluorescence at resting $[Ca^{2+}]_0$, $F_1$ is the peak fluorescence produced by the first action potential, $F_2$ is the fluorescence immediately before the second action potential, and $F_3$ is the peak fluorescence produced by the second action potential (Fig. 6 left). Use of this equation to estimate $\Delta[Ca^{2+}]_i$ assumes that the influxes produced by each action potential are the same (as confirmed by low affinity indicators (Fig. 6 right)[27,33,55,77,85] and that the decay in $[Ca^{2+}]_i$ between action potentials is small compared with the peak $[Ca^{2+}]_i$.

## Conclusion

The use of $Ca^{2+}$ sensitive fluorescent indicators has proven to be an indispensable tool for studying the spatio-temporal dynamics of intracellular $[Ca^{2+}]$, which plays a critical role in many cellular processes. In this chapter we have discussed commonly used fluorescent $Ca^{2+}$ indicators for the measurement of intracellular $[Ca^{2+}]$ in neurons and astrocytes in brain slice preparations. By reviewing the properties of the several operational indicator classes and discussing the experimental advantages and disadvantages of each, it is hoped that many of the experimental pitfalls may be avoided when attempting to accurately follow and quantify intracellular $[Ca^{2+}]$ changes.

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# CHAPTER 3

# A Brief History of Calcium Channel Discovery

**Richard W. Tsien and Curtis F. Barrett**

## The Key Role of $Ca^{2+}$ Channels in Cellular Signaling

### *The Stretch Reflex Exemplifies the Importance of Electrical-to-Chemical Transduction*

The stretch reflex represents the best-known and longest studied neural circuit. Unlike sensory circuits, for example in the visual system, the stretch reflex connects an external input to a behavioral output, both mechanical in nature, and is thus easily demonstrable without technical equipment. It provides an extremely reliable input–output relationship—hence, our view of the "knee-jerk" reaction as the epitome of automatic behavior. Yet, the underlying physiology of this stretch reflex is not so simple. Indeed, it consists of an extensive chain of events, characterized by many conversions between mechanical, electrical and chemical forms of energy, complex enough to rival a contraption by Rube Goldberg (Fig. 1). The stretch reflex invokes the burgeoning fields of excitation–secretion coupling and excitation–contraction coupling. Fortunately, after decades of study (and several Nobel Prizes, the most recent in 2003), the chain of causality between a tap on the tendon and the subsequent knee jerk is now understood in considerable detail. At three stages—two electro-chemical synapses and one electro-mechanical transducer—electrical impulse spread is linked to the flow of calcium ions, and, in one way or another, that flow of $Ca^{2+}$ is controlled by $Ca^{2+}$ channels. These $Ca^{2+}$ channels do not function in isolation—rather, they work in coordination with other varieties of ion channels, notably, voltage-gated sodium and potassium channels, as well as ligand-gated channels controlled by the neurotransmitters glutamate and acetylcholine. But the critical and specific role of $Ca^{2+}$ channels in signal transduction is unique: in every instance, the conversion of an electrical signal to a chemical message requires the activation of $Ca^{2+}$ channels. This is a nearly universal rule in excitable cells.

### *Why Are Calcium Channels So Powerful, Pervasive and Fascinating?*

Let us begin with the issue of how $Ca^{2+}$ channels are generically well suited to their unique task, and return later to the issue of $Ca^{2+}$ channel types and their specialization for specific roles. The chemistry of calcium puts it into a different category than alkali metal elements such as sodium or potassium, or other alkaline earth elements such as magnesium.[1-4] It has been suggested that once cells opted to use high-energy phosphate compounds as metabolic currency, they faced great evolutionary pressure to maintain an unusually low intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i < 100$ nM). Otherwise, salts of calcium and phosphate would precipitate, turning the cytosol into a bone-like solid.[1] Accordingly, special homeostatic mechanisms such as calcium pumps evolved. In turn, the low $[Ca^{2+}]_i$ made it possible to produce a significant change in concentration with only a small $Ca^{2+}$ flux across the cell membrane; this kind of

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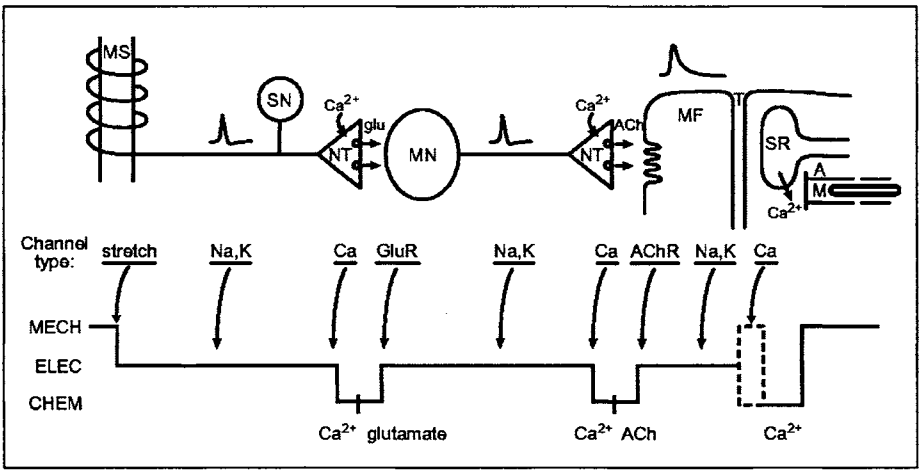



Figure 1. The classical "knee-jerk" stretch reflex illustrates the critical role of voltage-gated $Ca^{2+}$ channels in converting electrical signals to biochemical and mechanical signals. Upper panel, sequence of events in the stretch reflex depicted schematically. Lower panel, corresponding mechanical, electrical and chemical steps in the pathway, with the participating channel types, ions and transmitters indicated. Note that $Ca^{2+}$ channels are required in every case of conversion from membrane electrical activity (ELEC) to intracellular biochemical signaling (CHEM). A, actin (thin filament); ACh, acetylcholine; AChR, acetylcholine receptor; Ca, calcium; glu, glutamate; GluR, glutamate receptor; K, potassium; M, myosin (thick filament); MF, muscle fiber; MN, motoneuron; MS, muscle spindle; Na, sodium; NT, nerve terminal; SR, sarcoplasmic reticulum; T, transverse tubule.

leverage for signaling is not shared by sodium or potassium ions. In a typical cell, the concentrations of $Na^+$ and $K^+$ on both sides of the membrane are in the millimolar range, so a much larger flux of ions would be needed to produce a proportional change in concentration; thus, the ion fluxes that generate $Na^+$ and $K^+$ spikes are not sufficient to deliver an ion-encoded message. Moreover, calcium has an additional advantage for signaling, in that receptor proteins that respond efficiently to $Ca^{2+}$ ions could be readily utilized because calcium's divalent charge provides the energy needed to help drive large conformational changes. Finally, inasmuch as $Ca^{2+}$ is a "hard" ion, it satisfies a more sharply defined set of requirements for high-affinity binding than a soft ion such as $Mg^{2+}$, thereby favoring highly specific interactions.

Why is it so significant that $Ca^{2+}$ channels are highly sensitive to membrane voltage? Coupling membrane potential to second-messenger signaling greatly expands cellular capability. Electrical events have intrinsic advantages in cell signaling, including the ability of action potentials to spread quickly and faithfully, and the possibility of summation of synaptic inputs. Finally, voltage-dependence offers a means of linking the activity of one channel (e.g., $Na^+$) to that of another (e.g., $K^+$ or $Ca^{2+}$ channels), even if the channels are not in close physical proximity.

Combining the ideas of $Ca^{2+}$ as a chemical messenger and change in membrane as an initiator of $Ca^{2+}$ influx brings us to the essence of the advantages offered by voltage-gated $Ca^{2+}$ channels. The opening of a voltage-gated $Ca^{2+}$ channel is a powerful mechanism for delivering the second messenger ($Ca^{2+}$) very quickly, as there is no lack of $Ca^{2+}$ in the external milieu, and the concentration gradient for generating a large net flux is provided by the same processes that keep cytosolic $Ca^{2+}$ low. Consequently, large fluxes translate to a rapid rise in intracellular $Ca^{2+}$. In addition, $Ca^{2+}$ channels in the plasma membrane have an added advantage relative to channels in intracellular membranes, in that they have access to the plasma membrane's voltage potential, a global indicator of cellular activity. A rapid, voltage-dependent closing of the channel allows for dissipation of the message by diffusion and strong, rapid local buffering, thereby

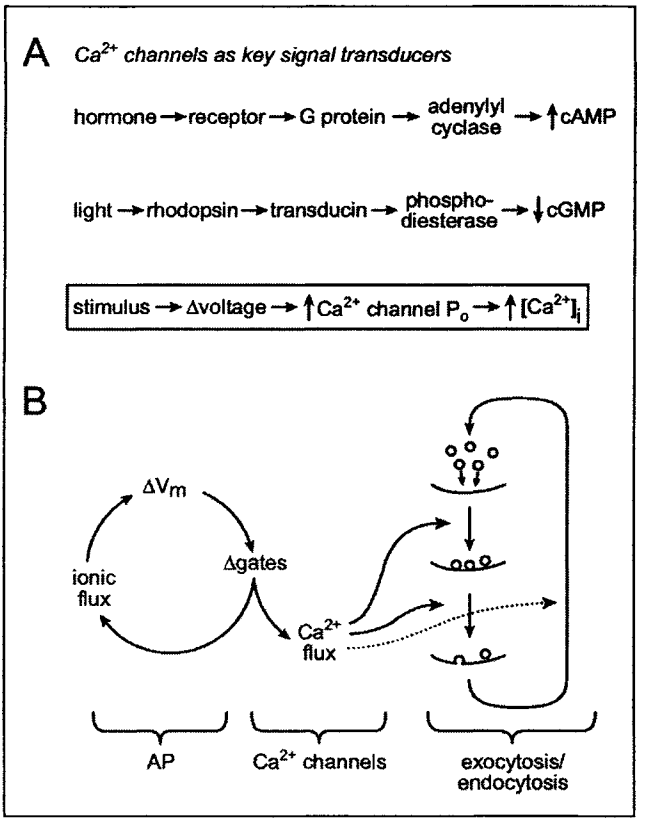



Figure 2. A) $Ca^{2+}$ channels can be modeled as key signal transducers, analogous to G protein–coupled receptors and other famous membrane-associated signaling systems. In this pathway, the stimulus (e.g., a sensory input) leads to a change in membrane voltage (e.g., an invading action potential), which in turn activates voltage-gated $Ca^{2+}$ channels. $Ca^{2+}$ influx through these channels serves as a diffusible second messenger to trigger cellular events. B) $Ca^{2+}$ forms the link between the classical Hodgkin cycle[32] and synaptic vesicle recycling of the kind envisioned by Heuser and Reese. AP, action potential at the presynaptic terminal; $V_m$, membrane potential.

achieving spatio-temporally precise signaling. In fact, $Ca^{2+}$ channels can be thought of as playing much the same role as other sources of second messengers in signaling cascades. Thus, $Ca^{2+}$ channel activity can be likened to renowned signaling systems like G protein–coupled receptors and adenylyl cyclase (Fig. 2A). In contrast, such a comparison would not be appropriate for $Na^+$, $K^+$ and $Cl^-$ channels.

To capitalize on all of these advantages, $Ca^{2+}$ channels must satisfy a number of structural and biochemical requirements. First, they should be highly voltage-dependent, making them quick to both open and close in response to changes in membrane potential (e.g., action potentials and excitatory postsynaptic potentials). Second, they must be highly permeable to $Ca^{2+}$, but not to other ions such as $Na^+$, or $K^+$. Calcium channels must also be properly localized near the relevant targets of $Ca^{2+}$ regulation, allowing for local increases in $Ca^{2+}$ concentration. Finally, they must be subject to modulation, and diverse enough to allow for selective regulation of different classes of calcium channels. All of these requirements seem to have been fulfilled by the $Ca^{2+}$ channels one finds in excitable cells. It is thus perhaps no wonder that calcium channels are critical for so many cellular processes.

### *Nevertheless, $Ca^{2+}$ Channels Were Relative Latecomers to Cellular Electrophysiology*

Considering the pivotal role of $Ca^{2+}$ channels in so many cellular processes, as well as the near-ubiquitous presence of difference types of $Ca^{2+}$ channels in excitable cells, it would seem that they would have been intensely studied from the very beginning of the era of intracellular recording and channel biophysics. But this was hardly the case. Why was the calcium channel nearly overlooked at first, and why did it receive relatively little attention even after it was first discovered? Neural circuits like the stretch reflex were very much on the minds of Alan Hodgkin, Andrew Huxley, and John Eccles, the winners of the first Nobel Prize in modern electrophysiology. However, they focused primarily on electrogenesis by sodium channels and the postsynaptic manifestations of neurotransmission, indisputably important elements of neuronal communication. In the case of Hodgkin, Huxley and Bernard Katz, a sensible preference for large preparations and large currents, attributes suitable for propagation over long distances, led them to study the squid giant axon. Here, the electrophysiological study of excitability became rightfully synonymous with the Sodium Theory. In the case of Eccles, the focus was on postsynaptic recordings and the nature of excitatory and inhibitory transmission, questions passed down from Charles Sherrington.

One glimpse of the central focus of the era can be obtained by revisiting a thick compendium of classical literature, gathered in 1972 by Ian Cooke and Mack Lipkin. Their *Cellular Neurophysiology* [5] offered a generation of neuroscience students easy access to the original classic studies. Reflective of the ethos of the time, there was a huge disparity in Cooke and Lipkin's choice between amounts of space devoted to $Na^+$ versus $Ca^{2+}$ entry; indeed, not a single paper is devoted to $Ca^{2+}$ channels as such. Today, however, it is apparent that $Ca^{2+}$ channels have reached the forefront of the field of ion channel research, as judged by the number of abstracts, meetings and publications devoted to $Ca^{2+}$ channels as opposed to $Na^+$ channels. Indeed, entire international symposia are now dedicated exclusively to current research on calcium channels; due to their vital role in cellular signaling, their diversity, and great susceptibility to modulation, $Ca^{2+}$ channels have attracted considerable interest. It may be satisfying for some of us to see such fervent attention to the molecular object of our affections, but the question still remains: how did we get from there to here?

## The Winding Road of Calcium Channel Discovery

### *Bernard Katz and Colleagues: Early Beginnings at University College London*

Where did the notion of a calcium channel first start? Clearly, it was in the Biophysics Department at University College London, founded by A.V. Hill and later led by Bernard Katz (Fig. 3A). Most investigators would credit Katz, Paul Fatt and Bernard Ginsborg for pioneering the calcium channel field with their work in large muscle cells of crab[6] and crayfish.[7] These three were charter members of a small group of electrophysiologists who clarified many aspects of nerve propagation and synaptic transmission. But despite the pedigree of these investigators, calcium channels were not a major source of attention in the early 1950s, despite their now clearly critical role in even the simplest neuromuscular circuit. Indeed, the discovery of $Ca^{2+}$ spikes aroused no great excitement in the 1950s, nor even many years later, when the accumulated triumphs of electrophysiology were reviewed.

Given their functional importance, why were $Ca^{2+}$ channels so slow to catch on? Several reasons come to mind.

#### The First Paper Drew Little Attention because the Investigators Themselves Were Unsure of What They Had Found

Fatt and Katz entitled their 1953 paper "The Electrical Properties of Crustacean Muscle Fibres", not exactly a title destined to be a Citation Classic! In fact, the discovery itself was merely an accident. Fatt and Katz were after bigger game—the electrical properties of inhibi-




Figure 3. Three of the principal founders of the field of voltage-gated calcium channels. A) Sir Bernard Katz. B) Susumu Hagiwara. C) Harald Reuter.

tory transmission—and were proceeding through a checklist of resting potential, membrane resistance and capacitance, the current–voltage relationship, and the basis of electrogenesis. They were not expecting to find electrical responses that persisted in sodium-free media, for example when $Na^+$ was replaced by tetrabutylammonium (TBA). The data at hand were limited, so their conclusions were suitably cautious. They discussed in considerable detail the "choline action potential" and the "tetrabutylammonium action potential", but were quite circumspect about concluding that $Ca^{2+}$ was the charge carrier: "The mechanism of the action potential, and the species of ions involved in the movement of charge across the membrane, remain a puzzling problem...(i) it may be that TBA remains adsorbed to the fibre surface, but is mobilized during excitation and temporarily transferred into the cell interior; (ii) alternatively, influx of calcium or magnesium, or outflux of some internal anion may be responsible for transport of charge."

## The Next Advance in Understanding Was Slow in Coming

It took a full five years before Fatt, now working with Ginsborg, published a follow-up study, showing that full-blown action potentials could be generated in the absence of $Na^+$, even without quaternary ammonium ions, provided the external solution contained strontium or barium ions.[7] After soaking the preparation in tetraethylammonium-containing solution and subsequent washout of TEA, $Ca^{2+}$ could serve as a charge carrier (later, it was found that the quaternary ammonium compounds entered the fiber and served to dampen outward potassium currents). This led to a cautious but clear statement that the movement of $Sr^{2+}$, $Ba^{2+}$ or even $Ca^{2+}$ itself could support action potentials across the membrane.

## Interest in Excitability Revolved around Proposed Mechanisms in Squid Axons

At the time, the results in crustacean muscle were of interest mainly because they seemed so unusual. The "Sodium Theory" had already been proven in myelinated nerve,[8] skeletal muscle[9] and heart tissue.[10] The behavior of all tissues was judged against the standard of squid axons, where the recordings were most reliable. Exceptions were therefore of growing importance, if for no other reason than an ongoing debate with Ichiji Tasaki, who opposed the Sodium Theory of Hodgkin and colleagues. Since most of the 1953 Fatt & Katz paper was devoted to nailing down the fact that the action potential was not due to sodium entry, spikes dependent on $Ca^{2+}$ entry were seen mainly as a curious variant on the dominant $Na^+$ entry mechanism.

## The Studies in Crustacean Muscle Focused on Electrogenesis Only, without Reference to Muscle Contraction

A direct relationship between $Ca^{2+}$ entry and muscle contraction was not mentioned. In those days, the role of $Ca^{2+}$ in contractile activation was not fully appreciated. This was despite

Heilbrunn and Wiercinski's experiments with $Ca^{2+}$ injection,[11] as well as the much earlier work of Sydney Ringer, who showed the critical dependence of cardiac contraction on external $Ca^{2+}$. Even the discovery of troponin-C as a $Ca^{2+}$ sensor for muscle contraction [12] didn't bring to full light the importance of $Ca^{2+}$ in contractile activation.

### The Uncovering of $Ca^{2+}$ Spikes Appeared Amidst a Crowded Field of Discovery

Even at its publication, Fatt and Katz's paper on the electrical properties of crab muscle was unfortunately but understandably overshadowed by its companion paper,[13] their classic work on inhibitory transmission, a study establishing that inhibitory impulses can increase the post-junctional membrane conductance, thereby shunting excitatory neurotransmission whether or not the membrane actually hyperpolarizes. Bernard Katz himself was partway along a marvelous intellectual trajectory, ranging from excitability[14] to mechanisms of excitatory transmission[15] and the quantal basis of transmitter release.[16]

Each of the above factors contributed to the slow entrance of calcium channels into the burgeoning field of ion channel electrophysiology. However, despite their relative lateness entering the field, once their importance became clear, calcium channels quickly assumed a prominent position in the arena of ion channel electrophysiology.

## *The Inescapable Influence of Alan Hodgkin and Squid Axons*

As an aside to the story of Katz and his associates in the Department of Biophysics, it is interesting to ask the question of whether Alan Hodgkin and Andrew Huxley, the principal pioneers in the field of membrane excitability, ever actually studied $Ca^{2+}$ channels. As co-founders of the Sodium Theory (together with Katz), Hodgkin and Huxley never directly worked on calcium channels, although they came much closer than people usually think. Limitations on availability of squid to certain seasons and certain locations, and perhaps restlessness while the rest of the world caught up to the 1952 papers, spurred their interest in solving other problems. Thus, Hodgkin and Huxley turned eventually to excitation–contraction coupling, the terminal step in the stretch reflex, in vertebrate skeletal muscle. In classic experiments performed with Bob Taylor, Huxley used focal stimulation to demonstrate that voltage-dependence of contraction arose at regularly spaced "hot-spots" within the Z-line of frog skeletal muscle, corresponding to the openings of transverse tubules. Concurrently, working with Paul Horowicz, Hodgkin applied $K^+$-rich solutions to depolarize single muscle fibers and described the very steep voltage-dependence of contraction. We now know that E–C coupling is critically dependent on voltage-gated $Ca^{2+}$ channels (comprised of the $\alpha_{1S}$ ($Ca_v1.1$) subunit along with ancillary subunits) in the transverse tubules. So in fact Hodgkin and Huxley both implicitly studied $Ca^{2+}$ channels.

While we are on the subject of $Ca^{2+}$ channels and muscle contraction, it is worth noting that several scientific heirs of Hodgkin and Huxley's have made vital contributions to the field of E–C coupling, some directly or indirectly involving $Ca^{2+}$ channels. Knox Chandler (a postdoc of Hodgkin's) and Martin Schneider (a graduate student with Horowicz, thereby Hodgkin's scientific "grandson") are good examples. They co-discovered a charge movement in the transverse tubules that mediated the voltage-dependence of contraction.[17] That charge movement was really a form of gating current, now known to be critical for controlling the gating of almost all voltage-dependent channels. What wasn't clear then, and was only shown much later by Eduardo Rios, a postdoc of Schneider's, was that the charge movement observed by Schneider and Chandler actually originates in the skeletal L-type ($\alpha_{1S}$) $Ca^{2+}$ channel.[18]

Certainly one of the most fascinating parts of the story of Alan Hodgkin and calcium channels is as follows. Hodgkin and Keynes used squid axons to make the first measurements of activity-dependent $^{45}Ca^{2+}$ flux.[19] They found that the net entry of tracer calcium was 600-fold less than the entry of sodium, making $Ca^{2+}$ unlikely to contribute to the spike. However, they included this prophetic statement in their discussion:

"The finding of a greater uptake of calcium by stimulated nerve may be relevant in considering the mode of release of chemical transmitters by nerve endings. In addition to its action in dispersing squid axoplasm, calcium has been found to have a disruptive effect on

other intracellular structures, such as the sarcosomes of heart muscle, and it is interesting to speculate whether a penetration of calcium at the nerve ending might not be one of the factors involved in breaking up the intracellular vesicles near the membrane[20] and releasing acetylcholine from them. There is some indication that $Mg^{2+}$ ions do not have the same action as $Ca^{2+}$ on intracellular structures, so that a competition between them to enter the nerve terminals, together with a failure of $Mg^{2+}$ to disrupt the vesicles, might help to explain the inhibitory effect of magnesium." This was a clear (and accurate) assessment of the importance of $Ca^{2+}$ entry in mediating many cellular processes, such as transmitter release and E–C coupling.

Katz's group was also very familiar with the importance of $Ca^{2+}$ in transmission[21] and the antagonistic effect of $Mg^{2+}$.[22] Although in Katz's department, these authors did not mention Fatt and Katz's 1953 paper; after all, at that time the working hypothesis, (del Castillo and Katz and later, Jenkinson)[23,24] was to envision $Ca^{2+}$ and $Mg^{2+}$ competing for an unidentified surface receptor X, which upon binding ligand converted to an active form X', allowing release of acetylcholine. So Hodgkin and Keynes were breaking new ground by suggesting that neurosecretion might be triggered by $Ca^{2+}$ influx. They further suggested that Fatt and Katz might have been studying excitability that depended on some kind of divalent cation influx. So the first insight into the notion of the calcium channel's role in excitation-response coupling may actually have originated with Hodgkin and Keynes in 1957; the follow year, Fatt and Ginsborg nicely credited this paper in their study of divalent cation spikes,[7] thereby adding favor to the notion of $Ca^{2+}$-dependent excitability.

## *Katz and Miledi Re-Enter the Fray by Focusing on the Squid Giant Synapse*

So when did it become accepted that $Ca^{2+}$ entry through voltage-gated calcium channels triggers transmitter release? Katz's classic text *Nerve, Muscle, and Synapse*[25] provided a retrospective snapshot of his views more than a dozen years after his 1953 paper on crab muscle electrophysiology. Katz quoted Fatt and Ginsborg's evidence in crustacean muscle for "entry of a divalent cation like Ca", but only as a curious exception to the generalization of $Na^+$ influx as the inward current during excitation. With characteristic modesty, there was no reference to Fatt and Katz.[6] The birth of calcium channels was tucked away in a short paragraph alongside the efflux of an internal anion in the impulse-conducting plant cell *Nitella*, both deemed illustrative exceptions. It appears that the provocation from Hodgkin and Keynes[19] about synaptic transmission had not yet taken effect. However, something was clearly brewing, because there is a forward-looking statement in the very last paragraph of Katz's book:

> *It is interesting to note certain features that the initiation of muscle contraction shares with the initiation of an action potential and with the release of a transmitter substance at a nerve terminal. In all three cases, the primary event is a depolarization of the cell membrane. Depolarization, however, is not a sufficient stimulus; it becomes effective in producing its diverse results only if calcium is present, whether the final result be increase of sodium permeability,[26] the facilitated release of acetylcholine quanta,[27] or the activation of myosin molecules.[28]*

The germ of an idea about a messenger role for $Ca^{2+}$ was there, but Katz continued to focus on the presence of calcium, not on $Ca^{2+}$ flux per se.

Of course, in the long run, Katz made further giant contributions to understanding the significance of $Ca^{2+}$ influx for triggering downstream cellular events. This was revisited in 1967, after the momentous period of clarifying the quantal nature of neurotransmission and the key role of calcium. Working with Ricardo Miledi at the Marine Station in Naples, Katz returned to the subject of $Ca^{2+}$ entry, but now looking in nerve terminals. Using the squid giant synapse, Katz and Miledi showed: that transmission required membrane depolarization, but not sodium channels; that $Ca^{2+}$ spikes could be generated at the terminal if outward $K^+$ currents were blocked; and that transmitter release required repolarization when initiated by strong depolarization, consistent with the requirement for both channel opening and a strong electrical driving force for $Ca^{2+}$ influx. Interestingly, in his papers describing his work on the squid synapse, Katz refers back to the work on crustacean muscle, but once again only to the

paper of Fatt & Ginsborg,[7] and not his own earlier work.[6] Nevertheless, the studies of Katz and Miledi were to provide a foundation for elegant studies in the 1970s by Llinás and colleagues[29] and later by Augustine, Charlton and Smith.[30]

### *Susumu Hagiwara: $Ca^{2+}$-Based Electrogenesis in Full Bloom*

Another significant contributor to the birth of the field of calcium channels was Susumu Hagiwara (Fig. 3B). Known affectionately as "Hagi", Hagiwara was a zoologist whose roots were in Japan, but who worked mainly in the States. Although he held an M.D. (as well as a Ph.D.), Hagiwara was not particularly obsessed about working strictly with mammals or even vertebrates; nowadays he might be classified a comparative neuroethologist. His interests were extremely broad, ranging from the singing of cicadas to echolocation in bats, from the electrical properties of eggs to taste sensation in cats. Within this expansive range of topics, Hagiwara is best known for his studies on "calcium spikes", first in barnacle giant muscle fibers, then later in many other preparations.

Interestingly, the Chilean barnacle was actually a substitute for the squid at the Monte Mar marine station on the Chilean coast. The squid were displaced from their usual territory by a large storm, forcing Hagi and his colleagues Alan Grinnell and Jared Diamond to find another preparation. As described earlier by Hoyle, the giant muscle fibers of the barnacle were up to a millimeter in diameter and five centimeters in length. So here was a large preparation in which $Ca^{2+}$ channels could be brought to center stage under appropriate experimental conditions (even though these fibers don't have full-blown spikes under physiological conditions).

Hagiwara is often credited with helping to establish the ubiquity of $Ca^{2+}$ channels because of his work on so many different preparations. Ted Bullock and Alan Grinnell, two of Hagiwara's long-time colleagues, provided this quote from Bertil Hille:[31] "Hagi [was] a research scientist of peerless distinction... He is remembered as the champion who brought the calcium channel to its rightful respected place and in the process discovered blocking ions, flux saturation, inactivation dependent on internal calcium, and many other unanticipated phenomena."

This well-deserved praise for Hagiwara does not change the history of the calcium channel itself, or its slow emergence from obscurity. Once again, Bullock and Grinnell on Hagiwara: "He recounts, with characteristically self-deprecating humor, how, during this period when 'the calcium channel was only found in miserable animals like crustaceans and was thought to play no important function in the mammalian brain... I suffered tremendously from a minority [*sic*] complex.'"

Along with its humble position in the phylogenetic tree, the barnacle had an additional handicap: the complicated morphological cable properties of its giant muscle fiber made it very difficult to study in a rigorously biophysical way. As Hagiwara and Byerly stated, "At first it appeared that barnacle giant muscle fibers might become the 'squid axon' of Ca currents... however, there are deep, long invaginations of the sarcolemma in all crustacean muscle fibers...[which] cause barnacle muscle to fail to satisfy criteria about reliability and speed of voltage control." In his classic book on ion channels,[32] Hille judiciously skips over these cable complications, but chooses instead to introduce the gigaohm seal (patch-clamp) recording methods of Neher and Sakmann and Sigworth,[33] right in the middle of the chapter on calcium channels. This seems fair enough, as this was one field that benefited enormously from patch-clamp techniques.

Despite the obscure idiosyncrasies of the invertebrate preparations of those early days, the interactions between investigators were typified by collegiality. Alan Grinnell described the relationship between Katz and Hagiwara as follows: "Hagi and BK met several times, and I know they admired each others' work. One of the reasons I went to UCLA after my postdoc in London was Katz's strong recommendation of Hagi. And in 1967, when Hagi and I went to Chile to study the giant synapse in squids there—which proved abortive because a major storm came up, following which the squid were unobtainable for several years, so we worked on giant barnacles instead—we were later grateful for the storm, since that fall Katz & Miledi published

their beautiful J Physiol paper on Naples squid establishing the role of $Ca^{2+}$ in synaptic transmission. As Hagi said at the time, we would have been scooped by the best."

Around the same time, Hagiwara and Nakajima[34] published a fundamental paper describing the pharmacology of $Ca^{2+}$ spikes, neatly combining work on both barnacle muscle and heart muscle. The agents tetrodotoxin (TTX) and procaine, normally effective on $Na^+$ spikes of other tissues, failed to block the barnacle $Ca^{2+}$ spike. On the other hand, manganese acted as an inhibitor, competing with $Ca^{2+}$ to block the $Ca^{2+}$ spike. In heart, TTX and procaine suppressed the initial rate of the rising phase, but spared the plateau; in contrast, manganese acted primarily on the plateau phase. As Hagiwara and Nakajima recognized, the heart provides a clear example of a setting where $Ca^{2+}$ channels are absolutely essential for maintaining life; we know now that all heart cells use $Ca^{2+}$ channels for one or more aspects of their electrical activity. $Ca^{2+}$ channels are critical for pacemaker activity in the sino-atrial node, whose depolarization is aided in its last stages by an increasing permeability to $Ca^{2+}$ ions. Slow propagation in the atrio-ventricular node absolutely requires $Ca^{2+}$ channels. Finally, in the atria and ventricles, where the pumping action of the heart takes place, both the action potential plateau and contraction rely on $Ca^{2+}$ channels.

## *Harald Reuter: First $Ca^{2+}$ Currents under Voltage Clamp*

Enter the next major father figure in this saga, Harald Reuter (Fig. 3C). Born in Germany, Reuter has spent the bulk of his illustrious scientific career in Bern, Switzerland, but with brief and sometimes nearly annual peregrinations to the States. Reuter first came to calcium channel research as an M.D., with a non-quantitative training in cardiac pharmacology and an abiding interest in how adrenaline affects the heart. Reuter's voltage-clamp recordings of shortened Purkinje fibers, using the method of Trautwein and associates, were the first to provide evidence for a $Ca^{2+}$ current.[35] Interestingly, the same Journal of Physiology volume that featured Reuter's paper on the putative $Ca^{2+}$ current also contained Katz and Miledi's study of synaptic transmission in the absence of nerve impulses, using TTX to block $Na^+$ channels.[36] One year following his presentation of $Ca^{2+}$ currents, Reuter, together with Seitz, found evidence linking external $Na^+$ to $Ca^{2+}$ efflux, now known as the $Na^+$–$Ca^{2+}$ exchange.[37]

The work in cardiac Purkinje fibers was later followed by recordings from bundles of ventricular or atrial heart muscle,[38,39] which also showed inward currents supported by $Ca^{2+}$ influx. In magnitude and kinetics, the putative $Ca^{2+}$ channel currents were smaller and slower than sodium currents. This conclusion soon came under attack on technical grounds, mainly from Ted Johnson and Mel Lieberman, who suggested that the "calcium current" or "slow inward current" might be an artifact arising from the methodologies that needed to be applied to try controlling the membrane potential while the membrane current was generated.[40] The multi-cellular cardiac preparations were regarded as far inferior to experimental systems such as the squid giant axon, where techniques for uniformly controlling the membrane potential were more rigorously applicable. For example, narrow extracellular spaces in the cardiac tissue could support unwanted and immeasurable voltage drops in series with the excitable membrane of interest, invalidating the voltage clamp. The next few years were particularly confusing for the study of cardiac $Ca^{2+}$ channels, which was a world unto itself for a while, with little crossover from those interested in nerve or skeletal muscle. The controversy about the very existence of $Ca^{2+}$ currents began to resolve with cardiac preparations with more favorable geometries, such as the rabbit Purkinje fiber (wide clefts)[41] combined with three-microelectrode voltage clamp, which measured and capitalized on cable non-uniformity but nevertheless verified the existence of bona fide $Ca^{2+}$ currents.[42] The ultimate resolution came with the advent of methods for studying single cells, first with large suction electrodes and later with modern patch-clamp methodology. The ability to alter the intracellular ionic composition, first tediously developed in multicellular preparations,[43] became much easier at the level of single cells.[44] Interestingly, Harald Reuter was very active in the use of both suction and patch-clamp electrodes, and made many additional contributions to the field of cardiac electrophysiology and $Ca^{2+}$ channels using these newer techniques.

In hindsight, most would agree that the first sets of voltage-clamp experiments had not paid sufficient attention to possible technical difficulties, but were nevertheless correct in uncovering a new component of inward excitatory current. It is clear now that the problems of spatio-temporal control of membrane potential were actually less serious than originally thought, whereas issues of overlapping currents were more severe. An experiment in which extracellular [$Ca^{2+}$] varied could undoubtedly evoke changes in $Ca^{2+}$-dependent currents as well as in the flux through $Ca^{2+}$ channels themselves.

The mid-1960s also marked the advent of pharmacological approaches to identifying $Ca^{2+}$ currents using channel blockers such as verapamil, nifedipine and diltiazem, thanks to the efforts of Albrecht Fleckenstein and others (for review, see ref. 45). Application of such agents buttressed emergent ideas about the distinct nature of sodium and calcium channels in cardiac cells. Today, $Ca^{2+}$ channel blockers are effective in a number of cardiovascular syndromes and represent a multibillion-dollar business; who would have thought that good could result from channel blockade that could in principle reduce the strength of cardiac contraction? In fact, the main therapeutic target for the $Ca^{2+}$ channel blockers is the L-type $Ca^{2+}$ channel in vascular smooth muscle, where relaxation favors blood flow and lowers blood pressure; this arises from the ability of the blockers to act more potently on vascular smooth muscle than on heart (a piece of good fortune for both the patient and the drug industry).

## The Diversity of Native $Ca^{2+}$ Channel Currents

### *Early Studies on the Diversity of $Ca^{2+}$ Channel Types*

In the early days of $Ca^{2+}$ channel electrophysiology using morphologically simple preparations, the original presumption (with or without credit to Gertrude Stein) was that "a $Ca^{2+}$ channel is a $Ca^{2+}$ channel is a $Ca^{2+}$ channel", with little regard for the possibility of multiple $Ca^{2+}$ channel types. Peter Baker, one of the leading scientists in the study of squid axon excitability, was an example of someone with little patience for studies of multiple $Ca^{2+}$ channels. To him, the important issues were whether $Ca^{2+}$ channels truly existed, how they were linked to the process of vesicle exocytosis, and how vesicle fusion itself took place. This was fair enough, and perhaps fitting for someone who began his career at a time when the very nature of excitability and the role of $Na^+$ influx were still under fire.

However, Hagiwara was ahead of his time in recognizing the multiplicity of channels and their possible importance in excitability. Working with starfish eggs, he and his colleagues Ozawa and Sand described two inward currents, labeled I and II, both supported by $Ca^{2+}$ influx;[46] (see also Fox and Krasne[47] for their work on egg cells of a marine worm). Today, their "I" and "II" would be more descriptively termed low-voltage activated (LVA) and high-voltage activated (HVA), respectively. As Hagiwara and Byerly stated in 1981: "...at present it seems most objective to give up the prejudice that the Ca channel is like the Na channel and allow that there may be various types of Ca channels." But not everyone was convinced: Lux's group[48] summarized their patch-clamp recordings of unitary $Ca^{2+}$ channel activity from bird, snail and rat by saying "...we conclude that $Ca^{2+}$ channels everywhere are basically the same."

The distinction between LVA and HVA channels gained momentum through several studies in the early 1980s, mostly in biological systems less obscure than starfish eggs. In beautiful recordings of action potentials in inferior olivary neurons, Llinás and Yarom[49] discovered low-threshold $Ca^{2+}$ spikes that depended on an underlying conductance; this conductance required strongly negative potentials to become de-inactivated. This was the current-clamp equivalent of LVA channels, later termed T-type channels. Carbone and Lux,[50] as well as Fedulova, Kostyuk and colleagues,[51] provided further evidence of LVA $Ca^{2+}$ channels in the form of single-channel recordings of chick and rat sensory neurons. Finally, Matteson and Armstrong[52,53] demonstrated slow deactivation of T-type channels upon sudden repolarization, a key property of these channels. In 1985, Bean obtained similar evidence for the

distinctiveness of T-type channels in atrial myocytes, showing that only the HVA population of channels was responsive to $Ca^{2+}$ channel blockers and β-adrenergic stimulation.[54]

In that period, the evidence supporting the separation of voltage-gated $Ca^{2+}$ channels into LVA and HVA channels was mostly biophysical. This evidence included voltage-dependence of gating (LVA channels require a more negative holding potential, and activate at much more negative test potentials than HVA channels), speed of deactivation (LVA channels deactivate slowly compared to HVA channels), resistance to run-down (loss of activity) following patch excision or dialysis of the cytosol (LVA channels are more resistant to run-down), and differential sensitivity to $Cd^{2+}$ (LVA channels are much less sensitive to $Cd^{2+}$). Taken together, it became increasingly evident that voltage-gated calcium channels could be accurately separated into two broad categories on the basis of biophysical properties.

## *A Tripartite Classification of Native $Ca^{2+}$ Channels*

Our group's own interest in multiple types of $Ca^{2+}$ channels took an interesting turn when Martha Nowycky and Aaron Fox teamed up to examine the properties of single $Ca^{2+}$ channels in cell bodies of chick sensory neurons.[55] Based on published research, for example Aaron's previous work on marine worm egg $Ca^{2+}$ channels, we fully expected to find two categories of $Ca^{2+}$ channels, LVA and HVA. However, the observed pattern of unitary channel properties—their slope conductances and activation and inactivation properties—fit more neatly into *three* distinct categories, leading Martha to postulate that there must be a third category. Upon closer examination of our whole-cell and single-channel recordings, it became clear that she was correct, and we soon found other criteria for distinguishing the three channel types, which we then called T-type (LVA) and N- and L-type (both HVA). The L-type channels were so named because they had a *l*arge unitary conductance to $Ba^{2+}$, supported a *l*ong-lasting $Ba^{2+}$ current (different properties were found with $Ca^{2+}$ as the charge carrier, but the $Ba^{2+}$ currents provided the most distinctive channel profiles) and were similar to single channels found in heart cells.[56] The T-type channels generated *t*iny unitary $Ba^{2+}$ currents, gave rise to a *t*ransient average current, showed characteristically slow deactivation following sudden repolarization,[57] and were also found in heart cells.[54,58] Finally, the N-type channels were largely specific to *n*eurons, had an *i*ntermediate conductance to $Ba^{2+}$, and, although they required negative holding potentials to be available for opening, they were activated at high voltages, indicating that they were *n*either T- nor L-type. Contrary to recurrent rumors, the N-type channel was not named by or for Martha Nowycky, although there would have been some justice had this been the case.

In coming up with this tripartite classification, the trickiest distinction was between L-type and N-type channels. Their profiles of unitary $Ba^{2+}$ conductance and voltage-dependent gating were distinctive enough, but the most decisive evidence came from experiments with the drug Bay K8644; this dihydropyridine compound acts as a stimulator (agonist) of L-type $Ca^{2+}$ channels, greatly increasing divalent cation influx by promoting a mode of activity with very long openings (Fig. 4A). Indeed, the behavior and pharmacology of neuronal L-type channels[59] were very similar to those of cardiac L-type channels.[60] In contrast, N-type channel activity was not affected by Bay K8644 (Fig. 4B). Once again, pharmacology provided essential tools for studying $Ca^{2+}$ channels, and confirmed distinctions derived from biophysical comparisons.

Shortly after the first presentation of the tripartite classification of voltage-gated calcium channels, a peptide toxin derived from marine snail venom, ω-conotoxin-GVIA, became available through the excellent work of Baldomero Olivera and Doju Yoshikami. Working together with them, we found that this peptide blocks HVA channel activity, particularly that of N-type channels.[61] We then used this peptide in collaboration with Richard Miller's group to demonstrate that N-type channels play an important role in the release of norepinephrine from sympathetic neurons.[62] Soon thereafter, the groups of Peter Hess and Bruce Bean showed that ω-conotoxin-GVIA was actually quite selective for N-type channels and thus completely spared L-type channels,[63,64] providing further evidence to support distinctions between N- and L-type $Ca^{2+}$ channels.

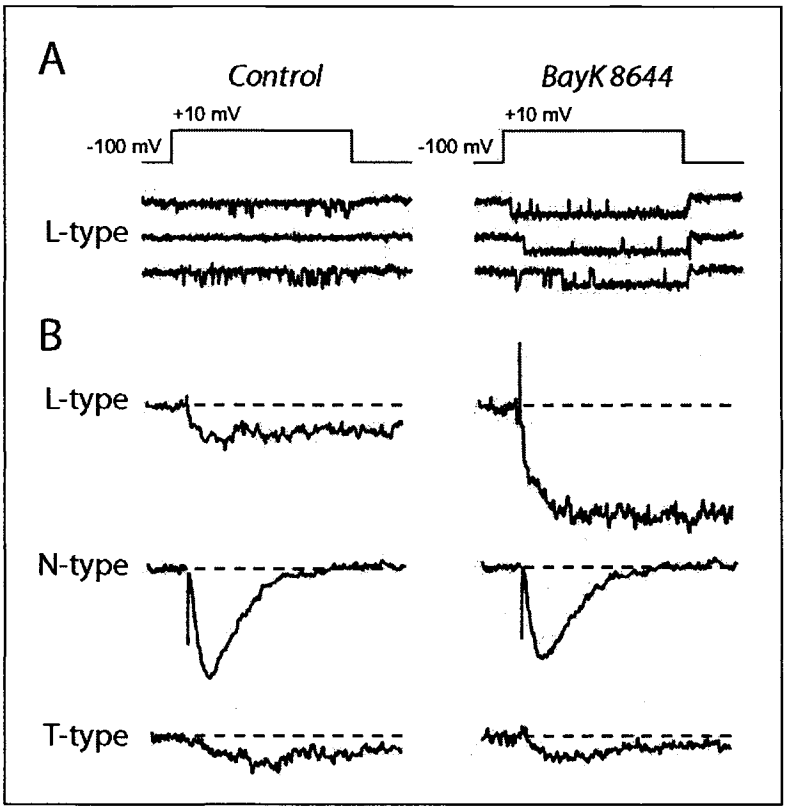



Figure 4. Distinguishing between three classes of $Ca^{2+}$ channel activity in sensory neurons with distinct biophysical and pharmacological profiles. Use of the dihydropyridine BayK8644 as a selective stimulator of L-type voltage-gated $Ca^{2+}$ channels. A) unitary L-type $Ca^{2+}$ channel activity recorded from a cell-attached patch of a guinea pig ventricular cell, using the voltage protocol indicated. By convention, downward deflections represent inward current through the channel. Left panel, in control solution, the channel displays brief openings (mode 1 activity), with occasional null (mode 0) activity. Right panel, application of 5 μM BayK8644 greatly increases open probability by inducing long-lasting openings (mode 2). Adapted from Hess et al (1984).[60] B) In recordings of macroscopic currents, 5 μM BayK8644 dramatically increases the activity of L-type $Ca^{2+}$ channels, but not N- or T-type channels. Left panel, control single-channel current averages recorded from cell-attached patches of chick dorsal root ganglion neurons. Right panel, the same patches recorded after application of 5 μM BayK8644. Adapted from Nowycky et al (1985).[55]

## *The Tripartite Classification of Neuronal $Ca^{2+}$ Channels Was Not Universally Accepted*

Some of the experts in the field, including Richard Miller and Harald Reuter, embraced the distinction between T-, N-, and L-type channels, noting that it resolved many puzzling aspects of findings on neurotransmitter release.[65,66] However, the idea of three categories of voltage-gated $Ca^{2+}$ channels was slow to catch on with the groups that had contributed to the original LVA–HVA distinction. The greatest resistance to the tripartite classification came from Swandulla and Armstrong,[67] who found the separation between L-type and N-type channels unconvincing, based on their favorite criterion, the speed of tail deactivation.[67] Carbone and Lux also found it difficult initially to accept the pharmacological separation between L- and N-type channels, in part because they were unable to elicit convincing responses to dihydropyridine

***Table 1. A few significant events in the brief history of N-type $Ca^{2+}$ channels***

| | |
|---|---|
| 1985 | N-type $Ca^{2+}$ channels discovered |
| 1987 | Specific block by ω-conopeptide first described |
| 1987 | N-type channel identified as target of modulation by G proteins |
| 1988 | Role of N-type channels in norepinephrine release established |
| 1992 | Isolation of the cDNA clone for the $\alpha_{1B}$ subunit |
| 1993 | N-type channels found to play key role in CNS transmission |
| 1995 | Block of N-type channels as therapeutic intervention for intractable pain |

antagonists such as nifedipine, and even the agonist Bay K8644, despite these compounds' carefully-verified effectiveness in heart cells.[68] In contrast, the Kiev group of Platon Kostyuk and colleagues were rather receptive to the idea that the HVA channels could indeed be split into two groups; they confirmed the specificity of Bay K8644 for one of those groups, and even proposed a distinction between high-threshold, inactivating (HTI) and high-threshold, non-inactivating (HTN) channels, in keeping with the N- and L-type subdivisions, respectively.

It is interesting to look back over the rapid progression of developments in the study of the N-type channel (Table 1). In the decade following its initial discovery, we witnessed the emergence of a specific blocker (ω-conotoxin-GVIA) as well as the cloning of the underlying $\alpha_1$ subunit, $\alpha_{1B}$ ($Ca_v2.2$; for a review of voltage-gated calcium channel nomenclature) (see refs. 69 and 70). Other highlights include the recognition of the key role of N-type channels in neurotransmitter release in peripheral and central neurons, and their susceptibility to modulation by G-protein–coupled receptors. More recently, the N-type channel has been examined as a possible therapeutic target for treatment of intractable pain, though this has yet to receive full FDA approval.

## *Beyond T, N and L: The Identification of P/Q-Type Channels*

Not long after the debut of N-type channels, Rodolfo Llinás and colleagues discovered a non-inactivating, DHP-insensitive current in the cell bodies of cerebellar Purkinje neurons; because of this localization, they named these channels P-type $Ca^{2+}$ channels.[71,72] Bruce Bean, Michael Adams and colleagues then demonstrated that these P-type channels were exquisitely sensitive to the funnel web spider toxin peptides ω-Aga-IVA and ω-Aga-IVB. Around the same time, a full-length brain $Ca^{2+}$ channel gene called BI (for brain-1) was cloned and expressed by Yasuo Mori and Shoshaku Numa in Kyoto.[73] Many call this $\alpha_{1A}$, following the general classification of brain $Ca^{2+}$ channels from Terry Snutch, Henry Lester and Norman Davidson. Because the message for this $Ca^{2+}$ channel was abundant in cerebellum, and specifically in cerebellar Purkinje cells, Rudolfo Llinás felt sure that it encoded the P-type channel. However, Numa was reluctant to commit to this, as the properties of the cloned $\alpha_{1A}$ subunits, when expressed in *Xenopus* oocytes, deviated greatly from those of native P-type channels recorded from cerebellar Purkinje neurons. Rather, the behavior of the cloned $\alpha_{1A}$ subunit in oocytes was much more similar to a current component labeled Q-type by our group.[74] Several lines of evidence, including antibodies, antisense techniques[75] and gene knockout have now convincingly established that both P- and Q-type currents both arise from $\alpha_{1A}$. For example, both current types are completely eliminated in cerebellar Purkinje and granule neurons of $\alpha_{1A}^{-/-}$ mice.[76] Thus, the currently accepted terminology of "P/Q-type" channel seems appropriate, as the original distinctions between the P and Q components have been mostly accounted for by splice variation in the $\alpha_{1A}$ gene.[77]

This is an appropriate point to acknowledge the enormous contributions of those who have provided the molecular tools that played a critical role in identifying diverse $Ca^{2+}$ channels and dissecting their contributions to neuronal function (see later chapters in this book). Baldomero Olivera has been widely recognized as a pioneer in discovering the ω-conotoxins, but what is not

commonly known is that he came to the field as a molecular biologist and adapted to the limited resources available in his native Philippines by studying a local menace, the deadly marine snails. His careful and thorough examination of these snails led to the discovery of a large number of invaluable neurotoxins.[78] Michael Adams, who discovered the peptide ω-Aga-IVA, began his work as an entomologist, not a neuroscientist. The availability of ω-conotoxin GVIA and ω-Aga-IVA helped settle debates over the distinctiveness of N- and P/Q-type channels, clarified their relationship to their respective $\alpha_1$ subunits, and greatly accelerated our examination of these channels' roles in neuronal processes such as $Ca^{2+}$ signaling and neurotransmission.

### *The Uncovering of Yet another Kind of HVA Neuronal $Ca^{2+}$ Channel: R-Type*

By the early 1990s, the actions and specificity of the newly available toxins and pharmacological compounds were understood sufficiently to allow a separation of current components based primarily on pharmacology. Andy Randall used a combination of nimodipine (L-type–specific), ω-conotoxin-GVIA (N-type–specific) and ω-Aga-IVA (P/Q-type–specific) to tease apart the various $Ca^{2+}$ current types in cerebellar granule neurons.[74] Because of the homogeneous nature of the neurons in this preparation, as well as their favorable passive properties, a clean, reproducible dissection of HVA $Ca^{2+}$ current types was obtained (Fig. 5). Present were currents carried by the three usual suspects, L-, N- and P/Q-type channels. However, a fourth current type, displaying properties distinct from the other three, was revealed upon application of inhibitors of the three known channel types. Because this current was *r*esistant to the three inhibitors (making it *r*esidual after inhibition of the other three current types), it was named R-type. This current type showed unique properties, including very rapid decay and an unusual sensitivity to block by $Ni^{2+}$. Moreover, there was a strong resemblance to the properties of a novel $\alpha_1$ subunit (*doe*-1) cloned from a marine ray by our group in collaboration with Tom Schwarz and his colleagues.[79]

The designation of another channel type seemed problematic to several experts in the field, who initially dismissed the notion of a fourth HVA $Ca^{2+}$ channel type, suggesting that the residual current might result from incomplete block of L-, N- or P/Q-type channels. Even the distinct kinetics of this R-type current might have been explained by alternative splicing of one of the other channel type's message (as was the case with P- and Q-type channels), or perhaps by associating with a different accessory subunit. Lacking a mammalian homolog for the $\alpha_1$ gene encoding this current, the smoking gun we needed was a specific inhibitor of this residual current, ideally one selective enough to spare L-, N- and P/Q-type currents. Fortunately, this was provided by Robert Newcomb, George Miljanich and colleagues, who isolated another spider toxin peptide, designated SNX-482.[80] This inhibitor blocks R-type currents in several neuronal systems.

### *Issues Surrounding $\alpha_{1E}$ and Its Initial Identification with T-Type Channels*

Another interesting episode in the unveiling of the $Ca^{2+}$ channel family occurred when matching $\alpha_{1E}$, the most recent member of the $Ca_v2$ subfamily to be cloned, with a $Ca^{2+}$ channel phenotype defined by electrophysiological properties. When Tuck-Wah Soong, Terry Snutch, and their colleagues first isolated $\alpha_{1E}$, they suggested somewhat provocatively that this gene might encode low-voltage–activated T-type channels.[81,82] Of all the voltage-gated $Ca^{2+}$ channels, T-type currents were the easiest to isolate biophysically, yet the hardest to study biochemically (perhaps their role in generating relatively slow pacemaker depolarizations required only small currents and thus low levels of protein in the membrane); therefore, successfully cloning this channel would represent a major step forward in the study of T-type calcium channels. On the other hand, our group[74,83] favored the notion that $\alpha_{1E}$ encodes the R-type current that remained after blockade of neuronal L-, N-, and P/Q-type channels. This controversy was ultimately laid to rest by the cloning of novel $\alpha_1$ subunits by Edward Perez-Reyes, LeAnn Cribbs and their colleagues.[84-86] These genes were classified $\alpha_{1G}$, $\alpha_{1H}$ and $\alpha_{1I}$, in keeping with the convention of the time (these genes comprise their own subfamily, called $Ca_v3$). The isolation of these clones stands as a testament to the persistence of the researchers and their willingness to try in silico approaches. When these three genes were expressed and recorded, the currents matched nicely with native T-type

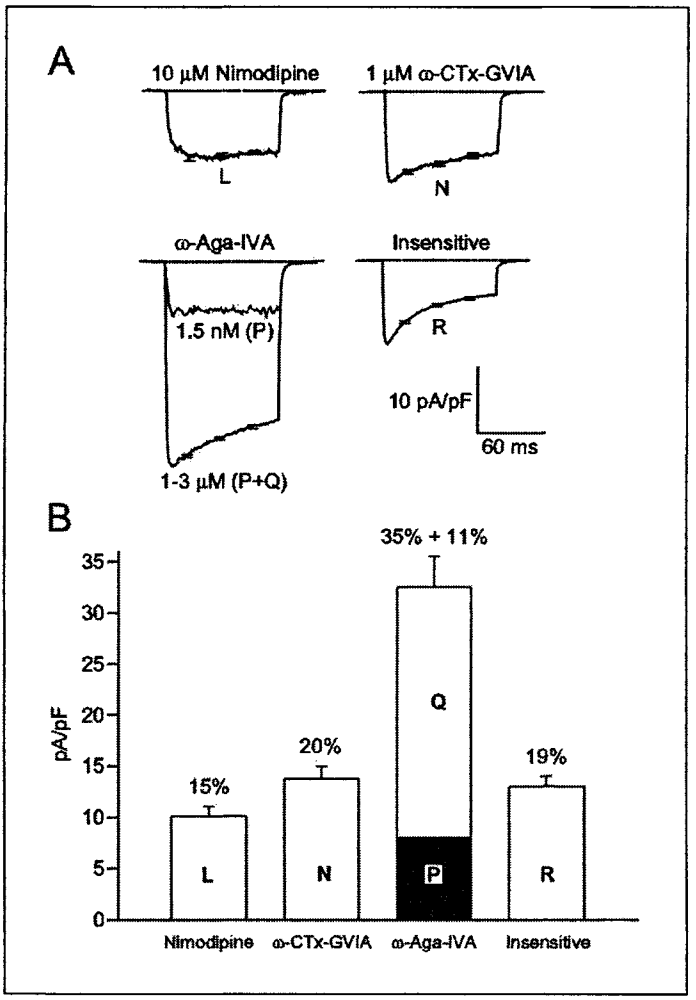



Figure 5. Pharmacological dissection of high-voltage–activated (HVA) whole-cell $Ca^{2+}$ current types in rat cerebellar granule neurons. A) L- and N-type currents were isolated by application of 10 μM nimodipine or 1 μM ω-conotoxin-GVIA (ω-CTx-GVIA), respectively. P- and Q-type currents were isolated by application of 1.5 nM or 1–3 μM ω-agatoxin-IVA (ω-Aga-IVA), respectively. R-type current was isolated by combined application of nimodipine, ω-CTx-GVIA and ω-Aga-IVA. B) summary of the current densities and relative contributions of each current type, determined from the data in A. Adapted from Randall and Tsien (1995).[74]

(LVA) currents; none of these three clones gave rise to R-type currents. On the other hand, it is now generally accepted that $\alpha_{1E}$ is the major contributor to R-type current. Recently, the interest in R-type channels has sharply increased due to their ability to support neurotransmitter release[87,88] and their contribution to dendritic $Ca^{2+}$ homeostasis[89] and synaptic plasticity.[90] In the discovery of the R-type channel, one sees repeated the familiar history of an initial distinction of a $Ca^{2+}$ channel type based on biophysical and pharmacological criteria, followed by an emerging consensus about its molecular basis, driven in part by cloning. What began as a curiosity or anomaly (and to many investigators, an artifact) rapidly transitioned from controversy to general acceptance, proving to be more interesting than its discoverers could have anticipated.

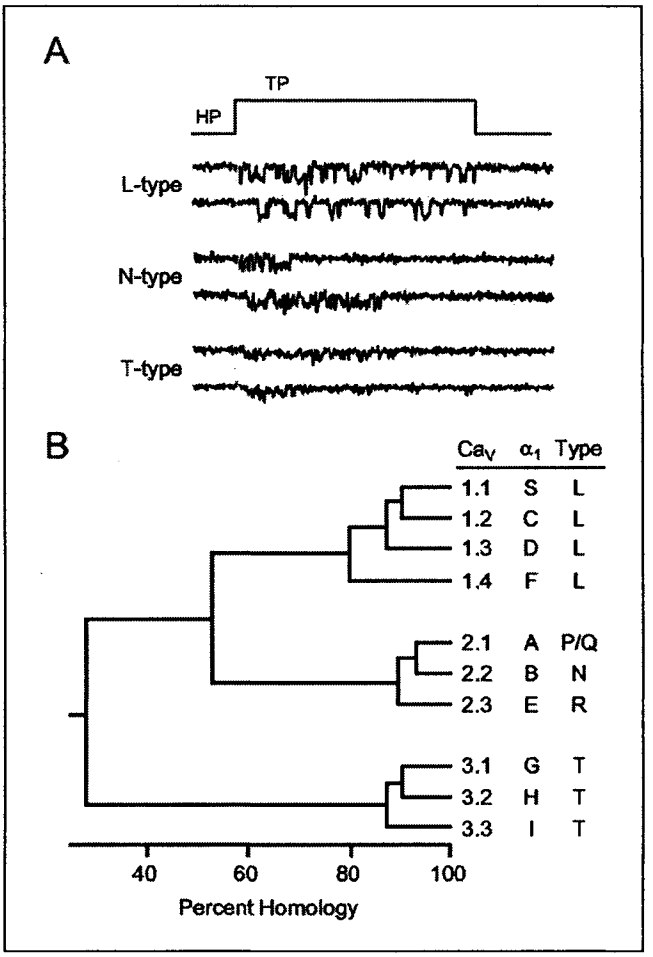



Figure 6. Convergence of physiological and molecular biological evidence for a tripartite classification of voltage-gated $Ca^{2+}$ channels. A) unitary activity of three voltage-gated $Ca^{2+}$ channel types, recorded from cell-attached patches of chick dorsal root ganglion neurons. Activity was elicited by stepping from a given holding potential (HP) to the appropriate test potential (TP), as follows: L-type, –20 mV to +20 mV; N-type, –80 mV to +20 mV; T-type, –80 mV to –20 mV. Adapted from Nowycky et al (1985).[55] B) tripartite classification of voltage-gated $Ca^{2+}$ channels, based on biophysical properties, pharmacological sensitivity and sequence homology. The $Ca_v1$ and $Ca_v2$ subfamilies comprise the high-voltage–activated channels, while the $Ca_v3$ subfamily contains low-voltage–activated (T-type) channels.

## *The Current State of the Three-Branched Calcium Channel Family Tree*

A new set of questions comes to mind now that the diverse nature of the $Ca^{2+}$ channel family, first proposed on the basis of patch-clamp recordings, has received corroboration from molecular biology. Sequence analysis has confirmed and extended our original tripartite classification (Fig. 6). The members of the $Ca_v2$ subfamily, first typified by N-type channels (N-type = $\alpha_{1B}$; P/Q-type = $\alpha_{1A}$; R-type = $\alpha_{1E}$), appear functionally quite distinct from the $Ca_v1$ subfamily of L-type channels ($\alpha_{1S}$, $\alpha_{1C}$, $\alpha_{1D}$ and $\alpha_{1F}$) and the $Ca_v3$ subfamily of T-type channels ($\alpha_{1G}$, $\alpha_{1H}$ and $\alpha_{1I}$). Each of the cloned channels shows a steep voltage-dependence, highly similar to a first approximation, and a strong selectivity for $Ca^{2+}$ and $Ba^{2+}$ rather than $Na^+$. In

this regard, truly "a $Ca^{2+}$ channel is a $Ca^{2+}$ channel is a $Ca^{2+}$ channel". If so, why might such diversity have evolved? The most obvious answer is that the individual family members evolved to fill distinct physiological or cell biological niches. The employment of more than one type of channel allows greater flexibility with regard to time- and voltage-dependence, cellular localization, and responsiveness to different forms of modulatory regulation. Diverse as they are, $Ca^{2+}$ channel types are far less numerous than the signaling roles for voltage-gated $Ca^{2+}$ entry itself.

## *Conclusions: Some Lessons and Ironies*

The history of calcium channel discovery offers many insights and a few interesting ironies. Although first identified in the heyday of classical neurophysiology, $Ca^{2+}$ channels did not receive much initial attention, despite their now-evident importance in processes such as chemical neurotransmission and excitation-contraction coupling. The timeline of $Ca^{2+}$ channel discovery is remarkable when one considers the extraordinarily long gestation period from first sighting (1953/1958) to full-blown focus (1970s for multicellular approaches, 1980s for patch-clamp recordings). The circuitous path to understanding how $Ca^{2+}$ entry triggers neurosecretion, reviewed in this chapter, is indicative of a more general disconnect between the participation of $Ca^{2+}$ in electrogenesis and the role of $Ca^{2+}$ as a second messenger. Squid giant axons, the preeminent preparation for studying axonal conduction, do not express high levels of $Ca^{2+}$ channels. Despite pioneering work by Reuter and others, this paucity of $Ca^{2+}$ channels in squid axons had a strong influence: Johnson and Lieberman were not alone in asking why the heart should use calcium channels to make action potentials when the reigning preparation, the squid giant axon, did not. The course of scientific discovery might have been quite different if physiologists had focused on crustacean muscles, which appear to lack sodium channels, rather than squid giant axons. Another irony is that full-blown $Ca^{2+}$ spikes, the first indication of the presence of $Ca^{2+}$ channels, are not actually critical for excitation–contraction coupling in the muscle fibers of crab, crayfish or barnacle, and are evident only when $K^+$ channels are blocked or $Ca^{2+}$ channel inactivation is suppressed.

In mammalian preparations, the sino-atrial and atrio-ventricular nodes offer the clearest examples of $Ca^{2+}$ channel–dependent electrogenesis, but these systems are rather difficult to study, even today. On the other hand, skeletal muscle is one of the richest sources of homogeneous $Ca^{2+}$ channels, and thereby facilitated the early biochemical characterization of $Ca_v1.1$ ($\alpha_{1S}$) subunits by William Catterall, Kevin Campbell, Michel Lazdunski and others, as well as the first cloning of a $Ca^{2+}$ channel by Shoshaku Numa and colleagues. Yet, despite much elegant work,[91] the steps between $Ca^{2+}$ channel activation and subsequent internal $Ca^{2+}$ release from the sarcoplasmic reticulum remain incompletely understood even a decade later.

In recent years, rapid progress in the fields of $Ca^{2+}$ channel structure, function, diversity and regulation can be attributed to several key factors. First, investigators have approached the study of calcium channels from many angles, ranging from hardcore biophysics and biochemistry to clinical perspectives—recall that Harald Reuter and Albrecht Fleckenstein both began their careers as cardiac pharmacologists. Second, even with the advent of improved patch-clamp technologies, and the availability of a veritable cornucopia of potent, selective $Ca^{2+}$ channel drugs, the field would not have experienced such a rapid rate of progress without a shift in the prevailing attitudes of the individual investigators. The sharing of information and resources between scientists has greatly contributed to the speed of new discoveries. In earlier times, compartmentalization stood as a barrier to collaborative discovery. For example, an early paper about $Ca^{2+}$ channels in cardiac preparations would credit Hagiwara and Nakajima as well as Niedergerke and Orkand for circumstantial evidence of such channels in heart, but not cite Fatt and Katz or Fatt and Ginsborg's work! On the other hand, working from an invertebrate vantage point, Hagiwara and Byerly entitled their 1981 review "Calcium Channel", but stated disarmingly that they were not familiar with the literature in vertebrate heart or smooth muscle. As Rodolfo Llinás put it in a 1983 letter: "We need to unify our fields a bit more. Nature seems to be trying to tell us something and we continue stubbornly to think that muscles and nerve

cells are not next of kin." Thankfully, this wish for unification has now largely been realized, and we now understand a great deal more about what nature can tell us about $Ca^{2+}$ channels, their diversity, and their critical roles in cellular processes.

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# CHAPTER 4

# Biochemical Studies of Voltage-Gated $Ca^{2+}$ Channels

**William A. Catterall**

## Abstract

Voltage-gated $Ca^{2+}$ channels mediate $Ca^{2+}$ entry into cells in response to membrane depolarization. Electrophysiological studies reveal different $Ca^{2+}$ currents designated L-, N-, P-, Q-, R-, and T-type. The high-voltage-activated $Ca^{2+}$ channels that have been characterized biochemically are complexes of a pore-forming $\alpha_1$ subunit of about 190 to 250 kDa, a transmembrane, disulfide-linked complex of $\alpha_2$ and $\delta$ subunits, an intracellular $\beta$ subunit, and in some cases a transmembrane $\gamma$ subunit. The $\alpha_1$ subunits form the transmembrane pore. The $\alpha_2$ and $\delta$ subunits are glycoproteins encoded by the same gene and produced posttranslational proteolytic processing. The $\gamma$ subunits are transmembrane glycoproteins, whereas the $\beta$ subunits are hydrophilic subunits located on the cytosolic face of the channel. The $Ca_v1$ family of $\alpha_1$ subunits conduct L-type $Ca^{2+}$ currents, which initiate muscle contraction, endocrine secretion, and gene transcription. The $Ca_v2$ family of $\alpha_1$ subunits conduct N-type, P/Q-type, and R-type $Ca^{2+}$ currents, which initiate rapid synaptic transmission. Both of these families of $Ca^{2+}$ channels are regulated by protein phosphorylation and interact with intracellular signal transduction proteins that mediate $Ca^{2+}$ -dependent regulatory events and in turn regulate the channels.

## Introduction

$Ca^{2+}$ channels have been studied intensively by electrophysiological methods since the initial recordings of $Ca^{2+}$ currents by Reuter in 1967.[1] In the early 1980's, stimulated in part by success at purification and reconstitution of sodium channels,[2] a new focus of $Ca^{2+}$ channel research developed aimed at identification of the $Ca^{2+}$ channel proteins and analysis of their biochemical properties. In this chapter, I briefly review the biochemical studies that led to the first identification of the $Ca^{2+}$ channel proteins and provided the foundation for the subsequent analysis of the structure and function of the channel subunits by molecular biological methods. I have restricted consideration here primarily to experiments in which biochemical methods were applied to purified protein preparations. For this reason, only the $Ca^{2+}$ channels of the $Ca_v1$ and $Ca_v2$ families are considered. Readers are directed to subsequent chapters in this book for consideration of more recent studies that have employed a combination of molecular biological and protein expression methods to further analyze these questions.

## Purification and Biochemical Characterization of Skeletal Muscle $Ca^{2+}$ Channels

The $Ca^{2+}$ channels in the transverse tubule membranes of skeletal muscle have served as a primary biochemical preparation for studies of $Ca^{2+}$ channels because of their abundance. These channels serve two critical physiological roles. Like other $Ca^{2+}$ channels, they mediate $Ca^{2+}$ entry in response to depolarization. The primary voltage-gated $Ca^{2+}$ currents in skeletal muscle

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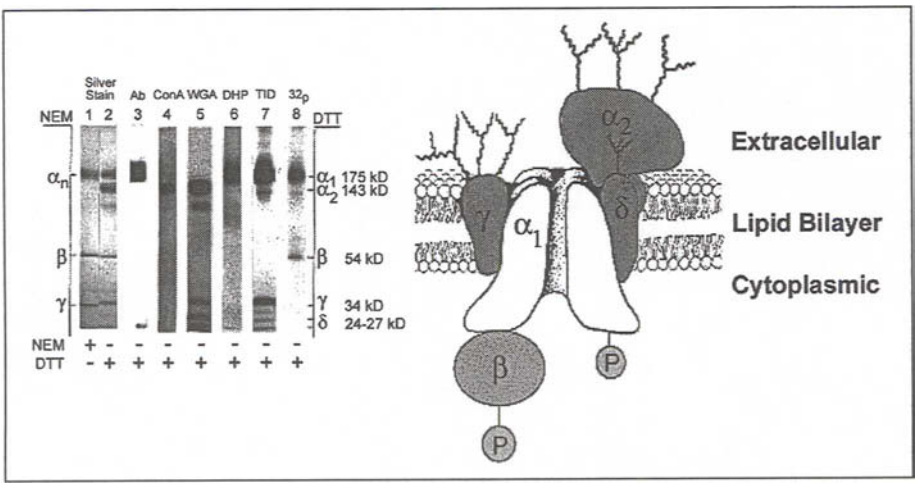



Figure 1. Biochemical properties of skeletal muscle $Ca^{2+}$ channels. A. Summary of the biochemical properties of purified skeletal muscle $Ca^{2+}$ channels. Lanes 1 and 2, silver stain of polypeptides; lane 3, staining with an antibody against the $\alpha_1$ subunit; lane 4, staining with concanavalin A, a lectin binding high mannose N-linked carbohydrate chains; lane 5, staining with wheat germ agglutinin, a lectin staining N-linked complex carbohydrate chains; lane 6, photoaffinity labeling with azidopine, a photoreactive dihydropyridine; lane 7, photoaffinity labeling with TID, a hydrophobic probe of the transmembrane regions of proteins; lane 8, phosphorylation by cAMP-dependent protein kinase (adapted from ref. 14). B) The subunit structure of $Ca^{2+}$ channels purified from skeletal muscle is illustrated. The model is updated from the original description of the subunit structure of skeletal muscle $Ca^{2+}$ channels.[14] This model also fits biochemical and molecular biological results for neuronal $Ca^{2+}$ channels. P, sites of phosphorylation by cAMP-dependent protein kinase and protein kinase C. Ψ, sites of N-linked glycosylation.

are L-type,[3-5] characterized by slow voltage-dependent inactivation, large single channel conductance ( about 25 pS), high voltage of activation, and specific inhibition by dihydropyridine $Ca^{2+}$ channel antagonists. These channels activate very slowly, and the $Ca^{2+}$ entering vertebrate skeletal muscle through voltage-gated $Ca^{2+}$ channels is not required for muscle contraction. It appears to replenish cellular $Ca^{2+}$ during periods of rapid activity and to increase intracellular $Ca^{2+}$ in response to tetanic stimulation, leading to increased contractile force. The primary physiological role for the skeletal muscle $Ca^{2+}$ channel is to serve as a voltage sensor in excitation-contraction coupling. Voltage-gated $Ca^{2+}$ channels in the transverse tubule membranes are thought to interact physically with the $Ca^{2+}$ release channels located in the sarcoplasmic reticulum membrane. Voltage-driven conformational changes in the voltage-gated $Ca^{2+}$ channels then activate the $Ca^{2+}$ release from the sarcoplasmic reticulum by protein-protein interactions.[6-8]

Purification of $Ca^{2+}$ channels from skeletal muscle began with isolation of the transverse tubule membranes, which are highly enriched in $Ca^{2+}$ channel protein, and specific labeling by high affinity binding to dihydropyridine $Ca^{2+}$ channel antagonists to identify the channel protein.[9,10] $Ca^{2+}$ channels were solubilized in the mild detergent digitonin to retain native subunit associations and purified by a combination of ion exchange chromatography, affinity chromatography on wheat germ agglutinin-Sepharose, and sedimentation through sucrose gradients.[10] A heterogeous α subunit band[9,10] and associated β subunits of 50 kD and γ subunits of 33 kD[10] were identified as components of the $Ca^{2+}$ channel in the initial purification studies, as assessed by comigration during column chromatography and sucrose gradient sedimentation. Subsequent experiments demonstrated that the heterogenous α subunit band contained not only the principal $\alpha_1$ subunits with an apparent molecular mass of 175 kD but also a disulfide-linked dimer of $\alpha_2$-δ subunits with apparent molecular masses of 143 kD and 27 kD, respectively, as illustrated in the SDS-PAGE results in Figure 1A.[11-15] The specific association

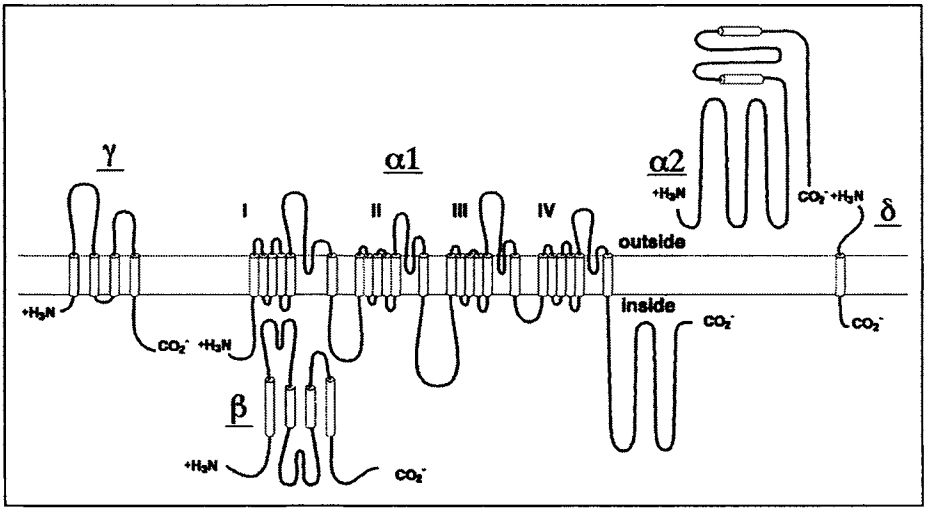



Figure 2. Structures of the Subunits of $Ca_v$ Channels. Transmembrane folding models and site of regulation of the $Ca^{2+}$ channel subunits. Predicted alpha helices are depicted as cylinders. The lengths of lines correspond approximately to the lengths of the polypeptide segments represented.

of these proteins as a multisubunit complex was supported by the copurification of each subunit with the dihyropyridine binding activity and $Ca^{2+}$ conductance activity of the $Ca^{2+}$ channel,[10,14,16,17] by coimmunoprecipitation of all these proteins by antibodies directed against the $\alpha_1$ subunits,[12,14,18] and by co-immunoprecipitation of the $Ca^{2+}$ channel complex by antibodies against each auxiliary subunit.[19-21] Estimates of stoichiometry indicated that each mol of $Ca^{2+}$ channel complex contains approximately 1 mol of each of the 5 subunits. The biochemical and molecular properties of each of the subunits of skeletal muscle $Ca^{2+}$ channels are considered below.

## *$\alpha_1$ Subunit*

The $\alpha_1$ subunit of skeletal muscle $Ca^{2+}$ channels was cloned by library screening based on amino acid sequence[22] (Fig. 2). The cDNA predicts a protein of 1873 amino acids with a molecular weight of 212 kD, considerably larger than the estimate of 175 kD for the α1 subunits of purified $Ca^{2+}$ channels. Analysis of the $\alpha_1$ subunits of purified $Ca^{2+}$ channels and $Ca^{2+}$ channels in transverse tubule membranes using sequence-directed antibodies showed that most (>90%) contained $\alpha_1$ subunits that were truncated in their carboxyl terminal domain between residues 1685 and 1699 resulting in a 190 kD form that runs anomalously in SDS gels at 175 kD.[23,24] Only a small fraction (<10%) of skeletal muscle $Ca^{2+}$ channels contained the full length $\alpha_1$ subunit encoded by the cDNA. Both forms were detected in rat skeletal muscle cells in culture suggesting that both may be present in vivo.[25] Since no mRNA encoding the more abundant, truncated form has been identified, the truncated form is probably produced by specific proteolytic processing in vivo. A similar cleavage product can be produced by calpain treatment, suggesting calpain as a candidate for in vivo processing of the $\alpha_1$ subunits.[26]

## *$\alpha_2$ Subunit*

The $\alpha_2$ subunit of skeletal muscle $Ca^{2+}$ channels is a hydrophobic glycoprotein with an apparent molecular mass of 143 kD before deglycosylation and 105 kD after deglycosylation (Fig. 1A, lanes 1 and 2).[14,27,28] It contains both high mannose and complex carbohydrate chains (Fig. 1A, lanes 4 and 5). Cloning and sequencing cDNAs encoding the $\alpha_2$ subunit defined a protein of 1106 amino acids with a molecular mass of 125 kD, multiple potential

transmembrane segments, and multiple consensus sites for N-linked glycosylation[29] (Fig. 2). The predicted $\alpha_2$ protein was 20 kD larger than the apparent molecular mass of the deglycosylated $\alpha_2$ subunit suggesting that a portion of the protein encoded by the $\alpha_2$ cDNAs may not be present in the mature $\alpha_2$ subunit that had been characterized biochemically. Subsequent studies showed that both $\alpha_2$-$\delta$ subunits are encoded by the same mRNA (see below).

### *β Subunit*

The β subunits are hydrophilic proteins that are not glycosylated and therefore are likely to be located on the intracellular side of the membrane (Fig. 1).[14,20] cDNA cloning and sequencing revealed a protein of 524 amino acids with a predicted molecular mass of 58 kD.[30] In agreement with biochemical data, the primary structure does not include any potential transmembrane segments but contains multiple consensus sites for phosphorylation by different protein kinases (Fig. 2B).

### *γ Subunit*

The γ subunit of skeletal muscle $Ca^{2+}$ channels is a hydrophobic glycoprotein with an apparent molecular mass of 30 kD without deglycosylation and 20 kD following deglycosylation.[14,21] Cloning and sequencing cDNAs encoding γ subunits revealed a protein of 222 amino acid residues with a molecular mass of 25 kD.[31,32] The deduced primary structure contained four predicted hydrophobic transmembrane segments and multiple sites for N-linked glycosylation.

### *δ Subunit*

The δ subunit appears on SDS gels as a doublet of 24 and 27 kD proteins, which are both hydrophobic and glycosylated.[14,28] Determination of the amino acid sequences of peptides derived from the δ subunit showed that it was encoded by the same mRNA as the $\alpha_2$ subunit.[33,34] The mature $\alpha_2$ subunit is truncated at alanine 934 of the $\alpha_2\delta$ precursor protein; residues 935-1106 constitute the disulfide-linked δ subunit. This sequence comprises a protein of 16 kD and contains a single transmembrane segment and three consensus sequences for N-linked glycosylation. The doublet on SDS gels represents two differently glycosylated forms of the δ subunit.

### *Membrane Association of Subunits*

The transmembrane organization of the $Ca^{2+}$ channel complex was intially investigated by labeling of potential transmembrane segments with a hydrophobic photoaffinity probe.[14] By this criterion, the $\alpha_1$, $\alpha_2\delta$ complex, and γ subunits were identified as probable integral membrane proteins, with the labeling of the $\alpha_2$ subunits the weakest despite its large size (Fig. 1A, lane 7, and B).[14] Subsequent hydropathy analysis of the primary structures revealed 24 transmembrane segments in the $\alpha_1$ subunit, four in the γ subunit, and one in the δ subunit (Fig. 2), but the prediction of transmembrane alpha helices in the $\alpha_2$ subunits has been uncertain.[29] Analysis of association of the $\alpha_2$ subunit with the membrane by expression and biochemical extraction procedures led to the conclusion that the $\alpha_2$ subunit does not have a true transmembrane segment.[35] Instead, it is likely to be associated with the membrane primarily by virtue of its disulfide linkage to the δ subunit and perhaps also by superficial ionic and hydrophobic interactions with the membrane bilayer (Fig. 1B).

### *Sites of Protein Phosphorylation*

Activation of skeletal muscle L-type $Ca^{2+}$ channels is enhanced by phosphorylation by cAMP-dependent protein kinase.[36,37] In cultured skeletal muscle cells, repetitive depolarization causes a dramatic enhancement of $Ca^{2+}$ currents,[38,39] up to 10-fold in the critical membrane potential range near -20 mV. This potentiation of $Ca^{2+}$ currents is strongly voltage-dependent and also is dependent on the activity of cAMP-dependent protein kinase.[39] This may result from interaction between voltage-dependent gating and phosphorylation of the $Ca^{2+}$

channel itself. This novel regulatory mechanism greatly increases $Ca^{2+}$ channel activity during tetanic stimulation of skeletal muscle cells and may play a critical role in the regulation of contractile force of skeletal muscle in response to hormones and to the frequency of stimulation of the motor nerve.

The $\alpha_1$ subunit and the $\beta$ subunit of skeletal muscle $Ca^{2+}$ channels are substrates for phosphorylation by cAMP-dependent protein kinase and a number of other protein kinases (Fig. 1A, lane 8).[14,40-44] $Ca^{2+}$ flux through the purified skeletal muscle $Ca^{2+}$ channel is regulated by cAMP-dependent protein phosphorylation.[17,45-47] Ion flux studies in reconstituted phospholipid vesicles show that phosphorylation of the $\alpha_1$ and $\beta$ subunits can greatly increase the number of functional $Ca^{2+}$ channels in purified preparations.[46,47] Single channel recording experiments in planar bilayer membranes detect both increases in the number of functional $Ca^{2+}$ channels and increases in the activity of single $Ca^{2+}$ channels after phosphorylation by cAMP-dependent protein kinase.[17,45] Thus, the $\alpha_1$ and $\beta$ subunits of the purified $Ca^{2+}$ channel contain the sites at which cAMP-dependent protein phosphorylation modulates channel function in vitro.

Two size forms of the $\alpha_1$ subunit of approximately 190 kDa and 212 kDa are present in purified preparations of skeletal muscle $Ca^{2+}$ channels, T-tubule membranes, and intact skeletal muscle cells in culture, and both are phosphorylated by PKA in intact cells.[23-25] Antibody mapping of the C-terminal region of $\alpha_{1190}$ placed the C-terminus between residues 1685 and 1699.[24] The most rapidly phosphorylated site in the truncated form of the $\alpha 1$ subunit in purified $Ca^{2+}$ channel preparations is Ser 687, located in the intracellular loop between domains II and III.[48,49] In contrast, time course experiments indicated that Ser 1854 near the C-terminal portion of full-length $\alpha_{1212}$ is the most intensely and rapidly phosphorylated.[49,50] To date, the site(s) of phosphorylation that regulate skeletal muscle $Ca^{2+}$ channel activity have not been directly identified.

### *Phosphorylation of the β Subunit of Skeletal Muscle $Ca^{2+}$ Channels*

Like the $\alpha_1$ subunit of the skeletal muscle $Ca^{2+}$ channel, the $\beta$ subunit is stoichiometrically phosphorylated by cAMP-dependent protein kinase in purified preparations[40] and in reconstituted $Ca^{2+}$ channels that are regulated by cAMP-dependent protein kinase.[17,47] Both serine 182 and threonine 205 have been shown to be phosphorylated in vitro.[23,30] Since phosphorylation of both $\alpha_1$-$\beta$ subunits have been shown to be correlated with regulation of the ion conductance activity of skeletal muscle $Ca^{2+}$ channels, both are candidates for sites of channel modulation by phosphorylation.

### *Sites of Drug Binding*

A defining feature of $Ca_v1$ channels and the L-type $Ca^{2+}$ currents they conduct is block by dihydropyridines, phenylalkylamines, and other $Ca^{2+}$ channel antagonist drugs. Photoaffinity labeling studies established that the binding sites of dihydropyridines,[13-15,21] phenylalkylamines,[51] and benzothiazepines[52] are located in the $\alpha_1$ subunits. Detailed mapping of the dihydropyridine receptor site by proteolytic digestion and mapping of the digested fragments with sequence-directed antibodies identified the IIIS6 and IVS6 segments as important components of the binding site.[53] These results led to a **domain interface** model of drug binding of dihydropyridines, in which these drugs bind at the interface between domain III and IV at the point of interaction of the S6 segments.[54] Subsequent extensive mapping of the dihydropyridine receptor site by mutagenesis and functional analysis has given a detailed view of this important receptor site (see Chapter 17) and allowed construction of high affinity dihydropyridine receptor in non-L-type $Ca^{2+}$ channels.[55-57] Similarly, the receptor sites of phenylalkylamines and benzothiazepines have been extensively mapped by photoaffininty labeling and peptide mapping[58,59] and subsequently by mutagenesis and functional analysis (see Chapter 17). All three receptor sites are close in the expected three-dimensional structure of the channel and have both distinct and overlapping molecular determinants of their binding.

## Biochemical Properties of Other $Ca_v1$ Channels

### *Subunits of Purified Cardiac $Ca^{2+}$ Channels*

Like the skeletal muscle $Ca^{2+}$ channel, the principal cardiac $Ca^{2+}$ channel conducts L-type $Ca^{2+}$ currents.[60,61] Antibodies against $\alpha_2\delta$ subunits of skeletal muscle $Ca^{2+}$ channels detect corresponding subunits in cardiac preparations.[62,63] Partially purified cardiac $Ca^{2+}$ channels contain $\alpha_1$, $\alpha_2\delta$, and $\beta$ subunits.[64-68] However, the relatively low abundance of $Ca^{2+}$ channels in cardiac tissue and the difficulty of controlling proteolysis during lengthy purification procedures have frustrated attempts at complete purification of an intact cardiac $Ca^{2+}$ channel complex. The $\alpha_1$ subunit with an apparent mass of 165 kD to 190 kD has been directly identified by photoaffinity labeling with photoreactive dihydropyridines.[64,66,69] As in skeletal muscle, most of the $\alpha_1$ subunits of cardiac $Ca^{2+}$ channels are truncated at the C-terminus by proteolytic processing.[70] The primary site for phosphorylation by PKA in vitro and in cultured cells is Ser1928 (in rabbit $Ca_v1.2$ sequence numbering), which is located in the distal C-terminus beyond the site of proteolytic cleavage,[70] as observed for the skeletal muscle $Ca^{2+}$ channel.[49,50] Therefore, it is possible that there is a functional relationship between proteolytic cleavage of the C-terminal domain and regulation of the cardiac $Ca^{2+}$ channel by protein phosphorylation. Consistent with this idea, truncation of the C-terminal domain by expression of cDNAs with appropriate deletions yields increased $Ca^{2+}$ channel activity,[71] and perfusion of C-terminal peptides into the cells expressing truncated channels inhibits the increase in $Ca^{2+}$ channel activity.[72]

### *Molecular Basis for Modulation of Cardiac $Ca^{2+}$ Channels by PKA*

Although regulation of the cardiac $Ca^{2+}$ channels by the cAMP pathway was the first example of ion channel regulation through second messenger pathways,[73,74] the molecular basis for this regulation is still not clearly resolved. Early biochemical studies of the cardiac $Ca^{2+}$ channel resulted in purification of a short, 160 to 195 kDa form of the $\alpha_1$ subunit that was not a substrate for cAMP-dependent protein kinase.[64,66] As for skeletal muscle $Ca^{2+}$ channels, more recent results on cardiac $Ca^{2+}$ channels have revealed a full-length $\alpha 1$ subunit form with a molecular mass of approximately 220 kDa, as expected from the cDNA sequence.[70] This full-length $\alpha_1$ subunit is phosphorylated on a single serine residue (Ser1928) in the C-terminal domain.[70] Similarly, expression of the C-terminal domain in bacteria yields a protein that is phosphorylated primarily on Ser1928.[75] This site is missing in the truncated from of the $\alpha_1$ subunit isolated in early biochemical preparations. Primarily the full-length form of $Ca_v1.2$ is present in cardiac tissue.[76,77] Both $\beta_1$ and $\beta_2$ isoforms are expressed in cardiac tissue[76-79] and likely are both associated in $Ca_v1.2$ channels.

$Ca^{2+}$ channels in a CHO cell line that expresses only the full-length form of the $Ca_v1.2$ $\alpha_1$ subunit can be regulated by activation of endogenous PKA, by intracellular perfusion of PKA, and by voltage-dependent potentiation, which requires the activity of PKA.[80,81] These results indicate that at least part of the regulation of $Ca^{2+}$ channel activity is due to phosphorylation of the $\alpha_1$ subunit. Since the $\alpha_1$ subunit is only phosphorylated on Ser1928 in vitro or in transfected cells,[70] this regulation likely involves phosphorylation of this site. In support of this conclusion, mutation of this serine residue to alanine reduces the regulation of $Ca^{2+}$ channel activity caused by activation of PKA with 8-Br-cAMP in transiently transfected human embryonic kidney cells from a 35% increase to a 7% increase.[76,77] Although these results are promising, the extent of regulation of $Ca^{2+}$ channel activity in these experiments in transfected cells falls well short of the magnitude recorded in native cardiac cells (2- to 4-fold increase), and regulation of cloned $Ca^{2+}$ channels analyzed in this way is not observed in some of the major laboratories in the field.[82] Therefore, it seems likely that additional regulatory influences that have not yet been reproduced and characterized in transfected cells are important for control of the activity of cardiac $Ca^{2+}$ channels in vivo.

### *Phosphorylation of the β Subunit of Cardiac $Ca^{2+}$ Channels by PKA*

The β subunits of cardiac $Ca^{2+}$ channels are phosphorylated by PKA in intact hearts treated with β-adrenergic agonists.[83] Bunemann et al[84] have reported substantial (greater than 2-fold) regulation of a C-terminal truncated form of $Ca_v1.2$ lacking Ser1928 by intracellular perfusion of activated PKA in human embryonic kidney cells co-transfected with $Ca_v1.2$ and $\beta_{2a}$ subunits. This regulation required phosphorylation of Ser 478 and/or Ser 479, two serine residues in non-classical PKA phosphorylation sites that are specific to the $\beta_{2a}$ subunit. These results provide the best evidence to date for an important role of the β subunits in regulation of cardiac $Ca^{2+}$ channels. It will be important to further analyze $Ca^{2+}$ channel regulation when full-length α1 subunits are co-expressed with $\beta_{2a}$ and other β subunits expressed in cardiac myocytes and to restore regulation by physiological stimulus procedures rather than intracellular perfusion of activated PKA.

### *Subunits of Purified Neuronal $Ca_v1$ Channels*

Because the concentration of $Ca^{2+}$ channels in skeletal muscle transverse tubules is much higher than in neuronal membranes, the biochemical properties of these channels in neurons are not as well established. Immunoprecipitation of $Ca^{2+}$ channel complexes labeled with dihydropyridines using specific antibodies against $\alpha_2\delta$ subunits revealed a complex of polypeptides with sizes corresponding to $\alpha_1$, $\alpha_2\delta$, and β subunits of dihydropyridine-sensitive L-type $Ca^{2+}$ channels in the brain.[19,85,86] A potentially novel 100 kD protein was also identified as a specifically associated component of the L-type $Ca^{2+}$ channel complex from brain.[19] Further biochemical characterization of brain $Ca_v1$ channels will require development of a method for purification of larger quantities of channel protein.

## Biochemical Properties of the $Ca_v2$ Family of $Ca^{2+}$ Channels

Multiple types of $Ca^{2+}$ channels, which differ in physiological and pharmacological properties, are expressed in neurons. At least three types of high-voltage-activated $Ca^{2+}$ channels have been distinguished in addition to L-type (see Chapter 3).[87-90] N-type, P-type, and Q-type channels all have intermediate single channel conductances (about 15 pS) and can mediate $Ca^{2+}$ currents with varying rates of voltage-dependent inactivation depending on their subunit composition and on other factors (see below). They are best distinguished by their pharmacological properties: N-type channels are specifically inhibited by ω-conotoxin GVIA whereas P/Q-type are most sensitive to ω-agatoxin IVA and ω-conotoxin MVIIC. These specific peptide toxins have provided experimental tools for analysis of the protein subunits of neuronal $Ca^{2+}$ channels.

### *$Ca_v2.2$ Channels*

The ω-conotoxin-sensitive N-type $Ca^{2+}$ channels purified from rat brain contain an $\alpha_1$ subunit, a 140 kD $\alpha_2$-like subunit, and β subunits of 60 kDa to 70 kD as identified by antibodies against the skeletal muscle forms of these subunits.[19,91-94] Both L-type and N-type $Ca^{2+}$ channels from brain appear to lack a γ subunit, but a protein of approximately 100 kD is specifically associated with N-type $Ca^{2+}$ channels as well as L-type $Ca^{2+}$ channels from brain and may be an additional, brain-specific associated protein.[19,91,92,94] The $\alpha_1$ subunit of N-type $Ca^{2+}$ channels was identified as $Ca_v2.2$ by homology cDNA cloning, co-immunoprecipitation, and functional expression.[95,96] As for the $Ca_v1$ family channels, analysis of the $Ca_v2.2\alpha_1$ subunit using sequence-specific antibodies revealed two size forms (240 kD and 210 kD) that differ in their C-termini and in their phosphorylation by specific protein kinases.[97,98] It is unknown at present whether these $Ca_v2.2\alpha_1$ subunits are derived from proteolytic truncation, alternatively spliced mRNAs or both (see Chapter 5).

### *$Ca_v2.1$ Channels*

Agatoxin-sensitive P/Q-type $Ca^{2+}$ channels purified from brain are also composed of $\alpha_1$, $\alpha_2\delta$, and $\beta$ subunits.[99,100,101] In addition, recent experiments have unexpectedly revealed a novel $\gamma$ subunit, which is the target of the *stargazer* mutation in mice.[102] This $\gamma$-subunit-like protein can modulate the voltage dependence of expressed $Ca^{2+}$ channels containing $Ca_v2.1$ subunits, so they may be associated with these $Ca^{2+}$ channels in vivo.[102] If this new $\gamma$ subunit is indeed associated with neuronal $Ca^{2+}$ channels, their subunit composition would be identical to that of skeletal muscle $Ca^{2+}$ channels defined in biochemical experiments.[14] The cDNA encoding the $Ca_v2.1$ subunit was isolated by homology cloning and identified by functional expression.[103,104] Analysis of the $\alpha_1$ subunit peptides present in brain has revealed a wide range of size forms, including C-terminal truncations and internal deletions, and these isoforms are differentially phosphorylated by protein kinases.[105,106] These results continue the theme that all $Ca^{2+}$ channels studied to date have multiple forms due to varying C-terminal domains and that they are differentially phosphorylated by protein kinases.

## Interactions of $Ca^{2+}$ Channels with Intracellular Regulatory Proteins

$Ca^{2+}$ entering cells through voltage-gated $Ca^{2+}$ channels initiates many intracellular processes through activation of effector mechanisms. Often these effector mechanisms are highly localized to respond to high local concentrations of $Ca^{2+}$. In addition, local regulation of $Ca^{2+}$ channel function is also modulated by specifically bound regulatory proteins. The interactions of these intracellular effector and regulatory proteins with $Ca^{2+}$ channels have been studied by biochemical approaches as outlined here and by molecular biological methods as described in Chapters 8, 9 and 11. These studies show that a functional $Ca^{2+}$ channel complex not only has four auxiliary subunits but also contains multiple associated regulatory proteins that modify its activity.

### *Ryanodine Receptors*

In skeletal muscle fibers, excitation-contraction coupling involves direct conformational coupling of the voltage-gated $Ca^{2+}$ channel in the transverse tubule membrane to the ryanodine-sensitive $Ca^{2+}$ release channel (RyR) of the sarcoplasmic reticulum at the transverse tubule/sarcoplasmic reticulum junction.[6,7,107] Conformational coupling is mediated by the intracellular loop connecting domains II and III of the $\alpha_1$ subunit.[108,109] Depolarization of the transverse tubules by the conducted action potential activates L-type voltage-gated $Ca^{2+}$ channels. A complex of ryanodine receptor and $Ca^{2+}$ channels can be identified by detergent extraction and resolution by sucrose gradient sedimentation and antibody labeling.[110] This complex is required for excitation/contraction coupling and for feedback regulation of $Ca^{2+}$ channel function.[109,111] and Chapter 21.

### *SNARE Proteins*

$Ca^{2+}$ entry through voltage-gated $Ca^{2+}$ channels is thought to initiate exocytosis by triggering the fusion of secretory vesicle membranes with the plasma membrane through actions on the SNARE protein complex of syntaxin, SNAP-25, and VAMP/synaptobrevin (reviewed in refs. 112,113). The function of the SNARE protein complex is regulated by interactions with numerous proteins, including the synaptic vesicle $Ca^{2+}$ -binding protein synaptotagmin. The SNARE proteins syntaxin and SNAP-25 are associated with N-type and P/Q-type $Ca^{2+}$ channels and can be co-purified and co-immunoprecipitated from brain.[91,100,114,115] Presynaptic N-type and P/Q-type $Ca^{2+}$ channels interact directly with the SNARE proteins through a specific synaptic protein interaction (synprint) site in the large intracellular loop connecting domains II and III.[116,117]and Chapter 8. This binding interaction is regulated by $Ca^{2+}$ and protein phosphorylation.[117,118] Synaptotagmin also binds to the synprint site of $Ca_v2$ $Ca^{2+}$ channels.[119-121] Injection of peptide inhibitors of this SNARE protein interaction into

pre-synaptic neurons inhibits synaptic transmission, consistent with the conclusion that this interaction is required to position docked synaptic vesicles near $Ca^{2+}$ channels for effective fast exocytosis.[116,122,123] SNARE protein interaction at this site is also important for G protein regulation of $Ca^{2+}$ channels (see Chapter 9).

### *Kinase Anchoring Proteins*

cAMP-dependent protein kinase is often anchored to specific subcellular compartments or specific kinase substrates by A Kinase Anchoring Proteins (AKAPs).[124,125] These proteins contain a targeting domain that directs the AKAP to a specific cellular site and a kinase anchoring domain containing an amphipathic alpha helix that binds the regulatory subunit dimer of cAMP-dependent protein kinase. Because regulation of the skeletal muscle $Ca^{2+}$ channel by membrane depolarization and cAMP-dependent protein kinase is very rapid, with observable effects in 50 ms, it was an attractive candidate for regulation through PKA bound to AKAPs. Biochemical studies of skeletal muscle $Ca^{2+}$ channels revealed a novel 15-kDa AKAP (AKAP-15) associated with purified $Ca^{2+}$ channels and with specifically immunoprecipitated $Ca^{2+}$ channels.[126] This AKAP is an 81-residue protein with N-terminal palmitoyl and myristoyl moieties that serve as membrane anchors and an amphipathic helix that binds PKA[127] (also designated AKAP-18).[128] AKAP-15, PKA, and $Ca^{2+}$ channels are co-localized in the specialized junctions formed between sarcoplasmic reticulum and transverse tubule membranes, where excitation-contraction coupling takes place.[127,129] AKAP-15 binds to the skeletal muscle $Ca^{2+}$ channel through a specific modified leucine zipper motif in the distal C-terminal domain.[130] In this way, the bound AKAP directly targets PKA to its sites of phosphorylation in the C-terminal of the full-length $\alpha_1$ subunit.

### *$Ca^{2+}$ Binding Proteins*

$Ca^{2+}$ entering cells through $Ca^{2+}$ channels is involved in initiation of intracellular signaling processes, and these often involve $Ca^{2+}$ binding proteins like calmodulin, which is expressed ubiquitously, and neuro-specific $Ca^{2+}$ binding proteins, which are expressed in specific neurons and in distinct subcellular locations. Research reviewed in Chapter 11 shows that both calmodulin and neuro-specific $Ca^{2+}$ binding proteins interact with $Ca^{2+}$ channels at specific binding sites in the C-terminal domains and modulate their function.

## Conclusion

Voltage-gated $Ca^{2+}$ channels are complex proteins containing five distinct protein subunits, $\alpha$ , $\alpha$ , $\beta$, $\gamma$, and $\delta$, that specifically associate with each other. In addition to subunit assembly, their biosynthesis involves extensive glycosylation as well as proteolytic processing and disulfide linkage of subunits. Each $\alpha 1$ subunit that has been studied is present in multiple isoforms that differ in their C-terminal domains and in phosphorylation by specific kinases. $Ca^{2+}$ channels provide intracellular $Ca^{2+}$ to initiate local signaling events, and they are directly associated with both effector proteins that initiate $Ca^{2+}$ -dependent processes and with regulatory proteins that control their activity. $Ca^{2+}$ channel signaling complexes are a crucial element of local regulation of cellular events.

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# CHAPTER 5

# Molecular Properties of Voltage-Gated Calcium Channels

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## Native Voltage-Gated $Ca^{2+}$ Channels

Early electrophysiological recordings from neurons, muscle and endocrine cells revealed voltage-activated calcium ($Ca^{2+}$) currents with distinct characteristics, suggesting the existence of two major classes of $Ca^{2+}$ channels based upon the membrane potentials at which they first open (see Chapter 3); low-voltage activated (LVA) and high-voltage activated (HVA). The LVA (or T-type) channels, typically have a small conductance (8-12 pico Siemens (pS)), open in response to small changes from the resting membrane potential and inactivate rapidly. In contrast, the HVA currents generally possess larger conductances (15-25 pS), are activated by stronger depolarizations and display variable inactivation kinetics. To date, multiple types of HVA $Ca^{2+}$ channels (L-, N-, P/Q- and R-type) have been categorized on the basis of a number of criteria including, single channel conductance, kinetics, pharmacology, and cellular distribution.[1-4]

## High Voltage-Activated $Ca^{2+}$ Channels

### *L-Type Channels*

L-type $Ca^{2+}$ channels were initially described in peripheral neurons and cardiac cells, but appear to be present in all excitable as well as many types of non-excitable cells.[4] In certain cells, L-type channels have been shown to be preferentially localized to specific subcellular regions. For example, the L-type channels responsible for skeletal muscle contraction are concentrated on the transverse tubule membrane,[5] while neuronal L-type channels are located primarily on cell bodies and proximal dendrites.[6] The L-type channel is the primary route for $Ca^{2+}$ entry into cardiac, skeletal, and smooth muscles.[2] The skeletal muscle L-type channel acts as a voltage sensor for excitation-contraction (E-C) coupling in skeletal muscle, presumably linking membrane depolarization to $Ca^{2+}$ release from intracellular stores. While $Ca^{2+}$ entry through this channel is not required for the initiation of contraction in skeletal muscle, it may provide a source of $Ca^{2+}$ to replenish internal stores.[2,4,7,8] There is some evidence that L-type channels are involved in exocytotic release from endocrine cells and some neurons[9-13] and the localization of L-type channels on the cell soma[6] has also implicated these channels in the regulation of gene expression.[14-16]

Much is known about the pharmacological properties of L-type $Ca^{2+}$ channels. The three main classes of organic L-type channel blockers are the phenylalkylamines (verapamil), benzothiazapines (diltiazem), and 1,4-dihydropyridines (DHPs) (e.g., nitrendipine, nifedipine, nimodipine). The DHP antagonists bind preferentially to channels in the active conformation, a state favored by depolarization (producing more potent inhibition at depolarized potentials). A number of DHP agonists have also been developed, the most highly utilized of which is

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(-)-Bay K 8644 which increases both the open time and the single channel conductance (see chapter by Striessnig for more detail). L-type channels are also blocked by certain native peptide toxins such as ω-agatoxin IIIA (ω-Aga IIIA), isolated from the venom of the funnel web spider *Agelenopsis aperta*.[17,18] ω-Aga IIIA reduces the current amplitude without affecting the time course and unlike the DHPs, ω-Aga IIIA inhibition is voltage-independent and blocks L-type channels at all potentials[2] (see chapter by Adams and Lewis for more detail).

L-type channels have a unitary conductance ranging from 20 and 27 pS using 110 mM barium ($Ba^{2+}$) as the charge carrier. L-type channels require large departures from resting potential to become activated and typically begin to open at potentials positive to -10 mV, although they can activate at significantly more negative potentials in chromaffin cells, sensory neurons, and cardiac cells. In the presence of $Ba^{2+}$ as the charge carrier, once open, L-type channels do not inactivate significantly during depolarizations of hundreds of milliseconds.[2,3,19] Compared to $Ba^{2+}$ currents, using $Ca^{2+}$ as the charge carrier L-type currents are smaller and inactivate rapidly. This $Ca^{2+}$-dependent inactivation has a number of characteristic properties and inactivation attributable to $Ca^{2+}$ influx is greatest at depolarizations at which $Ca^{2+}$ entry through the channel is maximal.[20] While the degree of inactivation is slowed by the addition of BAPTA and other $Ca^{2+}$ chelators, it is not completely abolished. $Ca^{2+}$-dependent inactivation can however be eliminated by intracellular applications of trypsin, suggesting that the mechanism through which $Ca^{2+}$ acts to inactivate the channel is in close proximity to, if not part of, the channel complex itself.[2,3,21,22] Because the rate of $Ca^{2+}$-dependent inactivation does not change with channel density, Neely et al (1994)[22] proposed a "local domain" hypothesis, in which $Ca^{2+}$ affects only the channel through which it enters (see Chapter 11 for more detail). Moreover, current models view calmodulin to be constitutively bound to the C-terminus forming part of the $Ca^{2+}$ sensing machinery, ultimately leading to the signal transduction of $Ca^{2+}$-dependent inactivation.[23]

## *N-Type Channels*

In addition to L- and T-type $Ca^{2+}$ channels, recordings from chick dorsal root ganglion (DRG) cells revealed a third type of single channel $Ca^{2+}$ conductance of 13 pS (in 110 mM $Ba^{2+}$), intermediate between that of the T- (8 pS) and L- (25 pS) type channels.[1,2,19,24,25] Although this conductance shares some general electrophysiological characteristics with currents through both T- and L-type channels, it could not be attributed to either. Consequently, the corresponding channel was designated as N (neither)-type.

Although first identified in chick DRG neurons, N-type channels have also been detected in mammalian DRG cells,[26-29] mammalian and amphibian sympathetic neurons,[30-33] and other cells of the peripheral and central nervous systems.[34-39] N-type channels appear to be expressed only in neuronal tissues,[31,40] although an N-type current has been reported in rat thyroid C-cell line.[41] Electrophysiologically, N-type channels are most easily distinguished from L-type channels by their inactivation properties. Unlike L-type channels, N-type channels display time-dependent inactivation (with $Ba^{2+}$ as the charge carrier). N-type currents decay with a time constant ($\tau$) ranging from 50 to 110 ms, significantly slower than the rapid ($\tau$ = 20-50 ms) inactivation of the LVA T-type channels, but much faster than the non-inactivating L-type channels. In chick DRG neurons, the N-type current decays almost completely during a test depolarization of 140 ms, while L-type current shows little inactivation over the same period of time.[1] However, N-type currents do not always inactivate rapidly. In sympathetic neurons, the decay rate of N-type currents is much slower ($\tau$ = 500 to 800 ms) and can be incomplete, even over depolarizations lasting longer than one second.[2,41-42] Thus, there appears to be at least two distinct components to N-type current inactivation. These differences in inactivation kinetics could reflect different subtypes of N-type channel. Alternatively, a single N-type channel could support both currents by switching between the slow- and fast-inactivating states.[40,43]

In addition to the time-dependence parameter, there is also a voltage-dependent aspect to N-type channel inactivation.[1,24,25,29] Holding the cell membrane at potentials between -60

and -40 mV results in significant inactivation of the N-type current, and strongly negative potentials are required to reprime the channels. N-type channels are markedly more sensitive to the effects of holding potential on inactivation than are L-type channels. At resting membrane potentials of -20 mV, N-type channels are completely inactivated while L-type channels remain available for opening.

Theoretically, the different inactivation properties of N- and L-type channels provides two parameters that can be used to dissect the relative contributions of the two channel types to the whole cell HVA current.[2,24] One method takes advantage of the different inactivation rates. The component of whole cell current that decays during a prolonged depolarization can be attributed to the inactivating N-type channels, while the non-inactivating portion is identified as L-type current. The second approach exploits the different ranges over which voltage-dependent inactivation takes place. The contribution of each type to the whole cell current may be determined by analyzing the differences in whole cell currents elicited by depolarizations from resting potentials of -40 and -90 mV. Because L-type channels are relatively resistant to the effects of holding potential on inactivation while N-type channels inactivate at depolarized membrane potentials, the difference under these two conditions should reflect the contribution of N-type channels to the whole-cell current. However, neither method may be adequate to properly distinguish these currents. Some N-type channels can inactivate quite slowly and inactivation may not be complete. In addition, voltage-dependent inactivation of N-type channels can be highly variable and takes place over a wide range of holding potentials between -80 to -20 mV.[24,25] If N-type channels predominate in a cell, the residual current through incompletely inactivated N-type channels may be significant. Thus definitions of N- and L-type current based solely on these criteria may not be valid.

Pharmacologically, N-type channels are sensitive to inhibition by a class of native peptide toxins called the ω-conotoxins, which are a family of small (13-29 amino acid) peptides found in the venom of predatory marine snails of the genus *Conus*.[44,45] All known ω-conotoxins inhibit N-type $Ca^{2+}$ channels, although their specificities and blocking affinities for this particular channel vary significantly. To date, ω-conotoxin GVIA (ω-CgTx), a 27-amino acid peptide from *Conus geographus*[46] is the most specific ω-conotoxin peptide for N-type channel inhibition. ω-CgTx produces complete and irreversible inhibition of N-type currents in DRG, hippocampal, sympathetic, and sensory neurons at concentrations of approximately 100 nM to 1 μM.[47-49] At higher concentrations (5-15 μM), ω-CgTx also inhibits L- and T-type currents, although unlike N-type channels, the effects are incomplete and reversible[47-52] (see chapter by Adams and Lewis for more detail).

ω-CgTx binding sites (and by extension N-type $Ca^{2+}$ channels) are distributed throughout the PNS and CNS, including the cortex, hippocampus, olfactory bulb, and cerebellar cortex, and appear especially concentrated in regions of high synaptic density.[2,53-58] Although N-type channels were first identified by single channel recordings from the cell bodies of DRG neurons,[1] they appear to be more abundantly localized on dendrites and axon terminals. In muscle, ω-CgTx binding occurs at the active zones of presynaptic cells in spatial register with the postsynaptic acetylcholine (ACh) receptors on the muscle. Labeling is rarely found between active zones, nor is it localized to areas of the presynaptic membrane that do not face the muscle. N-type channels have also been observed to cluster in areas of synaptic contact on hippocampal CA1 neurons.[54]

The presence of N-type channels on the presynaptic membrane suggests that $Ca^{2+}$ entry through these channels is responsible for triggering neurotransmitter release. An early study[59] demonstrated that ω-CgTx blocks electrically-induced release from the frog NMJ and numerous subsequent studies have demonstrated that the application of ω-CgTx inhibits neurotransmitter release in the central and peripheral nervous system.[60-67] Furthermore, biochemical studies indicate that N-type channels are physically associated with proteins such as synaptotagmin and syntaxin which are part of the exocytotic machinery.[68-70] There appear to be species- and cell-specific differences in N-type-channel-regulated neurotransmission. For example, while

ω-CgTx completely abolishes neurotransmission at the avian and amphibian NMJ, it has no effect on the mammalian motor nervous system.[59,65,71-73] The ability of this toxin to inhibit neurotransmission also varies depending on the type of synapse within a given species.[52,65-67] For example, inhibitory synaptic transmission in hippocampal CA1 neurons is strongly reduced by the application of ω-CgTx, whereas the toxin blocks excitatory transmissions to a much lesser extent. In addition, while ω-CgTx inhibits release of ACh from both autonomic and central neurons in the rat, release from central neurons is approximately 20-fold less sensitive.[65]

In spite of the complete and irreversible inhibition of N-type channels produced by ω-CgTx, application of the toxin to many types of neurons only partially inhibits neurotransmitter release, suggesting that other types of $Ca^{2+}$ channels contribute to neurotransmitter release from both central and peripheral neurons.[58,74,75] In fact, while regulation of transmitter release from peripheral neurons appears to predominantly involve N-type channels, release in the central nervous system appears to be controlled primarily by other types of $Ca^{2+}$ channels that are insensitive to both ω-CgTx and DHPs.[76,77]

The presence of N-type channels in regions other than the synapse indicates that these channels have other functions in addition to neurotransmitter release. N-type channels localized to dendritic branch points may be involved in integration or amplification of neural inputs.[56] N-type channels may also play a role in nervous system development as evidenced by the expression of N-type channels on postmitotic cerebellar granule cells. These cells only begin migration after the appearance of N-type channels and ω-CgTx causes a cessation of migration.[78]

## *Other HVA $Ca^{2+}$ Channels: P-, Q-, and O-Types*

The original classification system of $Ca^{2+}$ channels, which was expanded from the simple LVA/ HVA dichotomy to encompass T-, L- and N-channels, was subsequently found to be too restrictive to adequately describe all types of $Ca^{2+}$ conductances. The availability of blocking agents that target L- and N-type channels revealed other HVA currents that could not be defined according to this scheme.[36,37,45,79-81] These novel channel types, variously named P-, Q-, O-, and R-, have primarily been defined on the basis of their distinctive pharmacological properties rather than electrophysiological characteristics.

The P-type current was originally identified as an HVA current in Purkinje cells that is insensitive to the agents typically used to inhibit L- and N-type channels.[82] These channels are thought to support the $Ca^{2+}$-dependent action potentials in the dendrites of cerebellar Purkinje cells, which are unaffected by DHPs and ω-CgTx, but are potently blocked by components of the venom of the funnel web spider *Agelenopsis aperta*.[82-85]

Whole cell recordings from Purkinje cells reveal an HVA current that peaks at voltages between -30 and -20 mV and inactivates slowly over the duration of the depolarization.[37,52,81,86] Single channel analysis of P-type channels reveals conductances in ranges similar to those of N- and L-type channels. Multiple unitary conductance levels of 9, 14, 19 pS in 110 mM Ba have been reported for P-type channels in the Purkinje cell soma and dendrites,[87] and a P-type current in hypoglossal motorneurons has a unitary conductance of 20 pS.[39]

The venom of the funnel web spider, like that of the cone snail, is a cocktail of toxins that target different elements of the synaptic machinery. FTX, a non-peptide component of the venom (arginine polyamine and a synthetic analog of FTX, sFTX), was initially reported to be specific blockers of P-type channels,[3,82,83] but subsequently shown to produce inhibition of other $Ca^{2+}$ currents in conjunction with the P-type block.[3,45]

ω-Aga IVA, a 48-amino acid peptide also found in the venom of *A. aperta* potently inhibits P-type $Ca^{2+}$ channels.[52,84,86] In Purkinje cells, complete inhibition is observed at concentrations below 200 nM, with half-maximal block produced at concentrations between 2 and 10 nM. Inhibition is rapid, occurring within two minutes of application, and while the inhibition is poorly reversible by wash-out, it can be removed by a series of strong depolarizations (i.e., to

+70 mV). Block of P-type currents by ω-Aga IVA in other neurons is qualitatively similar to that in Purkinje cells although the kinetics vary slightly. For example, while inhibition of P-type current in spinal cord interneurons and neurons in the visual cortex occurs as rapidly as that in the Purkinje cells, the rate of block is several times slower in CA1 and CA3 hippocampal neurons.[86]

A peptide toxin isolated from the cone snail *Conus magus* has also been shown to inhibit P-type channels.[88] This toxin, ω-CgTx MVIIC, blocks P-type channels with an $IC_{50}$ of 1-10 μM. However, ω-CgTx MVIIC also inhibits N-type channels as well as the Q- and O-type conductances[88] (see chapter by Adams and Lewis for more detail).

P-type channels do not account for all of the DHP- and ω-CgTx-resistant current in neurons since a substantial fraction of current remains even after exposure to saturating concentrations of DHPs, ω-CgTx, and ω-Aga IVA. Cultured rat cerebellar granule cells express an HVA current that is unaffected by these inhibitors at concentrations which block L-, N- and P-type channels, respectively.[45,89,90] However, the channels supporting this novel current (termed Q-type) are partially blocked by ω-Aga IVA at concentrations 10 to 100 times that required for P-type inhibition and are completely blocked by ω-CgTx MVIIC ($IC_{50}$= 30 to 300 nM). In addition to differing sensitivities to these toxins, Q-type channels partially recover from ω-Aga IVA-induced inhibition within minutes of toxin washout. Q-type channels also display electrophysiological properties distinct from those of P-type channels. While P-type currents in Purkinje cells and cerebellar granule cells show no inactivation over a 100 ms test depolarization, the Q-type current in granule cells decays to approximately 65% of the peak current over the same time period.

The existence of O-type channels has been inferred solely from pharmacological studies. O-type channels were identified as high affinity ω-CgTx MVIIC binding sites.[91] These channels are significantly more sensitive to the toxin than are other ω-CgTx MVIIC-sensitive channels[45] It is possible that P-, Q-, and O-type channels are members of the same channel family which possess slightly different pharmacological and electrophysiological properties as a result of alternative splicing of the $\alpha_1$ subunit gene and/or different complements of auxiliary subunits. While O-type channels have not been localized immunohistochemically, evidence from binding studies suggests that they are widely distributed in the mammalian CNS.[45] Estimates of O-type bindings sites in rat brain preparations suggest that O-type channels are more prevalent than N-type channels in the CNS, and it has been proposed that O-type channels are localized exclusively to synaptic termini, which would largely prevent their detection through electrophysiological means.

Many studies implicate P-, Q, and O-type channels in neurotransmitter release.[45] While N-type channels mediate release at some synapses in the mammalian CNS, the ω-Aga IVA-sensitive P- and Q-type channels appear to play a more prominent role.[52,92] ω-Aga IVA potently blocks $Ca^{2+}$ uptake into synaptosomes[84] and partially inhibits the release of dopamine and glutamate from synaptosomes[93,94] and at CA1-CA3 synapses in the hippocampus.[58,74,76] In the peripheral nervous system, ω-Aga IVA has little or no effect on the autonomic nervous system,[95] but P-type channels are probably responsible for neurotransmitter release at the mammalian NMJ.[96,97] As ω-Aga IVA blocks both P- and Q-type channels, it is possible that both channel types are involved in neurotransmission. Wheeler and colleagues found that the pharmacological properties of the ω-Aga IVA-sensitive channels supporting neurotransmission in the hippocampus and for ω-Aga IVA-induced block of inhibitory postsynaptic potentials in the cerebellum were more similar to Q- than P-type channels.[58,98] Finally, O-type channels may also mediate neurotransmission at certain synapses, as norepinephrine release in the hippocampus is inhibited by subnanomolar concentrations of ω-CgTx MVIIC.[45]

### *R-Type Channels*

A component of the HVA current in cerebellar granule cells remains even after the application of nimodipine, ω-CgTx , ω-Aga VIA, and ω-CgTx MVIIC. This current, categorized as

R (residual or resistant)- type,[89] comprises approximately 15% of the HVA current in these cells. R-type current may not necessarily reflect a single channel type, but a family of molecularly distinct channels with similar pharmacological and electrophysiological characteristics.

R-type currents begin to activate around -40 mV and reach a peak amplitude at 0 mV. The current inactivates rapidly, and the increased rate of inactivation with $Ca^{2+}$ as the charge carrier suggests that the channels supporting the R-type current inactivate in a $Ca^{2+}$-dependent manner. R-type channels are equally sensitive to block by $Cd^{2+}$ and $Ni^{2+}$ ions. The exact nature of the channels supporting this current is currently unknown. See the below section on Class E channels for further discussion.

## Cloned Calcium Channels

### *HVA $Ca^{2+}$ Channels Are Multi-Subunit Complexes*

Biochemical studies have established that high threshold voltage-gated $Ca^{2+}$ channels are multi-subunit complexes. Taking advantage of the high-affinity binding of organic antagonists, several groups purified the L-type channel from skeletal muscle. Four distinct polypeptides, designated $\alpha_1$ (175-kDa), $\alpha_2\delta$ (170-kDa), $\beta$ (52-kDa), and $\gamma$ (32-kDa), co-migrate with the ligand-binding activity.[99-102] A minor 212-kDa band also co-purified and was shown to represent a larger, much less abundant form of the skeletal muscle $\alpha_1$ subunit.[103] Similar approaches have been used to isolate the cardiac L-type[104] and brain N-type channels[105] These complexes also consist of an $\alpha_1$ subunit associated with $\beta$ and $\alpha_2\delta$ subunits. The $\beta$ and $\alpha_2$-$\delta$ are highly similar, if not identical to, the subunits associated with the skeletal muscle $\alpha_1$.[106,107] However, unlike the skeletal muscle L-type channel, no $\gamma$ subunits appeared as part of either complex. A novel 95-kDa polypeptide was found to comigrate with the N-type channel although it is unclear whether this represents a bona fide channel subunit or a proteolytic fragment.[105]

While subunit composition differs slightly depending on channel type, a general model has been proposed for HVA channels in which four to five proteins form a multisubunit complex (Fig. 1A). In this model, the $\alpha_1$ subunit forms the channel proper, comprising both the voltage-sensing mechanism and the $Ca^{2+}$ selective pore, and the remaining proteins interact with the $\alpha_1$ subunit to modulate activity.

### *Primary Structure and Properties of $Ca^{2+}$ Channel $\alpha_1$ Subunits*

The first cDNAs encoding $Ca^{2+}$ channel $\alpha_1$ subunits were isolated from rabbit skeletal muscle.[5,108] The $\alpha_{1S}$ L-type subunit is an 1873-residue protein that bears a high degree of amino acid similarity to the voltage-gated $Na^+$ and potassium ($K^+$) channels (see Fig. 1). The $\alpha_1$ subunit is predicted to consist of four homologous, mainly hydrophobic domains (designated domains I, II, III and IV). Each of the four domains is comprised of six putative membrane-spanning segments (S1-S6). The S4 segment in each domain contains positively-charged residues every third or fourth position and is believed to form part of the voltage-sensing mechanism of the channel. Between the S5 and S6 segments of each domain are two hydrophobic segments, SS1 and SS2, which are predicted to form the channel pore (Fig. 1B).

Based upon similarity to voltage-gated $Na^+$ channels, Tanabe et al (1987)[5] speculated that the $\alpha_1$ subunit may form both the $Ca^{2+}$-selective pore and the voltage sensor of the channel complex. This hypothesis was supported by studies demonstrating that expression of the $\alpha_{1S}$ in myotubes from dysgenic mice restored normal skeletal muscle-type E-C coupling and the slow $Ca^{2+}$ current absent in these cells.[7] In addition, $\alpha_{1S}$ expression in dysgenic myotubes restored the charge movement observed in normal myotubes upon membrane depolarization.[109] These results indicated that the skeletal muscle $\alpha_{1S}$ subunit acts both as a voltage-sensor, providing a physical connection between membrane depolarization and $Ca^{2+}$-release from intracellular stores for the initiation of muscle contraction, and is also part of a functional VGCC.

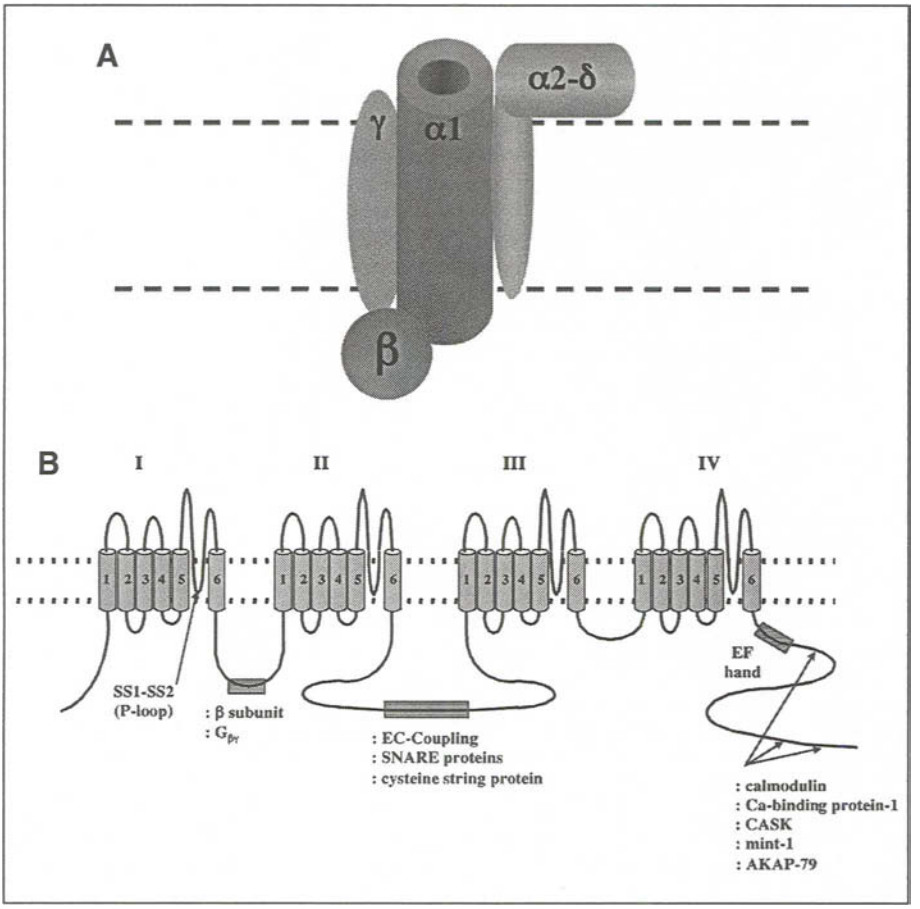



Figure 1. Composition of a VGCC complex and structure of the $\alpha_1$ subunit. A) Diagram of a high-voltage-activated VGCC complex, indicating the $\alpha_1$, $\alpha_2/\delta$, $\beta$, and $\gamma$ subunits. The $\alpha_1$ subunit forms the channel proper, comprising the voltage-sensing mechanism, the Ca selective pore, and target of identified pharmacological agents. B) Predicted structure and transmembrane topology of the $\alpha_1$ subunit. Each domain possesses six putative membrane-spanning segments (1-6) and pore-forming P-loop (SS1-SS2). All high voltage-activated channel $\alpha_1$ subunits possess a conserved region in the domain I-II linker that binds the Ca $\beta$ subunit as well as a conserved EF hand motif in the carboxyl terminus. Other structural elements identified amongst the various types of high voltage-activated $Ca^{2+}$ channels includes: a high affinity G-protein $\beta\gamma$-subunit binding site in the I-II linker ($Ca_v2.1$ and $Ca_v2.2$); distinct regions in the domain II-III linker responsible for functional interaction with the synaptic release machinery ($Ca_v2.1$ and $Ca_v2.2$), cysteine string protein ($Ca_v2.2$) and the skeletal muscle excitation-contraction coupling machinery ($Ca_v1.1$); as well as carboxyl terminal regions shown to interact with calmodulin ($Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$, $Ca_v2.3$), binding to AKAP-79 ($Ca_v1.2$), Ca-binding protein-1 ($Ca_v1.2$, $Ca_v2.1$), CASK ($Ca_v2.2$) and mint-1 ($Ca_v2.2$).

Using the skeletal muscle clone as a probe, cDNAs encoding homologous L-type $\alpha_1$ subunits have been subsequently cloned from cardiac[110] and smooth muscle.[111,112] Injection of the cardiac $\alpha_1$ subunit into dysgenic myotubes resulted in the expression of a VGCC which differed markedly in terms of activation rate, $Ba^{2+}$ permeability, and E-C coupling from the current conducted through channels formed by the skeletal muscle clone.[113] Expression of

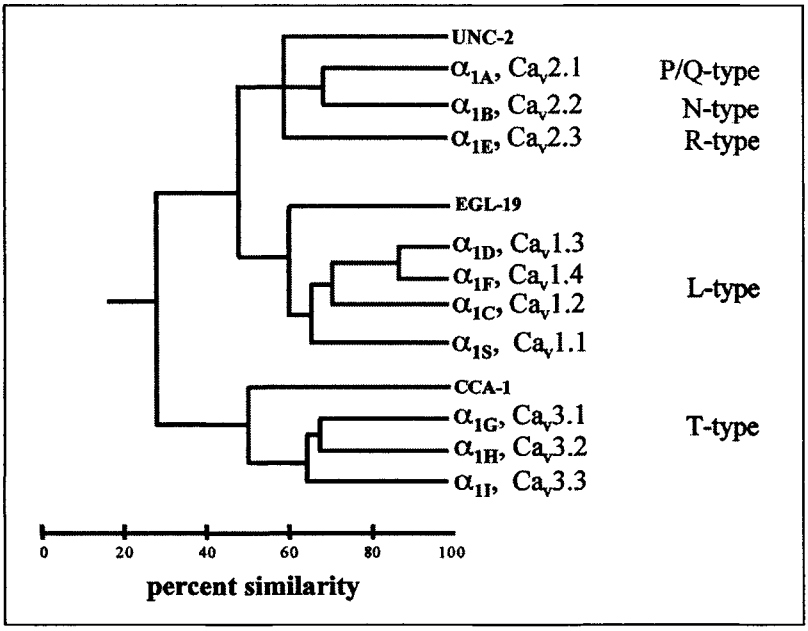



Figure 2. Similarity tree of mammalian VGCC $\alpha_1$ subunits. The predicted amino acid sequences of representatives of each class of VGCC $\alpha_1$ subunit were compared pairwise and the percent similarities plotted. *unc-2*, *egl-19* and *cca-1* represent the *Caenorhabditis elegans* ancestoral homologues of the mammalian subunits. GenBank Accession Numbers for VGCCs: rat $\alpha_{1A}$, M64373; rat $\alpha_{1B}$, M92905; rat $\alpha_{1C}$, M67515; rat $\alpha_{1D}$, AF370009; rat $\alpha_{1E}$, L15453; human $\alpha_{1F}$, AJ224874; rat $\alpha_{1G}$, AF290212; rat $\alpha_{1H}$, AF290213; rat $\alpha_{1I}$, AF290214; rabbit $\alpha_{1S}$, M23919.

cardiac and smooth muscle $\alpha_1$ subunit clones in *Xenopus* oocytes[110,111,114,115] resulted in large inward currents that were sensitive to the organic channel agonists and antagonists, thereby identifying them as L-type channels. Co-expression of skeletal muscle-derived $\alpha_2$-$\delta$ and $\beta$ subunits, while affecting the amplitude and voltage dependence of the currents, was not required for channel activity or drug binding, suggesting that the $\alpha_1$ subunit is capable of forming a functional channel in the absence of the other subunits. However, because some VGCC subunits may be endogenously expressed by *Xenopus* oocytes,[116,117] it is possible that the $\alpha_1$ protein forms a complex with these endogenous auxiliary subunits. This prompted several groups to examine the properties of the $\alpha_1$ subunit in cells lacking these proteins. Murine L-cells[118,119] and Chinese Hamster Ovary (CHO) cells[114] stably transformed with $\alpha_1$ subunits express voltage activated $Ca^{2+}$ currents sensitive to L-type channel blockers. While $Ca^{2+}$ currents in cells expressing the smooth muscle $\alpha_1$ subunit displayed similar drug sensitivities and kinetics to the native currents, the currents supported by $\alpha_{1S}$ activated considerably more slowly than currents recorded from skeletal muscle cells.

At least nine different $\alpha_1$ subunit genes are now known to be expressed in the mammalian nervous system (see Fig. 2, Table 1). Initially, four distinct classes of $\alpha_1$ subunits were isolated from a rat brain library on the basis of their homology to the rabbit skeletal muscle $\alpha_{1S}$ subunit.[120] Each cDNA hybridized to one of four distinct banding patterns on Northern blots of rat brain mRNA, allowing them to be grouped into four classes, designated $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1C}$ and $\alpha_{1D}$. Subsequently, a fifth $\alpha_1$ subunit ($\alpha_{1E}$) was isolated from rat brain.[121] Southern blot analysis and DNA sequencing indicated that the five classes are separate members of a multigene family with the $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1E}$ channels being more similar to one another than they are to

***Table 1. Voltage-gated calcium channel subunits***

| Native Channel Type | $\alpha_1$ Subunit | Activation | Pharmacological Range | Localization Characteristics |
|---|---|---|---|---|
| P/Q | $\alpha_{1A}$, ($Ca_v2.1$) | HVA | ω-Aga IVA (P-type), ω-MVIIC (Q-type); insensitive to DHPs | CNS, heart, pituitary; some cell bodies, many dendrites and presynaptic terminals |
| N | $\alpha_{1B}$ ($Ca_v2.2$) | HVA | ω-CgTx GVIA, insensitive to DHPs | CNS; subset of cell bodies, dendrites and presynaptic terminals |
| L | $\alpha_{1C}$ ($Ca_v1.2$)<br>$\alpha_{1D}$ ($Ca_v1.3$)<br>$\alpha_{1F}$ ($Ca_v1.4$)<br>$\alpha_{1S}$ ($Ca_v1.1$) | HVA | ω-CgTx (reversible; $\alpha_{1D}$), DHPs, benzothiazapines, phenylalkylamines | $\alpha_{1C}$: CNS, smooth and cardiac muscle; $\alpha_{1D}$: CNS, endocrine cells $\alpha_{1F}$: retina, immune system $\alpha_{1S}$: skeletal muscle; ($\alpha_{1C}$, $\alpha_{1D}$) cell bodies and proximal dendrites |
| R | $\alpha_{1E}$ ($Ca_v2.3$)* | HVA | $Ni^{2+}$; insensitive to DHPs, ω-CgTx, and w-Aga- IVA | CNS; cell bodies, some distal dendrites and presynaptic terminals |
| T | $\alpha_{1G}$ ($Ca_v3.1$)<br>$\alpha_{1H}$ ($Ca_v3.2$)<br>$\alpha_{1I}$ ($Ca_v3.3$) | LVA | mibefradil, amiloride | CNS, heart, placenta, lung, kidney |

* While the $\alpha_{1E}$ subunit is often cited as encoding the R-type current first described in cerebellar granule cells, mice with a targeted gene deletion in the $\alpha_{1E}$ gene retain significant amounts of whole cell R-type currents.

the class C and D channels (Fig. 2). The class C clone is almost identical to the cardiac $\alpha_1$ subunit, suggesting that the class C and D clones represent members of the DHP-sensitive L-type channels, while the A, B, and E clones are DHP resistant. Four other VGCC $\alpha_1$ subunit genes have been identified in the mammalian genome. The $\alpha_{1F}$[122,123] shares the most sequence identity with the L-type channels. The $\alpha_{1G}$, $\alpha_{1H}$, and $\alpha_{1I}$ clones represent the LVA branch of the VGCC family.[124-126]

The individual $\alpha_1$ subunit clones share the most homology in the transmembrane domains with the majority of sequence divergence occurring in the putative cytoplasmic regions of the channels. The loop between domains II and III, and the cytoplasmic tail vary in size as well as sequence. The DHP-sensitive channels (classes C, D, F, and S) have relatively short (≈130 amino acid) sequences linking domains II and III while the analogous region in the class A and B channels are significantly larger (≈ 430 amino acid). However, despite the size similarity between the linkers, the class A and B clones show little sequence homology in this region.[127] While the LVA channel clones (classes G, H, and I) share much less sequence identity with the other classes, the voltage-sensing S4 region and the loop that forms the channel pore are well conserved. Other motifs, such as the β-subunit binding site and the E-F hand, which are found in the HVA classes of VGCCs are absent in the LVA channels. Whole cell and single channel electrophysiological techniques have provided information about the functional and pharmacological properties of the cloned channels and allowed researchers to assign the individual clones to channel types (Table 1).

## $\alpha_{1A}$/ $Ca_v$ 2.1

Class A $\alpha_1$ subunits have been cloned from both rabbit (BI-1, BI-2)[128] and rat (rbA-I)[129] brain and *Drosophila melanogaster* (Dmca1A).[130] Northern blot analysis identified a single RNA transcript of 9.4 kb in rabbit brain, while two transcripts of 8.3 and 8.8 kb were detected in rat brain. $\alpha_{1A}$ transcripts are widely distributed throughout the nervous system, as well as being present in the heart and pituitary, but not in skeletal muscle, stomach, or kidney. In brain, the highest levels of class A transcripts were found in the cerebellum, suggesting that this clone might encode a P-type channel and initial expression studies supported this hypothesis. Studies showed that $\alpha_{1A}$ clones expressed in *Xenopus* oocytes supported HVA currents which were insensitive to DHPs and ω-CgTx but inhibited by ω-Aga VIA.[128,131,132] However, a number of discrepancies between currents elicited in oocytes expressing class A clones and native P-type currents have called this into question.[131,132] The $\alpha_{1A}$ currents display prominent time- and voltage-dependent inactivation, yet P-type currents show little time-dependent inactivation and are relatively insensitive to holding potential. Furthermore, the pharmacological sensitivities of $\alpha_{1A}$ channels are quite different from those of P-type channels. While these currents are blocked by ω-Aga IVA, they are approximately 200-fold less sensitive to the toxin ($IC_{50} \approx$ 200 nM) than are P-type currents ($IC_{50} \approx$ 2-10 nM). In addition, $\alpha_{1A}$ channels are markedly more sensitive to block by the snail toxin ω-CgTx MVIIC than P-type channels ($IC_{50} \approx$ 150 nM vs. 1-10 μM for P-type channels). Sather et al (1993)[131] noted that the kinetic and electrophysiological features of the $\alpha_{1A}$ current were more similar to the Q-type current described in cerebellar granule cells by Randall et al (1993).[133] Based on these results, some researchers have suggested that the class A clones represent Q-type channels, and that P-type channels are the product of a different gene. However, Stea et al (1994)[132] noted that the high correlation between the localization of $\alpha_{1A}$ transcripts and P-type channel immunoreactivity implied a possible structural similarity between P- and Q-type channels and the $\alpha_{1A}$ gene product. They further proposed that the functional differences between the two channel types may arise as a result of differential post-translational processing of the proteins (which could affect toxin binding), subunit composition of the channel complex, and/or alternative splicing of the $\alpha_{1A}$ gene.

The auxiliary subunits of the VGCC complex are known to modulate the properties of the $\alpha_1$ subunit (see below). The inactivation kinetics of the $\alpha_{1A}$ subunit are dramatically affected by the type of β subunit with which it is associated.[132] Expression of the $\alpha_{1A}$ subunit from rat brain in the absence of the β subunit results in a current that inactivates considerably (40% remained after a 400 ms test pulse). Co-expression of either the $\beta_{1b}$ or $\beta_3$ subunit increased $\alpha_{1A}$ current inactivation to a rate similar to that of the Q-type current. In contrast, currents recorded from oocytes expressing the $\alpha_{1A} + \beta_{2a}$ combination show significantly slower inactivation kinetics, such that the waveform is more similar to that of native P-type currents. The β subunit also appears to affect voltage-dependent inactivation of the $\alpha_{1A}$ subunit. The $\beta_{2a}$ subunit shifted the steady-state inactivation of the $\alpha_{1A}$ approximately 15 to 20 mV more depolarized, thus reducing the sensitivity of the channel to holding potential.

Multiple isoforms derived from the alternative splicing of $\alpha_{1A}$ transcripts have been detected by several groups.[128,134-136] Bourinet et al (1999)[135] isolated an $\alpha_{1A}$ variant which possessed an number of sequence differences when compared to the rbA-1 clone and examined the functional implications of these splicing events. A valine insertion in the I-II linker both slowed time-dependent inactivation and altered steady-state inactivation. $\alpha_{1A}$ variants containing this valine have inactivation properties similar to P-type channels, while valine-less isoforms, such as rbA-1, appeared more Q-like. A second splice site consisted of the insertion of an asparagine-proline (N-P) pair in the IVS3-IVS4 linker. This affects the electrophysiological properties of the $\alpha_{1A}$ channel by producing a depolarizing shift in the current-voltage relationship. The N-P insertion also had the effect of decreasing the affinity of the channel for ω-Aga IVA by decreasing the on-rate of the toxin and increasing the off-rate. Thus, it is likely that the $\alpha_{1A}$ gene encodes both P- and Q-type channels, and the distinct channel properties reflect

differences both in subunit composition and alternative splicing. P-type channels may be comprised of splice variants that contain the valine insertion in the I-II linker, but not the N-P pair in the IVS3-IVS4 loop. Conversely, Q-type currents may be produced by channels lacking the valine, but containing the N-P insertion. In addition, the association of different β subunits may also be an important determinant of the P- versus Q-type phenotype.

### $\alpha_{1B}$/ $Ca_v$ 2.2

Class B $\alpha_1$ subunits have been cloned from rat (rbB-I)[137] or ($\alpha_{1B\text{-}I}$),[138] human ($\alpha_{1B\text{-}1}$, $\alpha_{1B\text{-}2}$),[139] and rabbit brain (BIII),[140] as well as from the forebrain of the marine ray *Discopyge ommata* (doe-4).[141] The various clones encode proteins of 2336 to 2339 amino acids with predicted molecular weights of ≈260 to 262 kDa.[142] The $\alpha_{1B}$ amino acid sequence is more similar to that of the $\alpha_{1A}$, with the majority of the sequence divergence occurring in the cytoplasmic loop between domains II and III and in the cytoplasmic carboxyl tail.

Initial indications that this class of $\alpha_1$ subunit corresponds to N-type channels came from the work of Dubel et al (1992)[137] who showed that a polyclonal antibody (CNB-1) raised against the II-III loop region of the rbB-I clone immunoprecipitated almost 50% of the high-affinity ω-CgTx binding sites, but none of the DHP-binding sites from rat brain. Furthermore, Northern blot analysis of experimental cell lines showed that rbB-I expression was correlated with the presence of N-type channels in nerve tissues and cell lines that express N-type channels.[137,139,140,143] Northern blotting and in situ immunohistochemistry experiments have localized the rbB-I transcripts to the cerebral cortex, hippocampus, forebrain, midbrain, cerebellum, and brainstem. At the subcellular level, rbB-I protein is found on dendrites, at presynaptic terminals and, to a lesser extent, neuronal cell bodies. The localization pattern of the $\alpha_{1B}$ compares well with that observed with a monoclonal antibody against ω-CgTx,[56] although the ω-CgTx antibody staining was more widely distributed.

Molecular cloning and biochemical studies have also provided evidence for the existence of multiple isoforms of the $\alpha_{1B}$ subunit.[138-140,143-146] At least two of these isoforms represent channels with differentially spliced carboxyl tails, and the inability of CNB-1 to immunoprecipitate all of the ω-CgTx binding sites might suggest the existence of additional isoforms with distinct II-III loop sequences. In addition, $\alpha_{1B}$ clones with small insertions and deletions scattered throughout the channel have been identified, and expression studies indicate that these sequence variations have a profound influence on the properties of the channel[132] (see below). These include a variant of the human N-type calcium channel that lacks the synaptic protein interaction site in the domain II-III linker[145] and can therefore not associate with synaptic proteins such as syntaxin 1A and SNAP-25.

Transient expression of both human $\alpha_{1B\text{-}1}$ in HEK cells[147] and rabbit brain BIII in dysgenic myotubes[140] produced HVA currents that first activated between -10 and -30 mV and reached a maximum between +10 and +30 mV. Currents partially inactivated over the time course of the depolarization and were sensitive to holding potential (showing 50% current inactivation at approximately -60 mV). At a holding potential of -40 mV, the bulk of the current (90%) was inhibited. In agreement with binding studies, the $\alpha_{1B}$-induced currents were irreversibly blocked by 1 μM ω-CgTx and were insensitive to DHPs.

The properties of currents generated in *Xenopus* oocytes by expression of the rbB-I clone agreed well with those seen with the $\alpha_{1B\text{-}1}$ and BIII clones in terms of pharmacological sensitivities and voltage-dependence of activation. However, there were some notable discrepancies in other properties. For example, the rbB-I channel was less sensitive to holding potential, and the rates of activation and inactivation of the rbB-I clone were markedly slower, resulting in significantly different current waveforms.[148] Co-expression of the $\beta_{1b}$ subunit shifted the voltage-dependence of inactivation to more negative potentials similar to those observed with the human and rabbit clones. The β subunit also increased the rate of activation such that the current attained peak magnitude in approximately 120 ms (compared to 150-250 ms for the rbB-I subunit alone), and increased the rate of inactivation of the rbB-I current. After 800 ms,

current through rbB-I alone had decreased by 15-20%, whereas co-expression of the β subunit resulted in a 65-70% reduction in peak current. Despite the rate increases produced by β subunit co-expression, these parameters remained dramatically different from those displayed by the other clones. The rate of activation of rbB-I (110 ms to peak) was still significantly slower than that of $\alpha_{1B\text{-}I}$ currents (10 ms). In addition, the $\alpha_{1B\text{-}I}$ clone showed biphasic inactivation; the first, rapid phase had a τ of 46-105 ms. The τ of the slow phase ranged between 291 and 453 ms. In contrast, decay of rbB-I currents was monophasic and much slower (τ= 700 ms).

Evidence obtained by Stea et al (1999)[138] from a second rat brain clone (rbB-II or $\alpha_{1B\text{-}II}$) found that the differences in channel kinetics were the result of small amino acid alterations that are most likely the product of alternative splicing and/or cDNA cloning artifacts. While $\alpha_{1B\text{-}II}$ differs from $\alpha_{1B\text{-}I}$ in four regions, they found that the substitution of a glycine for a glutamate in transmembrane segment IS3 was sufficient to speed the activation and inactivation kinetics. It has been noted that, while N-type channels are typically described as having fast kinetics, this is not always the case (see N-type channels, above). It may be that isoforms containing a glutamate in IS3, such as the rbB-I ($\alpha_{1B\text{-}I}$) clone may account for the slow and incomplete inactivation of N-type current that has been described in sympathetic neurons. As is the case with the $\alpha_{1A}$ gene, alternative splicing and differential subunit composition may combine to produce slight modifications in channel characteristics with tissue or developmental specificities. Just recently, a newly discovered tissue specific splice isoform variant of the N-type channel has been discovered in dorsal root ganglia (DRG).[149] This splice variant arising from the presence of exon 37a has the unique property of being expressed exclusively in nociceptive neurons of the DRG and ultimately may serve as a novel target for pain management.

### $\alpha_{1C}$/ $Ca_v$ 1.2

The first complete class C $\alpha_1$ ($\alpha_{1C}$) subunit to be cloned was isolated from cardiac muscle (CARD1).[110] The cardiac and skeletal muscle L-type VGCCs arise from separate genes and are approximately 66% identical at the amino acid level. Subsequently, $\alpha_{1C}$ clones were isolated from rabbit lung (pSCaL)[111] and rat aorta (990: VSM$\alpha_1$)[112] which shared 95% identity with the cardiac clone. Class C clones later isolated from rat (rbC-I, rbC-II)[150] and mouse (mbC)[151] brain are also more closely related to the cardiac and smooth muscle $\alpha_1$ subunits than to the skeletal muscle clone. The $\alpha_{1C}$ clones code for proteins of 2140 to 2171 amino acids with predicted molecular masses of 235 to 239 kDa. Antibodies directed against the II-III loop of the neuronal class C channel also identify a truncated form with an approximate mass of 195 kDa.[6]

The high degree of similarity amongst these proteins suggest that they are products of a single gene, and this is supported by genomic Southern blotting experiments. However, there are regions of considerable diversity in these clones which are the result of alternative splicing of the primary transcript.[150,152,153] pSCaL, the smooth muscle channel isolated by Biel et al (1990)[111] differs from the cardiac form in the amino terminus, the IS6 and IVS3 transmembrane segments, and by a 25-amino acid insertion in the I-II linker. In contrast, the VSM$\alpha_1$ clone, also isolated from smooth muscle, contains a cardiac channel-like IS6 segment and a 68 residue substitution in the carboxyl tail. This sequence is also found in the neuronal clones, rbC-I and –II.[150] Finally, rbC-I and rbC-II contain regions of identity with both the smooth muscle and cardiac clones, but also contain many substitutions, primarily in cytoplasmic regions of the protein and the IIIS2 transmembrane segment. Notably, many of these substitutions are localized to the cytoplasmic linker between domains II and III, and may reflect cell-specific functions of the channels. The truncated form of the neuronal protein may also be the result of alternative splicing, or may be due to post-translational processing as is the case with the skeletal muscle channel.[6]

As would be expected considering the diverse nature of the tissues from which class C cDNAs have been cloned, the $\alpha_{1C}$ gene has a widespread pattern of expression. Transcripts of 8.9 kb have been detected in heart.[110] In smooth muscle and brain, hybridizing transcripts were slightly smaller (8.6 and 8 kb, respectively).[112,150] Additional transcripts of 15.5 kb (cardiac) and 12 kb (smooth

muscle and brain) were also detected which were proposed to represent stable processing intermediates.[112] Northern blot analysis indicate that the class C gene is expressed in heart, smooth muscle (e.g., uterine, lung, stomach, and intestine), and throughout the CNS.[112,150,151] Within the brain, high expression levels are detected in the olfactory bulb, cerebellum, striatum, thalamus, hypothalamus and cortex, and at much lower levels in the pons/medulla and spinal cord.[6,150] Thus far, there is no evidence for exclusive expression of $\alpha_{1C}$ splice variants in specific tissues. cDNAs containing both variants of the IVS3 transmembrane segment have been isolated from heart, smooth muscle, and brain[112,150,152] and the alternate carboxyl tail is expressed in both smooth muscle and neuronal tissues.[112,150] However, a more detailed study of the expression pattern of class C variants in rat has revealed tissue-specific differences in expression of the rbC-I and rbC-II proteins.[150] Overall, rbC-II is the more abundant form, and generally more prevalent in any given tissue, although the relative amounts of the two transcripts vary between brain regions and tissue types.

The subcellular localization of class C $\alpha_1$ subunits was studied using the polyclonal antibody, CNC1.[6] Immunoprecipitation and Western blotting experiments indicated that class C $\alpha_1$ subunits comprise approximately 75% of the DHP binding sites in rat cerebral cortex and hippocampus. CNC1 immunoreactivity was distributed at low levels on cell bodies and proximal dendrites, with staining diminishing along the length of the dendrite. In addition, clusters of high levels of immunoreactivity were observed on the surface of cells (as opposed to representing a cytoplasmic pool of channels).

Expression of $\alpha_{1C}$ clones in *Xenopus* oocytes resulted in currents with electrophysiological and pharmacological properties characteristic of L-type channels.[110,111,115,154] In $Ba^{2+}$, depolarizations to -10 to -30 mV elicited large inward currents that inactivated slowly, if at all, over the course of a several hundred millisecond the test pulse. The currents peaked between +10 and +30 mV and were inhibited by $Cd^{2+}$ (100-200 μM) and were sensitive to DHPs. Like native L-type currents, the cloned L-type channels showed little sensitivity to holding potential. At holding potentials as high as -20 mV, half of the channels remained available for opening.

The class C channels were shown to be modulated by the auxiliary subunits in much the same manner as the class A and B channels. Co-expression of $\alpha_{1C}$ with $\beta_{1b}$ and $\alpha_2$-δ significantly increased the magnitude of the whole cell currents. This increase appeared to be mediated primarily through interaction with the β subunit, while the addition of $\alpha_2$-δ had a slight synergistic effect. In addition, co-expression of rbC-II with the auxiliary subunits $\beta_{1b}$ and/or $\alpha_2$-δ caused a small hyperpolarizing shift in the voltage dependence of activation of the channel and altered channel kinetics.[154] The rate of activation for rbC-II varied substantially among oocytes. Time constants of activation ranged between 4 and 50 ms with an average of approximately 10 ms. Coexpression with $\beta_{1b}$ and $\alpha_2$-δ both increased the rate of activation and reduced the degree of variability. Furthermore, the $\beta_{1b}$ and $\alpha_2$-δ subunits increased the rate of inactivation. Unlike the $\alpha_{1B}$ rbB-I channel (see above), neither auxiliary subunit had a significant effect on the voltage dependence of inactivation. With the exception of the voltage dependence of channel activation, the modulatory effects on the kinetics and voltage dependent parameters of the channel appear to be mediated primarily through interaction with the β subunit, while the addition of $\alpha_2$-δ had slight synergistic effects.

Whole cell recording using $Ca^{2+}$ as the charge carrier resulted in current traces with markedly different waveforms. The magnitude of the whole cell current was significantly smaller in $Ca^{2+}$ than $Ba^{2+}$, indicating that the channels are more permeable to $Ba^{2+}$ than to $Ca^{2+}$. In addition, instead of eliciting currents that are essentially non-inactivating, depolarizing pulses produce currents that decay rapidly by more than 50%.[154,155] The increase in inactivation seen with $Ca^{2+}$ as the permeant ion retains all the hallmarks of $Ca^{2+}$-dependent inactivation (see above).

Calcium dependent inactivation (CDI) is predominantly linked to two regions of the Alpha1 subunit C-terminus. The EF hand,[156] located from aa 1526 to1554, is responsible for CDI and may also regulate voltage-dependent inactivation (VDI).[157] The second region, (found downstream of the EF hand), is comprised of three distinct binding motifs. Peptide A;

(aa1588to1610) and Peptide C (1615to1636aa),[158] and IQ(1649to1669),[159] all work together to form a calcium bound Calmodulin binding site. In the absence of calcium, $Ca^{2+}$ free calmodulin (ie Apo Cam) is pre-associated with the channel at a site localized between the EF motif and IQ region.[23,160] Calcium entering through the channel binds to calmodulin, thus inducing a conformational change that relieves an inhibitory action of the calmodulin/C-terminus complex on the voltage-dependent inactivation machinery.[161]

### $\alpha_{1D}/Ca_v1.3$

A class of VGCC cDNAs sharing about 70% amino acid identity with the cardiac clones has been cloned from a variety of species including rat (RB$\alpha_1$;[162] rCACN4A, rCACN4B[163]), human ($\alpha_{1D}$;[51] neuroendocrine or β-cell $\alpha_1$ or hCACN4:[164] HCa3a[165]), and chicken.[166] A cDNA encoding an invertebrate ortholog of the class D $\alpha_1$ subunit has been isolated from *Drosophila melanogaster* (Dmca 1 D).[167] These clones retained little similarity (~40% amino acid identity) with the non-DHP-sensitive class A clones,[51,164] but were almost identical to the partial rat brain clone designated class D.[120] In spite of the sequence divergence between cDNAs generated from class C and class D genes, the two channel types are remarkably similar in certain regions. As with all VGCC $\alpha_1$ subunits discussed thus far, the transmembrane regions tend to be highly conserved, while the intracellular loop sequences are much more divergent. In addition to these regions, the $\alpha_{1D}$ clones are almost identical to the DHP-sensitive class C and S clones in the segments predicted to form the DHP and phenylalkylamine binding sites, suggesting that the class D $\alpha_1$ subunit cDNAs also encode members of the DHP-sensitive L-type family of VGCCs. The exception to this lies in the DHP-binding region in the *Drosophila* Dmca 1D clone which contains a number of non-conserved changes. This finding, however, is consistent with the pharmacology of phenylalkylamine and DHP binding in *Drosophila* head membranes,[167] and provides further support for the role of these regions in drug binding.

The cloned $\alpha_{1D}$ subunits range in size from the 1634-amino acid (187-kDa) rat brain isoform to the 2516-amino acid (276-kDa) *Drosophila* channel clone. The range in protein sizes is due primarily to the truncated carboxyl terminal ends of the RB$\alpha_1$, HCa3a, and rCACN4B clones.[162,163,165] The rCACN4B clone is a full 535 residues smaller than its rCACN4A counterpart and is proposed to result from the use of an alternative splice acceptor site.[163] In addition to the truncation of the carboxyl tail, a number of other regions have been identified in which variants have been produced through alternative splicing.[51,152,162,163] These regions include insertions in the cytoplasmic linker between domains I and II, the extracellular linker connecting IVS3 and IVS4, and the transmembrane segments IS6 and IVS3. In addition, Kollmar et al (1997)[166] have reported that the chicken brain and cochlear $\alpha_{1D}$ proteins differ in the IIIS2 segment and IVS2-IVS3 loops, as well as in the carboxyl tail. Presumably, these splice variants impart functional differences to the channel. While it is not yet clear what these functional differences may be, Ihara et al (1995)[163] note that RB$\alpha_1$, HCa3a and rCACN4B are all truncated at different sites. Furthermore, a number of potential PKA sites are eliminated by the truncations, which may result in the differential regulation of these isoforms by phosphorylation.

The class D channels, often termed "neuroendocrine" because of their presence in brain and pancreatic cells, have also been detected in the retina, ovaries, and cochlear hair cells, but not in heart, skeletal muscle, spleen, colon, or liver. Reports differ on whether $\alpha_{1D}$ transcripts are present in kidney.[164,166] Within the CNS, class D expression is found in the hippocampus, habenula, basal ganglia, and thalamus.[51] The subcellular localization of the class D $\alpha_1$ subunit was characterized using the polyclonal antisera anti-CND1.[6] Anti-CND1 was generated against a peptide homologous to the unique II-III loop of the rat brain $\alpha_{1D}$ clone.[162] (Hui et al, 1991). Class D channels appear to be far less abundant in the rat CNS than class C channels. The sera labeled the cell bodies and proximal dendrites of both projection neurons and interneurons throughout the brain. In contrast to the punctate staining pattern seen with the class C antibody, anti-CND1 staining was evenly distributed over the cell body. The staining pattern of anti-CND1 was typical for neurons in all regions of the CNS with the notable exception of the

cerebellar Purkinje cells. While the cell bodies of these neurons were labeled, there was a marked absence of staining on the Purkinje cell dendrites.

Transient expression of human $\alpha_{1D}$[51] in *Xenopus* oocytes and stable expression of the rat CACN4A and CACN4B clones (Ihara et al, 1995)[163] in CHO cells gives rise to DHP-sensitive currents, confirming the notion that class D channels are members of the L-type family. In both systems, functional expression of the $\alpha_{1D}$ subunit required co-expression of the $\beta$ subunit. In *Xenopus* oocytes, transient expression of $\alpha_{1D}$ with the $\beta$ and $\alpha_2$ subunits yielded larger currents than those produced by expression of $\alpha_{1D}$ plus $\beta$ alone.[51] $Ba^{2+}$ currents in oocytes expressing $\alpha_{1D}$+ $\beta$ + $\alpha_2$ first activated upon depolarizations positive to -30 mV and peak current attained with depolarizations to 0 mV, thus the current-voltage relationship of the $\alpha_{1D}$ is somewhat more negative than that of the $\alpha_{1C}$[154] (see above). $\alpha_{1D}$ channels activated rapidly and inactivated little over depolarizations lasting as long as 700 ms. $\alpha_{1D}$ channels inactivate to a considerably lesser degree over long test pulses than do $\alpha_{1C}$ channels.[154]

As indicated, class D channels fall under the heading of DHP-sensitive L-type channels. $Cd^{2+}$ produces substantial block, while $Ni^{2+}$ has a minimal effect on the current. The DHP agonist Bay K8644 increases current magnitude and shifts the voltage-dependence of activation by approximately -10 mV. In addition, the current is inhibited by the DHP antagonist nifedipine.[51,163] However, the affinity of DHPs for $\alpha_{1D}$ channels is generally lower than that observed with $\alpha_{1C}$.[168,169] Moreover, unlike other DHP-sensitive channels, the cloned $\alpha_{1D}$ is partially and reversibly blocked by high concentrations of $\omega$-CgTx (10-15 $\mu$M).[51]

The predominance of the $\alpha_{1D}$ subunit-containing VGCCs in the cochlear hair cells and in the $\beta$-cells of the pancreas suggest that these channels may be involved in tonic exocytotic release in these cells[163,164,166,170,171] Kollmar et al (1997)[166] suggest that the electrophysiological properties of the $\alpha_{1D}$ subunit, such as its lack of inactivation during depolarizations may render it suitable for mediating tonic release. In addition, as suggested by the localization of $\alpha_{1D}$ channels on the cell body and at the base of dendrites of neurons in the CNS, these channels may be involved in integrating signals impinging upon the neuron from multiple sources.[6]

### $\alpha_{1E}$/ $Ca_v$ 2.3

The class E gene encodes a VGCC $\alpha_1$ subunit ($\alpha_{1E}$) that does not fall neatly into either the HVA or LVA categories. $\alpha_{1E}$ cDNAs have been isolated from rabbit (BII-1, BII-2)[172] and rat (rbE-II)[121] brain, and from *Dyscopyge ommata.*[141] These clones code for proteins between 2178 and 2259 amino acids with predicted molecular masses of approximately 250 kDa. Splice variants of the rabbit brain channel, BII-1 and BII-2, differ from one another in their carboxyl tails, resulting in the addition of a putative PKA site.

The class E clones appear to be more closely related to the DHP-insensitive non-L-type channels (54-60% amino acid identity) than to the L-type channels (less than 45% similarity). However, class E channels are less similar to either class A or B channels than these two classes are to one another, suggesting that the class E channels are members of a novel, more distantly related subgroup of DHP-insensitive channel (Fig. 2).[121,141,172]

Northern blotting studies have identified transcripts ranging in size from 10.5 to 12 kb in the mammalian CNS.[121,172] High levels of expression was identified in the cerebral cortex, hippocampus, and striatum, while lower levels were detected in the olfactory bulb, midbrain, and Purkinje and granule cell layers of the cerebellum. While $\alpha_{1E}$ appears abundant in brain, none was detected in skeletal muscle, heart, stomach or kidney. At the subcellular level, $\alpha_{1E}$ protein was localized nearly exclusively to the cell body of neurons throughout the CNS. Dendritic staining varies across brain regions. For example, in the cortex and hippocampal formation there is barely perceptible staining of the dendritic branches, while in Purkinje cells, $\alpha_{1E}$ antibodies labeled the distal dendritic branches, but not the main dendritic trunks.[173]

The $\alpha_{1E}$ channel was initially reported to be a novel member of the LVA family of $Ca^{2+}$ channels.[121] Expression of rbE-II in *Xenopus* oocytes produced a channel that activated rapidly at low membrane potentials (threshold≈ -50 mV) and inactivated significantly during

the depolarization. Other voltage-dependent parameters of this channel (current-voltage relationship, voltage-dependence of inactivation) were also considerably more negative than those of other cloned HVA channels. The rbE-II current magnitude increased steeply with increasing depolarizations, peaking at around 10 mV, and steady state inactivation analysis indicated that the channels were inactivated near the resting membrane potential of the cell. In addition, rbE-II channels were equally permeable to $Ca^{2+}$ and $Ba^{2+}$, a property reported to be unique to the LVA channels. Another similarity with LVA channels was the high sensitivity of the current to block by Ni. Furthermore, the channel was found to be expressed in many of the cells that have been shown to possess T-type currents. However, Soong et al (1993)[121] noted a number of discrepancies between rbE-II and native T-type currents. For example, although the voltage-dependent properties of rbE-II currents were more negative than those of the other cloned HVA $Ca^{2+}$ channels, the activation and peak current potentials were not as hyperpolarized as for typical T-type channels.[121] Analysis of the electrophysiological properties of other class E channels[174-176] have produced some results that contradict those of Soong et al (1993).[121] In these studies, the $\alpha_{1E}$ clones formed HVA channels, activating at approximately -10 mV and peaking at +30 mV. The single channel conductance of $\alpha_{1E}$ channels is also much larger than that of T-type channels (12-14 pS vs. ~8 pS).[141,177] As $\alpha_{1E}$ channels share properties with LVA as well as HVA channels, the detection of pure $\alpha_{1E}$ currents in native cells may difficult.

It has been suggested that the class E channels may be one of a group of channels comprising the R-type current.[89,178,179] The two currents share some electrophysiological and pharmacological characteristics, such as strong voltage-dependence of activation and insensitivity to DHPs, ω-CgTx, and ω-Aga IVA. However, the R-type current is smaller in $Ca^{2+}$ than in $Ba^{2+}$, whereas the $\alpha_{1E}$ channels support the two currents equally.[89,177] Most relevant, mice lacking the $\alpha_{1E}$ gene entirely still exhibit significant R-type current.[180] Thus, while class E channels may comprise a component of R-type current in cerebellar granule cells, the R-type current may actually result from incomplete block of other $Ca^{2+}$ channels by applied pharmacological agents, the expression of additional splice variants of already identified $Ca^{2+}$ channel subtypes, or as yet to-be-identified $\alpha_1$ subunits.

### *$\alpha_{1F}$/ $Ca_v$ 1.4*

The human class F gene (CACNA1F) was identified through genetic studies in which the X-linked visual disorder Congenital Stationary Night Blindness (CSNB) was mapped to a locus containing a putative VGCC gene.[122,123] The predicted CACNA1F gene product ($\alpha_{1F}$) is between 1912 and 1966 amino acids (alternatively spliced forms have been detected) with an estimated molecular mass of 219 kDa. Sequence analysis indicates that $\alpha_{1F}$ is 55-70% identical at the amino acid level to the L-type channel $\alpha_1$ subunits, sharing the most similarity with $\alpha_{1D}$, and 35% identical to the P- and N-type channels. In addition, the putative DHP-binding domains in IIIS6 and IVS6 appear relatively well conserved. These results suggest that the $\alpha_{1F}$ is an L-type channel that diverged from the $\alpha_{1D}$ subunit gene.[122]

$\alpha_{1F}$ expression was initially reported to be restricted to the retina in situ hybridization experiments indicate high levels of $\alpha_{1F}$ transcript in the two retinal layers containing the photoreceptors, and horizontal, bipolar, and amacrine cells, but not the ganglion-cell layers,[122,123] however, recent reports indicate a more global distribution that includes the immune system and skeletal muscle.[181] L-type $Ca^{2+}$ channels have been implicated in synaptic release from photoreceptors[182] and the correlation of the hereditary visual disorder CSNB with mutations in the $\alpha_{1F}$ gene[122,123] suggests that the $\alpha_{1F}$ channel mediates neurotransmitter release at these synapses. Functional expression of $\alpha_{1F}$ calcium channels reveals that these channel encode a non-inactivating L-type calcium channel that is DHP sensitive,[183] and which is not regulated by ancillary subunits.[181] Moreover the channel appears to lack CDI, and displays a large window current, thus making this channel ideally suited to support tonic glutamate release from photoreceptors.[181]

## Low Voltage-Activated (T-Type) Channels

LVA (T-type) channels were first described in rat and chick sensory neurons,[1,184] but also are present in other excitable tissues, including cardiac sinoatrial cells, smooth and developing skeletal muscle, neuroendocrine cells, and thalamic neurons, as well as non-excitable cells such as fibroblasts, osteoblasts, and astrocytes.[2,4,185] Other cell types, such as sympathetic neurons, superior cervical ganglion cells, and adrenal chromaffin cells, appear not to express significant T-type currents.[2,186] T-type $Ca^{2+}$ channels typically first activate at potentials more positive to -70 mV and whole-cell currents are usually maximal by ~ -40 mV. T-type $Ca^{2+}$ channels are fully inactivated at resting potentials greater than -40 mV, inactivate rapidly in a voltage-dependent manner, and deactivate, or close, relatively slowly. Because these channels are inactivated at positive holding potentials, very negative holding potentials (-80 mV or more negative) are required for full availability of the channels. The kinetics of activation and inactivation of T-type channels also display voltage dependency; rates are slow near threshold potentials and accelerate with increasing potentials.[1,24]

While direct evidence linking T-type channels to specific physiological roles is limited, their electrophysiological profiles and cellular and subcellular localizations suggest a number of likely functions. For example, their expression in many cell types that display spontaneous electrical activity (sinoatrial nodal cells of the heart, neuroendocrine cells, and thalamic neurons) together with their low threshold of activation and requirement for hyperpolarized membrane potentials to overcome inactivation, suggests that T-type channels play a role in pacemaker activity and bursting behavior. T-type channels may also exert an effect by generating a resting inward current which could in turn mediate the gating of $Ca^{2+}$-dependent ion channels and regulate $Ca^{2+}$-dependent enzymes and gene expression. Finally, T-type currents are highly expressed in developing muscle and nervous tissue, suggesting that these channels may play a developmental role.[2,187-189]

The study of T-type $Ca^{2+}$ channels has lagged behind that of other $Ca^{2+}$ channel subtypes, in part due to the lack of cDNA clones representative of this type (see below), but primarily because of the lack of selective pharmacological agents. T-type channels are generally sensitive to the divalent cations nickel (Ni), cadmium ($Cd^{2+}$), and zinc ($Zn^{2+}$), with $Ni^{2+}$ being the most potent. However, in some cell types, low concentrations of these cations fail to block LVA currents or also block other HVA $Ca^{2+}$ currents.[190,191] A number of organic compounds inhibit T-type channels, but often at concentrations that block other $Ca^{2+}$ channels. For example, octanol and the sodium (Na) channel blocker amiloride have been utilized as T-type channel antagonists, although these compounds also inhibit some components of whole cell HVA currents.[2,4,186,190,191] Ethosuximide, a drug used to treat absence epilepsy, has been shown to reduce current through T-type channels with little effect on HVA channels although the concentrations required for complete T-type block are quite high.[186,190] The antihypertensive mibefridil may be the most potent T-type channel blocker identified to date (IC50 in the submicromolar range)[191] although even mibefradil however has recently proven to be a relatively non-specific $Ca^{2+}$ channel blocker.[192]

### $\alpha_{1G}$, $\alpha_{1H}$ and $\alpha_{1I}$

Low stringency library screening strategies such as the ones used to isolate the HVA channels discussed above proved unsuccessful for cloning the T-type $Ca^{2+}$ channels. The first members of the T-type $Ca^{2+}$ channels were identified by screening data banks for sequences with similarity to previously cloned $Ca^{2+}$ channels.[124,193,200] Subsequently, other classes of T-type $Ca^{2+}$ channels were identified by screening cDNA libraries.[125,126,194]

Thus far, T-type clones have been isolated from rat ($\alpha_{1G}$;[124] $\alpha_{1G}$, $\alpha_{1H}$, $\alpha_{1I}$;[200] $\alpha_{1I}$[126]), mouse ($\alpha_{1G}$[193]), human brain ($\alpha_{1G}$, $\alpha_{1I}$),[194-196] and human heart ($\alpha_{1H}$).[125] The $\alpha_{1G}$ and $\alpha_{1H}$ subunits are approximately 65% identical, whereas the $\alpha_{1I}$ subunit shares only 53% identity with the $\alpha_{1H}$ and 47% with the $\alpha_{1G}$. As expected from the failure to identify T-type channels in the low stringency hybridization screens used to isolate many of the HVA channels,

the T-type channels share limited sequence homology with HVA $Ca^{2+}$ channels. The highest level of sequence similarity is found in the four membrane-spanning domains. Most of the amino acid changes in these regions are conservative, thereby maintaining structural elements common to voltage-gated ion channels. The charges located in the fourth transmembrane segment of each domain are conserved, as are the pore-forming loops between the fifth and sixth transmembrane segments. In HVA channels, a glutamate residue located in each of these four loops is believed to determine the ion selectivity of the channels.[197,198] All T-type channels cloned thus far contain aspartate residues instead of glutamates in the domain III and IV P-regions.[124] This difference may account for the difference in the permeation properties seen between high and low voltage activated channels. The intra- and extracellular linkers joining the transmembrane domains share little homology with either HVA channels or with other T-type channels. Furthermore, the T-type channels do not seem to possess specific functional motifs that have been identified in HVA channels, including the binding site in the I-II linker or the putative EF-hand motif in the carboxyl tail.[124]

The three classes of T-type channel have been localized using Northern blotting, in situ hybridization, and RT-PCR techniques.[124-126,193,195,200,201] The $\alpha_{1G}$ subunit appears to be expressed abundantly throughout the brain and to a lesser degree in heart. Low levels have also been detected in placenta, lung, and kidney. High levels of transcript are observed in the cerebellum, hippocampus, thalamus, and olfactory bulb, with lesser amounts localized to the cerebral cortex and septal nuclei. Initially, the $\alpha_{1H}$ was detected only in cardiac tissue, kidney, and liver, with very little, if any, expression in the brain.[125] However, a subsequent study[199] suggests that the $\alpha_{1H}$ subunit may be responsible for a large proportion of the T-type current in sensory neurons, and another study indicates the expression of $\alpha_{1H}$ in all areas in the rat brain.[201] $\alpha_{1I}$ transcripts have only been detected in brain,[126,195] with one study showing specific expression in the striatum of adult rats.[200]

Expression of these three subunits in *Xenopus* oocytes[124,126] and HEK-293 cells[125,193,195,196,200-203] demonstrates that they support currents with most of the characteristics expected of T-type channels. Currents activated upon weak depolarizations from negative holding potentials. In one study, the three T-type channel classes had differing permeability properties.[200] As has been noted for classic T-type currents, $\alpha_{1G}$ channel was more permeable to Ca than to Ba. However, $\alpha_{1H}$ channels were more permeable to $Ba^{2+}$ than $Ca^{2+}$, while $\alpha_{1I}$ channels were equally permeable to the two ions. In most cases, currents were inhibited by mibefradil and Ni, although the $IC_{50}$ of each T-type class varied significantly.[193,200] The activation and inactivation kinetics of the T-type channels are strongly voltage-dependent. While rates of activation and inactivation are slow near threshold potentials, they accelerate as the strength of depolarization increases. Deactivation is also voltage-dependent, increasing at more hyperpolarized potentials. Steady-state inactivation analysis indicates that the majority of the channel population would be inactivated at the resting potential of most cells. However, because all the channels are not inactivated at the resting potential and the threshold of activation is so negative, a small proportion of channels are capable of opening at the resting potential, thus producing a "window" current. The window current refers to a small, but sustained influx of $Ca^{2+}$ that occurs even when the cell is ostensibly at rest. This current can contribute to the overall excitability of the membrane and may contribute to the bursting and pacemaker activities attributed to the T-type channels. Finally, as anticipated for T-type channels, the three exogenously expressed channels have small single channel conductances of 5 ($\alpha_{1H}$), 7.5 ($\alpha_{1G}$), and 11($\alpha_{1I}$) pS.[126]

Similar to the different classes of channels within the HVA subfamily, the biophysical properties of the three T-type channels vary considerably.[126,200] The $\alpha_{1G}$ and $\alpha_{1H}$ possess very similar activation and inactivation potentials, while those of the $\alpha_{1H}$ appear to be slightly more negative. Rates of activation and inactivation of $\alpha_{1G}$ and $\alpha_{1H}$ currents also are quite similar. In contrast, activation and inactivation rates for $\alpha_{1I}$ currents are significantly slower. In addition, the activation threshold of $\alpha_{1I}$ channels also differs from the values obtained for $\alpha_{1G}$ and $\alpha_{1H}$ channels. However, varying results have been reported concerning the direction of the observed voltage

shift. The rat brain $\alpha_{1I}$ clone studied by J.-H. Lee et al (1999)[126] activated at more positive potentials than did the $\alpha_{1G}$ and $\alpha_{1H}$ channels, while McRory et al (1999)[200] reported $\alpha_{1I}$ current activation at considerably more negative potentials. That the properties of the $\alpha_{1I}$ channel differ from those of the $\alpha_{1G}$ and $\alpha_{1H}$ is not entirely unexpected when one takes into account the degrees of similarity seen amongst the three channels. Furthermore, multiple splice variants of the $\alpha_{1I}$ have been identified[194,195] and may account for the contrasting results reported for $\alpha_{1I}$ currents. Finally, the properties of the three LVA channel clones do not account for all of the T-type characteristics in native cells and there may be additional classes of T-type channels and/or a set of as yet unknown auxiliary subunits specific to LVA channels which further modulate the properties of the LVA $\alpha_1$ subunit. For example, robust alternate splicing for $\alpha_{1G}$ channels had been reported and shown to result in significantly altered biophysical characteristics.[203]

## Auxiliary $Ca^{2+}$ Channel Subunits

Biochemical studies have shown that in addition to the pore-forming $\alpha_1$ subunit, HVA $Ca^{2+}$ channel complexes include two or three other proteins: a $\beta$ subunit, an $\alpha_2$-$\delta$ subunit, and in some cases, a $\gamma$ subunit (Fig. 1).

### *$\beta$ Subunits*

The $\beta$ subunit is the most extensively studied of the auxiliary subunits and appears to have the most profound effects on the functional properties of the $\alpha_1$ subunit. In mammals, there are at least four different $\beta$ subunits ($\beta_1$, $\beta_2$, $\beta_3$, and $\beta_4$) which are encoded by distinct genes. The transcripts of at elast two of these genes, the $\beta_1$ and $\beta_2$, are alternatively spliced to give rise to $\beta_{1a}$ $\beta_{1b}$, and $\beta_{1c}$ and $\beta_{2a}$ and $\beta_{2b}$.

Biochemical and primary sequence analyses indicate that the $\beta$ subunits are hydrophilic with no transmembrane segments or glycosylation sites.[204-210] The $\beta$ subunits contain potential phosphorylation sites for both protein kinase C and cAMP- dependent protein kinase. The modulatory effects of these enzymes on VGCC function may, in part, be the result of their actions on this auxiliary subunit.[211]

Although the specific effects of channel modulation depend upon the $\beta$ subunit isoform, all $\beta$ subunits appear to have the same general impact on the properties of HVA $\alpha_1$ subunits. Coexpression of the $\alpha_1$ and $\beta$ subunits in both L-cells and *Xenopus* oocytes increased whole-cell currents and DHP binding without affecting the level of $\alpha_1$ message. This suggests that rather than enhancing expression of the $\alpha_1$ subunit, the $\beta$ subunit may promote insertion of the $\alpha_1$ subunit into the membrane and/or stabilize a specific conformation of the protein[212-215] have proposed that the $\beta$ subunit potentiates coupling of the gating-charge movement caused by changes in membrane potential with the opening of the pore thereby increasing the probability of channel activation and, in turn, increasing the peak current.

In addition to increasing the magnitude of the current through the $\alpha_1$ subunit, co-expression of the $\beta$ subunit alters channel kinetics. For most $\beta$ subunits, the rate of inactivation is increased and there is a shift in the voltage-dependence of activation to more negative potentials.[148,154,207,209,216-219] The effect on kinetics of inactivation, however, varies depending upon the class of $\beta$ subunit expressed. The $\beta_1$ and $\beta_3$ proteins increase the rate on inactivation, while the $\beta_2$ subunit significantly slows inactivation.

These modulatory effects are observed regardless of which $\alpha_1$ and $\beta$ subunits are co-expressed, suggesting that the mechanism through which the $\beta$ subunit acts is common to all HVA VGCCs. The region required for $\beta$ subunit modulation of the $\alpha_1$ subunit has been localized to a stretch of 30 amino acids at the amino-terminal side of the second of two conserved domains.[220] This region, known as the BID ($\beta$ subunit interaction domain) is also responsible for anchoring the $\beta$ subunit to the $\alpha_1$. The $\beta$ subunit has been shown to bind to a conserved motif of 18 amino acids in the intracellular loop between domains I and II of the $\alpha_1$ subunit (the AID: $\alpha_1$ subunit interaction domain).[221] The observation that the $\beta$ subunit from skeletal muscle dramatically increases the magnitude of the current through brain $\alpha_1$ subunits when co-expressed in *Xenopus* oocytes[128] further supports the idea of a common mechanism of $\alpha_1$- $\beta$ subunit interaction.

The β subunits exhibit homology with the Src homology 3-guanylate kinase domain of membrane associated guanylate kinases and this region appears to regulate inactivation of HVA VGCCs.[289] A more detailed account of β subunit physiology is provided in the Chapter from the Charnet lab.

## *$\alpha_2$-δ Subunits*

Purification studies indicate that the VGCC $\alpha_2$-δ subunit consists of two distinct subunits ($\alpha_2$ and δ that are disulfide-bonded in the native state.[8,222] The $\alpha_2$ and δ subunits are derived from a single gene product that is proteolytically cleaved to form 143-kDa $\alpha_2$ and 27-kDa δ subunits.[223] There are currently four genes that code for the $\alpha_2$-δ family; $\alpha_2$-$\delta_1$, $\alpha_2$-$\delta_2$, $\alpha_2$-$\delta_3$, and $\alpha_2$-$\delta_4$.

Both proteins are heavily glycosylated,[224] supporting the prediction that the $\alpha_2$-δ subunit is predominantly extracellular and a recent study has determined that no more than five residues comprise the cytoplasmic portion of the protein.[225] The complex is anchored in the membrane by a single transmembrane segment formed by a portion of the δ subunit. The transmembrane domain is thought to interact with other subunit(s) in the VGCC complex while the extracellular domain is responsible for the modulatory effects.[8,142,223-225]

There are several splice variants of the $\alpha_2$-$\delta_1$ auxiliary subunit, due primarily to the alternative splicing of three specific regions. The total splicing combination of these three regions reveals 5 unique isoforms which are all expressed in a tissue specific manner.[226] Skeletal muscle and brain express the isoforms $\alpha_2$-$\delta_{1A}$ and $\alpha_2$-$\delta_{1B}$ respectively. The heart expresses mainly $\alpha_2$-$\delta_{1C}$ and $\alpha_2$-$\delta_{1D}$, and smooth muscle expresses $\alpha_2$-$\delta_{1D}$ and $\alpha_2$-$\delta_{1E}$. Interestingly the cardiovascular system expresses all five isoforms.[227]

The $\alpha_2$-$\delta_2$ subunit is expressed in many different tissues including heart, brain, pancreas, testis lung, liver, kidney.[227] The $\alpha_2$-$\delta_2$ subunit has two regions of alternative splicing. The first region is found on the $\alpha_2$ subunit between residues 661/663 and involves the addition of eight amino acid residues. The second is located on the δ protein and is characterized by the addition of three different residues.[228] The resulting three isoforms of the $\alpha_2$-$\delta_2$ subunit are all expressed in hMTC (human medullary thyroid cells.) Alternately, in the human heart, only $\alpha_2$-$\delta_{2A}$ is expressed. cDNA cloning of the $\alpha_2$-$\delta_3$ subunit did not uncover additional splice variants, and this particular $\alpha_2$-δ gene appears to be expressed exclusively in the brain.

In 2002, a new member of the $\alpha_2$-δ auxiliary subunit family was described. $\alpha_2$-$\delta_4$ demonstrated a unique property in that it was shown to be expressed primarily in endocrine tissues. Immunohistochemical studies revealed that the $\alpha_2$-$\delta_4$ subunit has limited distribution in special cell types of the pituitary, adrenal gland, colon, and fetal liver.[229] Whether the $a_2$-$d_4$ subunit plays a physiological role in certain endocrine tissues remains to be seen. Alternative splicing of the $\alpha_2$-$\delta_4$ gene gives rise to four potential variants (called a-d.[229]

The functional effects of the $\alpha_2$-δ are thought to be more subtle that those of the β subunit and are highly dependent on the class of $\alpha_1$ subunit and the cell type used for exogenous expression. For example, Singer et al (1991)[217] found that the $Ca^{2+}$ current in *Xenopus* oocytes expressing the cardiac $\alpha_{1C}$ protein was greatly enhanced by co-expression of the $\alpha_2$-δ subunit from skeletal muscle. In addition, the rates of activation and inactivation were increased and the voltage dependence of inactivation was shifted to more negative potentials. In contrast, Varadi et al (1991)[218] did not observe these effects when they co-expressed the skeletal muscle $\alpha_1$ subunit with the $\alpha_2$-δ subunit in L cells.[230] Unlike *Xenopus* oocytes, L cells do not express endogenous calcium channel subunits.[219] Thus, it is possible that the $\alpha_2$-δ subunit acts synergistically with other auxiliary subunits to modulate the properties of the $\alpha_1$. Finally, there is some evidence that the $\alpha_2$-δ subunit is required for efficient expression and/or trafficking of the $\alpha_1$ subunit to the cell membrane,[110] an idea that is supported by recent studies in tsA-201 cells showing that $\alpha_2$-δ subunits are key modulators of current densities of $\alpha_{1C}$, $\alpha_{1B}$ and $\alpha_{1E}$ channels.[231]

## *γ Subunits*

### $\gamma_1$ (Cacng1)

The VGCC $\gamma_1$ subunit protein was first identified in guinea pig skeletal muscle during the purification of 1,4-dihydrpyridine receptors. This VGCC heteromultimeric protein consisted of five different subunits. The $\gamma_1$ subunit, a 28-35 kDa on SDS-Page[232] proved to be one of them. Several years later Jay et al (1990)[233] isolated rabbit skeletal muscle cDNA and uncovered the primary sequence the $\gamma_1$ subunit. The cDNA was a 666-nucleotide clone, with a reading frame that would yield a 222 amino acid glycoprotein containing four transmembrane domains. $\gamma_1$ has been shown to be expressed primarily in skeletal muscle,[234] but recently has also been shown to be weakly expressed in the aorta.[235] Mice lacking the $\gamma_1$ subunit display altered skeletal muscle calcium current. Functional effects of the $\gamma_1$ protein include a hyperpolarized shift in the steady state inactivation properties in skeletal muscleVGCC.[236,237]

### Stargazin or $\gamma_2$ (Cacng2)

For nearly a decade the presence of other γ–like subunits remained undetected. It was the discovery of the mouse stargazin gene Cacng2 by Letts et al (1998)[238] that ultimately led to the description of seven new γ subunits. The stargazin gene and protein were named after the Stargazer mouse, a mouse strain that is prone to absence seizures including an upward neck tilt and prolonged gaze. These mice had acquired a transposon in a 1.5kb region on chromosome 15 and were heterozygous recessive.[238,239] The stargazin gene, renamed $\gamma_2$, had inherited a stop codon rendering the protein inactive and truncated. The etiological consequences of a mutated Cacng gene responsible for the absence epilepsy phenotype of the allelic stargazer (stg) and waggler (wag) mutant mice.[238,240]

$\gamma_2$, has been classified as a γ subunit based on its structural similarity to $\gamma_1$, despite having only weak protein sequence identity (25%).[238] At the tissue expression level, unlike $\gamma_1$, the $\gamma_2$ subunits are found to be expressed in the brain. Letts et al (1998)[238] found mouse $\gamma_2$ mRNA to be expressed in adult mouse brain abundantly with highest expression in cerebellum, olfactory bulb, cerebral cortex, thalamus, and CA3 and dentate gyrus regions of the hippocampus.

### $\gamma_3$/$\gamma_4$ (Cacng3/4)

Despite the low sequence homology of $\gamma_2$ with its original $\gamma_1$ counterpart, many of the newly discovered γ subunits demonstrate high similarity with the $\gamma_2$ subunit. $\gamma_2$, $\gamma_3$ and $\gamma_4$ are of closest similarity and make up a subfamily of neuronal γ subunits. Klugbauer et al (2000)[241] used Northern blots to show that $\gamma_3$ and $\gamma_4$ expressions were exclusively restricted to the brain. However, Chu et al (2001)[235] found $\gamma_4$ to be additionally present in the atrium, aorta, and lung.

### $\gamma_5$ (Cacng5)

The $\gamma_5$ subunit is unique in that it is expressed not only in the brain and skeletal muscle, but also in different types of endocrine tissues, primarily the liver, kidney, heart, lung and testes. There is some disagreement on the categorization of this subunit as a bona fide γ subunit, and it is also referred to as the "pr protein". In exogenous expression experiments the mouse pr protein has been shown to the modulate properties of the $\alpha_{1G}$ T-type channel.[241]

### $\gamma_6$, $\gamma_7$, $\gamma_8$

In 2001 Chu et al[235] described three new human and rat γ–like subunits (human $\gamma_6$, $\gamma_7$, and $\gamma_8$). $\gamma_6$ was found in the atrium, ventricle, skeletal muscle and a short splice variant of its kind was found in atrium, ventricle, aorta, brain, and lung. $\gamma_7$ is expressed in all tissues except aorta, kidney, liver, and spleen. $\gamma_8$ was found only in brain and testis.

In summary, the eight γ subunits are technically considered to be part of the Claudin family of proteins, and are differentially expressed among a variety of different tissues. They all display four membrane-spanning regions with both their C and N termini located intracellularly, while

***Table 2. Spontaneous mutations in calcium channel subunit genes***

| Gene | Channel Subunit | Disease State/Phenotype | Reference |
|---|---|---|---|
| CACNA1A | $\alpha_{1A}$ | Human: episodic ataxia type 2, familial hemiplegic migraine, spinal cerebellar ataxia type 6<br>Mouse: tottering (tg), leaner ($tg^{la}$) | 240, 250-257 |
| CACNA1S | $\alpha_{1S}$ | Human: hypokalemic periodic paralysis, malignant hypothermia susceptibility type 1<br>Mouse: muscular dysgenesis (mdg) | 258-260 |
| CACNA1H | $\alpha_{1H}$ | Human: childhood absence epilepsy; idiopathtic generalized epilepsy | 261, 262, 288 |
| CACNA1F | $\alpha_{1F}$ | Human: Incomplete X-linked congenital stationary night blindness | 123, 181, 122 |
| CACNA2D | $\alpha_2$-$\delta_2$ | Mouse: ducky (du), ataxia, paroxysmal dyskinesia | 263-265 |
| CACNB4 | $\beta_4$ | Mouse: lethargic (lh), ataxia, focal seizures | 266 |
| CACNG2 | $\gamma_2$ | Mouse: stargazer (stg), waggler $stg^{wag}$, ataxia, spike wave seizures | 240, 267 |

all extracellular regions display N-glycosylation sites. All the Cacng genes have four exons (with the exception of Canga5 and 7 which have five exons),[242] and exon1 is predominately the largest. The carboxyl terminus is of particular interest given that $\gamma_2$, $\gamma_3$, $\gamma_4$ and $\gamma_8$ have a PDZ binding motif in this region.[243,244] The C-termini of the $\gamma_7$ and $\gamma_5$ are longer and lack this consensus motif, however it is interesting to note that they have inherited an SS/TSPC site, probably designated for protein interactions.[235] Of particular note, stargazin and other member of the $\gamma$ subunit family ($\gamma$3, $\gamma$4 and $\gamma$8) have recently been shown to define a novel family of transmembrane AMPA receptor regulatory proteins (TARPs).[290] The TARPs appear to regulate two distinct roles in AMPA receptor signaling: trafficking of AMPA receptors to the plasma membrane and an agonist-mediated dynamic interaction that may contribute to synaptic plasticity. [291]

## Effects of $\gamma$ Subunits on Channel Biophysics

Over the years, many different combinations of all of the hetermultimer subunits with different accompanying $\gamma$ subunits have been tested. Wei et al (1991)[219] co-expressed rabbit Cardiac $\alpha_{1C}$ with $\gamma_1$, and observed increased peak currents. Letts et al, (1998)[238] demonstrated the modulatory effects of neuronal $\gamma_2$, by co-expressing the subunit with $\alpha_{1A}$-$\beta_1$-$\alpha_2$-$\delta$. The effects of $\gamma_2$ included a hyperpolarizing shift of the steady state inactivation properties of the channel. Similar results were obtained by Klugbauer et al (2002)[245] with $\alpha_{1G}$ and $\alpha_{1C}$. The same hyperpolarizing effects on channel SSI properties were observed with $\gamma_4$, but were not with $\gamma_5$. It is interesting to note that $\gamma_2$ shifted the activation potential to a more depolarized level, when co-transfected with $\alpha_{1A}$-$\beta_1$-a2-d heteromultimers, while $\gamma_3$, $\gamma_4$, and $\gamma_5$ did not.

Rousset el al (2001)[246] studied the electrophysiological properties of $\alpha_{1A}$- $\alpha_2$-$\delta$ channels expressed in Xenopus oocytes in the presence and absence of various $\gamma$ subunits (i.e., $\gamma_{1,2,3,}$ or $_4$), and found that $\gamma_2$ and $\gamma_3$ induced a small negative shift of the inactivation curve and an acceleration of inactivation kinetics. Green et al (2001)[247] studied the functional effects of $\gamma_2$, $\gamma_3$, and $\gamma_4$ on $\alpha_{1I}$ T-type channels in HEK293 cells. Their results revealed a significant slowing of deactivation with $\gamma_2$ and a slight but insignificant increase in peak calcium current. $\gamma_2$ was further explored by Kang et al (2001).[248] These authors reported that $\gamma_2$ decreased current amplitude of $\alpha_{1B}$ and $\alpha_{1A}$ calcium channels when co-expressed with $\beta_3$-$\alpha_2$-$\delta$ subunits. These inhibitory effects were dependent upon the presence of the $\alpha_2$-$\delta$ subunit. Both $\gamma_1$ and $\gamma_2$ slowed the activation kinetics of $\alpha_{1B}$.

***Table 3. Induced mutations in calcium channel subunit genes***

| Gene | Channel Subunit | Disease State/Phenotype | Reference |
|---|---|---|---|
| CACNA1A | $\alpha_{1A}$ ($Ca_v$ 2.1) | Mouse: targeted gene deletion – die at ~ three weeks postnatal | 268 |
| CACNA1B | $\alpha_{1B}$ ($Ca_v$ 2.2) | Mouse: targeted gene deletion – viable, reduced ability to induce neuropathic & inflammatory pain | 269-272 |
| CACNA1E | $\alpha_{1E}$ ($Ca_v$ 2.3) | Mouse: targeted gene deletion – viable, altered sensitivity to pain | 273 |
| CACNA1F | $\alpha_{1F}$ ($Ca_v$ 1.4) | Mouse: targeted gene deletion – viable, blind | 274 |
| CACNA1D | $\alpha_{1D}$ ($Ca_v$ 1.3) | Mouse: targeted gene deletion – viable, deaf, cardiac arrhythmias | 170 |
| CACNA1G | $\alpha_{1G}$ ($Ca_v$ 3.1) | Mouse: targeted gene deletion – viable, reduced spike and wave discharges | 275, 276 |
| CACNA1H | $\alpha_{1H}$ ($Ca_v$ 3.2) | Mouse: targeted gene deletion – viable, impaired relaxation of vasculature | 277 |
| CACNA1C | $\alpha_{1C}$ ($Ca_v$ 1.2) | Mouse: targeted gene deletion – die at about day 12 pc, no heartbeat after day 14 pc | 278 |
| CACNA1S | $\alpha_{1S}$ ($Ca_v$ 1.1) | Mouse: targeted gene deletion – non viable, die at birth due to inability to contract diaphragm | 279 |
| CACNB1 | $\beta_1$ | Mouse: targeted gene deletion - perinatal lethality, no E-C coupling | 280, 281 |
| CACNB2 | $\beta_2$ | Mouse: targeted gene deletion - embryonic lethal | 282 |
| CACNB3 | $\beta_3$ | Mouse: targeted gene deletion - no visible phenotype, viable | 283, 284 |
| CACNG1 | $\gamma_1$ | Mouse: targeted gene deletion - no visible phenotype, viable | 236 |
| *unc*-2 | $\alpha_1$ | *C. elegans*: multiple alleles - mild to severe movement defects, egg laying constitutive, defecation defects, thin | 285, 287 |
| *egl*-19 | $\alpha_1$ | *C. elegans*: multiple alleles - null allele is embryonic lethal; reduction of function mutants are egg-laying defective, slow, flaccid | 286 |
| *unc*-36 | $\alpha_2\delta$ | *C. elegans*: multiple alleles - very slow movement, almost paralyzed, thin | 287 |

Recently, Moss et al (2002 – EMBO J)[249] showed that when $\alpha_7$ was co-expressed transiently in either Xenopus oocytes or COS-7 cells, N-type current was completely abolished. This effect appears to be mediated by blocking expression rather than interfering with trafficking or the biophysical properties of the channel.

## Summary

From the initial identification of native $Ca^{2+}$ channel subtypes, tremendous progress has been made in our understanding of the molecular biology and physiology of voltage-gated

$Ca^{2+}$ channels, including the cloning and expression of representatives of all known $Ca^{2+}$ channel types, the elucidation of their tissue distribution, and an in depth understanding of their structure and function. Moreover, we have come to understand the pathophysiology of $Ca^{2+}$ channels through naturally occurring channelopathies in humans and mice (see Table 2), and through targeted gene deletions (see Table 3). The ensuing chapters in this book will provide additional details of our understanding of voltage-gated $Ca^{2+}$ channels.

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# CHAPTER 6

# Role of β Subunits in Voltage-Gated Calcium Channel Functions

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## Abstract

$Ca^{2+}$ channel auxiliary subunits have been suspected to play fundamental roles in channel function from their early biochemical characterization (see Chapter 4 ). Their strong association with the main $\alpha_1$ subunits and their role in the reconstitution of proper channel activity, were strong evidences for key functions. Later, the discovery of a whole family of β subunit genes, now counting four members, with regions of high similarity including channel binding sites as well as more ubiquitous protein-protein interaction domains, and expression studies challenging different combinations of calcium channel subunits, confirmed their modulatory functions in every aspect of channel activity, from expression and targeting to regulation by G-proteins. The discovery of neuropathologies linked to genetic defects in the β subunit gene constitutes one more argument for assigning to this auxiliary subunit a central role in channel function and regulation.

## Isolation, Characterization and Cloning

A so-called β ($Ca_v\beta$) subunit of molecular mass of 55 kDa was first isolated as one of the main subunits associated with the dihydropyridine receptor purified from rabbit skeletal muscle.[1-8] This subunit was later shown to be necessary for proper channel function, but also to be a substrate for various protein kinases. In 1989, Ruth et al,[9] using peptide sequences derived from the purified proteins, were the first to report the complete cDNA and deduced amino-acid sequences of this skeletal muscle β subunit (now called $\beta_{1a}$). The 1835 nucleotides sequence encoded a protein of 524 amino-acids of predicted molecular mass of 57.8 kDa. This protein lacked a hydrophobic sequence signal as well as putative transmembrane domains, but displayed 4 major α helical regions, each containing a similar stretch of eight residues with negative charges (Fig. 1) and assumed to play a role in divalent cation binding.[9] Thus the $\beta_{1a}$ subunit was associated to the membrane only through its strong interaction with the α1 pore-forming subunit. PKA, PKC, PKG and casein kinase phosphorylation sites were also found, in accordance with in vitro phosphorylation studies. Tissue distribution and northern-blot analysis of the mRNA encoding this subunit however, soon suggested the existence of splice variants as well as other homologous, but not identical, β subunits.

Soon after, a novel β subunit cDNA ($\beta_{1b}$) was isolated from a rat brain cDNA library using a nucleotide probe derived from the skeletal muscle β subunit sequence.[10] This 597 amino acid subunit showed a high homology with $\beta_{1a}$ (82%), but with divergence in the central and C-terminal regions. Genomic analysis identified a third $\beta_1$ subunit and demonstrated that these three subunits derived from the same gene through alternative splicing.[11] While the $\beta_{1a}$ subunit was exclusively expressed in skeletal muscle (with possible species differences[12]), the two other-splice variants were found in brain, heart and spleen.[13] Four alternatively spliced

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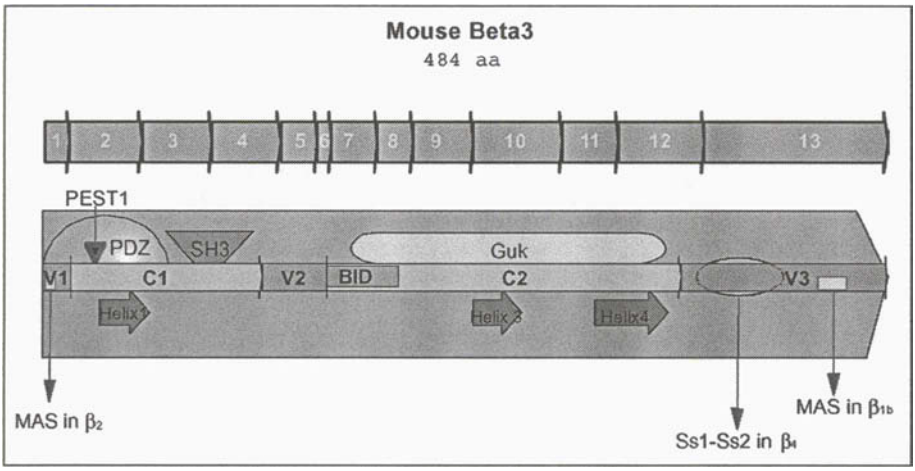



Figure 1. A functional map of the β ($Ca_v\beta$) subunit. Schematic representation of experimentally-identified or putative functional sites localized within the primary structure of the β subunit. The localization of the 13 exons of the $\beta_3$ subunit is shown on the top, and the sites identified in the four β subunits have all been placed on the homologous region of the $\beta_3$ subunit. C1, C2: regions of high homology between subunits V1, V2, V3: variable regions MAS, PEST, PDZ, SH3, BID, GuK, Helix, Ss1, Ss2 see text for details.

variants of a second β subunit gene ($\beta_{2a}$, $\beta_{2b}$, $\beta_{2c}$ and $\beta_{2d}$) were then isolated from a rabbit heart library,[14] which display ~70% homology with the $\beta_{1a}$ subunit. These variants vary in size from 566 ($\beta_{2c}$) to 632 amino acids ($\beta_{2b}$), with splicing occurring in the central and amino-terminal regions of the protein.[14] Putative phosphorylation sites for PKA, PKC and casein kinase were also found.[15] This subunit is expressed in heart, aorta, brain, lung and trachea.[16]

Two new β subunit genes, predominantly expressed in brain, but also in other tissues, were then identified from a rat brain cDNA library: $\beta_3$[15] and $\beta_4$.[17] Again, they showed a high percentage of homology (> 70%) with the two other β subunits in two conserved regions, the N and C-termini and a central domain being more variable (see ref. 18 for review). Three of the four α helical domains (with their stretch of negatively charged residues), and one PEST domain (a proline-rich region proposed to be implicated in protease sensitivity[9]), found in the $\beta_{1a}$ subunit are conserved.[17] These proteins, of 484 and 519 amino-acids respectively, possess several potential phosphorylation sites for PKC and casein kinase II, but as opposed to $\beta_1$ and $\beta_2$, no consensus sequence for cAMP-dependent protein kinase A. An ATP/GTP binding site (motif A) is also found specifically on the $\beta_4$ subunit.[17] Structure prediction studies have also identified a PDZ, an SH3 and a guanylate kinase-related potential domain[19] in all four β subunits (see Fig. 1). A similar arrangement is also found in the members of the membrane-associated guanylate kinase (MAGUK) family, known to be implicated in ion channel and receptors clustering.[20] Although the roles of these domains are not yet known, they may participate to specific protein-protein interactions.

The chromosomal location places the $\beta_1$, $\beta_2$, $\beta_3$ and the $\beta_4$ subunit genes on human chromosomes 17q11-q22, 10p12, 12q13 and 2q22-q23 respectively.[21-23] Analysis of the genomic sequence of the β subunit gene reveals an organization highly conserved through species (rat, rabbit, mouse and human) and through β subunit genes.[24] The $\beta_3$ subunit gene spreads over 8kb and 13 exons of various sizes, from 20 base-pairs to 2.1 kb. The N- and C-termini and the central variable domain of the β subunit are contained in single exons: 1, 13 and 5, respectively, subject to alternative splicing (Fig. 1, refs. 24,25). The two conserved domains however, are split in several exons. Exons 5, 6[24] and 7[26] can be skipped, leading to a shift in the reading frame and a premature stop codon. It should be noted that short forms of the $\beta_1$, $\beta_2$, $\beta_3$ and $\beta_4$

subunits, without the Beta-Interaction Domain (BID, see below), have also been identified, without assignment of a specific functional role.[26,27]

## Association with $Ca^{2+}$ Channels

The co-purification of the $Ca^{2+}$ channel β subunit with the pore-forming $\alpha_1$ subunit, as well as their functional effects on channel properties, strongly suggested a direct association between the α1 and the β subunits. Indeed a short sequence of 30 amino acids (Beta Interaction Domain, or BID), located in the amino terminal end of the second conserved domain of all β subunits (V2, Fig. 1), was found to interact with the AID (Alpha Interaction Domain) an 18 amino acids segment present on the I-II loop of all high-threshold α1 subunits.[28] This interaction is necessary and sufficient to produce most of the functional effects of the β subunit (see below) and does not appear to be sensitive to pH, phosphorylation, ionic strength or $Ca^{2+}$ concentration.[29] Despite a high percentage of homology between the BID of the four β subunits (> 85%), each β subunit displays a distinct apparent affinity for the AID of the $\alpha_{1A}$ subunit, with the following rank order $\beta_4 > \beta_2 > \beta_1 >> \beta_3$ (Kd from 3 to 55nM). This strong interaction has been shown to be potentially reversible, either in vitro, through the use of synthetic competitive peptides mimicking the AID sequence,[30] or in vivo, thereby regulating most of the processes which depend on this α1/β association such as activation, inactivation, and regulation by G-proteins.[31] These results are consistent with recent evidence suggesting a decrease in the affinity of the AID/$Ca_v$β interaction[32] following insertion of the $\alpha_1$ subunit in the polarized plasma membrane. It should be noted, that neither AID-like sequences, nor modifications in channel properties upon expression of β subunits, are observed in low threshold $Ca^{2+}$ channels in heterologous systems or in neurons.[33,34]

After initial unsuccessful attempts to characterize other sites of interaction between these α1 and β $Ca^{2+}$ channel subunits,[35] Walker and collaborators[36] identified two overlapping sequences in the C-terminal tail of the $\beta_4$ subunit that specifically interact, in a competitive manner, with the C- and N-terminal ends of some variants of the $\alpha_{1A}$ ($Ca_v2.1$) $Ca^{2+}$ channel subunit (see also ref. 37). These sequences, called Ss1 and Ss2, could be the molecular support for some of the specific effects recorded with the β4 subunit. These sites are of low affinity (Kd>90nM), and can take place only when the primary AID/$Ca_v$β interaction is present. While the N-terminal interaction site (Ss1) has been precisely located on the $\beta_4$ subunit (aa 446-482), the Ss2 site appears to rely on multiple microdomains within the complex tertiary structure of the C-terminal tail of the $\beta_4$ subunit.[36] Similar interactions, albeit weaker, are also found between the C-tail of the $\beta_{2a}$ subunit and the $\alpha_{1A}$ and $\alpha_{1E}$ ($Ca_v2.3$) subunits. [38] These secondary interactions are involved in the precise tuning of the voltage-dependent activation (for Ss2) and inactivation (for Ss1) of the $\alpha_{1A}$ subunit and may play a role in G-protein dependent regulation. There is still some controversy as to whether these interacting sequences form 3 spatially separate $\alpha_1$/β binding sites or cooperate in a large interacting surface.

Post-translational modifications of the β subunits, other than phosphorylation, have only been described for specific variants of the $\beta_2$ subunit, where palmitoylation occurs on two cysteines at positions 3 and 4.[39] This palmitoylation site is required to anchor the $\beta_{2a}$ subunit to the membrane without the necessity of the $\alpha_1$ subunit (Fig. 4).[40-43] Three positive charges, located just downstream of cysteines 3 and 4, participate to the translocation of the palmitoylation site to the membrane; possibly via an initial electrostatic interaction with the polar groups of the phospholipids, as already suggested for G-proteins.[44] Unaltered SH3 and BID domains (see Fig. 1) are also necessary for this membrane localization of the $\beta_2$ subunit.[42,45] Such anchoring of the $\beta_{2a}$ subunit has important consequences on $Ca^{2+}$ channel inactivation and facilitation[43] (see Figs. 1, 4 and text below), possibly via interaction with the N-terminus of the $\alpha_1$ subunit.[46] Another membrane anchoring site has also been found on the C-terminus of the $\beta_{1b}$ subunit,[47] although in this case, anchoring occurs without palmitoylation.

However, despite this important amount of knowledge on the properties of the $\alpha_1$/β subunit interaction, little is known about their physiological association and the actual pairs of $\alpha_1$/β

***Table 1. Example of preferential association between $\alpha_1$ and $\beta$ subunits in different tissues***

| Tissue | $\alpha_1/\beta$ combinations |
|---|---|
| Skeletal muscle | $\alpha_{1S}$ - $\beta_{1a}$ |
| Cardiac muscle | $\alpha_{1C}$ - $\beta_{2c}$ |
| Brain | $\alpha_{1B}$ - $\beta_3$ |
| Cerebellum | $\alpha_{1A}$ - $\beta_4$ , $\alpha_{1A}$ - $\beta_2$ |

subunits existing in vivo. In some cases only, where either the quantity of material, and/or the limited numbers of different subunits expressed are particularly favorable, these combinations have been resolved and they suggest that, even if one type of $\alpha_1$ subunit can be purified with different types of $\beta$ subunits,[48] preferential associations do exist (see Table 1) that could be regulated during development.[49]

Altogether, the existence of conserved and specific sites of interaction between the $\alpha_1$ and $\beta$ subunits fits well with the report of generic and specific functional effects on $Ca^{2+}$ channel properties.

## Role in Channel Expression and Targeting

A role for the $\beta$ subunit in channel trafficking was first suspected by Perez-Reyes et al[14] on the basis of an increase in both, the number of DHP binding sites and the expressed current, upon co-expression of the $\alpha_{1C}$ ($Ca_v1.2$) and $\beta_2$ subunits. Despite an early controversy suggesting that $\beta$ subunits were only increasing the effective coupling between gating-charge displacement and $\alpha_1$ $Ca^{2+}$ channel opening,[50] this involvement of $\beta$ subunits in $\alpha_1$ subunit expression and/or trafficking to the membrane is now widely supported by experimental evidence and has been found for all four $\beta$ subunits. A 2 to 100 fold increase in both $Ca^{2+}$ current and amount of surface membrane toxin binding-sites was found upon co-expression of the $\beta$ subunit with the $\alpha_{1A}$, $\alpha_{1B}$($Ca_v2.2$), $\alpha_{1C}$, $\alpha_{1D}$($Ca_v1.3$), $\alpha_{1S}$($Ca_v1.1$), and $\alpha_{1E}$ subunits in L cells, CHO, COS, HEK293 cells and Xenopus oocytes[14,51-58] illustrating the conservation of the stimulatory mechanim. This effect is potentiated by co-expression of the $\alpha2$–$\delta$ and $\gamma$ subunits[52,56,59] (see Chapter 7) and clearly establishes a role for $\beta$ subunits in channel surface expression. The increase in toxin binding sites, current gating-charge and current amplitude seems to be the result of a combination of an increase in channel gating, channel routing to the membrane, and protein stabilization, since immunocytochemistry, biochemistry, electrophysiology, and toxin binding all bring arguments in favor of one or several of these hypotheses.[50,58,60] No changes in single channel conductance, or protein expression, however, were detected.[50,53,58,60] Interestingly, in the flatworm schistosomes, the same AID/($Ca_v\beta$) interaction induced a reduction in current amplitude, and conferred to the channel a new sensitivity to the antiparasitic drug Praziquantel.[61] This differential action has been tantalizingly assigned to modifications of the schistosome $\beta$ subunit BID.[61]

In fact, the increase in plasma membrane localization of $\alpha_{1C}$ recorded upon co-expression of the $\beta_{2a}$ subunit arises through a direct inter-subunit association,[41] probably in the endoplasmic reticulum,[62-64] and a chaperone-like mechanism. This effect is also found, albeit with different efficacy, with the non-plasma membrane-associated, non-palmitoylated $\beta_1$, $\beta_3$ and $\beta_4$ subunits,[45,65] conferring upon palmitoylation of $\beta_2$ another role besides targeting the $\alpha_1$ subunit to the membrane. Direct injection of $\beta$ subunit proteins into $\alpha_{1C}$ expressing Xenopus oocytes, reproduced both the alteration of the properties of the $Ca^{2+}$ channel and also the chaperoning of the $\alpha_1$ subunit, the latter effect only, being sensitive to inhibitors of intracellular protein transport.[62] These two regulatory roles thus represent two separate functions of the $\beta$ subunit, involving different structures, protein-interactions and mechanisms,[63,64] as suggested by the existence of secondary interaction sites.

Recently, an interesting mechanism has been proposed for the role of the β subunit in channel expression. In this scheme, an ER retention signal is present in the I-II loop of the α1 subunit, close to the AID. This signal is suspected to severely limit plasma membrane insertion of the channel, in the absence of an expressed β subunit. Expression and rapid binding of the β subunit to the AID will favor masking of the ER retention sequence, thereby decreasing its efficacy,[66] and allowing channel trafficking. Regulation of the $\alpha_1/\beta$ interaction is thus of primary importance for channel expression, and not surprisingly, has been shown to be under the control of other intracellular partners. Kir/GEM, a member of the Ras-related small G-protein family, can bind $Ca^{2+}$/calmodulin, GTP, phosphate and $Ca^{2+}$ channel β subunits.[67] The association of Kir/GEM with $Ca^{2+}$/calmodulin induces a re-localization of the complex from the nucleus to the cytoplasm, where the release of calmodulin will permit GTP-induced activation of Kir/GEM and association with the $Ca^{2+}$ channel β subunit. The consequent destabilization of the $\alpha_1/\beta$ subunit association reverses the inhibition of the ER retention signal, and restricts cell surface expression of the $\alpha_1$ subunit (see Fig. 2).[67] The long-term regulation of $\alpha_1$ subunit trafficking by Kir/GEM small GTPases is observed with all four β subunits.

More specific and compartmentalized trafficking of the $\alpha_1$ subunits, can also be induced by β subunits as discovered using polarized cell systems, where the $\alpha_{1A}$ subunit can be targeted to different plasma membrane areas depending on the β subunit expressed.[47,68] This suggests the existence of other specialized interactions and partners that remain to be discovered. The concentration dependence of the effects of the β subunits on channel targeting and channel properties differs in one order of magnitude (17 nM, versus 120 nM, respectively).[32] This difference in potency is suspected to arise from modifications in the affinity of the AID for the β subunit, a consequence of the polarization of the channel environment, when the $\alpha_1$ subunit becomes inserted in the plasma membrane.[32] Such a modification fits well with the recent finding of the existence of channels composed of $\alpha_1$ subunits alone, even in conditions where β subunits are also expressed.[31]

## Role in Channel Properties: Activation, Inactivation and Facilitation

Expression of the pore forming α1 subunit alone, in an expression system devoid of any noticeable amount of the other regulatory subunits (α2–δ, β or γ) is sufficient to promote the apparition of voltage-gated $Ca^{2+}$ current.[54,69] However, the voltage-dependence and kinetics of both activation and inactivation, of the currents expressed do not fit with those of the native currents, such as can be recorded in skeletal muscle, cardiac myocytes or neurons.[69,70] Normalization of these parameters can be obtained by co-expression of a regulatory β subunit. Co-expression of any β subunit with any $\alpha_1$ subunit (except low-threshold T-type $Ca^{2+}$ channel $\alpha_1$ subunits) can promote acceleration of the activation kinetics and hyperpolarization of the current-voltage curve (10-15 mV),[71-73] suggesting that, in addition to regulating channel expression and trafficking, β subunits also play an important modulatory role in channel gating. This effect is found with $\beta_1$, $\beta_2$, $\beta_3$ and $\beta_4$ on L ($Ca_v1.1$, $Ca_v1.2$ and $Ca_v1.3$),[15,52,72,74,75] as well as non-L type $Ca^{2+}$ channel $\alpha_1$ subunits ($Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$)[51,70,73,76-78] and in different expression systems (L-cells, HEK293, Xenopus oocytes...). It is worth noting that for a same combination of subunits, or for combinations using the same β subunit, slight differences in the reported functional effects may exist between work performed in different laboratories. Although these differences may reflect truly different modes of regulation, they could also be the result of different experimental procedures (concentration and nature of the permeant ion used, temperature, channel protein processing by host cells, nature of the channel isoform used, contamination by endogenous conductances, or tonic G-protein regulation which is known to be influenced by the channel composition...see above).

A careful study of both ionic current and gating charge movement associated with pore opening demonstrated that the β subunit only slightly modified the voltage dependence, the effective valence or the kinetics of charge movement,[79] but increased the total gating charge movement.[50,76,80] Therefore the modifications in voltage dependence and kinetics of $Ca^{2+}$ current

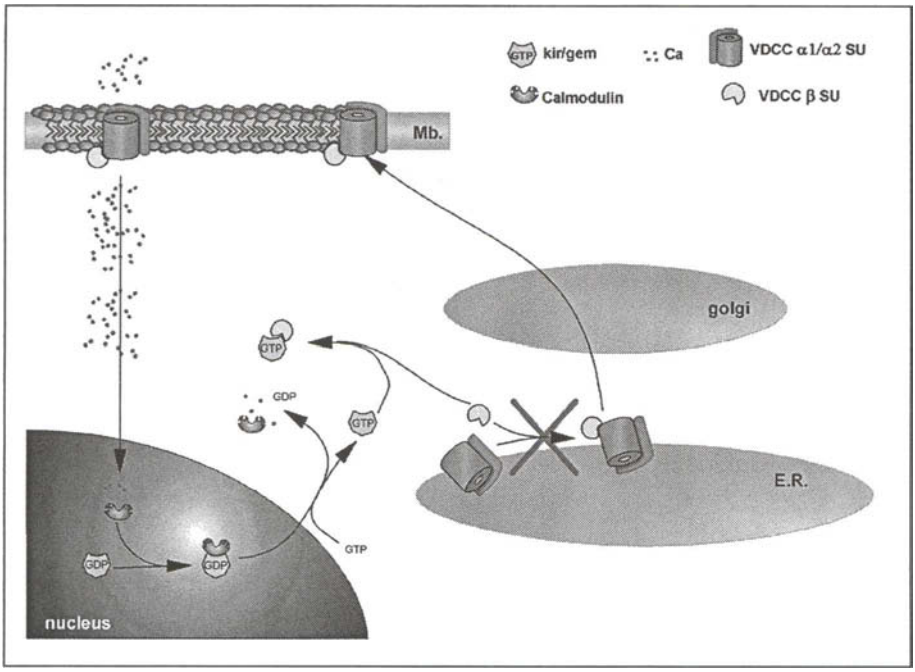



Figure 2. Proposed mechanism for β subunit and Kir/Gem action on channel expression.

activation induced by β subunits must take place after the gating charges have moved, and probably reflect a decrease in the energy barrier of the last, weakly voltage-dependent, closed-to-open transition, i.e., a better coupling between the voltage-sensor and the opening of the channel, as illustrated by both, the increase in the ratio of current over total charge movement and the smaller shift between the gating- and current-voltage curves.[50,76,79] A similar shift and acceleration of activation is observed with all four β subunits, and may depend on the sole AID/BID, or at the least, on the I-II loop/β C2 domain interaction.[73,81-83] However, variations of this modulation can be obtained by using different splice variants of the same β subunit, suggesting that additional N- and C-terminal sequences are also implicated in setting the voltage-dependent gating parameters.[25,36,84]

A negative shift in the voltage dependence of inactivation is also recorded when the $\beta_1$, $\beta_3$ or $\beta_4$ subunits are co-expressed with $Ca_v1.2$,[52] $Ca_v2.1$,[73,85,86] $Ca_v2.2$ [83,87] or $Ca_v2.3$ (Fig. 3).[76,88] In most cases, the $\beta_2$ subunit has been reported to modulate inactivation in the opposite direction (i.e., a positive shift). It should be noted that the magnitude of the shift can vary with the $\alpha_1$ and β subunits expressed,[73] but also, in a β-specific manner, with the nature and the concentration of the permeant ion (Ba, Sr, Ca at 1.8-40 mM).[85,89] Hence in different experimental conditions, a given β subunit can displace the inactivation curve in the opposite direction, thus explaining part of the contradictory results reported in the literature. The regulation of inactivation occurs independently of the regulation of channel activation by the β subunit,[81] which suggests the existence of β subunit-specific modifications of the transmembrane electric- field in the vicinity of the voltage-sensor. The displacement of the inactivation curve is accompanied by drastic, and β-subunit specific, changes in current inactivation kinetics,[73,76,81,82,90,91] with faster inactivation rates recorded with $\beta_{1b}$, $\beta_3$ or $\beta_4$ subunits versus slower inactivation observed with $\beta_{2a}$ (see Fig. 3). These specific modifications are conserved not only between $\alpha_1$ subunits but also between voltage- or $Ca^{2+}$-dependent inactivation,[16,82,92] demonstrating the conservation of the molecular mechanisms by which β subunits regulate channel inactivation. An ultra-slow component of inactiva-

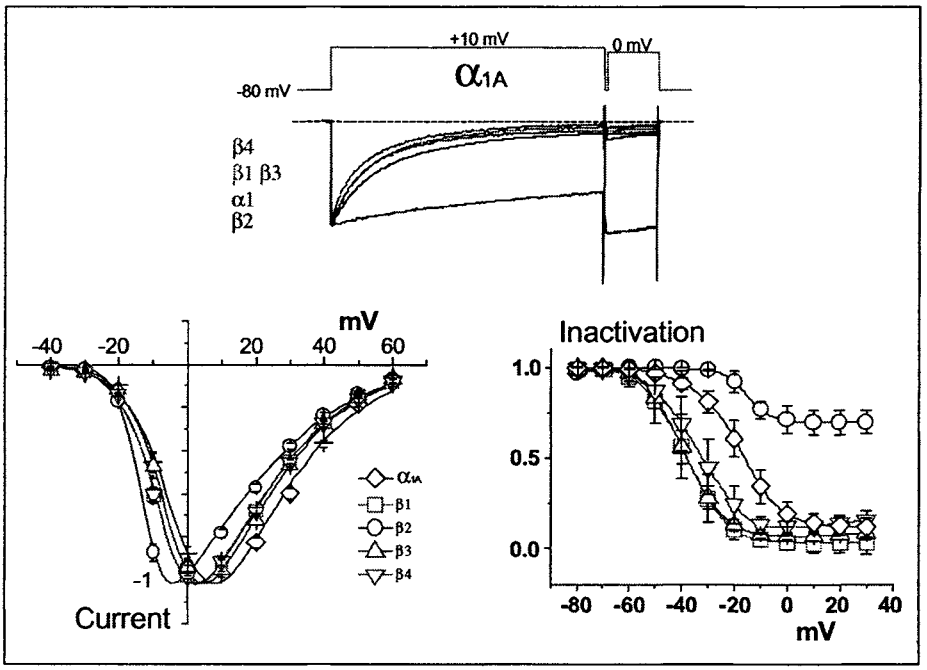



Figure 3. Effect of β subunits on channel activation and inactivation. Top) Scaled traces recorded from Xenopus oocytes expressing the $\alpha_{1A}$ subunit in combination with different β subunits. Currents were recorded in the presence of 10 mM Ba in response to the protocol shown on the top. Note the marked slowing of current decay induced by expression of the $\beta_2$ subunit. Bottom-left) Current-voltage relationship of $Ca^{2+}$ channels composed of the $\alpha_{1A}$ subunit alone, or co-expressed with the $\beta_1$, $\beta_2$, $\beta_3$ or $\beta_4$ subunits. Recordings were performed in the presence of $Ba^{2+}$ 10 mM. Bottom-right) Steady-state inactivation curves recorded with the same subunit combinations. Note the positive shift, and the decreased inactivation obtained upon expression of the $\beta_2$ subunit.

tion, present in $Ca_v1.2$ and $Ca_v2.1$ channels, has also been recently shown to be under the indirect control of the β subunit. [93] In fact, the ultra-slow mode of inactivation is more likely to occur from the open conformation of the channel, thus being promoted by β subunits that delay the fast inactivation (such as $\beta_{2a}$).[93] Fast and slow inactivation induced by β subunits are under the control of at least 3 different sequences:

1. the C2 domain, which is required for the attachment of the β subunit to the channel,
2. a palmitoylation site, only present at the very N-terminus of some variants of the $\beta_2$ subunit.[39,42] This site, also called Membrane Anchoring Site (MAS, Fig. 1)[43] is responsible for the slow inactivation produced by the $\beta_{2a}$ subunit[81,94] through its interaction with the membrane, probably by immobilization of the I-II loop of the channel (Fig. 4)[43] and
3. an internal sequence in the V2 region of the β subunit (Fig. 1).[95] This site can also induce the slow inactivation mode, but only when encoded by a long alternatively spliced form and when MAS is deleted.[95] This region might therefore play a regulatory role only under physiological conditions where the MAS site is either proteolysed or depalmitoylated as recently suggested.[96] Such situation may directly influence neurotransmitter release and neuronal excitability.

Finally it should also be noted that the recovery from inactivation is under the influence of the β subunit too. In this case acceleration or slowing of recovery can be recorded depending only on the type of $\alpha_1$ subunit, all four β subunits behaving qualitatively in a similar way.[97]

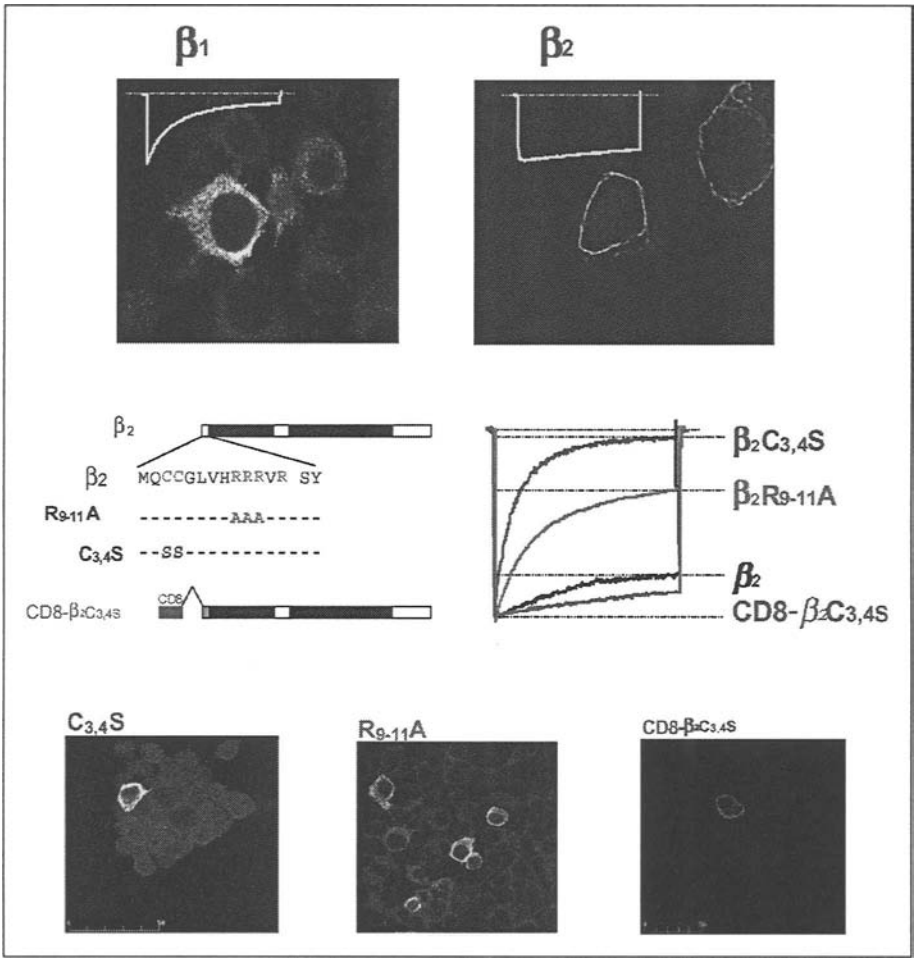



Figure 4. A proposed mechanism for the $\beta_2$ subunit-induced Ca channel slow inactivation. Top) Traces showing the slow inactivation induced by expression of the $\beta_2$ subunit and its membrane localization when it is expressed alone in tsA-201 cells (right). Fast inactivation and cytoplasmic localization are obtained with the $\beta_1$ subunit expressed in the same conditions (current recorded in Xenopus oocytes with the $\alpha_{1A}$ subunit). Middle and Bottom) Mutations in the membrane anchoring site of the $\beta_2$ subunit (either the Cys 3 and 4, where palmitoylation occurs, or Arg9-11, that helps membrane insertion) attenuate both slowing of inactivation and membrane localization. Chimeric constructs that restore the membrane localization via addition of a transmembrane segment (CD8), restore also the slow inactivation (adapted from ref. 43, see text for details).

In addition to these classical effects on $Ca^{2+}$ channel activation and inactivation, β subunits are also involved in more dynamic regulations of the $Ca^{2+}$ influx occurring during sustained activity. A reversible enhancement of $Ca^{2+}$ channel activity, also called $Ca^{2+}$ channel facilitation, can be recorded following strong depolarizations, or short trains of activity.[98] Such facilitation has been recorded in different tissues including muscle (cardiac or skeletal), chromaffin cells and neurons,[99-103] and has important consequences on cellular $Ca^{2+}$ homeostasis, contraction, secretion, or neurotransmitter release. In these cases, are grouped under the term facilitation, different types of $Ca^{2+}$ current enhancements that are characterized by specific

modulations by voltage, frequency of stimulation, phosphorylation, permeant ions and/or $Ca^{2+}$ channel subunit composition.[99,103-109] In the case of the cardiac/neuronal L-type $Ca_v1.2$ $Ca^{2+}$ channel subunit, short conditioning depolarizations of moderate amplitude (50-100mV) produce a 100 to 300% increase in $Ca^{2+}$ or $Ba^{2+}$ current amplitude that most probably reflects a switch of the channel to a willing mode of high activity,[104] lasting several seconds to minutes and involving a phosphorylation step that has not been precisely characterized.[107,110-112] Reproduction of such depolarizations in Xenopus oocytes, expressing the $Ca_v1.2$ $\alpha_1$ subunit with various combinations of regulatory subunits has underlined the permissive role of the β subunit,[94,107] at least for some splice variants of the $\alpha_{1C}$ subunit.[110,111] The neuronal $\beta_{2a}$ subunit, plays here again a particular role, since it inhibits the normal development of facilitation.[113] This property has allowed, via the construction of mutations and chimera with permissive β subunits, the characterization of the permissive role of the C2 domain, conserved in all β subunits, and the inhibitory role of the two palmitoylated cysteines located at the N-terminus of the $\beta_{2a}$ subunit.[94,113,114] Indeed, the cardiac $\beta_{2c}$ subunit, devoid of these two cysteines, is permissive for facilitation.[16,94,111] The involvement of multiple domains of the $\alpha_1$ subunit,[115] and the capacity of the α2–δ subunit to occlude facilitation by driving the channel into a conformational state where openings occur easily even without prepulse,[111,116] suggest that the molecular mechanism underlying L-type $Ca^{2+}$ channel facilitation is not restricted to the $\alpha_1/\beta$ interaction.

## Role in Channel Regulation by Heterotrimeric G-Proteins

The use of heterologous expression systems, where specified $Ca^{2+}$ channel subunits, seven-helix transmembrane receptors and G proteins can be expressed in the absence of other contaminating conductances, has been a key element in the analysis of the role of the β subunits in this type of regulation. Fast, membrane-delimited, G-protein inhibition appears to be mainly restricted to presynaptic $Ca^{2+}$ channels ($Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$) and involves the G-proteins sensitive to pertussis toxin, either Gi or Go.[117,118] This inhibition of $Ca^{2+}$ influx is mediated by a 1:1 interaction between the βγ subunit of the G-protein and the channel $\alpha_1$ subunit, favoring the transition of the channel from a "willing" to a "reluctant state", characterized by slow activation and fewer channel openings.[118-122] The opposite transition can be favored by strong membrane depolarizations, which are proposed to induce a rapid (time constant in the order of 2-5 ms) unbinding of the βγ dimer from the channel. Thus cellular activity may play an important modulatory role in this type of regulation. The direct binding of Gβγ, and the subsequent functional effects, rely primarily on the I-II loop of the sensitive $\alpha_1$ subunit, and comprise two sites that overlap with the AID, one of which contains a QXXR motif previously identified as a Gβγ binding-site on adenylyl cyclase.[120,123-126] Such results suggest a regulatory role for the $Ca^{2+}$ channel β subunit, an hypothesis that was previously suspected based on the enhancement of G-protein regulation following injection of ansisense oligonucleotides against β subunits in neurons. It was proposed at the time, and later confirmed using isolated neurons or expression systems, that $Ca^{2+}$ channel β subunits and G protein βγ subunits can compete for two overlapping sites and produce, via an allosteric mechanism, antagonistic functional effects.[119,120,127-129] Interestingly, expression of $Ca^{2+}$ channel β subunits reduces both, the tonic and the receptor stimulated, G-protein dependent inhibition,[127,128,130] at least after expression in Xenopus oocytes, in a $Ca^{2+}$ channel $\alpha_1$ and β and G-protein βγ subunit specific manner.[130-132] In addition, the $Ca^{2+}$ channel β subunit, by allowing PKC-dependent regulation,[133] introduces an additional level of regulation via the negative coupling of G-protein regulation to PKC modulation (the so-called cross-talk).[124,134] The PKC phosphorylation site, located close to the BID/Gβγ subunits binding site, operates as a molecular switch, triggering both PKC up-regulation and inhibition of the G-protein block.[135,136]

Several explanations have been put forward to explain the antagonistic properties of the $Ca^{2+}$ channel β subunit, including an increased rate and hyperpolarization of the voltage-dependent reversal of G-βγ inhibition,[132,137] and/or a β-subunit dependent facilitation

of the GTPase activity of the G-protein.[138] Moreover, other interaction sites between the $\alpha_1$ and G$\beta\gamma$ subunits have been found in the N and C-termini of the channel, two regions already known to interact with some $\beta$ subunits.[46,130,136,139-142] G$\beta\gamma$ binding to the C-terminus of the $\alpha_1$ sensitive subunits can be prevented by prior interaction with the C2 domain of the $\beta_{2a}$ subunit, (that contains the BID),[141] further supporting the notion of antagonism between G$\beta\gamma$ and $Ca^{2+}$ channel $\beta$ subunits. Whether these additional sites form a separate binding site or participate, with the I-II loop, to the formation of a unique, larger pocket, accommodating $Ca^{2+}$ channel $\beta$ or G-protein $\beta\gamma$ subunits is not yet known, but they surely influence G-protein inhibition of the channel.[130,139,141]

The key question of the simultaneous presence of the G$\beta\gamma$ and the calcium channel $\beta$ subunits on the channel, has been addressed by different group, but remains partly unanswered. Indeed, by recording changes in Fluorescence Resonance Energy Transfert between the $\alpha$1 and the $\beta$ subunits during G-protein activation, evidences have been found for either a stronger association or a complete dissociation of the $\beta$ subunit from the channel[143,144] when G$\beta\gamma$ is bound.

Direct implication of the $\beta$ subunit in the regulation of $Ca^{2+}$ channels by PKA or PKC-induced phosphorylation has also been proposed, in the case of the $\alpha_{1C}$, $\alpha_{1B}$ and $\alpha_{1E}$ subunits.[133,145-148] The presence of the $\beta$ subunit, either allows the functional effects of phosphorylation ($Ca_v2.2$, $Ca_v2.3$, $Ca_v1.2$, PKA or PKC), or carries itself in some cases, the phosphorylation sites ($Ca_v1.2$, $\beta_{2a}$).[145-150] The role of the $Ca^{2+}$ channel $\beta$ subunit on the other regulatory pathway of the channels involving tyrosine kinase (src, PYK2 or the Mitogen-Activated-Protein Kinase)[151-155] has not yet been determined.

Recently two series of articles have brought new lights on the structural requirement necessary for the $\beta$ subunit function. Using mutations in both the SH3 and the GK domains of the $\beta$ subunit, it has been demonstrated that, similar to the proteins of the MAGUK family, these two domains form intramolecular interactions responsible (and necessary) for the stabilisation of the $\beta$ subunit structure (interaction 2 in Fig. 5A).[156-158] Mutation of either one of these two domains severely blunted the modulation of the current by the $\beta$ subunit and the $\alpha$1-$\beta$ interaction (interaction 1 in Fig. 5A). This core complex (C1+C2 without variable regions 1, 2 and 3) was perfectly able to function as a whole $\beta$ subunit, and mutation or deletion of the first domain could be "trans-complemented" by intermolecular interaction with another subunits mutated or deleted in the second domain. Alternatively, Takahashi et al[157] have proposed that the SH3 domain could also participate to the transduction of a functional response by a direct interaction with the $\alpha$1 subunit (interaction 3, Fig. 5B).[157]

The core complex has now been crystallized alone and in association with the AID by 3 groups[159-161] (see Fig. 5B). They confirmed the interaction between the SH3 and GK domains. However, the BID was found to be buried in the $Ca_v\beta$ core structure and unavailable for protein-protein interaction while being essential for the SH3-GK interaction. A hydrophobic pocket, located at one end of the $\beta$ subunit, was largely responsible for the interaction with the AID. This interaction may be transduced deep in the channel structure via the S6 helice of the first domain and then affect channel gating by influencing the conformational changes of the channel protein that occurred during channel opening or inactivation. This arrangement refutes a direct role of the BID in the $\alpha$1-$\beta$ association, but ignores the influence of the secondary interactions between the V3 domain and the N or C-terminus of the channel, present at least in $\beta$2 and $\beta$4 subunits. From a structural point of view, neither polyproline nor ATP binding in the SH3 and GK modules respectively seemed to be functional in $\beta$ subunit.[160] The V2 domain however, connecting the SH3 and the GK modules, has been suggested to play the role of the HOOK domain in MAGUK proteins and be involved in protein-protein interaction.[161]

These new data thus extend the role of the $\beta$ subunits beyond their regulatory action on $Ca^{2+}$ influx and strongly suggest a major role for these protein-interacting modules in the construction of a complete signalling unit around the channel main pore subunit.

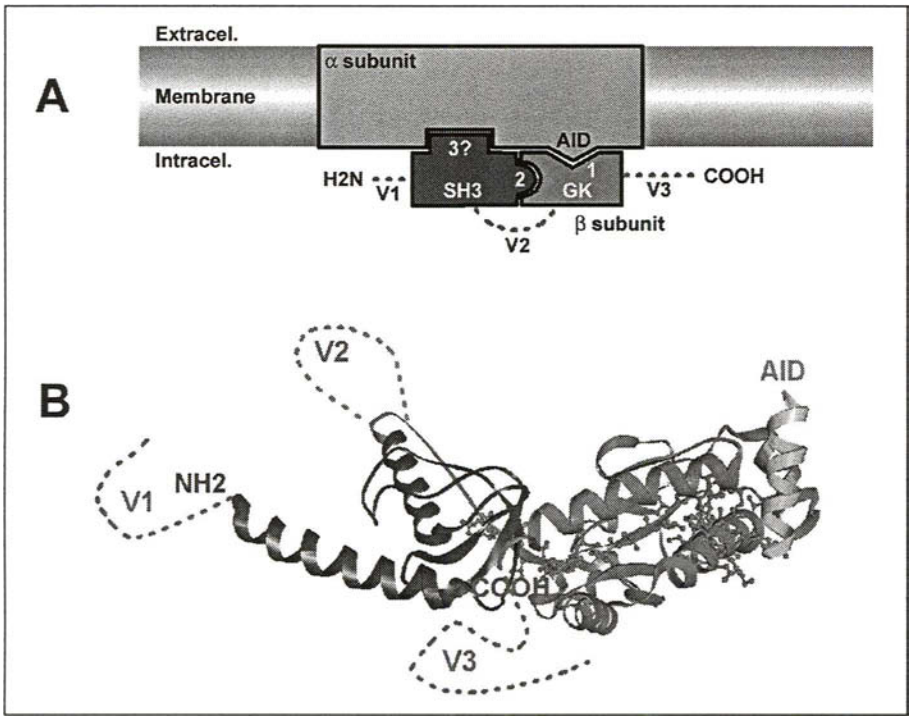



Figure 5. Structural analysis of the α1-β subunits interactions. A) Schematic representation of the functional interactions underlying the b subunit-induced modulation of α1. Interactions 1 and 2 between the GK (C2) domain and the α1-AID or β-SH3 respectively, have been demonstrated in vitro.[28,157,158] Interaction 3 has been proposed.[157] B) Three dimensional structure of the β subunit core complex (C1+C2) in association with the AID. The SH3 (C1) domain is represented in blue, the GK (C2) domain in pink and the AID in green. The BID sequence, represented with atoms in ball and stick, is buried in the GK domain and does not seem to participate directly to the interaction with the AID. The positions of the deleted V1, V2 and V3 sequences are shown as red doted lines (adapted from ref. 160, protein data bank accession code 1T3L).

## Conclusions

The in vivo role of β subunits can been assessed by the analysis of a "naturally" occurring mutation which suppresses expression of a particular β subunit (see lethargic mice, in Chapter 16), or, more directly, by constructing β subunit KO mice or through the use of antisense oligonucleotides or adenoviral gene delivery.[49,162-164] Most of these studies have confirmed the effects of the β subunit on channel activity, including the increase in total gating charge,[165] the increase in current amplitude, the shift of the voltage-dependence of activation and the regulation of inactivation. They also suggest a half life for the β subunit greater than 50 hours.[165-167] The lack of β1 subunit in β1-/- mice muscles, where compensation by other β subunits does not occur, induces an important decrease in L-type of $Ca^{2+}$ current that is accompanied by a loss of gating charge, membrane-expressed $\alpha_{1S}$ subunit, intracellular $Ca^{2+}$ transient and hence spontaneous or evoked contraction.[168] All these effects are reversed after transfection of the $\beta_1$-/- myotubes with a $\beta_{1a}$ subunit.[165] However, restoration of normal excitation-coupling specifically requires the $\beta_{1a}$ subunit, acting through its C-terminal region, possibly favoring colocalization of DHP and ryanodine receptors.[169] A role for the β subunit in calcium channel maturation during postnatal rat brain ontogeny has also been proposed,[49] underlying the important and specific regulatory roles played by β subunits not only in the precise tuning of the

calcium influx at the second or millisecond time-scale, but also in more integrated functions during development.

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# CHAPTER 7

# Distribution and Targeting Mechanisms of Voltage Activated $Ca^{2+}$ Channels

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Voltage dependent $Ca^{2+}$ channels control critical parameters of cell function. Their involvement in excitation contraction (EC)-coupling in muscle, their role in secretion in pancreas, their involvement in the acrosome reaction in sperm and their function in fast and slow signal transduction in brain predicts a variety of different $Ca^{2+}$ channel complexes with defined biophysical properties. Since all these actions occur within specialized subcellular structures like the triad in muscle cells or the presynaptic terminal in neurons, $Ca^{2+}$ channel complexes have to be targeted to their specific site of action. Thus mechanisms have to exist to control for subunit assembly and transport of the $Ca^{2+}$ channel complex.

The human genome predicts more than 20 different $Ca^{2+}$ channel subunits, which may assemble to a large variety of channel combinations. Functional $Ca^{2+}$ channels consists of three to four subunits designated $\alpha_1$, $\beta$, $\alpha_2$-$\delta$ and $\gamma$. The $\alpha_1$ subunit is the pore forming subunit consisting of four channel domains, which are connected via intracellular protein or peptide bridges. The ancillary subunits $\beta$, $\alpha_2$-$\delta$ and $\gamma$ interact with the intracellular, extracellular and transmembrane regions of the $\alpha_1$ subunit and modulate the channel function. Three large $Ca^{2+}$ channel families can be distinguished according to their sequence homology of the $\alpha_1$ subunit and their electrophysiological and pharmacological differences. These families are L-type channels, P/Q/N/R-type channels and T-type channels. Further characterization of the predicted $Ca^{2+}$ channel types obtained from genome projects will likely extend each subgroup and may define new $Ca^{2+}$ channel types. We will first describe the distribution of these different families in brain and periphery and will then discuss what we know about the transport mechanisms to the subcellular destiny of the $Ca^{2+}$ channel subunit.

## Distribution of $Ca^{2+}$ Channel Types Specified by Their Pore Forming $\alpha_1$ Subunit

Voltage dependent $Ca^{2+}$ channels appear already in the most primitive animals like the single cell ciliate *Paramecium* where they trigger regenerative $Ca^{2+}$ action potential necessary for environment sensing.[1,2] In mammals $Ca^{2+}$ channels are expressed in almost every cell throughout all tissues and organs such as brain, muscle, pancreas and testis.

## Distribution of L-Type Channels

### *$Ca_v1.1$ and $Ca_v1.4$*

L-type channels are encoded by the $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ $\alpha_1$ subunits. L-type channels were first identified in large diameter muscle fibers of crab leg.[3] Due to its high concentration in the T-tubular system of skeletal muscle it was the first $Ca^{2+}$ channel complex purified.[4] This complex consists of the $Ca_v1.1$ $\alpha_1$, $\beta_{1a}$, $\alpha_2$-$\delta_1$ and $\gamma_1$ subunit.[5] The $Ca_v1.1$ $\alpha_1$subunit contains the DHP binding site and together with the other channel subunits are

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located in the skeletal muscle transverse tubules, where it mediates the EC-coupling. Surprisingly, $Ca_v1.1$ mRNA and protein were also detected in cardiac myocytes.[6-8]

$Ca_v1.1$ as well as $Ca_v1.4$ seem to have a very restricted distribution in comparison to the other members of the L-type channel family. The $Ca_v1.4$ $\alpha_1$ subunit was identified, because loss of function mutations in this $\alpha_1$ subunit caused congenital stationary night blindness.[9,10] $Ca_v1.4$ has been first postulated to be retina specific and is localized in punctate clusters in the outer and inner plexiform layer. The clusters in the outer plexiform layer are most likely in the rod active zones, suggesting that these channels are involved in the glutamate release from rod photoreceptors.[11] Recently, $Ca_v1.4$ $\alpha_1$ subunit mRNA has also been detected in dorsal root ganglion neurons.[12]

## *$Ca_v1.2$ and $Ca_v1.3$*

$Ca_v1.2$ and $Ca_v1.3$ $\alpha_1$subunits encode for most of the L-type currents in brain, heart, smooth muscle, pancreas, adrenal gland, kidney, ovary, testis and cochlea.[13-18] The relative contribution of either $Ca_v1.2$ or $Ca_v1.3$ to L-type current is difficult to address since both channel types have similar pharmacological and biophysical properties and different splice variants.[16] However, $Ca_v1.3$ seems to activate at more negative potentials and is less sensitive to dihydropyridines.[19,20] Thus, the correlation between the mRNA transcript and/or protein expression levels in certain tissues and cells with the reconstruction of the currents in heterologous expression systems will eventually lead to a clear distinction between these two channel types and their splice variants.

L-type $Ca^{2+}$ currents in heart have two major functions: They determine the long plateau phase of the heart action potential and mediate EC-coupling.[21] EC-coupling occurs at interaction sites (junctions) between the invaginations of the plasma membrane (T-tubules) and the internal $Ca^{2+}$ stores (sarcoplasmic reticulum (SR)) (Fig.1).[22] In cardiac muscle L-type $Ca^{2+}$ channels are clustered in these junctional domains, where they trigger $Ca^{2+}$ induced $Ca^{2+}$ release from SR necessary for muscle contraction. $Ca_v1.2$ and $Ca_v1.3$ mRNAs are present in atria, but only $Ca_v1.2$ is expressed in ventricles. Atria expresses a long form (splice variant in extracellular loop in repeat IV) of the $Ca_v1.3$ $\alpha_1$ subunits.[23] Three splice variants of $Ca_v1.2$ have been detected, which reveal a tissue specific distribution.[15,24] The alternative splicing in the domain IS6 region of $Ca_v1.2$ $\alpha_1$ subunits, which creates L-type channel isoforms with different DHP sensitivity determine the tissue specific distribution of the cardiac and vascular smooth muscle L-type channel. While the $Ca_v1.2a$ form is expressed in heart and ventricular myocytes, the $Ca_v1.2b$ $\alpha_1$ subunit is expressed in aorta and smooth muscle myocytes. Embryonic cardiac cells express both splice variants (2a and 2b).[8] In neonatal $Ca_v1.2$ (-/-) myocytes, $Ca_v1.1$ and two splice variants of $Ca_v1.3$ were detected by RT-PCR, however their functions remain yet to be determined. Alternative splicing of $Ca_v1.3$ has been described in cochlea, pancreas and other tissues.[25-28]

L-type channels, which are involved in the pacemaker activity, are found in the sinoatrial node. The main mRNA found in sinoatrial node is $Ca_v1.2$ , while $Ca_v1.3$ mRNA is expressed at lower levels.[23,29] According to their voltage dependence of activation it seems reasonable that $Ca_v1.3$ containing channels, which activate at more negative potentials, contribute to the upstroke of the action potential, while $Ca_v1.2$ channels are responsible for the long diastolic depolarization of the heart action potential.[30,31]

According to the finding that L-type channel blockers cause male infertility, numerous isoforms of $Ca_v1.2$ $\alpha_1$ subunits in human and rat testis and spermatozoa have been detected.[32] Interestingly, the splicing of the testis L-type $Ca_v1.2$ $\alpha_1$ subunit occurs in regions which are responsible for the gating kinetics and dihydropyridine sensitivity, suggesting that these channel types will express L-type currents different from the ones detected in heart or brain. In addition, $Ca_v1.3$ $\alpha_1$ subunit transcripts were detected via RT-PCR in testis. Clustered pattern of localization was observed for the $Ca_v1.2$ $\alpha_1$ subunit in mouse sperm. The L-type channels are clustered at the acrosome and at the principal piece of the flagellum. In addition, they are diffusely distributed in spermatogenic cells.[33,34]

L-type channels have been described to be involved in triggering hormone secretion in (neuro)endocrine cells, like pancreatic β cells.[35,36] Secretion of neurotransmitter and hormones occurs at a much slower time scale than fast excitatory transmission in the CNS and PNS. It involves the release from low density secretory granules. This release is sensitive to L-type channel blockers suggesting that $Ca_v1$ family members are responsible for the slow $Ca^{2+}$ dependent hormone and transmitter release. $Ca_v1.2$ and $Ca_v1.3$ transcripts[17,25,26,37] and $\beta_{1b}$, $\beta_2$ and $\beta_3$[26,38,39] have been identified in insulin secreting pancreatic β cells. In the insulin secreting cell line RINm5F $Ca_v1.2$ $\alpha_1$ subunits (existing in two lengths) assembled with $\beta_{1b}$ and $\beta_3$ subunits are the major constituents of DHP-labeled $Ca^{2+}$ channels in these cells.[40] L-type channels and secretory granules colocalize in β cells.[41] While both L-type channel transcripts were detected in mouse pancreas, the $Ca^{2+}$ current underlying insulin secretion in mouse β cells is most likely mediated by $Ca_v1.2$ $\alpha_1$ subunits rather than $Ca_v1.3$ $\alpha_1$ subunits. In $Ca_v1.3$ (-/-) β cells the L-type channel amplitude is unaltered and in wild type β cells $Ca_v1.3$ protein is only weakly expressed.[42]

L-type channel currents, transcripts and proteins were described in various brain regions, including hippocampus, cerebral cortex, cerebellum and retina to contribute to signal integration in dendrites and soma.[13-18,28,43-46] L-type channels are also involved in excitation transcription coupling, linking signaling cascades from the plasma membrane to the nucleus. For example in cortical neurons, L-type channels are responsible for the CREB phosphorylation,[47] which drives the expression of various genes involved in neuronal plasticity and survival.[48] Early immunocytochemical studies revealed the presence of DHP sensitive $Ca^{2+}$ channels in the cell bodies and dendrites (or the corresponding inner segments of retinal photoreceptors) of various brain regions including dentate granule cells and many different classes of interneurons.[49,50] In particular in hippocampal pyramidal cells, L-type channels are clustered at the base of the major dendrites.[51] Further analysis of the distribution of $Ca_v1.2$ and $Ca_v1.3$ $\alpha_1$ subunits indicated that both subunits reveal a similar distribution across the CNS with a high density of expression in cell bodies in particular at the origin of dendrites. In addition, weak immunoreactivity was detected in the distal dendrites.[52] $Ca_v1.2$ channels appear to be more clustered than the $Ca_v1.3$ channels, which have smoother distribution along the cell surface. The differential distribution was not observed in GABAergic mouse cortical neurons. Here both $Ca_v1.2$ and $Ca_v1.3$ $\alpha_1$ subunits were localized at the proximal dendrites and the cell bodies.[53]

Both $Ca_v1.2$ and $Ca_v1.3$ transcripts are expressed in cochlea.[18] In hair cells of the chick basilar papilla the $Ca_v1.3$ $\alpha_1$ subunits are predominantly expressed[27] suggesting a role in afferent synaptic transmission in these cells. In fact, $Ca_v1.3$ knock out mice are deaf due to a loss of L-type $Ca^{2+}$ current in the cochlear inner hair cells.[30] Thus, L-type currents couple sound-evoked depolarization to neurotransmitter release.

L-type channels also play a role in immature neurotransmitter release. In embryonic chick ciliary ganglion neurons acetylcholine (ACh) release is sensitive to L-type and N-type channel blockers, while after hatching ACh release becomes DHP insensitive. In newly developed regenerated neuromuscular junctions but not in the adult synapse, L-type channel blockers increase the evoked but not the spontaneous release, suggesting a role of L-type channels in immature transmitter release.[54] For example in NCAM (-/-) mice, L-type channels are expressed at the adult mouse NMJ and are most likely responsible for the immature transmitter release observed in these mice.[55] In addition, L-type channels were localized on developing axons and growth cones, where they probably also trigger the immature release.[56,57]

Recently, L-type channels ($Ca_v1.2$ and $Ca_v1.3$) have been localized on axons of the adult rat optic nerve using anti-$\alpha_1$-antibodies.[58] The channels are not clustered at the nodes of the axons, since a diffuse rather than a punctate staining was detected along the axon. In addition, L-type $Ca^{2+}$ channels were also detected in GFAP-positive glial cells with high expression of $Ca_v1.2$ in the cell bodies and processes. While $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels contribute to anoxic injury,[58-60] the precise physiological role of the axonal $Ca^{2+}$ channel is unknown. $Ca^{2+}$ might sense axonal activity and could play a role in the regulation of axonal

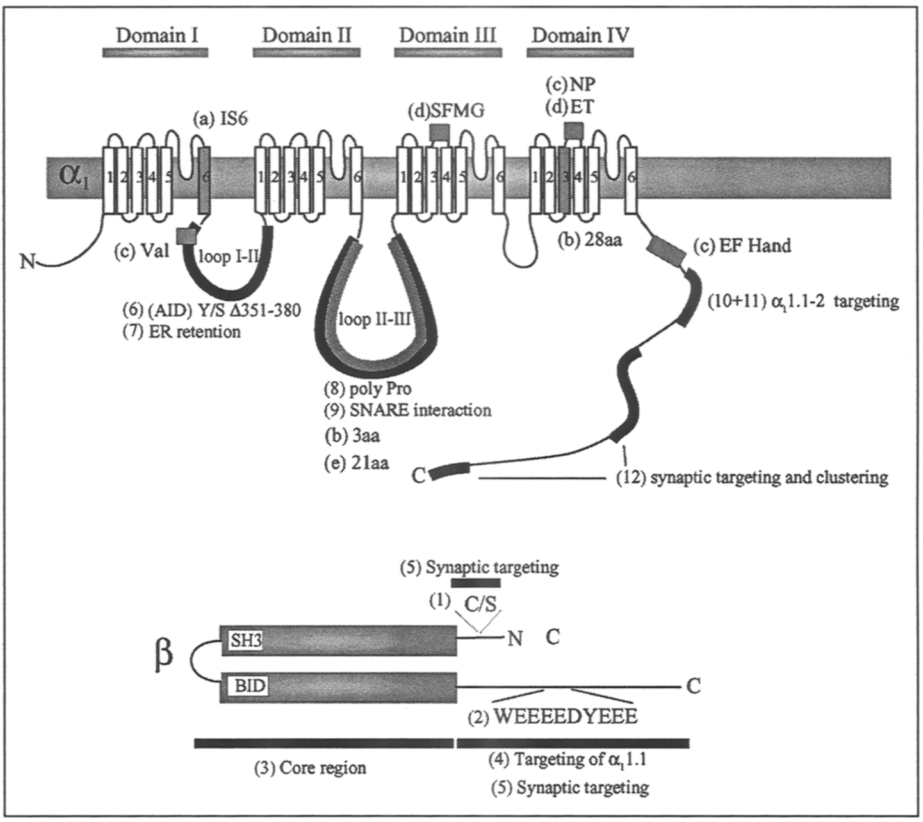



Figure 1. See legend next page.

transport. In addition to L-type channels, N-type channels have been also suggested to play a role in anoxic injury and might therefore also be found in the axon.[59,60] However, the localization of N-type channels is controversial.[58]

## Distribution of P/Q-, N- and R-Type Channels

The second group of voltage gated $Ca^{2+}$ channels contains the P/Q-type channels encoded by the $Ca_v2.1$ $\alpha_1$ subunit, the N-type channels encoded by $Ca_v2.2$ $\alpha_1$ subunit, and R-type channels encoded by the $Ca_v2.3$ $\alpha_1$ subunit.

### *$Ca_v2.1$ (P/Q-Type Channels)*

P-type channels were first identified in and named after the cerebellar Purkinje cells.[61,62] They were distinguished from Q-type currents in cerebellar granule neurons,[63,64] which differ in their inactivation properties and their sensitivity to the spider toxin ω-Aga-IVA. Later studies revealed that P- and Q-type channels most likely are encoded by $Ca_v2.1$ splice variants.[65]

Northern blot analysis and antibody labeling revealed that $Ca_v2.1$ $\alpha_1$ subunits are expressed throughout the brain including hippocampus, dorsal cortex, olfactory bulb and at highest levels in the cerebellum.[44,45,66-69] Within the cerebellum both Purkinje and granule cells are stained. Punctate staining suggesting presynaptic localization of $Ca_v2.1$ $\alpha_1$ subunits was observed along dendrites of many types of neurons in the CNS and in particular along dendrites and cell bodies of cerebellar Purkinje cells, hippocampal neurons (in particular mossy fibers of

Figure 1. Protein domains involved in tissue specific distribution and membrane targeting of $Ca^{2+}$ channels. Schematic representation of protein domains within the $\alpha_1$ and $\beta$ subunit involved in membrane and subcellular targeting of voltage dependent $Ca^{2+}$ channels (1-12, black) and tissue specific distribution (a-d, grey). $Ca^{2+}$ channel $\alpha_1$ subunits consist of 4 domains, which are connected by intracellular peptide loops. These protein domains are targets for intracellular protein-protein interactions. The $\beta$ subunit can be divided into 5 domains. $\beta$ subunits are highly conserved in the middle of the protein (thick grey bars, high homology domains), while the N- and C-termini are not conserved among the subunit members (thin lines, low homology domains). 1) The $\beta_{2\alpha}$ subunit is the only $\beta$ subunit that is palmitoylated. Palmitoylation occurs at Cys 3 and 4 within the $\beta_2$ N-terminus. Exchange of both Cys against Ser results in loss of palmitoylation and decrease in L-type current. Palmitoylation involves also the SH3 domain and the $\alpha_1$ interaction domain.[194] 2) An 11 amino acid long acidic motif (WEEEEDYEEE) within the C-terminal region of $\beta_{1b}$ is responsible for membrane localization in COS-7 cells.[191] 3) Point mutations in the $\beta$ subunit, which disrupts the $\alpha_1/\beta$ subunit interaction results in perinuclear staining of $\alpha_1$ subunits. The conserved core region of the $\beta$ subunit excluding N- and C-terminus is sufficient to localize $\alpha_1$ in punctate channel clusters at the plasma membrane.[195] 4) The C-terminus of $\beta_{1a}$ is important for sufficient transport and localization of $Ca_v1.1\alpha_1/\beta_{1a}$ channel in muscle cells.[227] 5) Synaptic targeting and clustering of $\beta_4$ subunits involves the nonconserved N- and C-terminus.[238] 6) Point mutations within the intracellular I-II linker of the $Ca_v1.2$ $\alpha_1$ subunit, which disrupt the $\alpha_1/\beta$ interaction,[196, 197] interferes with membrane localization of $\alpha_1$.[190] 7) The I-II linker contains an ER retention signal, which is shielded by the $\beta$ subunit for ER-Golgi-plasma membrane transport.[213] 8) L-type channel expression can be upregulated by A-kinase anchoring proteins (AKAP). It involves a functional, but not physical interaction of AKAP79 with the intracellular II-III linker domain of the $Ca_v1.2$ $\alpha_1$ subunit. Within the C-terminal half of the II-III linker, a poly proline sequence is conserved among the L-type channel subunits and has been suggested to act as a suppressor retention of surface expression of L-type channels.[220] 9) $Ca_v2.1$ splice variants differ in their interaction with SNARE proteins and their subcellular distribution. The isoform which binds only to SNAP-25 ($Ca_v2.1$a, rbA) is localized predominantly in cell bodies and presynaptic terminals, while the isoforms that bind to syntaxin and SNAP-25 ($\alpha_1$2.1a, B-I, $\alpha_1$2.1b, B-II) are located along the surface of dendrites as well as in cell bodies and nerve terminals.[53, 70] 10) Deletion mutants $Ca_v1.2$-$\Delta$1623 and $Ca_v1.2$-$\Delta$1623-1673 within the 664 amino acid long C-terminus of $Ca_v1.2$ abolish membrane targeting.[198] 11) A 27 amino acid long sequence which contains a consensus PDZ binding motif within the C-terminus of $Ca_v1.1$ is responsible for $\alpha_1$ subunit targeting.[224, 225] 12) The interaction between $Ca^{2+}$ channel $\alpha_1$ subunit with the modular adaptor proteins Mint-1, CASK and Veli involves the sequence motif (E/D-X-W-C/S-COOH) which is located at the C-terminal end of the long splice variant of $Ca_v2.1$ and $Ca_v2.2$ but not from $\alpha_1$ subunits from the R- or L-type channel. CASK interaction involves a proline rich region in the middle of the $\alpha_1$ subunit C-terminus. The binding motif within the C-terminus of the N-type $\alpha_1$ subunit is also necessary for sufficient targeting and clustering of the N-type channels, when exogenously expressed with $\beta_3$ subunits in hippocampal cells. Deletion of the C-terminus results in somatodendritic localization of the channel complex.[258] a) The alternative splicing in IS6 of $Ca_v1.2$ $\alpha_1$ subunits determines the tissue specific distribution of the cardiac and vascular smooth muscle L-type channel. The $Ca_v1.2$a form is expressed in heart and ventricular myocytes and the $\alpha_1$1.2b subunit is expressed in aorta and smooth muscle myocytes. Embryonic cardiac cells express both splice variants (2a and 2b).[8] b) A three amino acid insertion in the II-III linker and a 28 amino acid substitution in domain IV S3 region creates two alternatively spliced forms of $Ca_v1.2$c. Both splice forms are detected in the tissues analyzed. The $Ca_v1.2$c-II variant is the predominant transcript in brain. In pituitary $Ca_v1.2$c-I is expressed higher.[15] c) The $Ca_v2.1$ splice variants underlying P- and Q-type current characteristics differ in three sites. The $Ca_v2.1$b variant contains a valine (Val421) insertion in the I-II linker region, an asparagine-proline (NP) insertion in the domain IV S3-S4 linker and a stretch of 10 substituted amino acids in the C-terminus within the EF hand region (EF hand). Val421 containing $Ca_v2.1$ transcripts are widely expressed in brain and are also detected in kidney. According to the distribution of the EF-hand sequence, $Ca_v2.1$a shows high levels of expression in cerebellar cortex and lower levels in cortex, hippocampus and olfactory tubercule. $Ca_v2.1$b reveals strong expression in CA1-CA2 pyramidal and dentate granule cells in the hippocampus. NP carrying $Ca_v2.1$ $\alpha_1$ subunits are expressed in hippocampus, while only low levels of NP carrying $Ca_v2.1$ $\alpha_1$ subunits mRNA are detected in cerebellum.[65] d) N-type channel isoforms containing a SFMG tetrapeptide sequence in the IIIS3-S4 region are primarily expressed in rat brain, while the other isoform, which lacks the SFMG sequence but contains an ET dipeptide sequence in IVS3-S4 is highly expressed in peripheral neurons like dorsal root ganglia and superior cervical ganglia.[89, 90] e) A N-type channel splice variant within the II-III linker region (additional 63 bases or its absence) are predominantly expressed in sympathetic ganglia and significant amount of both splice variants are detected in more caudal regions of the CNS (spinal cord and brain stem).[91]

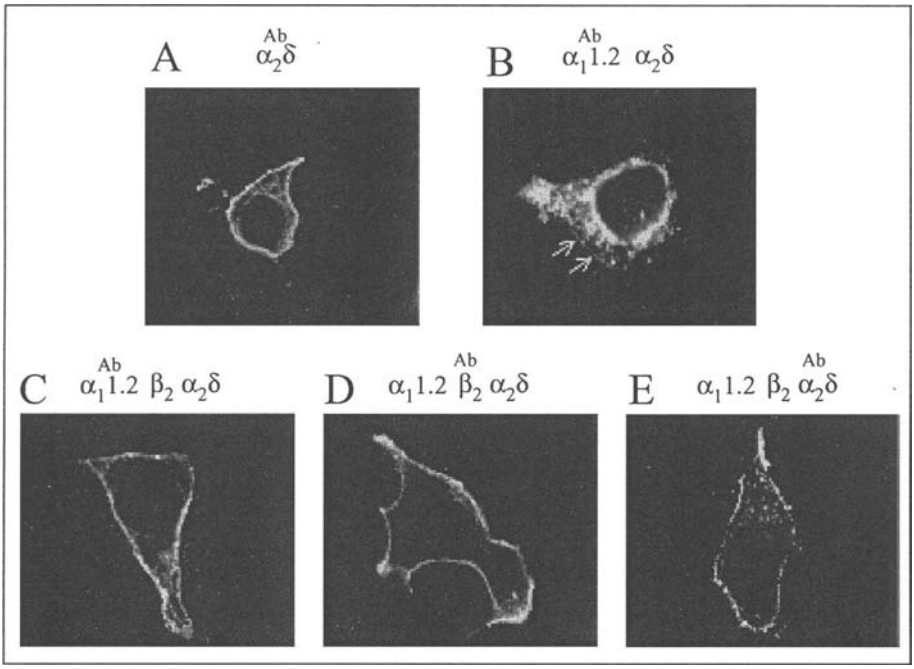



Figure 2. Targeting and distribution of L-type $Ca^{2+}$ channels in HEK-tsA201 cells.[195] Confocal images of tsA cells expressing (A) $\alpha_2$-$\delta$ (detection with antibody against $\alpha_2$-$\delta$), (B) $Ca_v1.2$, $\alpha_2$-$\delta$ (detection with antibody against $Ca_v1.2$), (C) $Ca_v1.2$, $b_{2a}$, $\alpha_2$-$\delta$ (detection with antibody against $Ca_v1.2$), (D) $Ca_v1.2$, $\beta_{2a}$, $\alpha_2$-$\delta$ (detection with antibody against $\beta_{2a}$) (E) $Ca_v1.2$, $\beta_{2a}$, $\alpha_2$-$\delta$ (detection with antibody against $\alpha_2$-$\delta$). Expression pattern of the subunits within these cells were detected with subunit specific antibodies as indicated. The antibody staining reveals that $\alpha_2$-$\delta$ alone targets to the plasma membrane (A), but is not able to transport the $Ca_v1.2$ $\alpha_1$ subunit sufficiently to the membrane (B). However, coexpression of all three subunits results in localization of all three subunits to the plasma membrane (C-E).

the dentate granule cell synapses onto the proximal dendrites of CA3 pyramidal cells) and cortical pyramidal neurons. In addition to the presynaptic staining, $Ca_v2.1$ protein was detected along the primary dendrites and their arborizations.[68]

The $Ca_v2.1$ splice variants underlying P- and Q-type current characteristics differ in three sites. The $Ca_v2.1b$ variant contains a valine (Val421) insertion in loopI-II, an asparagine-proline (NP) insertion in domain IV S3-S4 linker and a stretch of 10 substituted amino acids in the C-terminus within the EF hand region (EF hand). Analysis of the distribution of the splice variants according to the existence of Val421 insertion and C-termini substitution revealed that Val421 containing $Ca_v2.1$ transcripts are widely expressed in brain and were also detected in kidney. In situ hybridization with the different EF hand sequences revealed that $Ca_v2.1a$ and $Ca_v2.1b$ are differentially expressed. $Ca_v2.1a$ showed high levels of expression in cerebellar cortex and lower levels in cortex, hippocampus and olfactory tubercule. Both $Ca_v2.1a$ and $Ca_v2.1b$ were expressed in cerebellar granule cells with predominant expression of $Ca_v2.1a$ in Purkinje cells. $Ca_v2.1b$ revealed strong expression in CA1-CA2 pyramidal and dentate granule cells in the hippocampus. NP carrying $Ca_v2.1$ subunits were expressed in hippocampus and only low levels of NP carrying $Ca_v2.1$ subunits mRNA were detected in cerebellum. The differential expression of certain combinations of splice variants and subsequent assembly of the cell type specific $Ca_v2.1$ splice variants with different $\beta$ subunits may account for the expression of P-, Q- or intermediate P/Q-type currents in various neurons. In addition to the $Ca_v2.1a$

and b splice variants, other isoforms are described for the $Ca_v2.1$ $\alpha_1$ subunit with different localization in hippocampal pyramidal cells[70] and different interactions with presynaptic membrane proteins. These isoforms differ in their loop II-III region and in particular in their C-termini. The isoform which binds only to SNAP-25 ($Ca_v2.1a$, rbA) is localized predominantly in cell bodies and presynaptic terminals, while the isoforms that bind to syntaxin and SNAP-25 ($Ca_v2.1a$, B-I, $Ca_v2.1b$, B-II) are located along the surface of dendrites as well as in cell bodies and nerve terminals. The same distribution pattern of the $Ca_v2.1$ isoforms was observed in GABAergic mouse cortical neurons.[53]

Most studies performed at the adult mammalian neuromuscular junction suggest that ACh release is mediated by P/Q-type channels.[71-78] Immunolocalization studies of the human and rat neurosmuscular junction revealed that $Ca_v2.1$ subunits (P/Q) are localized in discrete puncta at the presynaptic terminals of motorneurons.[79,80] Surprisingly, low levels of N-type channels were detected in terminals form motorneurons of tibialis anterior, gastrocnemius, and soleus muscles but not at the NMJ.[80]

## *$Ca_v2.2$ (N-Type Channels)*

N-type channels were first discovered in sensory neurons as channels which are neither L- nor T-type channels according to their biophysical and pharmacological properties.[81,82] They are blocked specifically by the snail venom toxin ω-conotoxin GVIA (ω-CTx GVIA),[83] and are involved in neurotransmitter and hormone release. Hence, they are found in neurons and secretory cells. Indeed, Northern blot analyses and in situ hybridization assays using specific antibodies against the $Ca_v2.2$ subunit revealed that N-type channels are widely expressed in the brain, as well as the spinal cord.[44,46,84-87] N-type channels are highly expressed within the cerebral cortex (layers 2 and 4), cerebellum, olfactory bulb, hippocampus (pyramidal cell layers CA1, CA2 and CA3), dentate gyrus, in the subiculum and habenula, hypothalamus and thalamus.[88] Interestingly, several N-type channel splice variants have been identified, which differ in their tissue distribution and their biophysical properties. One of these isoforms containing a SFMG tetrapeptide sequence in IIIS3-S4 is primarily expressed in rat brain, while the other isoform, which lacks the SFMG sequence but contains an ET dipeptide sequence in IVS3-S4, is highly expressed in peripheral neurons like dorsal root ganglia and superior cervical ganglia.[89,90] Another splice variant within the II-III linker also revealed cell type specific expression. mRNAs containing the additional 63 bases were predominantly expressed in sympathetic ganglia and significant amount of both splice variants were detected in more caudal region of the CNS (spinal cord and brainstem).[91] Other studies concerning the expression pattern of the II-III linker splice variants suggest high levels of expression of N-type channels in monaminergic neurons.[92]

Dendritic $Ca^{2+}$ channels have been suggested to be involved in the generation of $Ca^{2+}$ dependent action potentials and in the integration and amplification of synaptic inputs within the dendritic arbor.[93] $Ca_v2.2$ channels are localized along the entire length of the dendrites. The distribution of the channels appears to be patchy rather than even suggesting clustering of these channels at specific sites.[87] It seems that L- and N-type channels reveal a complementary distribution. In Purkinje cells N-type channel levels are increased in the branch point of dendrites.[84,87] In addition, N-type channels are localized in a subset of neuronal cell bodies, such as pyramidal neurons in the dorsal cortex and Purkinje cells in the cerebellum.

N-type channels have been suggested to play a prominent role in synaptic transmission and have been therefore detected in various presynaptic terminals, like the presynaptic active zones of the frog but not the mammalian neuromuscular junction,[71,94-98] in cultured hippocampal neurons[99,100] or in calyx type synapses.[101,102] Punctate staining of N-type channels, suggesting nerve terminal localization, was observed along cell bodies and dendrites of many neurons in particular of cortical and hippocampal pyramidal neurons.[87] In mouse GABAergic cortical neurons, N-type channels seem to be exclusively localized in presynaptic nerve terminals.[53] Interestingly, these nerve terminals expressed either N-type channels alone or in combination with the P/Q-type channels.

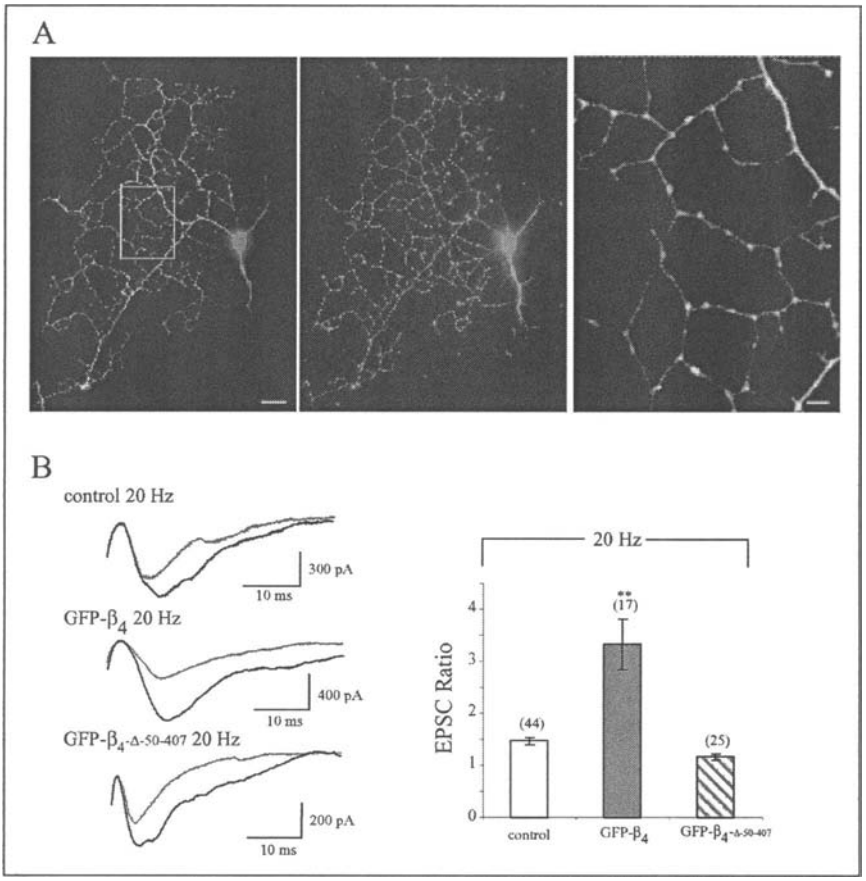



Figure 3. Distribution and function of $Ca^{2+}$ channel $\beta_4$ subunits in cultured hippocampal neurons.[238] A) distribution of GFP-tagged $Ca^{2+}$ channel $\beta_4$ subunits. Hippocampal neurons were infected with Semliki forest virus carrying GFP tagged $\beta_4$ subunits (left), stained with an anti-synaptobrevin II antibody (visualized with an Alexa546 coupled secondary antibody) (middle). Overlay in the indicated area demonstrates that GFP-$\beta_4$ is partly colocalized with the synaptic vesicle marker synaptobrevin II (Scale bar in left and middle 10 µm and right 2 µm). B) (left) Representative autaptic EPSC traces from hippocampal neurons (control) and neurons infected with synaptically targeted GFP-$\beta_4$ or somatodendritically targeted GFP-$\beta_4$(Δ50-407), respectively. 30 EPSCs were elicited at 20 Hz stimulation and the 1st and 4th EPSC of one representative experiment are shown. (right) EPSC ratios for 20 Hz stimulation are significant different between noninfected neurons (control; white bar) or GFP-$\beta_4$(Δ50-407)- (striped bar) and GFP-$\beta_4$- (grey bar) infected neurons ($p<0.01$, two tailed t test). EPSC ratios were calculated by dividing the amplitude of the 1st EPSC by the 4th EPSC within each set of experiments.

N-type channels have also been identified in growth cones indicating a role of N-type channels in neurite outgrowth and migration of immature neurons.[103-106] Activity and developmental dependent alterations in the expression of T-, L- and N-type channels have been described, which may be correlated with the occurrence of certain $Ca^{2+}$ channel types in the neurites of the developing neuron.[105,107,108] Interestingly, N-type channels ($Ca_v2.2$ $\alpha_1$ and $\beta_3$ subunits) were diffusely distributed within immature hippocampal neurons in soma, neurites and growth cones and became clustered and colocalized with synaptotagmin after neuron-neuron contact formation.[106]

### *$Ca_v2.3$ R-Type Channels*

$Ca^{2+}$ currents resistant to the common L- and N/P/Q-type blockers were detected in cerebellar granule cells[64,109,110] and designated R-type currents. Since the $Ca_v2.3$ $\alpha_1$ subunit when coexpressed with the auxiliary subunits $\beta$ and $\alpha_2$-$\delta$ resembles some of the properties of these currents, it has been suggested that $Ca_v2.3$ $\alpha_1$subunits encode for R-type currents.[63,64,110] Indeed, the majority of R-type currents were absent in central amygdala neurons in $Ca_v2.3$ knock out mice.[111] However, a significant R-type current component was still observed in cerebellar granule neurons,[112] suggesting that R-type currents may be encoded by additional $\alpha_1$ subunits. Several different $Ca_v2.3$ isoforms have been described in brain, pancreas and kidney.[113] R-type channels are described to be involved in neurotransmitter release and secretion[102,114,115] and might be a major source of $Ca^{2+}$ influx at dendritic spines in response to presynaptic action potentials.[116-119]

$Ca_v2.3$ protein is distributed all over the brain including both projection neurons and interneurons. The highest staining was observed in the deep ventral structures (caudate putamen, globus pallidus, thalamus, hypothalamus and amygdala.[46,113,120-126] $Ca_v2.3$ channels in Purkinje cells are expressed in cell bodies and dendritic trees, and to a lower extent in axons. Lower expression was also observed in the molecular layer.[113] Using a $Ca_v2.3$ driven lacZ reporter, $Ca_v2.3$ expressing cells were visualized in particular in regions involved in the control of pain transmission (e.g., dorsal root ganglia and dorsal horn of the spinal cord).[127] The highest expression was observed in the cell soma but staining was detected also in dendrites.[80,127] In cortical neurons, $Ca_v2.3$ staining extends only into the proximal dendrites, while in the olfactory bulb extensive dendritic labeling is observed in the external plexiform layer. In the plexiform layer, dendrodendritic synapses between granule and mitral cells are formed. The globus pallidus and thalamus neurons revealed dendritic and somatic R-type channel staining. In addition, the surrounding neuropil is also stained suggesting the participation of $Ca_v2.3$ at the presynaptic terminal. In fact at Calyx type neurons, R-type channels were described to be involved in synaptic transmission and immunostaining revealed the existence of these channel types at the presynaptic terminal.[102,114] Punctate distribution in the neuropil and along dendritic branches was also observed for neurons in the amygdala.

### *$Ca_v2$ in Testis and Sperm*

All $Ca_v2$ transcripts were detected in testis with RT-PCR with high expression of $Ca_v2.3$ in spermatogenic cells.[128] $Ca_v2.1$ and $Ca_v2.3$ $\alpha1$ subunits are clustered in mouse sperm.[34] While $Ca_v2.1$ $\alpha1$ subunits are found in the flagellum and apical tip and equatorial segment of the sperm head, $Ca_v2.3$ $\alpha1$subunits are clustered in the acrosomal segment and the principal piece of the flagellum. The role of these $Ca^{2+}$ channels in sperm is still unclear. However, due to their clustered localization in the acrosome and flagellum, these $Ca^{2+}$ channels are probably involved in the flagellar beat, acrosome reaction and gamete fusion. Indeed, involvement of N- and R-type channels have been reported in the depolarization-evoked $Ca^{2+}$ entry into mouse sperm.[129]

## $Ca_v2$ in Pancreas

Besides L-type channels, N- and P/Q-type channels have also been identified in pancreatic $\beta$ cells.[130-134] In fact, P/Q-type channel variants were identified, which mediated the DHP and $\omega$-CTx resistant component of insulin secretion suggesting a corelease mechanism between L-, N- and P/Q-type channels.[131] In addition, $Ca_v2.3$ protein is also expressed in endocrine cells of the pancreas and in the gut suggesting also a role of R-type currents in hormone secretion.[113]

## Distribution of T-Type Channels

Three $\alpha_1$ subunits encoding T-type channels have been identified so far, designated $Ca_v3.1$ through $Ca_v3.3$. Extensive in situ hybridization studies and northern blot analysis have been performed to describe the localization of mRNA transcripts in several tissues including brain, heart and kidney.[135,136]

All three subunits are expressed abundantly in brain.[136-140] High concentrations of all three subtypes are found in the olfactory bulb, granule cells and hippocampal pyramidal cells. $Ca_v3.1$ is the most abundant T-type channel subunit in the brain. It is highly expressed in the claustrum and bed nucleus of the stria terminalis of the basal forebrain, in the amygdala, layers IV and VI of the cerebral and piriform cortex, in hypothalamus, spinal cord, principal relay nuclei, intralaminar nuclei and lateral habenula of the thalamus, in the tegmental nuclei of the midbrain, in Purkinje cells of the cerebellum and in the inferior olive. $Ca_v3.2$ $\alpha_1$ subunits are highly expressed in the island of Calleja in the olfactory system, pineal gland, pituitary and sensory ganglia. The brain specific $Ca_v3.3$ $\alpha_1$ subunits are highly expressed in the olfactory bulb and the islands of Calleja in the olfactory system, the subthalamic nucleus in the basal forebrain and the reticular and lateral habenula of the thalamus.[140] However, using RT-PCR to detect $Ca_v3.3$ mRNA it was found that $Ca_v3.3$ transcipts are ubiquitously expressed in juvenile rat brain, but are selectively expressed in striatum in the adult rat.[136] According to their function in neurons, e.g., $Ca^{2+}$ dependent burst firing, neuronal oscillation, and lowering threshold for spike generation, T-type channels are expected to be localized within the somatodendritic membrane. Indeed, T-type currents were recorded in the soma of relay cells and from the dendrites of thalamic reticular, hippocampal CA1 and Purkinje cells. To date, T-type channels have not been detected in presynaptic terminals and thus may not play a role in synaptic transmission.[141]

In heart T-type currents are involved in pacemaker activity during the heart action potential[135] and are therefore found in the sinoatrial node. Here, $Ca_v3.1$ is the most prominently expressed LVA channel, while $Ca_v3.2$ is only moderately expressed.[29] T-type currents have been identified in several cell types from heart, such as atrial myocytes, sino-atrial nodal cells, latent atrial pacemaker cells and Purkinje cells. In adult heart $Ca_v3.1$ and $Ca_v3.2$ are the predominant T-type channel subunits, while only $Ca_v3.1$ transcripts and protein were identified in fetal mouse ventricular myocytes. $Ca_v3.1$ protein and transcripts were detected in the ventrical as well as vasculature and trial walls, indicating that T-type $Ca^{2+}$ currents are encoded by $Ca_v3.1$ and contribute to contraction in the developing myocardium.[142]

T-type channels have also been suggested to play a role in the acrosome reaction in spermatozoa and are therefore important for fertilization. mRNA transcripts of $Ca_v3.1$ and $Ca_v3.2$ are expressed in human, rat and mouse testis, Sertoli cells and in particular germ cells.[143-146] Several splice variant of $Ca_v3.1$ and two splice variants of $Ca_v3.2$ were also detected.[146]

## Distributions of the Ancillary Subunits β, $\alpha_2$-δ and γ

The $Ca^{2+}$channel ancillary β, $\alpha_2$-δ and γ subunits assemble with the $\alpha_1$ subunit to form functional $Ca^{2+}$channel complexes.

### *β Subunits*

Four β subunits and several splice variants have been identified in mammals. β subunits modify, in a subunit specific manner, the gating properties of $Ca^{2+}$channels probably through their direct interaction with the pore forming $\alpha_1$ subunit.[147] Thus assembly of $\alpha_1$ and β subunits will characterize in particular the biophysical properties of the channel complex. β subunits assemble with all $\alpha_1$ subunits of the L and P/Q/N/R-type family but not with T-type channels.

In skeletal muscle, $\beta_{1a}$ is associated with $Ca_v1.1$ $\alpha_1$ subunits as part of the DHP-sensitive L-type channel.[148] This $\beta_{1a}$ subunit splice variant is tissue specific. Skeletal muscle from $\beta_1$ (-/-) mice lack EC-coupling and therefore die after birth.[149]

The most dominant subunit in heart is the $\beta_2$ subunit,[38,45,150,151] which assembles with the $Ca_v1.2$ $\alpha_1$ subunit to form functional L-type channels in the T-tubule system (Fig.1). In addition to $\beta_2$ subunits, $\beta_4$ subunits are temporally expressed in the developing heart.[152] Exogenous expression of GFP-tagged β subunits in heart cells revealed that only $\beta_{2a}$-tagged subunits

localize at the surface sarcolemma, thus having the proper structural information to target to the physiological site of action.[153] In addition, only $\beta_2$ subunits were found to form L-type channels in heart muscle.[154] In smooth muscle, $\beta_2$ and $\beta_3$ subunits seem to be the major constituent of L-type channels. $\beta_2$ and $\beta_3$ are expressed in uterus and trachea, while only $\beta_3$ is expressed in aorta.[151]

In kidney hormone regulated $Ca^{2+}$channels are involved in controlling renal $Ca^{2+}$ secretion, which probably involves P/Q-type channels. Several splice variants of $\beta_2$, $\beta_3$ and $\beta_4$ were identified by PCR. Northern blot analysis revealed that $\beta_4$ is preferentially expressed in the kidney cortex, while all three $\beta$ subunits are expressed in the nephron segments. Only $\beta_4$ was found in distal convoluted tubule and connecting tubule, where it colocalizes with the $Ca_v2.1$ $\alpha_1$ subunit.[155] $\beta_1$ transcripts were detected in spleen,[156] and all four $\beta$ subunits were found in testis (germ cells).[33] $\beta$ subunits reveal a diffuse distribution in spermatogenic cells and appear clustered in sperm.

The expression of all four $\beta$ subunits has been extensively investigated in the brain. Two $\beta_1$ splice variants, $\beta_{1b}$ and $\beta_{1c}$, are expressed in brain. $\beta_{1b}$ is highly expressed in the cerebral hemispheres and hippocampus.[157] Moderate to high mRNA and protein levels were found in olfactory bulb, cortex, hippocampus, caudate putamen, amygdala, habenula, nucleus intermeduncularis, superior colliculus, inferior colliculus and cerebellum. $\beta_2$ subunits reveal the weakest expression of all $\beta$ subunits in brain. $\beta_3$ is highly expressed in olfactory bulb and habenula, which overlaps with a high expression of $Ca_v2.3$ $\alpha_1$ subunits, suggesting that R-type channels in this brain region might be assembled with $\beta_3$ subunits. $\beta_4$ subunits show the highest expression in the cerebellum (granule cells and Purkinje neurons) overlapping with a high expression of $Ca_v2.1$ (P/Q-type) and $Ca_v2.3$ (R-type) $\alpha_1$ subunits.[45,154] According to the high expression of $\beta_4$ in cerebellum and the association with P/Q-type channels, $\beta_4$ seems to play a prominent role at the presynaptic terminal.[154,158] Interestingly in the hippocampus, $\beta_1$, $\beta_2$, and $\beta_3$ subunits have similar patterns of distribution, while $\beta_4$ reveals a different expression pattern. $\beta_1$ and $\beta_2$ subunits are found in all regions of the hippocampus. $\beta_4$ is absent from CA1 in human brain but is present in rat. In human $\beta_4$ is highly expressed in the subiculum. The subcellular distribution of $\beta$ subunits in hippocampal neurons revealed that in general all $\beta$ subunits were found in the soma, dendrites and neuropil suggesting that $\beta$ subunits are not excluded from certain subcellular compartments. $\beta_4$ subunits were intensively expressed in dendrites, while the remaining $\beta$ subunits revealed a higher relative expression in the soma.[159,160]

A particular role for the $\beta_2$ subunit has been suggested in the assembly of the retinal specific L-type channel.[161] CNS $\beta_2$(-/-) mice revealed an altered retinal morphology with an altered pattern of $Ca_v1.4$ protein expression in the thinner outer plexiform layer.

Subunit specific correlation of brain expression pattern between $\alpha_1$ and $\beta$ subunits suggest that P/Q-type channels coexpress with $\beta_4$ subunits, $Ca_v1.2$ $\alpha_1$ subunit containing L-type channels coexpress with $\beta_3$ subunits and L-type channels containing $Ca_v1.3$ $\alpha_1$ subunits coexpress with $\beta_1$ subunits.[44] All $\beta$ subunits can assemble with $Ca_v1$ and $Ca_v2$ family members. The purification of the N-, P/Q and L-type channel complexes from rabbit, guinea and/or rat brain membranes indicates the association of all channel types with all four $\beta$ subunits.[154,158,162,163] In general, $\beta_3$ and $\beta_4$ subunits were found in a higher fraction than $\beta_{1b}$ and $\beta_{2a}$ subunits. However, fractional contribution of the $\beta$ subunits for channel assembly was brain region dependent. In addition, the relative fraction of $\beta$ subunits assembled with at least N-type channels is developmentally regulated. While N-type channels assemble with $\beta_1>\beta_3>\beta_4$ subunits at postnatal day 2, at day 14 N-type channels contain $\beta_3>\beta_1=\beta_4$.[163] In particular $\beta_1$ and $\beta_4$ subunit expression is unregulated during development, while $\beta_2$ and $\beta_3$ subunit expression remain constant from birth to the adult stage.[164] The developmental regulation of the assembly and expression of $Ca^{2+}$ channel subunits is probably also responsible for the discrepancy in distribution observed for example between rat and human brain regions.

## $\alpha_2$-$\delta$ Subunits

The $\alpha_2$-$\delta$ subunit transcript is posttranslationally cleaved into a disulfide-linked $\alpha_2$ and $\delta$ portion.[165,166] The highly glycosylated extracellular $\alpha_2$ domain is less conserved than the $\delta$ domain, which contains the transmembrane anchor.[167-169] The $\alpha_2$ domain increases the membrane expression of functional channels and facilitates the assembly of $\alpha_1$ at the cell surface. In contrast the $\delta$ protein is necessary for assembly with the $\alpha_1$ subunit.[167,170]

Four different $\alpha_2$-$\delta$ genes ($\alpha_2$-$\delta_{1-4}$) have been identified in mammals.[171,172] $\alpha_2$-$\delta_1$ is highly expressed in brain, heart, lung, spleen, kidney, liver, testis and skeletal muscle, while $\alpha_2$-$\delta_2$ and $\alpha_2$-$\delta_3$ are highly expressed in brain with lower levels of expression in kidney and testis.[173] Northern blots analysis of human tissue indicated a high expression of $\alpha_2$-$\delta_2$ in lung and testis and lower expression in heart and brain.[174] Functional splice variants of $\alpha_2$-$\delta_2$ have been described, which differ in the $\alpha_2$ and $\delta$ protein in several amino acid positions and appear to be differentially expressed in cardiac tissue and human medullary thyroid carcinoma cells.[175,176] Differences in the tissue distribution between humans and mice were observed for $\alpha_2$-$\delta_2$ and $\alpha_2$-$\delta_3$. In humans, all $\alpha_2$-$\delta$ isoforms are highly expressed in brain, heart and skeletal muscle, with highest expression of $\alpha_2$-$\delta_2$ in lung (note that highest expression in heart, pancreas and skeletal muscle and lower levels in kidney, liver, placenta and brain were reported by Klugbauer et al).[172] Mouse $\alpha_2$-$\delta_3$ was only detected in brain and $\alpha_2$-$\delta_2$ was not expressed in lung.[177] The differences in $\alpha_2$-$\delta$ subunit expression among the different groups might be due to cross hybridization of the probes used, age and/or species dependent expression of the various subunits.

$\alpha_2$-$\delta_2$ and $\alpha_2$-$\delta_3$ revealed similar expression patterns in brain. $\alpha_2$-$\delta_2$ mRNA had high levels in cerebellum, moderate levels in medulla, pons and striatum. $\alpha_2$-$\delta_2$ is also expressed in cortex, hippocampus, habenula and nucleus reticularis thalami.[173] Within the cerebellum, $\alpha_2$-$\delta_2$ revealed high levels in Purkinje cells and lower levels in granule cells. Correlated with the high expression of $\alpha_2$-$\delta_2$ in Purkinje cells is the reduction of $Ca^{2+}$ currents in these cells observed in the $\alpha_2$-$\delta_2$ mouse mutant ducky, which suffers from ataxia, wide-based gait and paroxysmal dyskinesia.[173] $\alpha_2$-$\delta_1$ has high levels of expression in the granule cell layers and $\alpha_2$-$\delta_3$ in the molecular layer. Thus, the $\alpha_2$-$\delta$ subunits have distinct pattern of expression in cerebellum.[173,176]

A complementary pattern of distribution was described for $\alpha_2$-$\delta_1$/$\alpha_2$-$\delta_2$ and $\alpha_2$-$\delta_3$ in DRG neurons. While high amounts of $\alpha_2$-$\delta_1$ and $\alpha_2$-$\delta_2$ mRNAs were found in small c-type sensory neurons, $\alpha_2$-$\delta_3$ was highly expressed in large-diameter cells.[12] Since the $\alpha_2$-$\delta$ proteins are highly glycosylated, glycosylation levels may play a role for tissue specific and functional expression of this subunit. Indeed, differences in the glycosylation levels between two forms of the $\alpha_2$-$\delta_2$ subunit have been observed in DRG neurons.[178]

## $\gamma$ Subunits

$\gamma$ subunits are integral membrane proteins with four transmembrane regions and cytoplasmatically located N- and C-termini. 8 different $\gamma$ ($\gamma_{1-8}$) subunits have been identified in mammals, which are divided into two different subgroups: subfamily one includes $\gamma_1$ and $\gamma_6$ and subfamily 2 consists of $\gamma_{2-5}$ and $\gamma_7$.[179-183-185]

The $\gamma_1$ subunit is highly expressed in skeletal muscle. Since this subunit is part of the skeletal muscle DHP sensitive $Ca^{2+}$ channel complex, the $\gamma_1$ subunit is localized in the triads. In addition, $\gamma_1$ subunit transcripts were detected in aorta using RT-PCR. The related $\gamma_6$ subunit is expressed in various tissues including cardiac and skeletal muscle, aorta, brain and lung. RT-PCR detected two isoforms of $\gamma_6$ (long and short, which differentially distribute among the tissues.[186]

The other subfamily members were all detected in brain by RT-PCR.[186] Western blot analysis of mouse brain using $\gamma$-specific antibodies as well as northern blot and dot-blot hybridization revealed that $\gamma_{2-4}$ protein and $\gamma_{3-4}$ mRNA were exclusively detected in brain[183,187] (note: RT-PCR products for $\gamma_2$ and $\gamma_4$ were also detected outside of the brain in testis ($\gamma_2$) and cardiac muscle, aorta and lung ($\gamma_4$)).[186] High mRNA levels for $\gamma_2$ were found in cerebellum, moderate levels in hippocampus, cerebral cortex and olfactory bulb. $\gamma_3$ mRNA was detected in hippocampus, cortex, olfactory bulb and caudate putamen. $\gamma_4$ was highly expressed in caudate putamen,

olfactory bulb, habenula and at lower levels in the thalamus and cerebellum.[183] High levels of $\gamma_2$ protein were found in cerebellum and cortex, moderate levels are detected in midbrain, thalamus, hippocampus and striatum and low levels in pons and brain stem. $\gamma_2$ subunit was enriched in mouse synaptic plasma membranes indicating its role in synaptic function.[181] Electron microscopy of hippocampal mossy fiber synapses detected $\gamma_2$-immunoreactivity in dendrites.[187] $\gamma_2$ subunits interact here at least with AMPA receptors and PSD95. The interaction has been shown to be required for transport of AMPA receptors to the synapses.[1,2] Thus, $\gamma_2$ subunits are localized at the postsynaptic side rather than the presynaptic terminal.

$\gamma_3$ revealed high levels of expression in cortex, moderate in midbrain and striatum and low levels in thalamus and hippocampus. $\gamma_4$ was high in cortex and midbrain, hippocampus and striatum and low in thalamus, pons, brainstem and cerebellum.[187]

$\gamma_5$ has high mRNA levels in liver, moderate levels in kidney, heart and lung and low levels in testis[183], while RT-PCR products for $\gamma_5$ were only found in brain.[186]

$\gamma_7$ RT-PCR products were detected in cardiac muscle (atria and ventricle), brain, skeletal muscle, lung and testis,[186] and northern blots only detected two $\gamma_7$ subunit isoforms in brain. Here, a high concentration of $\gamma_7$ was found in the amygdala, hippocampus, thalamus, cerebellum and specifically in cerebral cortex.[188] $\gamma 8$ was detected in brain and testis.[186]

## Mechanisms of $Ca^{2+}$ Channel Targeting: Transport from the ER to the Plasma Membrane and Specified Subcellular Structures

Transmembrane proteins like $Ca^{2+}$ channels are synthesized at endoplasmic reticulum (ER) ribosomes and transported through the ER and Golgi. Eventually, they are packed into transport vesicles and moved to the plasma membrane. Depending on the cell type and the specific function of the $Ca^{2+}$ channel complex within the cell, a decision has to be made to which subcellular structure the channel complex will be transported. In neurons for example, P/Q- and N-type channels may be transported to the synaptic terminals, while L-type channels may remain at the soma. In epithelial cells, certain ancillary subunits might be excluded from the apical or basolateral membrane, when assembled with a specific $\alpha_1$ subunit. In any case, once the channel arrives at the plasma membrane, the protein complex has to be anchored at the subcellular structure through interaction with intracellular proteins or assembly within a certain signaling complex. The combination of electrophysiological measurements to detect ion channel currents with imaging of fluorescently tagged ion channel subunits creates the possibility to monitor the life time targeting events of a channel complex and its subsequent physiological effects.

## $Ca^{2+}$ Channel Complex Formation in Heterologous Expression Systems

$Ca^{2+}$ channel complex formation was in particular studied in heterologous expression systems. Expression systems like *Xenopus* oocytes, HEK293 or COS-7 cells contain low levels of endogenous $Ca^{2+}$channels and are therefore suitable to exogenously express various $Ca^{2+}$ channel subunit combinations and analyze their distribution and function. Early studies of recombinant $Ca^{2+}$ channel complexes expressed in these expression systems already revealed that the whole cell current amplitude as well as the number of drug binding sites measured was drastically increased, when pore forming $Ca^{2+}$ channel $\alpha_1$ subunits were coexpressed with ancillary $Ca^{2+}$ channel $\beta$ and/or $\alpha_2$-$\delta$ subunits. These early results suggested, that $\alpha_1$, $\beta$ and/or $\alpha_2$-$\delta$ subunits are critical for efficient transport of the $Ca^{2+}$ channel complex.

The transport and localization of L-type and P/Q-type channels in heterologous expression systems revealed an important role of the $Ca^{2+}$ channel $\beta$ subunits for transporting the $\alpha_1$ subunit to the plasma membrane. When expressed alone in HEK293 cells, $Ca_v1.2$ $\alpha_1$ subunits revealed a predominantly intracellular and perinuclear staining.[189] 80% of $\alpha_1$ subunits expressed alone in these cells were localized in the ER, while 15 % were transported to the plasma membrane.[190] A similar expression pattern was observed when P/Q-type channel $Ca_v2.1$ $\alpha_1$ subunits were expressed alone in COS-7 cells. Coexpression of $\beta$ subunits in both cell types

caused dramatic increase in plasma membrane staining and punctate channel clustering of $Ca_v1.2$ containing L-type and $Ca_v2.1$ containing P/Q-type channels.[189-191] Interestingly, $\beta_{1a}$ and $\beta_3$ subunit were only detected in the ER in the presence of $\alpha_1$ subunits.[190] The time course of β subunit action on functional $Ca^{2+}$ channel expression was also analyzed. In pulse chase studies, the half life of the channel complex was estimated to be 3 h. β subunit mediated $Ca^{2+}$channel expression measured as whole cell currents increased up to 50 h and then slowly declined. In $Ca_v1.2$ expressing *Xenopus* oocytes, whole cell currents could be measured 1-4 hours after injection of purified his-tagged $\beta_3$ subunits. The increase in whole cell current was mediated by an increase in the amount of $\alpha_1$ subunits at the plasma membrane involving the intracellular glycoprotein transport.[192] β subunits might also be involved in the maturation of the $\alpha_1$ subunit containing channel complexes. This was suggested by the finding that coexpression of $\beta_{2a}$ subunits with $\alpha_1$ subunits increased DHP binding of the L-type channels.[189]

In contrast to $\alpha_1$, $\beta_3$ and $\beta_4$ subunits, $\beta_1$ and $\beta_{2a}$ subunits alone are able to localize to the plasma membrane, which probably involves different mechanisms. The $\beta_{2a}$ subunit is the only β subunit which is palmitoylated. Palmitoylation occurs at Cys 3 and 4 within the $\beta_2$ N-terminus. Exchange of both Cys against Ser results in loss of palmitoylation. All non palmitoylated β subunits (i.e., $\beta_{1b}$, $\beta_3$ and $\beta_2$-palmitoylation deficient splice variants and mutants) showed a diffuse intracellular staining pattern in comparison to the plasma membrane staining of palmitoylated $\beta_{2a}$. The non palmitoylated β subunits still fractionated with the membrane but not with the cytosolic fraction, indicating that β subunits are associated with intracellular membranes. Mutated $\beta_{2a}$ subunits decreased the whole cell currents when coexpressed with $Ca_v1.2$ $\alpha_1$ subunit. Surprisingly, the size of the charge movement was not altered, suggesting that the same amount of $Ca_v1.2$ $\alpha_1$ subunits are located at the plasma membrane. Interestingly, exchange of the N-terminus of $\beta_{1b}$ and $\beta_3$ against the $\beta_{2a}$ palmitoylated N-terminus did not result in plasma membrane localization of the particular β subunit, indicating that other parts of $\beta_{2a}$ are involved in plasma membrane localization.[193,194] While no membrane staining of $\beta_{1b}$ subunits was observed in HEK293 (tsA201) cells, expression of this subunits in COS-7 cells revealed plasma membrane targeting of $\beta_{1b}$. Chimeras between the membrane localized $\beta_{1b}$ subunit and the diffused distributed $\beta_3$ subunit identified an 11 amino acid long acidic motif (WEEEEDYEEE) within the C-terminal region of $\beta_{1b}$ responsible for membrane targeting. This sequence motif might be important for cell type specific localization and clustering of $\beta_{1b}$ assembled channels.[191]

The targeting of $\alpha_1$ subunits to the plasma membrane is mediated by all four β subunits and involves the direct interaction between these two proteins. The interaction between $\alpha_1$ and β subunits occurs at subunit specific protein domains. Point mutations in the β subunit, which disrupt the $\alpha_1/\beta$ subunit interaction, results in perinuclear staining of $\alpha_1$ subunits. In addition, mutations within the SH3-motif of β, which also interfere with binding of $\alpha_1$ to β, disrupt plasma membrane targeting of $\alpha_1$. Interestingly, the conserved core region of the β subunit excluding N- and C-terminus still localizes $\alpha_1$ in punctate channel clusters at the plasma membrane.[195] Point mutations within the intracellular I-II linker of the $Ca_v1.2$ $\alpha_1$ subunit, which disrupt the $\alpha_1/\beta$ interaction,[196,197] interfere with membrane localization of $\alpha_1$, leaving 90% of the subunit within the ER.[190]

The importance of the $\alpha_1$ subunit C-terminus for channel targeting was identified by using deletion mutants of the $Ca_v1.2$ L-type channel.[198] The C-terminus of the $Ca_v1.2$ $\alpha_1$ subunit contains 664 amino acids (i.e., it starts at amino acid position 1507 and ends at 2171). Truncation of the C-terminus reduced membrane staining as detected with immunocytochemical methods. Two mutations were of particular interest, since they completely abolished membrane staining and functional expression of the channel (i.e., $Ca_v1.2$-Δ1623 and $Ca_v1.2$-Δ1623-1673). This region overlaps with the calmodulin binding site suggesting a role for calmodulin in channel targeting. However, mutations, which reduce or abolish calmodulin binding, do not abolish L-type channel expression.[199-202] Interestingly, at the more C-terminal end of the $Ca_v1.2$ $\alpha_1$ subunits, sites were identified which increased the intracellular accumulation

of the protein. These channels seemed to be functional, since no differences in the PN200 binding were detected. The protein domain might be involved in the regulation of the constitutive recycling of the channel complex between the plasma membrane and the intracellular compartments.

Several studies also suggested a role of $\alpha_2$-$\delta$ in membrane targeting of the $Ca^{2+}$ channel in heterologous expression systems. However, the precise function of these subunits on channel targeting are not as clear and as characterized as for the $\beta$ subunits. Coexpression of $\alpha_2$-$\delta$ with different $\alpha_1$ and or $\alpha_1$/$\beta$ combinations in *Xenopus* oocytes, HEK 293 and COS-7 cells increased N-, P/Q-, L-type current and even T-type currents.[66,167,172-174,176,203-205] The increase in current could be correlated with an increase in gating current[205-207] or PN2001 binding (L-type channel specific).[208] While the extracellular part of $\alpha_2$-$\delta$ in its glycosylated form interacted with the extracellular region of the L-type $\alpha_1$ subunits ($Ca_v1.2$ and $Ca_v1.1$), it was necessary but not sufficient for increase in current density and drug binding affinity (PN2001). It has to be mentioned, that other studies could not detect increase in current levels and drug binding for coexpression of $\alpha_1$ and $\alpha_2$-$\delta$ subunits.[121,209]

Visualization of $\alpha_2$-$\delta$ targeting events using fluorescent antibodies or tags also yielded controversial results. $\alpha_2$-$\delta$ subunits expressed alone showed plasma membrane staining in HEK-tsA201 cells[195] but not in COS-7 cells.[210] No increase in membrane fluorescence was observed for coexpression of $\alpha_2$-$\delta$ with $Ca_v1.2$ or $Ca_v2.1$ in some studies, while other investigators reported that coexpression of $\alpha_2$-$\delta$ and $Ca_v1.2$ increased membrane fluorescence of $Ca_v1.2$ $\alpha_1$ subunits and L-type currents. Increase in fluorescence intensity was maximal, when all three subunits $\alpha_1$, $\beta$ and $\alpha_2$-$\delta$ were coexpressed.[211] The results suggest, that the environment within the cell may play an important role for the targeting process. The $\alpha_2$ part is highly glycosylated and interacts with the extracellular domains of $\alpha_1$ subunit. Thus, glycosylation may play an important role in $Ca^{2+}$ channel targeting.[212] Since purified $Ca^{2+}$ channel complexes are assembled of at least $\alpha_1$, $\beta$ and $\alpha_2$-$\delta$ subunits, it seems likely that $\alpha_2$-$\delta$ and $\beta$ subunits may cooperatively affect channel assembly and targeting to the plasma membrane.

## Up and Down Regulation of $Ca^{2+}$ Channel Expression: $\beta$ Subunits, $\gamma$ Subunits, AKAP and kir/Gem

By using the I-II linker of the $Ca_v2.1$ $\alpha_1$ subunit (the interaction site with the $\beta$ subunit) and analyzing its way through the cell membrane system a mechanisms was suggested as to how $Ca^{2+}$ channel complex targeting to the plasma membrane may occur.[213] Fusion of this intracellular loop to an unrelated ion channel, the *Shaker* potassium channel, drastically decreased whole cell potassium currents of *Shaker* when expressed in *Xenopus* oocytes. This current was rescued by coexpression of the $Ca^{2+}$ channel $\beta$ subunit, suggesting that $\beta$ subunits antagonized the suppressor effect of the I-II linker on *Shaker* channel expression. In addition, fusion of the transmembrane protein CD8 with I-II linker revealed restricted distribution of the fusion construct to the ER. Coexpression of $\beta_3$ subunits targeted the CD8-I-II linker to the plasma membrane. Pulse chase experiments indicated that the association between $\beta$ and the I-II linker occurs very early during the biosynthetic pathway of the $Ca^{2+}$ channel complex mostlikely at the ER level. ER retention and ER retention signal shielding seem to be a common mechanisms for ion channel complex assembly and membrane targeting as described for several other ion channels like $K_{ATP}$ channels and NMDA receptors.[214]

Recently, it has been shown that $Ca^{2+}$ channel $\beta$ subunits also interact with the small G protein kir (kir/Gem), a member of the Ras-related small G-protein family.[215] Kir/Gem exists as an active GTP and inactive GDP bound form and binds $Ca^{2+}$/CaM. It has been suggested, that $Ca^{2+}$/CaM bind to the GDP form where it localizes kir/Gem close to $\beta$ subunits within the cell. Once activated, GTP bound kir/Gem binds to the $\beta$ subunit and prevents trafficking of the $\alpha_1$ subunit through the ER to the plasma membrane. Indeed, coexpression of $Ca_v1.2$ or $Ca_v1.3$ plus $\beta$ subunits and kir/gem in *Xenopus* oocytes or BHK cells suppresses $Ca^{2+}$ currents. In addition, exogenous expression of kir/gem in secretory PC12 and MIN6 cells decreased

endogenous L-type currents and hormone secretion. Since kir/Gem is expressed in secretory cells like the pituitary, adrenal cells and pancreatic islets the small G protein might be involved in the regulation of L-type channel surface expression necessary for secretion of hormones.

In addition to kir/Gem, the stargazin-like γ subunits ($\gamma_{1,2 \text{ and } 7}$) also reduced N-type, P/Q and L-type channel expression in *Xenopus* oocytes and COS cells.[188,216] Interestingly, $\gamma_7$ almost completely abolished N-type current expression in COS-7 cells (coexpression of $Ca_v2.2/\beta_{1b}/\gamma_7$) via lowering $\alpha_1$ but not β protein levels. However, exogenous expression of $\gamma_7$ did not affect endogenous N-type channel currents in sympathetic neurons. This suggests, that $\gamma_7$ does not directly act on the channel but rather alters the degradation, folding, synthesis or targeting of the $Ca^{2+}$ channel complex.

One of the mechanisms for L-type channel upregulation involves the A-kinase anchoring proteins. L-type channels are phosphorylated by PKA, which increases the channel activity in brain, skeletal and heart muscle.[217-219] The postsynaptic scaffolding protein AKAP79 increases L-type but not non L-type currents in heterologous expression systems. These effects are independent of PKA and involve a functional, but not physical interaction of AKAP79 with the intracellular linker domain (loop II-III) of the $Ca_v1.2$ subunit. Within the C-terminal half of the loop II-III a poly proline sequence is conserved among the L-type channel $Ca_v1.1$, 1.2 and 1.3 $\alpha_1$ subunits and has been suggested to act as a suppressor retention of surface expression of L-type channels.[220]

## Targeting of $Ca^{2+}$ Channels in Skeletal and Cardiac Muscle

L-type channels in striated muscle mediate EC-coupling via activating of RyR. These L-type channels consists of $Ca_v1.1$ $\alpha_1$ subunits and $\beta_{1a}$ subunits in skeletal muscle and $Ca_v1.2$ $\alpha_1$ and $\beta_{2a}$ subunits in heart and are located in the plasma membrane invaginations, i.e.,the surface membrane of the T-tubuli.[22] Cell lines derived from the dysgenic muscle (GLT cells) do not contain functional $Ca_v1.1$ $\alpha_1$ subunits and lack EC-coupling. Therefore, these cell lines were extensively used to study the targeting and function of $Ca_v1.1$ $\alpha_1$ subunits. Exogenous expression of GFP tagged $Ca_v1.1$ and $Ca_v1.2$ or untagged $Ca_v1.1$ $\alpha_1$ subunits revealed that the pore forming $Ca^{2+}$ channel $\alpha_1$ subunits cluster in GLT cells, suggesting incorporation of the subunit into the T-tubule SR junction. Electrically evoked contraction was restored only in cells, which overexpressed L-type $\alpha_1$ subunits but not P/Q or N-type $\alpha_1$ subunits.[221] In contrast to heterologous expression systems, the $Ca_v1.1$ $\alpha_1$ subunit was responsible for targeting the β subunits to its precise localization. GFP tagged $\beta_{1a}$ subunits redistributed to the T-tubule/SR junction only in the presence of $Ca_v1.1$ $\alpha_1$ subunits.[222] The targeting involves also the direct interaction between $\alpha_1$ and β subunits, since mutations within the $\alpha_1$ subunit, which disrupted the $\alpha_1/\beta$ interaction, abolished the redistribution of $\beta_{1a}$.[223] The role of β subunits for the localization of the muscle specific $Ca_v1.1$ $\alpha_1$ subunit was also investigated in heterologous expression systems.[197] Coexpression of $Ca_v1.1$ and GFP-tagged $\beta_{1a}$ subunits attracted the $\beta_{1a}$ subunit to the intracellular cytoplasmic membrane system. The targeting was again dependent on the interaction between $\alpha_1$ and β subunits as demonstrated with $Ca_v1.1$ $\alpha_1$ subunits deficient in $Ca^{2+}$ channel β binding. In contrast to $\beta_{1a}$ subunits, $\beta_{2a}$ subunits, which physiologically do not interact with $Ca_v1.1$, targeted $Ca_v1.1$ $\alpha_1$ subunits to the plasma membrane but could not modulate $Ca_v1.1$ channel function.

Targeting and precise localization of L-type channels into the triads involves the C-terminus of $Ca_v1.1$ $\alpha_1$ subunits. C-terminal deletion mutants of $Ca_v1.1$ $\alpha_1$ subunits identified a 27 amino acid long sequence which contains a consensus PDZ binding motif. Point mutations within these motif disrupted the interaction with muscle specific proteins as shown in a yeast two hybrid assay and reduced the EC-coupling in dysgenic myotubes after nuclear injection of the mutated constructs.[224] The reduction in EC-coupling was due to non localization or targeting of the mutated subunit, since these subunits were localized close to the injected nucleus and did not distribute like the full length $Ca_v1.1$ $\alpha_1$ subunit along the length of the myotube. Chimeras between the targeted $Ca_v1.1$ $\alpha_1$ subunit and the nontargeted $Ca_v2.1$ $\alpha_1$ subunit,

which is retained in the ER/SR system, revealed that transfer of the C-terminus but not exchange of the other intracellular protein loops transferred triad targeting from L-type channels to P/Q-type channels. Triad targeting of P/Q-type channels also restored the EC coupling in the dysgenic myotubes.[225]

Since dysgenic myotubes still contain functional $\beta_{1a}$ subunits, the role of $\beta$ subunits for skeletal muscle distribution and function was studied in $\beta_1$ subunit knockout mice, which die at birth from asphyxia.[149] $\beta_1$(-/-) mice reveal a drastic decrease in L-type current, charge movement and lack EC-coupling. The effects are mostlikely due to a drastic reduction in $Ca_v1.1$ $\alpha_1$ subunits. The loss of punctate distribution of L-type $Ca^{2+}$ channels suggests, that $\beta$ subunits are important for function and localization of $\alpha_1$ in the triads.[149,226] Coexpression of either $\beta_{1a}$ or $\beta_{2a}$ subunits in these $\beta_1$(-/-) muscles restored L-type currents. However, $\beta_{1a}$ transfected cells revealed higher density of charge movement and a larger amplitude of $Ca^{2+}$ transients, suggesting that EC coupling is more efficient for $\beta_{1a}$ assembled channels. Transfer of the C-terminus of $\beta_{1a}$ to $\beta_{2a}$ subunits reversed the effects.[227] Due to the C-terminus of $\beta_{1a}$, $Ca_v1.1\alpha_1/\beta_{1a}$ channels may thus couple more effectively to RyR or are more sufficiently transported and localized at the triads than $Ca_v1.1\alpha_1/\beta_{2a}$ assembled channels.

## Sorting of $Ca^{2+}$ Channels in Polarized Cells: A Correlation between Polarized Sorting in Epithelial Cells and Neurons

N-, P/Q- R-, L- and T-type channels are differently distributed within neurons. While N-, P/Q- and R-type channels are found in presynaptic terminals, L- and T-type channels are localized primarily on cell bodies and proximal dendrites. In addition, $\beta$ and $\alpha_2$-$\delta$ subunits have been observed in neuronal cell bodies, dendrites and neuropil. Thus, mechanisms have to exist to account for the different sorting of various channel complex combinations in neurons. The Madin-Darby canine kidney (MDCK) epithelial cell line has been widely used to study the polarized transport of neuronal proteins. This appears to be due to a correlation between apical versus basolateral transport in epithelial cells and somatodendritic versus axonal sorting within neurons.[228,229] Thus, it was interesting to analyze whether L-type channels distribute differently from N- and P/Q-type channels as would be predicted from their neuronal distribution. In MDCK cells, coexpression of $Ca^{2+}$ channel subunits $\alpha_1$, $\beta$ and $\alpha_2$-$\delta$ was necessary to study the channel complex targeting.[230] Interestingly, L-type channels targeted to the basolateral side (comparable to somatodendritic targeting) and N-type channels to the apical side (comparable to the presynaptic targeting). However, P/Q-type channels distributed to the apical side when assembled with $\beta_1$ and $\beta_4$ subunits, while $\beta_2$ assembled channels were transported to the basolateral side. $\beta_3$ subunit assembled channels were distributed equally to both poles of the cell.[230] The results indicate that the channel type as well as the subunit combination determines channel targeting within polarized cells, pointing to a complex pattern of protein interactions necessary to decide if a channel is transported to dendrites, the soma or axons within neuronal cells.

Neurons transport newly synthesized membrane proteins along axons by microtubule- mediated fast axonal transport.[70] Using GFP tagged proteins various vesicle types were identified, which are involved in the transport process. For example Nakata et al[231] identified punctate structures as tubulovesicular organelles with various incorporated GFP fusion proteins like GAP-43, SNAP-25, synaptophysin, trkA and TGN-3, which moved along the axon. Other studies in sympathetic nerve fibers revealed that synaptobrevin, a highly enriched presynaptic vesicle protein involved in transmitter release,[232] was present in large dense-core vesicles and in lighter vesicles, whereas SNAP-25, syntaxin and two types of $Ca^{2+}$ channels (N- and L-type) were only found in lighter vesicles.[233] In hippocampal neurons GFP-tagged synaptobrevin was found in so called cytoplasmic transport packets consisting of dense-core vesicles, pleiomorphic small vesicles and tubulovesicular structures. Colocalization revealed the existence of P/Q-type $Ca^{2+}$ channels, synaptic vesicle proteins SV2 and synapsin I and a component of the endocytosis machinery normally localized at presynaptic terminals (amphiphysin I).[234] In addition, N-type channels could be localized to membranes of secretory granules in neurosecretory cells.[235-237]

Colocalization of the GFP-$\beta_4$ construct with endogenous synaptobrevin II and the presynaptic P/Q-type $Ca^{2+}$-channel $Ca_v2.1$ $\alpha_1$ subunit in hippocampal neurons suggests that GFP-$\beta_4$ is also targeted and clustered at presynaptic terminals. Costaining between the dendritic marker MAP2 and GFP-$\beta_4$ revealed that $\beta_4$ is found in the axon as well as in the dendrites. The localization and targeting involves the nonconserved N- and C-terminus of $\beta_4$ subunits. Deletion of $\beta_4$ N- and C-termini localized $\beta_4$ to the soma with drastically reduced punctate clustering.[238] So far several synaptic targeting signals have been described.[239-241] Synaptobrevin contains a 92 amino acid long N-terminal region,[242] mGluR7 a 60 amino acid domain,[243] the neuronal glycine transporter contains a di-leucine motif[244] and the transferrin receptor a tyrosine based motif,[242] but so far no truly conserved motif has been described. However, a di-leucine motif is conserved in all $\beta$ subunits, while a tyrosine based motif (EEDY) is found in $\beta_1$, $\beta_3$ and $\beta_4$ but not $\beta_2$.

Mutations in the ancillary subunits cause severe malfunctions. A mutation in $\beta_4$, which results in a loss of $\beta_4$ subunit in the mouse, leads to an epileptic phenotype (lethargic; lh/lh).[245] Excitatory synaptic transmission in lethargic mouse is reduced, suggesting a presynaptic function for $\beta_4$ subunits in transmitter release.[246] Indeed, overexpression of GFP-tagged $\beta_4$ subunits in hippocampal cells but not the non targeted $\beta_4$ deletion mutant altered short term synaptic plasticity, i.e.,paired pulse facilitation.[238] Paired pulse facilitation involves an increase in the presynaptic $Ca^{2+}$ levels at low release probability synapses.[247,248] Thus, changes in paired pulse facilitation reflects a presynaptic function of $\beta_4$ subunits. The change in paired pulse facilitation might be explained by a change in the biophysical properties of the presynaptic $Ca^{2+}$ channel (i.e.,inactivation from the open state), assuming that the overall population of presynaptic $Ca^{2+}$ channels have assembled with $\beta_4$ rather than with $\beta_1$ or $\beta_3$ subunits.

Fast synaptic transmission at specific synapses in the CNS depends on the concerted action of the $Ca^{2+}$ channel $Ca_v2$ family, including N-, P/Q- and R-type channels.[249-254] It has been suggested, that different types of channels are required for kinetically distinct phases of release, depending on their precise location relative to the fusion machinery and the electrophysiological characteristics of the channel type involved.[102,251,255] Distant $Ca^{2+}$ channels located far from release sites may contribute little to the $Ca^{2+}$ concentration necessary to trigger transmitter release but may increase the residual $Ca^{2+}$ following an action potential influencing short term plasticity like paired pulse facilitation.[256] For example, P/Q-type $Ca^{2+}$ channels at granule to Purkinje cell synapses or calyx type terminals in the rat medial nucleus couple more efficiently to release sites than N-or R-type channels, as suggested by the steeper $Ca^{2+}$ dependence of transmitter release.[102,251] Therefore, the precise localization of $Ca^{2+}$ channel complexes within the synaptic terminal will determine the synaptic output. Recently, it has been shown that N- and P/Q-type channels interact with modular adaptor proteins. The modular adaptor proteins Mint-1, CASK and Veli form a tripartite complex within the presynaptic terminal, which may underlie the precise localization of the presynaptic terminals. The interaction with the $Ca^{2+}$ channel $\alpha_1$ subunit involves the sequence motif (E/D-X-W-C/S-COOH), which is located at the C-terminal end of the long splice variants of $Ca_v2.1$ and $Ca_v2.2$ but not in the $\alpha_1$ subunit C-termini of the R- or L-type channels. This sequence interacts with the PDZ domain of Mint-1 and the SH3 domain of CASK. The CASK interaction also involves a proline rich region in the middle of the $\alpha_1$ subunit.[257] The binding motif within the C-terminus of the N-type $\alpha_1$ subunit is also necessary for sufficient targeting and clustering of the N-type channels, when exogenously expressed with $\beta_3$ and $\alpha_2$-$\delta_1$ subunits in hippocampal cells. Deletion of the C-terminus and/or disruption of the PDZ and SH3 binding motif within the C-terminus results in disruption of channel clustering.[258]

The understanding of $Ca^{2+}$ channel assembly and targeting in particular in brain is at its infancy. The development of new fluorescent tags and dyes for life time imaging with combined new microscopic techniques and physiological recordings will shine more light into our brain and other mysterious organs like testis and pancreas.

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# CHAPTER 8

# The Calcium Channel and the Transmitter Release Site

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## Summary

The $Ca^{2+}$ channel exhibits multiple interactions at the presynaptic terminal. Not only is the channel critical for the action potential-gated influx of $Ca^{2+}$ ions that leads to the activation of the transmitter release mechanism, a feed-forward pathway, but it is in itself modulated by a complex web of molecular interactions that involve both transmitter release site and non-release site proteins. Clearly, we do not yet nearly understand the full complexity of these molecular interactions since entirely new facets are being discovered apace. However, the picture is emerging of a highly structured and yet subtly designed molecular machine that is involved in complex forward and backward regulatory mechanisms in the control of transmitter release and, hence, of synaptic strength.

## Background

### *Involvement of $Ca^{2+}$ Ions in Transmitter Release*

The importance of $Ca^{2+}$ in the transmission of the neural impulse was recognized initially by Locke in a classic publication in 1894.[1] This paper which occupied 2 sides of Pflugers Archiv demonstrated that the indirect (via the motor nerve) activation of muscle occurred in the presence of external $Ca^{2+}$ ions but was not supported by an alternative divalent cation, barium. Further elucidation of the role of $Ca^{2+}$ occurred in tandem with the development of the chemical basis of neurotransmission and its key features, including the release of transmitter from secretory vesicles and the demonstration and acceptance of the ion channel hypothesis. Key discoveries were made by Katz and colleagues. They first recorded the miniature potential and demonstrated that it must be composed of more than one transmitter molecule and proposed the hypothesis that each quantum of transmitter corresponded to the release of the contents of the secretory vesicle.[2] In 1967 Katz and Miledi[3] carried out the critical experiment of applying external $Ca^{2+}$ and depolarizing pulses separately, demonstrating that evoked transmitter release only occurred when external $Ca^{2+}$ was combined with nerve terminal depolarization. This strongly supported the hypothesis that $Ca^{2+}$ ion entry into the nerve terminal during the action potential triggers transmitter release. This hypothesis was established on demonstrations in the squid giant synapse that transmitter release could be triggered by the direct injection of $Ca^{2+}$ ions into the nerve terminal, in the absence of membrane depolarization,[4] and that $Ca^{2+}$ ions actually enter the terminal during action potentials, as demonstrated by the $Ca^{2+}$ sensitive luminescent dye Aequorin.[5] The basic features of presynaptic $Ca^{2+}$ channel behaviour were established by Llinas in the first voltage clamp analysis of $Ca^{2+}$ currents in the presynaptic nerve terminal[6] which was followed by an analysis of the input/output kinetics of $Ca^{2+}$ entry and transmitter release.[7,8]

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### *$Ca^{2+}$ Channel Diversity*

Until the mid 1970s it was generally assumed that there was only one $Ca^{2+}$ channel species which was blocked by drugs such as verapamil and nifedipine. Indeed, the idea that there were might be more than one $Ca^{2+}$ channel type was strongly resisted. The eventual dissection of the distinct types was achieved based on two main criteria: the biophysical characteristics of the current,[9] and, more importantly, with toxins and drugs that exhibit differential $Ca^{2+}$ current block. It was established that verapamil or nifedipine-like drugs blocked the L-type (now $Ca_v1.2/.3/.4$) channel. The T-type ($Ca_v3.1/.2/.3$) $Ca^{2+}$ current component was relatively easily distinguished by its low voltage thresholds and inactivation range and also its barely resolvable single channel current events.[10] Tsien and colleagues proposed the existence of N-type ($Ca_v2.2$) $Ca^{2+}$ channel[11-13] on the basis of whole cell and single channel properties. The discovery of the N-type $Ca^{2+}$ channel blockers from cone shell venom, ω-conotoxin GIVA (ω-CTX), provided a highly specific tool to dissect these channels from their close relatives.[14] The P type $Ca^{2+}$ channel ($Ca_v2.1$), was discovered in cerebellar Purkinje cells[15] and was found to be blocked by a spider toxin, Agatoxin GIVA.[16] The demonstration that there were two classes of $Ca^{2+}$ currents with respect to their Agatoxin GIVA sensitivity led to the notion that there were two distinct channel types, dubbed the P and Q types. However, once it was agreed that these subtypes derive from the same fundamental $Ca^{2+}$ channel species the channel became known as the 'P/Q type'. The $Ca_v2.3$ (R-type) channel was named to account for the $Ca^{2+}$ current that could not be blocked with the L, N- or P/Q type specific toxins. Further characterization and nomenclature of $Ca^{2+}$ channels is dealt with in detail elsewhere in this volume (Chapters 3 and 4).

## Presynaptic $Ca^{2+}$ Channel Types

As discussed above, during the early 70s it was generally accepted that there was only one type of $Ca^{2+}$ channel and also that transmitter release required $Ca^{2+}$ influx through $Ca^{2+}$ channels. However, verapamil, which blocked smooth muscle (L-type) $Ca^{2+}$ current did not affect transmitter release.[17,18] Thus, the pharmacology of transmitter release indicated that there was more than one type of $Ca^{2+}$ channel several years prior to the recognition that there was more than one $Ca^{2+}$ channel species.[9] Indeed, in most nerve terminals transmitter release is not regulated by L-type $Ca^{2+}$ channels,[19-21] the type blocked by verapamil, and it was demonstrated both at the vertebrate chick ciliary ganglion calyx synapse[22] and at the invertebrate squid giant synapses[23] that the presynaptic $Ca^{2+}$ current was insensitive to this drug. However, the L-type channel is often predominant at synapses that mediate tonic secretion, non-neuronal presynaptic structures, such as at hair cells[24] and retinal receptors[25,26] but also at tonically secreting retinal ganglion neurons.[25-28] In contrast, N-type channels are commonly associated with transmitter release. This was recognized very soon after the discovery of ω-CTX.[14,19,29-31] It appears, however, that evolution appears to have gradually favored P/Q-type $Ca^{2+}$ channel as the predominant $Ca^{2+}$ channel at presynaptic terminals. This channel type was first associated with presynaptic terminals and transmitter release by Llinas and colleagues[15,32] and has been localized to a great number of mammalian presynaptic terminal types, as determined by the sensitivity of these terminals to specific blockers,[33,34] most notably Agatoxin GIVA.[34,35] Many synapses exhibit a predominance of the N and P/Q types but in others a combined treatment of these blockers for L, N and P/Q still does not eliminate transmitter release with residual attributed to the R type $Ca^{2+}$ channel.[36-38] Which channel type predominates at any specific synapse appears to be highly regulated with complex combinations at different synapses in any one species. It should be noted that much of this analysis has relied on toxin block: the actual $Ca^{2+}$ current combination has only been examined with direct voltage clamp techniques at a handful of synapse types. These range from pure L-type[27] to virtually pure N-type[39] and to terminals where the $Ca^{2+}$ channel complement is complex[40,41] and changing with time.[41] It is also of interest that the T-type $Ca^{2+}$ channels are very uncommon at presynaptic terminals, with compelling evidence only at the non-neuronal rod photoreceptor.[42] The general absence

of this channel type from neuronal nerve terminals raises the question whether the T-type channel group lacks a molecular feature required for targeting to nerve terminals.

## $Ca^{2+}$ Channel Localization at the Presynaptic Nerve Terminal

### *Synaptic Delay Places the $Ca^{2+}$ Channels at the Release Sites*

Since nerve terminals are generally exceedingly small structures the location of the $Ca^{2+}$ channels that gate transmitter release was never considered an issue. Definitive evidence for a localization of the $Ca^{2+}$ channels at the release site was concluded from measurements of synaptic delay. In a classic experiment at the voltage clamped presynaptic terminal of the squid giant synapse Llinas triggered transmitter release using a pure $Ca^{2+}$ tail current.[7] The membrane potential was rapidly depolarized to beyond the $Ca^{2+}$ current reversal potential so that the $Ca^{2+}$ channels opened but, due to the lack of a net driving force, no $Ca^{2+}$ influx occurred. When the membrane potential was repolarized an instantaneous rapid influx of $Ca^{2+}$ ions occurred through the open channels as the channels started to close. The latency from the onset of $Ca^{2+}$ influx to the first detected transmitter release in the postsynaptic axon was 0.2 ms. Since it can be presumed that in order to trigger release the $Ca^{2+}$ ions bind directly to the release site (and not some remote trigger) and, hence, must diffuse from the open channel to their binding sites, the available time limits the distance from one to the other to less than 100 nm. The subsequent demonstration that a single release site could be activated by a single $Ca^{2+}$ channel[43] argued for an even tighter co-localization, estimated to be <25 nm (see below).

### *Morphological Evidence*

The first glimpse of nerve terminal structure at the molecular level was obtained by the application of the freeze-fracture experimental technique to the frog neuromuscular junction. This technique takes advantage of the fact that when biological tissue is frozen the water-filled spaces are harder than the lipid-filled ones and when the tissue is literally 'fractured' the rupture plane occurs primarily along the bimolecular lipid membranes. The fractured tissue is shadowed with platinum vapor, the tissue is digested off and the resulting surface replica examined in an electron microscope. The membranes are generally seen as smooth flat surfaces. However, proteins that extend through the membrane remain associated with one or other membrane leaflet and can be observed as particles with a characteristic size in the freeze fracture replica. At the frog neuromuscular junction the transmitter release site is observed as two parallel arrays of large particles separated by a smooth hump.[44] Within these linear arrays the particles are distributed with a periodicity of ~16 nm.[45] Secretory vesicle exocytosis was observed immediately external to these particle arrays and it was hypothesized that the large particles correspond to the $Ca^{2+}$ channels themselves.[46] An approximate correlation was noted at the squid giant synapse between the number of functional channels, as assessed by $Ca^{2+}$ current recording, and the number of large particles at the release sites.[47] However, it remains possible that these particle arrays may include other large proteins known to be located close to the release sites, including the calcium-activated potassium channel (BK).[48] Haydon et al[49] used atomic force microscopy to examine the distribution of $Ca^{2+}$ channels at the release face of the chick ciliary ganglion calyx synapse. The channels were identified with biotinylated ω-conotoxin GVIA and then tagged with avidin-coated colloidal gold particles. At this synapse the channels do not form the orderly array observed at the frog neuromuscular junction but instead form clusters at the release site. However, linear arrangements were observed within the cluster and a nearest-neighbor analysis revealed an inter-channel distance of ~16 nm, in close correspondence with the inter-particle distance in freeze-fracture.[45] Recently Harlow et al [45] have applied computer-assisted tomography to electron micrographs of the frog neuromuscular junction to explore the structure of the protein 'scaffold' underlying the releases sites. This scaffold included longitudinal proteins, beams, within the inter-particle hump region and transverse proteins, ribs

that could be superimposed on the freeze fracture array of particles and that appear to terminate on the secretory vesicles. In addition, small processes projected from the ribs to the surface membrane in a pattern that was similar to the particle array in freeze fracture images, suggesting that these 'pegs' represent the anchors for the $Ca^{2+}$ Channels.

## The Calcium Channel and the Triggering of Transmitter Release

### *Action Potential Gated $Ca^{2+}$ Influx*

$Ca^{2+}$ ions, as with any other ions, move across the cell membrane subject to two forces: a concentration gradient, in this case a high $Ca^{2+}$ ion concentration on the outside drives the ions inward, and, since the ion is charged, a membrane potential gradient. Thus at resting membrane potentials (~-65 mV in a nerve terminal) there is a strong double force attracting the ions into the cell. However, admission in depends on a diffusion pathway and for a cell membrane this is usually (but not exclusively) via the opening of voltage sensitive $Ca^{2+}$ channels. The voltage sensitivity of $Ca^{2+}$ channel opening is quite steep ensuring that at the normal negative resting membrane potentials these channels are very unlikely to open. Thus, there is a very low probability that spontaneous $Ca^{2+}$ channel flickering will trigger transmitter release. However, this steep dependence ensures that during a depolarization, even a very rapid one, a significant fraction of the channels will open. What fraction that is depends on multiple factors: the amplitude and shape of the depolarization; the biophysical properties of the channel (rate constants, cooperativity) and whether the channels are modulated by other factors. The typical nerve terminal action potential has a waveform that triggers what is essentially a $Ca^{2+}$ channel tail current. Thus, the abrupt depolarization at the beginning of the spike to a very positive membrane potential, ~+60 mV opens the channels but also removes the driving force for $Ca^{2+}$ entry. The action potential ends as abruptly as it begins triggering a rapid influx of $Ca^{2+}$ ions as the membrane potential moves away from the reversal potential—but also the closure of the channels—resulting in a pulse of $Ca^{2+}$ ion influx that gates the secretory mechanism. Note that action potential triggered tail current $Ca^{2+}$ entry has the very distinct advantage of being very rapid **and** very abrupt and the total duration of the inward $Ca^{2+}$ current is typically less than 0.5 ms.[50] Note that the amplitude of the action potential-triggered $Ca^{2+}$ pulse can be affected by several different factors: the $Ca^{2+}$ concentration gradient (such as by external $Ca^{2+}$ depletion); the duration of the action potential (such as by the excitability of K channels); the number of available $Ca^{2+}$ channels (such as by voltage-insensitive block); the voltage dependence of the $Ca^{2+}$ channels opening (such as by G protein inhibition, Chapter 10) and by the voltage dependence of $Ca^{2+}$ channel closing.

### *Latency of Secretion*

As discussed above, for most nerve terminals we can approximate $Ca^{2+}$ ion entry as a very brief duration inward pulse. The latency between this pulse and the onset and peak of transmitter release is an important value with respect to the understanding of the transmitter release mechanism since it sets a time limit to the reaction sequence between binding of the $Ca^{2+}$ ion to its receptor and fusion of the vesicle with the membrane. As discussed above, the first accurate estimate of this latency was by Llinas and colleagues giving a minimum latency of 0.2 ms. Since this time interval includes both vesicle fusion, diffusion of the transmitter across the cleft and binding and activation of the postsynaptic receptor it can be seen that the latency between $Ca^{2+}$ binding to the release mechanism and the onset of fusion must be very fast indeed. This time interval could be termed the 'killer amendment' since it sets such a rigorous limit to any model designed to explain how the release mechanism works.

### *$Ca^{2+}$ Dependency of Transmitter Release—Multiple Ion Gating of Release*

One of the extraordinary features of transmitter secretion at a presynaptic nerve terminal is the element of control. The nerve terminal goes from a spontaneous vesicular fusion frequency

of ~1/sec (as with miniature endplate potentials at the neuromuscular junction) to rates that are several orders of magnitude higher and then abruptly falls to very low levels again. This acceleration depends on two main factors: first the very brief $Ca^{2+}$ influx pulse discussed above, and second, the finding that it appears to take the binding of multiple $Ca^{2+}$ ions to the transmitter release site to actually gate the fusion mechanism. This conclusion is based on the early work of Robinson[51] and Dodge and Rahamimoff.[52] These authors noted that when the external $Ca^{2+}$ ion concentration is varied the transmitter release triggered by an action potential does not go up as a linear function but far more steeply. Dodge and Rahamimoff described this dependence by a power relation such that transmitter release was the fourth power of the external $Ca^{2+}$ ion concentration. If it could be assumed (and as has been shown for $Ca^{2+}$ channels in general and at the presynaptic nerve terminal of the squid giant synapse) that within the low external $Ca^{2+}$ ion concentration range $Ca^{2+}$ ion entry is essentially a linear function of external $Ca^{2+}$ ion levels the fourth power dependency would seem to reflect the binding of the $Ca^{2+}$ ions to the release site itself. Thus, at least four $Ca^{2+}$ ions must bind to the release site to trigger the fusion of the secretory vesicle. Since this discovery the steep dependence of release on cytoplasmic $Ca^{2+}$ concentration has been described as $Ca^{2+}$ cooperativity. However, this term has caused some confusion since the term cooperativity can be used both for a process triggered by the action of several independent ligands, as here, and for a change in the binding characteristics of the target protein for a second ligand upon binding of the first, as is the case for $O_2$ binding to hemoglobin. It is not known whether the gating of the release site involves one or both of these so called 'cooperative' actions.

## *$Ca^{2+}$ Dependency of Transmitter Release—Domain Size*

The above consideration of cooperativity refers solely to the titration of $Ca^{2+}$ ion concentration against the activation of the transmitter release site, it does not consider the complications introduced by the distribution of $Ca^{2+}$ channels and the fact that at nm resolution $Ca^{2+}$ does not enter the terminal evenly across the surface membrane but does so only at the open mouth of the ion channel. Thus, when the channel is open there is a sharp drop-off in $Ca^{2+}$ ion concentration radially away from the pore in all directions, both inwardly into the cell and along the inner surface membrane.[53] It is believed that this concentration gradient is established and dissipates virtually instantaneously after channel opening and closing. This area of elevated $Ca^{2+}$ ion concentration was originally called a 'domain' but is usually referred to as a 'microdomain'. Unfortunately, some confusion has arisen by the use of this term for both the $Ca^{2+}$ plume generated by a single channel or the plume resulting when a cluster of nearby $Ca^{2+}$ channels open. This double use is unfortunate since the two are functionally different in that when a single channel opens the domain can be thought of as essentially an instantaneously all-or-nothing, or a quantal, event whereas with multiple channels the amplitude and radial dimensions of the domain are graded. For this reason I will use the term 'nanodomain' here for the $Ca^{2+}$ influx through a single channel and 'microdomain' for that through multiple simultaneously opening, and colocalized channels.

One can increase the amount of $Ca^{2+}$ entering the cell in two fundamentally different ways that have very different implications as to how the secretion sites in the cell are organized: first, as above, by a graded external $Ca^{2+}$ concentration or, second, by maintaining external $Ca^{2+}$ constant and recruiting an increasing number of $Ca^{2+}$ channels (such as with depolarizing pulses of different amplitude). The contrast between these methods is that in the former there is a true and continuous titration of $Ca^{2+}$ influx into the cell, even at the highest spatial resolution. However, when the number of channels activated is increased, locally $Ca^{2+}$ influx goes up **quantally** since current through an open channel is an all-or-nothing event. If we compare these methods at the level of the nanodomain the fundamental difference is that with graded external $Ca^{2+}$ the number of nanodomains remains constant while the amplitude through each is varied whereas with graded $Ca^{2+}$ channel recruitment the amplitude of the $Ca^{2+}$ nanodomain is constant but the number of such domains varies.

Evidence suggests that in most secretory cells the ion channels appear to be sufficiently remote from the transmitter release sites that secretion can be modeled as if the individual channels are essentially irrelevant: the $Ca^{2+}$ ions from each channel pool so that in effect in both cases there is a global increase in concentration. In such cells it would make no difference if the $Ca^{2+}$ dependence of transmitter release were titrated by graded or quantal $Ca^{2+}$ influx.

### *Where Is the Magic Button? $Ca^{2+}$ Binding Sites on the Release Mechanism*

The initial demonstration that transmitter release required both the depolarization of the nerve terminal and the presence of external $Ca^{2+}$ ions[3] was one of the critical pieces of evidence indicating that an influx of $Ca^{2+}$ ions is required to gate the release mechanism. This immediately begged the question of what comprises the $Ca^{2+}$ binding site? Katz and Miledi speculated that the divalent $Ca^{2+}$ ion simply cross links the two membranes to trigger fusion, an attractive, if simplistic hypothesis. However, this idea could not account for the tight linkage between $Ca^{2+}$ influx and transmitter release nor the consistent $4^{th}$ power cooperativity. These key features are more compatible with a specific protein-based gating mechanism. Other ideas that have been proposed include transmitter release triggered by a calcium-gated ion channel that either releases the transmitter directly from the cytoplasm[54] or the secretory vesicle[55] or that leads to the intracellular rupture of the secretory vesicle.[56] Such ideas have been generally dropped in favor of hypotheses based on the role of a 'SNARE' complex fusion mechanism and $Ca^{2+}$ binding sites on synaptotagmin, a secretory vesicle integral membrane protein (but see ref. 57). The fact that release is blocked upon cleavage of any of the SNAREs with botulinum toxins[58] is compelling evidence that these proteins are critical to the fusion mechanism. However, whether this complex comprises the fusion mechanism itself or simply forms a frame to enable fusion by some other mechanism has not as yet been resolved. The C2B domain of synaptotagmin, an integral protein of the secretory vesicle, is an attractive putative $Ca^{2+}$ binding site:[59,60] the protein is known to interact with both the $Ca^{2+}$ channel itself (see below) and with the SNARE complex while synaptotagmin knockouts exhibit a greatly reduced $Ca^{2+}$-dependent transmitter release,[61] as does molecular modification of the putative $Ca^{2+}$ binding site.[59] Other studies have suggested more complex $Ca^{2+}$ binding sites resulting from the tertiary structure of the intertwined SNARE protein complex. It should be noted that all of the putative four cooperating binding sites may not have the same $Ca^{2+}$ binding characteristics[62,63] and may not even be located on the same protein.

## Calcium Channel Interaction with the Proteins of the Transmitter Release Site

Early concepts of the presynaptic nerve terminal organization did not recognize that the location of the $Ca^{2+}$ channel within the nerve terminal is critical for rapid transmitter release. The demonstration that transmitter release occurred at a sub-millisecond interval after $Ca^{2+}$ entry[7] was critical to change this concept. Since the $Ca^{2+}$ ions can only diffuse a fraction of a μm within this time interval the $Ca^{2+}$ channel had to be located within a small distance of the transmitter release site. The subsequent finding that the transmitter release site could be gated by the opening of a single $Ca^{2+}$ channel[43] provided an even greater restriction to the channel-release site distance and provided functional evidence that $Ca^{2+}$ channels must be an integral component of the release site complex.

In the early 90s a number of studies demonstrated that proteins which were then or subsequently identified as associated with the release site complex could be co-immunoprecipitated with N-type[64-66] and P-type[67] $Ca^{2+}$ channels. It was recognized early on that this protein link, in particular via the protein syntaxin, might represent a mechanism whereby the channels were actually attached to the release site.[66,68] Sheng et al[69] demonstrated that syntaxin, and subsequently several of the proteins involved in exocytosis, bound to a defined 'synprint' region of the N-type $Ca^{2+}$ channel on its II-III linker region (the cytoplasmic loop between the 2nd and 3rd of the four transmembrane domains). SNAP-25, a surface membrane-associated

protein, has also been reported to link to the $Ca^{2+}$ channel as has the vesicle protein synaptotagmin[65,68,70,71] and RIM,[72] a potential release site scaffolding protein that interacts with Rab3. While the synprint region plays an important role in $Ca^{2+}$ channel-release site protein interactions it is not obligatory for nerve terminal function since since invertebrate ion channels that lack this region still exhibit normal rapid transmitter release.[73]

Ion channels are finely tuned devices and are sensitive to changes in their immediate environment. The demonstration of a specific protein binding site raised the possibility that this interaction might have functional consequences on the activity of the $Ca^{2+}$ channel. Cysteine string protein (CSP) was the first transmitter release site-associated protein suggested to modulate the $Ca^{2+}$ channel. This protein was discovered independently both as a *Drosophila* mutant[74] and as a potential $Ca^{2+}$ channel subunit on the grounds that coexpression of CSP with $Ca^{2+}$ channels greatly enhanced the net $Ca^{2+}$ current.[75] However, the protein was subsequently localized to the secretory vesicle.[76,77] This observation led to a novel hypothesis linking these two findings: that when the secretory vesicle docks at the transmitter release site CSP increases the excitability of nearby $Ca^{2+}$ channels.[76] Thus, when an action potential subsequently invades the nerve terminal the CSP-bound, and hence, secretory vesicle associated, $Ca^{2+}$ channels will be preferentially recruited. While CSP appears to have multiple functions in transmitter release,[78,79] the $Ca^{2+}$ channel modulation hypothesis is supported by the observation that CSP greatly enhances the $Ca^{2+}$ current at intact presynaptic nerve terminals.[80]

Recent studies have identified what may be a different interaction between CSP and $Ca^{2+}$ channels. In these studies the effect of CSP on N-type $Ca^{2+}$ channels was examined by co-expression of the proteins in a cell line. The $Ca^{2+}$ channels were inhibited by a mechanism that likely involves G proteins since the effect was relieved by a strong depolarizing prepulse (see below for an introduction to G protein inhibition of $Ca^{2+}$ channels).[81] Biochemical studies indicated that CSP can bind to both the G protein $\alpha$ and $\beta\gamma$ subunits. While this interaction is interesting there is no clear evidence as yet that CSP generates a tonic G protein-dependent inhibition at the presynaptic nerve terminal.

A different effect has been noted when $Ca^{2+}$ channels are co-expressed with the release site-associated protein, syntaxin I. The evoked $Ca^{2+}$ current exhibited a negative shift in its 'steady state inactivation characteristics (refs. 82-90, but see ref. 91, an effect that is seen with both syntaxin 1A and syntaxin1B.[92] In practical terms, this means that at a given resting membrane potential and in the presence of syntaxin I the $Ca^{2+}$ channel is likely to be less recruited during a transient depolarization. This effect has attracted a considerable degree of attention (see reviews, ref. 87) since it has been demonstrated that the negative shift in the inactivation curve can be reversed by the simultaneous expression of other presynaptic proteins including the integral vesicle protein, synaptotagmin. This latter observation led to the following hypothesis.[82] At the intact release site the $Ca^{2+}$ channel is generally inhibited by its interaction with syntaxin I but when the secretory vesicle docks one of its associated proteins relieves the syntaxin I effect making the local $Ca^{2+}$ channel preferentially excitable. Thus, when an action potential invades the nerve terminal the $Ca^{2+}$ channel next to a 'cocked' secretory vesicle will be more likely to open. This hypothesis is essentially the same as that suggested for CSP and could be complementary since syntaxin could be the 'off' switch while CSP is the 'on'. However, two findings are not entirely consistent with this conclusion. First, the finding that SNAP-25, a non-vesicular, surface membrane release site protein and a functional partner with syntaxin I,[93] could also relieve the inhibition has made this hypothesis less attractive. Second, the observations that $Ca^{2+}$ currents in intact presynaptic nerve terminals, where the $Ca^{2+}$ channel should be syntaxin I associated, are very resistant to steady-state inactivation and the finding that cleavage of syntaxin I with botulinum toxin C1 at intact nerve terminals fail to cause large shifts in the $Ca^{2+}$ current inactivation curve raise questions about the biological relevance of this effect at the release site.[94,95] In contrast, a study using $Ca^{2+}$ sensitive dyes detected an enhanced influx of depolarization-triggered $Ca^{2+}$ influx into synaptosomes after botulinum treatment.[96]

## Modulation of Presynaptic $Ca^{2+}$ Channels

The recognition that $Ca^{2+}$ channel activity can be up or down regulated via second messenger pathways has led naturally to the question as to whether these pathways play a role in the regulation of transmitter release at synapses. Since the numbers of presynaptic terminals is in vast excess over that of the postsynaptic neurons the potential additional processing complexity afforded by regulating transmitter release is enormous.

Interestingly, only a few second messenger pathways have been proposed to regulate presynaptic $Ca^{2+}$ channels. The most important, ubiquitous and generally accepted of these is the membrane delimited G protein linked pathways. This mechanism is of particular interest since it affords a potentially highly rapid and effective means of controlling transmitter release. A second pathway that has been implicated in the modulation of transmitter release is via protein kinase C (PKC) which has been reported to enhance N type $Ca^{2+}$ current.

### *G Protein Mediated Inhibition*

The inhibition of $Ca^{2+}$ channels via neurotransmitter-induced,[97] G-protein mediated pathways has long been recognized as a potentially critical mechanism for the modulation of transmitter release at synapses.[97,98] This mechanism of inhibition is described to be 'membrane delimited' and 'voltage sensitive'. The membrane delimited term results from the observation that $Ca^{2+}$ channels recorded in a cell-attached patch are not inhibited when the GPCR agonist is applied to the extra-patch regions of the cell. This isolation is attributed to the fact that the active component of the trimeric G protein, the $\beta\gamma$ subunit, is membrane anchored and, hence, can not traverse the pipette/membrane seal region. The 'voltage sensitivity' term recognizes that the G protein-dependent inhibition of the $Ca^{2+}$ channel is rapidly relieved by a preceding depolarization. In excitatory synapses the most common GPCR mediating this type of inhibition is the Adenosine type1 receptor though several other GPCR types inhibit presynaptic $Ca^{2+}$ channels. As for most N-type $Ca^{2+}$ channels[99,100] the G proteins involved in this modulation at the presynaptic terminals are usually, if not exclusively, of the $G\alpha_{O/I}$ family,[101] despite the fact that other $G\alpha$ protein types can mediate this pathway in experimental models.[100,102]

There has been some debate as to whether the inhibition of presynaptic $Ca^{2+}$ channels via the G protein pathway actually plays a physiological role in the release of neurotransmitter and whether, since the inhibition is voltage sensitive, it can be relieved by trains of impulses. The answer to these questions are complex and may depend on the cell type or cellular structure examined. Thus, when DRG neurons were used as an experimental model, G protein activation clearly reduced the evoked $Ca^{2+}$ current when tested with short duration action potentials.[103] Many studies have reported that activation of presynaptic GPCRs will inhibit transmitter efflux but few have tested if this inhibition can be attributed to an effect on $Ca^{2+}$ channels. In a study at the parallel fiber to Purkinje cell synapse activation of GABA(B) or adenosine(A1) receptors reduced both transmitter release and $Ca^{2+}$ influx.[104]

If G protein activation can indeed inhibit transmitter release by reducing $Ca^{2+}$ influx then this raises the question as to whether this inhibition be relieved by a train of impulses. This question is complicated by two issues: intrinsic changes in the biophysical properties of the $Ca^{2+}$ channel, such as by inactivation, and, where transmitter release is used as a monitor of channel behaviour, superimposed train-dependent facilitation or depression of the release mechanism. Studies in the DRG cell model, as above, indicated G protein-dependent $Ca^{2+}$ channel inhibition could indeed be relieved during an action potential train.[102] However, in the parallel fiber/Purkinje cell presynaptic model treated with GPCR agonists very little increase (15%) was noted in the amplitude of the action potential-induced $Ca^{2+}$ influx during a train, suggesting that action potential trains were unlikely to result in much inhibitory relief.[104] These two studies can be reconciled by the fact that the action potentials used in the DRG neurons were probably of significantly longer duration than those normally occurring in nerve terminals.

Hence, unless a specific synapse type utilizes action potentials of unusually long duration, inhibitory relief due to action potential trains may play a minor factor in neural coding.

The G protein-mediated inhibition pathway is known to be highly rapid. This speed results in part from the involvement of a minimum number of second messenger elements and to the membrane delimited nature of the pathway. The speed from GPCR activation to $Ca^{2+}$ current inhibition has been reported to be as fast as ~1 sec in rat superior cervical ganglion neurons.[105] We do not as yet know how fast this inhibitory pathway is in nerve terminals. The finding that cleavage of syntaxin, a protein associated with the transmitter release site, interferes with the G protein-dependent inhibition of presynaptic $Ca^{2+}$ channels suggests that at the nerve terminal this inhibitory mechanism may be an integral element of the release site itself. Thus, it is possible that the speed of inhibition may be much faster than that determined in somata experimental models.

The finding that cleavage of syntaxin using botulinum toxin C1 interferes with the G protein-dependent inhibition of presynaptic $Ca^{2+}$ channels suggested a structural and functional coupling between G-protein action and the transmitter release site.[94] It was subsequently found that expression of syntaxin 1A in tsA-201 cells induced a tonic G-protein inhibition that could be greatly attenuated with botulinum toxin C treatment.[106] Recent evidence has suggested that in vitro syntaxin is capable of linking the Gβ subunit to the $Ca^{2+}$ channel resulting in tonic inhibition. Of particular interest is the finding that this action appears to occur independently of Gγ suggesting that syntaxin may act as a Gγ substitute.[91]

### *Protein Kinase C*

Initial findings that PKC stimulation could increase cellular $Ca^{2+}$ currents suggested that this may represent a unique direct mechanism for the enhancement of transmitter release. However, a detailed study on rat superior cervical ganglion neurons concluded that this increase in transmitter release mediated by PKC could be attributed entirely to the relief of inhibition via the G protein pathway.[107] Few studies have contradicted this conclusion but a recent report suggests that the PKC activator enhances the N-type $Ca^{2+}$ current via changes in channel kinetics, even in the presence of the tonic G protein inhibitor GDPβS and hence, independently of the G protein pathway.[108] The interaction of these pathways is of particular interest and recently protein kinase C has been reported to antagonize syntaxin binding to the channel II-III linker[109] while phorphorylation of the I-II linker upregulates channel activity and antagonizes G protein inhibition.[110]

## Commentary

Since we still have a relatively limited knowledge of how the transmitter release site functions with respect to vesicle exocytosis it is highly likely that we are also barely scraping the surface with respect to the complexities of presynaptic $Ca^{2+}$ channel function. This field has been hampered by the general inaccessibility of presynaptic nerve terminals to direct experimentation. The emergence of immunoprecipitation as a means of detecting biochemically interacting proteins in neural extracts and cellular expression systems as a means to analyze $Ca^{2+}$ channel functional interactions with potential molecular neighbors has resulted in a surge of findings with respect to the modulation of $Ca^{2+}$ channels. However, enthusiasm with these approaches must be tempered with the simple fact that these approaches alone demonstrate only what **can** happen. In order to determine if these phenomena actually do occur it is essential to return to the intact presynaptic terminal, despite its complexities. At this stage it is not extreme to hypothesize that the $Ca^{2+}$ channel responds to a host of competing influences: synapse development, incorporation into the release site, docking and exocytosis of secretory vesicles and extraneous modulatory influences, to name but a few. Resolving which particular molecular interactions mediate these processes and how they are suppressed or override each other will be the primary challenge for work in this field.

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# CHAPTER 9

# Determinants of G Protein Inhibition of Presynaptic Calcium Channels

**Aparna Nirdosh and Gerald W. Zamponi**

## Summary

The modulation of presynaptic calcium channels following the activation of G-protein coupled receptors is a key regulatory mechanism of synaptic transmission. The past two decades have yielded a tremendous advance in our understanding of this phenomenon at the molecular level. It is now widely accepted that the closed conformation of the channel is stabilized upon binding of G protein $\beta\gamma$ dimers directly to the cytoplasmic region linking domains I and II, and perhaps also to the C-terminus region, of the calcium channel $\alpha_1$ subunit. The channels consequently become reluctant to open, resulting in inhibition of current activity. The molecular mechanisms that control G protein regulation of calcium channels are immensely complex, as the extent of modulation depends on the membrane potential, G$\beta$ subunit subtype, the presence of ancillary calcium channel subunits, PKC-dependent phosphorylation of the channel, RGS proteins, and on interactions with the presynaptic vesicle release protein complex. These intricate interactions between second messenger pathways, synaptic release proteins, calcium channels and G proteins allow for the potential of fine tuning the entry of calcium into the presynapse, and consequently, neurotransmitter release.

## Introduction

The influx of calcium ions through voltage gated calcium channels triggers a wide array of cytoplasmic processes, including excitation contraction coupling in heart muscle, the activation of calcium dependent enzymes, calcium dependent gene transcription, and the release of neurotransmitter from presynaptic nerve terminals.[1-6] Most neurons express multiple types of calcium channels (i.e., L-, N-, T-, P-, Q-, and R-types), of which the N-type and P/Q-type are targeted predominantly to presynaptic nerve terminals.[7-10] Calcium entry through these channels is triggered by incoming action potentials, which have propagated along the axon, and culminates in the release of neurotransmitter. Hence, any inhibition of these channels by either pharmacological agents, or cytoplasmic messengers, results in decreased neurotransmission. One of the key cellular mechanisms regulating presynaptic calcium influx is the activation of G protein coupled receptors (GPCRs) linked to either $G\alpha_o$ (the main neuronal G$\alpha$ isoform) or $G\alpha_i$ proteins (see ref. 11). Agonist binding to these receptors activates a cytoplasmic messenger response that ultimately depresses calcium channel activity. The significance of this inhibitory pathway is illustrated by the opioid receptor control of pain transmission in dorsal root ganglion cells. Activation of opioid or opioid like receptors downregulates N-type calcium channel activity, and hence, the transmission of pain signals in the spinal cord (for review, see ref. 12). Here, we shall provide an overview of our current state of understanding of the molecular mechanisms that underlie GPCR control of presynaptic calcium channels.

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## Functional Consequences of Direct G Protein Action on Calcium Channels

The phenomenon of G-protein inhibition of calcium currents was first discovered over two decades ago.[13] Using chick DRG neurons, Dunlap and Fischbach[13] elegantly showed that the application of serotonin or adrenergic receptor agonists resulted in a pronounced inhibition of calcium currents. In the ensuing years, a number of additional GPCRs have been shown to inhibit voltage gated calcium channels, including muscarinic, opioid and somatostatin receptors.[2,14-26] It was also noted that the receptor mediated inhibition could typically be abolished following treatment with pertussis toxin (an inhibitor of $G\alpha_o$ and $G\alpha_i$ proteins), and could be blocked with GDPβS. These results indicated that a G protein pathway was involved in calcium channel inhibition.[15] However, curiously, the inhibitory effects appeared to occur independently of a soluble second messenger, giving rise to the term "direct membrane delimited inhibition"[14] (for review, see ref. 11).

In whole cell patch clamp recordings, membrane delimited G protein inhibition of N-type and P/Q-type calcium channels is observed as a reduction in peak current amplitude, accompanied by an often dramatic slowing of the apparent rate of activation, and inactivation (see Fig. 1A, inset). Moreover, the degree of inhibition is strongly dependent on the membrane potential such that inhibition is more effective at hyperpolarizing voltages.[17,18,27] This effect arises predominantly from a rightward shift in the activation curve of the channel, which indicates that in the presence of G proteins, channels have a reduced probability of opening at any given potential. This pronounced voltage dependence results in a destabilization of the G protein inhibited channel conformation at depolarized voltages, such that G protein inhibition can be effectively relieved by application of strongly depolarizing voltage steps (or prepulses before the test depolarization, see Fig. 1B)[11,27-30] or following a rapid train of action potentials.[31,32] This has given rise to the term "prepulse facilitation," although the term "prepulse relief of G protein inhibition" is probably a more accurate reflection. It is noteworthy that the relief from inhibition following the rapid successive membrane depolarizations may contribute to the magnitude of paired pulse facilitation at the synapse, such that in the presence of G protein receptor agonists, the degree of paired pulse facilitation is augmented.[33,34] Thus, the de-inhibition of G protein modulated presynaptic calcium channels could significantly contribute to short term plasticity.

Single channel patch clamp recordings reveal that the first latency to opening is increased following GPCR activation, without any detectable effect on single channel conductance.[35] It is interesting to note that this can account for all of the functional consequences at the whole cell level. The increase in first latency to opening results in a slowing of the macroscopic time course of activation, and a reduced open probability at intermediate test potentials. At greater membrane depolarizations, the decrease in first latency becomes diminished, thus resulting in less inhibition. Moreover, due to the occurrence of late openings, the apparent macroscopic time constant for inactivation appears increased, thus accounting for the slower inactivation kinetics. The G protein induced change in the probability of opening gave rise to the terminology of "willing" (i.e., non inhibited) and "reluctant" (inhibited) gating modes of the channels.[17,25,27,36,37] Thus, in order for a channel to open, the channel needs to become disinhibited by the G protein, hence accounting for the increase in first latency to opening. However, this may not be universally true, as there is increasing evidence from modeling data that N-type channels (but apparently not P/Q-type channels) are able to undergo reluctant openings, albeit with a relatively low probability.[38,39] As will be discussed in the ensuing section, the disinhibition of channels appears to be linked to a physical dissociation of the G proteins from the channel complex. It may ultimately be interesting to attempt to correlate G protein dissociation with channel kinetics by combining single channel recordings with FRET analysis.

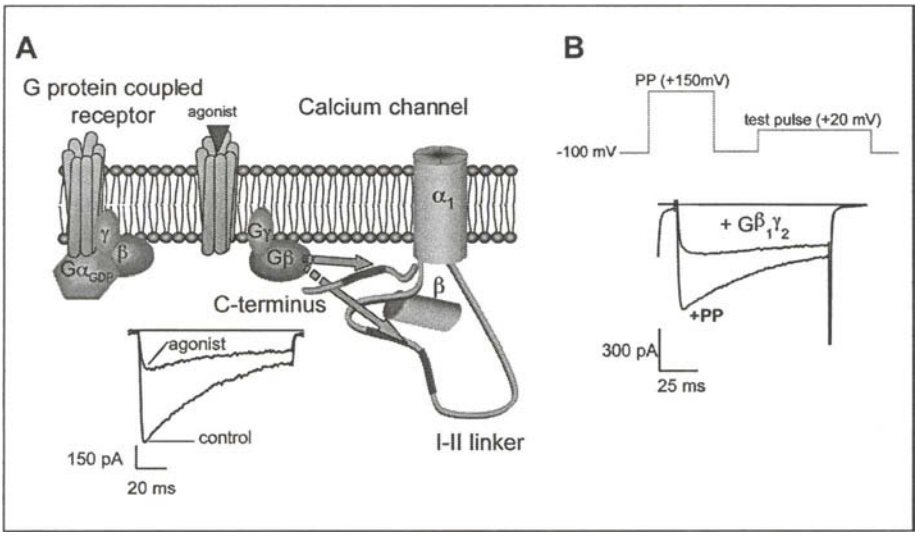



Figure 1. A) Schematic representation of GPCR signaling to N-type calcium channels. Activation of a GPCR results in the dissociation of the heterotriomeric G protein complex. The G protein βγ subunit physically interacts with binding sites in the calcium channel domain I-II linker, and in the C-terminus region. Inset: Whole cell patch clamp recording from a tsA-201 cell expressing $Ca_v2.2$ ($+\beta_{1b}+\alpha_2\text{-}\delta$) channels. Application of 100 nM somatostatin (agonist) activates endogenous somatostatin receptors and results in a peak current inhibition, as well as kinetic slowing. B) Prepulse relief of tonic G protein inhibition mediated by overexpression of $G\beta_1\gamma_2$ in tsA-201 cells expressing N-type channels. Top. Pulse paradigm to study prepulse relief. A 50 ms prepulse to +150 mV 5 ms prior to a typical test depolarization results in unbinding of the G proteins from the channels, thus revealing disinhibited N-type currents (see recordings +PP). In the absence of the prepulse, a smaller current with slower kinetics is observed.

## Stoichiometry between G Proteins and the Calcium Channel

Nearly a decade ago, there was a debate about the G protein/channel stoichiometry, and as to whether G proteins physically dissociate during prepulse relief.[11,17,20,25,36,37] Boland and Bean[36] suggested that G protein inhibition of N-type calcium channels involved a simultaneous interaction of the channel with 4 G-proteins (see review by ref. 11). Moreover, the authors suggested that prepulse relief required the dissociation of the G protein subunits.[36] Golard and Siegelbaum[25] proposed an analogous model based on two G protein molecules. Other groups suggested different models in which strong membrane depolarizations were proposed to result in a temporary conformational change in the channel protein, which decays over time.[17] This question is best addressed with a stoichiometric experiment examining the dependence of recover from prepulse relief on G protein concentration. One possible means to address this issue is to examine the G protein concentration dependence underlying the inhibition. In case of a conformational change, one would expect the recovery kinetics to be G protein concentration independent. This was examined by Zamponi and Snutch[28] by adding various concentrations of purified G protein subunits to the intracellular recording solution, showing that the time constant for re-inhibition of the channel following the application of the prepulse was inversely proportional to the G protein concentration in the pipette. Moreover, the concentration dependence was best described by a model based on a 1:1 stoichiometry between the G proteins and the channel.[28] Subsequently, this was supported further by elegant modeling data of Bertram and Behan.[34]

## G Protein Modulation Depends on the Nature of the Calcium Channel Subunits

Among native calcium channels, membrane delimited inhibition is typically not observed with L-type, T-type, or R-type calcium channels. In contrast both N-type and P/Q-type calcium channels can be inhibited by G proteins, albeit to different extents,[40] with the N-type channels typically showing a greater degree of inhibition. As with many other functional properties of voltage gated calcium channels, a detailed investigation into the molecular details of G protein inhibition is more effectively conducted using recombinant expression systems. For transiently expressed calcium channels, the selective modulation of presynaptic calcium channels, and more specifically, the differential inhibition of N-type and P/Q-type channels was first examined by Bourinet et al[41] in *Xenopus* oocytes. Using co-expressed μ-opioid receptors to activate G proteins, neither $Ca_v1.2$ nor $Ca_v2.3$ channels seemed to be susceptible to inhibition. In contrast, $Ca_v2.2$ (N-type) showed a robust (~55%) inhibition that was two-to three fold greater than that of $Ca_v2.1$ (P/Q-type) channels. However, the modulation of the $Ca_v2.1$ channels was dramatically enhanced when the calcium channel β subunit was omitted, consistent with data of Campbell et al[42] This important result was subsequently confirmed by a number of other groups.[43-45] Curiously, in the absence of the β subunit, $Ca_v2.3$ channels became sensitive to G protein inhibition,[46,47] indicating that the structural homology among the $Ca_v2$ calcium channel family allows for a fundamentally conserved ability of $Ca_v2$ channels to be regulated by G proteins. The fact that the presence of the calcium channel β subunit could regulate the extent of G protein inhibition of $Ca_v2$ channels raised that possibility that the type of calcium channel β subunit that is co-expressed could affect G protein regulation. Indeed, as shown recently by both the Dolphin laboratory (see refs. 48, 49) and our group,[50] this is indeed the case. Both for receptor mediated inhibition, and in the presence of co-expressed G proteins, the nature of the calcium channel beta subunit appears to regulate the extent of G protein inhibition of N-type calcium channels. In particular, in the presence of the rat $\beta_{2a}$ subunit, the extent modulation and particularly the degree of kinetic slowing became dramatically enhanced (Dolphin lab, also see refs. 50-52). The rat $\beta_{2a}$ subunit is unique as it contains two cysteine residues at its N-terminal and which can be palmitoylated.[53] Replacement of these cysteines with serines significantly attenuates the effect of $\beta_{2a}$ on kinetic slowing;[50] suggesting the possibility that palmitoylation of calcium channel subunits could be a regulatory mechanism of G protein inhibition. To date, it is unclear if the remaining calcium channel subunits ($\alpha_2$-δ, γ) are determinants of G protein inhibition of calcium channels.

## Gβ Subunits Mediate Calcium Channel Inhibition

The activation of GPCRs results in the dissociation of the Gα subunit from the Gβγ complex. Historically, the Gα subunit was considered to be the active G protein species, but it is now clear that the Gβγ dimer also acts as an essential signaling molecule (Fig. 1). An involvement of the Gβγ complex in N-type calcium channel inhibition was first suggested by Bourinet et al[41] but first demonstrated by Ikeda[54] and Herlitze et al[55] The authors showed that overexpression of Gβγ, but not Gα could mimic the effects of GPCR activation on calcium channel function (see also Fig. 1B). Along these lines, addition of purified Gβγ subunits to the intracellular solution in patch clamp experiments is sufficient to induce G protein regulation.[56] The degree of G protein inhibition appears to depend on the type of Gβ subunit present.[30,49,57-59] However, there is conflicting information in the literature as to the precise Gβ subunit dependence of the inhibition. Garcia et al[57] reported that in bullfrog sympathetic neurons, $G\beta_1$ and $G\beta_2$ both effectively inhibited N-type channel activity, with a smaller effect caused by $G\beta_3$, while no inhibition was observed with either $G\beta_4$ or $G\beta_5$. Using the same experimental setting, Ruiz-Velasco and Ikeda[58] determined that all of the Gβ isoforms with the exception of $G\beta_5$ inhibited N-type channels. However, both groups utilized native N-type channels, which due

to alternate splicing[60] and β subunit heterogeneity may not form a homogeneous population of channels. Using tsa-201 cells as an expression system to co-express individual types of calcium channels with cloned Gβ subunits, allows one to investigate the interaction between a homogeneous population of calcium channels with a single identified G protein species. Using this approach, Arnot et al[30] reported that rat $Ca_v2.2$ channels were effectively inhibited by $G\beta_1$ and $G\beta_3$, with less inhibition by $G\beta_4$ and $G\beta_2$, and no effect by $G\beta_5$. This contrasts with data obtained with human $Ca_v2.2$ channels[61] where a small degree of inhibition is also seen with $G\beta_5$. Rat $Ca_v2.2$ channels were most potently inhibited by $G\beta_4$,[30] but overall seemed to be less susceptible to inhibition, consistent with the earlier work of Bourinet et al.[41] More recently, Feng et al[50] showed that the relative rank order of the inhibitory effects of different Gβ subunits on N-type calcium channel activity is affected by the type of calcium channel β subunit, thus adding further complexity to the regulation of presynaptic calcium channels by Gβγ. Despite several attempts,[62,63] it remains to be determined whether individual types of GPCRs can couple to N-type calcium channels via specific subsets of Gβ subunits.

The Gγ subunit cannot by itself support G protein inhibition of voltage gated calcium channels; however, it is not clear if its presence is required for inhibition. While Herlitze et al[55] showed that in transient expression systems, the Gγ subunit is not needed, Ikeda's work[54] in intact neurons suggest a need for Gγ to be present. Transiently expressed Gβ might perhaps be able to combine with endogenous Gγ. Even if there is a need to co-express a Gγ subunit, the particular type of G protein γ subunit seems to be a relatively minor determinant of calcium channel inhibition.[58]

Taken together, the differential modulation of individual presynaptic calcium channel complexes, by different combinations of Gβγ subunits adds to the capability of neurons to fine tune presynaptic calcium channel activity via GPCR activation.

## Channel and G Protein Structural Basis of G Protein Modulation

The first attempt to delineate the calcium channel structural determinants of Gβγ modulation was based on a chimeric approach using $Ca_v2.2$ and $Ca_v2.1$ channels as a template.[64] This work suggested that domain I, and the carboxy terminal region of the channel, were important for G protein inhibition. By combining electrophysiological, molecular biological and biochemical techniques, Zamponi et al[56] and DeWaard et al[65] identified two separate sites in the domain I-II linker of $Ca_v2.1$ and $Ca_v2.2$ channels that could physically bind to Gβγ subunits (Fig. 1A). It is interesting to note that one of the two Gβγ binding sites in the I-II linker overlaps with the calcium channel β subunit interaction site, consistent with the observation that calcium channel β subunit subtype regulates G protein action.[41,42,48,50] Despite some contradictory results by Qin et al[47] who suggested that the I-II linker region was inconsequential for G protein action, a number of other groups have now shown that the domain I-II linker is of critical importance of G protein action.[66,44] However, additional evidence implicating the C-terminus region of the $Ca_v2.3$ calcium channel as a G protein target has been presented,[47] suggesting that there may be as many as three distinct G protein binding sites on the N-type calcium channel (Fig. 1A). To make matters even more complicated, work from the Mori laboratory[67-69] revealed that the C-terminus region of P/Q-type calcium channels could also associate with Gα subunits in vitro but the exact functional significance of this finding remains unclear. In addition, other regions of the N-type calcium channel have also been implicated in G protein inhibition, including the N-terminus.[45,51,70-72] However, as no direct binding interactions between Gβγ and this region of the channel have been shown, it seems likely that this region is involved in G protein inhibition of the channel in general, rather than in G protein binding per se. For example, it is possible that the N-terminus could be involved in translating G protein binding into a change in the activation properties of the channel.

Unlike with the channel structural determinants that control G protein inhibition, only limited information is available about the Gβ subunit structural determinants. A study by Ford et al[73] identified several important residues on Gβ that seem to regulate the ability of $G\beta_5$ to modulate N-type channels, but interestingly, these residues are entirely conserved in all type of Gβ subunits, and hence cannot account for the Gβ subtype dependent effects observed by us and other groups.[30,57,58] Thus, there must be other residues in Gβ that contribute to G protein inhibition of presynaptic calcium channels. Our group has recently elucidated G protein β subunit structural determinants that underlie the inhibition of N-type calcium channels. We showed that regulation of N-type channel activity is mediated by at least three distinct Gβ regions. Moreover, we identified a pair of asparagine residues in positions 35 and 26 of the $G\beta_1$ subunit that are unique to $G\beta_1$, and which allow this subunit to detect PKC-dependent phosphorylation of the N-type calcium channel I-II linker.[73a]

## Interactions between G Protein and Protein Kinase C (PKC) Pathways

The activation of PKC results in an intrinsic upregulation of N-type calcium channel activity.[56,74,75] In addition, however, PKC activation antagonizes inhibition of N-type calcium channels by a range of different GPCRs in both native neurons[76,77] and in transient expression systems.[56,59,75] Interestingly, the magnitude of this effect varies with GPCR subtype, indicating some specificity for the G protein cascades activated by these individual receptors. The putative Gβγ binding region of the N-type channel I-II linker contains two PKC consensus sites, T422 and S425.[56,75] Using site directed mutagenesis of these residues, Hamid et al[75] were able to show that showed that phosphorylation of either one of these two PKC sites resulted in an all or none upregulation of current activity. By contrast, PKC phosphorylation of the T422, but not the S425 residue, could antagonize G protein inhibition triggered by somatostatin receptor activation (see Fig. 2, ref. 75). The effect of phosphorylation of this residue differentially affected somatostatin and opioid receptor mediated inhibition of the channel.[59] Cooper et al[59] showed that only responses mediated by $G\beta_1$ were affected by phosphorylation of T422, while other types of Gβ subunits were not affected. Thus, depending on which Gβ subunit a particular GPCR can couple to, one may expect more or less G protein-PKC crosstalk, thereby perhaps proving a molecular explanation for the varying degrees of crosstalk seen in native cell.[77] and the differential sensitivity of opioid and somatostatin mediated inhibition to PKC activation.

In some types of native neurons, the effects of PKC dependent phosphorylation on N-type channel activity appear to be linked predominantly to a removal of tonic G protein inhibition rather than an intrinsic upregulation of N-type channel activity.[78] The differences with the work on expressed channels could perhaps be due to alternative splicing events, such as in the domain I-II linker region (see ref. 60), constitutive activation of other second messengers in these neurons, or perhaps even the type of calcium channel β subunit that is expressed.

## RGS Proteins and G Protein Modulation of Calcium Channels

A key regulatory mechanism of G protein signaling are regulator of G protein signaling (RGS) proteins. This family of proteins modulates G protein function by three pathways: First, they accelerate GTP hydrolysis of the Gα subunit,[79] thus favoring the reassociation of the Gαβγ complex and termination of receptor mediated G protein action.[80] Second, some members of the RGS family can interfere with the interactions between Gα subunits and their effectors.[81] Finally, several types of RGS proteins (RGS6, RGS7, RGS9, and RGS11) can associate with Gβ molecules (in particular, $G\beta_5$) through G protein gamma subunit like (GGL) domains, and interfere with Gβ action on effector systems.[82]

Effects of RGS proteins on G protein inhibition of calcium channels have only recently been described.[83-87] Muscarinic and adrenergic inhibition of N-type channels is attenuated by various RGS proteins (RGS3, RGS8, RGS4) via a reduction of free Gβγ levels.[84,86] Consistent

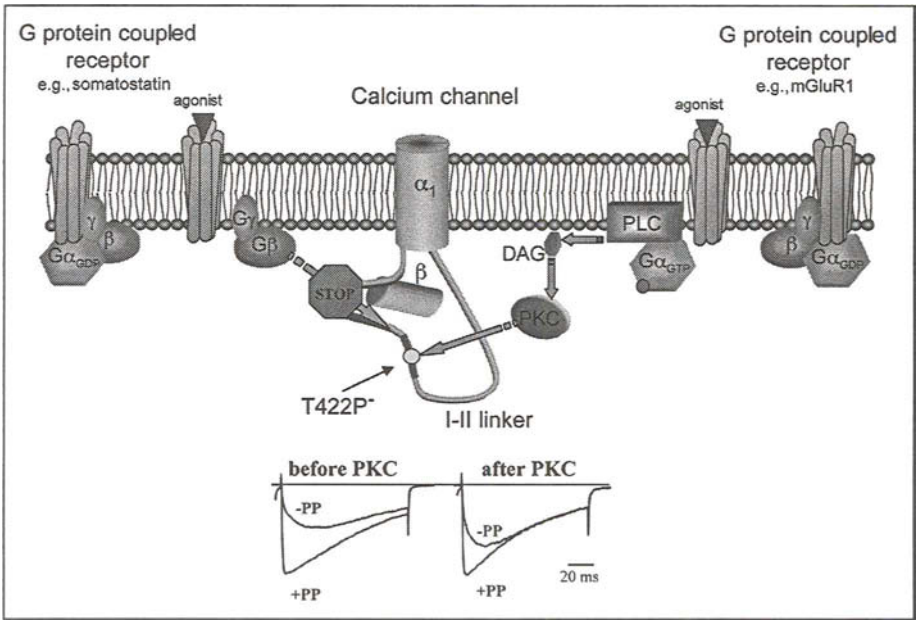



Figure 2. Top: Schematic illustration of G protein PKC crosstalk at the level of the N-type calcium channel. G protein modulation of mediated by somatostatin receptor activation is antagonized by activation of a receptor pathway coupled to PKC (i.e., mGluR1). Phosphorylation of residue T422 in the N-type channel domain I-II linker antagonizes G protein interactions with the channel. Bottom: In tsA-201 cells expressing the N-type calcium channel and $G\beta_1\gamma_2$, direct activation of PKC via phorbol esters reduces the degree of prepulse relief, thus indicating that G protein inhibition of the channels was antagonized by PKC activation (see ref. 59). The records were scaled to facilitate comparison. Note that the effect is specific for $G\beta_1$ mediated responses.

with such a mechanism, dialysis of neurons with an RGS4 antibody enhances norepinephrine inhibition of N-type channels in chick DRGs.[85] Finally, Zhou et al[61] showed that RGS proteins could regulate $G\beta_5$ mediated inhibition of heterologously expressed human N-type calcium channels. Taken together, these observations support an important role of RGS proteins in N-type calcium channel modulation.

## Interactions between G Proteins and Synaptic Release Proteins

Besides binding $G\beta\gamma$, presynaptic calcium channels also physically couple to proteins involved in neurotransmission, such as syntaxin 1 and SNAP-25. These proteins interact with the domain II-III linker regions of both $Ca_v2.1$ and $Ca_v2.2$ calcium channels.[88-90] This raised the possibility that there might be functional effects of synaptic proteins on G protein inhibition of voltage gated calcium channels. Indeed, experiments by Stanley and Mirotznik[91] showed that cleavage of syntaxin 1 via botulinum toxin C1 abolishes the ability of N-type calcium channels by G proteins (triggered by GTPγS application), suggesting that syntaxin 1A may be required in order for G protein inhibition of N-type channels to occur. And yet, in transient expression system that lack syntaxin (such as *Xenopus* oocytes or tsa-201 cells), G protein inhibition can be readily observed, raising the possibility that syntaxin 1A may perhaps only have a modulatory function. In accordance with such a mechanism, Jarvis et al[92] showed that the coexpression of syntaxin 1A with N-type calcium channels resulted in a tonic G protein inhibition of the channels in the absence of any exogenous activation of the G protein pathway (see

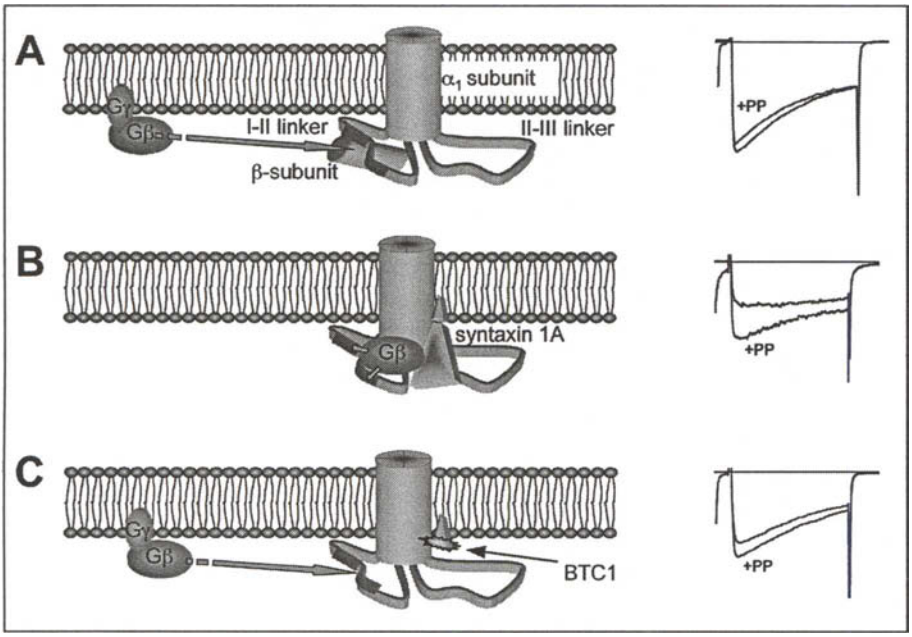



Figure 3. The presence of syntaxin 1A induces tonic G protein regulation of N-type channels expressed in tsA-201 cells. A) In the absence of syntaxin, endogenous Gβγ subunits are unlikely to associate with N-type channels, thus no prepulse relief can be observed (see whole cell tracings). B) Syntaxin colocalizes endogenous Gβ with the N-type channels, leading to tonic G protein inhibition which can be relived by application of a prepulse. C) Cleavage of syntaxin 1A with botulinum toxin C1 removes the tonic inhibition induced by syntaxin.

Fig. 3). Overexpression of a peptide corresponding to the syntaxin binding site on the channel, or cleavage of syntaxin 1 via botulinum toxin C abolished this effect (Fig. 3).[92,93] The authors also showed that syntaxin 1A could concomitantly bind to Gβ and to the N-type calcium channels, thus acting like a chaperone to colocalize free endogenous Gβ and the calcium channel. Interestingly, more recent work[94] showed that this binding interaction occurred in the absence of the Gγ subunit, raising the possibility that syntaxin 1A might perhaps act as a GGL protein. The syntaxin 1 induced modulation was maintained in the presence of other synaptic protein such as SNAP25 and n-Sec1,[93] and was also observed in intact chick DRG neurons.[95] Surprisingly, despite 85% sequence identity to syntaxin 1A, the syntaxin 1B isoform could not induce tonic modulation of N-type channels despite binding both to the channel and to Gβ.[94] Hence, depending on which syntaxin 1 isoform is expressed in a given neuron, tonic, receptor independent G protein regulation may or may not occur. Moreover, the notion that the expression of certain P/Q-type calcium channel isoforms can trigger the expression of syntaxin 1A through calcium dependent gene transcription[96] suggests the possibility that P/Q-type calcium channels can indirectly regulate N-type calcium channel activity. It is noteworthy that the syntaxin G protein interactions are not confined to calcium channels, but seem to play a major role in modulation of voltage gated potassium channels.[97]

Besides syntaxin 1A, cysteine string protein (CSP) also appears to chaperone G proteins towards to N-type calcium channel to induce tonic modulation.[98]

Moreover, both the J-domain and cysteine string domain of CSP appear to be able to stimulate G protein modulation of the channel, albeit through separate pathways, with the cysteine

string region serving to anchor G proteins close to the channel, whereas the effects of the J-domain do not depend on the interaction with the calcium channel per se, and may instead occur indirectly.[99] It remains unclear as to whether the binding of Gβ to CSP involves the Gγ subunit. The fact that synaptic proteins can interact with G protein subunits suggests the possibility that G proteins might be able to directly regulate synaptic activity, independently of their action on presynaptic calcium channels. Indeed, in lamprey neurons, the binding of Gβγ to syntaxin 1 and SNAP25 appears to regulate neurotransmitter release without involving voltage gated calcium channels,[100] thus adding to the complement of modulatory mechanisms in the presynapse.

## Conclusion

Over the past five years, tremendous advances in our understanding of G protein regulation of presynaptic calcium channels have occurred, including the identification of Gβγ as the active G protein species, novel insights into channel and G protein structure an function, and novel concepts of interactions between the synaptic release machinery and G proteins. This intricate web of complex interactions within the presynaptic nerve terminal provides for a plethora of avenues for precise control of calcium homeostasis, and exocytosis.

### *Notes Added in Proof*

Recently, we revealed that N-type calcium channels and opioid receptor like (ORL1) receptors form a signalling complex.[101] A low levels of constitutive receptor activity mediates tonic G protein inhibition of complexed channels in the absence of receptor agonist. As a result, N-type channel activity can be regulate by variations in ORL1 receptor density.

Two recent studies based on fluorescence resonance energy transfer measurements aimed to determine whether the calcium channel β subunit remains associated with P/Q-type calcium channels when G protein β subunits are activated. While the Herlitze laboratory[102] reached the conclusion that the calcium channel β subunit remains attached to the calcium channel in the presence of Gβγ, the deWaard laboratory[103] reported that the calcium channel β subunit dissociated from the channel following G protein activation. Our own electrophysiological measurements[50] support, in principle, the conclusions reached by the Herlitze group. Indeed, upon coexpression of different types of calcium channel β subunits with $Ca_v2.2$ calcium channels, the current kinetics seen in the presence of Gβγ vastly differ, which implies that the calcium channel β subunit can still regulate channel activity following G protein activation. This clearly argues against a dissociation of the calcium channel β subunit from the calcium channel complex.

Finally, work from the Barrett lab showed that $Ca_v3.2$ T-type calcium channels can be inhibited by G protein β2 subunits.[104]

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# CHAPTER 10

# Phosphorylation-Dependent Regulation of Voltage-Gated $Ca^{2+}$ Channels

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## Overview

Neurotransmitters, hormones, growth factors and extracellular matrix proteins bind to cell membrane receptors that, in turn, activate intracellular signaling cascades. Often, these cascades involve signaling by protein or lipid kinases and protein or lipid phosphatases. Protein kinases add phosphate groups to serine, threonine or tyrosine residues within proteins, and lipid kinases add phosphates to the inositol rings of certain lipids; phosphatases reverse this process. Phosphorylation or dephosphorylation can "switch-on" or "switch-off" protein or lipid activity. It has long been known that voltage-gated $Ca^{2+}$ channels are regulated through signaling events involving phosphorylation. In this chapter, we summarize recent findings in this field. We have attempted to minimize overlap with other recent reviews.[1-9]

### *Abbreviations*

AC= adenylyl cyclase; Akt/PKB= protein kinase B; AKAP= A kinase anchoring protein; β2AR= beta 2 adrenergic receptor; CaM= calmodulin; CaMKII= $Ca^{2+}$/calmodulin-dependent protein kinase II; GPCR= G-protein-coupled receptor; NCS-1= neuronal calcium sensor-1; nNOS= neuronal nitric oxide synthase; PDE= phosphodiesterase; PI3K= phosphoinositide 3-kinase; $PIP_2$= phosphatidylinositol 4,5-bisphosphate; $PIP_3$= phosphatidylinositol 3,4,5-trisphosphate; PKA= protein kinase A; PKC= protein kinase C; PKG= protein kinase G; PLC= phospholipase C; PP2A= protein phosphatase 2A; RTK= receptor tyrosine kinase; sGC= soluble guanylyl cyclase.

## Regulation of $Ca^{2+}$ Channels by Protein Kinase A (PKA)

L-type $Ca^{2+}$ channels display several different gating modes upon depolarization.[10] Mode 0 represents a closed state of the channel and is characterized by null sweeps, mode 1 is characterized by brief bursts of channel openings, and mode 2 is characterized by long openings and delayed deactivation upon repolarization. Mode 2 gating of L-type channels can be induced by strong depolarization, PKA-dependent phosphorylation, or some combination thereof (for review see ref. 6). As described by Hess et al[10] mode 2 can also be induced by 1,4-dihydropyridine (DHP) agonists such as Bay K 8644. Wilkens et al[11] recently demonstrated that strong depolarization and Bay K 8644 trigger mode 2 gating of cardiac L-type channels through distinct mechanisms. They mutated the DHP binding site within $Ca_v1.2a$, and showed that the mutant channel cannot respond to Bay K 8644, but can still respond to depolarization. They also constructed a chimera between $Ca_v1.2a$ and $Ca_v2.1$; the latter is a neuronal P/Q-type channel that does not exhibit mode 2 gating in response to either depolarization or Bay K 8644. This chimeric channel responded to Bay K 8644, but not to strong depolarization. Interestingly, the chimeric channel possesses all of the PKA concensus phosphorylation sites found within $Ca_v1.2a$,

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suggesting that the presence of these sites is by itself not sufficient for induction of mode 2 gating by strong depolarization.

Held et al[12] present data indicating that PKA-dependent regulation of skeletal muscle L-type channels ($Ca_v1.1$) involves the $\gamma_1$ subunit. In myotubes from $\gamma_1$-knockout mice ($\gamma_1$-deficient), steady-state inactivation is shifted by +15 mV and current densities are increased relative to wildtype myotubes. Both effects are reversed in $\gamma_1$-deficient myotubes by overexpressing $\gamma_1$. In contrast, overexpressing the distantly related $\gamma_2$ subunit (stargazin) had no effect. A membrane permeant cAMP analogue (8-Br-cAMP) increased L-type current amplitudes in wildtype but not in $\gamma_1$-deficient myotubes. Steady-state inactivation was unaffected by 8-Br-cAMP in either phenotype. Rp-cAMPs, an inactive cAMP-analogue that blocks activation of PKA, reduced current amplitude in $\gamma_1$-deficient myotubes without altering steady-state inhibition. Currents in wildtype cells were unaffected by Rp-cAMPs. Altogether, these findings suggest that $Ca_v1.1$ (or an associated protein, for example $\beta_{1a}$) is already phosphorylated by PKA in the absence of $\gamma_1$.

Wu et al[13] demonstrated that phosphatidylinositol 4,5-bisphosphate ($PIP_2$) affects the activity of neuronal P/Q-type $Ca^{2+}$ channels ($Ca_v2.1$) by two different mechanisms. First, application of exogenous $PIP_2$ slows rundown of $Ca_v2.1$ channels in giant inside-out patches pulled from *Xenopus* oocytes. Rundown is also slowed by intracellular ATP, but surprisingly, PKA-dependent phosphorylation does not mediate this effect. Instead, ATP is thought to replenish $PIP_2$ by activating membrane-associated lipid kinases. Second, $PIP_2$ causes voltage-dependent (VD) inhibition of $Ca_v2.1$. Thus, $PIP_2$ inhibits currents evoked by weak depolarizations more strongly than currents evoked by strong depolarizations. Interestingly, VD inhibition by $PIP_2$ is alleviated by PKA-dependent phosphorylation. The authors propose that $Ca_v2.1$ has two distinct binding sites for $PIP_2$. Binding of $PIP_2$ to a high-affinity site stabilizes channel activity (i.e., slows rundown), whereas binding of $PIP_2$ to a second, lower-affinity site shifts the channel into a "reluctant" gating mode. Binding of $PIP_2$ to the second site is thought to be antagonized by PKA-dependent phosphorylation.

## *AKAPs and $Ca^{2+}$ Channel Regulation*

A-kinase anchoring proteins (AKAPs) act as scaffolds that mediate colocalization of PKA and its substrates, including L-type $Ca^{2+}$ channels (for review see ref. 14). AKAPs are thought to enhance PKA-dependent regulation of native L-type channels.[15-18] Using a heterologous expression system (TsA-201 cells), Gao et al[16] showed that PKA-mediated regulation of $Ca_v1.2a$ could be reconstituted by coexpression of AKAP79. The reconstituted channel stimulation was considerably less robust than PKA-mediated stimulation of native L-type current in cardiac myocytes, however, suggesting that AKAPs may not be solely responsible for coupling PKA to L-type channels. Subsequent studies of AKAPs have generally supported an emerging model of ion channel regulation wherein scaffolding proteins coassemble molecular components of signal transduction cascades.[19,20] For example, coimmunoprecipitation assays demonstrate that AKAP15 and $Ca_v1.1$ form a complex in skeletal muscle.[17] From data obtained using a yeast two-hybrid system and coimmunoprecipitation from transfected TsA-201 cells, Hulme et al[21] proposed that AKAP15 interacts with $Ca_v1.1$ via a leucine-zipper (LZ) motif. An LZ motif within the carboxyl-terminus of $Ca_v1.1$ binds to a corresponding motif within AKAP15. Mutations within the LZ motifs of either AKAP15 or $Ca_v1.1$ disrupt this interaction. In mouse MM14 myotubes, dialysis of a peptide corresponding to residues 38-54 of AKAP15 blocks voltage-dependent facilitation of $Ca_v1.1$ as effectively as PKI (a pseudosubstrate inhibitor of PKA) or AP2 (a specific peptide inhibitor of the AKAP15 – PKA interaction). Interestingly, it has long been known that the carboxyl-terminal portion of $Ca_v1.1$ (the region that binds AKAP15, i.e., amino acids 1774-1821) is proteolytically cleaved from the rest of the $Ca_v1.1$ polypeptide.[22] This would seem to preclude close association between $Ca_v1.1$ and AKAP15. However, the corresponding region of the cardiac channel $Ca_v1.2a$, which is also thought to be proteolytically cleaved, remains associated with $Ca_v1.2a$ at the plasma membrane and regulates its function.[23,24] Thus, the cleaved carboxyl-terminus of the skeletal muscle $Ca_v1.1$ may also remain associated with the channel and thereby mediate its colocalization with AKAP15.

Davare et al[25] showed that cAMP-dependent stimulation of hippocampal L-type channels involves highly localized signaling. Previously, this group had reported that microtubule-associated protein 2B (an AKAP) coprecipitates with PKA and $Ca_v1.2$.[26] More recently, Davare et al[27] showed that phosphatase 2A (PP2A) is also constitutively associated with $Ca_v1.2$ and reverses stimulation of this channel by PKA. The new data of Davare et al[25] indicate that $Ca_v1.2$, PKA and PP2A form a macromolecular signaling complex with $\beta_2$-adrenergic receptors, G$\alpha$s and adenylyl cyclase. Thus, in cell-attached recordings from hippocampal neurons, endogenous L-type currents were enhanced only when $\beta_2$-receptor agonist was applied through the pipette, and not when it was bath applied. Importantly, these findings from neurons are consistent with demonstrations that cAMP signaling is tightly localized in cardiac myocytes.[28-30]

Recent evidence indicates that AKAPs may perform other important functions in addition to colocalizing PKA and its substrates. For example, Altier et al[31] showed that AKAP79 increases expression of $Ca_v1.2c$ at the cell surface. Unexpectedly, this effect of AKAP79 did not involve PKA. The increased surface expression of $Ca_v1.2c$ requires a polyproline region within its II-III linker, but direct binding of AKAP79 to this sequence was not detected. Altier et al[31] proposed that the polyproline region antagonizes surface trafficking of $Ca_v1.2c$, and that AKAP79 somehow counteracts this effect, possibly by interacting with $Ca_v1.2c$ through an unidentified adaptor protein.

## *Other Examples of PKA-Dependent $Ca^{2+}$ Channel Regulation*

Relatively little is known about regulation of $Ca_v1.3$ ($\alpha$1D), a neuroendocrine L-type channel. Chik et al[32] used RT-PCR to show that rat pinealocytes express primarily $Ca_v1.3$. Although whole-cell $Ba^{2+}$ currents were small (~50 pA), they were clearly inhibited in response to 8-Br-cAMP or activation of receptors ($\beta$-adrenergic or PACAP) that elevate intracellular cAMP. Channel inhibition was blocked by Rp-cAMPS. Even less is known about regulation of $Ca_v1.4$ ($\alpha_{1F}$), which has not yet been expressed in heterologous systems. However, Stella et al[33] showed that adenosine, acting through $A_2$ adenosine receptors, stimulates PKA to produce inhibition of L-type currents in salamander retinal rod cells. This effect was blocked by Rp-cAMPs. The L-type current in vertebrate retinal photoreceptors is presumably mediated by $Ca_v1.4$.[34]

Pemberton et al[35] reported that M3 muscarinic acetylcholine receptors stimulate native T-type channels in NIH 3T3 cells through PKA. Lenglet et al[36] presented evidence that 5-$HT_7$ receptors, G$\alpha$s, adenylyl cyclase, and PKA stimulate endogenous T-type $Ca^{2+}$ currents and aldosterone secretion in rat adrenal glomerulosa cells.

In freshly-dissociated rat amygdalar neurons, pharmacologically isolated P-type currents are potentiated by agents (isoproterenol or forskolin) that elevate cAMP.[37] When expressed in *Xenopus* oocytes from cerebellar mRNA, P/Q-type channels are increased by injection of cAMP.[38] Similar cAMP-dependent stimulation of P/Q-type current was observed by Fukuda et al[39] and Kaneko et al[40] in oocytes expressing $Ca_v2.1$. In each of the above studies, cAMP-dependent stimulation of P/Q-type currents was prevented by inhibitors of PKA. Stimulation of P/Q-type channels by PKA may be important in certain forms of neuronal plasticity.[37,41]

When expressed in *Xenopus* oocytes, N-type channels ($Ca_v2.2$; $\alpha_{1B}$) are only weakly potentiated by treatments that elevate cAMP.[39,40] However, such subtle PKA-dependent modulation of N-type channels may be physiologically important. For example, Herring and Paterson[42] showed that secretion of acetylcholine (ACh) by vagal nerve is increased by the nitric oxide donor sodium nitroprusside (SNP). This effect of SNP was blocked by $\omega$-conotoxin GVIA and by inhibitors of guanylyl cyclase or PKA, but not by a PKG inhibitor. The effect of SNP was also occluded by an inhibitor of phosphodiesterase 3 (PDE3). These results suggest that NO can stimulate N-type channels mediating vagal ACh secretion through a pathway involving (sequentially) NO, guanylyl cyclase, cGMP, PDE3, cAMP and PKA.

Activation of Gαs-coupled D1 dopamine receptors inhibits both N-type and P-type currents in rat nucleus acumbens medium spiny neurons.[43] Inhibition is mimicked by 8-Br-cAMP and is blocked by either PKI or okadaic acid. The authors suggest that channel inhibition results from dephosphorylation by a PKA-activated phosphatase. In summary, activation of PKA may produce either stimulation or inhibition of L-type, N-type, P/Q-type and T-type $Ca^{2+}$ channels. Additionally, there appears to be significant crosstalk between NO and cGMP-dependent signaling pathways and PKA-dependent pathways.

## Regulation of $Ca^{2+}$ Channels by Protein Kinase C (PKC)

A variety of membrane receptors activate phospholipase C (PLC), leading to production of 1,2-diacylglycerol (DAG). This signaling lipid activates both conventional (α, βI, βII, γ) and novel (δ, ε,η,θ) isozymes of PKC.[44] DAG is mimicked by phorbol esters such as 12-myristate, 13-acetate (PMA), and for this reason phorbol esters have been extensively used to activate PKC experimentally. However, it has recently become apparent that enzymes other than PKC are also activated by DAG.[45,46] Thus, effects of phorbol esters should always be confirmed using specific PKC inhibitors.

PKC-dependent regulation of cardiac and smooth muscle $Ca^{2+}$ channels has been the topic of numerous investigations (for reviews see refs. 5 and 8). Furthermore, "crosstalk" between PKC-dependent phosphorylation and G protein-dependent modulation of neuronal $Ca^{2+}$ channels has received considerable attention (see ref. 2 and Chapter 9 of this volume). We therefore limit coverage here to papers that have appeared since these previous reviews.

In nerve growth factor-differentiated PC-12 cells, neuropeptide Y inhibits L-type currents through a PKC-dependent pathway.[47] Using *Xenopus* oocytes, Blumenstein et al[48] expressed a human splice-variant of $Ca_v1.2a$ having a long amino-terminus. This L-type channel is stimulated by PMA through a pathway that is blocked by bisindolylmaleimide I (Bis I), a specific inhibitor of PKC. Love et al[49] demonstrated PKC-dependent stimulation of L-type channels in HIT-T15 cells, a clonal pancreatic β-cell line. Presumably, these channels are $Ca_v1.3$, because Scholze et al[50] cloned $Ca_v1.3$ from HIT-T15 cells and expressed it in *Xenopus* oocytes. Scholze et al[50] then demonstrated channel stimulation through a PKC-dependent pathway linked to coexpressed μ-opioid receptors. Thus, under some conditions both $Ca_v1.2$ and $Ca_v1.3$ channels can be stimulated by PKC.

PMA stimulates R-type $Ca^{2+}$ currents mediated by rat brain $Ca_v2.3$ expressed in *Xenopus* oocytes[51,52] or human brain $Ca_v2.3$ expressed in HEK 293 cells.[53] Rat brain $Ca_v2.3$ is also stimulated through activation of coexpressed metabotropic glutamate receptors (mGluR) that couple to PKC.[51] In these experiments, receptor-mediated stimulation of $Ca_v2.3$ was occluded by PMA, and staurosporine blocked channel stimulation by either PMA or mGluR activation. $Ca_v2.3$ cloned from rabbit brain, which contains a longer amino-terminus than the rat clone and is consequently susceptible to voltage-dependent inhibition by Gβγ dimers,[54] is also stimulated by either PMA or M2 muscarinic acetylcholine receptors.[55] Both rat and rabbit $Ca_v2.3$ channels are strongly stimulated through M1 muscarinic acetylcholine receptors[56] which couple strongly to PKC. Bannister et al[56a] showed that muscarinic stimulation of $Ca_v2.3$ involves signaling by Gαq/11, diacylglycerol, and a $Ca^{2+}$-independent PKC isozyme. Muscarinic stimulation of rabbit $Ca_v2.3$ decreases inhibition of these channels by Gβγ subunits, similar to the "crosstalk" previously reported for N-type ($Ca_v2.2$) $Ca^{2+}$ channels.

Wu et al[13] showed that $Ca_v2.1$ channels expressed in *Xenopus* oocytes are biphasically modulated by PKC. Application of PMA initially produces a modest stimulation of $Ca_v2.1$ currents, followed by a more substantial inhibition. Both effects are blocked by Bis I.

Morita et al[57] have identified a nifedipine-insensitive, rapidly-inactivating $Ca^{2+}$ current (NI-*ICa*) in guinea pig arteriole smooth muscle cells. Although NI-*ICa* is clearly not an L-type, N-type, P/Q-type or T-type channel, the authors argue that it is also not R-type (i.e., encoded by $Ca_v2.3$). Recently, Morita et al[58] showed that NI-*ICa* is stimulated through a purinergic pathway involving PKA, and inhibited through a separate purinergic pathway involving PKC.

### *PKC Can Regulate $Ca^{2+}$ Channels Indirectly by Triggering Receptor Desensitization*

Garcia et al[59] demonstrated that PKC phosphorylates, and thereby downregulates CB1 cannabinoid receptors in AtT-20 cells. PKC-dependent receptor downregulation significantly attenuates agonist-dependent inhibition of endogenous P/Q-type channels in these cells. Similarly, Wu et al[60] showed that a low concentration (10 nM) of PMA, which submaximally activates PKC, desensitizes 5-$HT_{1A}$ receptors and attenuates receptor-mediated inhibition of N-type channels in F11 rat dorsal root ganglion x mouse neuroblastoma hybrid cells. The attenuation of channel inhibition was mediated by a conventional PKC isozyme as it was prevented by the inhibitor Gö6976. Furthermore, mutation of a single PKC phosphorylation site (T149A) within the second intracellular loop of the 5-$HT_{1A}$ receptor abolished desensitization in response to 10 nM PMA.

## Regulation of $Ca^{2+}$ Channels by Protein Kinase G (PKG)

Protein kinase G (PKG) is activated by cyclic GMP (cGMP), which is generated by guanylyl cyclase (GC). Nitric oxide (NO) stimulates soluble guanylyl cyclase,[9,61] which can lead to $Ca^{2+}$ channel regulation by PKG. In an early study, Chen and Schofield[62] showed that NO donors (SNP and SNAP) increase whole-cell $Ca^{2+}$ currents (mostly N-type) in rat SCG neurons, but the involvement of cGMP was not documented in this study. NO donors or membrane-permeable cGMP analogues (e.g., 8-Br-cGMP) also stimulate whole-cell $Ca^{2+}$ currents (which include N-type) in rat DRG neurons.[63] In contrast, N-type currents in human neuroblastoma (IMR32) cells are inhibited by NO donors acting through cGMP and PKG.[64] Native L-type currents in NIH 3T3 fibroblast cells are inhibited by 8-Br-cGMP, and inhibition is prevented by KT-5823, a specific PKG inhibitor.[65] SNP also inhibits endogenous L-type channels in bovine adrenal chromaffin cells through a pathway involving cGMP.[66] Abi-Gerges et al[67] found that high concentrations of the NO donor DEANO (100 μM) antagonized β-adrenergic stimulation of cardiac L-type current through a pathway involving guanylyl cyclase, PKG and pertussis-toxin sensitive G proteins. Other NO donors (SIN-1, SNAP, SPNO) did not have the same effects as DEANO. In a subsequent paper, Abi-Gerges et al[68] reported that lower concentrations (1 pM – 1 μM) of both DEANO and SNAP potentiate, rather than inhibit, β-adrenergic stimulation of L-type current. This potentiating effect of NO donors appears to involve the adenylyl cyclase pathway, and to be independent of cGMP.

It is important to note that NO may also modulate $Ca^{2+}$ channel function through direct *S*-nitrosylation of $Ca^{2+}$ channels or their regulatory proteins. *S*-nitrosylation is independent of PKG and other enzymes. For an excellent review of this topic, see Ahern et al.[9]

It is worth mentioning here that internal perfusion with cGMP may produce effects not mediated by PKG. For example, Vandecasteele et al[69] showed that a low concentration (0.5 μM) of intracellular cGMP stimulated basal L-type currents in human atrial myocytes by inhibiting PDE3, a cGMP-inhibited phosphodiesterase. Inhibition of PDE3 results in elevated intracellular cAMP and activation of PKA, which enhances L-type current. In contrast, a high concentration of intracellular cGMP (5 μM) inhibited basal L-type currents by activating PDE2, a cGMP-activated phosphodiesterase.

In summary, regulation of $Ca^{2+}$ channels by cGMP may be complex, because in addition to altering the activity of PKG, changes in intracellular [cGMP] can alter the activity of PDEs, which can then produce changes in [cAMP] and PKA-dependent phosphorylation.

## Regulation of T-Type $Ca^{2+}$ Channels by $Ca^{2+}$/ Calmodulin-Dependent Protein Kinases (CaM Kinases)

Chen et al[70] showed that angiotensin II activates CaMKII in adrenal glomerulosa cells, and CaMKII also produces a hyperpolarizing shift in the activation of native T-type $Ca^{2+}$ channels. This hyperpolarizing shift results in enhanced $Ca^{2+}$ influx and enhanced aldosterone secretion in response to elevated serum [$K^+$]. In the same cell type, Barrett et al[71] showed that

$Ca^{2+}$-dependent increases in T-type current were blocked by inhibitors of CaMKII, and mimicked by constitutively-active mutant CaMKII. Wolfe et al[72] found that coexpression of CaMKIIγc stimulates recombinant T-type channels ($Ca_v3.2$; $\alpha_{1H}$) expressed in HEK 293 cells. CaMKIIγc increased channel activation at negative potentials without altering channel inactivation. Importantly, a closely-related T-type channel ($Ca_v3.1$; $\alpha_{1G}$) was not modulated by CaMKIIγc. These results indicate that some but not all T-type channels are stimulated through CaMKinases.

## Regulation of $Ca^{2+}$ Channels by Tyrosine Kinases

Regulation of ion channels through tyrosine kinases was recently reviewed by Davis et al.[7] Tyrosine kinases may be receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor, or nonreceptor tyrosine kinases such as Src. Studies concerning tyrosine kinases typically employ pharmacological inhibitors. Notably, certain inhibitors of tyrosine kinases block $Ca^{2+}$ channels directly.[55,73-75] For this reason, it is critical to employ control compounds that are chemically closely-related but which do not inhibit tyrosine kinase activity. Otherwise, direct channel block could be misinterpreted as basal channel regulation by tyrosine kinases.

Activation of α-adrenergic receptors antagonizes β-adrenergic stimulation of cardiac L-type $Ca^{2+}$ current ("α-adrenergic antagonism"). Belevych et al[76] showed that a tyrosine kinase-dependent signaling pathway underlies α-adrenergic antagonism in adult guinea pig ventricular myocytes. Intriguingly, Hool[77] presented evidence that α-adrenergic antagonism only involves tyrosine kinases under nonhypoxic conditions. During hypoxia, α-adrenergic antagonism appears to involve phospholipase $A_2$ instead.

Several growth factors are thought to signal through receptor tyrosine kinases. Long-term exposure to EGF increases the density of high voltage-activated $Ca^{2+}$ currents in pituitary GH3 cells without altering channel biophysical properties.[78] Exposure to nerve growth factor (NGF) maintains N-type and L-type current densities at their preisolation levels during long-term culture (6-15 days) of bullfrog SCG neurons.[79] Exposure to either NGF or brain-derived neurotrophic factor (BDNF) increases L-type currents in PC12 cells, but surprisingly, this effect is quite fast, requiring only 3-5 minutes.[80] These results imply that receptor tyrosine kinases can increase $Ca^{2+}$ channel expression and/or activity.

In chick DRG neurons, inhibition of N-type channels through $GABA_B$ receptors can be divided into two components: a voltage-independent, steady-state component (SSI) and a voltage-dependent kinetic slowing component (KS). Diversé-Pierliussi et al[81] showed that SSI could be selectively blocked by either genistein or srcKI (a specific peptide inhibitor of Src). These results suggest that Src mediates SSI but not KS.

Fitzgerald and Dolphin[82] showed that injection of the monomeric G protein p21-Ras or its downstream effector $pp60^{c\text{-}Src}$ increased whole-cell $Ca^{2+}$ current density in neonatal rat DRG neurons. Current density was also decreased by an antibody (Y13-259) directed against p21-Ras or a phosphopeptide (Trk490) that blocks Ras activation. $Ca^{2+}$ currents were also decreased following incubation of cells in serum-free media or media containing anti-NGFR antibody. Finally, currents were decreased by genistein but not its inactive analogue genistin. These results suggest that p21-Ras signals through the tyrosine kinase $pp60^{c\text{-}Src}$ to increase $Ca^{2+}$ current density in rat DRG neurons.

Blair et al[83] showed that native L-type channels in rat cerebellar granule neurons are potentiated by insulin-like growth factor-1 (IGF-1), which binds to a receptor tyrosine kinase. IGF-1 activates a signaling pathway involving the lipid kinase, phosphoinositide 3-kinase (PI3K) and the serine/threonine kinase Akt (also known as PKB). They show that Akt is responsible for potentiating L-type channels in these cells.

Regulator of G-protein signaling (RGS) proteins serve as GTPase-activating proteins or as effector antagonists for certain heterotrimeric G-proteins.[56,84-86] By accelerating GTP hydrolysis by Gα or by blocking interactions of Gα with downstream effectors, RGS proteins may influence signaling events that involve kinases or phosphatases. RGS proteins can significantly influence $Ca^{2+}$ channel regulation (see Chapter 9). Recently, Schiff et al[87] reported that RGS12

can directly associate with N-type $Ca^{2+}$ channels in chick DRG neurons. Intriguingly, this interaction appears to require channel phosphorylation by a tyrosine kinase. Experiments also indicate that RGS proteins themselves may be regulated through phosphorylation,[86] suggesting another indirect route by which kinases and phosphatases could influence $Ca^{2+}$ channel activity.

Strauss et al[88] showed that $pp60^{c\text{-}src}$ coprecipitates with neuroendocrine L-type channels ($Ca_v1.3$) isolated from rat brain or retinal pigmented epithelial (RPE) cells. In electrophysiological experiments in RPE cells, intracellular dialysis of $pp60^{c\text{-}src}$ produced a modest increase in L-type current. Thus, $Ca_v1.3$ is apparently stimulated through a pathway involving $pp60^{c\text{-}src}$. Similarly, Rosenthal et al[89] found that the fibroblast growth factor receptor 2 (FGFR2) mediates stimulation of L-type current in RPE cells and in central neurons. Stimulation is blocked by lavendustin A, implicating tyrosine kinases. Additionally, FGFR2 coprecipitate with $Ca_v1.3$, and vice versa.

$Ca^{2+}$ channels in bovine adrenal chromaffin cells are tonically inhibited through autocrine release of ATP and opioids. These agonists of G protein-coupled receptors inhibit P/Q-type channels through both voltage-dependent (VD) and voltage-independent (VI) mechanisms. Weiss and Burgoyne[90] showed that suramin and naxolone (purinergic and opioid receptor antagonists, respectively) increase P/Q-type current by relieving tonic autocrine inhibition. Relief from inhibition is not prevented by Bis I nor occluded by PMA, indicating that PKC is not involved. However, relief from VI inhibition is blocked by coexpression of a mutant, dominant-negative Neuronal Calcium Sensor-1 (NCS-1) protein; the effect of NCS-1 E120Q suggests that wildtype NCS-1 (also known as Frequenin) is somehow necessary for tonic inhibition. Furthermore, inhibition of Src (by PP1 or srcKI) relieves tonic VI inhibition as effectively as suramin and naloxone. Weiss and Burgoyne[90] also demonstrated that Src-containing cell extracts support direct phosphorylation of $Ca_v2.1$ channels. Altogether, their results suggest that both NCS-1 and Src mediate autocrine VI inhibition of $Ca_v2.1$ in adrenal chromaffin cells.

Tyrosine kinases may also be involved in signaling pathways not traditionally associated with ion channel regulation. For example, vascular smooth muscle L-type $Ca^{2+}$ channels ($Ca_v1.2b$) are stimulated through activation of $\alpha_5\beta_1$ integrins.[91] Channel stimulation is reduced by broad-spectrum tyrosine kinase inhibitors or by intracellular application of antibodies directed against focal adhesion kinase (FAK) or $pp60^{c\text{-}Src}$. In a related study, Waitkus-Edwards et al[92] found that a peptide (leucin-aspartate-valine; LDV) that mimics the integrin binding sequence of a fibronectin triggers vasoconstriction of rat skeletal muscle arterioles. Vasoconstriction is mediated by L-type current, and L-type currents are potentiated by LDV through a pathway blocked by PP2, another inhibitor of Src. These studies suggest that integrins activate a tyrosine kinase pathway that stimulates L-type channels, leading to smooth muscle contraction.

Wu et al[13] showed that $Ca_v2.1$ channels expressed in *Xenopus* oocytes are biphasically modulated by nerve growth factor (NGF) acting through TrkA, a receptor tyrosine kinase that activates phospholipase C-$\gamma$. Application of NGF initially produces a mild enhancement of $Ca_v2.1$ currents that is thought to reflect depletion of membrane $PIP_2$ and an accompanying shift of $Ca_v2.1$ channels from "reluctant" to "willing" gating modes. Subsequent inhibition of $Ca_v2.1$ in response to NGF is thought to reflect channel rundown arising from continuing depletion of membrane $PIP_2$. These effects of NGF are not prevented by Bis I, suggesting that PKC is not involved.

## Regulation of $Ca^{2+}$ Channels by Mitogen-Activated Protein (MAP) Kinases

MAP kinases can be activated by a wide variety of signaling pathways (for reviews, see refs. 93 and 94). Wilk-Blaszczak et al[95] reported that bradykinin receptors activate a particular MAP kinase (p38-2) in NG108-15 cells. They also presented pharmacological evidence that p38-2 inhibits N-type channels. Thus, bradykinin-mediated channel inhibition was blocked

by SB203580, an inhibitor of p38 MAP kinases, but not by its inactive analogue SKF106978. Inhibition was similarly not blocked by PD98059, which inhibits MAP kinases in the ERK pathway. In another study, Lei et al[96] showed that longterm exposure to PD98059 prevents NGF from maintaining $Ca^{2+}$ channel density in bullfrog sympathetic neurons kept in culture. Their results suggest that NGF may signal through MAP kinases in controlling $Ca^{2+}$ channel expression.

As mentioned above, Fitzgerald and Dolphin[82] showed that p21-Ras increases $Ca^{2+}$ currents in rat DRG neurons. Extending this observation, Fitzgerald[97] presented evidence that Ras increases $Ca^{2+}$ currents by signaling through MAP kinases. In her experiments, currents were potentiated by a mutant Ras protein (V12S35Ras) that specifically activates Raf-1 and a MAP kinase signaling pathway. Current density was decreased by UO126, a specific inhibitor of MEK, but not by UO124, its inactive control compound. More recently, Fitzgerald[98] showed that UO126 (but not UO124) decreases N-type currents produced by expression of $Ca_v2.2$ in COS-7 cells, suggesting that $Ca_v2.2$ activity is tonically stimulated by MAP kinases under these conditions.

## Regulation of $Ca^{2+}$ Channels by Lipid Kinases

Lipid kinases play important roles in a variety of cellular processes, including but not limited to cell survival, cell transformation, vesicle budding, cytoskeletal assembly, and activation of serine/threonine kinases such as PKC and Akt/PKB (for reviews see refs. 99 and 100). The roles of lipid kinases and phosphatases in $Ca^{2+}$ channel regulation are just beginning to be explored. Most existing studies have focused on the role of phosphoinositide 3-kinases (PI3Ks). PI3Ks can be activated by ligand binding to receptor tyrosine kinases, to G protein-coupled receptors, or by integrin-dependent cell adhesion. Activated PI3K converts plasma membrane phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$), otherwise known as $PIP_3$. A wide variety of signaling proteins contain domains that specifically bind $PIP_3$, resulting in protein translocation to the plasma membrane and interaction with effectors.[100] Significantly, certain PI3Ks (e.g., PI3K$\alpha$ and PI3K$\gamma$) can also phosphorylate serine or threonine residues within proteins. Inhibitors of PI3K activity, such as wortmannin and LY294002, cannot be used to distinguish between lipid and protein kinase activity.[99]

Evidence indicates that PI3Ks regulate L-type channel activity in smooth muscle cells. Thus, in rat portal vein myocytes, activation of $AT_{1A}$ angiotensin II receptors stimulates L-type channels through a pathway involving G$\beta\gamma$ released from G$\alpha_{13}$. Channel stimulation is blocked by intracellular dialysis with an antibody against G$\beta$, or by overexpression of a G$\beta\gamma$-sequestering peptide.[101] Viard et al[102] showed that L-type current is also stimulated in these cells following dialysis with purified G$\beta\gamma$. Channel stimulation by either purified G$\beta\gamma$ or angiotensin II is blocked by nanomolar wortmannin. Dialysis with activated, recombinant, G$\beta\gamma$-sensitive PI3K$\gamma$ also stimulated L-type channels. The stimulating effects of G$\beta\gamma$ or recombinant PI3K$\gamma$ were reduced, but not blocked, by inhibitors of PKC. These results indicate that PKC is involved in, but not completely responsible for, channel stimulation by G$\beta\gamma$ and PI3K$\gamma$.[102] Quignard et al[103] subsequently showed that stimulation of L-type channels through the G protein-coupled $AT_{1A}$ angiotensin II receptor involves signaling by PI3K$\gamma$. On the other hand, Macrez et al[104] reported that a different isozyme, PI3K$\beta$, mediates stimulation of L-type channels through the platelet-derived growth factor receptor, which is a receptor tyrosine kinase. The above studies demonstrate that PI3Ks stimulate vascular L-type $Ca^{2+}$ channels. However, the mechanism by which they act remains unclear: possibilities include direct effects of $PIP_3$ on $Ca^{2+}$ channels or proteins that regulate them, $PIP_3$–dependent activation of PDK1 and subsequent activation of PKC or Akt/PKB, direct channel phosphorylation by PI3K, or some combination thereof.[99]

Several recent studies indicate that lipid kinases can modulate ion channels by altering the concentrations of membrane phospholipids. Specifically, changes in membrane $PIP_2$ concentration following PLC activation have been implicated in regulation of several different ion

channel types.[105] It was recently proposed[106] that phosphoinositide 4-kinase (PI4K) restores membrane $PIP_2$ following its depletion by PLC, and that restoration of $PIP_2$ is necessary for recovery of M-type $K^+$ channels from slow muscarinic inhibition. This same signaling pathway is thought to produce slow muscarinic inhibition of neuronal L-type and N-type $Ca^{2+}$ channels.[107] As described earlier, other recent evidence supports the idea that membrane $PIP_2$ plays important roles in $Ca^{2+}$ channel regulation.[13]

## Regulation of $Ca^{2+}$ Channels by Cyclin-Dependent Kinase 5 (Cdk5)

Cdk5 has important neuronal functions and its absence or dysfunction may contribute to neurodegenerative diseases such as Alzheimer's and amyotrophic lateral sclerosis.[108] Liu et al[109] presented evidence that Cdk5 stimulates $Ca^{2+}$ currents in dissociated mouse neostriatal neurons. Activation of mGluRs stimulated whole-cell $Ca^{2+}$ currents in these cells, and stimulation was blocked by butyrolactone, an inhibitor of Cdk5. Subsequently, Tomizawa et al[110] reported that p35, a neuron-specific activator of Cdk5, inhibits neurotransmitter release in rat hippocampus by disrupting interactions between P/Q-type channels and SNARE proteins. Their conclusion was based in part on the finding that roscovitine, which at that time was thought to be a specific inhibitor of Cdk5, potentiates synaptic transmission mediated by P/Q-type channels. They[110] showed that $Ca_v2.1$ is phosphorylated in vitro by Cdk5, and that such phosphorylation effectively blocks interactions between $Ca_v2.1$ and SNAP-25 or synaptotagmin I. These findings led to a model in which Cdk5-mediated phosphorylation of P/Q-type channels uncouples $Ca^{2+}$ influx from neurosecretion. However, Yan et al[111] have more recently demonstrated that roscovitine is a direct agonist of P/Q-type channels. Extracellular roscovitine slows channel deactivation, thereby potentiating tail currents and $Ca^{2+}$ influx. This effect is independent of Cdk5, because roscovitine is ineffective when applied intracellularly, and roscovitine slows P/Q-type current deactivation in neurons from mice lacking p35 as much as in neurons from wildtype mice. Cdk5 activity is largely eliminated in neurons from mice lacking p35.

## Regulation of $Ca^{2+}$ Channels by Calcineurin

Protein and lipid phosphatases are activated through various membrane receptors and cell signaling mechanisms (for review see ref. 4). The concept that dephosphorylation activates $Ca^{2+}$ channel activity has been relatively neglected, and most information concerning the effects of phosphatases on $Ca^{2+}$ channel activity is derived from purely pharmacological studies. Protein phosphatase 2B, or calcineurin (CaN), has been of special interest to $Ca^{2+}$ channel physiologists because it is abundantly expressed in brain and is activated by $Ca^{2+}$ influx. CaN, like PKA and PKC, is thought to be tethered nearby its activators and substrates by AKAPs.[112] Zhu and Yakel[113] found that calcineurin inhibitory fragment (CIF) reduced the voltage-dependent (VD) inhibition of N-type channels in rat major pelvic ganglion cells through endogenous $\alpha_2$-adrenergic and somatostatin receptors. Inhibition through $\alpha_2$-adrenergic receptors was also reduced by CaN inhibitors (cyclosporin A and cyclophilin), but not by okadaic acid which does not inhibit CaN. Additionally, CIF relieved kinetic slowing and accelerated inactivation in cells dialyzed with GTPγS. Thus, CaN-dependent dephosphorylation may participate in the "crosstalk" between kinases and G proteins in the modulation of N-type, P/Q-type and R-type channels. Lukyanetz et al[114] also presented evidence for regulation of L-type and N-type channels by CaN in NG108-15 cells. They showed that both L-type and N-type currents are decreased by high-frequency trains of stimulation that elevate intracellular $Ca^{2+}$. This effect was blocked by FK-506, an inhibitor of CaN. Transfection with CaN antisense increased calcium currents, whereas overexpression of CaN triggered tonic channel inhibition that was partially relieved by FK-506 or EGTA. Their results suggest that basally phosphorylated L-type and N-type channels are inhibited through dephosphorylation by CaN in these cells.

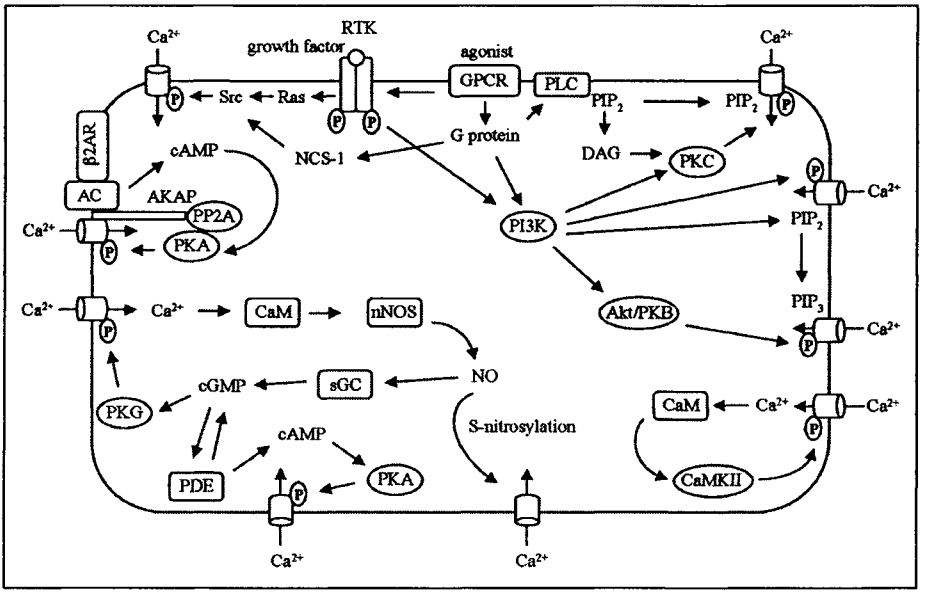



Figure 1. In this figure, we have attempted to graphically depict many of the phosphorylation-dependent interactions discussed in this chapter. Voltage-gated $Ca^{2+}$ channels are represented by transmembrane barrels, with penetrating arrows to indicate $Ca^{2+}$ influx. Arrows within the cell represent established or hypothesized interactions, which may be either stimulatory or inhibitory. The circled Ps represent phosphorylation of $Ca^{2+}$ channel proteins or associated molecules.

In rat striatal neurons, activation of D2 dopamine receptors produces inhibition of L-type channels through a pathway that includes, sequentially, $G\beta\gamma$, $PLC\beta_1$, elevated intracellular $Ca^{2+}$ and activation of CaN.[115] More recently, Day et al[116] showed that currents mediated by $Ca_v1.2$ in rat prefrontal pyramidal neurons are inhibited through an almost identical signaling cascade activated by 5-$HT_2$ receptors.

## Summary

Recent experiments indicate that a wide variety of protein and lipid kinases can regulate voltage-gated $Ca^{2+}$ channels. The examples summarized herein are mostly limited to the relatively well known kinases such as PKA, PKC, CaMKII, tyrosine kinases and PI3K (see also Fig. 1). However, the human genome encodes approximately 500 different protein kinases.[117] It therefore seems likely that voltage-gated $Ca^{2+}$ channels are regulated through many additional kinase-dependent signaling pathways. Furthermore, the potential for crosstalk between different kinase-involving pathways seems enormous. However, crosstalk may be highly restricted within living cells, because an emerging theme seems to be that $Ca^{2+}$ channels, receptors, G proteins and scaffolding proteins are tightly colocalized within "signaling complexes" that include kinases and phosphatases. Future studies will undoubtably strive toward a better understanding of the composition and function of such signaling complexes. Future work will also be required to elucidate the physiological roles of membrane phospholipids in $Ca^{2+}$ channel function. It will also be of interest to more fully understand how alternative splicing of $Ca^{2+}$ channel proteins determines their participation in signaling networks, and ultimately, their physiological roles.

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# CHAPTER 11

# $Ca^{2+}$-Dependent Modulation of Voltage-Gated $Ca^{2+}$ Channels

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## Abstract

The passage of $Ca^{2+}$ ions through voltage-gated $Ca^{2+}$ channels triggers a wide range of signaling pathways but also fundamentally regulates further $Ca^{2+}$ influx through these channels. $Ca_v1.2$ (L-type) and $Ca_v2.1$ (P/Q-type) channels undergo a dual feedback regulation by $Ca^{2+}$, which is manifested as enhanced facilitation and inactivation of $Ca^{2+}$ currents compared to $Ba^{2+}$ currents. $Ca^{2+}$-dependent facilitation and inactivation are distinct biophysical processes but are linked through a common molecular mechanism. The ubiquitous $Ca^{2+}$-sensing protein calmodulin binds directly to multiple sequences in the $Ca^{2+}$ channel $\alpha_1$ subunit, causing $Ca^{2+}$-dependent conformational changes that favor facilitated or inactivated states of the channel. In the nervous system, $Ca^{2+}$ binding proteins related to CaM also contribute to modulation of $Ca^{2+}$ channels, which may diversify $Ca^{2+}$ signaling in different neurons. In all excitable cells, $Ca^{2+}$-dependent facilitation and inactivation of voltage-gated $Ca^{2+}$ channels may be a general mechanism for fine-tuning $Ca^{2+}$ entry to protect against toxic $Ca^{2+}$ overloads and to control the specificity with which $Ca^{2+}$-dependent signaling pathways are activated.

## Introduction

Voltage-gated $Ca^{2+}$ channels (VGCCs) couple membrane depolarization to the influx of $Ca^{2+}$ ions, which initiate gene transcription, muscle contraction, and neurotransmitter release. While $Ca^{2+}$ is critical as a second messenger, excess $Ca^{2+}$ entry can cause cell death. Thus, the activity of VGCCs is tightly controlled by a number of factors. Of these, perhaps the most fundamental is a feedback regulation by the $Ca^{2+}$ ions that permeate the channel itself. Based on different properties of $Ca^{2+}$ and $Ba^{2+}$ currents ($I_{Ca}$ and $I_{Ba}$) through the same channels, it has been proposed that some VGCCs have a $Ca^{2+}$-sensing mechanism that promotes inactivation, and in some cases, also causes facilitation of further $Ca^{2+}$ entry. Such $Ca^{2+}$-dependent regulation of VGCCs may enhance excitation-contraction coupling in the heart and contribute to synaptic plasticity in the nervous system. Elucidating the molecular mechanisms mediating $Ca^{2+}$-dependent inactivation and facilitation has been the focus of intense research, culminating in the recent identification of calmodulin and related $Ca^{2+}$-binding proteins as $Ca^{2+}$ sensors that directly associate with and modulate various VGCC family members. This chapter summarizes the findings that have led to current views and remaining controversies about $Ca^{2+}$-dependent regulation of VGCCs.

## Feedback Regulation of VGCCs by $Ca^{2+}$ in Paramecium and Aplysia

The influx of $Ca^{2+}$ ions through VGCCs in the plasma membrane of *Paramecium* initiates the reorientation and beating of cilia to allow the organism to swim in reverse.[1] Voltage-clamp

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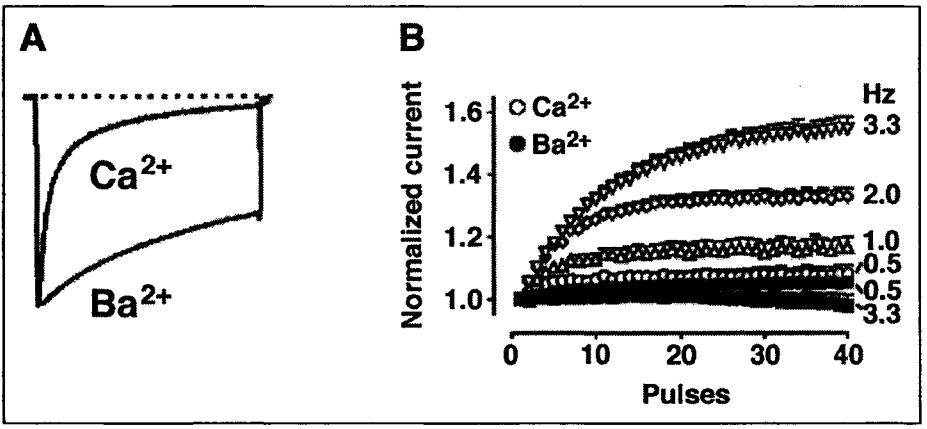



Figure 1. $Ca^{2+}$-dependent inactivation and facilitation of $Ca_v1.2$. A) $Ca_v1.2$ currents evoked by 400-ms test pulses from -90 to +20 mV using $Ca^{2+}$ or $Ba^{2+}$ as the permeant ion. B) Facilitation of $Ca^{2+}$ but not $Ba^{2+}$ currents evoked by repetitive depolarizations at the indicated frequencies in $Ca_v1.2$ channels containing the I/A mutation. (From Zuhlke et al, 1999).

studies of this $Ca^{2+}$ current in *P. caudatum* first characterized a major role for $Ca^{2+}$ ions in accelerating inactivation of a voltage-gated $Ca^{2+}$ conductance.[2,3] In these experiments, several lines of evidence favored a $Ca^{2+}$-dependent mechanism for inactivation that was distinct from the conventional mechanism for voltage-dependent inactivation. First, inactivation of test currents correlated with the amplitude of the peak $Ca^{2+}$ current. A plot of the amount of inactivation of $I_{Ca}$ vs. test voltage was U-shaped, with the greatest inactivation occurring at the voltage eliciting the maximal inward $Ca^{2+}$ current. By contrast, inactivation that depended solely on membrane potential should increase monotonically with the applied voltage. Second, the use of $Ba^{2+}$ rather than $Ca^{2+}$ as the permeant ion significantly reduced the amount of inactivation of test currents. Finally, chelating intracellular $Ca^{2+}$ by injection of EGTA greatly slowed inactivation of $I_{Ca}$, suggesting that $Ca^{2+}$ influx and accumulation in the cell was necessary for $Ca^{2+}$-dependent inactivation. Parallel experiments in giant neurons in the marine snail, *Aplysia californica*, revealed similar $Ca^{2+}$-dependent inactivation of $I_{Ca}$ that was prevented with $Ba^{2+}$ substitution for $Ca^{2+}$ and by injection of EGTA.[4,5] These classical studies defined standards for characterizing $Ca^{2+}$-dependent inactivation (CDI) of VGCCs, which would prove to be a fundamental form of negative feedback regulation in a diverse range of cell types and organisms.[6,7]

## $Ca^{2+}$-Dependent Modulation of $Ca_v1.2$ (L-Type) Channels

### *$Ca^{2+}$-Dependent Inactivation*

Dihydropyridine-sensitive $Ca_v1.2$ (L-type) channels mediate $Ca^{2+}$ influx required for the contraction of the heart in response to membrane depolarization. Electrophysiological recordings of cardiac myocytes revealed that inactivation of these channels is jointly regulated by both $Ca^{2+}$ ions and voltage.[8-11] An example of CDI measured for cloned and expressed $Ca_v1.2$ is illustrated in Figure 1A. [12] Unlike the feedback regulation of $Ca^{2+}$ channels in *Paramecium* and *Aplysia* neurons, CDI in heart cells is only partially removed by intracellular $Ca^{2+}$ chelators,[13] which led to the hypothesis that the underlying mechanism involved very local increases in $Ca^{2+}$ either in or near the channel pore.[14-17] Single-channel analyses of rat myocytes supported a model in which $Ca^{2+}$ entry through $Ca_v1.2$ channels shifts their gating to a mode with lower open probability.[18,19] In parallel whole-cell recordings, calmidazolium, a calmodulin inhibitor, did not block $Ca^{2+}$-dependent inactivation, suggesting that perhaps $Ca^{2+}$-binding directly to

the channel was the molecular switch that altered channel gating. However, calmidazolium does not competitively inhibit all CaM-target interactions, particularly those involving constitutive association of CaM,[20] which may explain its inefficacy in this case.

### *$Ca^{2+}$-Dependent Facilitation*

In addition to enhancing inactivation, $Ca^{2+}$ ions cause a positive feedback regulation of further $Ca^{2+}$ entry through $Ca_v1.2$ channels in cardiac myocytes, which may be important in amplifying intracellular $Ca^{2+}$ signals leading to contraction of the heart.[21,22,23] While CDI is evident during prolonged depolarizations, $Ca^{2+}$-dependent facilitation (CDF) of $Ca_v1.2$ currents is revealed in repetitive pulse protocols. In whole-cell recordings of cardiac myocytes, $Ca^{2+}$ currents during a train of depolarizations progressively increase in amplitude, reaching a steady state by the fifth pulse.[24] An example of CDF of the cloned and expressed $Ca_v1.2$ channel is illustrated in Figure 1B.[12] The facilitation of $Ca^{2+}$ currents is $Ca^{2+}$-dependent in that it can be induced by photolysis of caged-$Ca^{2+}$ compounds[25] and is abolished by substitution of extracellular $Ca^{2+}$ with $Ba^{2+}$ or $Sr^{2+}$.[24] CDF can still be observed with 20 mM EGTA or 5 mM BAPTA in intracellular recording solutions, suggesting that like CDI, CDF may depend on very local increases in $Ca^{2+}$ near the channel.[24] Interestingly, while both CDF and CDI characterize $Ca_v1.2$ channels in cardiac myocytes, recombinant $Ca_v1.2$ channels expressed in Xenopus oocytes and HEK 293 cells show primarily CDI and comparatively little CDF.[12] These results suggest that the mechanism for CDF may depend on cell-type specific differences in the processing of $Ca_v1.2$ channels or on cellular factors, such as $Ca^{2+}$-dependent kinases or phosphatases, which are more abundant in cardiac cells than in heterologous expression systems.

## Molecular Determinants of $Ca^{2+}$-Dependent Modulation in $Ca_v1.2$ Channels

Efforts to identify the molecular structures important for $Ca^{2+}$-dependent modulation of $Ca_v1.2$ have focused on the C-terminal domain of the pore-forming $\alpha_1$ subunit of these channels ($\alpha_1 1.2$)[26-28] (Fig. 2). Alternative splicing of exons 40-42 of the human $\alpha_1 1.2$ gene ($\alpha_1 1.2_{86}$) gives rise to channels in which $I_{Ba}$ inactivates rapidly with no further enhancement of inactivation of $I_{Ca}$ [29] Further analyses revealed that this loss of CDI depended on multiple sequences within the alternately spliced region of $\alpha_1 1.2_{86}$ (amino acids 1572-1651). Substitution of $^{1572}$IKTEG$^{1576}$ and $^{1600}$LLDQV$^{1604}$ of $\alpha_1 1.2$ with the corresponding residues of $\alpha_1 1.2_{86}$ eliminated CDI.[30] In addition, deletion of a more distal sequence (aa 1624-1631) also caused fast $Ca^{2+}$-independent inactivation of $\alpha_1 1.2$ .[31] This region partially encompassed the IQ-domain, a consensus site for CaM-binding.

### *The Role of $Ca^{2+}$ and CaM in CDI of $Ca_v1.2$.*

The emergence of CaM as a critical element in the $Ca^{2+}$-dependent regulation of many ion channels[32] foreshadowed the discovery that CaM binding to the IQ-domain was important for CDI of $Ca_v1.2$ channels. Peptides corresponding to the IQ-domain bind CaM in a $Ca^{2+}$-dependent manner and mutations of residues in the IQ-domain that prevent CaM binding also impair CDI.[12,33,34] Furthermore, CaM mutants in which at least three of the four $Ca^{2+}$-binding EF-hand motifs are rendered dysfunctional prevent CDI of coexpressed $Ca_v1.2$ channels.[12,34] Interestingly, inactivating mutations restricted to the C-terminal but not the N-terminal EF-hands of CaM block CDI.[34] The C-terminal lobe of CaM binds $Ca^{2+}$ ions with higher affinity than the N-terminal lobe,[35] which may be functionally significant for initiating CDI of $Ca_v1.2$ channels.

Although CaM binding to the IQ-site was $Ca^{2+}$-dependent , the ability of the CaM mutants to cause dominant negative inhibition of CDI suggested that $Ca^{2+}$ binding to CaM is not required for interaction with the channel, and that apoCaM must somehow be tethered to the channel, similar to the interaction of CaM with $Ca^{2+}$-activated $K^+$ channels.[20] Such constitutive association of CaM with $Ca_v1.2$ channels would explain the millisecond kinetics of CDI

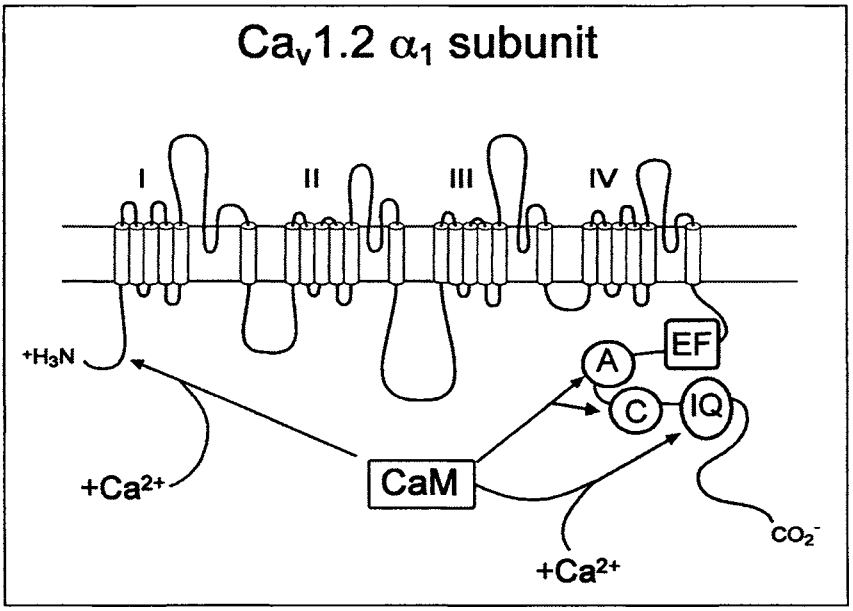



Figure 2. Molecular determinants for $Ca^{2+}$-dependent regulation of $Ca_v1.2$. Schematic showing $\alpha_1 1.2$ subunit with EF-hand and sites involved in CaM tethering (A and C) and the $Ca^{2+}$/CaM binding sites in the N- and C- terminal (IQ) domains.

and was confirmed in living cells using fluorescence resonance energy transfer (FRET) and in biochemical experiments.[36,37] Although peptides corresponding to the IQ-domain do not bind CaM at or below 10 nM $Ca^{2+}$, non-contiguous sequences upstream of the IQ-domain bind CaM at these levels of $Ca^{2+}$, which approximate the resting $Ca^{2+}$ concentration in cells.[36] These separate "A" and "C" peptides contain the $^{1572}$IKTEG$^{1576}$ and $^{1600}$LLDQV$^{1604}$ sequences, respectively, shown previously to bind CaM and to contribute to CDI of $Ca_v1.2$ channels.[30,31,38,39] Based on analyses of the $Ca^{2+}$-dependence of CaM interactions with A, C, and IQ-peptides, Pitt and colleagues (2001) propose a model in which CaM is tethered by the A and C peptides at resting $Ca^{2+}$ levels and elevations in $Ca^{2+}$ to the micromolar range cause a conformational change in CaM and/or $\alpha_1 1.2$ that results in additional contacts of CaM with the IQ-domain. In this way, the IQ-domain would act as a $Ca^{2+}$-dependent effector site. It is also proposed that CaM tethering to the A and C sites slows inactivation of $Ca_v1.2$ channels when $Ca^{2+}$ is low. This would neatly explain the unexpected enhancement of $I_{Ba}$ inactivation when either A or C sites are mutated.[30,31] Alternatively, the critical residues mediating CaM binding in the A and C sites may also control $Ca^{2+}$-independent mechanisms for inactivation, such that mutations in these regions would enhance both $I_{Ba}$ and $I_{Ca}$ inactivation regardless of whether CaM was bound.[40,41]

In addition to the CaM-binding sites in the cytoplasmic C-terminal domain of $\alpha_1 1.2$, other molecular determinants may play a significant role in CDI of $Ca_v1.2$ channels (Fig. 2). For example, CaM binds also to a site in the intracellular N-terminal domain of $\alpha_1 1.2$ and deletion of this site attenuates CDI.[42] Furthermore, a number of studies indicate a major role for a EF-hand motif upstream of the CaM-binding sites in the C-terminal domain of $\alpha_1 1.2$.[26,31,43] While the $Ca^{2+}$-binding capability of this EF-hand is clearly not required for CDI,[28,43] deletion of the entire EF-hand and mutations of residues other than those that would coordinate $Ca^{2+}$ ions severely limit CDI.[31,43] Other mutational analyses of the EF-hand indicate the importance of this region for voltage- as well as $Ca^{2+}$-dependent mechanisms for $Ca_v1.2$ inactivation.[44]

### CaM, CaM Kinase II and CDF of $Ca_v1.2$ Channels

Although CDI and CDF were thought to be distinct processes both kinetically and mechanistically, structure-function studies of the IQ-domain uncovered a surprising role for CaM binding to this site in both processes.[12] In *Xenopus* oocytes, facilitation of $Ca_v1.2$ channels is evident as a faster recovery from inactivation for $I_{Ca}$ compared to $I_{Ba}$. While mutating $^{1624}$Ile to glutamate abolished CDI as well as the $Ca^{2+}$-dependent enhancement of recovery from inactivation, substituting alanine for $^{1624}$Ile prevented CDI, but greatly enhanced CDF.[12] These results suggested that CDF was partially obscured in wild-type channels due to the strength of CDI. Although it is still not apparent how CaM binding to the IQ domain leads to both forms of modulation, exhaustive substitutions of $^{1624}$Ile with various amino acids indicate the importance of a bulky hydrophobic side-chain at this residue for CDI but not CDF.[45] It could be that minute changes in contacts between CaM and $^{1624}$Ile accompany transitions to inactivated and facilitated states. Clarification of this issue awaits crystallization of the CaM-IQ complex, as has been done for $Ca^{2+}$-activated $K^+$ channels.[46]

In addition to CaM binding to the IQ domain, other studies support a role for CaM kinase II (CaMK) in CDF of $Ca_v1.2$. In single-channel recordings of inside-out patches excised from cardiac myocytes, bath application of a constitutively active form of CaMK caused a marked increase in the probability of channel opening. This effect was blocked by a specific inhibitor of CaMK and by perfusion with non-hydrolyzable forms of ATP, suggesting that phosphorylation of the channel or some regulatory protein is essential for the facilitation.[47] CaMK not only enhanced $I_{Ca}$ but also reduced $Ca^{2+}$ release from, and increased $Ca^{2+}$ content of, the sarcoplasmic reticulum in cardiac myocytes, suggesting a role for CaMK and CDF of $Ca_v1.2$ channels in excitation-contraction coupling.[48] Moreover, CDF was not observed upon treating myocytes with thapsigargin, ryanodine, or caffeine, supporting a dependence of CDF on $Ca^{2+}$ from intracellular stores in these cells.[49] While it is not clear how CaMK might interact with CaM binding to the IQ-domain in promoting CDF, the prominence of CDF of $Ca_v1.2$ currents in cardiac cells compared to transfected cells may result from the stronger effects of CaMK regulation in the former compared to the latter system. These studies demonstrate the importance of studying CDI and CDF in native systems, in which the physiological regulation of $Ca_v1.2$ channels may be considerably more complex than in transfected cell lines.

## $Ca^{2+}$-Dependent Regulation of $Ca_v2.1$ (P/Q-Type) Channels

$Ca^{2+}$ influx through presynaptic $Ca_v2.1$ (P/Q-type) channels initiates neurotransmitter release at many central synapses. Compared to the prominent CDI of $Ca_v1.2$ channels, early experiments indicated that $Ca_v2.1$ channels expressed in Xenopus oocytes show relatively little regulation by $Ca^{2+}$. However, fluorometric analysis of $Ca^{2+}$ influx in rat brain synaptosomes revealed $Ca^{2+}$-dependent inactivation of $Ca_v2.1$ channels that could be suppressed by decreasing the extracellular $Ca^{2+}$ concentration.[50] CDI of presynaptic $Ca^{2+}$ channels was further supported in patch clamp recordings of presynaptic nerve terminals in the rat neurohypophysis[51] and at the calyx of Held synapse in the rat brainstem.[52]

Presynaptic $Ca^{2+}$ currents at the Calyx of Held are unusual in that their functional and pharmacological properties are consistent solely with $Ca_v2.1$ channels and not a mixed population of VGCCs.[52] During tetanic stimulation, $Ca_v2.1$ currents at this synapse undergo an initial facilitation followed by progressive inactivation. Both the facilitation and inactivation are diminished by intracellular perfusion with BAPTA and by $Ba^{2+}$ rather than $Ca^{2+}$ as the permeant ion.[53,54] Because of the steep dependence of neurotransmitter release on presynaptic $Ca^{2+}$ concentrations, this dual feedback regulation of $Ca_v2.1$ channels by $Ca^{2+}$ ions could significantly affect synaptic efficacy. Indeed, simultaneous recording of excitatory postsynaptic responses showed that CDF and CDI of $Ca_v2.1$ channels could lead to synaptic enhancement and depression, respectively.[52-54] CDF and CDI are also observed for cloned and expressed $Ca_v2.1$ channels expressed in mammalian cells, as illustrated in Figure 3.[55] Due to the general importance of presynaptic $Ca_v2.1$ channels in regulating neurotransmitter release throughout

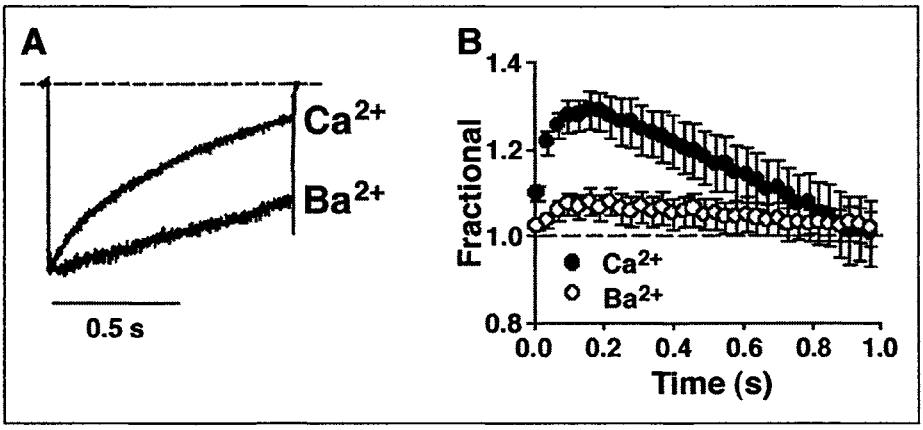



Figure 3. $Ca^{2+}$-dependent inactivation and facilitation of $Ca_v2.1$. A) $Ca_v2.1$ currents evoked by 1-s test pulses from -80 to +10 mV using $Ca^{2+}$ or $Ba^{2+}$ as the permeant ion. B) Facilitation of $Ca^{2+}$ but not $Ba^{2+}$ currents through $Ca_v2.1$ channels upon repetitive depolarizations given at 100-Hz.

the central nervous system, these results suggested a role for $Ca^{2+}$-dependent regulation of $Ca_v2.1$ channels in mechanisms of activity-dependent synaptic plasticity.

### *CaM Binding Domains in the C-Terminal Region of $\alpha_1$2.1*

A major clue to the mechanism underlying the $Ca^{2+}$-dependent regulation of presynaptic $Ca_v1.2$ channels in neurons came from the identification of a novel CaM-binding site in the C-terminal domain of the pore-forming $\alpha_1$2.1 subunit of $Ca_v2.1$ channels using yeast two-hybrid as well as in vitro binding and immunoprecipitation methods.[55] This CaM-binding domain (CBD) (Fig. 4) is located on the C-terminal side of the sequence in $\alpha_1$2.1 that corresponds to the IQ-domain of $Ca_v1.2$ channels.[12,33,34] The modified IQ domain of $\alpha_1$2.1 contains IM instead of IQ and has other changes that would also be predicted to substantially reduce its affinity for CaM.

Although CaM binding to the CBD was found to be $Ca^{2+}$-dependent,[55] the physiological significance of this interaction was not clear since $Ca_v2.1$ channels showed little $Ca^{2+}$-dependent regulation when expressed in heterologous cells.[56] With a relatively low (0.5 mM) concentration of EGTA in intracellular recording solutions, $I_{Ca}$ through transfected $Ca_v2.1$ channels inactivates significantly faster than $I_{Ba}$.[55] CDI is particularly evident in channels containing the auxiliary $\beta_{2a}$ subunit, in which voltage-dependent inactivation is minimal.[57] Moreover, $Ca_v2.1$ channels also show prominent CDF, which is evident as a significant increase in $I_{Ca}$ amplitude above initial levels during recovery from inactivation, in paired-pulse protocols, and during repetitive depolarizations designed to mimic trains of action potentials.[55,57] The enhanced facilitation and inactivation of $I_{Ca}$ are not seen for $I_{Ba}$ or for cells in which intracellular $Ca^{2+}$ is buffered with 10 mM BAPTA, and both CDF and CDI are blocked by coexpression of a CaM inhibitor peptide. The slower $Ca^{2+}$ buffer EGTA (10 mM) blunts CDI but not CDF, suggesting that CDF has greater $Ca^{2+}$ sensitivity due to faster binding or higher affinity binding of $Ca^{2+}$.[57] These results strongly suggested that the $Ca^{2+}$-dependent modulation of $Ca_v2.1$ channels in neurons is caused by two sequential interactions with CaM or perhaps a related $Ca^{2+}$-sensing protein.

As for $Ca_v1.2$ channels, the mechanism for CDI and CDF of $Ca_v2.1$ channels involves more than just CaM binding to a single site. Deletion of the CBD from the C-terminal domain of $\alpha_1$2.1 decreases but does not completely abolish $Ca^{2+}$-dependent regulation of transfected $Ca_v2.1$ channels.[57] CaM also binds to peptides corresponding to the modified IQ-domain of $\alpha_1$2.1, and mutation of the I and M residues of this motif to alanines prevents CDF, but not CDI.[58] (Lee, Scheuer, and Catterall, unpublished). This result is surprising if CaM binding to

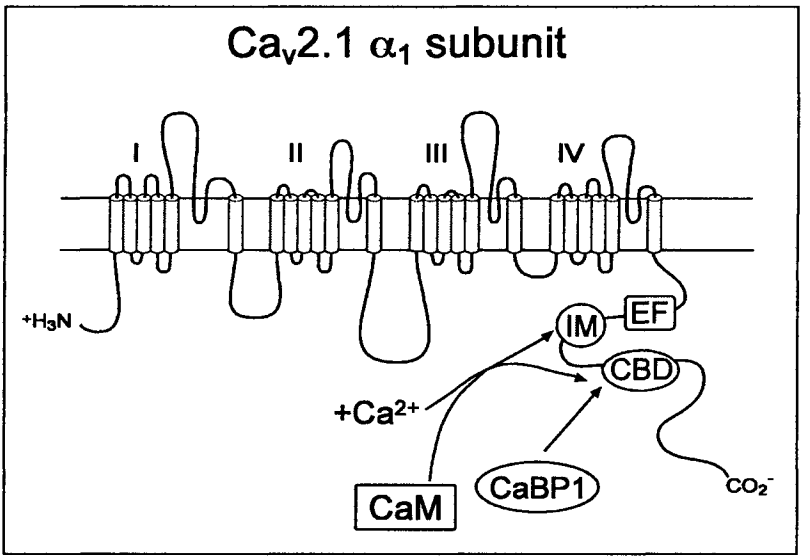



Figure 4. Molecular determinants for $Ca^{2+}$-dependent regulation of $Ca_v2.1$. Schematic showing $\alpha_1 2.1$ subunit with EF-hand and sites involved in binding $Ca^{2+}$/CaM and CaBP1.

the IQ-domain is essential for CDI since the first two residues of this motif are critical for virtually all target interactions with CaM.[59] Mutation of IM to negatively charged glutamate residues accelerates inactivation 40-fold[58] so that CDI is no longer detected as an increase over the much more rapid voltage-dependent inactivation. A similar effect was observed for $Ca_v2.1$ channels containing β1b subunits, which inactivate rapidly in the absence of $Ca^{2+}$ and have little CDI.[57] Thus, the apparent loss of CDI in the IM/EE mutant does not provide evidence for an essential role of the modified IQ motif in CDI, but does show clearly that these amino acid residues have crucial effects on the rate of voltage-dependent inactivation in the absence of $Ca^{2+}$. Overall, the results to date indicate that both the CBD and the modified IQ domain of $Ca_v2.1$ channels have significant roles in regulation by $Ca^{2+}$ and CaM, with the potential for different actions of these two distinct CaM binding motifs in CDF and CDI.

## *Mechanism for Dual-Feedback Regulation by CaM*

Alanine substitutions in the IQ-domain disrupt CDF but not CDI, while deletion of the CBD preferentially suppresses CDI.[57,58] These findings suggest that the CBD and modified IQ-domains may be important for CDI and CDF, respectively. Moreover, DeMaria et al have provided evidence that distinct molecular determinants for these two processes reside in the CaM molecule itself. CaM mutants containing inactivating mutations in the N-terminal lobe EF-hands (CaM 1,2) block CDI but spare CDF, while CaM mutants with the corresponding C-terminal mutations (CaM 3,4) prevent CDF but not CDI.[58] These results are consistent with the higher $Ca^{2+}$ sensitivity of CDF than CDI because the C-terminal EF hands have higher affinity for $Ca^{2+}$.binding than those in the N-terminus of CaM. The ability of these CaM mutants to compete successfully with the endogenous pools of CaM in the cell suggests that apoCaM may be tethered to $Ca_v2.1$. As for $Ca_v1.2$, FRET studies indicate that apoCaM can indeed bind to $Ca_v2.1$ channels in intact cells.[37] While regulatory interactions with other proteins depend on either the N- or C-terminal $Ca^{2+}$-binding capability of CaM, $Ca_v2.1$ channels would be the first example of lobe-specific bi-directional modulation within a single molecular target by CaM. An interesting hypothesis is that the two lobes of CaM interact differentially with the modified IQ domain and the CBD in order to effect bi-directional regulation, perhaps with the C-terminal

lobe primarily controlling interactions with the modified IQ domain and the N-terminal lobe primarily controlling interactions with the CBD.

## Differential Modulation of $Ca_v2.1$ by CaM and Neuronal $Ca^{2+}$-Binding Proteins

CaM is the most well-characterized member of a superfamily of $Ca^{2+}$-binding proteins, many members of which differ from CaM in neuron-specific localization and/or amino acid substitutions that prevent $Ca^{2+}$ binding to one or more of the EF-hands.[60,61] Although the physiological significance of many neuronal $Ca^{2+}$ binding proteins (NCBPs) remains elusive, some NCBPs have emerged as key regulators of ion channel function and synaptic transmission. Recent evidence indicates that NCBPs may confer $Ca^{2+}$-dependent and $Ca^{2+}$-independent regulation to VGCCs that differs significantly from their modulation by CaM.

### *Neuronal $Ca^{2+}$ Sensor-1*

Since its discovery in *Drosophila* mutants with aberrant synaptic function,[62] neuronal $Ca^{2+}$ sensor-1 (NCS-1) has been implicated in the regulation of neurosecretion, synapse formation, and neuronal circuits controlling associative learning.[63-65] NCS-1 acts indirectly through *src* tyrosine kinase activity to inhibit P/Q-type $Ca^{2+}$ channels in chromaffin cells, but enhances the activity of presynaptic $Ca_v2.2$ channels in cultured frog spinal cord neurons.[65-67] NCS-1 is localized in presynaptic nerve terminals at the calyx of Held synapse, and injection of purified recombinant NCS-1 into these nerve terminals causes a facilitation of $I_{Ca}$ that is blocked by intracellular perfusion with BAPTA and a dominant-negative NCS-1 inhibitor peptide.[68] Although a direct interaction between NCS-1 and the $Ca^{2+}$ channel was not demonstrated, these findings raise the possibility that NCS-1 rather than CaM may mediate activity-dependent facilitation of $Ca_v2.1$ channel at this, and potentially other, central synapses.

### *CaBP1*

$Ca^{2+}$-binding protein 1 (CaBP1) represents a novel family of NCBPs identified in retina and the brain.[69,70] Of all the NCBPs, CaBP1 and its variants show the highest degree of sequence homology with CaM, reflecting a potential to interact with and modulate CaM targets. Intriguingly, CaBP1 binds to the CBD and not the IQ-domain of $\alpha_1 2.1$, but unlike the interaction with CaM, CaBP1 binding does not require $Ca^{2+}$.[71] This $Ca^{2+}$-independent binding of CaBP1 causes a strong enhancement of the rate of inactivation, a positive shift in the voltage-dependence of activation, and a loss of $Ca^{2+}$-dependent facilitation of $Ca_v2.1$ channels in transfected cells.[71] All of these effects would combine to reduce the activity of $Ca_v2.1$ channels. These effects of CaBP1 do not depend on $Ca^{2+}$, but they are absent in channels lacking the CBD, consistent with the hypothesis that CaBP1 displaces CaM from this site in a way that stabilizes closed conformations of the channel. This idea is also supported by the loss of CaM-dependent facilitation when CaBP-1 is co-expressed.[71] The differences in modulation of $Ca_v2.1$ channels by CaBP1 and CaM may result from the structural differences between the two $Ca^{2+}$ binding proteins, which include an inactivated $Ca^{2+}$ binding site, a lipid anchor, and an extended central helix connecting the two lobes of CaBP-1.[69] For example, inactivation of the $Ca^{2+}$ binding activity of EF hand 1 in CaBP-1 may allow it to assume an active conformation and accelerate the rate of inactivation of $Ca_v2.1$ channels without $Ca^{2+}$ binding. Since it co-immunoprecipitates and co-localizes with $Ca_v2.1$ channels in the brain,[71] CaBP1 may be an important determinant of $Ca_v2.1$ channel function in neurons and may contribute to the diversity of function of these channels in the nervous system.

## Conclusion and Perspectives

Although $Ca^{2+}$-dependent regulation of VGCCs seems an elegantly simple way to control further $Ca^{2+}$ entry into cells, the underlying mechanism has proven deceptively complex. At resting levels of intracellular $Ca^{2+}$, CaM is prebound to regions upstream of the IQ-domain in

$Ca_v1.2$, and $Ca^{2+}$ permeating the channel orchestrates $Ca^{2+}$-dependent conformational shifts in the CaM binding pocket to include the IQ-domain, which leads to enhanced facilitation and/or inactivation of further $Ca^{2+}$ entry. CaM can also cause CDI and CDF of $Ca_v2.1$ channels, but the underlying mechanism appears to be fundamentally different from that for $Ca_v1.2$. First, the determinants for CaM binding and modulation of $Ca_v2.1$ function are unique. CaM binds in a $Ca^{2+}$-dependent manner to the modified IQ-domain and the CBD of $\alpha_1 2.1$, which both contribute to CDI and CDF, but are distinct from the corresponding sequences in $\alpha_1 1.2$. The modified IQ-domain of $\alpha_1 2.1$ differs from conventional IQ-motifs, including that in $\alpha_1 1.2$, in that the second residue is methionine rather than glutamine. Moreover, mutating the critical first two residues of $\alpha_1 2.1$ to alanine spares CDI for $Ca_v2.1$ but abolishes CDI in $Ca_v1.2$. By the same token, the CBD of $\alpha_1 2.1$ is not conserved in $\alpha_1 1.2$ and the A and C CaM tethering sites in $\alpha_1 1.2$ are not present in $\alpha_1 2.1$. How CaM binding to such distinct sites is transduced into similar forms of channel modulation is an intriguing question to resolve in comparative structure/function analyses.

Despite the remarkable advances in understanding how CaM interacts with VGCCs, in the nervous system, other $Ca^{2+}$ sensors may be physiologically more relevant than CaM in terms of channel modulation. NCBPs such as NCS-1 and CaBP1 may interact with and regulate $Ca_v2.1$ channels in neurons in ways that are distinct from CaM. Differences in subcellular targeting, $Ca^{2+}$ dependence of binding, or binding affinity may confer some NCBPs an advantage over CaM in competing for interaction with the same sites in the $\alpha_1$ subunit. Given that CaBP1, CaM, and potentially other NCBPs may be colocalized with VGCCs in neurons, it will be necessary to determine which $Ca^{2+}$-binding proteins preferentially regulate VGCCs and to understand the importance of these forms of VGCC modulation in vivo. Given the pathological consequences of VGCC defects, which include migraine, congenital deafness, and absence epilepsy, clarifying the physiological significance of $Ca^{2+}$-dependent modulation of VGCCs by CaM or other $Ca^{2+}$ sensors may reveal novel insights for the development of alternative therapeutic strategies for treating these and other disorders of aberrant VGCC function.

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# CHAPTER 12

# Voltage-Dependent Inactivation of Voltage Gated Calcium Channels

**Mary T. An and Gerald W. Zamponi**

## Summary

The fast inactivation of voltage-dependent calcium channels is an important, intrinsic regulatory mechanism that helps to precisely control the amount of calcium entering excitable cells during membrane depolarizations. The association with ancillary β subunits regulates the inactivation characteristics of the $\alpha_1$ subunit through functional (and possibly direct) interactions between the β subunit and N-terminal region of the $\alpha_1$ subunit. Moreover, palmitoylation of the β subunit N-terminus has emerged as a key regulatory mechanism of inactivation kinetics. Recent advances have provided novel insights into the calcium channel $\alpha_1$ subunit structural determinants of inactivation, revealing key roles for the S6 transmembrane regions, as well as intracellular linker such as the domain I-II and II-II linkers, and the C-terminus region. Current models include a mechanism of inactivation in which cytoplasmic loops may act as a gating particle that docks to the cytoplasmic end of the S6 transmembrane segments to block calcium flux. Here, we review the calcium channel structural determinants of fast, voltage-dependent inactivation of these channels.

## Introduction

The inactivation of ion channels is a fundamental biological process that prevents the breakdown of ionic gradients, and determines action potential duration and the refractory period of excitable tissues. It can be defined as a transition into a nonconducting state following channel opening. At the whole cell level, inactivation is seen as a decay of current levels during the course of the membrane depolarization, and a decreased availability for channel opening at more depolarized membrane potentials. In calcium channels, inactivation serves several unique purposes. It is a key mechanism by which cells are able to tightly control intracellular calcium levels. Such regulation of the temporal precision of calcium signals is of particular significance in view of the pivotal role of calcium as a cytoplasmic messenger in processes such as gene transcription or synaptic transmission.[1-4] For example, in nerve terminals, the inactivation of calcium currents may contribute to the short term depression of neurosecretion.[5-6] Conversely, calcium channel inactivation also helps to prevent the accumulation of excessive, cytotoxic levels of intracellular calcium.[7-9] Calcium channel inactivation also regulates cellular excitability. For example, in T-type $Ca^{2+}$ channels, voltage-dependent inactivation is of added significance as it is a key determinant of pattern behaviour and pacemaker activity in neurons. Calcium influx through T-type calcium channels during prolonged high frequency stimulation is critically dependent on the inactivation characteristics of the channels.[10-11] Changes in the inactivation properties of P/Q-type calcium channels are induced by naturally occurring mutations in the $Ca_v2.1$ $\alpha_1$ subunit (see Fig. 1),[12-14] raising the possibility that inappropriate inactivation of certain types of calcium channels may result in CNS malfunction. Finally, the

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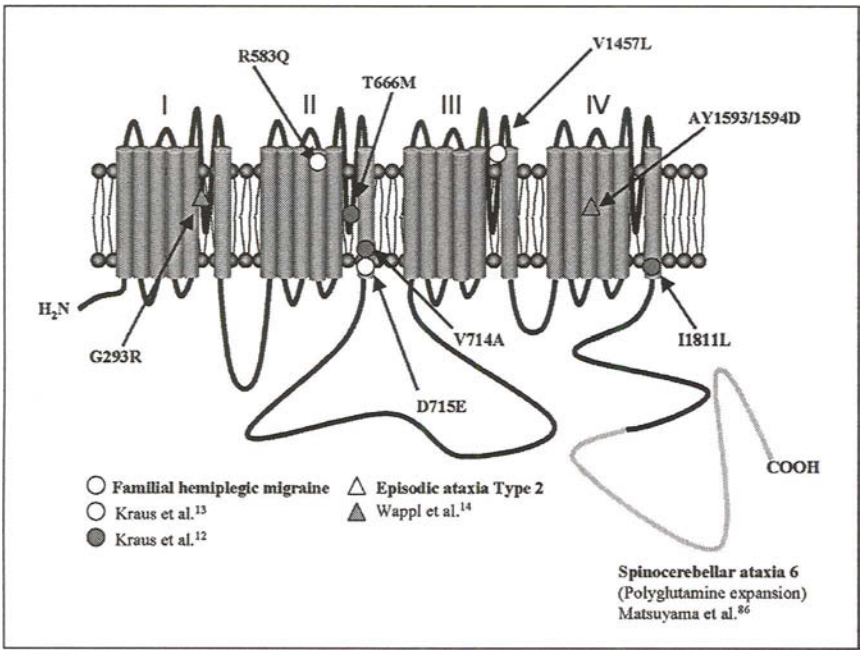



Figure 1. Schematic representation of the calcium channel $\alpha_1$ subunit depicting the loci of naturally occurring point mutations[12-14] and CAG repeats[86] that have been shown to affect inactivation properties of various types of voltage-gated calcium channels.

inactivated calcium channel conformation can result in dramatic increases in the affinity of the channel for a number of pharmacological agents, including many clinically used calcium channel therapeutics (see also Chapter 18).[15-17]

In principle, voltage gated calcium channels are capable of undergoing multiple types of inactivation processes. Calcium dependent inactivation can be observed predominantly with L-type channels,[18-19] although more recent evidence suggests that other types of high voltage-activated channels also undergo changes in inactivation kinetics in response to calcium entry.[20] This is reviewed in detail in Chapter 11, and will not be discussed further here. Slow inactivation occurs only after very prolonged membrane depolarizations (~1 minute) and remains poorly understood.[21-22] Here, we shall discuss aspects of fast voltage dependent inactivation.

## What Have We Learnt from Other Types of Voltage Gated Cation Channels?

It has been known for more than two decades that the exposure of sodium channels to cytoplasmically applied proteolytic enzymes results in a loss of inactivation.[23] Based on these initial findings, Armstrong et al[23] coined the "ball and chain mechanism of inactivation", in which inactivation was proposed to occur via occlusion of the channel pore by a cytoplasmic gating particle that was assumed to be physically tethered to the channel. Additional evidence for such a mechanism came from experiments that involved an anti-peptide antibody directed towards the short intracellular segment connecting sodium channel domains III and IV. Application of these antibodies to excised inside out patches resulted in the inhibition of inactivation.[24-25] The first conclusive molecular biological confirmation of the ball and chain mechanism was derived from experiments on the Shaker B potassium channels.[26-27] The amino terminus of the Shaker B $\alpha$ subunit contains a 22 amino acid cluster comprised

of hydrophobic and polar domains. Tryptic cleavage of this cluster, or deletion of the region by molecular biological means results in a dramatic slowing of inactivation kinetics.[26] But more strikingly, the application of synthetic peptides corresponding to the deleted regions restores the fast inactivation of mutated channels lacking the amino terminal,[27] with characteristics resembling those of typical open channel block with regard to concentration dependence of the open times.[28] Interestingly, the Shaker B amino terminal "ball" peptide is capable of inducing inactivation-like pore block of other types of potassium channels that do not inactivate under normal conditions.[29] Taken together, these results suggest a simple structural basis for the "ball and chain" inactivation model, with the N-terminus acting as the gating ball, and the remainder of the N-terminus serving as a ~50 amino acid long "chain" like tether for the ball.

Since voltage gated potassium channels are tetramers, each channel contains as many as four inactivation particles, but it is believed that only one may block the pore at a given time.[30] Less information is available on the exact binding site for the ball region, although functional studies indicate that the cytoplasmic linker between the fourth and fifth transmembrane segment of the $\alpha$ subunit may be involved.[31] However, the inactivation mechanism in potassium channels is complicated by the fact that certain types of potassium channel $\beta$ subunits (e.g., Kv $\beta$1.1 and Kv $\beta$3) contain regions that structurally and functionally resemble gating balls. Indeed, coexpression of these ancillary subunits with certain types of noninactivating Kv $\alpha$ subunits (i.e., Kv1.1, Kv1.2 or Kv1.5, but not Kv 1.6) produces rapidly inactivating currents.[32-33]

In the sodium channel field, it is more difficult to provide unequivocal evidence for the existence of a gating particle structure. It was shown that expression of the $\alpha$ subunit as two separate proteins (i.e., one corresponding to the first three domains, and one corresponding to the fourth domain) resulted in functional channels which inactivated, however, only very slowly.[34] Together with the antibody experiments mentioned earlier, this suggested a critical involvement of the domain III-IV linker region of the sodium channel $\alpha$ subunit in sodium channel inactivation. Indeed, site-directed mutagenesis of three adjacent hydrophobic amino acid residues in this region (I1488, F1489, M1490) to glutamine abolished fast inactivation,[35] which could be partially restored by cytoplasmic application of a pentapeptide containing the IFM motif.[36] This resembles in many ways the findings obtained with potassium channels, with the exception that a cytoplasmic loop rather than the N-terminal region is involved. Thus, the domain III-IV linker might act as a "hinged lid" that can dock to the pore and prevent current flow. There is evidence suggesting that the "hinge" might be formed by glycine and proline residues flanking the III-IV linker,[37] whereas the docking site for the "lid" structure may include the intracellular regions between the fourth and fifth transmembrane segments in domains III[38] and IV,[39] and the sixth transmembrane segment in domain IV.[40] The inactivation properties of the sodium channel $\alpha$ subunit are also modified through the noncovalent association of the auxiliary $\beta_1$ subunit which results in acceleration of inactivation kinetics, a speeding of the recovery from inactivation, and a negative shift in the voltage dependence of inactivation in both brain and skeletal muscle sodium channels.[41] Although the exact mechanism by which this occurs is not understood, it has been suggested that part of the effects of the $\beta$ subunits may be due to a switch in gating modes of the channel.[42]

Both sodium and potassium channels undergo a second, often slower inactivation process (termed slow inactivation in sodium channels and C-type inactivation in potassium channels) which is thought to occur via global conformational changes in channel structure in response to depolarization. For both sodium and potassium channels, this process appears to depend on regions flanking the pore[43-47] and may involve pore constriction.

In summary, in sodium and potassium channels, there is a common fast inactivation mechanism involving physical occlusion of the pore by an intracellular channel structure. Moreover, both sodium and potassium channel inactivation is regulated by ancillary subunits. As we will outline below, these two general principles may also apply to voltage gated calcium channels, although the molecular details are somewhat different.

## $\alpha_1$ Subunit Structural Inactivation Determinants in High Voltage Activated Channels

Based on work carried out in transient expression systems, the ability of voltage dependent calcium channels to inactivate appears to be an intrinsic feature of the $\alpha_1$ subunit, since expression of this subunit alone produces inactivating currents. Unlike in sodium and potassium channels, there have been no reports that show a loss of inactivation following intracellular application of proteolytic enzymes such as pronase or trypsin, suggesting the possibility that inactivation in calcium channels could perhaps be fundamentally different from that of other voltage-gated channels. This idea was supported by work of Zhang et al[48] who created a series of chimeric calcium channels that combined the structural features of rat $Ca_v2.1$ and marine ray $Ca_v2.3$ calcium channel $\alpha_1$ subunits (Zhang et al 1994). Based on functional studies carried out with these chimeras in *Xenopus* oocytes, the authors concluded that the differences in inactivation kinetics observed between the wild type channels could be attributed to the domain I S6 region. The apparent lack of involvement of a cytoplasmic loop led the authors to suggest the possibility that calcium channel inactivation might occur via a pore collapse mediated by the S6 segment, similar to what has been proposed for slow inactivation of potassium channels.[49-51] A similar possibility was raised by Spaetgens and Zamponi[52] based on chimera studies with hybrid $Ca_v1.2$ - $Ca_v2.3$ channels, which showed that all four transmembrane domains contributed to the rate and/or voltage-dependence of inactivation, with domains II and III mediating the largest effects. To test the hypothesis that the S6 segments might control inactivation in $Ca_v2.3$ channels, Stotz et al[53] exchanged the domain IIS6 and IIIS6 segments between $Ca_v1.2$ and $Ca_v2.3$ channels individually, or in combination. Consistent with the hypothesis, transfer of the domain IIS6 or the domain IIIS6 regions from the $Ca_v2.3$ channel onto the normally slowly inactivating $Ca_v1.2$ channel could confer the rapid inactivation kinetics seen with the rapidly inactivating wild type $Ca_v2.3$ channel. However, the converse experiment (i.e., slowing of inactivation of $Ca_v2.3$ with $Ca_v1.2$ sequence) did not alter inactivation rates, even when both the domain IIS6 and IIIS6 region were substituted concomitantly, suggesting that there had to be other structures that could independently maintain rapid inactivation kinetics. Indeed, only when the IIS6, IIIS6 and I-II linker regions were substituted together could $Ca_v2.3$ channel inactivation be slowed. Conversely, replacement of the $Ca_v1.2$ I-II linker with that of $Ca_v2.3$ conferred faster inactivation kinetics, suggesting that the domain I-II linker acts as a key structure in the inactivation process.[53] It is peculiar, however, that substitution of only the first two thirds of the $Ca_v1.2$ linker with $Ca_v2.3$ sequence mediates the opposite effect, namely a complete disruption of inactivation, yielding ultraslow inactivation kinetics (see also Fig. 2).[54-55] This may indicate that the structural integrity of this region may be critical for appropriate function. It is interesting to note that the domain IS6 chimera described by Zhang et al[48] involved a short stretch of the domain I-II linker being substituted, a notion that may perhaps serve to reconcile the apparently contradictory results with those of Spaetgens and Zamponi.[52] An involvement of the domain I-II linker region in calcium channel inactivation is also supported by a number of other studies on mutant calcium channels,[56-57] as well as from data obtained with a naturally occurring splice variant of $Ca_v2.1$ in which a single valine residue is inserted in this region which results in virtually complete loss of inactivation.[58] Indeed, this splicing event effectively converts $Ca_v2.1$ between a P-type and Q-type channel. Finally, Cens et al[59] reported that overexpression of peptides corresponding the domain I-II linker of $Ca_v2.1$ speeds the inactivation kinetics of transiently expressed $Ca_v2.1$ channels, which is consistent with the possibility of the domain I-II linker acting as a gating particle.

The involvement of the domain IIS6 and IIIS6 regions in calcium channel inactivation is supported by several other studies based on artificial mutants[60-61] and, perhaps more interestingly, based on naturally occurring mutations linked to disorders such as familial hemiplegic migraine (see Fig. 1).[12-14] A systematic investigation of residues involved in inactivation has been carried out for the domain II and IVS6 region of voltage gated calcium channels. Berjukov et al[62] reported that substitution of reissue M1811 in the domain IV S6 region of $Ca_v2.1$

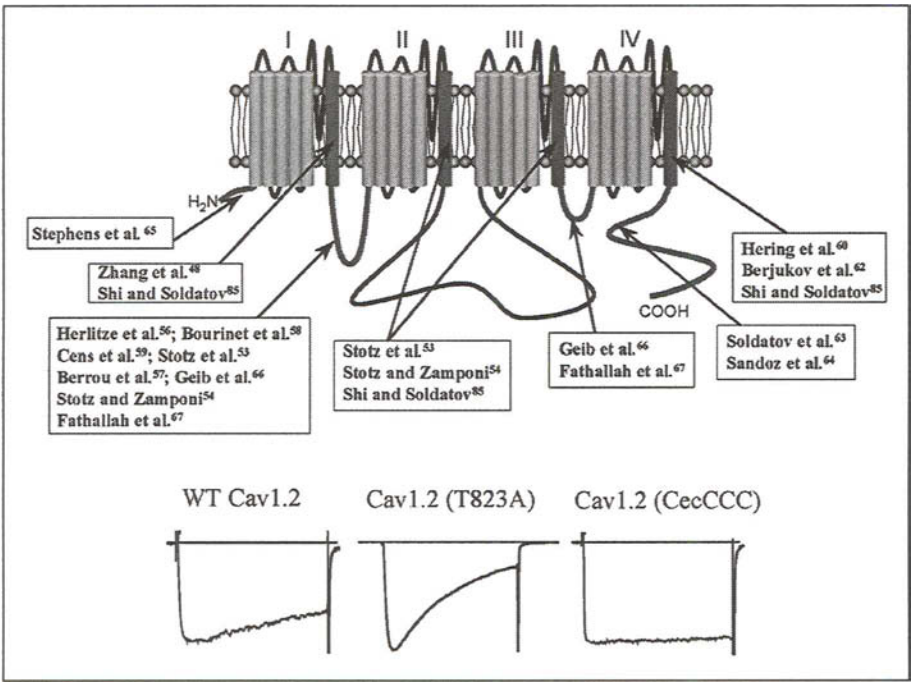



Figure 2. Regions of the calcium channel $\alpha_1$ subunits that have been implicated in inactivation (shown in dark gray—blue in online version). Inset: Current traces obtained from wild type $Ca_v1.2$ calcium channels, a $Ca_v1.2$ mutant in which residue T823 in IIS6 was replaced by alanine, and a $Ca_v1.2$ channel carrying part of $Ca_v2.3$ sequence in the domain I-II linker. Note that the S6 mutant shows dramatically faster inactivation, whereas substitution of the first two thirds of the I-II linker with $Ca_v2.3$ sequence (Cav1.2-CecCCC) completely disrupts inactivation.[54] A color version of this figure is available online at www.Eurekah.com.

calcium channels with glutamine results in a 75-fold increase in inactivation rates. Conversely, the authors showed that inactivation kinetics could be slowed by substitution of residue V1818. Stotz and Zamponi[54] systematically examined inactivation determinants in the IIS6 region of the $Ca_v1.2$ channel. The authors showed that replacement of two out of seven of amino acids in this region that were not conserved between $Ca_v1$ .2 and $Ca_v2.3$ could mimic the effect of exchanging the entire domain IIS6 segment between these channels. Moreover, a systematic replacement of these residues with a variety of different amino acids revealed that increasing size in position 823 slowed inactivation kinetics, whereas hydrophobicity was a more important determinant in position 829. Indeed, replacing F823 with alanine induced rapid inactivation kinetics resembling those typically only seen with T-type calcium channels, but neither recovery from inactivation, nor the voltage-dependence of inactivation were affected. These results indicate that only the rate of entry into the inactivated conformation, but not its stability was controlled by these residues. Taken together, the data of Berjukov et al,[62] Stotz et al,[53] Zhang et al,[48] and Stotz and Zamponi[54] suggest that all four S6 segments of the calcium channel $\alpha_1$ subunit contribute to inactivation.

Besides the domain I-II linker, three other cytoplasmic regions have been implicated in inactivation. Data from Soldatov et al[63] and Sandoz et al[64] have implicated the C-terminal region in voltage dependent inactivation of calcium channels, whereas Stephens et al[65] reported the N-terminal as an important regulator if inactivation. However, in both of these instances, this may be secondarily due to altered $\beta$ subunit interactions with the pore forming

$\alpha_1$ subunit. More recently, Geib et al[66] have reported that inactivation of $Ca_v2.1$ channels is regulated by an intramolecular interaction between the domain I-II and the domain III-IV linker regions. Site-directed mutagenesis, or intracellular application of peptides designed to disrupt this interaction slows the inactivation kinetics of the channel, provided that the channel complex does not contain a $\beta$ subunit. In follow up work, the authors[67] suggested that the domain III-IV linker region might structurally resemble the $G_\gamma$ subunit, and that this loop physically interacts with the $G_{\beta\gamma}$ binding site[68-69] in the domain I-II linker. The only remaining major intracellular loop, i.e., that connecting domains II and III, does not appear to be a major determinant of inactivation rate, since deletion of most of this region in a naturally occurring splice variant of the N-type channel produces normal current kinetics.[70] However, in these splice variants, the half-inactivation voltage is shifted towards more depolarized potentials compared to full length channels. This is consistent with the effects of syntaxin 1A and SNAP25, which bind to this region and induce hyperpolarizing shifts in the voltage dependence of inactivation (see Chapter 8).

Taken together, the inactivation of high-activated calcium channels is a complex process that involves a number of $\alpha_1$ subunit structural determinants (see Fig. 2), comprised predominantly of the S6 transmembrane segments. In addition, the domain I-II linker appears to be a key structure involved in this process, but its role in inactivation may be modulated by other cytoplasmic loops.

Members of the family of T-type calcium channels have only recently been cloned, therefore inactivation determinants have not yet been systematically examined for these channels. To date, there is conflicting information about what may be key structural requirements. Staes et al[71] reported that the C-terminus region of the $Ca_v3.1$ channel is important for inactivation. In contrast, Marksteiner et al[72] implicated the domain IIIS6 segment as a major inactivation determinant of these channels. Despite some apparent overlap in the structural determinants of inactivation, it remains at this point difficult to determine as to whether inactivation in low and high voltage activated calcium channel occurs via a functionally conserved mechanism. The fact that T-type calcium channel do not associate with calcium channel $\beta$ subunits,[73] and the low degree of sequence identity in the I-II linker regions of high versus low voltage activated channels may perhaps suggest that T-type channels could be unique among voltage-dependent calcium channels in terms of their inactivation mechanism. Future chimeric approaches will certainly shed light on this issue.

## Role of Ancillary Subunits in Calcium Channel Inactivation

Although $\alpha_1$ subunits can inactivate when expressed alone, the ancillary $\beta$ subunits are major modulators of the inactivation process.[74-75] For many types of high voltage activated channels, coexpression with $\beta_3$ or $\beta_{1b}$ tends to increase the rate of inactivation, whereas $\beta_4$, and particularly, the rat isoform of $\beta_{2a}$ slow inactivation kinetics.[76-77] Using a chimeric approach, Olcese et al and coworkers[78] showed that the N-terminal region of the rat $\beta_{2a}$ subunit was responsible for this subunit's dramatic slowing effect on inactivation. Deletion of the first 18 amino acids of the N-terminus removes the slowing effect.[79] What is unique about the N-terminus of the rat $\beta_{2a}$ subunit is the presence of two cysteine residues that form sites for palmitoylation. Replacement of the cysteines with serines,[77,80] or the block of palimitoylation via tunicamycin[81] blocks the kinetic slowing effect of the rat $\beta_{2a}$ subunit. Replacement of the N-terminus with a transmembrane region such as the CD8 receptor can restore the ability of the $\beta_{2a}$ subunit to slow calcium channel inactivation.[82] This is consistent with the role of palmitoylation, and suggests that membrane association of the N-terminus region is involved in regulating inactivation kinetics. Besides the N -terminus, additional interactions between the second variable region of the $\beta_{2a}$ subunit[79] and the N-terminus of the calcium channel $\alpha_1$ subunit[65] appear to contribute to the regulation of inactivation. Finally, for $\beta_4$ subunits, it has been shown that the highly variable C-terminal region can interact with the $Ca_v2.1$ carboxy terminus to regulate inactivation kinetics.

The effects of $\alpha_2$-$\delta$ and $\gamma$ subunit on inactivation have been less completely addressed, but the evidence to date suggests that both the $\alpha_2$-$\delta$[83] subunits and the $\gamma$[84] subunits can regulate inactivation. Coexpression of $Ca_v1.2$ and $Ca_v2.3$ calcium channels with different $\alpha_2$-$\delta$ subunits shifts voltage dependence of inactivation, and alters inactivation kinetics.[83] The $\gamma$ subunit appears to be able to regulate inactivation kinetics by enhancing a slowly inactivating current component seen with $Ca_v2.1$ calcium channels.[84] The mechanisms that underlie these effects have, however, not yet been fully explored.

Overall, of the ancillary calcium channel subunits, the $\beta$ subunit has by far the most profound effects on calcium channel inactivation. The picture emerges that these $\beta$ subunits interact in multiple ways with the calcium channel $\alpha_1$ subunit to regulate inactivation. Along these lines, the effect of mutagenesis of residues in the $\alpha_1$ subunit N-terminus[65] or C-terminus[63-64] may arise secondarily from a loss of $\beta$ subunit regulation rather than indicating a direct involvement of these regions in the core inactivation process.

## Possible Molecular Mechanism of Calcium Channel Inactivation

From the collective body of structure function studies, the picture emerges that the key structural determinants of inactivation are the domain I-II linker and the S6 segments in the four major transmembrane domains (see also ref. 85). The involvement of the a cytoplasmic domain and of transmembrane helices lining the inner vestibule of the pore is consistent with a pore blocking mechanism of inactivation similar to that seen with sodium and potassium channels. In such a hinged-lid mechanism, the domain I-II linker may act as the inactivation lid that may dock to the S6 segments to prevent current flow through the channels.[53-54] The intramolecular interaction with the domain III-IV loop,[66] or the association of the I-II linker with a membrane associated calcium channel $\beta$ subunit (such as rat $\beta_{2a}$) may restrict the mobility of this putative inactivation gate, thus modulating the inactivation kinetics. The additional interactions between regions on the $\beta$ subunit and the N-terminus[65] and C-terminus[64] regions of the $\alpha_1$ subunit may indirectly affect I-II linker function. Finally, a role of the I-II linker as an inactivation particle is consistent with the effects of overexpressed I-II linker peptides on inactivation rates.[59]

The observation that mutations in the domain IIS6 segment of $Ca_v1.2$ channels (i.e., F823, T829) modify the rate of inactivation, but not the recovery from inactivation suggests that only the rate of entry into the inactivated state is affected and once the channel is inactivated, the stability of the inactivated conformation does not involve these residues. Accordingly, Stotz and Zamponi[54] proposed that in response to membrane depolarization, residues 823 and 829 may be involved in a conformational change in the II S6 segment that culminates in the availability of a docking site for the inactivation gate. Once docking has occurred, the lifetime of the docked state would then no longer be influenced by these two residues (see Fig. 3). Based on work by several other groups,[62,48] the authors proposed that this principle would likely apply to all four S6 segments, suggesting a concerted action of the four pore lining transmembrane segments in the inactivation process. It is worth noting that at their cytoplasmic ends, these four transmembrane helices carry several highly conserved residues, which when mutated, result in slowing of inactivation.[22] Mutations in additional S6 segments produce incremental, additive effects. This raises the strong possibility that the S6 segments per se may form the docking site for the I-II linker region, but whether all four S6 segments act cooperatively to form a single site, or whether the inactivation gate has a choice of 4 different docking sites remains to be determined.

Taken together, the model proposed by Stotz and Zamponi[54] can account for the bulk of the structure function data reported in the literature to date. However, it is important to remember that this is merely a model whose validity is difficult to confirm without detailed structural information.

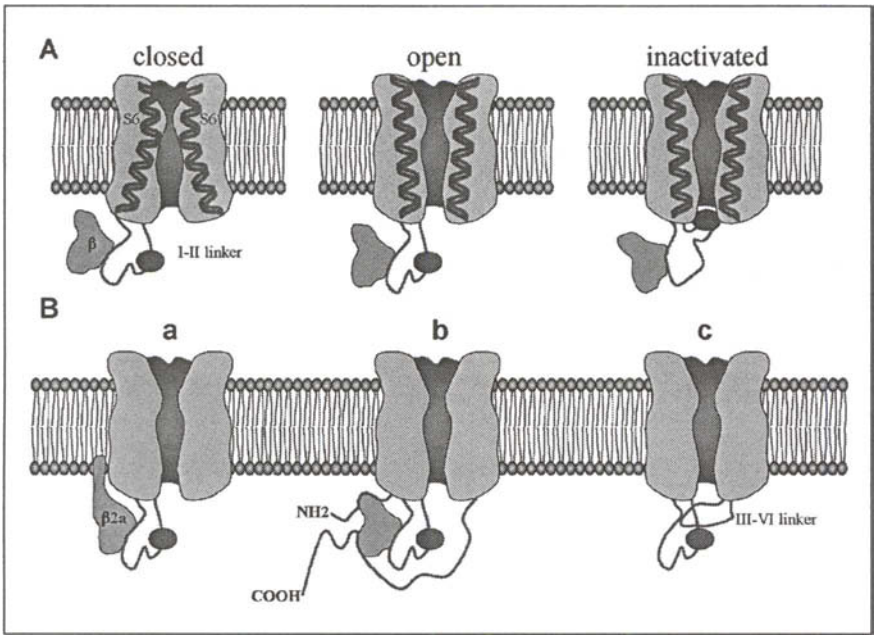



Figure 3. A) Possible model for calcium channel inactivation. Following membrane deploarization, the S6 segments undergo a conformational channel that unmasks a docking site for the inactivation gate formed by the domain I-II linker. B) Reconciliation of the model presented in panel A with data by a number of other laboratories (see main text). a: Membrane insertion of the palmitoylated N-terminus of the rat $\beta_{2a}$ subunit restricts the mobility of the inactivation gate. b: Interactions between the calcium channel β subunit with the N-terminus and/or the C-terminus region of the $\alpha_1$ subunit indirectly affects I-II linker function as the inactivation gate. c: Interactions between the I-II linker and the III-IV linker that occur in the absence of the β subunit directly regulate inactivation.

## Concluding Remarks

Many structural determinants of high voltage activated calcium channel inactivation have been identified over the past 8 years. To date, the model that is most consistent with the currently available literature is that of a hinged-lid mechanism of inactivation, in which the mobility of the lid is regulated by other intracellular loops and ancillary subunits. The basic mechanism of calcium channel inactivation therefore appears to be a common feature to voltage gated sodium and potassium channels, which reaffirms the fundamental importance of the inactivation process in the physiology of excitable cells.

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# CHAPTER 13

# Selective Permeability of Voltage-Gated Calcium Channels

**William A. Sather**

## Abstract

Advances based on experiment and theory are converging upon a structural description of the selectivity mechanism of voltage-gated calcium channels. The emerging description differs from that for potassium channels, which make use of a stiff filter lined with main chain carbonyl oxygens that snugly fit $K^+$ ions. Instead, calcium channels use a selectivity filter composed of the side chain carboxylate oxygen atoms of a cluster of four glutamate residues (EEEE locus). These carboxylate-bearing side chains are thought to project into the aqueous lumen of the pore where they sort $Ca^{2+}$ from other would-be permeant ions. The EEEE locus is evidently flexible: it can tightly bind a single $Ca^{2+}$ ion in order to block $Na^+$ flux but rearranges to accommodate multiple $Ca^{2+}$ ions in order to allow high $Ca^{2+}$ flux. The four glutamates are not functionally equivalent, an observation that is suspected to be important for $Ca^{2+}$ passage through the selectivity filter. This chapter summarizes the experimental results that support these conclusions and the theoretical models that have been proposed to explain how calcium channels can be at once highly selective and yet readily permeated.

## Introduction

The selective permeability of voltage-gated calcium channels is essential for transduction of cellular electrical activity into cytosolic $Ca^{2+}$ signals used by cells to initiate such processes as neurotransmitter release, contraction of cardiac muscle, and alteration of gene transcription. By opening in response to membrane depolarization, voltage-gated calcium channels permit $Ca^{2+}$ in the extracellular solution, which has a $Ca^{2+}$ concentration on the order of a few millimolar, to enter the cell and raise cytosolic $Ca^{2+}$ concentration above its resting level of ~0.1 micromolar.

Rapid transit of $Ca^{2+}$ through the pore of voltage-gated calcium channels is required to produce the cytosolic $Ca^{2+}$ transients that are necessary for release of neurotransmitter or contraction of cardiac muscle. Coincident with rapid $Ca^{2+}$ transit, voltage-gated calcium channels must be able to distinguish $Ca^{2+}$ ions from similarly sized $Na^+$ ions. Because $Na^+$ ions are much more abundant in the extracellular solution as compared to $Ca^{2+}$ ions, voltage-gated calcium channels have necessarily evolved a selectivity mechanism that places them among the most highly selective ion channels known.

As a consequence of their robust selective permeability, voltage-gated calcium channels have become a favored model system for investigation of ion selectivity mechanisms. Analysis of selective permeability in calcium channels has benefited from the wealth of information regarding structure of $Ca^{2+}$ binding sites in a variety of proteins and organic molecules. In addition, parallel study of calcium channels and potassium channels has greatly enriched the comparative biology of ion channel selectivity, with progress for each channel enhancing

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understanding of selective permeation in both. The present chapter considers the importance for selectivity of $Ca^{2+}$ binding within the pore, evidence that multiple ion occupancy of the pore is essential for rapid $Ca^{2+}$ throughput, what tinkering with pore structure has revealed about selectivity filter structure, and how theoretical approaches contribute to understanding selective permeability of $Ca^{2+}$ channels.

## Selectivity by Ion Binding Affinity

Some ions are simply too large to pass through a channel's selectivity filter: in calcium channels, cations larger than tetramethylammonium are too big to pass.[1] Among ions smaller than tetramethylammonium, various metal cations ($Ba^{2+}$, $Sr^{2+}$, $Li^+$, $Na^+$, $K^+$ and $Cs^+$, in addition to $Ca^{2+}$) will pass through the pore of a calcium channel under certain experimental conditions.[2-5] However, calcium channels strongly select among these cations when more than one of them is present. Most pertinent to the physiological situation, calcium channels reliably discriminate between $Ca^{2+}$ and the 100-fold more abundant $Na^+$ despite the fact that these two ions are nearly identical in diameter (2 Å). How does this happen?

In general, a selectivity filter must be able to stabilize permeant ions much as water stabilizes them in solution. In calcium channels, selective permeability is based on the fact that $Ca^{2+}$ ions are more effectively stabilized in the filter than are other ions, particularly $Na^+$ ions. This idea-that relative ion binding affinity underlies calcium channel selectivity among small metal cations-was first suggested by the finding that $Ca^{2+}$ reduced the amplitude and upstroke velocity of action potentials supported by $Sr^{2+}$ current through calcium channels, as though $Ca^{2+}$ blocked $Sr^{2+}$ current by competing with $Sr^{2+}$ for a binding site in the channel.[6] Other early evidence for the importance of binding affinity was the observation that $Co^{2+}$ blocks $Ca^{2+}$ current through calcium channels less potently than it blocks $Ba^{2+}$ current through these same channels: the greater resistance of $Ca^{2+}$ current to block by $Co^{2+}$ can be understood if the pore binds $Ca^{2+}$ more tightly than $Ba^{2+}$.[7]

Bi-ionic reversal potentials provide one means of estimating the relative permeability for divalent and monovalent cations.[8-11] From these kinds of measurements, the relative permeability sequence has been found to be $Ca^{2+} > Ba^{2+} > Li^+ > Na^+ > K^+ > Cs^+$. $Ca^{2+}$ is selected over $Na^+$ by >1000:1, which explains why $Ca^{2+}$ current is little influenced by extracellular $Na^+$ concentration.[12,13]

A permeability sequence can also be derived from measurements of single-channel conductance: a smaller conductance corresponds to a slower rate of ion passage through the pore and lower permeability. Single-channel conductance for $Ca^{2+}$ is small, indicating that this ion passes slowly through the pore, whereas conductance for $Na^+$ is larger because this ion passes through more rapidly. The permeability sequence obtained from single-channel conductance, $Ca^{2+} < Ba^{2+} < Li^+ < Na^+ < K^+ < Cs^+$, is opposite in order to that obtained from reversal potentials and seems to indicate that $Ca^{2+}$ is less permeant than, for example, $Na^+$.[12,14,15] However, this seeming contradiction can be resolved by considering the consequences of binding permeant ions in the pore. Permeant ions that bind with higher affinity will reside, on average, for a longer period of time on the binding site and will therefore travel through the pore more slowly, yielding smaller single-channel conductance.[16] This "sticky pore" concept thus predicts that ions with higher binding affinity ($Ca^{2+}$) have high relative permeability but small conductance, whereas ions with lower affinity ($Na^+$) have low relative permeability but high conductance.

## A Multi-Ion Pore Confers High Flux

For a single-file pore that cannot contain more than one ion, selection by affinity is ineffective.[16] If such a pore is confronted with an equimolar mixture of two permeant ions which differ by, for example, 1000-fold in affinity, the higher affinity ion traverses the pore 1000-fold slower, but it also blocks passage of the lower affinity, faster-throughput ion 99.9% of the time. The result is that the two ion species cross the pore at the same net rate and they are thus equally permeant. Pores that can contain more than one ion are not restricted in this way:

intrapore interactions between ions allow one high-affinity ion to elute another and so selectivity with high flux is achieved.

Three experimental observations indicate that calcium channels function as multi-ion pores. First, single-channel conductance is smaller in a mixture of $Ca^{2+}$ and $Ba^{2+}$ than in $Ba^{2+}$ or $Ca^{2+}$ alone.[4,17] The phenomenon is much larger for mixtures of $Ca^{2+}$ and $Na^{+}$.[18] This anomalous mole-fraction behavior[19] has been attributed to reciprocal, partial block by one ion species of flux by the other, which presupposes that the pore contains multiple ion binding sites and that ions move from site to site in single file.[4,18,20,21] The second line of evidence indicating that calcium channels are multi-ion pores follows from the fact that calcium channels bind $Ca^{2+}$ with two distinct affinities: $Ca^{2+}$ blocks $Na^{+}$ movement through calcium channels with a 1 μM dissociation constant ($K_D$), whereas single-channel $Ca^{2+}$ current saturates with a 14 mM $K_D$.[4,18] The two affinities have been thought to correspond to two $Ca^{2+}$ binding sites, and hence $Ca^{2+}$ channels appear able to contain at least two $Ca^{2+}$ ions.

The third and strongest piece of evidence that calcium channels can contain at least two divalent cations comes from study of the kinetics of $Cd^{2+}$ block of single-channel $Ba^{2+}$ currents.[22] $Cd^{2+}$ blocks calcium channel current by binding to a site in the pore that more usually binds $Ca^{2+}$ or other permeant ions, including $Ba^{2+}$. The rate of $Cd^{2+}$ unbinding (unblock) has been shown to depend upon the concentration of $Ba^{2+}$ in the bathing solution, so that raising $Ba^{2+}$ in the bathing solution—and thereby increasing the probability of $Ba^{2+}$ occupying the pore—speeds unbinding of $Cd^{2+}$. The ability of $Ba^{2+}$ to speed $Cd^{2+}$ exit must reflect an intrapore interaction between these ions because this is where the blocking $Cd^{2+}$ ion is located. The pore therefore seems able to accommodate at least two divalent cations. Moreover, the enhancement of $Cd^{2+}$ exit rate by $Ba^{2+}$ brings to mind the elution of one $Ca^{2+}$ ion by another, hypothesized to be the key feature supporting large $Ca^{2+}$ flux through the high-affinity selectivity filter.

How advantageous for flux is a multi-ion pore? Consider a one-site pore, for which the theoretical upper limit on flux is given by the rate of unbinding of the permeant ion: $k_{off} = k_{on}/K_D$ (Fig. 1A). In calcium channels, 1 μM $Ca^{2}$ half-blocks $Na^{+}$ current ($K_D = 10^{-6}$ M). This concentration is so low that only a single $Ca^{2+}$ ion would be bound in the selectivity filter during block, making this a one-ion situation. With this $K_D$, and assuming that $Ca^{2+}$ ions arrive at the site as fast as diffusion allows ($k_{on} \sim 10^{9}\ M^{-1}s^{-1}$), the maximal off-rate and hence flux is only $10^{3}$ $Ca^{2+}$ ions per second. In Figure 1A, arrow 2 indicates the size of the jump that a $Ca^{2+}$ ion must make in order to get out of a high-affinity, one-site pore. The large size of the jump explains the low rate of movement over the barrier and small flux through the one-site pore. But open calcium channels pass roughly $10^{6}$ $Ca^{2+}$ ions per second. One way high flux with high $Ca^{2+}$ binding affinity might work is based on interaction between pairs of $Ca^{2+}$ ions bound in the pore. Figure 1B illustrates such a scenario. When one $Ca^{2+}$ ion is bound tightly in either of the two postulated high-affinity sites, $Na^{+}$ flux is stopped, but when both sites are occupied by $Ca^{2+}$, electrostatic repulsion between the $Ca^{2+}$ pair decreases net affinity for pore and high flux results.[4,18] In this model, tight binding of one $Ca^{2+}$ ion maintains high selectivity while electrostatic repulsion provides the energy to push $Ca^{2+}$ ions through the avid selectivity filter 1000x faster than would be allowed in a one-ion, high-affinity pore.

Although high flux seems to require a multi-ion pore, such a pore need not necessarily possess more than one high-affinity site in order to support high flux. Indeed, the voltage-dependence of $Ca^{2+}$ block of monovalent cation current indicates that high-affinity binding occurs just within the extracellular entrance of calcium channels, as if only one high-affinity binding region is present in calcium channels.[14,15] But how do ions get out of this site? One possibility is that low-affinity sites flank the high-affinity region, and these might act as stair steps to allow an ion to climb out of the central high-affinity site.[23] Figure 1C illustrates this idea. Experimental evidence for such low-affinity sites has been obtained.[14] Another useful way of viewing the role of the low-affinity sites is that they act to increase the probability that the pore is multiply occupied, because ions reside in the high-affinity site, and though more briefly, at the low-affinity sites too. Calculations with the model show that substantial

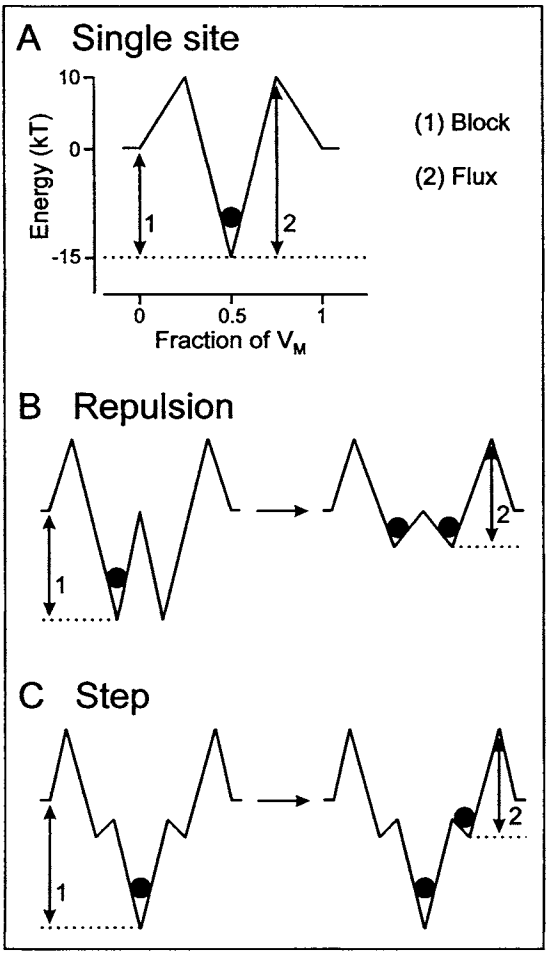



Figure 1. Three rate theory models for pores that have high affinity for $Ca^{2+}$. The fraction of the electric field ($V_M$) experienced by a $Ca^{2+}$ ion as it passes through the pore is indicated on the horizontal axis (0 = extracellular end, 1 = intracellular end). Chemical potential energy is indicated on the vertical axis. $Ca^{2+}$ ions are represented by solid black circles, and the energy profile along the permeation pathway is represented by solid lines. Lengths of vertical arrows represent (arrow 1) strength of the highest affinity site and (arrow 2) the size of the barrier that $Ca^{2+}$ must hurdle to exit the pore. The length of arrow 1 corresponds to the ability of $Ca^{2+}$ to block flux of foreign ions, and the length of arrow 2 corresponds to $Ca^{2+}$ flux, with a shorter arrow 2 indicating greater flux. In a one-site pore A), flux is very low because the energy barrier for exit is large (–22kT; arrow 2). In multi-ion pores, $Ca^{2+}$ flux is increased because the size of the rate-limiting barrier is reduced: in B), ion-ion interactions lower binding affinity, whereas in C) low-affinity sites flanking the selectivity site provide stair steps out of the central energy well. Modified with permission from: Dang TX, McCleskey EW. Journal of General Physiology 1998; 111:185-193. © 1998 The Rockefeller University Press.

occupancy of the low-affinity sites is required to achieve significant flux. Thus even with a single high-affinity site, the pore preserves high flux through multiple ion occupancy. Other models of a high-flux pore with a single high-affinity site have been developed as well.[24]

Summarizing ideas regarding binding and flux in calcium channels yields the following view. Ions move in single file through the pore, but several species of divalent and monovalent metal cations are able to pass through. Relative binding affinity determines, for a mixture of permeant ions, which will be permeant; higher affinity ions block flux by lower affinity ones. Multiple ion occupancy seems essential for high flux. These ideas also apply to potassium channels, for which ideas of single-file, multi-ion behavior were first developed.[24-29] For potassium channels, crystal structure shows a single file, multiply occupied pore.[30]

## Amino Acid Residues of the Selectivity Filter

The cloning of L-type calcium channels from skeletal[31,32] and cardiac[33] muscle allowed identification of structural features underlying ion binding and multi-ion behavior. Figure 2 illustrates the pore lining sequences of calcium channels.

The most obvious candidate binding site in the P-loops was a conserved cluster of four glutamate residues (0 position; Fig. 2) referred to as the EEEE locus. Each of the four P-loops

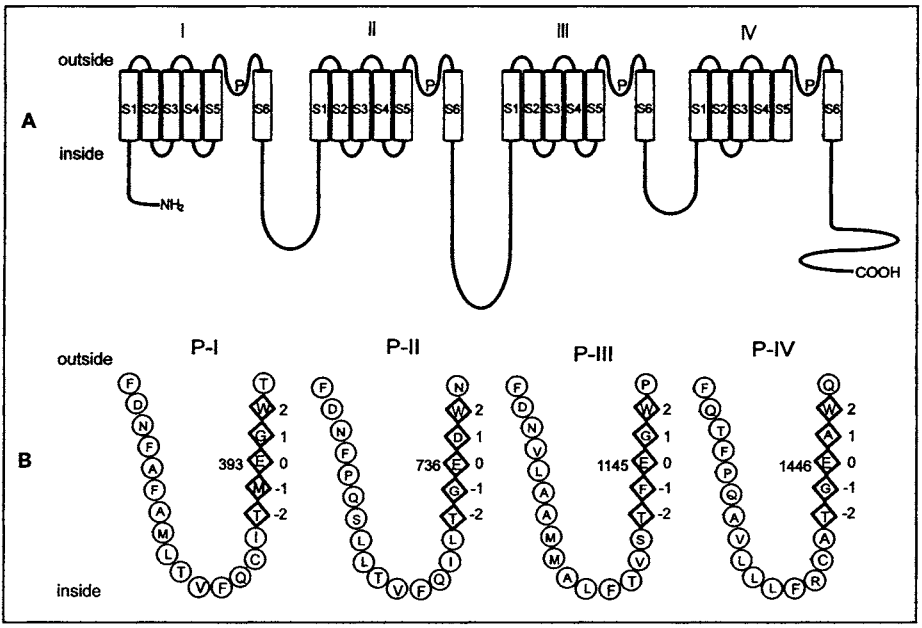



Figure 2. Membrane organization and amino acid residues that form the pore lining for an L-type calcium channel. In the four pore-lining loops (P-I through P-IV), the EEEE locus glutamate is designated as the 0 position. Amino acid sequence number is marked to the left of each EEEE locus glutamate.[31] Reprinted with permission from: Wu XS, Edwards HD, Sather WA. J Biol Chem 2000; 275:31778-31785. ©2000 The American Society for Biochemistry and Molecular Biology, Inc.

contributes a glutamate to the locus, and in analogy to $Ca^{2+}$ binding by chelators like EGTA[34], a tetracarboxylate binding site formed from the glutamate side chains was an immediately attractive idea. The first experimental evidence supporting the importance of the EEEE locus was obtained using a mutant sodium channel engineered to possess a DEEE locus: micromolar $Ca^{2+}$ blocked $Na^+$ current carried by this mutant and $Ca^{2+}$ was permeant, both hallmarks of calcium channels.[35] Single point mutations introduced into the EEEE locus of calcium channels severely compromised ion selectivity: measurement of bi-ionic reversal potentials revealed that the relative permeability of $Ca^{2+}$ was decreased in EEEE locus mutants, and block experiments showed that binding affinity for $Ca^{2+}$ was also decreased.[36-38]

Among calcium channel mutants in which only one EEEE locus glutamate was replaced, even relatively conservative substitutions such as aspartate or glutamine significantly decreased $Ca^{2+}$ binding affinity.[38] This finding, unexpected at the time, strongly suggested that calcium channels contain one—not two—high-affinity ($K_D$ ~ 1 μM) binding sites. Whether the EEEE locus represents the sole region of high-affinity interaction with $Ca^{2+}$ was therefore tested using constructs in which the entire EEEE locus was replaced with four glutamine or four alanine residues.[39] In these quadruple mutants (QQQQ or AAAA), half-block of monovalent current occurred at ~1 mM $Ca^{2}$) (Fig. 3). This weakened $Ca^{2+}$ block strongly implies that all high-affinity binding between $Ca^{2+}$ and the pore occurs in a region defined by the EEEE locus; there is apparently no other high-affinity site in the pore.

A potential loose end in this argument is that divalent cations are unable to carry currents of detectable size through the pore of the QQQQ or AAAA mutants.[39] Could the absence of potent block by $Ca^{2+}$ of monovalent current be attributed to an inability of $Ca^{2+}$ to pass through a hydrophobic QQQQ or AAAA locus and bind with high affinity to a putative second, and

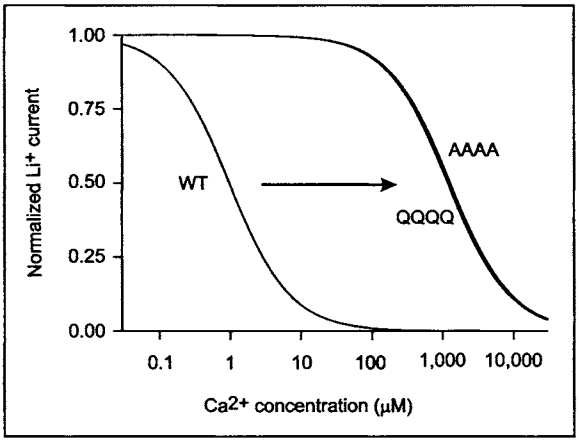



Figure 3. EEEE locus quadruple mutants lack high-affinity binding of divalent cations. The graph illustrates the concentration-dependence of block by $Ca^{2+}$ of normalized $Li^+$ current carried by calcium channels expressed in Xenopus oocytes. WT = wild type, Q4 = quadruple glutamine mutant, A4 = quadruple alanine mutant. Fitted curves are 1:1 binding functions. Modified with permission from: Ellinor PT, Yang J, Sather WA, et al. Neuron 1995; 15:1121-1132. ©1995 Cell Press.

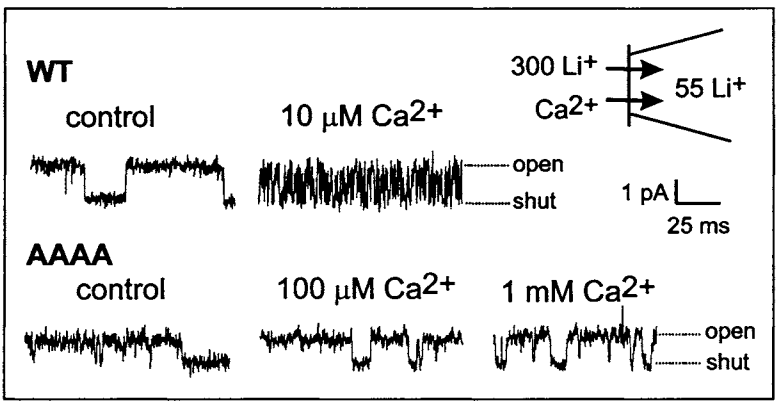



Figure 4. Recordings of single-channel currents in inside-out excised patches show that $Ca^{2+}$ applied to the intracellular pore entrance does not block monovalent current carried through EEEE locus quadruple mutants. WT = wild-type, AAAA = quadruple alanine mutant. The blocking ion ($Ca^{2+}$) and $Li^+$ current flow in the same, outward, direction (diagram at upper right). In the absence of $Ca^{2+}$, wild-type and mutant channel openings are long owing to the action of the dihydropyridine Bay K 8644. In the presence of 10 mM $Ca^{2+}$, Bay K 8644-prolonged openings of wild-type are interrupted at high frequency by brief $Ca^{2+}$ block events (top, middle). Openings of the quadruple alanine mutant are unaffected at up to 1 mM $Ca^{2+}$. Reproduced with permission from: Cibulsky SM, Sather WA. Journal of General Physiology 2000; 116:349-362. ©2000 The Rockefeller University Press.

more distally located high-affinity site? In pursuit of this idea, inside-out excised patches were used to examine whether $Ca^{2+}$ entering from the intracellularly-disposed pore entrance could block monovalent current.[40] As illustrated in Figure 4, these experiments failed to reveal high-affinity block by $Ca^{2+}$ entering from the intracellular pore mouth. The results thus support the conclusion, as first thought, that the EEEE locus constitutes the only region of high-affinity $Ca^{2+}$ binding in the pore.

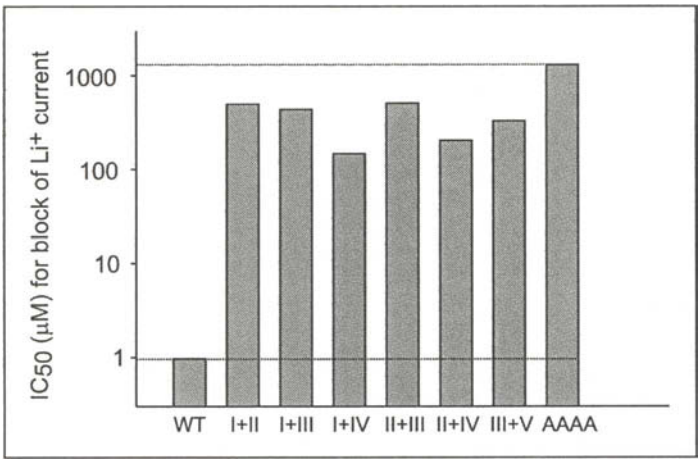



Figure 5. The EEEE locus does not house two high-affinity binding sites. $Ca^{2+}$ block of $Li^{+}$ current for channels with two alanine substitutions in the EEEE locus. Half-block values are compared for wild-type (WT), all six double alanine mutants (motifs bearing alanine substitutions are indicated by roman numerals), and the quadruple alanine mutant (AAAA). $Ca^{2+}$ block—binding affinity—was severely reduced in each of the six double alanine mutants, indicating that none of the pairs of EEEE locus glutamates forms a high-affinity binding site. Modified with permission from reference: Modified with permission from: Ellinor PT, Yang J, Sather WA, et al. Neuron 1995; 15:1121-1132. ©1995 Cell Press.

## A Single High-Affinity Locus

The attractiveness of the two-site model[4,18] was such that although mutagenesis experiments showed that no high-affinity site exists outside the EEEE locus, it was important to determine whether the EEEE locus might house two high-affinity sites itself. It was specifically postulated that the locus might be functionally subdivided into two working pairs of glutamates, with each pair forming a high-affinity site.[39] This seems sensible because each pair of glutamates would bear a charge of –2, exactly the charge needed to electrostatically balance one $Ca^{2+}$ ion. Pursuing this idea, pairs of alanine substitutions were introduced into the EEEE locus, on the idea that some of the double alanine mutations would knock out one putative high-affinity site (functional glutamate pair) but leave the other intact. Among the six ways that the glutamates might be paired, double alanine substitution would in two cases leave high-affinity binding intact, according to the hypothesis. The experimental results showed, however, that $Ca^{2+}$ block, and hence binding affinity, was severely reduced (>100x) in each of the six double alanine mutants (Fig. 5). The EEEE locus apparently does not contain two high-affinity sites.

In fact, even single point mutations in the EEEE locus significantly weaken $Ca^{2+}$ binding, by up to 50-fold for single alanine substitutions.[38,39] These results, combined with the fact that block of monovalent current occurs when only one $Ca^{2+}$ ion is bound in the pore, imply that each glutamate interacts with the single $Ca^{2+}$ ion in the EEEE locus. In other words, all four EEEE locus glutamates are involved in high-affinity binding, forming one high-affinity site.

From the standpoint of pore structure, how might $Ca^{2+}$ pass through this single high-affinity site at upwards of a million ions per second? One suggestion is that the EEEE locus is physically flexible, so that it can bind one $Ca^{2+}$ ion tightly or two $Ca^{2+}$ ions more loosely.[38] Figure 6 schematizes this view. Each of the EEEE locus glutamates contributes to tight binding of one $Ca^{2+}$ ion, the configuration underlying $Ca^{2+}$ block of $Na^{+}$ flux and ion selectivity. The glutamates can rapidly rearrange to hold more loosely two $Ca^{2+}$ ions, the configuration supporting high single-channel conductance. The EEEE locus is supposed to switch back and forth between these two configurations in response to the type of ion entering the pore, $Na^{+}$ or $Ca^{2+}$ in most instances. Figure 6 also suggests that additional conformational states may be important in

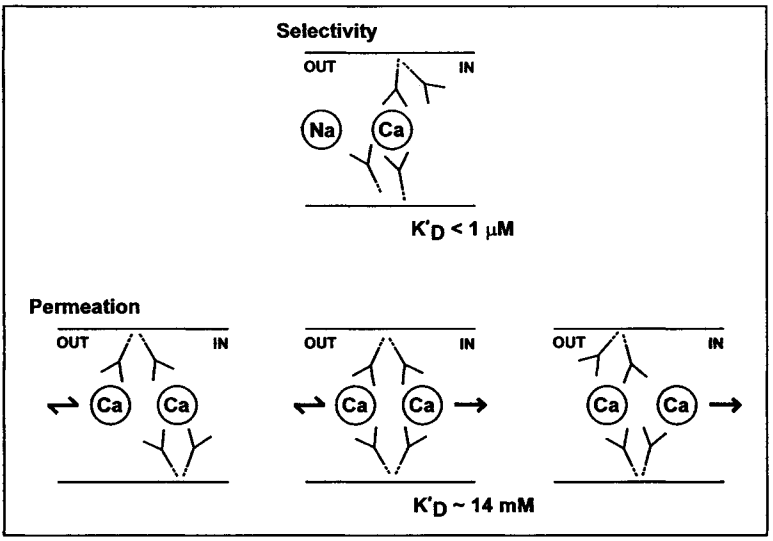



Figure 6. Cartoon depicting the hypothesized structural flexibility of the EEEE locus. Forks represent glutamate side chains. Adapted with permission from: Yang J, Ellinor PT, Sather WA, et al. Nature 1993; 366:158-161. ©2003 Nature Publishing Group.

fostering passage of $Ca^{2+}$ through the locus, with the glutamates working like a bucket brigade for $Ca^{2+}$. The behavior illustrated in Figure 6 is somewhat fanciful for calcium channels, but X-ray crystallographers have recently caught potassium channels in the act: images suggest that an ion can enter a pore already occupied by two ions, seeming to push one of the occupying ions out the other side.[41]

Despite the dominating importance of the EEEE locus, other regions of the channel do appear to have a role to play in ion conduction. Near the extracellular entrance of the channel and outside the narrow pore, site-directed mutagenesis work has identified a putative $Ca^{2+}$ binding domain that regulates calcium channel conductance, perhaps via allosteric interaction with the selectivity filter.[42] Other evidence that regions outside the EEEE locus are involved in control of ion conduction has been obtained by studying chimeras constructed between calcium channel isotypes that differ in single-channel conductance.[43,44] P-loops, S5 and S6 segments were swapped and single-channel conductance measured. Based on these experiments, regions that may form part of the external pore entrance or that compose much of the lining of the pore closer to the intracellular exit[30] are, in ways yet to be understood, involved in determining ion conduction. For selectivity, however, the EEEE locus appears to be the only structure of importance.

## Non-Equivalence of EEEE Locus Glutamates

The effect of an amino acid substitution in the EEEE locus depends upon which glutamate has been replaced.[36,38,39,45] Figure 7 presents examples of $Cd^{2+}$ block of single channel currents from four single alanine substitution mutants. The principal effect of the mutations, for the case of $Cd^{2+}$ versus $Li^{+}$, was reduced blocker on-rate (lower panels). The functional non-equivalence of the EEEE locus glutamates is evidenced by the differences between the four mutants in on-rate. Replacement of the glutamate in motif III ($E_{III}$) caused the largest change in on-rate. This fits the general pattern that substitution at $E_{III}$ has the greatest impact on block kinetics, reversal potential, or single-channel conductance[38,39] Substitution at $E_{II}$ typically follows replacement of $E_{III}$ in severity of effect; replacement of $E_{I}$ or $E_{IV}$ has lesser impact on function.

The significance of non-equivalence among EEEE locus glutamates is unknown, but an intriguing possibility is that it is important for high flux.[38] Figure 6 captures the idea: entry of

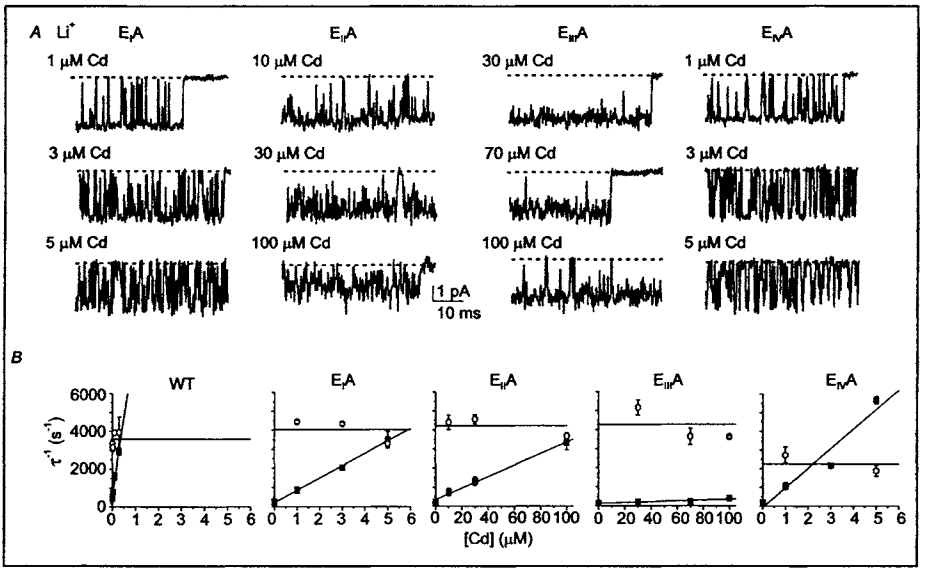



Figure 7. EEEE locus glutamates differ from one another in their interactions with divalent cations, as illustrated by the differing size of effect of single alanine substitutions on $Cd^{2+}$ block kinetics. Single-channel currents were carried by $Li^+$; dashed lines indicate closed level. The substituted glutamate is indicated by motif number (roman numerals), e.g., $E_IA$ indicates replacement by alanine of the EEEE locus glutamate in motif I. Lower panels show the concentration dependence of $Cd^{2+}$ block ($1/\tau_{open}$) and unblock ($1/\tau_{shut}$). Fitted lines represent $Cd^{2+}$ on-rate ($k_{on}$, through filled symbols) and off-rate ($k_{off}$, through open symbols). Reproduced from: Cloues RK, Cibulsky SM, Sather WA. The Journal of General Physiology 2000; 116:569-586. ©2000 The Rockefeller University Press.

a second $Ca^{2+}$ ion into an occupied locus, and departure of the first $Ca^{2+}$ ion, may rely upon distinctive interactions that each glutamate makes with $Ca^{2+}$ ions. In this view, each glutamate has a specialized role in transporting $Ca^{2+}$. The suggestion that some glutamates specialize in facilitating $Ca^{2+}$ entry into the locus or departure from it, and that other glutamates preferentially contribute to high-affinity binding of $Ca^{2+}$ recalls the stair steps model (low-affinity sites flanking a high-affinity site) described in Figure 1C. According to this thinking, the EEEE locus contains the central high-affinity site and the low-affinity flanking sites.

The non-equivalence of EEEE locus glutamates must originate from differences in structure between the four channel motifs. Focusing upon P-loop neighbors of the EEEE locus, residues that help determine the unique behavior of particular glutamates have been identified by exploiting differences between $Ca^{2+}$ channel isotypes in P-loop sequence and ion conduction. Site-directed mutagenesis at these positions has shown that neighbor residues can control ion conduction through cooperative interaction with the EEEE locus.[46]

## Functional Groups that Bind $Ca^{2+}$

The crystal structure of the KcsA potassium channel shows that high-affinity binding of permeant ions, potassium in this case, can be mediated by rings of carbonyl oxygen atoms projecting from the polypeptide main chain.[30] Although the EEEE locus carboxylates had generally been assumed to coordinate $Ca^{2+}$, the evolutionary relationship of calcium channels to potassium channels suggested that main chain carbonyl oxygens, rather than side chain carboxylates, might bind permeant ions in calcium channels as well. However, two kinds of experiments indicate that selectivity filter structure is different between calcium channels and potassium channels.

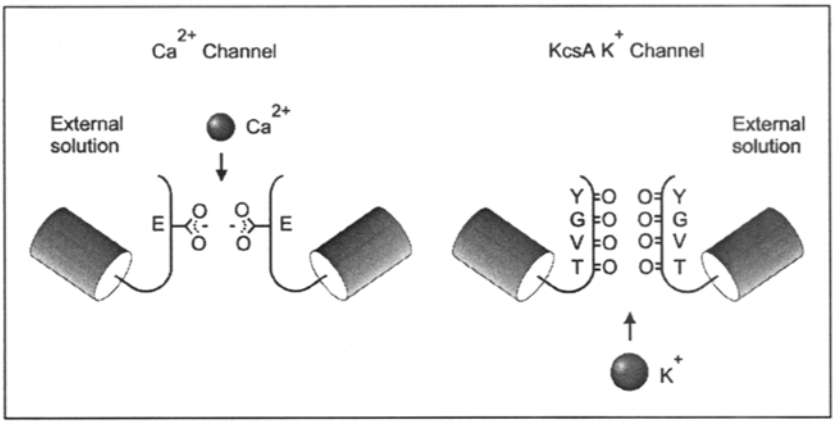



Figure 8. Contrasting models of selectivity filter structure for calcium and potassium channels. Only two of four pore lining regions are illustrated. Left, carboxylate oxygens from the EEEE locus glutamate side chains form a $Ca^{2+}$ binding locus in calcium channels. Right, carbonyl oxygen atoms from the polypeptide backbone form a series of high affinity $K^+$ binding sites. Pore helices[30] predicted from sequence analysis are illustrated as cylinders. Reprinted with permission from: Sather WA, McCleskey EW. Annual Review of Physiology, Volume 65 ©2003 by Annual Reviews.

The first experimental test was based upon the ability of protons to reduce the rate of ion conduction through calcium channels. The logic was this: if protonation of EEEE locus glutamate carboxylates underlies the proton-induced reduction in single-channel conductance, then site-directed mutation of the EEEE locus is predicted to alter proton action. Conversely, if EEEE locus substitutions affect proton action, then carboxylate oxygen atoms would seem to project into the pore.[47-49] The experimental results showed that the ability of protons to reduce single-channel conductance differed from one EEEE locus mutant to the next. The duration of single protonation events also varied according to the mutation introduced. These findings indicate that protonation within the EEEE locus is responsible for proton-induced reduction of single-channel conductance, and hence that locus carboxylate groups are solvent-accessible. The results also showed that multiple glutamate residues interact with a single proton,[48] much as they interact with a single $Ca^{2+}$ ion.

The second experimental approach to determining whether glutamate carboxylates reside in the pore lumen was based on the method of substituted cysteine accessibility.[50] In this approach, cysteine was serially substituted at P-loop positions and the susceptibility of the substituted-cysteine mutants to modification by methanethiosulfonate reagents was examined (Fig. 2; diamonds enclose substituted positions).[51,51] Methanethiosulfonates react very selectively with thiolate groups ($-S^-$), including the ionized sulfhydryl groups of solvent-exposed cysteine side chains. The thiolate form exists only in a high dielectric medium, such as saline, and thus only solvent-exposed sulfhydryl groups are susceptible to methanethiosulfonate modification. The reaction is covalent and results in attachment of moieties that can impede ion passage through narrow regions of a pore. Irreversible block by methanethiosulfonates thus reports the presence of cysteine side chains in the pore. The results of these experiments showed that consecutive P-loop residues were modifiable by methanethiosulfonates, including the substitutions made at the EEEE locus. Assuming that the cysteine mutants preserved basic pore structure, the conclusion is that carboxylate-bearing side chains of the EEEE locus project into the pore. Presumably, EEEE locus interaction with $Ca^{2+}$ is carried out by these carboxylates.

The picture of a tetracarboxylate-based high-affinity $Ca^{2+}$ binding domain in the calcium channel pore contrasts sharply with the known carbonyl-based structure of potassium channel selectivity filters (Fig. 8).[30,53] This difference in structure between channels sharing common

ancestry and exhibiting similarly high ion selectivity likely derives from the differing demands of binding divalent versus monovalent cations. In general, carboxylate groups make up binding sites selective for divalent metal ions, including in chelating agents like EGTA or EF hand domains of proteins. In these kinds of structures, $Ca^{2+}$ binds within a pocket of seven or eight oxygen atoms, many or all from carboxylate groups. Negatively charged binding sites such as these discriminate strongly against monovalent cations because these ions have an inadequate amount of positive charge to stabilize the closely packed carboxylate oxygens. Divalent metal cations, having greater positive charge, are able to overcome electrostatic repulsion among the carboxylate oxygens and hold such high-affinity sites intact.[34,54] A similar rationale may underlie the carboxylate-based selectivity filter of calcium channels.

## Structure-Based Selectivity Models

Rate theory models, of the sort illustrated in Figure 1, describe ion movement through pores using little information regarding pore structure. This was advantageous in the era prior to the cloning, and now crystallographic imaging, of ion channels. In counterpoint, however, rate theory has little to offer in linking channel structure to the energetics of selective ion permeation. Newer methods, considered below, promise greater insight into this issue. Two kinds of models are considered: ones using pores with simplified structure, and ones using predicted structure.

For model pores with simplified structure, both drift-diffusion and Brownian dynamics have been used. Drift-diffusion, developed originally to describe ion movement through semiconductors, represents ion movement as smooth diffusion through a homogeneous medium. Using experimental results to constrain pore dimensions (6 Å diameter, 10 Å length), and assuming reasonable values for the diffusion constant of $Ca^{2+}$ in the pore, the drift-diffusion model closely mimics most of the defining properties of ion permeation in real $Ca^{2+}$ channels.[55] An alternative approach of using Brownian dynamics considers ions as spheres that move according to Brownian motion. The ions are influenced by electrostatic forces in the pore. Water is considered a continuous, not particulate, medium of fixed dielectric value. The model pore used to analyze the calcium channel with Brownian dynamics starts with the wide filter known for calcium channels,[1] and the rest of the hourglass-shaped model pore is borrowed from the KcsA channel.[56] The charges of the EEEE locus glutamates are, in this model, buried in the wall of the selectivity filter rather than projecting into the pore lumen. Building into the model an ad hoc assumption that prevents ions from passing one another in the wide selectivity filter, the model is able to reproduce nearly all features of selective permeation in calcium channels.

Theory also suggests a structural basis for the ability of calcium channels to discriminate between $Na^+$ and $Ca^{2+}$. To approach this issue, the selectivity filter was modeled with the eight EEEE locus carboxylate oxygen atoms in the pore lumen. Each oxygen has a charge of –0.5 and is allowed to diffuse within the estimated volume of the selectivity filter. Metal cations entering the model filter must attempt to neutralize the charged locus. $Na^+$ has half the neutralizing power of $Ca^{2+}$ but the same size, and because space in the filter is limited, too few $Na^+$ ions can be fit inside to produce electroneutrality.[57,58] According to the model, this explains how calcium channels select with such fidelity between two different ions of essentially identical size.

Using predicted pore structure, structural biologists have addressed three fundamental questions for calcium channel pores: (i) is the selectivity filter flexible, (ii) does the pore contain more than one ion, and (iii) what force expels $Ca^{2+}$ from the high-affinity locus? Two approaches have been taken to answer these questions. One is static in nature, and considers the summed forces exerted by the channel on pore-localized ions at one instant.[59] The other has utilized molecular dynamics.[60] In this approach, the Newtonian forces exerted on each atom in a structure cause atoms to move a tiny amount. The new location of each atom is calculated, and then based on these new atomic coordinates, the calculation is carried out again. Each iteration corresponds to one picosecond in the journey of a $Ca^{2+}$ ion through the pore.

The selectivity filter is flexible, according to both approaches. The model pores show that in the absence of $Ca^{2+}$, the EEEE locus glutamates repel one another and open up a passageway 6 Å across. This is just the size needed to pass tetramethylammonium ions, the largest ion that passes rapidly through calcium channels.[1] When $Ca^{2+}$ enters the EEEE locus, however, the surrounding carboxylates are drawn close by electrostatic attraction, making a tight fit around the 2 Å ion. Using molecular dynamics or the static approach, both predicted structure models also find that a calcium channel can contain multiple ions within its pore.

Regarding forces that expel $Ca^{2+}$ from the locus during high flux, the models suggest that several ideas developed using rate theory may be involved. Molecular dynamics simulations using a pore of predicted structure reveal that $Na^{+}$ cannot displace $Ca^{2+}$ within the EEEE locus over a short time interval. $Ca^{2+}$ can displace $Ca^{2+}$ over the same time interval, however, suggesting ion-ion interaction may be involved in $Ca^{2+}$ flux.[60]

Using the static approach, several factors seem to be involved in mediating high $Ca^{2+}$ flux.[59] At millimolar (physiological) $Ca^{2+}$, three $Ca^{2+}$ ions are arranged in single file in the pore, one in the middle of the EEEE locus and the other two interacting with low-affinity sites located on the periphery of the EEEE locus. The low affinity interactions with $Ca^{2+}$ are mediated by two of the four EEEE locus glutamates. This arrangement of two low-affinity sites flanking a central high affinity site has experimental support[14] and corresponds to the stair steps rate theory model of Figure 1.[23] The stair steps appear to be involved in exit of $Ca^{2+}$ from the locus, but another factor is important as well. A region of strong negative electrostatic potential, or an "electrostatic trap," was found to surround the single bound $Ca^{2+}$ ion, preventing it from escaping from the EEEE locus. The situation changes when the pore becomes multiply occupied: in this case, the aligned positive charges of the ions in the EEEE locus push aside the webbing of the electrostatic trap. In this novel manner, $Ca^{2+}$ overcomes high-affinity binding and high $Ca^{2+}$ flux is permitted.

## Summary

Calcium channels use high-affinity binding for ion selectivity, and a multi-ion pore for high flux. The selectivity filter of calcium channels is composed of a cluster of four glutamate residues. This EEEE locus is organized as a single locus that supports high-affinity $Ca^{2+}$ binding but allows high $Ca^{2+}$ flux. Switching between configurations mediating high-affinity binding and high flux, the EEEE locus appears to have high structural flexibility. Functional non-equivalence of EEEE locus glutamates may be important in supporting high flux, with some glutamates providing stair steps for $Ca^{2+}$ passage through the locus. The selectivity filter of calcium channels differs in structure from that of related potassium channels: calcium channels use charged carboxylate oxygens of the EEEE locus to bind $Ca^{2+}$, whereas potassium channels use neutral carbonyl oxygens to bind $K^{+}$. Models of calcium channel selectivity suggest mechanisms that simultaneously allow selectivity by high-affinity binding and high ion throughput. More modern theories have begun to explain how pore structure may specify the selective permeability properties of calcium channels.

## *Acknowledgements*

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# CHAPTER 14

# The Run-Down Phenomenon of $Ca^{2+}$ Channels

**Klaus J.F. Kepplinger and Christoph Romanin**

## Introduction

The activity of many types of calcium channels decreases rapidly when contact between membrane and cytosol is disrupted. This phenomenon, called run-down, is wide spread among voltage gated calcium channels and appears to be related to regulatory factors present in the cytosol, which are rapidly lost upon patch excision. Even in whole cell experiments $Ca^{2+}$ currents exhibit a slow run-down, suggesting that endogenous cytoplasmic factors are subject to wash-out.[1,2] It is now generally recognized that run-down of ion channel activity is not due to an artificial artefact.[3] Run-down is time-dependent and generally fast, so that the complete closure of the channel occurs within a few minutes. Run-down has never been reported so far to show any voltage dependence. The loss of channel activity does not result from a lack of $Ca^{2+}$ channel integrity,[4,5] because of the following reasons:

a. The single channel current amplitude remains stable during run-down
b. The run-down in excised patches corresponds to the spontaneous drops in either the channel open probability ($P_O$) or the number of channels ($N$) present in multi-channel patches or both

After run-down, calcium channels can be re-activated excluding a proteolytic process interfering with channel activity.

## Abbreviations

LVA low voltage activated
HVA high voltage activated
PKA protein kinase A
PKI protein kinase inhibitor

## Which Native Calcium Channel Types Exhibit Run-Down?

There are many studies reporting from run-down of several calcium channel types. However, very few of them are dealing with the run-down phenomenon by itself. Low voltage activated (LVA)[1] T-type calcium channels do not show any run-down.[6-8] The high voltage activated (HVA) N-type calcium channels have been also found to lack run-down in the presence of ATP.[9,10] R-type HVA channels in hair cells exhibit a run-down,[11] whereas R-type calcium currents in smooth muscle cells[12] show a markedly reduced run-down. L-type calcium channels in general and particularly from cardiac cells exhibit run-down in the whole-cell and especially in the inside-out configuration.[4,13]

The mechanisms underlying run-down of calcium channel activity are not fully understood but are best studied on L-type channels. Hence we will focus here on the run-down phenomenon of L-type calcium channels.

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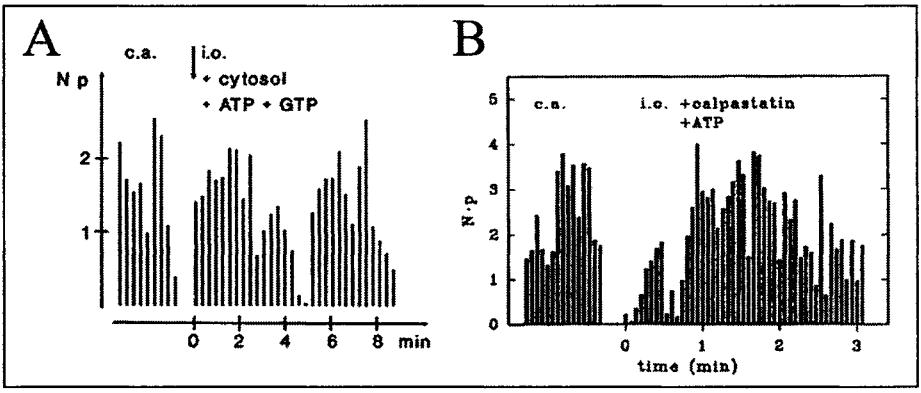



Figure 1. Prevention of run-down of cardiac $Ca^{2+}$ channel activity in single channel experiments after patch excision in the presence cytosol plus ATP and GTP (A) and treated with calpastatin plus ATP (B). Reproduced with permission from references 4 and 5.

## Prevention and Reversal of Channel Run-Down

Since the discovery of the run-down phenomenon, a lot of mechanisms have been proposed[14] which possibly are involved in the regulation of channel activity including direct ATP binding to, or phosphorylation/dephosphorylation of the $Ca^{2+}$ channels[15-18] and proteolysis by calcium dependent proteases of constituents involved in the regulation of $Ca^{2+}$ channel activity.[4,19,20] Kameyama et al[13] and Romanin et al[4] demonstrate that run-down of L-type channel activity in inside-out patches is prevented or can be reversed by cytoplasm prepared from cardiac tissue (Fig. 1A). A minimal solution containing the protease inhibitor calpastatin and ATP was equally effective in the prevention of run-down as cardiac cytosolic solution (Fig. 1B; Seydl et al).[5]

## The Nucleotides ATP and ADP

The modulatory effect of ATP on the activity of $Ca^{2+}$ channels has been discovered a long time ago.[21] In the sole presence of MgATP, run-down of L-type calcium channel activity is not prevented in the inside-out patch.[5,17] Hence, study of the effect of ATP requires the continuous presence of cytosolic solution. Yazawa et al[22] have examined recovery of calcium channel activity by the use of cytoplasmic preparations containing various levels of ATP. A reduced content of ATP (0.12 mM) in the cytoplasm results only in a recovery of 12 % of channel activity of that previously observed in the cell-attached patch, whereas 10 mM ATP restored activity to the same level as in the cell-attached configuration (Fig. 2). A similar result has been obtained when MgATP levels are varied in a cytoplasm fractionated on a diethylaminoethyl-sepharose (DEAE-sepharose) column ($Cp_{DEAE}$). Reduction of ATP to 0.055 mM markedly reduces the capability to recover channel openings. The same cytoplasm containing 10 mM MgATP increased channel activity to 60 % of that seen in cell-attached patches. Thus MgATP greatly increase the ability of cytoplasm to recover calcium channel activity in inside-out patches.

Replacing MgATP by $K_2ATP$ does not significantly change the efficiency of ATP to restore channel activity[22] suggesting a minor role of Mg in the regulation of calcium channels. Moreover, substitution of ATP by the non-hydrolysable AMP-PNP or AMP-PCP restored channel activity after run-down to 39 % of that observed in the cell-attached configuration,[22] suggesting that hydrolysis of ATP is only partially required in the regulation of channel activity.

In whole-cell patch clamp recordings, Elhamdani et al[23] have used bovine chromaffin cells with a mixed population of $Ca^{2+}$ channels (composed of about 50% current through L-type channels and 50% through N- and P-type channels) to study the effect of ATP in the regulation of calcium current run-down. They report that the presence of both ATP and $Mg^{2+}$ prevents run-down for recordings up to 60 min (Fig. 3A). This effect requires the presence of

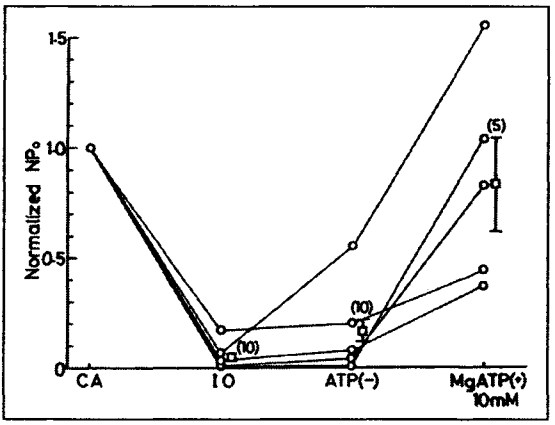



Figure 2. Restoration of calcium channel activity by cytoplasm is dependent on the presence of ATP. Channel activity ($NP_O$) under each condition (CA: cell-attached; IO: inside-out) as indicated is normalized to those obtained in the cell-attached patch. Reproduced with permission from reference 22.

$Mg^{2+}$ and is not mimicked by the non-hydrolysable ATP analogues AMP-PNP. This set of experiments suggests that hydrolysis of ATP seems to be essential to prevent $Ca^{2+}$ current run-down. The stabilizing effect of ATP on whole-cell $Ca^{2+}$ currents is not prevented by protein kinase inhibitors (a synthetic peptide inhibiting PKA; H7; calmidazolium; staurosporine). Furthermore, substitution of ATP by the cAMP analogue 8-Br-cAMP fails to reproduce the stabilizing effect of ATP. Additional experiments reveal that ADP is able to mimic the stabilizing effect of ATP in the presence of $Mg^{2+}$ (Fig. 3B). Conversion of ADP to ATP by adenylate kinase has been excluded by a lack of effect of the adenylate kinase blocker AP5A (di-(adenosine-5′)-pentaphosphate). Although the results of Elhamdani et al[23] are somewhat contrary to those of Yazawa et al,[22] they are consistent in that point that at least one effect of ATP in the regulation of L-type channel activity is independent of phosphorylation.

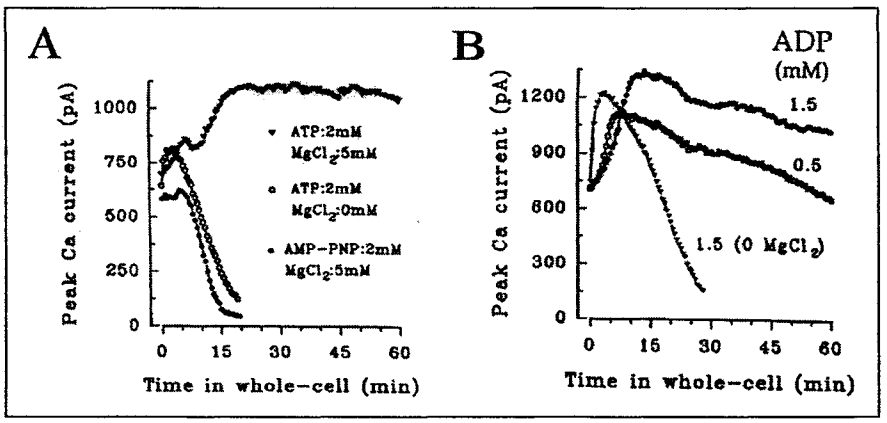



Figure 3. ATP or ADP stabilizes whole-cell calcium currents in the presence of Mg. A) A lack of run-down is observed in the presence of both ATP and $MgCl_2$ (Δ), whereas a fast run-down occurs in the absence of $Mg^{2+}$ (O) or when ATP is replaced by AMP-PNP (●). B) Mg-ADP at a concentration of 0.5 mM (□, 2 mM $Mg^{2+}$) and 1.5 mM (●, 3.5 mM $Mg^{2+}$) largely prevents calcium channel run-down, whereas 1.5 mM ADP and in the absence of $Mg^{2+}$ fails to prevent run-down (▲). Reproduced with permission from reference 22.

## Regulation by Phosphorylation/Dephosphorylation

It has been reported that up-regulation of L-type $Ca^{2+}$ currents is mediated by phosphorylation that involves distinct protein kinases (see ref. 57) such as protein kinases A, C as well as G and $Ca^{2+}$/calmodulin-dependent protein kinase. Functionally important phosphorylation sites have been already identified in L-type $Ca^{2+}$ channel forming proteins.[24-27] Dephosphorylation as possible mechanism underlying L-type $Ca^{2+}$ channel run-down is based on the finding that run-down of the channel can be slowed or prevented by agents that promote phosphorylation particularly cAMP or PKA.[16,17,28] L-type $Ca^{2+}$ channel activity that runs down within one minute following patch excision can be transiently restored by applying the catalytic subunit of PKA together with MgATP.[17] The stabilizing action of MgATP and PKA is inhibited by protein kinase inhibitor (PKI), and addition of PKI to the solution during stabilization abruptly stops channel activity. Okadaic acid, a protein phosphatase inhibitor, substantially slows the run-down process.

In summary, the effect of ATP in prevention of calcium channel run-down is apparently on the one hand a direct effect that appears to involve binding of ATP to the channel, and on the other hand an indirect effect via promotion of calcium channel phosphorylation.

## Is Calpastatin the Regulatory Protein in the Cytoplasm?

It has been extensively studied that channel activity in inside-out patches can be stabilized by superfusing cytosolic preparations.[1,2,4,29] Comparison of the stabilizing effect of different cytoplasmic preparations from various other tissues reveals that cytoplasm from skeletal muscle, brain and liver in contrast to that of kidney restores channel activity after run-down.[1] Partial purification of the relevant component(s) in the cytoplasm by ion-exchange chromatography yields a fraction, which is able to recover channel activity after run-down. This fraction subjected to two-dimensional electrophoresis shows several spots, of which the major has a Mr of about 100 kDa and an isoelectric point of 4.8. These properties resemble those of calpastatin (Table 1), an endogenous inhibitor of the protease calpain. The $Ca^{2+}$ dependent protease system[30] comprises μ-calpain, requiring μM [$Ca^{2+}$] and m-calpain, requiring mM [$Ca^{2+}$] for their activation and their specific inhibitor calpastatin. Reduction of the amount of calpastatin in the cytoplasm decreases the potency of maintaining $Ca^{2+}$ channel activity in the excised patch configuration, suggesting calpastatin as an important endogenous component regulating L-type $Ca^{2+}$ channels. Furthermore, a high molecular weight protein (>300 kDa) has been characterized that is required together with calpastatin and ATP to obtain long-term stabilization of L-type calcium channel activity similar to the efficiency of crude cytoplasm.[31]

***Table 1. Comparison of properties of the cytoplasmic factor and calpastatin***

| | Factor | Calpastatin |
|---|---|---|
| Gel filtration[a] | 250–300 kDa | 260–300 kDa |
| SDS-PAGE[b] | ~100 kDa | 110–120, 70 (RBC) kDa |
| pI[c] | 4.8 | 4.7, 4.8 (RBC) |
| DEAE column[d] | 100–360 mM (KCl) | 100–150 mM (NaCl) |
| Heat stability[e] | Partially stable | Stable |

[a] Apparent molecular mass estimated by gel filtration. [b] Apparent molecular mass estimated by sodium dodecylsulphate-polyacrylamide gel electrophoresis. [c] pI, isoelectric point. [d] Concentration range of salts for elution in diethylaminoethyl-ion exchange chromatography. [e] Heat stability of effect on channel activity (for the cytoplasmic factor, heated at 60°C for 10 min. and replenished with 3 mM MgATP [30]) and inhibitory effect on calpain (for calpastatin). Properties of calpastatin are taken from Takano and Murachi, 1982; Takano et al, 1986; Maki et al, 1987; Takano et al, 1988.[53-56] RBC refers to erythrocyte type, others are tissue type. Reproduced with permission from reference 29.

## Molecular Determinants of Calpastatin in the Regulation of Calcium Channel Activity

Calpastatin is composed of five domains containing an N-terminal L domain and four mutually homologous regions called domains 1-4.[32] Each of these domains 1 - 4 of calpastatin can inhibit calpain indicating that calpastatin can inhibit up to four calpain molecules at once. However, the domains in calpastatin, which interact with calpain, may not necessarily be required for the action on the $Ca^{2+}$ channel, particularly as the action of calpastatin in the regulation of calcium channel activity occurs independent of the inhibition of the calpains.[5] This has been tested by substitution of calpastatin by specific, synthetic calpain inhibitors.[33,34] Three inhibitors (calpain inhibitor I, II and MDL 28,170) that have been studied are clearly not able to substitute for calpastatin in the prevention of calcium channel run-down. Hence, calpastatin exerts an additional effect on L-type calcium channels, besides its inhibition of calpains.

Erythrocyte calpastatin, which lacks the N-terminal domains L and 1[35,36] is not efficient in the recovery of L-type calcium channel activity from run-down,[37] suggesting the importance of domains L and 1 for channel restoration. Among the different domains of calpastatin that were tested[37] for their ability to maintain channel activity, calpastatin domain L produces channel recovery that is similar to that of full-length calpastatin. Domain 1, however, is not sufficient to restore calcium channel activity, indicating that domain L rather than domain 1 of calpastatin is involved in calcium channel regulation.

## Which Subunits of the L-Type Calcium Channel Are Involved in the Run-Down Process?

The cardiac L-type calcium channel has emerged as a heteropentameric complex consisting of $\alpha_1$, $\beta$ and $\alpha_2$-$\delta$ subunits. The $\alpha_1$ subunits of calcium channels are able to serve as voltage gated ion channels when expressed alone. Although significant biophysical diversity of native $Ca^{2+}$ channels is conferred by the $\alpha_1$ subunits, tertiary structure and channel properties are modulated by associated auxiliary subunits, resulting in an enormous potential for functional and structural diversity.[38] A study by Hao et al focuses on different subunit compositions of the L-type calcium channel and their effect on channel activity and run-down. $Ca^{2+}$ channels in CHO cells stably expressing $\alpha_{1C}$, $\alpha_{1C}\beta_{1a}$, $\alpha_{1C}\beta_{1a}\alpha_2$-$\delta\gamma$ subunits have been studied in the inside-out patch configuration using the patch clamp technique. As observed with native $Ca^{2+}$ channels in guinea-pig cardiac myocytes,[1,4,13,29] excision of membrane patches results in run-down of calcium channel activity, independent of calcium channel subunit composition (Fig. 4). Thus, run-down of channel activity is not dependent on coexpression of auxiliary subunits, suggesting that the molecular determinant(s) for run-down is located in the $\alpha_{1C}$ subunit.

## Molecular Determinants for Run-Down in the $\alpha_{1C}$ Subunit

The pore forming $\alpha_1$ subunit consists of four homologous transmembrane domains, each composed of six transmembrane segments linked by variable cytoplasmic loops, and cytoplasmic domains of amino- and carboxy termini.[38,39,40] Alternative splicing of the human $\alpha_{1C}$ subunit generates multiple isoforms of the channel, including those with a structurally altered carboxyl terminal tail. Two human splice variants of the principal 2138 amino acid pore-forming $\alpha_{1C}$ subunit, an ubiquitous isoform $\alpha_{1C,77}$ and a hippocampal isoform $\alpha_{1C,86}$, show differences in their carboxyl terminal tail.[41,42] Due to alternative splicing 80 amino acid residues in the second quarter of the carboxyl tail are replaced with 81 non-identical amino acids yielding the $\alpha_{1C,86}$ splice variant (Table 2). In an attempt to analyze the role of these 80 amino acids, Kepplinger et al (2000)[43] studied the $\alpha_{1C,77}$ and $\alpha_{1C,86}$ subunits and the two sub-segmental mutants, $\alpha_{1C,77K}$ and $\alpha_{1C,77L}$ (Table 2; Soldatov et al)[44] expressed in HEK-tsA201 cells.

The $\alpha_{1C,77}$ channel exhibits a remarkably and significantly faster run-down compared to the $\alpha_{1C,86}$ channel (Fig. 5). The whole-cell data were substantiated by single-channel experiments in which $\alpha_{1C,86}$ shows a significantly slowed rundown within 1.5 - 2.5 min following patch excision, in contrast to a rapid rundown of $\alpha_{1C,77}$ within the same time period (Fig. 6).

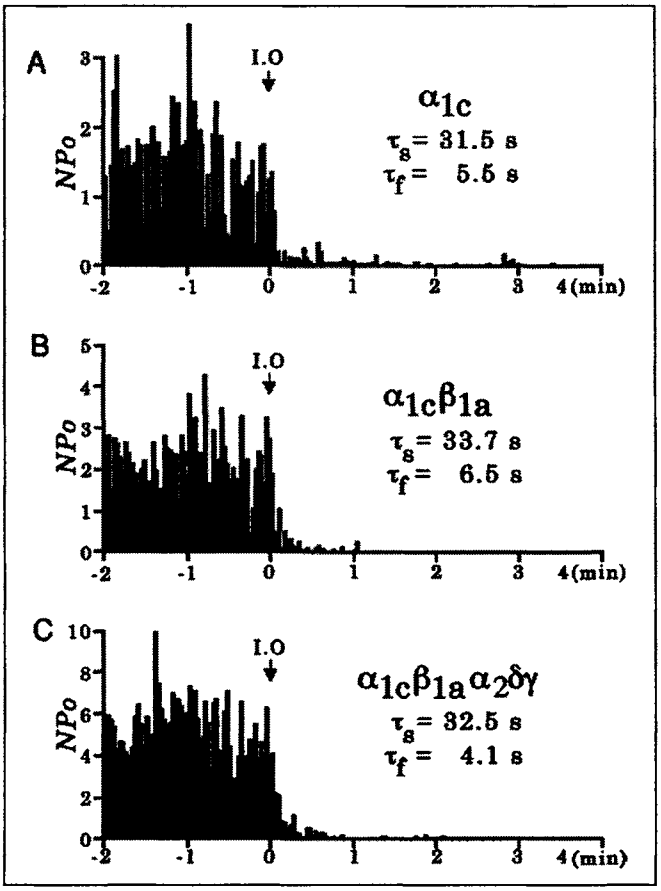



Figure 4. Run-down of $Ca^{2+}$ channels composed of different subunits. The $NP_O$ values of patches of $\alpha_{1C}$ (A), $\alpha_{1C}\beta_{1a}$ (B) and $\alpha_{1C}\beta_{1a}\alpha_2\delta\gamma$ (C) channels are summated, taking 0 min as the point of inside-out patch formation (indicated by I.O. and arrow). The decline in $NP_O$ is fitted to double exponential curves. $\tau_s$ and $\tau_f$ are the time constants. Reproduced with permission from BBRC, 1998; 247:844-850.

***Table 2. Structure of the variable parts in the carboxyl terminal tail of the $\alpha_{1C}$ subunits under investigation***

| | | |
|---|---|---|
| $\alpha_{1C,77}$ | IKTEGNLEQANEELRAIIKKIWKRTSMKLLDQVVPPAGDDEVTVGKFYATFL-IQEYFRKFKKRKEQGLVGKPSQRNALSL | (1572-1651) |
| $\alpha_{1C,77L}$ | ETELSSQVQYQAKEASLLERRRKSSHP | (1572-1598) |
| $\alpha_{1C,77K}$ | SSHPKSSTKPNKLLSSGGSTGWVEDARALEGQVLARGCGWLGSLEERERGPHHPPLGF | (1595-1652) |
| $\alpha_{1C,86}$ | ETELSSQVQYQAKEASLLERRRKSSHPKSSTKPNKLLSSGGSTGWVEDARALEGQVLARGCGWLGSLEERERGPHHPPLGF | (1572-1652) |

Amino acid sequences of $\alpha_{1C,77}$ (1572 - 1651) and $\alpha_{1C,86}$ (1572 - 1652) are shown in the top and bottom rows, respectively. Indicated amino acids of $a_{1C,86}$ replace the respective residues in the amino acid sequence of $\alpha_{1C,77}$ .In $\alpha_{1C,77L}$ and $\alpha_{1C,77K}$ subunits, indicated segments of $\alpha_{1C,86}$ replace the respective motifs L (1572 - 1598) and K (1595 - 1651) of the $\alpha_{1C,77}$ subunit. Residues in bold are located in identical positions between $\alpha_{1C}$ subunits. Reproduced with permission from reference 43.

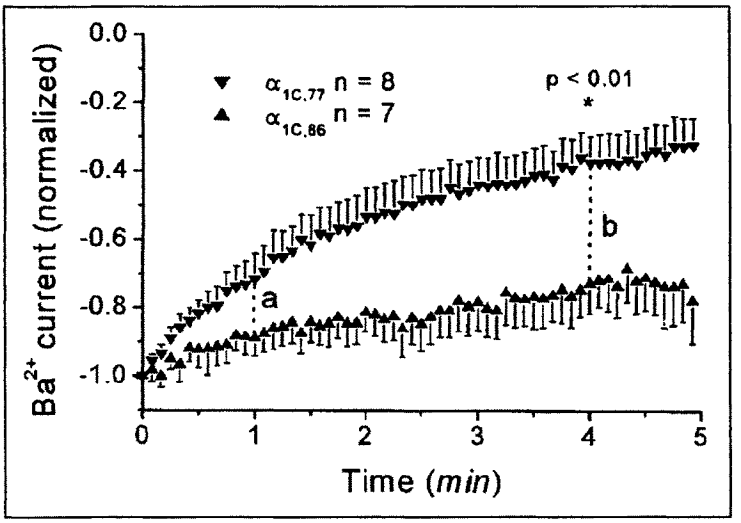



Figure 5. Time dependence of whole-cell $Ba^{2+}$ currents through $\alpha_{1C,77}$ and $\alpha_{1C,86}$ channels. Peak currents are generated by repetitive depolarizations to 25 mV ($\alpha_{1C,77}$) and 35 mV ($\alpha_{1C,86}$). Comparison of currents at 4 min indicated a significant (P < 0·01) difference in current size between the $\alpha_{1C,77}$ and $\alpha_{1C,86}$ channels. Reproduced with permission from reference 43.

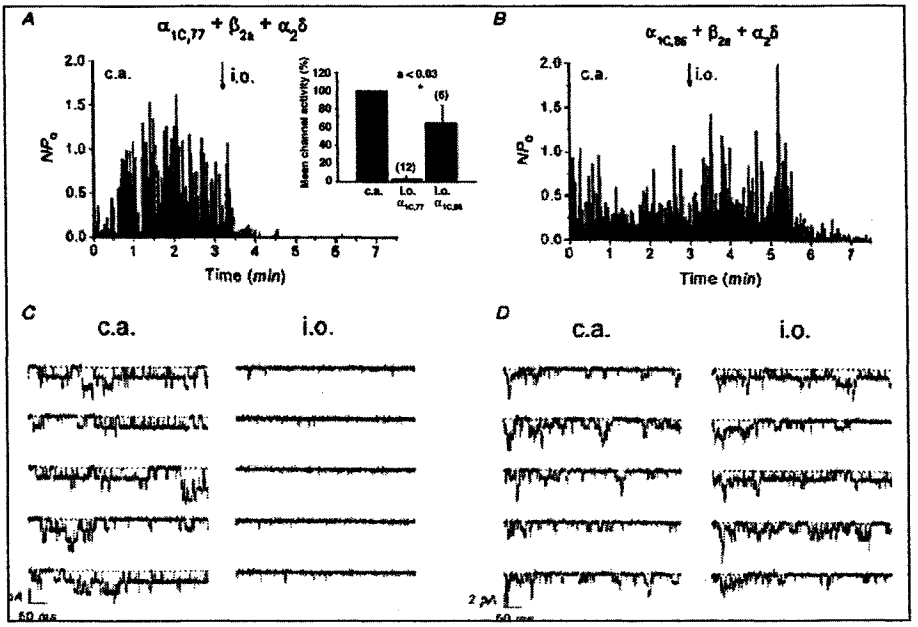



Figure 6. Time dependence of single channel currents of $\alpha_{1C,77}$ and $\alpha_{1C,86}$ channels in cell-attached and inside-out patch configurations. Average channel activity ($NP_O$) of $\alpha_{1C,77}$ (A) and $\alpha_{1C,86}$ channels (B) recorded in the cell-attached (c.a.) patch followed by inside-out (i.o.) patch configuration. Inset in A shows mean channel activity reached 1·5 - 2·5 min after patch excision normalized to the previous activity in the cell-attached patch. Consecutive single channel traces corresponding to the experiments in A and B are depicted in C and D both in the cell-attached and inside-out patch. Dotted lines indicate zero current level. Reproduced with permission from reference 43.

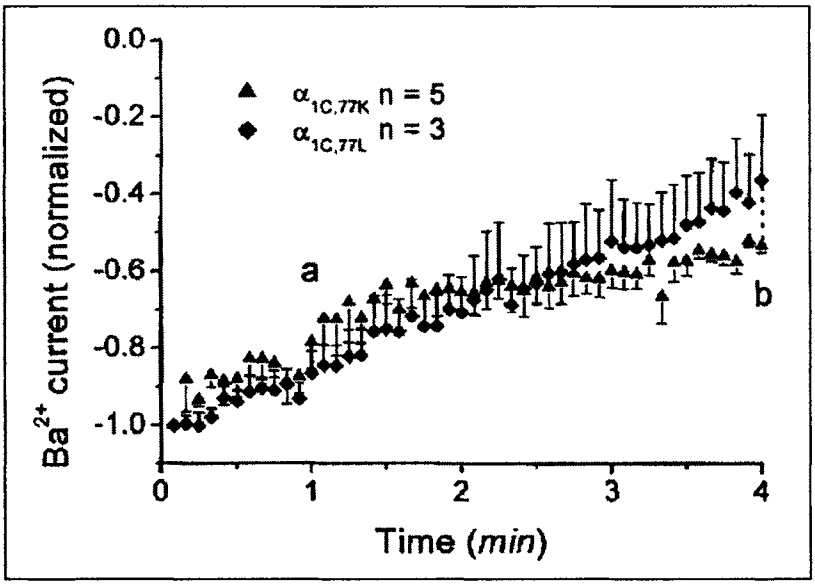



Figure 7. Time dependence of whole-cell currents of $\alpha_{1C,77K}$ and $\alpha_{1C,77L}$ channels. Peak currents normalized to initial currents are shown as dependent on time for $\alpha_{1C,77K}$ and $\alpha_{1C,77L}$ channels recorded at test potentials of 35 mV. Comparison of currents at 4 min indicated no significant ($P > 0.05$) difference in current size between the $\alpha_{1C,77K}$ and $\alpha_{1C,77L}$ channels. Reproduced with permission from reference 43.

As rundown has not been completely abolished, other structures within the $\alpha_{1C}$ subunit might additionally contribute to this process. In both whole-cell (Fig. 7) and single-channel experiments the segmental mutants $\alpha_{1C,77K}$ and $\alpha_{1C,77L}$ exhibit a similar and substantial rundown which resembles more that of the $\alpha_{1C,77}$ channel, indicating that a large stretch of the 81 amino acid sequence of the $\alpha_{1C,86}$ subunit is required to markedly reduce rundown of L-type $Ca^{2+}$ channels. As this sequence also determines channel inactivation,[44,45] both channel properties may be interdependent. However, the segmental mutants in contrast to the $\alpha_{1C,86}$ channel displayed a fast rundown, though they all showed rapid $Ba^{2+}$ current inactivation. In addition, the inactivation rate did not change during rundown. Thus, the mechanism of rundown seems to be different from that governing channel inactivation.

## Summary of the Molecular Mechanisms and Determinants of Run-Down

Dephosphorylation and washout of a regulatory factor have been suggested as prime mechanisms responsible for channel rundown. Proteolysis of the calcium channel protein can be excluded, as run-down has been shown to be fully reversible. Reversal of rundown by PKA is controversial. While Ono and Fozzard[17] reported a clear recovery from rundown by PKA application, Costantin et al[8] observed an effect only in a subset of patches, and Yazawa et al[22] as well as Elhamdani et al[23] found a stabilization of calcium channel activity by ATP, yet essentially independent of PKA. The functionally important PKA phosphorylation site[24,46] is not present within the sequence 1572 - 1651 in the $\alpha_{1C,77}$ channel, whereas a putative motif (R/KRXS) is found in the $\alpha_{1C,86}$ channel within the L segment (amino acids 1592 - 1595). However, a role of this putative PKA site in the sensitivity of $Ca^{2+}$ channels to rundown is rather unlikely as the $\alpha_{1C,77L}$ contains this motif and shows a rapid rundown similar to the $\alpha_{1C,77}$ channel. Hence, the major role of ATP in the regulation of calcium channel run-down is apparently a direct binding to the calcium channel, where the site of this interaction remains to be identified.

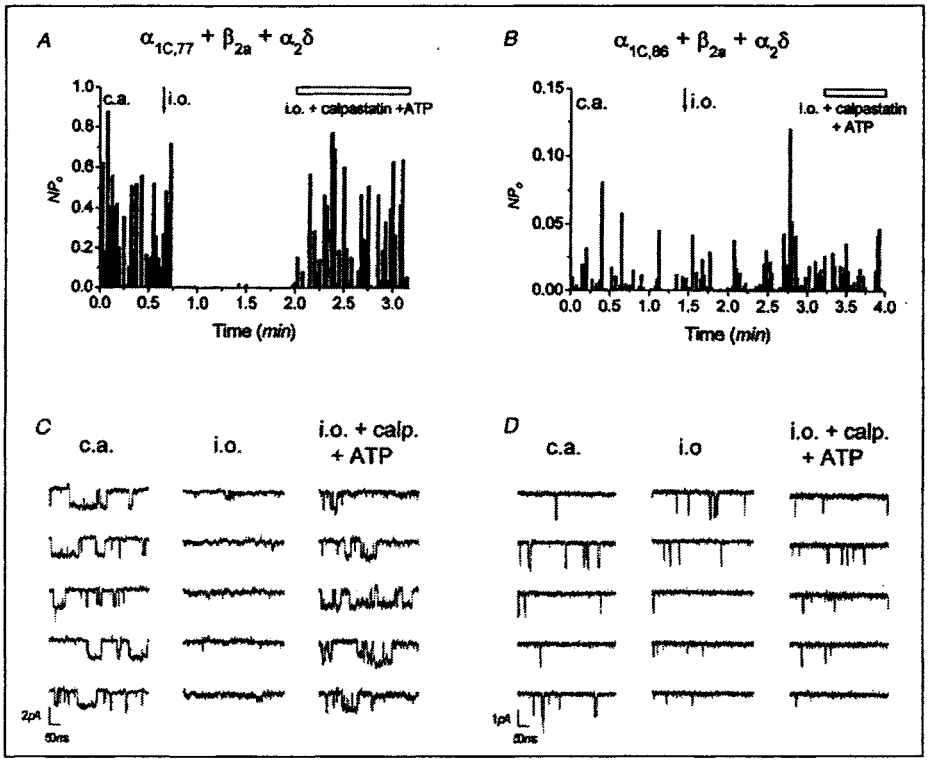



Figure 8. Effect of calpastatin + ATP on the activity of single $\alpha_{1C,77}$ and $\alpha_{1C,86}$ channels in the inside-out patch. Time-course of average channel activity ($N.p$) of $\alpha_{1C,77}$ (A) and $\alpha_{1C,86}$ (B) in the cell-attached (c.a.) and inside-out (i.o.) patch before and after addition of calpastatin (2 U/ml) + $Na_2ATP$ (1 mM). Single channel activity of $\alpha_{1C,77}$ almost completely recovered from run-down in the inside-out (i.o.) patch, whereas almost no effect was observed with channel activity of $\alpha_{1C,86}$ which was resistant to run-down in the i.o. patch. C, D) depict single channel traces for the corresponding experiments in A and B, respectively, recorded under the indicated conditions. Dotted lines denote zero current level. Reproduced with permission from reference 43.

Washout of a regulatory factor has been suggested as the second mechanism of rundown. It has been reported that the ubiquitous protein calpastatin is an important cytoplasmic factor regulating L-type $Ca^{2+}$ channel activity.[2,4,5,31] Calpastatin has been also detected in oocytes,[47] and HEK 293 cells,[43] where overexpression of calpastatin was found to increase L-type $Ca^{2+}$ channel activity (Leitner and Romanin, unpublished observations). Indeed, the activity of the $\alpha_{1C,77}$ channel subject to rundown following inside-out patch formation recovers by addition of calpastatin + ATP to the intracellular face of the membrane (Fig. 8). Among the five domains of calpastatin the N-terminal domain L may be particularly important in the regulation of calcium channel activity.[37]

Run-down has been reported to occur without changes in the gating currents[18,48] suggesting a disruption of the linkage between the voltage sensor and the opening of the ionic gate.[18] Thus it is tempting to speculate that the sequence 1572 - 1651 that determines run-down properties, represents the target site for the modulatory effects of calpastatin + ATP, which then restore coupling of the ionic gate to the voltage sensor, resulting in channel opening upon depolarization. It has been reported that L-type $Ca^{2+}$ channel activity, enhanced by intracellular application of trypsin, still exhibits the usual run-down, which is reversed by calpastatin

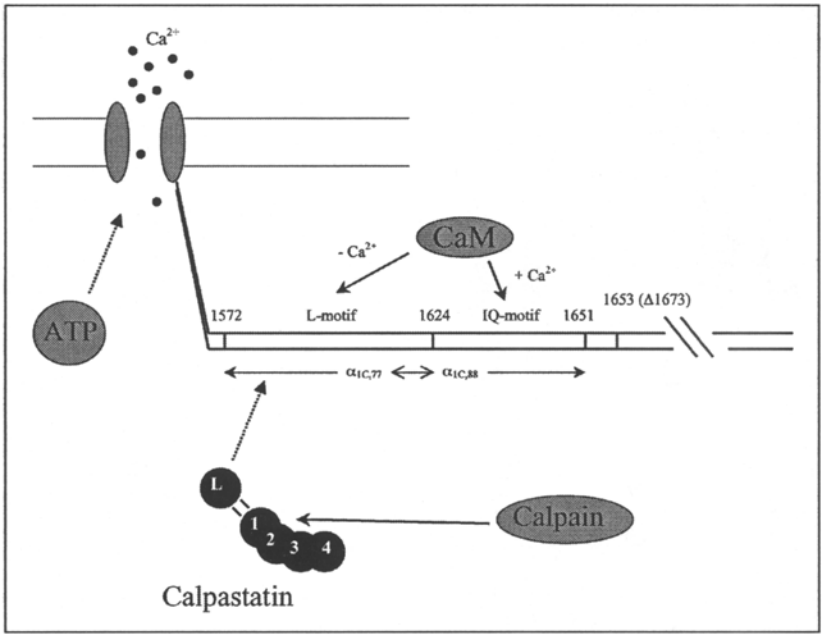



Figure 9. Molecular mechanism and determinants for regulating calcium channel run-down.

plus ATP.[49] Thus the molecular determinants for run-down and its reversal are not affected by the action of trypsin. The cleavage site of trypsin has been localized to the carboxyl terminus of $\alpha_{1C,77}$ distal to amino acid 1653 consistent with the involvement of the sequence 1572–1651 in the regulation and reversal of run-down. Further, a calmodulin binding IQ region (1624–1635) within this sequence 1572–1651 has been recently reported as a critical site for $Ca^{2+}$-induced inactivation.[50,51,52] Hence, a cross-talk between $Ca^{2+}$ channel regulation by calpastatin + ATP and $Ca^{2+}$ appears possible and remains to be investigated in future studies.

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# CHAPTER 15

# Calcium Channels As Therapeutic Targets

**Francesco Belardetti and Sian Spacey**

Voltage-dependent calcium channels link membrane potential changes of excitable cells to important intracellular processes, including regulated secretion of neurotransmitters and hormones, muscle contraction and gene transcription (see Chapters 1,8,20-22). This critical role makes them attractive targets to develop novel pharmacological agents for a broad variety of diseases originating from excitable tissues, from the central and peripheral nervous systems to the endocrine and cardiovascular apparatus. Currently, calcium channel-blockers are most widely used to treat cardiovascular ailments. Moreover, these agents act largely via block of just one type of calcium channel, the L-type (Table 1). Recent advances in the molecular and biophysical understanding of calcium channels (see Chapters 3-5) now make it possible to rationally design drugs that are selective for a particular molecular class of calcium channel. This chapter briefly reviews the established roles of L-type drugs and then focuses on the potential role of each class of channel as a therapeutic target.

## L-Type Channels

L-type channels play a unique role in exo- and endocrine secretion, as well as in excitation-contraction coupling in skeletal, smooth and cardiac muscle. Three pore-forming subunits $\alpha_{1C}$, $\alpha_{1D}$, $\alpha_{1S}$ are responsible for this type of calcium channel.

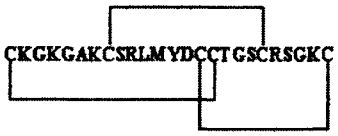
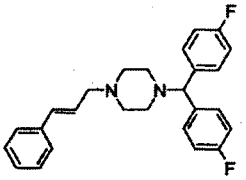
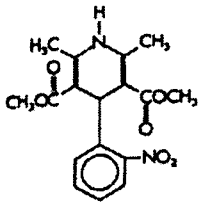
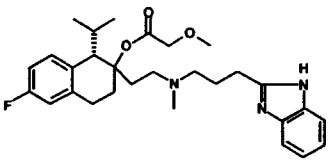
In addition to their expression in the myocardium and the vasculature, L-type channels are also expressed in the sinoatrial (SA) node and the atrioventricular (AV) node at the heart where they propagate action potentials. Their central role in controlling the activity of the heart and their regulation of peripheral vascular tone make them ideal targets for therapeutic intervention in the treatment of arrhythmia and hypertension.

Three classes of blockers that are selective for this group of calcium channels, which have been in use clinically for many years, include the dihydropyridines (nifedipine, amlodipine, felodipine; see Table 1), the phenylalkylamines (verapamil) and the benzothiazepines (diltiazem). In vitro experiments have demonstrated that these drugs decrease AV conduction velocity, heart rate and contractility in isolated hearts.[8] They also act in arterial smooth muscle to decrease peripheral vascular resistance.[8] The dilation of the vasculature combined with the negative inotropic and chronotropic effects on the myocardium result in decreased myocardial oxygen consumption.

Although these three classes of drugs block L-type calcium channels by interfering with channel gating, they achieve this outcome via different mechanisms. Dihydropyridines preferentially bind to the inactivated state of the channel, and therefore the potency of dihydropyridines increases at less negative membrane potentials.[8] Phenylalkylamines and benzothiazepines require open channels and their binding slows recovery of the channel from inactivation. Therefore, the inhibition by these drugs increases with stimulation frequency.[8] Indications for these drugs include hypertension, angina, supraventricular arrhythmia, migraine, and the prevention of vasospasm in subarachnoid hemorrhage. Unfortunately there are a number of limitations of these drugs.[3-6] There is controversy as to whether drugs such as

*Voltage-Gated Calcium Channels*, edited by Gerald Zamponi.
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***Table 1. Examples by class of drugs selective for calcium channels***

| Drug | Target | Therapeutic Indications | References |
|---|---|---|---|
| Ziconotide   | N-type | chronic pain | See ref. 1 |
| Flunarizine   | L-, P/Q-type | migraine | See ref. 2 |
| Nifedipine   | L-type | hypertension, angina, supra-ventricular arrhythmias | See refs. 3-6 |
| Mibefradil   | T-type | hypertension, chronic cardiac failure (withdrawn) | See ref. 7 |

diltiazem, felodipine, isradapine, nicardipine, nifedipine, nisoldipine verapamil and amlodipine, which are approved in the U.S.A. for the treatment of hypertension by lowering diastolic blood pressure, increase the risk of myocardial infarction (MI). A meta-analysis found a 26% increase in MI in patients who used calcium channel blockers compared to other therapies.[9] An increased sympathetic response seen with some calcium channel blockers may explain the increased risk of MI. Another limitation of L-type calcium channel blockers includes the unavoidable negative inotropic effects, which in turn can precipitate acute cardiac failure. In addition, the compensatory response of the sympathetic system can increase the risk of arrhythmia. These inherent problems have promoted the search of agents with more selective actions (see next section on T-type).

## N-Type Channels

The $\alpha_{1B}$ is the pore-forming subunit underlying the N-type current, which is a predominantly neuron-specific current involved in excitation-secretion coupling at many presynaptic terminals. In turn, presynaptic N-type current is extensively regulated by multiple G protein-coupled receptors, including μ-opioid receptors, the site of action of classic opioid analgesics[10] (Table 2).

Indeed, several lines of evidence support the view that the N-type current is prominently involved in the transduction of acute and chronic pain perception.[1,11-17] Strong, independent support for the critical role of the N-type channel in chronic neuropathic pain has been provided by the recent work with the $\alpha_{1B}$ null mice.[17] The evidence from this line of work concerning the involvement of this channel in inflammatory pain is less compelling, perhaps because of compensatory mechanisms.[17] These observations reinforce the idea that selective blockers of the N-type current might reproduce the analgesic properties of opioids without their side-effects, such as tolerance, addiction, respiratory depression and constipation. The ensuing effort has produced ziconotide, a derivative of the snail venom ω-conotoxin, which is currently awaiting approval by the FDA for use in chronic intractable pain[1,15] (Table 1). However, as ziconotide is a peptide, it is effective only by intrathecal application. Therefore, second-generation small molecular weight N-type blockers which are orally active for pain are currently under development.[18,19]

Interestingly, the $\alpha_{1B}$ null mice reportedly display a behavior consistent with lower anxiety levels than wild type animals,[17] a phenotype opposite to that shown by the $\alpha_{1E}$ null mice.[17] In retrospect, this finding may not be surprising given the intimate and often specific relationship between presynaptic calcium channels and transmitter release, and the well-established involvement of certain neurotransmitters in mood disorders.[20,21] More broadly, it is likely that calcium channels will emerge as targets for other psychiatric disorders. The possible link between T-type channels and certain symptoms of schizophrenia is discussed below.

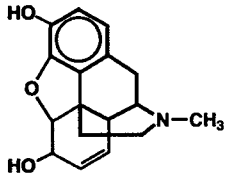
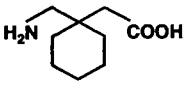
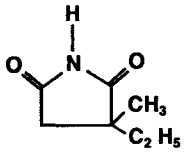
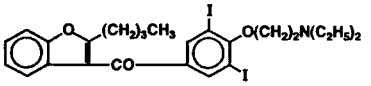
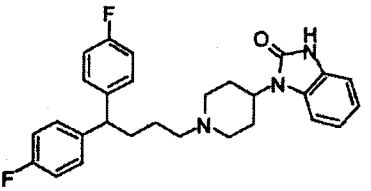
## P/Q-Type Channels

The $\alpha_{1A}$ is the pore-forming subunit underlying the P/Q calcium channel. Much like the $\alpha_{1B}$, it is also involved in presynaptic transmitter release. Several inherited neurological disorders, which include familial hemiplegic migraine, originate from mutations in either the CACNA1A gene (which codes for $\alpha_{1A}$), or in the accessory subunits of the P/Q channel[22,23] (see Chapter 16). Many, of these mutations result in an alteration of the P/Q current.[24]

These observations led to the view that common forms of migraine might also be related to dysfunction of this channel, and might benefit from pharmacological modulation of the P/Q current. A number of linkage studies were conducted to examine the role of the CACNA1A gene in migraine with or without aura. Linkage analysis was performed on four large Australian families with typical migraine and the disease locus was demonstrated to link to the CACNA1A region in one of the families.[25] A sibpair analysis looked at the occurrence of shared parental marker alleles in siblings with typical migraine with or without aura and demonstrated increased allele sharing in the CACNA1A gene region on 19p13 consistent with the involvement of this region in migraine.[26] However, in a subsequent study, two patients whose initial migraine locus was originally linked to the CACNA1A region were screened for mutations in the CACNA1A gene by sequencing the 47 exons and no mutations were identified.[27] Additionally, linkage and association studies in a large case control population and in 82 migraine pedigrees did not support a role for the CACNA1A gene in typical migraine.[27] These findings were further supported by a linkage study that demonstrated that chromosome 19p13 contains a locus which contributes to the genetic susceptibility of migraine with aura that is distinct from the CACNA1A gene.[28]

Linkage studies in classic migraine have not confirmed the involvement of the $\alpha_{1A}$ subunit, but the molecular etiology underlying migraine might involve a number of different genes. However, flunarazine, a P/Q type channel blocker,[29] is a proven migraine prophylactic.[2] This

***Table 2. Drugs that have partial action on calcium channels***

| Drug | Calcium Channel Target(s) | Therapeutic Indications | References |
|---|---|---|---|
| Morphine  | N-type, via $G_{\beta\gamma}$ | acute and chronic pain | See ref. 10, 75 |
| Gabapentin  | N-type? | chronic pain, epilepsy | See ref. 75 |
| Ethosuximide  | T-type, $_{1G}$? | absence seizures | See refs. 39, 40 |
| Amiodarone   | L-type | ventricular arrhythmias | See ref. 6 |
| Pimozide | T-type? | negative symptoms of schizophrenia, Tourette's syndrome | See ref. 71 |

suggests a potential role of P/Q type channels in migraine that is further supported by electrophysiological studies.[30] It is believed that the periaqueductal gray matter (PAG) plays an important role in migraine through its role in the descending pain modulation system.[31] Functional imaging studies have demonstrated that the periaqueductal gray matter is activated in migraine headache[32] and lesions of the periaqueductal gray matter have been associated with headache.[33,34] Facilitation of trigeminal nociceptive activity was reported after injection of the

P/Q type calcium channel blocker ω-agatoxin IVA into the ventrolateral subdivision of the periaqueductal gray matter in rats.[30] It has been postulated that in the periaqueductal gray matter the descending inhibitory neurons that target trigeminal regions of the dorsal horn are abnormally regulated by P/Q type calcium channel dysfunction in migraineurs.[30] Modulation of the P/Q type calcium channel may prove to be a rational therapeutic target in the treatment of migraine.

A relationship has also been demonstrated between mutations of the $\alpha_{1A}$ subunit and seizures, in particular absence seizures. Absence seizures are a form of non-convulsant childhood epilepsy, which are characterized by 3 Hz spike and wave electroencephalographic (EEG) activity generated by the hypersynchronous firing of neuronal populations in the thalamocortical circuit. A mutation in the P/Q type calcium channel has been associated with absence seizure phenotype in one human subject.[35] A heterozygous point mutation (C5733T) in this individual resulted in a premature stop codon. This mutation lies between the last transmembrane segment (IVS6) and the intracellular C terminus. Electrophysiological studies demonstrated that this mutation was non functional and exerted a dominant negative effect. As P/Q type channels are predominantly presynaptic and play a role in neurotransmitter release, it has been hypothesized that a reduction in current may disrupt the balance of excitation and inhibition within the thalamocortical loop.[35]

There are several animal models for absence epilepsy, which also involve a variety of voltage-dependant calcium channel subunits. The tottering mouse is the result of a mutation in the $\alpha_{1A}$ subunit gene on chromosome 8,[36] the lethargic mouse results from a mutation in the β4 subunit on chromsome 2[37] and the stargazer mouse from a mutation in the $\gamma_2$ subunit gene on chromosome 15.[38] Although these mutations are associated with absence seizure it is not clear how this is mediated. Several lines of evidence indicate that expression of this epileptic phenotype requires yet another calcium channel, a member of the low-threshold T-type.

## T-Type Channels

Succinimides have long been known as an effective treatment for absence seizures, and block of T-type calcium channels has been shown to be the likely mechanism of action[39,40] T-type calcium channels play a normal physiological role in the synchronous firing of thalamic neurons during slow wave sleep. This phase-locked synchronous firing, which results from the interplay of rhythmic synaptic inhibition and T-type calcium channel, is also active during absence seizures. Thalamic relay neurons are hyperpolarized by rhythmic inhibitory postsynaptic potentials, which remove the inactivation of the T current. Recently it has been demonstrated that the calcium dependent spike bursts in the thalamic relay neurons play a central role in the generation of absence seizures.[41] This was achieved through studies on $\alpha_{1G}$ T type calcium channel knockout mice. These mice had abolished T type calcium currents and calcium-dependent spike bursts in the thalamic relay neurons with no change in tonic firing. Systemic injection of $GABA_B$ agonists such as baclofen and γ-hydroxybutyrolactone which are known to produce thalamocortical spike wave discharges, did so in wild-type mice but not in the $\alpha_{1G}$ knockout mice. Importantly, $\alpha_{1G}$-null mice are resistant to absence seizures induced by deletion of the $\alpha_{1A}$ subunit.

Thus the primary mechanism directly responsible for the epileptic phenotype appears to be a T-type, $\alpha_{1G}$-induced current rather than a P/Q, $\alpha_{1A}$-generated current. A likely explanation of the absence seizures observed in $\alpha_{1A}$ null or mutant mice is over-expression of T-type currents. This mechanism has been recently confirmed.[42]

Thus $\alpha_{1G}$-induced T-type currents are clearly emerging as targets for at least one clinically relevant type of epilepsy. More broadly, because their gating properties are well suited to support rhythmic, high-frequency electrical activity, one can predict that T-type currents are targets in a variety of conditions such as chronic pain and certain cardiovascular disorders.[43,44]

The $\alpha_{1H}$ subunits (which is responsible for a second subtype of T-type current) might indeed be involved in pain. Support for this hypothesis has recently emerged from studies showing that these subunits are preferentially expressed in the dorsal root sensory neurons, and

***Table 3. Calcium channel knock-outs***

| Subunit | Phenotype | References |
|---|---|---|
| $\alpha_{1A}$ | ataxia, absence seizures | See ref. 76 |
| $\alpha_{1B}$ | higher threshold for neuropathic & inflammatory pain, higher threshold for anxiety,ortostatic hypotension | See ref. 17 |
| $\alpha_{1C}$ | lethal in utero | See ref. 77 |
| $\alpha_{1D}$ | deafness | See ref. 78 |
| $\alpha_{1E}$ | altered pain responses,lower threshold for anxiety | See ref. 73 |
| $\alpha_{1F}$ | N/A | |
| $\alpha_{1G}$ | resistance to absence seizures | See ref. 41 |
| $\alpha_{1H}$ | cardiovascular malformations | See ref. 49 |
| $\alpha_{1I}$ | N/A | |

would then be able to support the repetitive electrical discharges typical of painful stimuli.[45-48] However, a direct test of this hypothesis, as well as of the possible involvement of $\alpha_{1H}$ subunits in epilepsy, will have to wait for the development of selective blockers of the neuronal T-type current, or the detailed study of the $\alpha_{1H}$ knock-out mice.[49]

T-type currents are also attractive targets for cardiovascular disease for two sets of reasons. First, they are expressed in the vascular smooth muscle cells (mainly $\alpha_{1G}$ subunits),[50] and they are thought to contribute to the vascular tone.[44] Second, expression in cardiac tissues is relatively diffuse early in development and appears to be associated with the rate of growth; but then in adults they become restricted to the sino-atrial node.[51-55] Re-expression in ventricular myocytes is associated with chronic cardiac failure and cardiomyopathy.[56-60] Therefore, a T-type blocker would be an effective antihypertensive agent without some of the side effects of the L-type blockers, such as negative inotropy.[61-64] Based on data from experimental models, there are also expectations that T-type blockers might be effective in preventing the cardiovascular remodeling that accompany hypertension and chronic heart failure.[7,65-68] Mibefradil, a partially selective T-type blocker (Table 1), was recently introduced and then withdrawn due to extensive drug interactions.[69]

Some of the clinically available L-type blockers display moderate T-type blocking properties in-vitro, and at least one of them was reported to have antipsychotic properties in one double-blind clinical study.[70] Moreover, certain antipsychotic drugs active against the negative symptoms of schizophrenia are also blockers of the T-type current.[71] Taken together, these observations suggest that blockade of T-type currents might represent a novel class of anti-schizophrenic therapeutics without some of the side effects of the current medications. Interestingly, the $\alpha_{1I}$ subunit—which defines a third group of T-type currents—is almost exclusively expressed in the CNS and particularly in the striatum.[72] It will be important to define its role in the striatal dopaminergic system and the effects of the selective ablation and/or block of this subunit.

## Future Directions

We have only started to address the question of the variety of roles and functions originating from the multiple splice variants expressed by each calcium channel gene. Combined with the hetero-multimeric nature of these channels, this information will provide a rich playground for the next generation of high-throughput patch-clamp machines. More importantly, the search for splice variant-specific calcium channel blockers holds the promise of better drugs, active against a variety of neurological and psychiatric disorders and with fewer side effects.

Finally, the present attempt to understand calcium channels as therapeutic targets is based on the assumption that the research aim is to find a blocker. However, if one includes the

possibility of developing compounds that open the channels, additional and interesting possibilities can be envisioned. For example, $\alpha_{1E}$ null mice have a threshold for anxiety lower than normal animals:[73] thus, a selective opener of $\alpha_{1E}$ channels could be anxiolytic. Moreover, because the $\alpha_{1E}$ null mice are hyperglycemic presumably due to the role of these subunits in insulin secretion,[74] a selective opener of $\alpha_{1E}$ channels that does not cross the blood-brain barrier might be useful as antidiabetic.

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# CHAPTER 16

# Calcium Channelopathies

**Nancy M. Lorenzon and Kurt G. Beam**

Many cellular functions are directly or indirectly regulated by the free cytosolic calcium concentration. Thus, calcium levels must be very tightly regulated in time, space and amount. Intracellular calcium ions are essential second messengers and play a role in many functions including, action potential generation, neurotransmitter and hormone release, muscle contraction, neurite outgrowth, synaptogenesis, calcium-dependent gene expression, synaptic plasticity and cell death. Calcium ions that control cell activity can be supplied to the cytosol of the cell from two major sources: the extracellular space or intracellular stores. Voltage-gated and ligand-gated channels are the primary way in which $Ca^{2+}$ ions enter from the extracellular space. The sarcoplasm reticulum (SR) in muscle and the endoplasmic reticulum in nonmuscle cells are the main intracellular $Ca^{2+}$ stores: the ryanodine receptor (RyR) and inositol-triphosphate receptor channels are the major contributors of calcium release from internal stores.

Mutations of genes encoding calcium have been implicated in the etiology of a diverse group of nerve and muscle diseases. These mutations have been identified in humans, mice and other organisms. In this chapter, we will summarize calcium channelopathies of humans and mice. Of the ten calcium channel $\alpha_1$ subunits cloned and sequenced (see ref. 1), disease-causing mutations have been found in $\alpha_{1A}$ and $\alpha_{1F}$ in the nervous system, and $\alpha_{1C}$ and $\alpha_{1S}$ in muscle. Mutations in calcium channel auxiliary subunits ($\alpha_2$-$\delta$, $\beta$ and $\gamma$) have also been associated with both human and/or mouse neurological diseases. The disease-causing mutations may provide new insight into the cell biological roles of calcium channels as well as into relationships between structure and function. In addition, understanding how the mutations affect the physiology of the cell could lead to advances in disease treatment through relieving symptoms or slowing the progression of the disease. However, due to the multifaceted functions of calcium in the cell, the correlation between molecular mutation, physiological alterations and disease etiology is neither straightforward nor easily understood. Since calcium is an important intracellular signaling molecule, altered calcium channel function can give rise to widespread changes in cellular function. Indeed, serious diseases result from mutations that cause trivial alterations of calcium currents analyzed in vitro.

## Calcium Channelopathies of the Nervous System

Neuronal calcium channels have many cellular functions including; control of neurotransmitter release, regulation of gene expression, integration and propagation of postsynaptic signals, and neurite outgrowth. In addition to altering these signaling pathways, calcium channel mutations could in principle cause cytotoxicity. In particular, both increased (reviewed in ref. 2) and decreased[3,4,5] intracellular $Ca^{2+}$ have been reported to be cytotoxic to neurons. Neuronal calcium channels have been associated with several dominantly-inherited human diseases ranging from visual disorders to migraines, ataxia, and seizures. Mice have been described which have recessively-inherited phenotypes attributable to calcium channel defects. Some symptoms associated with human calcium channelopathies are similar to those observed in the

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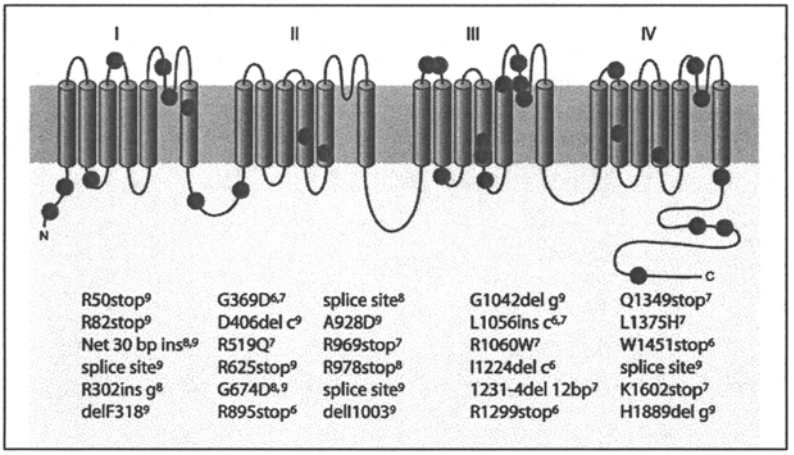



Figure 1. Mutations in $\alpha_{1F}$ causing incomplete X-linked congenital night blindness. Schematic representation of the $\alpha_{1F}$ subunit membrane topology and locations of mutations. Both the amino and carboxyl terminals are intracellular; Roman numerals indicate the four homologous repeats; each of which contains six putative transmembrane segments (S1-S6). In this and all subsequent figures mutations are indicated by the number of the affected amino acid. Superscript numbers indicate the references in which the mutations were identified and described. Amino acid numbering as in reference 9.

mutant mice; thus, the study of these murine disorders may lead to a better understanding of calcium channel function and etiology of calcium channelopathies in humans.

## *Mutations of $\alpha_{1F}$*

Based on its amino acid sequence, $\alpha_{1F}$ is an L-type channel, which is highly expressed in retina.[6,7] Human incomplete X-linked congenital stationary night blindness (incomplete xlCSNB) is a nonprogressive, dominantly-inherited disorder. Afflicted persons can show a range of symptoms including night blindness, decreased visual acuity, myopia, nystagmus, strabismus and abnormal electroretinogram results. These impairments are thought to result from altered synaptic transmission from photoreceptor cells to second-order neurons. Incomplete xlCSNB has been linked to at least 30 mutations in the $\alpha_{1F}$ subunit of a retinal L-type channel. The $\alpha_{1F}$ subunit is expressed only in retina; strongly in outer and inner nuclear layers and weakly in ganglion cells.[8] Mutations causing incomplete xlCSNB (Fig. 1) include missense changes, deletions, and frameshifts with premature stops.[6-9] The effects of these mutations on channel function have not yet been characterized. However, since synaptic transmission from photoreceptor cells to second-order retinal neurons is mediated by L-type current,[10] it seems likely that $\alpha_{1F}$ is at least partially responsible for this transmission and that the $\alpha_{1F}$ mutations may impair transmission.

## *Mutations in $\alpha_{1A}$*

The $\alpha_{1A}$ channels are expressed in cell bodies, dendrites and presynaptic terminals of most central neurons[11] with highest expression in cerebellar neurons.[11-15] As a result of alternative splicing, the $\alpha_{1A}$ gene produces both P- and Q-type currents (as defined by current kinetics and sensitivity to ω-Aga-IVA),[16] which mediate synaptic neurotransmitter release in many cell types.[17,18] (For detailed information regarding the expression, structure and function of $\alpha_{1A}$ channels see chapters 5 and 6) Important in regard to the channelopathies described in this chapter is that alternative splicing of several regions of the $\alpha_{1A}$ gene is known to produce striking alterations in the pharmacology and properties of the channel (Fig. 2). One alternative splicing inserts a valine residue in the domain I-II linker and causes alterations in inactivation

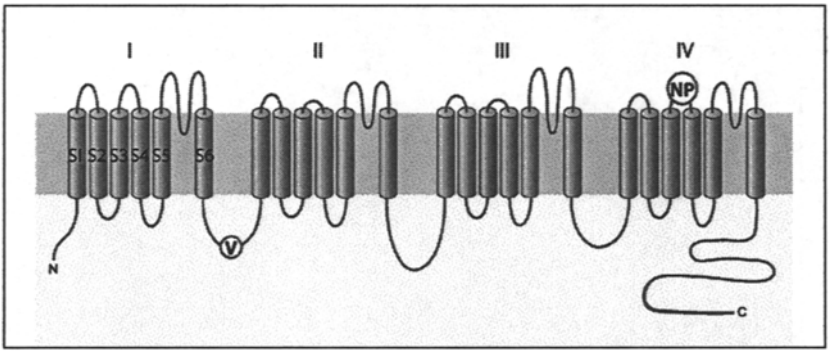



Figure 2. Alternative splicing of the $\alpha_{1A}$ subunit. Schematic representation of the $\alpha_{1A}$ subunit membrane topology and locations of 3 sites of alternative splicing. Valine 421, NP 1605-1606 insertion, and carboxyl-terminal sequence approximately 1853. See Bourinet et al[16] for details about these alternatively spliced regions.

and modulation by second messengers. A second alternative splicing inserts an asparagine and proline (+NP) into the S3-S4 extracellular linker in domain IV of $\alpha_{1A}$. This insertion alters the voltage-dependence of activation and the sensitivity to ω-Aga-IVA. The +NP splice isoforms display differential expression in the CNS.[16] For example, most cerebellar transcripts lack the NP insertion, while in the hippocampus the +NP and –NP isoforms are about equally expressed. The differential expression patterns of $\alpha_{1A}$ isoforms may mean that a particular $\alpha_{1A}$ mutation could have distinct functional consequences in different cell types. In addition, there are a number of alternatively spliced isoforms of the C-terminal domain of the $\alpha_{1A}$ subunit, including two major isoforms that have been designated $\alpha_{1A\text{-}1}$ and $\alpha_{1A\text{-}2}$ in humans[19] and B1-I and B1-II in rabbits[12] and rats.[14] Because of this alternative splicing, some mutations of $\alpha_{1A}$ are present in certain C-terminal isoforms, as described below for spinocerebellar ataxia-6.

Mutations in the $\alpha_{1A}$ gene have been shown to produce the human autosomal-dominant neurological disorders of familial hemiplegic migraine, episodic ataxia type-2, spinocerebellar ataxia-6, episodic-and-progressive ataxia and generalized epilepsy. Defects in the gene encoding the $\alpha_{1A}$ calcium channel subunit are also responsible for the recessively-inherited phenotypes of *tottering*, *leaner*, *rolling Nagoya*, and *rocker* in mice. In each of these diseases, alteration of the $\alpha_{1A}$ gene results in a host of neurological aberrations. In general, $\alpha_{1A}$ mutations have pronounced effects on the cerebellum, which is not surprising given that $\alpha_{1A}$ is highly expressed in cerebellar neurons. In particular, about 90% of rat Purkinje cell calcium current is blocked with high affinity by the toxin ω-Aga-IVA,[20] indicating that this large fraction of current arises from $\alpha_{1A}$.[16] The diseases associated with mutations in the $\alpha_{1A}$ subunit are discussed below.

Familial hemiplegic migraine (FHM) is a dominantly-inherited human disorder with symptoms that include headaches accompanied by aura and hemiparesis (which is typically transient lasting hours to days), ataxia and nystagmus. In some families, cerebellar atrophy may also occur. The onset of this disorder usually occurs in childhood or adolescence. Genetic studies of FHM patients have identified 14 missense mutations causing defects in the $\alpha_{1A}$ subunit[21-27] (Fig. 3), with T666M being the most recurrent mutation.[24] These missense mutations affect highly conserved residues[21] and are widely distributed throughout the protein.

Heterologous expression of $\alpha_{1A}$ containing FHM mutations has revealed changes in both macroscopic P/Q-type current density (which could arise from changes in surface protein expression, single channel conductance or open probability) and specific channel properties. Results from several studies are discussed below. Hans et al[28] and Tottene et al[29] analyzed human $\alpha_{1A\text{-}2}$ coexpressed with $\alpha_{2b}$-δ and either $\beta_{2e}$ or $\beta_{3a}$ in HEK293 cells, whereas Kraus et al[30,31] ex-

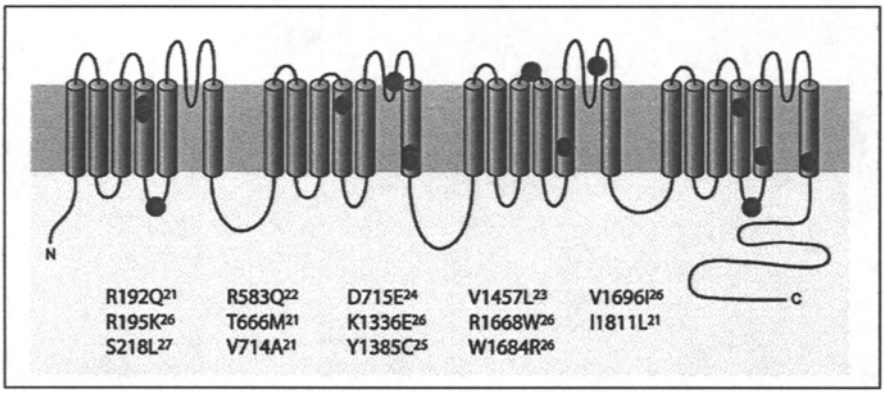



Figure 3. Mutations in $\alpha_{1A}$ causing familial hemiplegic migraine. Schematic representation of the $\alpha_{1A}$ subunit membrane topology and locations of mutations. Superscript numbers indicate the references in which the mutations were identified and described.

pressed rabbit $\alpha_{1A}$ (BI-2) together with $\alpha_2$-$\delta$ and $\beta_{1a}$ subunits in *Xenopus* oocytes or HEK293 cells (for simplicity, the rabbit mutations are renumbered according to the human sequence). Additionally, Tottene et al[29] analyzed expression of human $\alpha_{1A-2}$ in $\alpha_{1A}$ null mice cerebellar granule cells. In the human channel studies in HEK293 cells, the density of functional channel currents was increased for R192Q and decreased for T666M, V714A, I1811L[28] and V1457L.[29] Interestingly, in cerebellar granule cells, all of the five mutations above caused a reduction in the density of whole-cell calcium current.[29] At the single channel level (human $\alpha_{1A-2}$ in HEK293 cells), open probability was increased for R192Q, T666M, V714A, I1811L,[28] and V1457L.[29] Of these five mutations, single channel conductance was decreased for T666M and V714A[28] and V1457L.[29] Thus, Tottene et al[29] suggest that common features of FHM causing mutations are (i) a hyperpolarizing shift in activation and thus an increased $p_o$ at the single channel level, and (ii) a decrease in maximal current density at the whole cell level. A number of additional changes have been found in both the human and rabbit channel experiments. The mutations R583Q, T666M, V714A, D715E, V1457L, and I1811L all significantly shifted the voltage-dependence of activation to more hyperpolarized potentials (T666M, V714A, I1811L;[30] R583Q, D715E, V1457L;[31] V714A, I1815L;[28] V1457L.[29] This would result in a decrease in the threshold of calcium channel activation and an increase in the P/Q current in response to weak depolarizations. Two mutations (R583Q and D715E) caused a reduction in calcium channel current during pulse trains (slower recovery from inactivation for R583Q and faster inactivation for D715E), reflecting altered accumulation of channels in the inactivated state.[31] This could produce changes in calcium influx especially during high, but not low, neuronal activity, thus imposing the episodic nature of FHM with attacks triggered by sensory or emotional stimuli.[31]

Episodic ataxia-2 (EA-2) is a dominantly-inherited human disorder which results in symptoms of ataxia, nystagmus, dysarthria and vertigo. Cerebellar atrophy is common, and 50% of EA-2 patients report migraine-like symptoms. The ataxia is provoked by stress, exercise or fatigue and lasts hours to days. Between attacks, nystagmus on lateral or vertical gaze is the most prominent sign. Acetazolamide responsiveness is a common feature. The onset of this disorder is usually during late childhood or adolescence. Symptomatic mutation carriers show a broad range of phenotypes with appreciable variability in symptoms (including nonataxic manifestations) and severity of episodic as well as permanent neurologic abnormalities.[32-34] Jen et al[35] described an example of this heterogeneity in which significant variability in symptoms was present in a large pedigree. Some mutation carriers were totally asymptomatic (incomplete penetrance). Other family

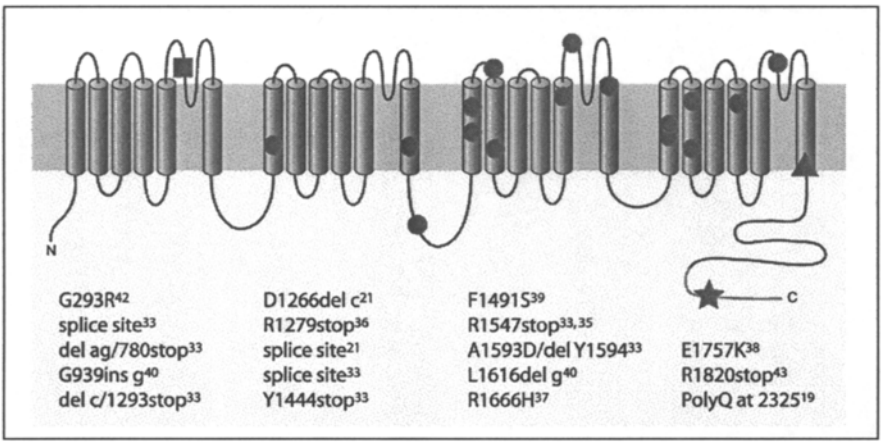



Figure 4. Mutations in $\alpha_{1A}$ causing ataxia disorders. Schematic representation of the $\alpha_{1A}$ subunit membrane topology and locations of mutations. The different mutation symbols signify different clinical diseases: circle= episodic ataxia type 2, square= episodic & progressive ataxia, triangle= generalized epilepsy and episodic and progressive ataxia, star= spinocerebellar ataxia 6. The indicated portion of the C-terminal is alternatively spliced. Superscript numbers indicate the references in which the mutations were identified and described. Amino acid numbering as in Ophoff et al[21] except as described by Denier et al[33] where the amino acid and/or nucleotide numbers were not described sufficiently for correlation with the Ophoff paper.

members exhibited episodic ataxia of varying severity, and two individuals additionally exhibited hemiplegia during ataxic episodes. Of these two patients, one suffered from migraines while the other did not. In general, males in this pedigree were more severely affected than the females (males showed earlier onset of the disorder and more frequent and severe ataxic episodes). One female showed exacerbation of her symptoms during pregnancy. The great intra-familial variability in penetrance and symptoms suggests that environmental, hormonal, and/or genetic factors other than $\alpha_{1A}$ mutation are important for the phenotype of EA-2.

Sixteen mutations causing EA-2 (Fig. 4) have been identified in the $\alpha_{1A}$ subunit.[21,33,35-40] Originally, the mutations found were predicted to lead to premature truncation of the protein; however, more recently, missense mutations have also been correlated with this disease. The mutations that cause early truncation of the protein include insertion or deletion mutations with frameshifts, single base mutations introducing premature stops, and aberrant splicing. The missense mutations are single nucleotide substitutions in various parts of the channel protein (F1491S;[39] R1666H;[37] E1757K[38]). An additional nontruncating mutation has been associated with EA-2 (A1593D/delY1594[33]).

Functional effects of one missense mutation (F1491S, located in IIIS6) have been studied by means of heterologous expression of mutated human $\alpha_{1A-2}$ (with $\beta_4$ and $\alpha_2$-$\delta$) in HEK293 cells.[39] Although immunostaining indicated that the mutant channel protein was expressed, calcium channel activity was abolished. Another study examined the effects of the R1279X and A1593D/delY1594 mutations on human $\alpha_{1A}$ function (coexpressed with $\beta_{1a}$ and $\alpha_2$-$\delta$) in tsA-201 cells and *Xenopus* oocytes.[41] The R1279X-mutated channel produced no current when expressed either in tsA-201 cells or *Xenopus* oocytes. The A1593D/delY1594 mutant did not mediate current in tsA-201 cells, but did mediate current when expressed in *Xenopus* oocytes. Compared to control $\alpha_{1A}$ currents, the currents from the mutated channel exhibited a depolarizing shift in the voltage-dependence of activation, an increase in inactivation during 3-s test pulses, and a slowing of recovery from inactivation. This latter study thus demonstrates the need to employ more than one type of expression system before concluding that channels are nonfunctional. In addition,

mutations need not lead to a complete loss of function to produce the symptoms of EA-2: dramatic reduction in current can be sufficient to cause these symptoms.

Episodic and progressive ataxia was originally categorized as a distinct clinical entity although its attributes overlap those of EA-2 and SCA6. Distinguishing features include earlier onset (5-15 years of age), more prominent cerebellar atrophy and, unlike EA-2, insensitivity to treatment with acetazolamide. Some family members exhibit severe progressive ataxia without episodic features, while others have a combination of progressive and episodic ataxia. The disease has been associated with a missense mutation (G293R; Fig. 4) of a conserved amino acid in the S5-S6 linker (pore region) of repeat I of $\alpha_{1A}$.[42] Heterologously expressed G293R-mutated $\alpha_{1A}$ (coexpressed with $\beta_{1a}$ and $\alpha_2$-$\delta$ in *Xenopus* oocytes) displayed a decrease in current density, a depolarizing shift in the voltage-dependence of activation, and enhanced inactivation compared to control $\alpha_{1A}$ currents. The mutant channels exhibited a decrease in mean open time.[41]

Primary generalized epilepsy and episodic and progressive ataxia has been associated with an $\alpha_{1A}$ mutation in an 11-year old patient.[43] The patient had generalized tonic-clonic seizures and frequent absence attacks. The episodic ataxia was similar to that in EA-2 patients. Molecular screening of $\alpha_{1A}$ identified a heterozygous point mutation (R1820X; Fig. 4) that would produce a premature stop codon and complete loss of the C-terminal region of the channel protein. Expression studies in *Xenopus* oocytes determined that the mutant $\alpha_{1A}$ was nonfunctional (coexpressed with $\beta_4$ and $\alpha_2$-$\delta$). When coexpressed with wild-type $\alpha_{1A}$, the mutant $\alpha_{1A}$ exhibited a robust dominant negative interaction with expression of the wild-type subunit.[43]

Autosomal dominant spinocerebellar ataxia 6 (SCA6) is a dominantly inherited disorder characterized by ataxia, nystagmus, dysarthria, and neuronal loss in the cerebellum (Purkinje and granule cells) and the dentate and inferior olivary nuclei. Symptoms initially appear at 40-50 years of age and become progressively more severe. Cerebella of SCA6 patients exhibit a severe loss of Purkinje cells, especially in the vermis, and various morphological changes in Purkinje cells and their dendritic arbors.[44]

Molecular analyses of patients with SCA6 demonstrated an expanded CAG repeat in exon 47 of the $\alpha_{1A}$ gene. Normal individuals have 4-16 CAGs and affected individuals have 21-27, which is much smaller than the trinucleotide repeat expansions associated with many other neurodegenerative disorders.[19] In addition, the repeat is more stable and anticipation (progressive expansion from generation to generation) is much milder than observed with other trinucleotide repeat disorders. Great clinical variability has been described in SCA6 symptoms. Similar length CAG expansions have been observed in individuals having symptoms of both SCA6 and EA-2[45] or only of EA-2,[32] which may indicate that the two diseases actually represent a continuum of symptoms. Repeat length effects have been found; however, other unknown factors must be important for phenotypic expression of SCA6. In support of this concept is that two sisters homozygous for SCA6 with identical CAG expansion length (25 repeats) and age of onset (27 years old) presented clinically with marked differences in disease progression and severity.[46] One patient had rapidly progressing ataxia, moderate dysarthria, mild dysphagia, nystagmus and moderate cerebellar atrophy. The other sister presented with milder and far slower progressing ataxia, slightly slurred speech, and moderate cerebellar atrophy, but no other clinical symptoms.

The CAG expansion in SCA6 introduces polyglutamines at a position approximately 100 amino acids from the carboxyl terminal (Fig. 4). Increased repeat size is associated with both greater disease severity and earlier onset. The pathological consequences of many diseases involving trinucleotide repeats are thought to be related to altered stability of the mutant protein, toxic metabolic breakdown products of the glutamines, or the interaction of the CAG repeats with proteins required for processing RNA. Alternatively, the polyglutamine expansion causing SCA6 might alter the biophysical properties, expression or protein-protein interactions of $\alpha_{1A}$.

One functional study expressed rabbit $\alpha_{1A}$ (BI-1) with 4, 24, 30 and 40 CAG repeats in BHK cells (coexpressed with $\alpha_2$-$\delta$ and $\beta_{1a}$).[47] Currents from cells expressing $\alpha_{1A}$-30 CAG or -40 CAG showed a significant (8 mV) hyperpolarizing shift in the voltage-dependence of inactivation. This would considerably decrease the number of available channels at resting potential.[47] In another study, heterologous expression of rabbit-human chimeric $\alpha_{1A}$ in *Xenopus* oocytes showed β-subunit specific alterations in electrophysiological properties of the mutant channels.[48] When coexpressed with $\alpha_2$-$\delta$ and $\beta_4$, the mutated $\alpha_{1A}$ exhibited a loss of regulation by G-proteins, a hyperpolarizing shift in the voltage-dependence of activation and a slowed rate of inactivation, although no changes in activation or inactivation occurred upon coexpression with $\alpha_2$-$\delta$ and $\beta_2$ or $\beta_3$. Similarly, after coexpression in HEK293 cells of human $\alpha_{1A}$ (homologous to the construct used in ref. 48) together with $\alpha_2$-$\delta$ and $\beta_{1c}$, increased surface expression was observed with only small alterations in voltage-dependence of activation and no changes in inactivation.[49]

Although the CAG repeat is in frame for some C-terminal splice isoforms of the $\alpha_{1A}$ subunit, it is out of frame for others.[19] Thus, understanding the consequences of the CAG repeat expansion requires information on the differential expression of the C-terminal splice isoforms. Immunological studies have shown that the exon 47-encoded polyglutamine tract is abundantly expressed in cell bodies and dendrites of cerebellar Purkinje cells, the cells most affected by this disease.[48] It appears that it will also be important to have additional information regarding the differential expression of isoforms with alternative splicing of regions other than the C-terminal tail. For example, the presence or absence of the NP insertion in repeat IV[16] (see Fig. 2) affected the functional consequences of polyglutamine expansions in channels heterologously expressed in HEK293 cells (coexpressed with $\alpha_2$-$\delta$ and $\beta_{1a}$).[50] The $\alpha_{1A}$(-NP) isoforms with 13, 24 and 28 polyglutamines exhibited a hyperpolarizing shift in the voltage-dependence of inactivation, which was proportional to the length of the polyglutamine repeat. However, for $\alpha_{1A}$(+NP), 13 polyglutamines had no effect on inactivation and 28 polyglutamines caused a depolarizing shift of 5 mV. Thus, in SCA6 patients, altered function of $\alpha_{1A}$(-NP), which is highly expressed in Purkinje cells, may cause Purkinje cell degeneration due to a decrease in $Ca^{2+}$ influx. Yet, other types of neurons that express both $\alpha_{1A}$(-NP) and $\alpha_{1A}$(+NP) (e.g., hippocampal neurons) may survive because a depolarizing shift in activation for $\alpha_{1A}$(+NP) might compensate for the alterations caused by $\alpha_{1A}$(-NP).[50]

*Tottering* (*tg*) is a recessively inherited neurological disease of mice characterized by seizures (absence and focal motor) and mild ataxia.[51] There is minor diffuse loss of cerebellar granule and Purkinje cells, an increased density of noradrenergic fibers from the locus coeruleus, and abnormal persistence of tyrosine hydroxylase expression in Purkinje cells.[52] *Tottering* results from a single nucleotide change that substitutes a leucine for proline (P601L; Fig. 5) in the IIS5-S6 linker of $\alpha_{1A}$.[53,54] In BHK cells heterologously expressing the *tg* mutated channel, gating was unchanged but current density was reduced by 56%; calcium channel currents were also reduced in cerebellar Purkinje cells of *tg/tg* mice.[55] The reduction in Purkinje cells was likely a consequence of reduced channel open probability because (i) levels of cerebellar $\alpha_{1A}$ mRNA were normal in *tg/tg* mice[54] and (ii) the mutation did not affect the amplitude of single channel currents in the BHK cells.[55] Defects in transmitter release in the neocortex have been observed in *tottering* mice.[56] The $Ca^{2+}$ transients in presynaptic terminals of hippocampal Schaffer collaterals exhibited a diminished contribution from P/Q-type currents and an increased contribution from N-type currents.[57] Concomittantly, neurotransmitter release at these synapses shifted from a predominant reliance on P/Q-type channels to an almost exclusive reliance on N-type channels.

*Leaner* ($tg^{la}$) is another recessively inherited murine disorder. The mutant phenotype includes severe ataxia[51] and a substantial loss of cerebellar Purkinje and granule cells.[58] The surviving Purkinje cells exhibit aberrant morphology[59] and aberrant tyrosine hydroxylase (TH) expression.[60] The $tg^{la}$ mutation has been identified as a single nucleotide substitution in a splice donor consensus sequence of the $\alpha_{1A}$ gene, which results either in a short or long isoform. The

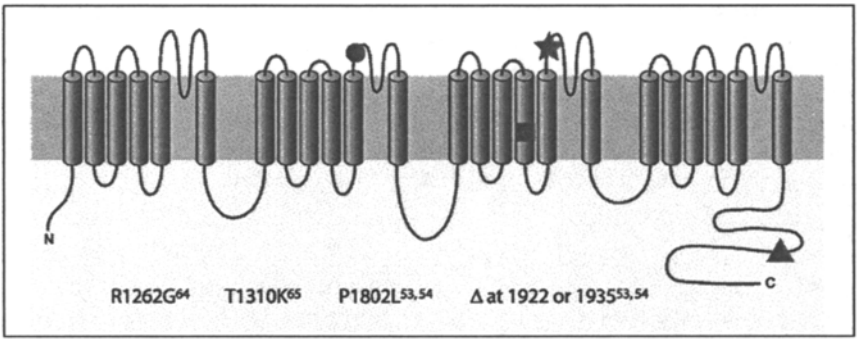



Figure 5. Mutations in $\alpha_{1A}$ associated with murine calcium channelopathies. Schematic representation of the $\alpha_{1A}$ subunit membrane topology and locations of mutations. The different symbols signify different mouse disease phenotypes: circle= *rolling Nagoya*, square= *rocker*, star= *tottering*, triangle= *leaner*. The *leaner* mutation predicts a novel sequence starting at 1922 or 1968 depending upon the isoform expressed. Superscript numbers indicate the references in which the mutations were identified and described.

short isoform has altered sequence that begins at amino acid 1922 (Fig. 5; numbered according to the mouse $\alpha_{1A}$) and terminates 57 amino acids later, and the long isoform has altered sequence that begins at amino acid 1968 and terminates 90 amino acids later.[53,54]

In Purkinje cells from $tg^{la}$ mice, whole cell calcium current was reduced in amplitude ~65%.[55,61,62] The reduction was specifically in the ω-Aga-IVA-sensitive component of current, and the remaining ω-Aga-IVA-sensitive current had unchanged gating properties.[61] Dove et al[62] suggest that the reduced current density results from decreased open probability. Interestingly, Wakamori et al[55] found that the behavior of the short and long $tg^{la}$ isoforms differed. In comparison with wild-type $\alpha_{1A}$ expressed in BHK cells, current density was unchanged for the long isoform although both activation and inactivation were shifted in the depolarizing direction (5-10 mV), whereas current density was reduced for the short isoform without any other changes. Physiological effects of the *leaner* mutation include defective neurotransmitter release at neocortical synapses.[56] In addition, marked alterations in rapid $Ca^{2+}$ buffering have been observed in *leaner* Purkinje cells. These neurons exhibited a diminished $Ca^{2+}$ buffering ability, attributed to reduced $Ca^{2+}$ uptake by the endoplasmic reticulum and a decrease in $Ca^{2+}$-binding proteins.[63]

*Rolling Nagoya* ($tg^{rol}$) is recessive, and affected mice have some phenotypic characteristics shared with their allelic counterparts *tottering* and *leaner*, and other characteristics that are distinct. *Rolling Nagoya* mice exhibit poor motor coordination of hindlimbs and sometimes stiffness of hindlimbs and tail, but they lack apparent seizures. The severity of the ataxia is between that of *tottering* and *leaner* mice. No significant numbers of apoptotic cells are observed in $tg^{rol}$ cerebella, with normal cell density and no obvious decrease in size of the granule cell layer. Calbindin immunostaining of mutant cerebella is normal (another indication that there is no significant loss of Purkinje cells), but TH expression in $tg^{rol}$ Purkinje cells fails to show the normal, postnatal decrease.[60]

*Rolling Nagoya* results from a single nucleotide change that substitutes a glycine for arginine 1262, which is located in the IIIS4 segment[64] (Fig. 5). Functionally, mutant cerebellar Purkinje cells and mutated recombinant rabbit $\alpha_{1A}$ expressed in BHK cells exhibited comparable alterations in electrophysiological properties.[64] Mutant Purkinje cells showed a reduced calcium current density and a depolarizing shift (8 mV) and reduced slope of the g-V curve. Mutant Purkinje cells recorded in cerebellar slices lacked $Ca^{2+}$ spike activity, displaying only abortive $Na^{+}$ spike activity. The altered voltage sensitivity and reduced activity of $\alpha_{1A}$ channels would significantly impair the integrative properties of cerebellar Purkinje cells, thus resulting in the locomotor deficits in *rolling Nagoya* mice.[64]

*Rocker* (*rkr*) mutant mice exhibit ataxic, unstable gait accompanied by intention tremor, typical of cerebellar dysfunction. The cytoarchitecture and gross morphology of the brain is normal; however, dendritic abnormalities have been found in mature cerebellar cortex. The Purkinje cell dendrites have a reduction in branching and show a "weaping willow" appearance of the secondary branches. This mutant phenotype is recessive and has been associated with a point mutation (T1310K) located between IIIS5-IIIS6 linker of the $\alpha_{1A}$ subunit[65] (Fig. 5). Functional studies of this mutation in $\alpha_{1A}$ have yet to be published.

### $\alpha_{1A}$ Null Mice

Two groups have used gene-knockout to examine the consequences of complete absence of $\alpha_{1A}$. There is consensus that mice homozygous for null mutation of $\alpha_{1A}$ are viable at birth but develop a rapidly progressive ataxia and dystonia before usually dying at approximately 3-4 weeks of age.[66,67] However, the electrophysiological correlates of these pathological changes are less clear based on analyses of cerebellar granule cells. Specifically, results from the two studies differed as to whether changes occurred in the density of L-, N- and R-type $Ca^{2+}$ currents in $\alpha_{1A}$-knockouts and as to whether P/Q-type $Ca^{2+}$ current density was reduced in heterozygotes compared to homozygous normals. One group[67] reported that occasional homozygous mice survived past weaning and that the cerebella in these animals (at 15 wks.) exhibited a striped pattern of Purkinje cell loss and a graded loss of granule cells, which was more severe in the anterior lobes. This neuronal loss was not observed before postnatal day 40. Thus, the neurodegeneration was similar to that seen in *leaner* cerebella.

## *Mutations in Auxiliary Subunits of Calcium Channels*

In addition to the $\alpha_1$ subunit, most calcium channels contain the auxiliary subunits, alpha$_2$-delta ($\alpha_2$-$\delta_{1\text{-}3}$), beta ($\beta_{1\text{-}4}$) and gamma ($\gamma_{1\text{-}8}$). The $\beta$ subunits are critical for surface expression of these calcium channels and also modulate the kinetics and voltage dependence; the other subunits also influence expression and properties of these channels, but to a lesser degree.[68] Channelopathies caused by mutations in each of these auxiliary subunits are summarized in Figure 6 and described below.

### *Mutations in the $\beta_4$ Subunit*

Juvenile myoclonic epilepsy has been found in the screening of a small pedigree to cosegregate with a single copy of the mutation R482X in the human $\beta_4$ subunit.[69] This mutation would result in a protein with a prematurely truncated C-terminus (thus, eliminating part of an interaction domain for the $\alpha_1$ subunit). Analysis by coexpression with $\alpha_{1A}$ in *Xenopus* oocytes revealed that rat R482X-$\beta_4$ produced calcium current with a small increase in current amplitude and a small acceleration of inactivation compared to control wild-type $\beta_4$. In addition, the R482X mutation would cause the deletion of two consensus phosphorylation sites that could affect channel function.[69]

Generalized epilepsy and praxis-induced seizures and episodic ataxia have been associated with a single missense mutation (C104F) in the human $\beta_4$ subunit.[69] An allele of $\beta_4$ bearing the C104F mutation was identified in two families which exhibited different clinical symptoms: one family with generalized epilepsy and seizures induced by playing complex strategic games and a second family with episodic ataxia. When coexpressed in *Xenopus* oocytes with the $\alpha_{1A}$ subunit, the C104F-$\beta_4$ subunit produced a calcium current with a small increase in current amplitude but no obvious alterations in channel kinetics compared to control wild-type $\beta_4$. Escayg et al[69] suggested that this amino acid substitution might affect channel clustering or targeting by disrupting a domain conserved among $\beta$ isoforms.

*Lethargic* (*lh*) is a recessive, murine disorder with behavioral traits overlapping those of human and murine $\alpha_{1A}$ diseases (ataxia and absence seizures), but without apparent neuronal degeneration.[70] The causative mutation is an insertion of four nucleotides into a splice donor site of the $\beta_4$ gene.[71] This results in two mutant isoforms of $\beta_4$, both of which are

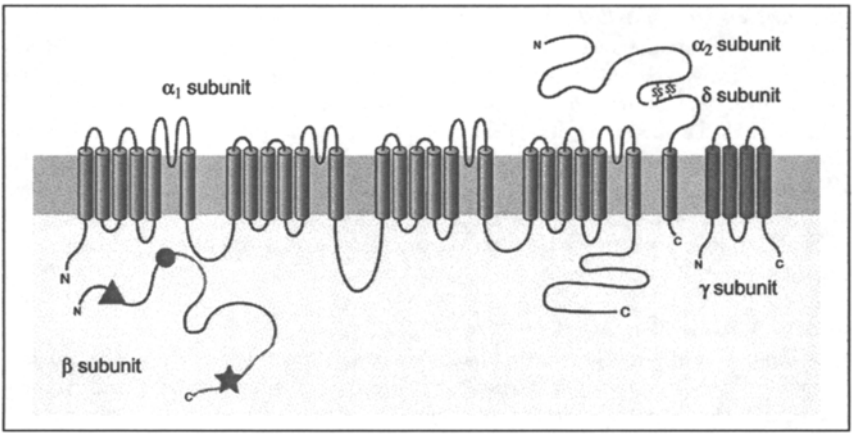



Figure 6. Mutations in calcium channel auxiliary subunits associated with human and murine channelopathies. Schematic representation of the membrane topology of the $\alpha_1$, $\alpha_2\delta$, β and γ subunits. The indicated central portion of β interacts with the α: I-II loop and the C-terminal portion of β4 interacts with the $\alpha_{1A}$ C-terminal. The mutations depicted in the β subunit are associated with the following diseases: triangle at C104F = human epilepsy and episodic ataxia,[69] circle= splice site insertion of 4 bp= *lethargic* mouse,[71] star= R482X= human juvenile myoclonic epilepsy.[69] The mouse mutant *stargazer* has been associated with a disruption in the γ subunit gene resulting in the total absence of the γ subunit protein. The *ducky* mutation in the $\alpha_2\delta$ subunit gene results in 2 different transcripts: one transcript with exons 1-3 and a novel sequence and a second transcript with exons 2-39 that is not translated. The allelic *du*$^{2J}$ is caused by a 2 base pair deletion in exon 9 causing premature truncation of the protein.

lacking the consensus sequence required for binding to $\alpha_1$ subunits (Fig. 6). McEnery et al[72] found that the forebrain and cerebellum of *lh* mice lack immunodetectable $\beta_4$ and have increased expression of $\beta_{1B}$. This upregulation of other β subunits could lead to rescue or compensation of function in cells expressing the mutated $\beta_4$ subunit. This mechanism may account for the observation that neither calcium channel subtypes nor calcium-dependent transmitter release were significantly altered at hippocampal synapses in *lethargic* mice.[57]

## *Mutations in the $\alpha_2$-$\delta$ Subunit*

*Ducky* (*du*) is a recessive murine disorder and is characterized by an ataxic, wide-based gait and paroxysmal dyskinesia. The mice are reduced in size and fail to survive beyond 35 days. In *du* mice, dysgenesis occurs in the medulla, spinal cord and cerebellum, with the Purkinje cells exhibiting thickened dendrites and reduced dendritic arbors having a "weeping willow" morphology.[73] The *du* mouse has been utilized as a murine model for epilepsy due to its phenotype of spike-wave seizures and ataxia. The *du* locus was identified as the $\alpha_2$-$\delta_2$ calcium channel subunit gene:[74] *du*-1 includes exons 1-3 and a novel sequence, and *du*-2 includes exons 2-39 and is not translated into protein.[73] Another *ducky* strain, *du*$^{2J}$ results from a 2-bp deletion within exon 9 causing premature truncation.[74]

Calcium channel currents in *du/du* cerebellar Purkinje cells were reduced by 35% compared to currents in wild-type cells.[74] When heterologously expressed in COS-7 cells, wild-type $\alpha_2$-$\delta_2$ caused no changes in biophysical parameters except for an increased surface expression of $\alpha_{1A}$ + $\beta_4$.[73] Surface expression in COS-7 cells (i.e., peak current) was reduced by 51% when wild-type $\alpha_2$-$\delta_2$ was replaced by its *du*-mutant,[73] without large changes in other properties at the macroscopic or single channel level. Thus, the *du* mutation appears to cause a reduction of the number of functional $\alpha_{1A}$ channels in the membrane.

### *Mutations in the $\gamma_2$ Subunit*

*Stargazer* (*stg*) is another recessive murine disorder and is characterized by ataxia, head-tossing and absence seizures.[75] In *stargazer*, the $\gamma_2$ gene has an insertion of an ETn retrotransposon in intron 2 causing premature transcriptional termination (Fig. 6) and total absence of $\gamma_2$ protein.[76] Although classified as a calcium channel subunit, $\gamma_2$ also interacts with AMPA receptors and synaptic PDZ proteins; moreover, cerebellar granule cells in *stargazer* mice lack functional AMPA receptors and voltage-gated calcium currents seem relatively unaffected.[77] However, heterologous expression indicated that $\gamma_2$ may have small effects on channels containing $\alpha_{1A}$ or $\alpha_{1B}$.[76,78] Thus, it is unclear whether altered properties of calcium currents contribute to the *stargazer* phenotype.

## Calcium Channelopathies of Muscle

Calcium channel defects have been linked to several human skeletal muscle disorders such as: hypokalemic periodic paralysis (HypoPP), malignant hyperthermia (MH) and central core disease (CCD). Recently, calcium channel defects have also been associated with two cardiac diseases, arrhythmogenic right ventricular cardiopmyopathy type-2 (ARVD2) and familial polymorphic ventricular tachycardia (FPVT). Causative mutations have been localized to calcium channel genes involved in excitation-contraction (E-C) coupling. Two key proteins involved in the process of E-C coupling are the dihydropyridine receptor (DHPR; an L-type calcium channel) in the plasmalemma and the ryanodine receptor (RyR) in the sarcoplasmic reticulum (SR). The DHPR expressed in muscle is a voltage-gated calcium channel containing the $\alpha_{1S}$ subunit (in skeletal muscle) or the $\alpha_{1C}$ subunit (in cardiac muscle). The RyR protein (RyR1 isoform in skeletal and RyR2 in cardiac muscle) is a calcium release channel composed of four identical subunits. In skeletal muscle, a depolarization-induced conformational change of the DHPR[79,80] results in the opening of the skeletal RyR channel in the SR. This activation may involve a direct molecular interaction between these two junctional components since E-C coupling in skeletal muscle does not require the entry of extracellular calcium. In cardiac muscle, opening of the DHPR channel causes calcium entry into the cell; the $Ca^{2+}$ ions then activate the immediately adjacent RyRs by Ca-induced calcium release. (For detailed information regarding E-C coupling see chapter 22)

*Muscular dysgenesis* (*mdg*) mice and *dyspedic* (*dys*) mice lack functional DHPR or RyR1, respectively. Cultured muscle cells from these animals can be used for expression of cDNAs and thus allow analysis of the effects of specific DHPR and RyR mutations in a near native environment. *Mdg* mice have a single nucleotide deletion in the gene encoding $\alpha_{1S}$ (skeletal DHPR) resulting in a truncated channel protein.[81] *Dyspedic* mice contain a targeted disruption of the gene encoding RyR1.[82,83] In *dyspedic* myotubes, there is a loss of E-C coupling as well as a large reduction in the density of L-type current compared to normal myotubes.[82,84-87]

### *Skeletal Muscle Channelopathies*

Hypokalemic periodic paralysis (HypoPP) is a dominantly-inherited human disorder presenting as episodic weakness of the trunk and limbs which can last hours to days and is generally accompanied by a reduction in serum $K^+$ levels. This disease usually becomes apparent during childhood or early adulthood and has reduced penetrance in females. Attacks can be provoked by rest after exercise and factors that lower serum $K^+$ levels (carbohydrate ingestion or insulin injection); other provoking factors include emotion, stress, cold exposure and alcohol ingestion. HypoPP has been associated with three missense mutations in the voltage-sensing (S4) segments of $\alpha_{1S}$ (Fig. 7): R528H located in IIS4,[88] and R1239H/G in IVS4.[89,90]

A small number of studies have been carried out on cultured myotubes derived from muscle biopsies of HypoPP patients carrying the R528H mutation. One study (based on three patients) found a small hyperpolarizing shift (~6 mV) in the voltage-dependence of both activation and inactivation of the L-type calcium current (i.e., the calcium current attributable to the $\alpha_{1S}$), but found no differences in depolarization-induced release of calcium from the SR.[91] A

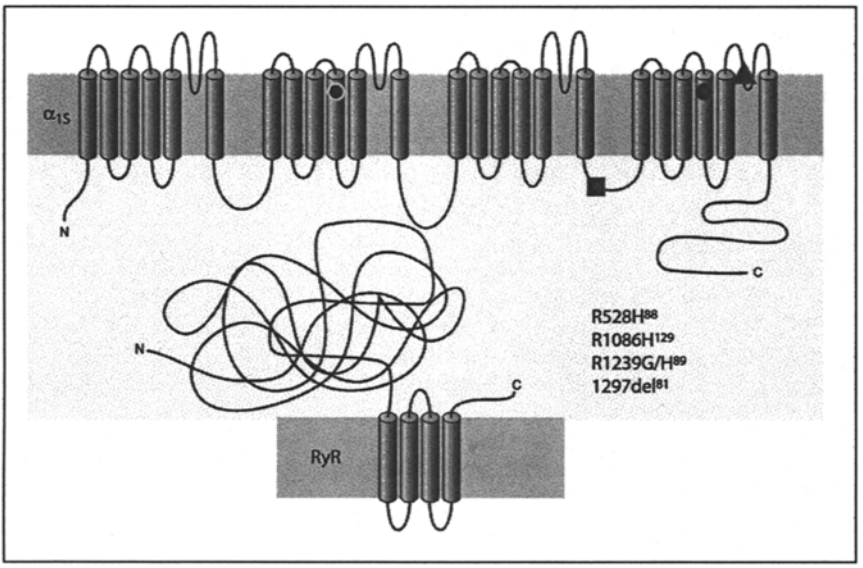



Figure 7. Mutations in $\alpha_{1S}$ subunit causing hypokalemic periodic paralysis and malignant hyperthermia. The different mutation symbols signify different clinical diseases: circle= hypokalemic periodic paralysis, square= malignant hyperthermia, triangle= *muscular dysgenesis* mouse. Superscript numbers indicate the references in which the mutations were identified and described. The topology of the RyR protein is not known; therefore, the schematic locations of the mutations in this protein are not noted in the diagram. See Table 1 for amino acid locations of the RyR mutations that have been associated with malignant hyperthermia.

second study (based on a single patient) found that the L-type current had a slightly reduced density and greatly (2-fold) slowed activation.[92]

Analyses by means of heterologous expression have not revealed any consistent functional changes in L-type calcium channels with introduced HypoPP mutations. Compared to wild-type $\alpha_{1S}$, expression in L-cells of rabbit R528H-$\alpha_{1S}$ produced current with a 70% decrease in density but no significant differences in gating.[93] Compared to wild-type $\alpha_{1C}$, expression in HEK293 cells of R650H $\alpha_{1C}$ (homologous to R528H in $\alpha_{1S}$) produced current with a 38% reduction in amplitude and a slight (5 mV) hyperpolarizing shift of both activation and inactivation.[94] In a preliminary report, Gonzales et al[95] described no significant differences in voltage-dependence or kinetics of either L-type current or calcium release in *dysgenic* myotubes after heterologous expression of rabbit $\alpha_{1S}$ containing the R528H, R1239H or R1239G mutations. Similarly, Jurkat-Rott et al[91] found no differences in calcium currents or calcium release between wild-type and R528H rabbit $\alpha_{1S}$ expressed in immortalized *dysgenic* muscle cells.

Even if heterologous expression had demonstrated consistent effects on calcium currents or E-C coupling, it would be difficult to explain the etiology of HypoPP. Specifically, when bathed in low $K^+$ solution, biopsied intercostal muscle fibers of HypoPP patients were depolarized by 20 mV (compared to a 10 mV hyperpolarization in normal muscle).[96] If a similar depolarization were to occur in vivo, it would inactivate voltage-gated sodium channels and thus induce muscle paralysis.

Because it is not obvious how altered calcium currents or E-C coupling would produce persistent depolarization triggered by low $K^+$, indirect mechanisms have been suggested (e.g., ref. 97). For example, a decrease in $Ca^{2+}$ entry could change activation of a $Ca^{2+}$-sensitive channel with direct control over resting potential (e.g., $Ca^{2+}$-activated $K^+$ channel). Alternatively, the expression or subcellular distribution of channels important for controlling membrane potential might be altered by small but long-term changes in cytoplasmic calcium levels.

Malignant hyperthermia (MH) and central core disease (CCD) are dominantly inherited muscle disorders that arise from altered handling of intracellular calcium [reviewed in ref. 98-100]. Thus, it is most convenient when discussing these diseases to combine the information about etiology and symptomatology. A number of mutations in RyR1 have been associated with these diseases, but mutations in other proteins can also be causal (see below). No matter what the causative mutation, RyRs represent an important trigger point for the pathophysiology, since RyRs play a key role in controlling intracellular calcium. In addition to being regulated via the DHPR, RyRs are activated by elevations in intracellular calcium ("calcium-induced-calcium-release" or "CICR"). This CICR is "overactive" in RyRs bearing MH mutations so that massive calcium release can be triggered by halogenated anesthetics (which decrease the threshold for CICR) or by depolarizing skeletal muscle relaxants (which trigger low amounts of calcium release via the normal E-C coupling pathway). An episode of MH can thus be triggered by pharmacological agents used during surgery. These episodes are characterized by skeletal muscle rigidity, tachycardia, unstable and rising blood pressure, and elevated body temperature. If not treated immediately, patients may die within minutes from ventricular fibrillation, within hours from pulmonary edema or coagulopathy, or within days from post-anoxic neurological damage and cerebral edema or obstructive renal failure, resulting from release of muscle proteins into the circulation [reviewed in ref. 101]. Individuals with CCD [reviewed in ref. 101] typically display a slowly progressive or nonprogressive myopathy (usually presenting in infancy) associated with hypotonia and proximal muscle weakness. Histologically, the central regions (cores) of both type I and type II skeletal muscle fibers display disintegration of the contractile apparatus, ranging from blurring and streaming of Z lines to total loss of myofibrillar structure. Many individuals with CCD also are susceptible to MH.

Thus far, 36 identified missense mutations and four deletion mutations in RyR1 have been associated with MH susceptibility and/or CCD[102-126] (see Table 1). While some mutations produce MH alone, other mutations cause both MH and CCD, and still others produce only CCD. The majority of the RyR1 mutations appear to be clustered in the N-terminal amino acids 35-614 (MH/CCD region 1) and the centrally located residues 2163-2458 (MH/CCD region 2).[127] Other RyR1 mutations located more distally have been associated with CCD. An MH-related porcine disorder [reviewed in ref. 98] is caused by the mutation R615C, which is homologous to the R614C mutation in humans. Swine heterozygous for R615C display increased muscle mass and increased calcium release in response to a variety of agents, whereas animals homozygous for the mutation suffer "porcine stress syndrome" with MH symptoms brought on by physical and emotional stress.[100] A canine form of MH, A547V, is inherited as an autosomal dominant trait and shares most of the clinical manifestations with MH in pigs and humans.[128] Mutations in genes other than RyR1 may also be responsible for MH. For example, in a single family, MH segregated with a missense mutation (Fig. 7) in the $\alpha_{1S}$ gene (R1086H).[129] Moreover, the demonstration of linkages between MH and several loci distinct from either RyR1 or $\alpha_{1S}$ indicate the possibility of additional gene targets.[99,130] The RyR1 mutations causing MH and CCD have been reported to have variable functional consequences. In part, this variability could be due to the study of different disease-causing mutations, which may truly have different effects. However, the variability most likely also arises from the use of a variety of experimental systems (muscle biopsies, myotube cultures obtained from affected individuals, expression of cDNAs in cell lines or *dyspedic* myotubes).

Muscle bundles biopsied from MH patients contracted in response to lower concentrations of caffeine and halothane than muscle from normal individuals. Muscle biopsies from patients with the deletion mutation, delE2347, exhibited unusually large electrically-evoked contractions compared to nonMH muscle.[124] Paralleling the contraction results from biopsied muscle, intracellular calcium release in cultured myotubes derived from MH patients showed an increased sensitivity to halothane[131] as well as to ryanodine.[132] The kinetic properties of the $[Ca^{2+}]_i$ rise in cultured human MH (G2435R) muscle differed significantly from controls.[132]

***Table 1. Mutations in RyR associated with disease***

| RyR1 Mutation | Disease | Reference |
|---|---|---|
| C35R | MH | Lynch et al. 1997 |
| R163C | MH, CCD | Quane et al.1993 |
| G248R | MH | Gillard et al. 1992 |
| G341R | MH | Quane et al. 1994b |
| I403M | CCD | Quane et al. 1993 |
| T522S | MH, CCD | Quane et al. 1994a |
| R552W | MH | Keating et al. 1997 |
| R614C | MH | Gillard et al. 1991 |
| R614L | MH | Quane et al. 1997 |
| D2129E | MH | Rueffert et al. 2001 |
| R2163C | MH | Manning et al. 1998b |
| R2163H | MH, CCD | Manning et al. 1998b |
| R2163P | MH | Fortunato et al. 2000 |
| V2168M | MH | Manning et al. 1998b |
| T2206M | MH | Manning et al. 1998b |
| T2206R | MH | Brandt et al. 1999 |
| V2214I | MH | Sambuughin et al. 2001b |
| Del E2347 | MH | Sambuughin et al. 2001a |
| A2367T | MH | Sambuughin et al. 2001b |
| D2431N | MH | Sambuughin et al. 2001b |
| G2434R | MH | Keating et al. 1994 |
| R2435H | MH, CCD | Zhang et al. 1993 |
| R2435L | MH | Barone et al. 1999 |
| R2452W | MH | Chamley et al. 2000 |
| R2454H | MH | Barone et al. 1999 |
| R2454C | MH | Brandt et al. 1999, Gencik et al. 2000 |
| R2458C | MH | Manning et al. 1998a |
| R2458H | MH | Manning et al. 1998a |
| RQF4214-6del | CCD | Monnier et al. 2001 |
| LS4647-48del | CCD | Monnier et al. 2001 |
| L4793P | CCD | Monnier et al. 2001 |
| Y4796C | MH, CCD | Monnier et al. 2000 |
| R4825C | CCD | Monnier et al. 2001 |
| T4826I | MH | Brown et al. 2000 |
| F4860del | CCD | Monnier et al. 2001 |
| R4861H | CCD | Monnier et al. 2001 |
| R4893W | CCD | Monnier et al. 2001 |
| I4898T | CCD | Lynch et al. 1999 |
| G4899E | CCD | Monnier et al. 2001 |
| R4914G | CCD | Monnier et al. 2001 |

Calcium release from SR vesicles of MH muscle also exhibited a lower threshold for activation by calcium, an enhanced sensitivity to caffeine, and a reduced sensitivity to inhibitory concentrations of calcium.[133] In addition to an increase in Ca-, halothane-, and TFP-induced calcium release, a reduction in the rate and capacity of calcium loading of heavy SR vesicles from MH patients has been observed.[134] Porcine MH muscle fibers also displayed an increased sensitivity to agonists such as halothane and caffeine[135] as well as a 15-mV negative shift in $Ca^{2+}$ release

induced by depolarization.[136] Introduction of a number of MH-causing missense mutations into RyR1 (expressed in HEK293 cells) caused an increased sensitivity to low concentrations of caffeine and halothane.[137] It has been suggested that the increased sensitivity of RyR1 to activators observed in many studies may result at least in part from elevated, resting intracellular $Ca^{2+}$.[138]

Two mutations associated with a severe and highly penetrant form CCD have been analyzed by means of heterologous expression. The CCD for one of these mutations (Y4796C) is accompanied by MH susceptibility,[121] whereas for the other (I4898T) it is not.[116] Rabbit Y4796C-RyR1 expressed in HEK293 cells exhibited a decreased $EC_{50}$ for activation by caffeine, a reduced amplitude of the caffeine response and an elevated resting $[Ca^{2+}]_i$, from which it was concluded that this mutation causes RyR1 to be "leaky".[121] Expressed in HEK293 cells, rabbit I4898T-RyR1 also resulted in an increased resting $[Ca^{2+}]_i$ but loss of responses to caffeine and halothane.[116] Expression of I4897T-RyR1 in *dyspedic* myotubes resulted in the complete uncoupling between electrical excitation of the plasma membrane and SR $Ca^{2+}$ release, but no change occurred in either resting myoplasmic $[Ca^{2+}]_i$ or in SR $Ca^{2+}$ content.[139] When I4897T-RyR1 was coexpressed with wild-type RyR1 (mimicking the situation in patients), voltage-gated SR release was depressed by 60%. Based on the studies above, it appears that CCD accompanied by MH may result from hyperactive (and thus leaky) RyRs, whereas CCD without MH can result from hypoactive RyRs.

### *Cardiac Muscle Channelopathies*

Inherited cardiac disorders that are associated with a propensity to malignant ventricular tachyarrhythmias constitute an important cause of sudden death in both young and adult individuals. Two such disorders, arrhythmogenic right ventricular cardiomyopathy type 2 and familial polymorphic ventricular tachycardia, are associated with mutations in RyR2.

Arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2) is an autosomal dominant disease characterized by partial degeneration of the myocardium of the right ventricle, electrical instability and sudden death. Thus far, four missense mutations in RyR2 have been correlated with ARVD2: R176Q, L433P, N2386I, T2504M.[140] These mutations are located at highly conserved amino acids in the cytosolic region of the protein and cluster in regions where mutations causing MH or CCD in RyR1 also cluster; R176Q exactly corresponds to the R163C mutation in RyR1 causing MH/CCD. The mutations N2386I and T2504M are located in the domain thought to interact with FKBP12.6, a regulatory subunit of RyR2.[140]

Familial polymorphic ventricular tachycardia (FPVT) is an autosomal dominant defect. This cardiac disease is characterized by volleys of bidirectional and polymorphic ventricular tachycardias in response to vigorous exercise, with no structural evidence of myocardial disease. This disease has a relatively early onset and a highly malignant course with estimates of mortality ranging from 30-50% by the age of 20-30 years. Thus far, seven mutations in RyR2 have been correlated with this disorder. Priori and colleagues[141] described three different missense mutations in the RyR2 gene of three distinct families: P2328S, Q4201R, and V4653F. A second study found four additional missense mutations: S2246L, R2474S, N4104K, and R4497C.[142] These mutations are located in regions of the protein that correlate with RyR1 mutation hot spots for MH and/or CCD.

It is not currently known how these mutations alter RyR2 channel function; however, several hypotheses have been suggested (e.g., ref. 141). These mutations may increase the $Ca^{2+}$ sensitivity of the channel. Thus, intense adrenergic stimulation or increased physical activity may cause massive SR $Ca^{2+}$ release. Alternatively, the mutations may alter the ability of the channel to remain closed, and on physical perturbation allow $Ca^{2+}$ to leak from the channel and promote massive SR $Ca^{2+}$ release. This excessive calcium release might alter the function of ion channels in the plasma membrane and thus cause severe and possibly fatal tachyarrhythmias.

## Summary

Mutations of genes encoding calcium channels are rapidly being identified as causing a diverse group of nerve and muscle diseases. Calcium channel mutations in $\alpha_{1F}$ and $\alpha_{1A}$ subunits and $\beta$, $\gamma$ and $\alpha_2$-$\delta$ auxiliary subunits have been associated with a number of neurological diseases, and defects in $\alpha_{1S}$ and $\alpha_{1C}$ subunits and RyR1 and RyR2 calcium release channels have been associated with several muscle disorders. The majority of the calcium channel mutations are associated with movement disorders (exceptions are night blindness and arrhythmias), perhaps because these are most readily observed. However, it is intriguing to speculate that linkage analysis will eventually establish correlations between calcium channel mutations and subtle behavioral disorders or even personality traits.

The calcium channelopathies differ from those of voltage-gated sodium and potassium channels, in which the disease phenotype is directly attributable to the changes in electrical excitability of nerve and muscle that result from altered biophysical properties of the channels. The only obvious mechanistic correlation for calcium channelopathies is that a given calcium channel mutation has the most profound impact on the organ or brain region in which that channel is most highly expressed (e.g., night blindness, cerebellar disorders, skeletal and cardiac muscle defects). However, there is often no clear link between disease severity and impact on channel function. Indeed, serious diseases can result from mutations that cause trivial alterations of calcium currents analyzed in vitro. In addition, the same disease phenotype can result from hyper- or hypo-activity of a particular calcium channel. Similarly puzzling is that seemingly comparable mutations of $\alpha_{1A}$ produce dominantly inherited diseases in humans and recessively inherited diseases in mice. Overall, the absence of clear phenotype-genotype correlations may reflect the fact that most analyses thus far have focused on the biophysical properties of channel function, whereas the mutations could well affect subcellular distribution of a channel or its association with other proteins important in signaling cascades. Moreover, genetic background is clearly important in determining the phenotypic consequences of channel mutations as evidenced by identical mutations producing very different clinical symptoms (e.g., disparate disease severity and progression in sisters with the same SCA6 mutation).[46]

Understanding genotype-phenotype correlation of calcium channelopathies is complicated by the fact that $Ca^{2+}$ plays such important and diverse roles as an intracellular regulatory molecule. Thus, even small changes in calcium channels may dramatically alter cellular development and homeostasis and thus result in widespread changes in cellular function. An important challenge for the future remains the identification of fundamental mechanisms involved in the calcium channelopathies: electrical behavior, transmitter release, enzyme cascades, channel targeting, or gene expression/development. Besides providing a better understanding of pathogenesis, future research on the disease-causing mutations may provide new insight into cell biological roles of calcium channels and into relationships between structure and function.

### *Note Added in Proof*

Since submission of this chapter, additional relevant studies have appeared.

One study (Neuton 2004; 41:701-710) found multiple gain-of-function effects in knockin mice carrying $\alpha_{1A}$ with the mutation R192Q, which causes familial hemiplegic migaine. These effects included increased current density in cerebellar granule cells, increased neuromuscular ACh release, and reduced threshold for, and increased velocity of, cortical spreading depression. This study illustrates the importance of examining the behavior of ion channels in their native environment rather than only after heterologous expression.

Mutations of a T-type channel (Cav3.2) now also appeaer to be associated with neurological disease, specifically childhood epilepsy (Ann Neurol 2003; 54:239-243). Effects of some of these mutations have been examined in heterologously expressed channels (J Biol Chem 279:9681-9684).

Heterologous expression has now been used to characterized wild-type $\alpha_{1F}$ channels (J Neurosci 2003; 23:6041-6049) as well as channels bearing mutations causing X-linked congenital night blindness (J Neurosci 2004; 24:1707-1718). In the latter study, it was found that $\alpha_{1F}$ channels have a much broader tissue distribution than originally thought.

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# CHAPTER 17

# The Molecular Basis of $Ca^{2+}$ Antagonist Drug Action-Recent Developments

**Jörg Striessnig, Jean-Charles Hoda, Edwin Wappl and Alexandra Koschak**

## Introduction

Nifedipine and other dihydropyridines (DHPs), verapamil (a phenylalkylamine, PAA) and diltiazem (a benzothiazepine, BTZ) are widely clinically used to treat cardiovascular disorders, such as hypertension and angina pectoris. Their therapeutic benefits result from vasodilation and cardiodepressive actions due to block of depolarization-induced $Ca^{2+}$ influx through voltage-gated L-type $Ca^{2+}$ channels (LTCCs) in vascular smooth and cardiac muscle.[1,2] As outlined in other articles of this series these "$Ca^{2+}$ antagonists" possess high affinity for $\alpha_1$ subunits (Fig. 1A) forming LTCCs but low (verapamil, diltiazem) or very low (DHPs) affinity for non-L-type $\alpha 1$ subunits.[3,4] Due to their high selectivity for LTCCs, DHPs are important pharmacological tools to isolate LTCC current components in neurons and other excitable cells. Most of their pharmacotherapeutic actions are mediated by $Ca_v1.2$ $Ca^{2+}$ channels which are densely expressed in the cardiovascular system.[5] However, block of $Ca_v1.3$ channels may be important for their bradycardic effects[6] (for $Ca^{2+}$ channel classification, nomenclature of the $\alpha 1$ subunits and their diverse functional roles see other chapters of this series).

Tritiated derivatives of these $Ca^{2+}$ antagonists were found to reversibly bind to LTCCs in membrane preparations of various tissues with dissociation constants in the picomolar or low nanomolar range.[7] This allowed the biochemical isolation and subsequent cloning of LTCC subunits. Together with fluorescent derivatives it could be shown that these chemically distinct classes of organic $Ca^{2+}$ antagonists affect each others binding to the channel complex[8] (for review see refs. 9,10). For example, DHPs can slow the association and dissociation kinetics of BTZs for the LTCC complex, whereas verapamil destabilizes binding. Although these interactions are of no clinical relevance, they provided valuable insight into the molecular organization of these drug binding domains. Originally it was believed that separate high affinity, stereoselective binding domains exist for the different chemical classes of $Ca^{2+}$ antagonists ("allosteric model", Fig. 1B). As will be outlined below, such a model does not account for the available structural data which postulate closely associated and even overlapping rather than separate binding domains.

Radioactive photoreactive $Ca^{2+}$ antagonists enabled the covalent labeling of the binding domains. It was found that they exclusively reside on the $\alpha_1$ subunits of the LTCC complex ($Ca^{2+}$ channel structure is discussed in more detail in other chapters of this series). Photoaffinity labeling experiments could even localize them within the pore-region of the $\alpha 1$ subunits (Fig. 1A, peptides a,b,c) and thus guided further mutational analysis of these drug binding domains on the single amino acid level. The resulting model of the $Ca^{2+}$ antagonist drug binding domains is at present the most detailed one among the family of voltage-gated ion channels. Further research in this field is warranted because the high structural homology between L-type and non-L-type $\alpha_1$ subunits may help to develop derivatives of $Ca^{2+}$ antagonists with decreased affinity for L-type (e.g., $Ca_v1.2\alpha_1$) but increased affinity for non-L-type channels. Small or-

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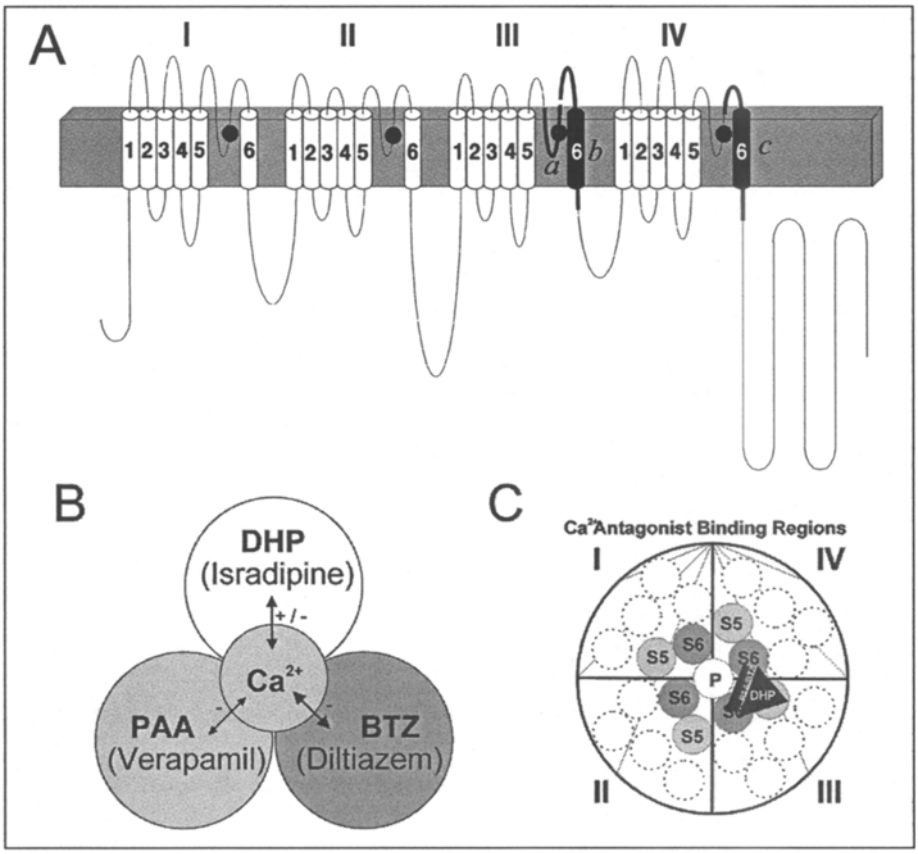



Figure 1. Transmembrane topology of $Ca^{2+}$ channel $\alpha 1$ subunits and the drug binding domains. A) 24 transmembrane helices (segments S1-S6) are organized in 4 homologous repeats (about 2000 amino acid residues). Proteolytic portions of the drug binding domains (indicated by bold lines and dark segments; fragments a, b, c) were initially identified by photoaffinity labeling. Photoincorporation of DHPs occured into all three fragments, of PAA only into fragment c and of BTZs into fragments b and c. The circles indicate the position of glutamate residues forming a common $Ca^{2+}$ selectivity filter. The segments S5, S6 and the connecting linkers ("pore-loops") assemble in the center of the folded $\alpha 1$ subunit molecule thus creating the ion conducting pathway. B) Radioactive DHPs, PAAs and BTZs possess high affinity drug binding domains on LTCC $\alpha 1$ subunits. These are modulated by $Ca^{2+}$. Micromolar $Ca^{2+}$ conentrations stabilize DHP binding (+), whereas millimolar concentrations inhibit binding of all three chemical classes of $Ca^{2+}$ antagonists (-). C) Instead of forming separate "allosterically coupled" domains B), recent structural data indicate that these binding domains overlap within a "multisubsite domain" [10,12] involving the photoaffinity labeled regions plus transmembrane segment S5.

ganic modulators of non-L-type channels (e.g., active on $Ca_v2.1\alpha_1$ or $Ca_v2.2\alpha_1$ subunits) are urgently needed to test such compounds for their efficacy in the treatment of diseases such as neuropathic pain or migraine.

The process of the antibody-based localization of photoaffinity labeled peptides and mutational analysis of all three LTCC drug binding domains has been reviewed in previous articles.[9-12] In this chapter we therefore discuss more recent data which nicely extent these previous findings and start to provide a more three-dimensional view of the drug binding pocket.

The L-type amino acid numbering will be according to their position in a rat brain splice variant of $Ca_v1.2\alpha_1$ ($\alpha_1$C-II, genbank accession number M67515[13]).

## Abbreviations

BTZ benzothiazepine
DHP dihydropyridine
LTCC L-type $Ca^{2+}$ channels
PAA phenylalkylamine

## A Multisubsite Model of the $Ca^{2+}$ Antagonist Drug Binding Domains

### *Interaction of Drug an $Ca^{2+}$ Binding Sites-How Drug Binding Can Affect Channel Function*

In addition to the above mentioned non-competitive interactions between the different chemical classes of $Ca^{2+}$ antagonist drugs, radioreceptor assays also revealed that high affinity DHP binding requires the presence of micromolar concentrations of $Ca^{2+}$ [8] (Fig.1B). In contrast, millimolar concentrations of divalent cations decreased the affinity for all chemical classes of $Ca^{2+}$ antagonists. This was the first biochemical proof for the presence of a high affinity $Ca^{2+}$ binding site on the channel. From these studies it was clear that a model for drug-channel interaction would also have to incorporate a binding site for $Ca^{2+}$. A major breakthrough for understanding the drug -$Ca^{2+}$ ion interaction came from the discovery that four conserved glutamate residues form the major determinants of the channel's $Ca^{2+}$ selectivity filter.[14] It could be demonstrated that this selectivity filter represents the high (and low) affinity binding site which modulates drug binding.[15]

L-type $Ca^{2+}$ channels require the association with at least a $\beta$ and an $\alpha_2$-$\delta$ subunit. Both subunits are required not only for proper plasma membrane targeting but they also stabilize the pore region. Thereby they not only fine-tune the physiological gating properties of $\alpha_1$ subunits[16] but also $Ca^{2+}$ coordination in the pore.[15] As the drug binding domains interact with the $Ca^{2+}$ binding site and are also localized in the pore region (see below), they are also stabilized by $\beta$ subunits.[15]

### *Drug Binding Residues Are Associated with the Ion Conducting Pathway in Repeats III and IV*

Sequence stretches photoaffinity labeled by radioactive $Ca^{2+}$ antagonists included the domain III pore loop (peptide a in IIIS5-IIIS6 linker, Fig. 1A) and peptides containing transmembrane segments IIIS6 and IVS6 (peptides b and c, Fig. 1A). These regions were therefore the major target of subsequent mutational studies undertaken not only to confirm these biochemical findings but to identify individual amino acid residues participating in drug interaction. For this purpose recombinant $\alpha_1$ subunits carrying specific mutations or chimeric constructs between L- and non-L-type $Ca^{2+}$ channel $\alpha_1$ subunits were heterologously expressed in Xenopus laevis oocytes or in mammalian cells. Drug sensitivity was then quantified by either electrophysiological recordings of channel currents in intact cells or by radioligand binding studies (restricted to DHP binding after expression in mammalian cells). "Loss-of-function" mutations/constructs were created in which L-type sequence was systematically changed to alanine ("alanine-scanning mutagenesis") or to the corresponding residues in non-L-type $\alpha_1$ subunits. In addition, "gain-of-function" mutants were constructed in which L-type sequence was engineered into non-L-type $\alpha_1$ subunits thereby introducing high $Ca^{2+}$ antagonist sensitivity.

The major findings of these experiments can be summarized as follows:

1. Drug binding residues are indeed localized within the photolabeled regions and therefore close to the channel pore: This established the usefulness of photoaffinity labeling techniques and argued against the criticism that the photoreactive side chains photolabeled regions outside the drug binding pocket.[17] In addition, transmembrane segment IIIS5 was discovered as an important determinant of DHP binding.[4,18,19] Note that the localization of the drug binding pocket within pore forming regions does not allow to conclude that $Ca^{2+}$ antagonists are simply pore blockers which occlude the pore. Instead, all of them can be shown to alter the gating behavior of the channel.[20] They are able to preferentially stabilize or promote entry

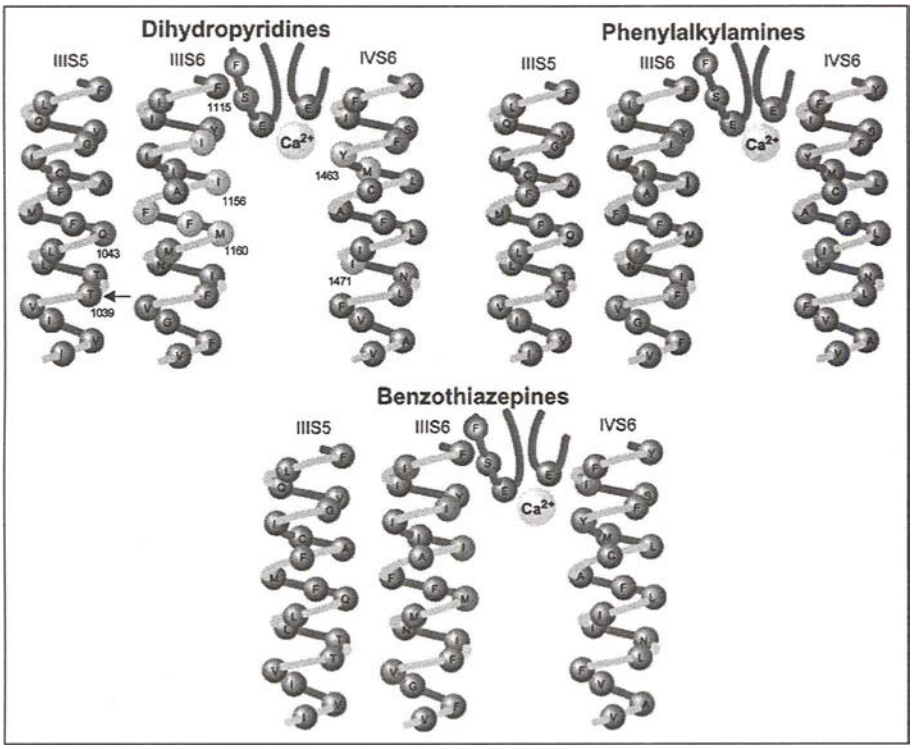



Figure 2. Individual amino acid residues (blue) participating in the formation of the DHP, PAA and BTZ binding domains. Residues interacting with drugs are highlighted in blue. Dark blue indicates stronger contribution. The numbers indicate the position of some of the residues within the $Ca_v1.2\alpha1$ subunit sequence (see text). The arrow in IIIS5 (DHPs) indicate Thr1039 which when replaced to tyrosine removes DHP sensitivity. The domain III and IV glutamate residues forming part of the $Ca^{2+}$ selectivity filter are also shown. They directly contribute to the binding of the cationic PAAs (and maybe also BTZs) and indirectly to DHP binding (conformational stabilization of the DHP binding domain when one $Ca^{2+}$ ion is bound, see text). A color version of this figure is available online at http://www.Eurekah.com.

into the inactivated channel state (channel blockers) or stabilize open channel conformations (channel activators like the DHP BayK 8644 or the benzoyl pyrrole FPL 64176[20-22]).
The amino acid residues participating in the formation of the individual drug binding domains are highlighted in Figure 2. Most of them were already discussed in previous reviews. The discovery of DHP binding residues Ser1115 and Phe1112 in the IIIS5-S6 linker[23,23a] and more recently discovered BTZ interaction sites[24,25] will be discussed in more detail below.

2. The drug binding domains are localized close to the $Ca^{2+}$ selectivity filter of the channel: Figure 1 highlights the close localization of drug and $Ca^{2+}$ binding domains which provided the relevant clue for explaining the $Ca^{2+}$ channel modulating effects of these drugs. $Ca^{2+}$ permeation through the channel requires selectivity (i.e., selection by high affinity) but, at the same time, high permeability. These apparently contradictory properties can both be achieved by sequential $Ca^{2+}$ binding to a single $Ca^{2+}$ selectivity filter. When one $Ca^{2+}$ is bound to the four glutamates it does so with high affinity (single occupancy, micromolar $K_D$). Obviously, the high stability of this complex prevents efficient permeation of the $Ca^{2+}$ ion through the pore into the cytoplasm. This monoliganded state corresponds to the high affinity $Ca^{2+}$ binding site previously found to stabilize the DHP antagonist interaction.[8] Permeation of $Ca^{2+}$ is initiated by the binding of a second $Ca^{2+}$ ion whose association is driven by the millimolar

extracellular $Ca^{2+}$ concentration. When two $Ca^{2+}$ ions are bound they do so with lower affinity (double occupancy, millimolar $K_D$). This results in the dissociation of one ion which exits on the cytoplasmic face of the open channel. The double occupancy state represents the low affinity $Ca^{2+}$ binding site inhibiting DHP (and also PAA and BTZ) antagonist binding. As the ternary complex of $\alpha_1$ with a bound Ca2+ ion and a DHP molecule seems very stable, this explains why $Ca^{2+}$ stabilizes high DHP affinity. It also explains why DHP thereby stabilizes the single occupancy of $Ca^{2+}$, i.e., a non-conducting state. Although experimental evidence is lacking it can be proposed that $Ca^{2+}$ channel activators, such as BayK 8644, facilitate double occupancy and thus promote the open channel state.[12]

In contrast to DHPs, PAAs (and most likely also BTZs; both are mainly positively charged at physiological pH) directly interact with the $Ca^{2+}$ selectivity glutamates in domain III and IV.[26] Their block of $Ca^{2+}$ channel currents can therefore be explained by a more direct interference with $Ca^{2+}$ binding and permeation.

3. As illustrated in Figure 2 the DHP, PAA and BTZ drug binding domains are located in close proximity to each other and even overlap. This is in good agreement with the results of binding experiments with radioactive[24] and fluorescent[27] DHP and BTZ derivatives demonstrating the presence of steric interactions which occur when two molecules bind simultaneously to the channel. Adjacent binding of DHPs and BTZs could explain e.g., the stablilization of BTZ binding and slowing of its binding kinetics[27] by DHPs but also destabilization of DHP binding by bulky BTZ side chains.[24] Many residues participate in the formation of more than one binding domain (Fig. 2). The previous "allosteric model" of $Ca^{2+}$ antagonist binding was therefore replaced by a " multisubsite domain" model.[9,10]
4. $Ca^{2+}$ antagonist sensitivity can be transferred to non-L-type $Ca^{2+}$ channel $a_1$ subunits. Transfer was facilitated by the fact that amino acid residues conserved between L-type and non-LTCC $\alpha_1$ subunits also participate in drug binding (e.g., Tyr1152 in IIIS6, Asn1472 in IVS6 in the case of DHPs; see also sequence comparison in Fig. 9 below). That means that a portion of these binding domains is already present in non-LTCCs but insufficient to support high affinity interaction. In the case of DHPs this prompted further studies to investigate how the L-type specific residues contribute to drug binding within a non-LTCC environment.

## Towards a Three-Dimensional Model of the DHP Binding Domain

### *The DHP Binding Domain—The Most Attractive Candidate to Understand $Ca^{2+}$ Channel Modulation by Drugs*

The most detailed molecular analysis has so far been provided for the DHP binding domain. There are several reasons for this:

1. DHPs discriminate more between L-type and non-LTCCs (> 100-fold selectivity[4] than PAAs and BTZs (about 10-fold selectivity[25]).
2. Mutational consequences on DHP interaction can directly be studied using radioligand binding to heterologously expressed recombinant channels. However, high nonspecific binding components prevent such assays with PAAs or BTZs (I. Huber, J. Striessnig, unpublished observations).
3. PAAs and BTZs block LTCCs in a highly use- (or frequency-) dependent manner. Quantification of their channel blocking activity requires repetitive channel stimulation, their apparent $IC_{50}$ becoming strongly dependent on experimental conditions. Some mutations were found to dramatically decrease the (apparent) sensitivity to PAAs and BTZs but mainly by altering channel kinetics (e.g., voltage-dependent inactivation and recovery from channel block) rather than decreasing binding affinity.[24,28] Although gating changes can also affect apparent DHP sensitivity it is easier to establish experimental conditions which allow to quantify real changes in binding affinity in functional experiments.[29]
4. Some DHPs, such as (S)-BAYK 8644 or (+)-SDZ202-791, act as $Ca^{2+}$ channel activators. Mutational analysis therefore allows also to identify residues which mediate the stabilization of open channel conformations by these compounds.

## *Three Dimensional Drug DHP Binding Models: Help Required from $K^+$-Channels*

Due to the high selectivity of DHPs for LTCC $\alpha_1$ subunits, the finding that DHP sensitivity could be transferred to non-LTCC $Ca_v2.1\alpha_1$ ($\alpha_{1A}$)[30,31] and $Ca_v2.3\alpha_1$ ($\alpha_{1E}$)[32] was surprising. This was achieved by transfer of only those 8 -9 DHP binding residues in the transmembrane segments IIIS5, IIIS6 and IVS6 which are L-type-specific (Fig. 2). But did this DHP binding pocket retain all its typical properties? If this would be the case one could argue that the folding structure between L-type and non-LTTCC $\alpha_1$ subunits must be highly preserved. Otherwise the DHP binding residues could not be arranged appropriately to accomodate the DHP molecule in a non-L-type $\alpha_1$ environment. A detailed analysis of the DHP-sensitive $Ca_v2.1\alpha_1$ ($\alpha_1A^{DHP}$, $Ca_v2.1\alpha_1{}^{DHP}$) in functional[30] and radioligand binding studies[33] showed that most characteristics typical for DHP binding to native LTCCs were preserved in the artificial construct: (i) DHPs still bound with low nanomolar affinity and in a highly stereoselective manner; (ii) (+)-[$^3$H]isradipine binding was stimulated by (+)-cis-diltiazem, (+)-tetrandrine and mibefradil; and (iii) $Ca^{2+}$ channel currents through $Ca_v2.1\alpha_1{}^{DHP}$ still showed the typical activation by BAYK 8644 (stimulation of peak inward current, slowing of tail currents, hyperpolarizing shift of voltage-dependence of activation). In order to obtain a precise three dimensional model of the DHP binding pocket the folding structure of LTCC $\alpha_1$ subunits must be known. Despite the enormous progress in solving the X-ray structure of different ion channels,[34,35] (see also notes added in proofs) no $Ca^{2+}$ channel has yet been analyzed. Therefore Huber et al[33] constructed a three-dimensional model of the DHP binding domain by using the three dimensional structural information of the KcsA $K^+$-channel.[35] This was the most closely related ion channel published at this time. It is likely that the folding structure of the pore-forming regions of the KcsA channel, at least within its membrane-spanning segments, is similar to $Ca^{2+}$ and perhaps other cation channels.[33] Note that this was the only assumption of this model. After sequence alignment, 12 of the most important DHP binding residues within the transmembrane segments, capable of forming the recombinant high affinity binding pocket[30,32] were introduced into the corresponding 3D structure of KcsA. No docking experiments with the DHP molecule were performed. Figure 3 shows the resulting model with only the "mutated" (i.e., DHP binding) residue side chains highlighted. Pore loops were not included in the model. KcsA was crystallized as a homotetramer, whereas $\alpha$1 subunits consist of four homologous but not identical repeats tethered together by intracellular linkers. So the first question that the model could address was if the $\alpha_1$ subunit sequence can be assigned to the KcsA tetramer by either a clockwise (Fig. 3A) or counterclockwise (Fig. 3B) arrangement. Figure 3A,B (top view along the axis of the pore) illustrates that the DHP binding residues in IIIS5, IIIS6 and IVS6 can participate in the formation of a single binding pocket only after clockwise arrangement of repeats I-IV. In contrast, in the counterclockwise arrangement (Fig. 3B) IIIS5 and some of the IIIS6 from a very narrow cleft from which IVS6 residues are completely excluded. Therefore one interesting prediction from this model was that the homologous repeats of $Ca^{2+}$ channel $\alpha_1$ subunits must be arranged in a clockwise manner. Meanwhile a clockwise arrangement has also been predicted for voltage-gated sodium channels.[36]

The clockwise model can form a binding pocket with dimensions that allow coordination of a DHP. Taking into account the flexibility of their side chains, all amino acids could be oriented towards a common binding pocket. An exception was IIIS6 Phe1159 (Fig. 3A). However, this residue contributes only very weakly to DHP sensitivity.[37] The narrow inner portion is formed by residues Met1161 (IIIS6) and Asn1472 (IVS6), whose side chains are approx. 5.5 Å apart. This is close enough to form a hydrogen bond that could participate in the conformational stabilization of the pore region. The interaction of the DHP with these residues could therefore contribute to the modulation of channel gating. The wider outer portion is formed by residues Tyr1152 (IIIS6), Gln1043 (IIIS5), Tyr1463 and Met1464 (IVS6). Gln1043-Met1464 and Gln1043-Tyr1463 are separated by approx. 10 Å. This is not close enough for hydrogen-bonding but both distances can easily be spanned by a DHP molecule (9 Å between the DHP ring nitrogen and the isopropyl side chain).

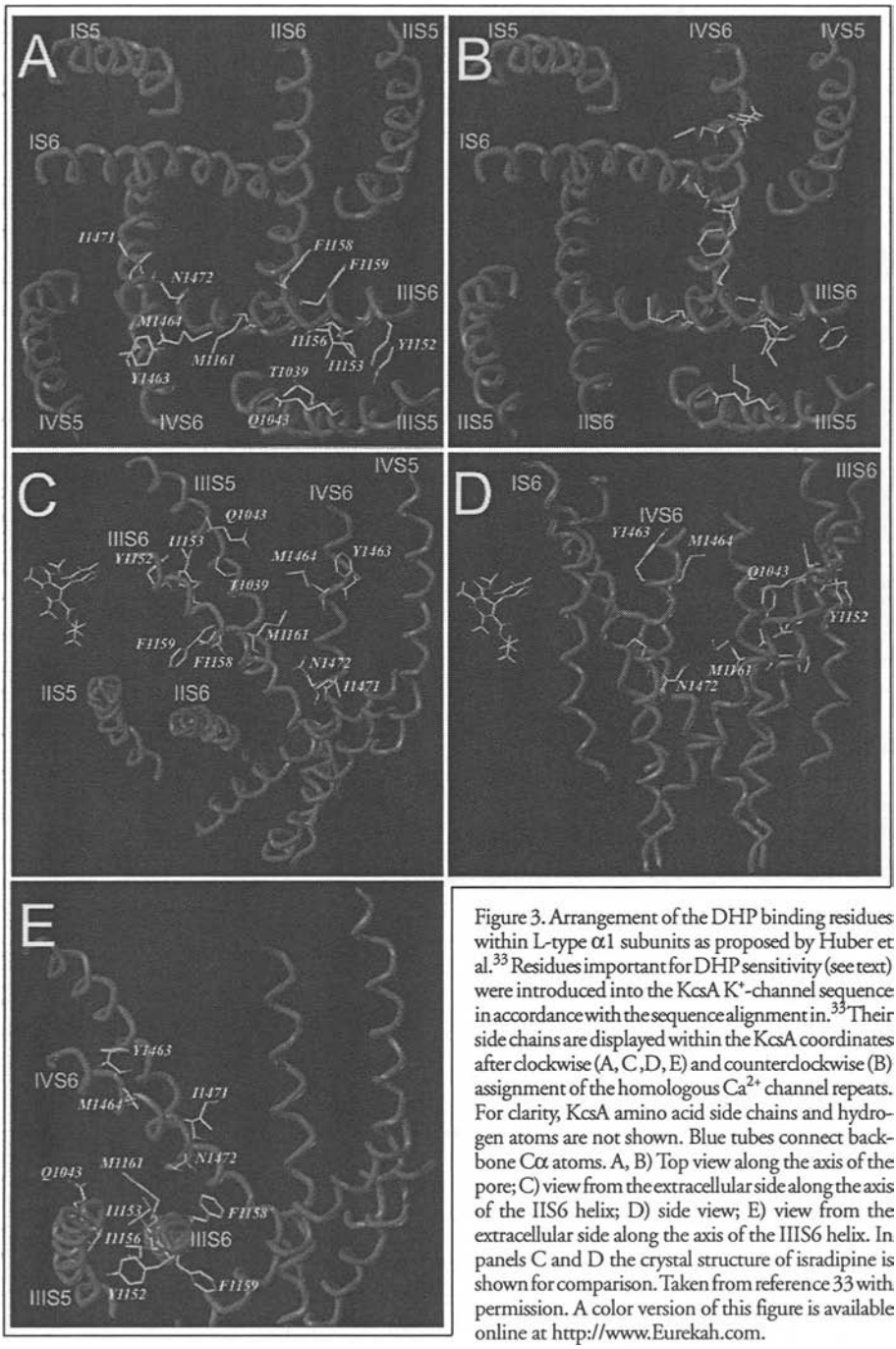



Figure 3. Arrangement of the DHP binding residues within L-type α1 subunits as proposed by Huber et al.[33] Residues important for DHP sensitivity (see text) were introduced into the KcsA $K^+$-channel sequence in accordance with the sequence alignment in.[33] Their side chains are displayed within the KcsA coordinates after clockwise (A, C, D, E) and counterclockwise (B) assignment of the homologous $Ca^{2+}$ channel repeats. For clarity, KcsA amino acid side chains and hydrogen atoms are not shown. Blue tubes connect backbone Cα atoms. A, B) Top view along the axis of the pore; C) view from the extracellular side along the axis of the IIS6 helix; D) side view; E) view from the extracellular side along the axis of the IIIS6 helix. In panels C and D the crystal structure of isradipine is shown for comparison. Taken from reference 33 with permission. A color version of this figure is available online at http://www.Eurekah.com.

A more detailed model was provided by Zhorov et al.[38] It also exploited the KcsA coordinates but attempted to predict the orientation of the DHP agonist and antagonist molecules within the binding pocket. For this purpose more than one sequence alignment between KcsA and the

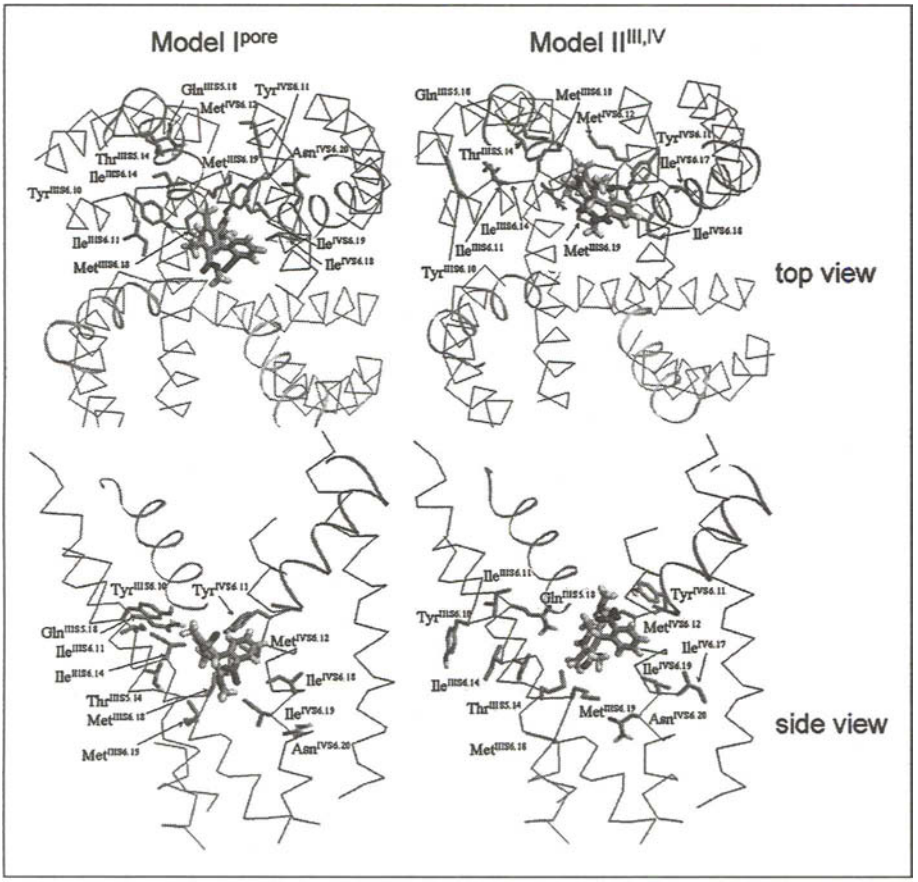



Figure 4. Nifedipine docked to different test models according to Zhorov et al.[38] Side and top view of nifedipine in test model $I^{pore}$ and test model $II^{III/IV}$ as described in the text. Shown are the lowest-energy complexes with bound ligand. Transmembrane segments and pore-loops are presented, respectively, by Cα tracing and ribbons whose darkness increases with repeat number. Side chains of DHP-sensing residues are shown as thin sticks, and nifedipine by thick sticks. At side views most of repeats I and II is removed for clarity. Test model $I^{pore}$ with nifedipine in the pore is better in terms of ligand–receptor energy and the number of ligand contacts with the DHP-sensing residues than test model $II^{III/IV}$ in which nifedipine is placed in the domain III-IV interface. Amino acid numbering is according to their position within the indicated helices. $Gln^{IIIS5.18}$ (18th residue in IIIS5 helix) corresponds to Gln1043 in Figure 2; other examples: $Tyr^{IIIS6.10}$ corresponds to Tyr1152 and $Tyr^{IVS6.11}$ to Tyr1463 in Figure 2. Taken from reference 38 with permission.

L-type $\alpha_1$ subunits was used to determine the positions at which L-type sequence was introduced. In addition, a portion of the four pore loops containing the selectivity filter glutamates was included in the model which also allowed to test for DHP $Ca^{2+}$ ion interactions. Nifedipine and BayK 8644 enantiomers were then docked into the proposed binding regions at different positions. Monte Carlo minimization was used to search the optimal conformations of drug and amino acid side chains. Cα-atoms (with the exception of some pore-loop residues) were restrained to kcsA coordinates. Four models were tested. Using two different alignments the calculated binding energy was calculated either with the DHP placed within the pore or within the domain III/IV interface. When the same alignment was used as in the model described above[33] optimal ligand coordination was obtained when placed in the domain III/IV interface (Fig. 4, model $II^{III/IV}$). A

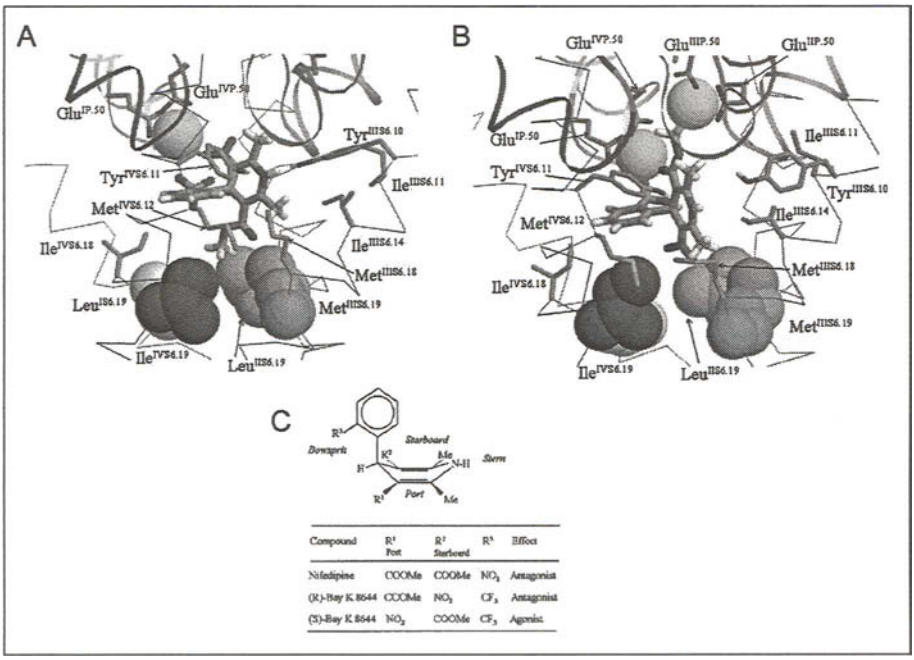



Figure 5. Energy minimized complexes of the DHP binding domain with BayK 8644 enantiomers. Monte Carlo-minimized complexes of the binding domain DHP with the enantiomers of BayK 8644 bound portside down. As in the case of nifedipine (not shown) the NH group of the ligand approaches the hydroxy groups of $Tyr^{IIIS6.10}$ and $Tyr^{IVS6.11}$. Interaction with $Tyr^{IVS6.11}$ stabilizes the portside-down orientation. $Ca^{2+}$ ion is coordinated between the ligand and two conserved glutamates. A) Antagonist (R)-Bay K 8644 approaching the hydrophobic gate by the hydrophobic methyl group would stabilize its closed conformations. B) Agonist (S)-Bay K 8644 approaching the hydrophobic gate by the hydrophilic group $NO_2$ would destabilize its closed conformations and, hence, facilitate the open conformation. C) Structure and activity of DHP ligands. Note designations of the different sides of the boat-like structure. For amino acid numbering in comparison to Figure 2 see legend to Figure 4. The hydrophobic residues forming the bracelet (see text) are illustrated in space-filling style (gray spheres). Taken from reference 38 with permission.

total ligand-receptor energy of -31.4 kcal/mol was obtained through interaction with Gln1043, Met1161, Tyr1463, Met1464 and Ile1471 (for the corresponding amino acid numbering in Figs. 4 and 5 see figure legends). An even more stable complex resulted from a different sequence alignment and with the DHP placed in the pore (Fig. 4, model $I^{pore}$). Interaction with Tyr1152, Ile1153, Ile1156, Met1161, Tyr1463, Ile1460 and Ile1471 provided a ligand-receptor energy of -38.7 kcal/mol. Therefore the latter model was pursued further and also analyzed with $Ca^{2+}$ ion binding in the pore. Figure 5 illustrates how DHP agonists and antagonists could affect channel function. Like nifedipine in model $I^{pore}$ the (R)-Bayk 8644 (antagonist) molecule (for basic structure see Fig. 5C) is positioned in the pore with the portside facing downwards, i.e., towards the inner opening of the channel.

Based on the KcsA structure the S6 segments form an inverted teepee structure crossing on the inner side of the channel thereby forming an inner barrier for ion permeation.[35] In the model this hydrophobic bracelet is formed by Met1161 (IIIS6), Ile1471 (IVS6) and two leucine residues in the corresponding positions of IS6 and IIS6 (Fig. 5). This narrow part of the bracelet keeps the permeation pathway closed. One could speculate that even small rearrangements upon membrane depolarization may be sufficient to open it. The hydrophobic bracelet faces a water filled cavity inside the channel in which the DHP binds. Figure 5A,B shows that

the NH groups of the DHP ring of the antagonistic (R) and agonistic (S) enantiomer interact with Tyr1152 and Tyr1463. The aromatic ring of Tyr1463 stabilizes the portside-down conformation. In case of the antagonistic (R)-enantiomer a hydrophobic methyl group of the portside COOMe (Fig. 5C) substituent interacts with residues of the hydrophobic bracelet. This can stabilize its hydrophobic interactions and favor a closed channel conformation. The polar (starboardside) $NO_2$ substituent faces up and participates in $Ca^{2+}$ ion coordination. In the agonistic (S)-enantiomer the hydrophilic $NO_2$ group is on the portside and thus faces the bracelet. Thus the closed hydrophobic bracelet becomes unfavorable allowing the agonist to destabilize the closed state. It can be envisaged that the close association of the DHP molecules with the bound $Ca^{2+}$ ion allows the antagonist to stabilize a ternary complex in which a single $Ca^{2+}$ becomes tightly bound and the DHP portside substituent simultaneously favors closure of the channel.

### *Steric Factors Determine Interactions of DHPs with the IIIS5 Segment*

In transmembrane segment IIIS5 only two residues contribute to DHP sensitivity, Thr1039 and Gln1043. Mutation of Thr1039 to tyrosine, the corresponding residue in non-LTCCs $\alpha_1$ subunits, completely removed high affinity for DHPs.[18,19] A surprising finding was that replacement by alanine did not affect DHP antagonist sensitivity.[29] Therefore the Thr1039 side chain is not required for direct antagonist interaction. Instead, the bulky tyrosine side chain seems to sterically prevent the coordination of the DHP within the binding pocket.[29]

The contribution of this residue for DHP sensitivity was analyzed in more detail in the gain-of-function mutant $Ca_v2.1\alpha_1^{DHP}$.[30] 10 μM isradipine blocked about 70% of the inward current in X. laevis oocytes. Unlike in LTCC $\alpha_1$ subunits (such as $Ca_v1.2\alpha_1$) block was independent of the holding potential. In Thr1039Tyr loss of DHP sensitivity was independent of the holding potential. It was absent at both negative (channels in resting state) and more positive (large fraction of channels in inactivated state) holding potentials.[29] When Thr1039 was replaced by a phenylalanine instead of tyrosine an interesting difference became evident. Like Thr1039Tyr, Thr1039Phe also removed block by isradipine at negative holding potentials (-100 mV). However, at more positive potentials Thr1039Phe showed almost normal DHP sensitivity. This suggested that the mutation prevents DHP binding only in resting channels but can exert its blocking effect once the channels inactivate. An interesting observation was that the onset of DHP block at more positive potentials was slower than for $Ca_v2.1\alpha_1^{DHP}$, as if the phenylalanine side chain would compete for access of the DHP to its site. As Thr1039Phe lacks the side chain hydroxyl of Thr1039Tyr, this must be responsible for the observed difference. It is likely that this hydroxyl can participate in the formation of a hydrogen bond, thereby locking the tyrosine side chain in a position preventing DHP access independent of channel conformation. Using the KcsA based DHP binding model described above,[33] the carbonyl oxygen of a IIIS6 residue (Met1160 in $Ca_v1.2\alpha_1$; replaced by a phenylalanine in $Ca_v2.1\alpha_1^{DHP}$) was identified as a potential candidate (for details see legend to Fig. 6). The absence of the hydroxyl could therefore prevent this stabilization and provide the side chain with more flexibility. In the inactivated (but not resting) channel conformation the flexibility of the side chain would then allow the DHP to slowly overcome its steric hindrance explaining the observed slow onset of DHP block. Taken together these results indicate that Thr1039 is not directly participating in DHP antagonist binding but that no bulky side chains are tolerated in this position. In model $I^{pore}$ a Thr1039 is not close to the ligand and its substitution by tyrosine could only affect DHP coordination via an indirect conformational effect (Fig. 4). Instead, these experimental data fit nicely to model $II^{III/IV}$ in which Thr1039 does not directly contribute to DHP interaction but is located close enough to the DHP to allow steric interference with the bulkier tyrosine side chain (Fig. 4).

A more detailed analysis was also carried out for Gln1043,[19,29] which was replaced by alanine (to reduce the side chain to a methyl group), methionine (the residue in non-LTCCs), glutamate or aspartate (to remove the amide) and asparagine (to shorten the side chain by a C-atom). Methionine and alanine reduced DHP antagonist sensitivity but glutamate, aspartate

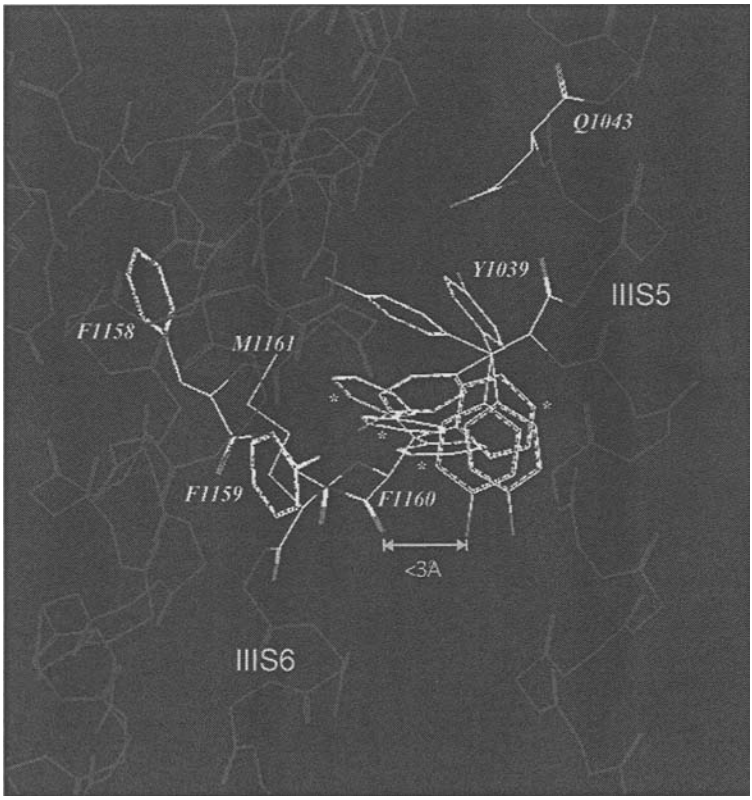



Figure 6. Proposed interaction of tyrosine side chain of mutant Thr1039Tyr in the model of Huber et al.[33] DHP binding residues of the Thr1039Tyr mutant were introduced into the model as described in Figure 3. Only the L-type amino acids Phe1158, Phe1159, Met1161, Gln1043 are highlighted. Possible conformations of the Tyr1039 side chain (which removes high DHP sensitivity in this mutant) were calculated and four representative conformations are shown. Note that this mutant was constructed within the $Ca_v2.1\alpha1$ sequence environment. Therefore the side chain of a $Ca_v2.1\alpha1$ phenylalanine (which replaces the corresponding Met1160 in $Ca_v1.2\alpha1$) was also included in the conformational analysis. Four representative conformations of this Phe1160 are also illustrated (highlighted by yellow asterisks). The Tyr1039 side chain is able to form a hydrogen bond with the carbonyl of Phe1160 in IIIS6. This restriction may fix this side chain in a conformation that prevents DHP coordination independent of channel conformation. Removal of the hydroxyl may restore its flexibility and allow DHPs to overcome its steric hindrance at least when channels inactivate (see text). A color version of this figure is available online at http://www.Eurekah.com.

and asparagine were tolerated.[18,29] This provided evidence that a carbonyl in this position is required for full antagonist sensitivity. It also questions the validity of model $I^{pore}$ and again favors model $II^{III/IV}$ in which this residue can participate in DHP binding. None of these Gln1043 mutations conferred full agonist sensitivity. The glutamine amide group seems therefore crucial for mediating BayK 8644 effects.

## *Activation of LTCCs by BAYK 8644 Seems to Be More Sensitive to Structural Changes Than Block by Antagonists*

As DHPs act as gating modifiers it is not surprising that some of them, like BAYK 8644, are able to stabilize open rather than closed (inactivated) channel conformations.[21] Do activators require a special interaction with the channel to accomplish this task? The answer seems to be

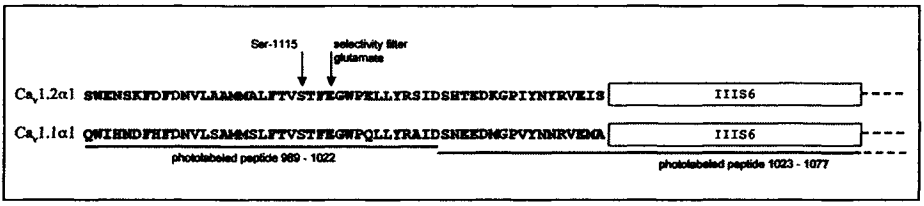



Figure 7. Sequence of $Ca_v1.1\alpha1$ and $Ca_v1.2\alpha1$ within the photolabeled regions in domain III. The positions of DHP photolabeled peptides, DHP binding residue Ser1115 and the domain III pore region glutamate are indicated.

"yes". A number of mutations affect agonist more than antagonist action. These are Gln1043Met, Gln1043Asn, Gln1043Glu (IIIS5), Tyr1152Ala (IIIS6) and Met1161Val (IIIS6) in construct $Ca_v2.1\alpha_1^{DHP}$,[29] a combined mutation of Tyr1463/Met1464 to isoleucine/phenylalanine, in $Ca_v1.2\alpha_1$[39] and Ser1115Ala in the IIIS5-S6 linker in $Ca_v1.2\alpha_1$ (see below). In some of these mutations the agonistic effect was even converted into an inhibitory one. This inhibitory effect seems to reflect the action of the antagonistic (R)-enantiomer of BayK 8644 in the racemic mixture. Although agonists seem to bind competitively with antagonists they may need to stabilize some amino acid side chains in defined conformations in order to promote open channel states.

## *Pore Loop Residues Also Contribute to DHP Interaction*

As outlined above, the mapping of the position of photoaffinity labeled proteolytic fragments within the $\alpha_1$ subunit sequence was a powerful tool to localize the drug interaction sites of the channel. Figure 7 illustrates the proteolytic peptides photoaffinity labeled by the DHP isradipine in the pore region of repeat III. In addition to a fragment containing the transmembrane segment IIIS6, isradipine photoincorporation was also mapped to an adjacent tryptic fragment within the IIIS5-S6 linker which contains the domain III selectivity filter glutamate. However, replacement of the L-type sequence stretch with non-L-type $\alpha_1$ sequence did not change DHP sensitivity.[3] More recently a series of nice experiments directly confirmed the contribution of this region to DHP binding. These experiments were prompted by the cloning of two non-mammalian $\alpha_1$ subunits from Halocynthia roretzi ($TuCa_1$) and the jelly fish.[23] Both were closely structurally related to mammalian L-type $\alpha_1$ subunits and also contained all known DHP binding residues in the transmembrane regions. Nevertheless their DHP sensitivity was very low. Yamaguchi et al[23] therefore reasoned that residues could contribute to DHP sensitivity which are conserved among all cloned mammalian $\alpha_1$ subunits but are replaced by other residues in the $TuCa_1$ and the jellyfish $\alpha_1$. From a series of potential candidates (Fig. 8B) Ser1115 and Phe1112 were found to mediate DHP sensitivity. Mutation of Ser1115 to alanine caused a 57-fold decrease in nitrendipine sensitivity (Fig. 8A,C). Agonist effects were affected even stronger. Stimulation by BayK 8644 was converted to inhibition and the stimulatory effect of FPL 64,176 was dramatically reduced (not illustrated). Mutation to threonine increased nitrendipine $IC_{50}$ 25-fold but stimulation by Bayk 8644 was almost completely removed (not illustrated). As Ser1115 is located only 3 amino acid residues away from the domain III selectivity filter glutamate, it should participate in conformational changes occurring when $Ca^{2+}$ binds to the selectivity filter (seee above). Thus it is likely that Ser1115 represents the (or part of the) effector mechanism allowing DHPs to allosterically interfer with $Ca^{2+}$ binding and flux (see above). It is also surprising that mutation of this residue did not affect verapamil and diltiazem sensitivity,[23] although at least PAAs directly bind to the domain III and IV selectivity filter glutamates[26] (Fig. 2).

A role for Ser1115 for DHP binding was successfully predicted by the DHP model shown in Figure 5.

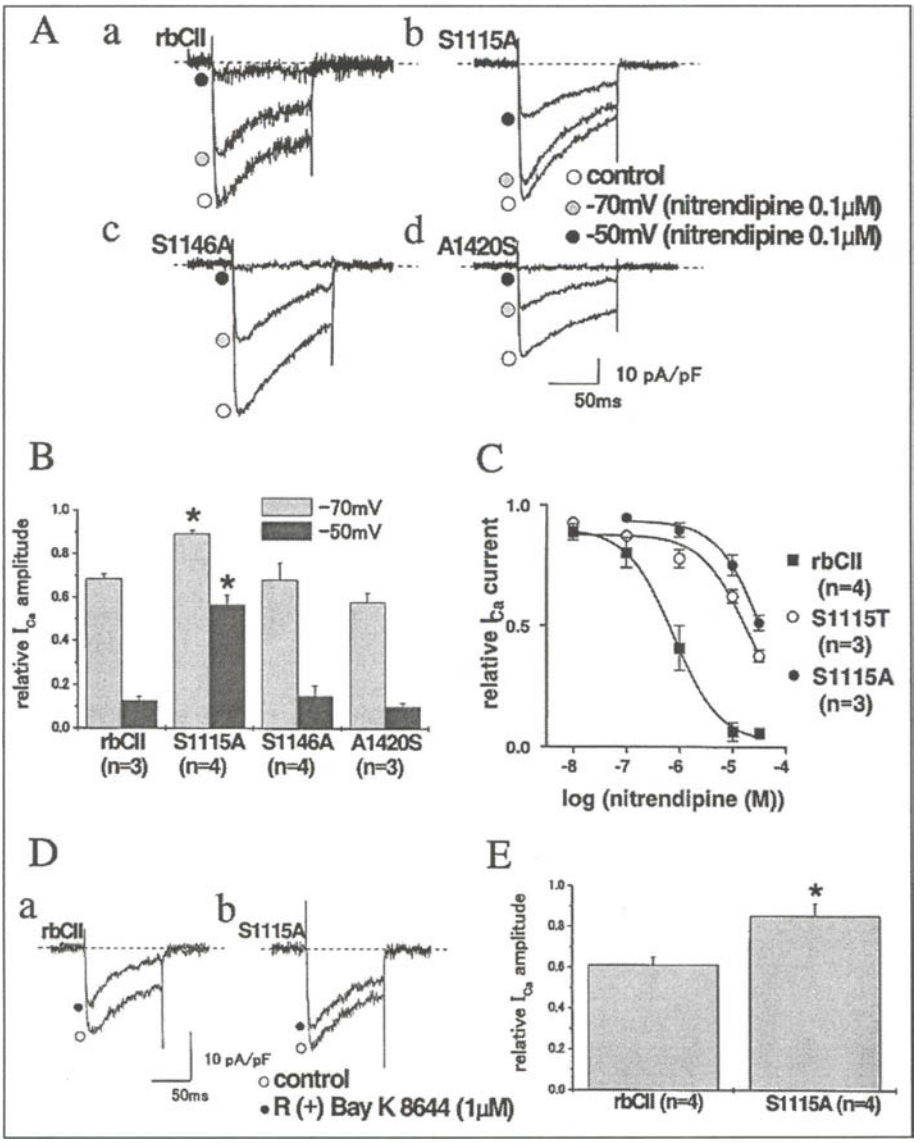



Figure 8. Voltage-dependent block of $Ca^{2+}$ channel currents by nitrendipine (0.1 μM) and R-(+)-BayK 8644 (1 μM). A) Representative current traces of wildtype $Ca_v1.2\alpha1$ (rbCII) (a), mutant Ser1115Ala (S1115A) (b), Ser1146Ala (S1146A) (c), and Ala1420Ser (A1420S) (d) elicited by a 100-ms test pulse to 0 mV at 0.1 Hz. Open circles represent control $Ca^{2+}$ channel currents at a holding potential of 270 mV. Gray and black circles represent $Ca^{2+}$ channel currents blocked by nitrendipine (0.1 μM) at holding potentials of -70 mV and -50 mV, respectively. B) Inhibition of $Ca_v1.2$ channel currents by nitrendipine at holding potentials of -70 mV and -50 mV. *p < 0.05 versus S1146A, A1420S, and wildtype (rbCII). C) Concentration-response curve for nitrendipine of wildtype (rbCII) and S1115A. The $IC_{50}$ value for S1115A and S1115T are 57.5 and 25 times higher than that of wildtype, respectively. D) Representative current traces of wildtype (a) and S1115A (b). Open and black circles represent control $Ca^{2+}$ channel currents and their block produced by R-(+)-BayK 8644 (1 μM), respectively. E) Inhibition by R-(+)-BayK 8644 of $Ca^{2+}$ channel current through wildtype and S1115A. The error bars show S.E. *p , 0.05 versus wildtype. Taken from reference 23 with permission.

## PAA and BTZ Binding Residues

Figure 2 shows that, in agreement with photoaffinity labeling studies employing photoreactive verapamil and diltiazem derivatives,[24,40] IIIS6 and IVS6 also harbor residues interacting with PAAs and BTZs. Again their binding determinants overlap considerably. This explains the non-competitive and competitive type of binding interactions observed between these two chemical classes of $Ca^{2+}$ antagonists.[41]

As outlined above, changes in the inactivation kinetics of the channel have profound effects on the apparent sensitivity towards use-dependent block by PAAs and BTZs. That means that mutations decreasing inactivation during a depolarization can decrease drug-induced use-dependent block without necessarily changing the affinity of the drug for its binding domain. For example, mutations within segment IVS5, a region unlikely to contribute to the formation of the binding domain according to the available folding models (Figs. 3-5), slow channel inactivation and decrease use-dependent block by diltiazem.[28] In binding experiments these mutants did not affect diltiazem stimulation of (+)-[$^3$H]isradipine binding suggesting that the mutations do not cause a decrease of diltiazem binding to its binding pocket.[28] Obviously, such effects on channel kinetics complicate the analysis of the PAA and BTZ binding residues in functional studies.

A similar finding was reported when the consequences of alanine mutations of IIIS6 residues Phe1164 and Val1165 on diltiazem sensitivity were investigated. The mutations reduced use-dependent diltiazem block when analyzed in $Ca_v1.2\alpha_1$[25] or in a chimeric construct.[24] A detailed kinetic analysis in the latter study revealed that diltiazem was still able to potently interact with the Phe1164Ala mutant. The drug accelerated inactivation of Phe1164Ala during long depolarizations and slowed its recovery from inactivation indistinguishable from wildtype. This demonstrated that the binding domain must still be intact to mediate these actions (this residue was therefore not marked as a potential BTZ interaction site in Fig. 2). In contrast, in Val1165Ala these effects of diltiazem were diminished and the drug only slightly slowed recovery from inactivation. This was interpreted as a lower affinity for the binding domain and/or an accelerated dissociation of the drug from blocked channels.[24] A model was proposed in which the Val1165 side chain controls dissociation of the drug from its binding domain such that its removal facilitates diltiazem dissociation. If this was true then bulkier diltiazem derivatives should escape at a lower rate and be less sensitive to this mutation. This was indeed confirmed with the bulky diltiazem derivative benziazem. It is therefore likely that Val1165 interacts with the diltiazem molecule but it remains unclear to which extent it directly contributes binding affinity.

## Differences in DHP Sensitivity between $Ca_v1.2$ , $Ca_v1.3$ and $Ca_v1.4$ L-Type $Ca^{2+}$ Channels

Based on recent progress in defining the differential roles of $Ca_v1.2$ , $Ca_v1.3$ and $Ca_v1.4$ L-type $Ca^{2+}$ channels (see other chapters in this series) it appears challenging to develop selective modulators of these L-type channels. It is therefore important to determine already existing differences in their $Ca^{2+}$ antagonist sensitivity.

### *DHP Sensitivity of $Ca_v1.3\alpha_1$*

Work from $Ca_v1.3\alpha_1$-deficient mice (see other chapters in this series) revealed a somewhat lower (but still high) DHP sensitivity for $Ca_v1.3\alpha_1$ mediated currents as compared to $Ca_v1.2$.[6] This was then proven by systematic comparison of DHP effects on heterologously expressed $Ca_v1.3\alpha_1$ and $Ca_v1.2\alpha_1$ subunits under identical experimental conditions in functional experiments. $Ca_v1.3\alpha_1$ showed an about 10-fold lower sensitivity for block by the DHP antagonist isradipine than $Ca_v1.2\alpha_1$;[42,43] but see reference 44).

Within the DHP binding regions $Ca_v1.3\alpha_1$ differs in only one amino acid position from $Ca_v1.2\alpha_1$ (Fig. 9), corresponding to Ile1156 in segment IIIS6 (Fig. 2). Substitution by a valine instead of isoleucine results in a modest reduction of the side chain size. Despite the existence

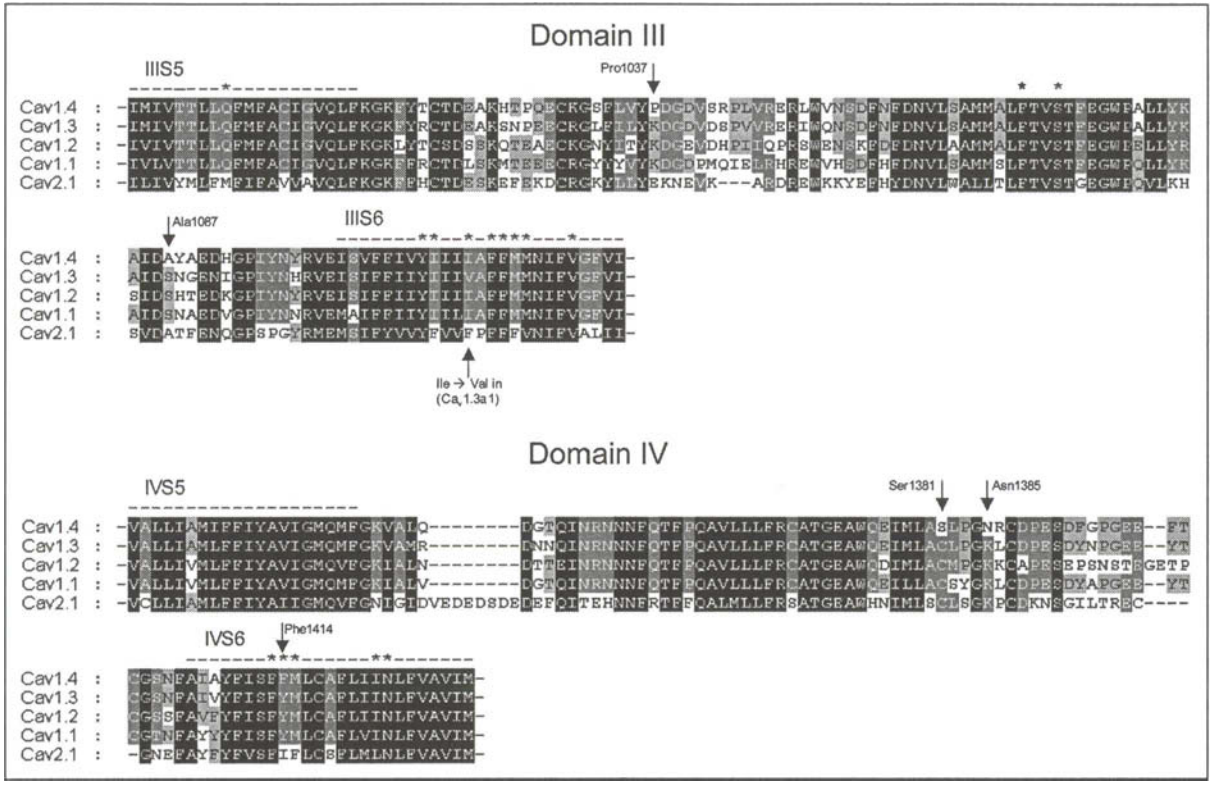



Figure 9. DHP binding residues in $Ca_v1.4\alpha1$ subunits. Clustalw sequence alignments between human Cav1.4α1, DHP-sensitive $Ca_v1.3\alpha1$, $Ca_v1.2\alpha1$, $Ca_v1.1\alpha1$ and DHP-insensitive $Ca_v2.1\alpha1$ (Genbank accession numbers: NM_005183, NM_000720, NM_000719, NM_000069, and NM_023035, respectively) are shown. The GONNET weight matrix was used for alignment. Residues participating in DHP interaction according to Figure 2 are marked with asterisks. Residues which could lower DHP-sensitivity of Cav1.4 are indicated by arrows.

of contradictory findings[37] considerable evidence exists suggesting that the contribution of Ile1156 for DHP sensitivity is small.[29,45] This is in accordance with radioligand binding experiments demonstrating that the affinity of heterologously expressed $Ca_v1.2\alpha_1$ and $Ca_v1.3\alpha_1$ subunits for (+)-[$^3$H]isradipine was indistinguishable.[42] This rules out major differences in the DHP affinity for the $Ca_v1.3\alpha_1$ binding pocket[42] when membrane voltage is absent. Therefore the different DHP sensitivity of $Ca_v1.2$ and $Ca_v1.3$ channels in functional studies must be due to the presence of membrane voltage. As previously shown for DHP antagonist block of $Ca_v1.2$ channels,[46] $Ca_v1.3$ is also blocked in a voltage dependent manner with higher apparent DHP sensitivity when the holding potential is more depolarized.[42,47] Isradipine sensitivity for $Ca_v1.3$ was increased by an order of magnitude when the holding potential was changed from -80 mV to -50mV.[42] The lower sensitivity of $Ca_v1.3$ for DHP antagonists as compared to $Ca_v1.2\alpha_1$ in functional experiments is therefore likely due to a difference in the voltage-dependence of DHP block, as previously described for splice variants of cardiac and smooth muscle $Ca_v1.2\alpha_1$ L-type $Ca^{2+}$ channels,[48] also reviewed in reference 1. Differences in their inactivation properties may also affect apparent DHP sensitivity. This is discussed in detail in a separate chapter of this series by S. Hering (Chapter 18).

## *DHP Sensitivity of $Ca_v1.4\alpha_1$*

$Ca_v1.4$ is exclusively expressed in retinal neurons and loss-of-function mutations cause incomplete X-linked congenital stationary night blindness in humans.[49,50] New generations of selective modulators of other L-type channels could cause side effects by modulating $Ca_v1.4\alpha_1$. On the other hand modulation of $Ca_v1.4$ channels may also be of therapeutic interest. Meanwhile $Ca_v1.4$ channels have been heterologously expressed in mammalian cells and shown to be DHP agonist and antagonist sensitive. However, their sensitivity for DHP channel blocker, such as isradipine, seems to even lower than for $Ca_v1.3$ in functional experiments.[51-53] Again, one explanation may be a difference in voltage-dependence of block. However, another explanation is that the binding pocket binds DHPs with somewhat lower affinity. So far this has not been tested by radioligand binding.

The amino-acid sequence of $Ca_v1.4\alpha_1$ is 63% identical to the $Ca_v1.3\alpha_1$ sequence, and is more than 83% identical in the DHP binding regions of domains III and IV. Most importantly, all residues identified to substantially contribute to DHP sensitivity (Fig. 2) are conserved except for Tyr1463, which is replaced by a phenylalanine in the human sequence (Phe1414 in $Ca_v1.4\alpha_1$, Fig. 9). Previous studies[3,37,45] have already quantified the role of Tyr1463 by mutational analysis. In $Ca_v1.1\alpha_1$ replacement by phenylalanine and alanine caused a 3.5 and 6.1-fold decrease in affinity, indicating that both the hydroxyl and the phenyl ring contribute binding affinity. Similar changes were found for Tyr1463Ala and Tyr1463Ile in $Ca_v1.2\alpha_1$.[37,45] Single mutations of Tyr1463 to isoleucine (the non-L-type residue) did also not prevent agonist stimulation.[39,45] It is therefore unlikely that this substitution would have a major effect on the DHP sensitivity of $Ca_v1.4\alpha_1$.

An important question is therefore if $Ca_v1.4\alpha_1$ contains amino acids within the DHP binding regions which are not present in all other L-type channel $\alpha_1$ subunits and may reduce binding affinity. The sequence alignment illustrates that this is indeed the case: Of special interest are Pro1037 ($Ca_v1.4\alpha_1$ numbering) and Ala1087 in the IIIS5-IIIS6 pore-loop. L-type specific conserved lysine and serine residues, respectively, are present in the other L-type $\alpha_1$ subunits. Pro1037 is also present in the barely DHP-sensitive jellyfish $\alpha_1$ (not shown). Proline is known to act as an $\alpha_1$-helix breaker and could introduce conformational changes affecting DHP sensitivity.

Two relevant differences to L-type sequence are also present in the IVS5-IVS6 pore-loop, Ser1380 and Asn1385 ($Ca_v1.4\alpha_1$) which are cysteine and lysine, respectively, in all L-type $\alpha_1$ subunits.

## Summary

Exciting new insight has been obtained into the structure of $Ca^{2+}$ antagonist binding domains within the $\alpha_1$ subunits of L-type $Ca^{2+}$ channels. On the basis of this structural information the data from earlier binding and functional studies can already be nicely combined to understand the basic molecular mechanism underlying block or activation by DHPs. This was supported by the development of three dimensional models derived from the structural homology of $Ca^{2+}$ with a bacterial $K^+$-channel. Due to expected structural differences between these ion channels these models cannot yet account for all experimental findings. However, they can be used to generate new hypotheses which can be tested experimentally in future mutational studies.[38] High resolution 3D models of more closely related, voltage-gated cation channels will help to refine these models.

### *Acknowledgements*

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### *Notes Added in Proof*

After the submission of this manuscript (August 2002) the X-ray structure of a voltage-gated bacterial $K^+$-channel has been reported by the MacKinnon group (Jiang et al. Nature 2003; 423:33-41). The folding structure of the pore-forming S5 and S6 segments is almost identical to KcsA.

### *Appendix*

Publication permission of the publishers must be seeked for the following material. The permission of the authors has already been granted to the corresponding author of this book chapter: Figures 4 and 5: material used for book chapter: Figures 3 and 5 of the following article. Authors: Zhorov BS, Folkman EV, Ananthanarayanan VS. Title: Homology model of dihydropyridine receptor: implications for L-type $Ca^{2+}$ channel modulation by agonists and antagonists. Published in: Archives of Biochemistry and Biophysics 2001; 393(1):22-41. Publisher: Academic Press. Contact: http://www.academicpress.com/

Figure 8: material used for book chapter: Figure 4 of the following article. Authors: Yamaguchi S, Okamura Y, Nagao T, Adachi-Akahane S. Title: Serine residue in the IIIS5-S6 linker of the L-type $Ca^{2+}$ channel alpha 1C subunit is the critical determinant of the action of dihydropyridine $Ca^{2+}$ channel agonists. Published in: Journal of Biological Chemistry 2000; 275(52):41504-11. Publisher: American Society for Biochemistry and Molecular Biology. Contact: http://www.jbc.org/misc/terms.shtml

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# CHAPTER 18

# Calcium Channel Block and Inactivation:
## Insights from Structure-Activity Studies

**Steffen Hering, Stanislav Sokolov, Stanislav Berjukow, Rainer Marksteiner, Eva Margreiter and Evgeni N. Timin**

## Introduction

During an action potential calcium ($Ca^{2+}$) ions enter the cell through voltage-gated $Ca^{2+}$ channels ($Ca_v$). $Ca_v$ channels first open and subsequently close before recovering to the resting state (Fig. 1A). The process of channel closure during maintained membrane depolarization is called "inactivation". During inactivation $Ca_v$ channels undergo several conformational changes: one is mediated by intracellular $Ca^{2+}$ interacting with a channel-calmodulin complex ($Ca^{2+}$-dependent inactivation), two other conformational changes are voltage-dependent (fast and slow voltage-dependent inactivation, Fig. 1A, see refs. 1,2 for review, and other chapters in this book).

Evolution has designed high-voltage-activated (HVA) $Ca_v$ channel families (also known as L-type [$Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, $Ca_v1.4$], P/Q-type [$Ca_v2.1$], N-type [$Ca_v2.2$] and R-type [$Ca_v2.3$] channels) and low-voltage-activated (LVA) $Ca^{2+}$ channel family $Ca_v3$.[3] The different family members display different inactivation properties.[1,2] HVA $Ca_v$ channels are heterooligomeric protein complexes composed of an $\alpha_1$-subunit, auxiliary $\beta$-, $\alpha_2$-$\delta$- and in some channels of an additional $\gamma$-subunit.[4] The $\alpha_1$-subunits sense the membrane voltage, form the pore and define the basic pharmacological properties of the channels[5-8] (Fig. 1C, D). Four $\beta$-subunit genes ($\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$) and various splice variants have been identified. These subunits modulate the inactivation properties of HVA $Ca_v$ (Fig. 1B).[6] HVA and LVA $Ca_v$ channels undergo fast and slow voltage-dependent inactivation.[9-12] $Ca^{2+}$/calmodulin-dependent inactivation is exclusively observed in HVA ($Ca_v1.2$, $Ca_v2.1$ and other HVA $Ca_v$ channels).[13-17]

The blocking action of many $Ca^{2+}$ antagonists such as the 1,4-dihydropyridines (DHPs), phenylalkylamines (PAAs), diltiazem (DIL) and mibefradil (MIB) in myocardial, smooth muscle and neuronal cells is enhanced by membrane depolarizations.[18-23] This is conventionally explained by a higher affinity of these drugs for open or inactivated channels (Fig. 1A). The precise mechanisms of channel block by these compounds remains to be elucidated.

Recent studies on chimeric and mutant $Ca_v$ channels provided new insights into the underlying molecular events. In particular the design of channels with different inactivation properties may help us to understand the molecular role of inactivation in channel inhibition by $Ca^{2+}$ antagonists.

### *Voltage-Dependent Inactivation and Drug Sensitivity of Chimeric $Ca_v$*

First evidence for a link between structural determinants of $Ca_v$ channel inactivation and drug-sensitivity came from studies on chimeric channels. In order to localise the drug-binding determinants for $Ca^{2+}$ antagonists Grabner et al[24] inserted various sequence stretches of a PAA, DHP and DIL-sensitive $\alpha_1$ subunit of an L-type channel into the poorly sensitive $\alpha_1 2.1$ (Fig.

*Voltage-Gated Calcium Channels*, edited by Gerald Zamponi.
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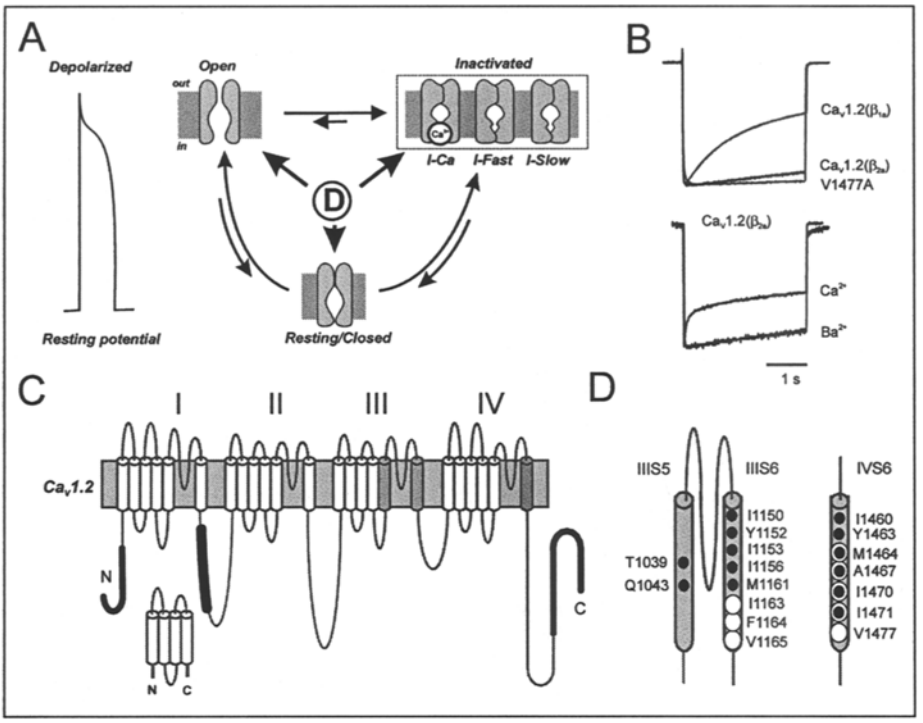



Figure 1. $Ca^{2+}$ channel conformational states as potential drug targets. A) Voltage-gated $Ca^{2+}$ ($Ca_v$) channels reside in closed (resting), activated (open) and several inactivated (I) states (i.e., $Ca^{2+}$-dependent inactivation [I-Ca], fast [I-fast] and slow [I-slow] voltage-dependent inactivation). Transitions to I-fast and I-slow occur upon membrane depolarizations, transitions to I-Ca are dependent on the intracellular $Ca^{2+}$ concentration. Negative shifts of the membrane potential promote transitions of open and/or inactivated channels to the resting state. It is hypothesised that some $Ca^{2+}$ channel blockers (e.g., DHPs) interact with an inactivated channel conformation.[20,21] Recent data from Berjukow et al[34] and Sokolov et al[32] suggest that transitions to the fast voltage-dependent inactivated state (I-fast) are of particular importance for the drug-channel interaction. B) $Ca_v2.1$ inactivation is affected by β-subunit composition and point mutations introduced into the $\alpha_1$-subunit. Representative barium and calcium currents through a $Ca_v1.2$ construct composed of $\beta_{1a}$- or $\beta_{2a}$-subunits[32] and currents through a mutant $Ca_v1.2(\beta_{1a})$ where a single valine in segment IVS6 was substituted by alanine (V1477A[34]). Holding potential -80 mV, test pulses to 20 mV. C) Putative topology of a $Ca_v$ channel $\alpha_1$-subunit. β-Subunits modulate $Ca_v$ channel properties by interacting with the intracellular linker between domains I and II, carboxy and amino terminus (highlighted in black).[6] D) Segments IIIS5, IIIS6 and IVS6 of $Ca_v1.2$. The putative drug-binding determinants (according to refs. 5,40) are indicated as black dots (L-type channel numbering here and further on according to $\alpha_{1C\text{-}II}$; accession number: M67515). Amino acids that have been shown to modulate channel inactivation are shown in white circles.

2A). Transfer of $Ca_v1.2$ segments IIIS5, IIIS6 and IVS6 to $Ca_v2.1$ confered PAA- and DIL-sensitivity to the resulting construct AL12 (Fig. 2B).[25]

Analysis of the inactivation of some of the chimeric channels revealed a correlation between inactivation kinetics and drug sensitivity. Substitution of domains IIIS5, IIIS6 and the connecting linker (chimera AL20, Fig. 2C) diminished both inactivation and block by DIL and PAA.[25] This was surprising because AL20 carries more than half of the PAA- and DIL-determinants (Fig. 1D). The almost complete loss of drug sensitivity raised the suspicion that the process of channel inactivation might interfere with drug action. This hypothesis was further supported by chimera AL22 lacking all PAA- and DIL-determinants in segment IIIS6 but inactivating significantly faster than AL12 and $Ca_v2.1$ (Fig. 2D). This channel exhibited

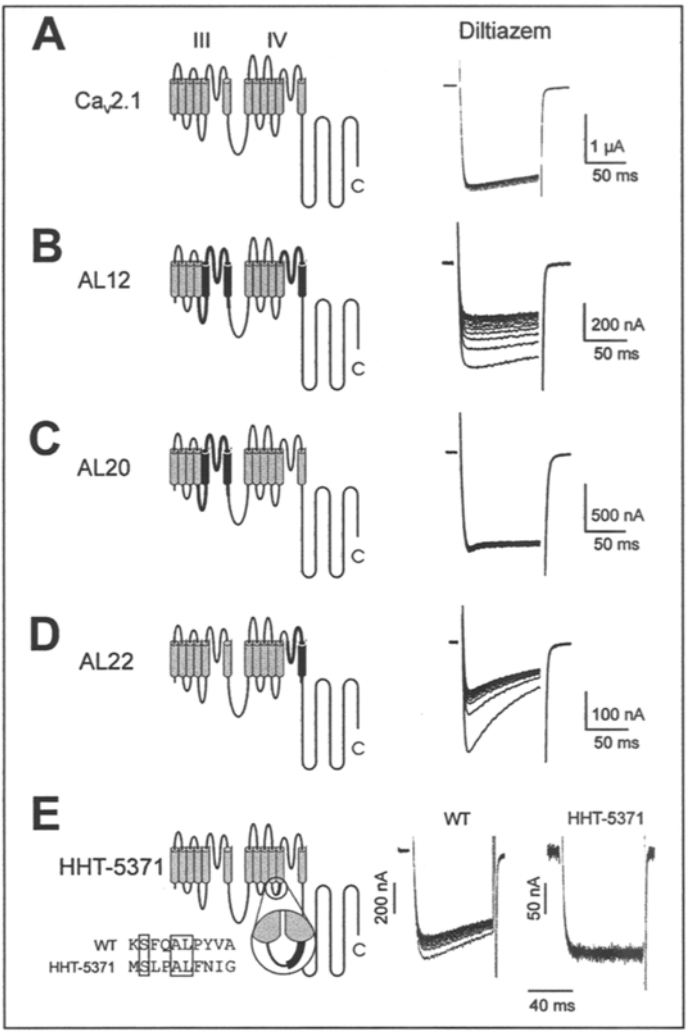



Figure 2. Inactivation properties of chimeric $Ca_v$ affect drug sensitivity. Domains III and IV of wild type $Ca_v2.1$ and $Ca_v2.1/Ca_v1$ chimeric channels. $Ca_v2.1$ sequence is shown in white and L-type sequence as black transmembrane segments, corresponding barium currents in the presence of ditiazem are shown on the right. A) Wild type $Ca_v2.1$ are almost insensitive to diltiazem. Drug-sensitivity was estimated as peak current inhibition during a train of 15 depolarizing 100-ms pulses applied at 0.1 Hz from –80 mV to 20 mV in the presence of 100 µM d-cis-diltiazem (same protocol used in B, C and D). B) Transfer of segments IIIS5, IIIS6 and IVS6 from the highly sensitive L-type channel into $Ca_v2.1$ enhances diltiazem-sensitivity to the level of $Ca_v1.2$ (see also Fig. 4C). C) Transfer of segments IIIS5 and IIIS6 (chimera AL20) does not enhance channel block as evident from negligible current inhibition during the pulse train. The construct comprises, however, all 6 amino acid determinants of diltiazem-sensitivity from $Ca_v1.2$ in segment IIIS6 (Fig. 1D). D) Substitution of segment IVS6 and part of the S5 – S6 linker of $Ca_v2.1$ by $Ca_v1.2$ sequence (chimera AL22) results in an enhanced rate of inactivation and strong use-dependent channel block by diltiazem. Data reproduced from ref. 25 with permission. E) Substitution of part of the IVS4-S5 linker (a sequence stretch that is unlikely to contribute to the drug receptor) in $Ca_v1.2$ by the rat brain sodium channel sequence diminishes inactivation and simultaneously reduces the apparent diltiazem sensitivity (300 µM d-cis diltiazem). Use-dependent block during 0.5 Hz train of 15 pulses from holding potential –60 mV in the presence of DIL for wild type $Ca_v2.1$ (left) and the chimera HHT-5371 (right) are reproduced with permission from reference 26.

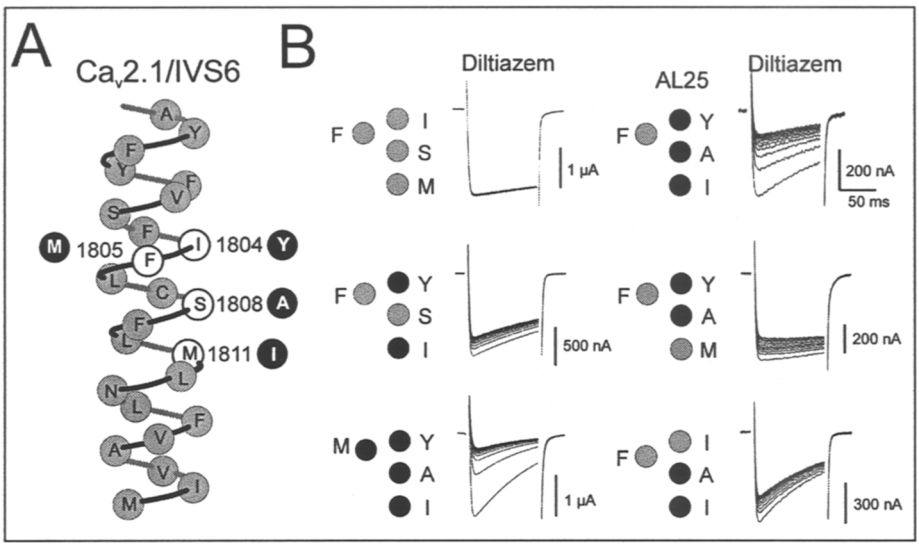



Figure 3. Single amino acids in segment IVS6 of a diltiazem-sensitive $Ca_v2.1$ mutant affect drug sensitivity and channel inactivation. A) α-Helical representation of $Ca_v2.1$ segment IVS6. Transfer of four $Ca_v1.2$ amino acids (Y, A, I, M, highlighted as black circles, $\alpha_{1A}$ numbering, accession number: P27884) enhances DIL- (and PAA- ) sensitivity.[25] B) Use-dependent $I_{Ba}$ inhibition by 100 µM d-cis-diltiazem (DIL) during a train of 15 pulses applied at 0.1 Hz. Wild type $Ca_v2.1$ (upper left traces) is barely drug-sensitive. A triple mutant AL25[25] (Y, A, I from $Ca_v1.2$) shows a high sensitivity for d-cis-diltiazem and displays a faster inactivation than wild type (upper right traces). Transfer of an additional L-type residue (F1805M) further increases the rate of inactivation and apparent drug-sensitivity of quadruple mutant (Y,M,A,I; bottom left traces). Mutating A1808 back to S (double mutant Y, I; middle left) or I1811 back to M (double mutant Y, A; middle right) impaires inactivation and apparent DIL-sensitivity. Substitution of Y1804 back to I in double mutant A, I (bottom right) reduces use-dependent block without considerably slowing inactivation kinetics (compared to AL25, top right). Reproduced from reference 25 with permission.

PAA- and DIL-sensitivity comparable to (or even higher) than chimera AL12 and wild type $Ca_v1.2$.[25]

A further example of a correlation between inactivation and drug-sensitivity in a chimeric channel comes from the work of Motoike et al[26] who substituted a small sequence stretch outside the putative drug binding region of $Ca_v1.2$ in the intracellular loop between IVS4 and IVS5 by the homologous residues from the rat brain $Na^+$ channel (chimera HHT-5371). As illustrated in Figure 2E, this substitution simultaneously diminished channel inactivation and DIL-sensitivity.

## Amino Acid Residues Located in the Putative Drug-Binding Region Affect Drug-Sensitivity and Channel Inactivation

Deeper insights into the interdependence between inactivation and channel block were enabled by the design of mutant $Ca_v$ channels with different inactivation properties. Hering et al[25] demonstrated that transfer of only three $Ca_v1.2$-specific amino acids in segment IVS6 to $Ca_v2.1$ (Y1804, A1808 and I1811 $\alpha_{1A}$ numbering, named chimera AL25[25], Fig. 3A) confers high sensitivity for (-)gallopamil and DIL (Fig. 3B, compare top left and top right traces). The authors paid, however, attention to the fact that these mutations significantly accelerated channel inactivation (Fig. 3B, top left and right traces). Furthermore, mutating either A1808 back to serine or I1811 back to methionine (Fig. 3B, middle panels) reduced not only PAA- and DIL-sensitivities but, simultaneously, the rate of channel inactivation.

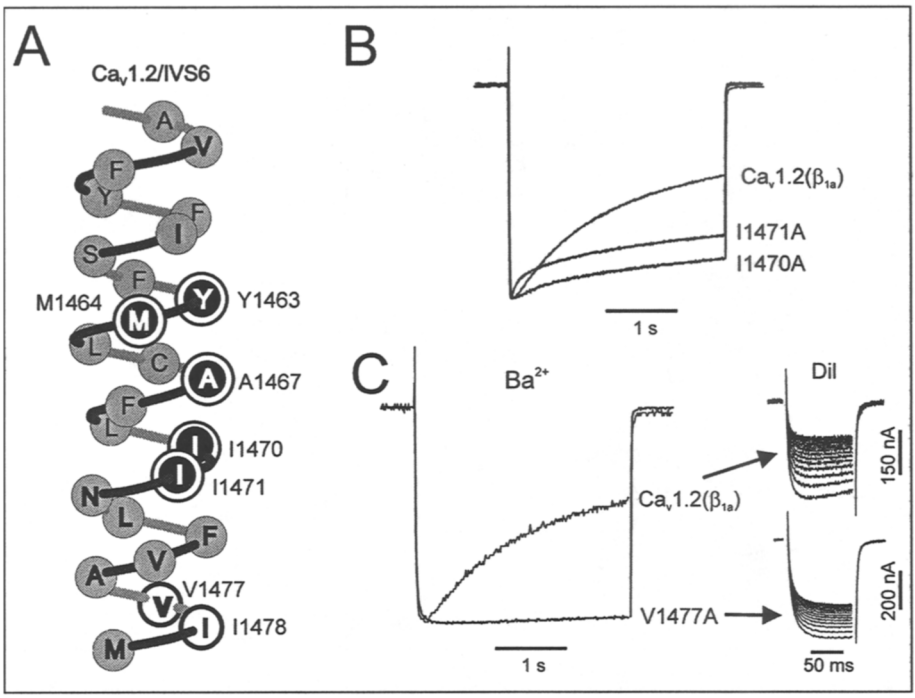



Figure 4. Inactivation determinants in $Ca_v1.2$ segment IVS6 modulate drug-sensitivity. A) α-Helical representation of $Ca_v1.2$ segment IVS6. Amino acid residues contributing to PAA- and DIL-sensitivity[5,40] (see Fig. 1D) are shown in black. A hot spot of inactivation determinants close to the inner channel mouth (I1471 and V1477[42]) and the inactivation determinant in position "-1" (I1478[43]) from the inner end of the helix are illustrated as white circles. B) Mutating the putative PAA- (I1470A) and DHP- (I1471A) determinants slows channel inactivation. C) Normalised superimposed barium currents of $Ca_v1.2$ and mutant V1477A. Substitution V1477A strongly reduces voltage-dependent inactivation and use-dependent block by d-cis-diltiazem during a train of 15 pulses (-80 mV to +10 mV, 100 ms) applied at 1 Hz (traces in diltiazem are unpublished data from Berjukow et al). Data reproduced from references 34,42 with permission.

On the other hand, insertion of a fourth $Ca_v1.2$ residue (M1805) into the corresponding position of AL25 further enhanced inactivation and sensitivity for (-)gallopamil and DIL (Fig. 3B, bottom left). These data confirmed a close relation between the rate of intrinsic inactivation and the apparent sensitivity for $Ca^{2+}$ antagonists. We hypothesise that the augmentation of the use-dependent block at least for some of the mutants is mediated by enhanced inactivation rather than by transfer of specific binding determinants.

Interestingly, mutation Y1804I decreased DIL- (and PAA-) sensitivity without significantly slowing the rate of channel inactivation (Fig. 3B bottom right). Y1804 is, therefore, more likely to contribute drug-binding energy than the other IVS6 residues that could equally modulate drug sensitivity in an indirect manner by affecting inactivation. A possible key role of Y1804 in drug binding is also supported by data from Degtiar et al who demonstrated low PAA-sensitivity of a chimeric construct (AL30) inactivating even faster than AL25 but lacking Y1804.[27]

It was therefore interesting to study if the putative drug binding determinants in the drug sensitive wild type $Ca_v1.2$ would affect inactivation. A first analysis of the impact of IVS6 amino acids in equivalent positions (Fig. 4) revealed that substitution of I1470 (corresponding to the $Ca_v2.1$ M1811, (Fig. 3A) and the adjacent DHP-determinant I1471 by alanine both substantially slow channel inactivation (Fig. 4A,B).

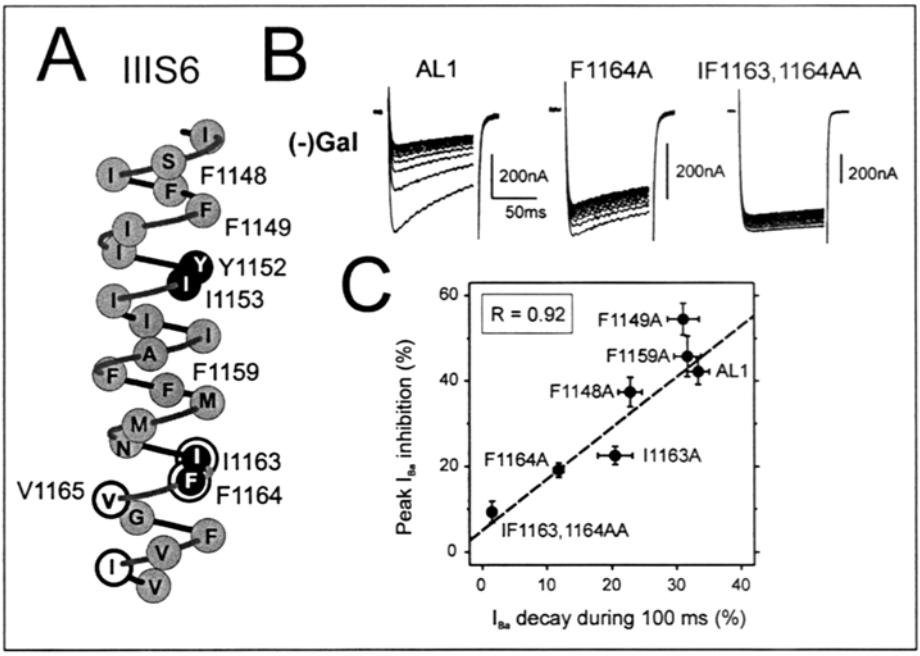



Figure 5. Point mutations in $Ca_v1.2$ segment IIIS6 reveal correlation between inactivation and PAA-sensitivity. A) α-Helical representation of $Ca_v1.2$ segment IIIS6 forming part of the highly PAA sensitive $Ca_v1.1/Ca_v2.1$ chimera AL1.[24] IIIS6 amino acid residues contributing to PAA- and DIL-sensitivity[5,40] (Fig. 1D) are shown in black. Inactivation determinants close to the inner channel mouth (I1163, F1164 and V1165 corresponding to IFV-inactivation motif[39]) are highlighted in open circles. B) PAA-sensitivity of chimera AL1 and mutants F1164A and IF1163,1164AA (inactivating at a slower rate) were estimated as use-dependent channel inhibition by 100 μM (-)gallopamil (10 100 ms-pulses from -80 mV to 10 mV at 0.1 Hz). The most rapidly inactivating construct AL1 displays the highest apparent PAA-sensitivity. I1163A and to a greater extend double mutation IF1163,1164AA gradually slow the rate of channel inactivation and channel inhibition by (-)gallopamil (see ref. 41 for a similar role of V1165 in DIL-sensitivity). C) A correlation (R=0.92) between the rate of channel inactivation (current decay during 100 ms) and drug-sensitivity (use-dependent channel block) of various AL1 mutants. Data reproduced from reference 39 with permission.

## Drug-Sensitivity Is Affected by Inactivation Determinants Located Outside the Putative Drug-Binding Region

A similar correlation between channel inactivation and apparent sensitivity for $Ca^{2+}$ antagonists was observed for amino acids substitutions outside the putative drug-binding region. As illustrated in Figure 4C, substitution of valine (V1478) in the lower part of segment IVS6 of $Ca_v1.2$ by alanine significantly reduced both inactivation and diltiazem sensitivity. A systematic analysis of amino acids in segment IIIS6 of a $Ca_v1.1/Ca_v2.1$ chimera revealed a strong correlation between the two parameters (Fig. 5C).[28] Substitution of two residues in segment IIIS6 localised close to the inner mouth of the channel pore by alanine (I1163A and F1164A, Fig. 5) and to an even greater extent the corresponding double mutation (IF1163,1164AA) gradually reduced the rate of current inactivation and simultaneously diminished use-dependent block by (-)gallopamil (Fig. 5B).

By investigating the effect of a single amino acid substitution in the I-II linker (R387E in $Ca_v2.1$) Sokolov et al[29] observed a similar trend: the slower the channel inactivated the lower was the apparent sensitivity for (-)gallopamil. In addition, Jimenez et al[30] reported that ultraslow

inactivation generated by a single valine insertion in to the I-II linker of the $Ca_v1.2$ $\alpha_1$ subunit strongly reduced use-dependent channel block by MIB. Together these data illustrate that $Ca^{2+}$ channel block is modulated by inactivation determinants that are located inside as well as outside of the putative drug-binding region.

## β-Subunits Modulate Inactivation and Channel Inhibition

It is well established that the β-subunit composition of HVA $Ca_v$ channels affects their inactivation properties.[6] According to a widely accepted hypothesis many $Ca^{2+}$ antagonists bind predominately (with high affinity) to the inactivated channel state.[20,21] Since β-subunits either enhance or reduce $Ca_v$ channel inactivation, they have emerged as important tools for the analysis of state dependent drug action. Coexpression of the $\alpha_1$ subunit with 'accelerating' β-subunits ($\beta_1$- or $\beta_3$-) was, therefore, expected to enhance channel block.

In line with this hypothesis Lacinova et al[31] reported an almost 14-fold higher apparent sensitivity of $Ca_v1.2$ for the PAA verapamil if the $Ca_v1.2$ $\alpha_1$-subunit was coexpressed with a $\beta_3$-subunit ($Ca_v1.2(\beta_3)$).

(-)Gallopamil inhibited $Ca_v1.2(\beta_3)$ 3.4-fold stronger than $Ca_v1.2$ $\alpha_1$ subunits expressed without β-subunits.[31] Sokolov et al[32] studied the underlying molecular mechanism and compared PAA-sensitivity of $Ca_v1.2$ composed of either an 'accelerating' $\beta_3$-subunits or a 'decelerating' $\beta_{2a}$. These studies revealed a relation between the rate of β-mediated inactivation and PAA-sensitivity (Fig. 6A-C).

Jimenez et al[30] demonstrated that inhibition of $Ca_v2.1$, $Ca_v2.3$ and $Ca_v1.2$ by MIB is significantly stronger if the corresponding $\alpha_1$-subunits are coexpressed with an 'accelerating' $\beta_{1b}$ rather than with a 'decelerating' $\beta_{2a}$-subunit. The concentration of drug required to block $Ca_v2.1$ and $Ca_v2.3$ composed of $\beta_{2a}$ was approximately 10-fold higher (Fig. 6D, left, middle). A smaller difference was observed for $Ca_v1.2$ (Fig. 6D, right). Further evidence for a role of the β-subunit interaction in $Ca^{2+}$ channel block comes from Zamponi et al[33] who reported that coexpression of the $Ca_v2.3$ $\alpha_1$ subunit with $\beta_{2a}$ impaired channel block by piperidines compared to $Ca_v2.3$ coexpressed with the 'accelerating' $\beta_{1b}$. In summary, these data suggest that a β-subunit-mediated increase or reduction in voltage-dependent inactivation modulates $Ca_v$ channel inhibition by PAAs, MIB and piperidines.

## Inactivation Determinants and DHP Sensitivity

Evidence for a key role of inactivation in channel inhibition by DHPs came from the drug-induced shift of $Ca_v1.2$ availability to hyperpolarized voltages in myocardial and smooth muscle cells. This is conventionally explained as high affinity binding of drugs such as nifedipine or isradipine to the inactivated channel conformation.[20,21] $Ca_v1.2$ composed of 'accelerating' $\beta_{1a}$- ($Ca_v1.2(\beta_{1a})$) or $\beta_3$-subunits ($Ca_v1.2(\beta_3)$) were, therefore, expected to enhance DHP-binding and channel block.

However, Lacinova et al[31] reported almost identical sensitivities of $Ca_v1.2(\beta_3)$ and $Ca_v1.2$ channels lacking the β subunit for (+)isradipine, despite the considerable differences in inactivation.

Berjukow et al[34] came to a similar conclusion by comparing the DHP sensitivities of $Ca_v$ channels containing either $\beta_{1a}$ or $\beta_{2a}$ subunits (Fig. 7). A DHP-sensitive $Ca_v2.1$ mutant composed of the 'accelerating' $\beta_{1a}$ ($\alpha_{1A\text{-}DHP}/\beta_{1a}$) displayed at –80 mV a 3-fold higher steady-state fraction of channels in an inactivated state compared to slowly inactivating $\alpha_{1A\text{-}DHP}/\beta_{2a}$. This larger fraction of hypothetically high affinity drug receptors did, however, not promote additional channel block by (+)isradipine (Fig. 7A). Analogous observations were made for $Ca_v1.2$, the prime target of DHP in vascular smooth muscle cells (Fig. 7).[34]

There is, nevertheless, evidence for an important role of the inactivation machinery in channel block by DHPs. The more rapidly inactivating mutant $\alpha_{1A\text{-}DHP}$ displays a higher sensitivity for (+)isradipine than the slower inactivating wild-type $Ca_v1.2$, irrespective whether an 'accelerating' $\beta_{1a}$ or a 'decelerating' $\beta_{2a}$-subunit were coexpressed (Fig. 7A). It is, therefore, tempting to

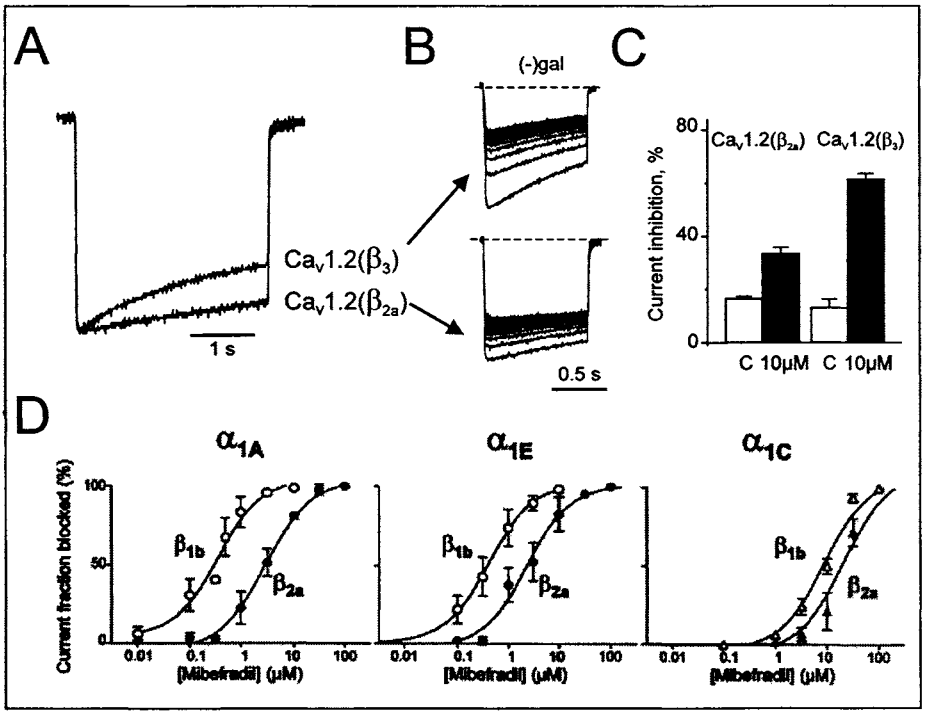



Figure 6. Auxiliary β-subunits modulate inactivation and apparent sensitivity for gallopamil and mibefradil. A) Representative currents through $Ca_v1.2$ composed of either $\beta_3$ ($Ca_v1.2(\beta_3)$) or $\beta_{2a}$-subunits ($Ca_v1.2(\beta_3)$). Barium currents were elicited by a 3-second depolarizations from –80 mV to +10mV. B) Use-dependent current inhibition of $Ca_v1.2(\beta_3)$ and $Ca_v1.2(\beta_{2a})$ by 10μM (-)gallopamil during a train of 10 1s-pulses (–80 mV to +10 mV) applied at 0.5 Hz. C) Apparent (-)gallopamil-sensitivities estimated as a peak current inhibition during the train in %. White bars represent the accumulation of channels in intrinsic inactivation (control, C), black bars show the current inhibition by 10 μM (-)gallopamil. Data reproduced with permission from ref.[32] D) $Ca_v2.1$, $Ca_v2.3$ and $Ca_v1.2$ inhibition by mibefradil is dependent on the β-subunit composition (reproduced from ref. 30 with permission). The 'accelerating' $\beta_{1b}$-subunit enhances whereas the 'decelerating' $\beta_{2a}$-subunit reduces channel inhibition.

speculate that the higher DHP-sensitivity of $\alpha_{1A\text{-}DHP}$ is related to their faster inactivation compared to $Ca_v1.2$ (Fig. 7B,C).

Furthermore, Berjukow et al[34] demonstrated that substitution of the putative DHP-binding determinant I1471 in segment IVS6 (Fig. 1D) by alanine slowed the rate of fast voltage-dependent inactivation (Fig. 8A,B) and additionally accelerated recovery of drug modified channels from inactivation. It appears that mutation I1471A destabilises the intrinsic fast inactivated state and the DHP-induced state.

A detailed study of kinetics of entry into and recovery from inactivation and DHP-inhibition of various $Ca_v1.2$ and $Ca_v2.1$ mutants led us to a hypothesis that these drugs promote fast voltage-dependent inactivation without binding selectively to this state. This idea was further supported by (+)-isradipine action on $Ca_v1.2$ mutant V1477A that is almost completely lacking fast voltage-dependent inactivation. (+)-Isradipine accelerated the current decay in a voltage-dependent manner comparable to voltage dependence of inactivation of wild type $Ca_v1.2$ (Fig. 8C). The effect of (+)-isradipine on this channel can, therefore, be interpreted as a restoration of fast voltage-dependent inactivation.[35]

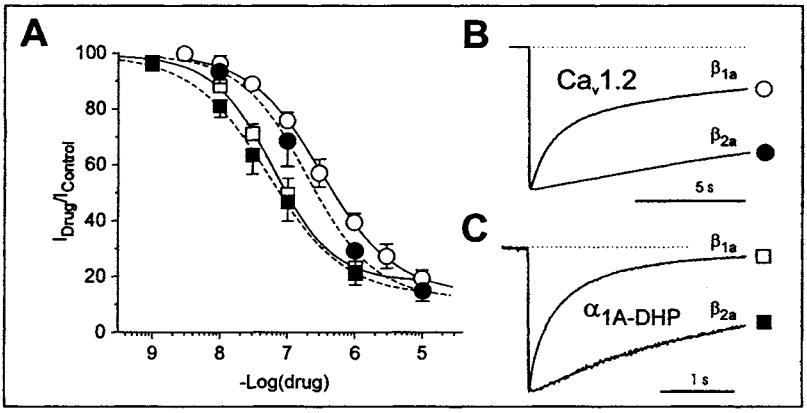



Figure 7. The rapidly inactivating $Ca_v2.1$ mutant $\alpha_{1A\text{-}DHP}$ displays a higher DHP-sensitivity than wild-type $Ca_v1.2$. A) Concentration-response relationships of peak barium current inhibition of $\alpha_{1A\text{-}DHP}(\beta_{1a})$ (open squares), $\alpha_{1A\text{-}DHP}(\beta_{2a})$ (filled squares), $Ca_v1.2(\beta_{1a})$ (open circles) and $Ca_v1.2(\beta_{2a})$ (filled circles) by (+)-isradipine ($IC_{50}(\alpha_{1A\text{-}DHP}/\beta_{1a})$ = 61 nM, $IC_{50}(\alpha_{1A\text{-}DHP}/\beta_{2a})$ = 52 nM *vs.* $IC_{50}(Ca_v1.2(\beta_{1a}))$ = 327 nM and $IC_{50}(Ca_v1.2(\beta_{2a}))$ = 198 nM). B) Representative scaled currents of $Ca_v1.2$ and $\alpha_{1A\text{-}DHP}$ (C) of the indicated subunit composition (holding potential –80 mV, test pulse to 20 mV). Data are reproduced from reference 34 with permission.

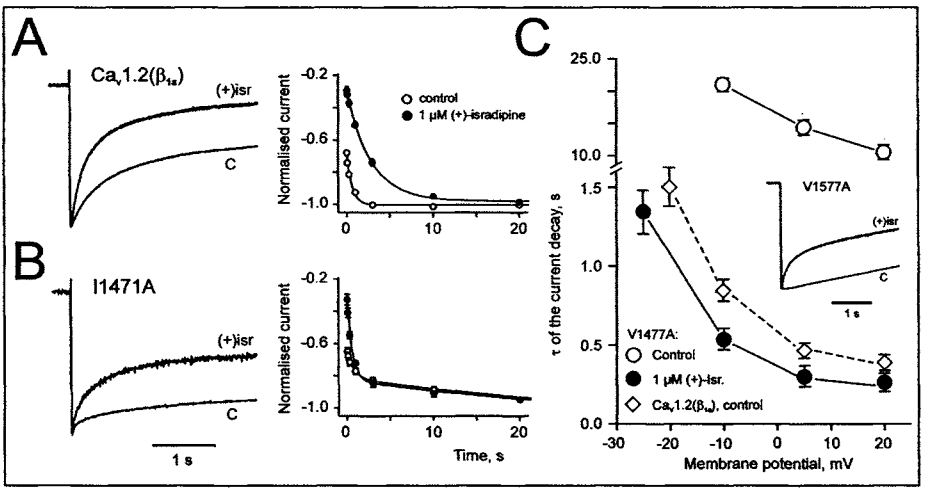



Figure 8. Onset of DHP-induced inactivation is voltage-dependent and recovery is modulated by inactivation determinants. A) (+)Isradipine (1μM) accelerates barium current decay through L-type construct $Ca_v1.2(\beta_{1a})$ (traces on the left) and slows recovery from inactivation/block (right panel) about 4,5-fold ($\tau_{recovery,control}$ = 0.63 s vs. $\tau_{recovery,\ isradipine}$ = 2.85 s). B) Amino acid residue I1471 is believed to form part of the DHP receptor. Substitution I1471A slows inactivation (Fig. 4B). Traces in the left panel illustrate barium currents through I1471A in control and the presence of (+)isradipine (1 μM). The right panel illustrates faster recovery of mutant I1471A from inactivation at rest compared to $Ca_v1.2(\beta_{1a})$ (compare with A). Recovery of I1471A in drug was faster than in $Ca_v1.2$ and not significantly different from control ($\tau_{recovery,control}$ =0.50 s, $\tau_{recovery,\ isradipine}$ = 0.46 s compare to A). Data from reference 34 with permission. C) Voltage-dependent acceleration of the barium current decay of slowly inactivating mutant V1477A by 1 μM (+)isradipine (inset). Voltage-dependencies of the time constant of fast $Ca_v1.2(\beta_{1a})$ inactivation and the rate of (+)isradipine-induced current decay are illustrated. The time constant of current decay of V1477A in the absence of drug (open circles) ranged between 10 and 25 seconds. Data reproduced from reference 35 with permission.

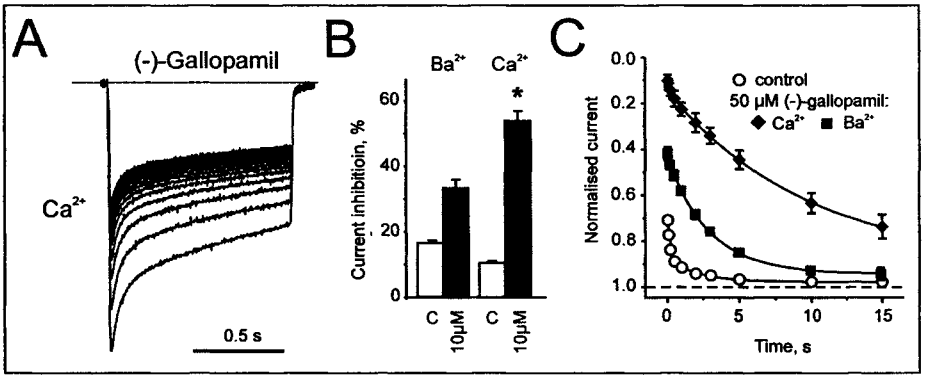



Figure 9. On the role of $Ca^{2+}$ dependent inactivation in (-)gallopapil action. A) Normalised $Ca^{2+}$ currents through $Ca_v1.2(\beta_{2a})$ elicited by a 0.5 Hz train of 10 pulses (-80 mV to 0 mV) in the presence of 10 μM (-)gallopamil. B) Use-dependent peak current inhibition with $Ca^{2+}$ or $Ba^{2+}$ as charge carriers (black bars) compared to peak current inhibition in absence of drug (white bars). Note the stronger use-dependent block in $Ca^{2+}$. C) (-)Gallopamil slows $Ca_v1.2(\beta_{2a})$ recovery kinetics. Barium current recovery in drug is significantly faster than recovery of calcium currents (measured after 3s conditioning pulses to +10mV ($Ba^{2+}$) or +20mV ($Ca^{2+}$) ($Ba^{2+}$: $\tau_{Control}$=1.7±0.2 s (not shown) vs. $\tau_{gallopamil}$=2.8±0.3 s; $Ca^{2+}$: $\tau_{Control}$= 2.3±0.2 s vs. $\tau_{gallopamil}$= 15±3 s, reproduced with permission from ref[32]). Note that $Ca^{2+}$ currents through $Ca_v1.2$ are more efficiently blocked by (-)gallopamil than $Ba^{2+}$ currents.

## On the Role of $Ca^{2+}$-Dependent Inactivation in Drug Sensitivity

$Ca^{2+}$-dependent inactivation causes an additional rapid component in current decay through $Ca_v1.2$ channels (Figs. 1B and 9A). Sokolov et al[32] reported that accelerated inactivation with $Ca^{2+}$ as charge carrier (compared to $Ba^{2+}$) is accompanied by enhanced use-dependent block by (-)gallopamil (Fig. 9B). This effect was apparently caused by a slower recovery from channel block in the presence of $Ca^{2+}$ (Fig. 9C). Its molecular basis has yet to be elucidated.

## Simulation of the Drug-Channel Interaction

Different versions of the "modulated receptor paradigm"[36] explain the cumulative $Ca^{2+}$ channel block by PAA, DIL and MIB during a train of test pulses ("use-dependent block", Fig. 10) by selective drug binding to either open or inactivated channels.[20,21,37]

Such a scenario would imply that during a single pulse the drug "has not enough time" to bind to all channels. Thus, due to the slow drug binding only a small fraction of channels would be blocked. Upon repolarization the channels recover to the low affinity resting state where the drug dissociates from its receptor site (Fig. 10). Drug unbinding is, however, slow as well and a certain fraction of channels will remain blocked until the next pulse is applied leading finally to cumulative (use-dependent) channel inhibition.

Alternatively, drug binding could occur independently of the channel state. In such a scenario that we call "drug inactivation synergism,"[38] $Ca^{2+}$ antagonists accelerate channel transitions into the fast inactivated state like 'accelerating' β-subunits or point mutations.

Figure 10 illustrates the two competing models. Both of them satisfactory reproduce the experimental data. In the "drug inactivation synergism" model the effect of (-)gallopamil on $Ca_v1.2(\beta_3)$ and $Ca_v1.2(\beta_{2a})$ is simulated by an about 7-fold increase in the on-rate of fast voltage-dependent inactivation (rate α(PAA)) and an 8-fold decrease in recovery from drug-induced inactivation (rate β(PAA)). The difference in use-dependent inhibition of the two β-subunit compositions arise, therefore, solely due to the differences in intrinsic inactivation in $Ca_v1.2(\beta_3)$ and $Ca_v1.2(\beta_{2a})$. I.e., the "drug-inactivation synergism" model reproduces the differences in apparent drug sensitivity without the necessity to assume different drug affinities for $Ca_v1.2(\beta_3)$ and $Ca_v1.2(\beta_{2a})$.

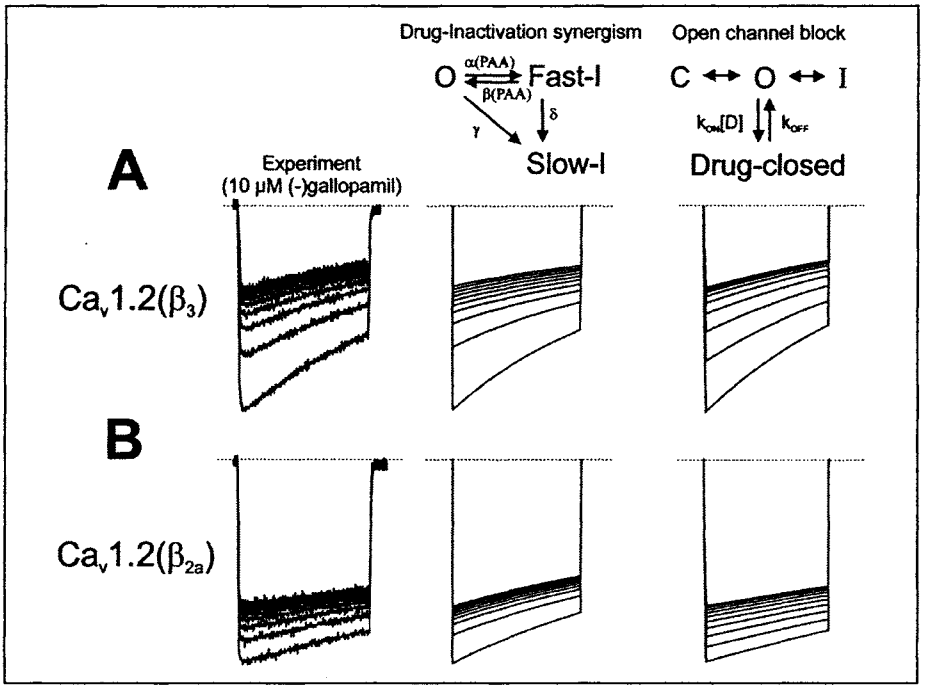



Figure 10. Simulation of use-dependent inhibition of $Ca_v1.2$ by (-)gallopamil. A, B) Use-dependent $Ca^{2+}$ channel block of $Ca_v1.2(\beta_3)$ (A, left panel) and $Ca_v1.2(\beta_{2a})$ (B, left panel) by 10μM (-)gallopamil (same experiments as in Fig. 6B). The smooth traces are simulations of the experimental data. The kinetic models of the respective PAA-channel interaction are shown above the simulated current traces. The "open channel block" model assumes high affinity drug binding to the open state (O) ($k_{on}(Ca_v(\beta_3))$ = $5*10^4$ $M^{-1}s^{-1}$; $k_{on}(Ca_v(\beta_{2a}))$ = $1.5*10^4$ $M^{-1}s^{-1}$). The "Drug-Inactivation-Synergism" model assumes that the drug promotes transitions to the (modified) fast inactivated state (rate α: $\alpha(control)\beta_3 = 0.13\ s^{-1}$, $\alpha(PAA)\beta_3 = 0.85\ s^{-1}$; $\alpha(control)\beta_{2a} = 0.07\ s^{-1}$, $\alpha(PAA)\beta_{2a} = 0.5\ s^{-1}$) and slow recovery from this state (rate β: $\beta(control)\beta_3 = 0.25\ s^{-1}$, $\beta(PAA)\beta_3 = 0.03\ s^{-1}$; $\beta(control)\beta_{2a} = 0.4\ s^{-1}$, $\beta(PAA)\beta_{2a} = 0.05\ s^{-1}$). Both models satisfactory reproduce use-dependent block during the 0.5 Hz train of 1s pulses (from ref. 32 with permission, see ref. 44 for simulation of β-subunit effects on inactivation).

In the "open channel block" model we had to presume a higher affinity of (-)gallopamil for rapidly inactivating $Ca_v1.2(\beta_3)$ compared to $Ca_v1.2(\beta_{2a})$ ($k_{on}[D](Ca_v(\beta_3))$ = $0.5\ s^{-1}$ *vs.* $k_{on}[D](Ca_v(\beta_{2a}))$ = $0.15\ s^{-1}$). This model reproduces the data only if we postulate a direct effect of the β-subunit on the drug binding process. Moreover, if we hypothesise equal affinities this model predicts enhanced inhibition of slowly inactivating (open) $Ca_v$ channels and not—as observed in most experiments—stronger block of faster inactivating channels (Figs. 2-7).

## Summary and Outlook

Point mutations in different parts of the $Ca_v$ $\alpha_1$-subunits that either enhance or impair channel inactivation simultaneously increase or reduce channel inhibition by PAA, DIL, MIB and to a certain extent by DHPs. Fast inactivating channels tend to be more efficiently inhibited than slow-inactivating ones. This simple correlation has been confirmed by independent groups for wild type, chimeric or mutant HVA $Ca_v$.[25-33] A similar conclusion can be drawn the drug action on $Ca_v$ channels containing either 'accelerating' (e.g., $\beta_1$ or $\beta_3$) or 'decelerating' ($\beta_{2a}$) β-subunits.[30,32] Subunit compositions promoting faster channel inactivation increase the apparent drug sensitivity.

A possible scenario of a "drug inactivation synergism" is illustrated in (Fig. 10) This model does not exclude state-dependent drug access for partially charged compounds. However, once these drugs dock within their binding pockets they are more likely to promote inactivation than to plug the open channel pore (Fig. 10). Some amino acids (e.g., I1470, I1471 in segment IVS6 and the IFV motif close to the inner channel mouth in segment IIIS6 of $Ca_v1.2$ as well as M1464 and A1467 in $Ca_v2.1$ environment (Fig. 1D)) are strong inactivation determinants.

It is currently not entirely clear which of the determinants of drug sensitivity in segments IIIS5, IIIS6 and IVS6 form part of the drug binding pocket and which of them affect the drug-channel interaction in an indirect manner via inactivation (Fig. 1D). In functional studies the effects of mutations on PAA-, DIL- and MIB-sensitivity is indistinguishable from mutations localised outside the putative drug-binding region. The respective impact of IIIS5, IIIS6 and IVS6 amino acids in inactivation and drug binding has yet to be thoroughly elucidated.

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# CHAPTER 19

# Block of Voltage-Gated Calcium Channels by Peptide Toxins

**Christina I. Schroeder, Richard J. Lewis and David J. Adams**

## Introduction

Structurally, the calcium channels are closely related to sodium channels, with the main structurally significant difference being the positioning and nature of the residues that line the selectivity filter in the pore of the channel. There are at least six pharmacologically distinct calcium channels types, including L-, N-, P/Q-, T, and R-type calcium channels (Table 1).[1] Within each group are multiple subtypes that are presently less easy to distinguish pharmacologically. In the nervous system, several types of ion channels may contribute to processes such as neurotransmitter release, with the ratio and role for each type varying among different nervous tissues[2] (see Table 1). A number of different peptide toxins from marine snails (conotoxins) and spiders (agatoxins, grammotoxin and DW13.3 *Filistata hibernalis*) are known to potently inhibit the activities of specific voltage-dependent calcium channels.[3,4] The conotoxins are usually smaller in size comprising around 22–30 amino acid residues and are believed to act by physically blocking the pore of the channel.[5] Spider toxins such as ω-Aga IVA[6] typically larger consisting of 60–90 residues and ω-grammotoxin[7] around 30–40 residues affect channel gating.[8]

The diversity of peptide toxins provides the possibility for selective modulation of nerve function that may allow the selective treatment of diseases such as pain and stroke. The first examples of calcium channel inhibitors being useful therapeutically are the ω-conotoxins MVIIA (Ziconotide or Prialt, Elan) and ω-CVID (AM336, AMRAD) which target the N-type calcium channel and are currently in clinical trials for chronic pain. ω-Conotoxins are a large family of structurally related peptides with a wide range of specificities for different subtypes of the VSCC (Table 2).[9-18] Their high selectivity has made them enormously valuable as physiological tools. A remarkable feature of the six cysteine/four-loop framework exemplified by the ω-conotoxins is the presence of a cystine knot within the structures. This motif consists of an embedded loop in the structure formed by two of the disulfide bonds and their connecting backbone segments. This loop is penetrated by the third disulfide bond in a remarkable example of nature's engineering designs. Although the structure of the core of ω-conotoxins is rigid due to the knotted disulfide structure, NMR studies have revealed conformational flexibility in the exposed loops that might influence receptor binding. This review will describe structural aspects of peptide toxins from cone snails as well as those from snakes and spiders that selectively inhibit different voltage-sensitive calcium channels (VSCCs). Several excellent general reviews describing peptide toxin interactions with voltage-gated ion channels including calcium channels have been published recently.[19-21]

*Voltage-Gated Calcium Channels*, edited by Gerald Zamponi.
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***Table 1. Subunit composition and function of $Ca^{2+}$ channel types[1]***

| $Ca^{2+}$ Channel | $Ca^{2+}$ Current Type | Primary Localisation | Previous Name of $\alpha_1$ Subunit | Specific Blocker | Functions |
|---|---|---|---|---|---|
| $Ca_v1.1$ | L | Skeletal muscle | $\alpha_{1S}$ | DHPs | Excitation-concentration coupling, Calcium homeostasis |
| $Ca_v1.2$ | L | Cardiac muscle Endocrine cells Neurons | $\alpha_{1C}$ | DHPs | Excitation-concentration coupling, Hormone secretion |
| $Ca_v1.3$ | L | Endocrine cells Neurons | $\alpha_{1D}$ | DHPs | Hormone secretion, Gene regulation |
| $Ca_v1.4$ | L | Retina | $\alpha_{1F}$ | | Tonic neurotransmitter release |
| $Ca_v2.1$ | P/Q | Nerve terminals Dendrites | $\alpha_{1A}$ | ω-Agatoxin | Neurotransmitter release Dendritic $Ca^{2+}$ transients |
| $Ca_v2.2$ | N | Nerve terminals Dendrites | $\alpha_{1B}$ | ω-GVIA | Neurotransmitter release Dendritic $Ca^{2+}$ transients |
| $Ca_v2.3$ | R | Cell bodies Dendrites Nerve terminals | $\alpha_{1E}$ | SNX-482 | $Ca^{2+}$-dependent action potentials |
| $Ca_v3.1$ | T | Cardiac muscle Skeletal muscle Neurons | $\alpha_{1G}$ | None | Repetitive firing |
| $Ca_v3.2$ | T | Cardiac muscle Neurons | $\alpha_{1H}$ | None | Repetitive firing |
| $Ca_v3.3$ | T | Neurons | $\alpha_{1I}$ | None | Repetitive firing |

## L-Type VSCC Antagonists

Toxins from several species of snails and snakes block the L-type VSCC. For example, ω-conotoxin TxVII from the mollusc hunting *Conus textile* targets dihydropyridine-sensitive L-type calcium channels.[17] TxVII is similar to other ω-conotoxins, including GVIA, MVIIA and CVID, having 26 residues including six Cys which form three disulphide bonds (Table 2). Thus TxVII shares a similar four-loop framework with the other ω-conotoxins (Fig. 1). However, TxVII is negatively charged and has a large hydrophobic patch unlike other ω-conotoxins that target N-and P/Q-type VSCCs which are highly positively charged (+5 to +6), These positive charges contribute to N-type VSCC binding, with Lys2 being the most critical charge[22-24] The conserved Lys2 is the only positively charged residue in TxVII and there are several negative charges in the proximity of Lys2. It is also missing a Tyr in position 13, which has been shown by several groups to be crucial for activity at the N-type VSCC.[22,25,26] Instead TxVII has a hydrophobic Leu13. The hydrophobic patch in TxVII, which includes Phe11 and Trp26, is not found in other ω-conotoxins and could be part of the face that interacts with the L-type VSCC. These differences presumably act in concert to explain why TxVII does not bind to the N-type VSCC, despite having the consensus structure of ω-conotoxins. Interestingly, TxVII reversibly inhibits snail but not rat L-type currents,[27] indicating that it has evolved to selectively inhibit calcium channels found in its prey. The sequence of TxVII resembles δ-conotoxin TxVIA, an inhibitor of sodium channel inactivation and despite the fact that they act on different ion channels, they share the common

***Table 2. Sequences of native ω-Conotoxins from different Conus species***

| Toxin | Sequence | Target | Net Charge | Ref. |
|---|---|---|---|---|
| ***C.geographus*** | | | | |
| GVIA | CKSOGSSCSOTSYNCCR-SCNOYTKRCY* | N-type VDCC | +5 | 9 |
| GVIB | CKSOGSSCSOTSYNCCR-SCNOYTKRCYG* | ? | +5 | 10 |
| GVIC | CKSOGSSCSOTSYNCCR-SCNOYTKRC* | ? | +5 | 10 |
| GVIIA | CKSOGTOCSRGMRDCCT-SCLLYSNKCRRY* | ? | +7 | 11 |
| GVIIB | CKSOGTOCSRGMRDCCT-SCLSYSNKCRRY* | ? | +7 | 11 |
| ***C.magus*** | | | | |
| MVIIA | CKGKGAKCSRLMYDCCTGSC-R-SGKC* | N-type VDCC | +6 | 11 |
| MVIIC | CKGKGAPCRKTMYDCCSG-SCGR-RGKC* | P/Q-type VDCC | +7 | 12 |
| ***C.striatus*** | | | | |
| SVIA | CRSSGSOCGVTSI-CC-GRC-YRGKCT* | not known | +5 | 13 |
| SVIB | CKLKGQSCRKTSYDCCSG-SCGR-SGKC* | N- and P/Q-type | +6 | 13 |
| ***C.tulipa*** | | | | |
| TVIA | CLSOGSSCSOTSYNCCR-SCNOYSRKCR* | ? | +4 | 14 |
| ***C.consors*** | | | | |
| CnVIIA | CKGKGAOCTRLMYDCCHG-SCSSSKGRC* | N-type VDCC | +5 | 15 |
| ***C.catus*** | | | | |
| CVIA | CKSTGASCRRTSYDCCTG-SCR--SGRC* | N-type VDCC | +5 | 16 |
| CVIB | CKGKGASCRKTMYDCCR-GSCR--SGRC* | N- and P/Q-type | +7 | 16 |
| CVIC | CKGKGQSCSKLMYDCCTG-SCSR-RGKC* | N- and P/Q-type | +6 | 16 |
| CVID | CKGKGAKCSKLMYDCCSGSCSGTVGRC* | N-type VDCC | +5 | 16 |
| ***C.textile*** | | | | |
| TxVII | CKQADEPCDVFSLDCCTGIC--LGV-CMW | L-type VDCC | -3 | 17 |
| ***C.radiatus*** | | | | |
| RVIA | CKPOGSOCRVSSYNCCS-SCKSYNKKCR* | N-type | +6 | 18 |

O = Hydroxyproline; * = C-terminal amidated

Cys four loop framework (Fig. 2).[28,29] Conotoxins with this conserved cysteine framework highlight the cysteine knots as an excellent structural scaffold from which to present different side chains to achieve diverse biological activities.[27]

Recently a conotoxin member of the Contryphan family, glacontryphan-M from *C. marmoreus* was reported to be the first contryphan peptide containing two γ-carboxylatedglutamic to be a selective L-type calcium channel blocker.[30] Interestingly the NMR solution structure of glacontryphan-M superimposes with the backbone of Contryphan-R[31] and Contryphan-Vn.[32,33] Similar to Contryphan-Vn, which was recently reported to be a $Ca^{2+}$-dependent $K^+$ channel inhibitor, glacontryphan-M requires calcium binding to the N-terminal of the peptide to be active at the L-type calcium channel. The binding of calcium to the peptide induces a structural perturbation in the Gla-containing part of the peptide believed to be important for the activity of the peptide.

The venoms from the snakes of the families *Elapidae* and *Hydrophidae* contain a variety of toxins with distinct pharmacologies. A large portion of these toxins are either neurotoxins[34] that act postsynaptically to block the nicotinic acetylcholine receptor causing flaccid paralysis of the muscle,[34] or cytotoxins[35] which appear to change membrane permeability and/or

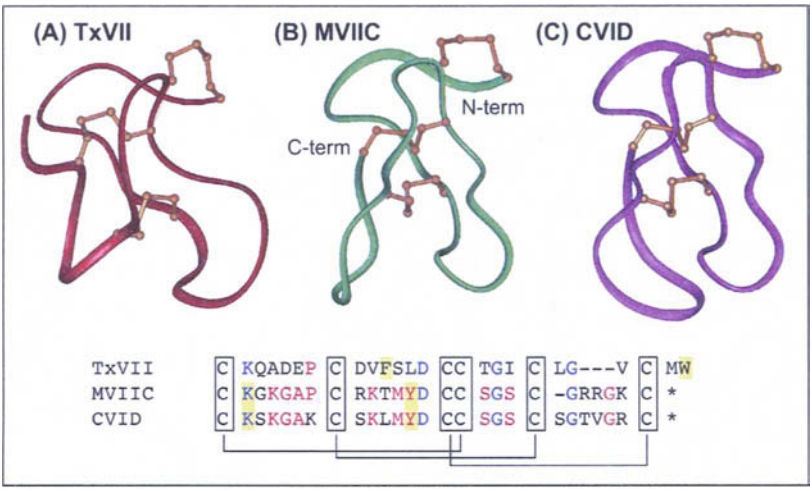



Figure 1. Three different ω-conotoxins that inhibit different voltage-sensitive calcium channels. Ribbon diagram and sequnence alignments for A) L-type VSCC inhibitor TxVII from *C. textile*, B) P/Q-type VSCC inhibitor MVIIC from *C. magus* and C) N-type VSCC blocker CVID from *C. catus*. superimposed across the backbone heavy atoms residue 1–12. The three disulfide bonds are in yellow. The cysteine knot disulfide bond connectivity is indicated in the sequence alignment of the three peptides. Yellow boxes indicate residues identified as important for binding to their VSCC target. Sequence homology across two peptides (red) and three peptides (blue) are indicated. O= hydroxyloproline; *= amidated C-terminal.

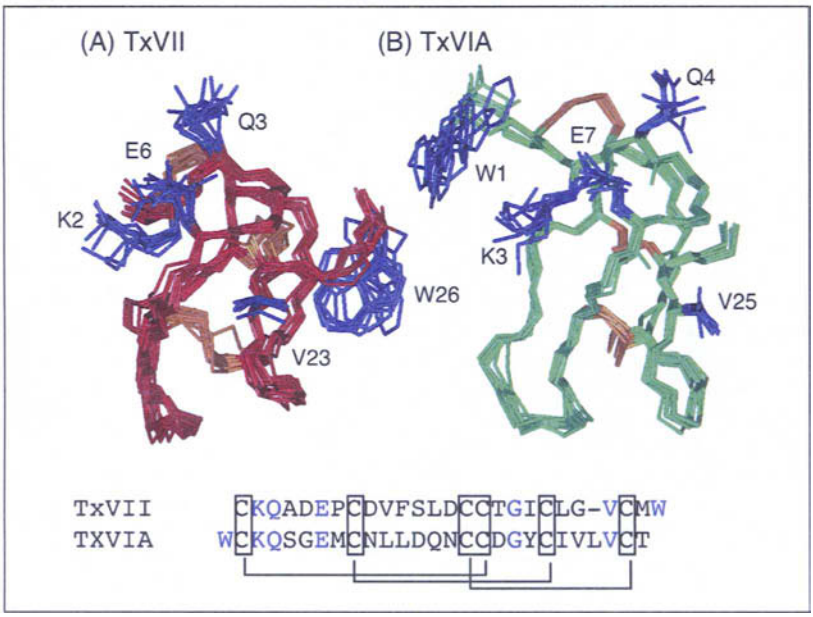



Figure 2. Superimposition of solution structures of A) ω-conotoxin TxVII, a L-type calcium channel inhibitor from *Conus textile* and B) δ-conotoxin TxVIA, a sodium channel inhibitor. Disulfides are indicated in orange and corresponding residues are indicated in blue. The cysteine framework is boxed in the sequence alignment and sequence homology across the two peptides is shown in blue.

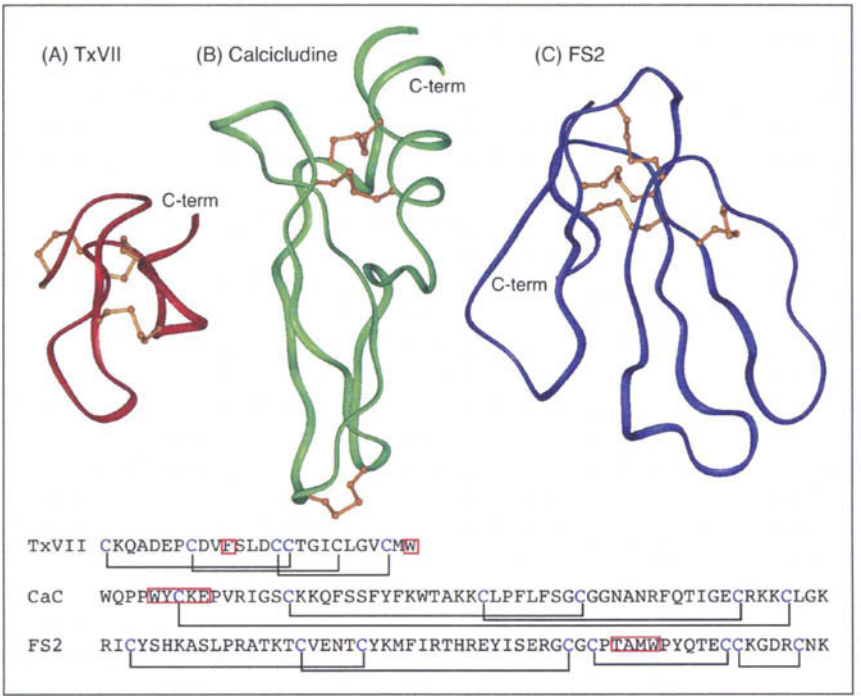



Figure 3. Three different L-type calcium channel blockers from different venomous species. A) TxVII, a 26 residue peptide from the cone snail *C. textile*; B) Calcicludine (CaC), a 60 residue peptide from the green mamba snake *Dendroaspis augusticeps* and C) FS2, a 60 residue peptide isolated from the venom of black mamba snake *Dendroaspis poluepis polyepis*. Stabilising disulfide bonds shown in yellow. Sequence alignments and disulfide bond connectivity are indicated.

membrane organisation. These two very distinct pharmacological classes share a similar structural motif, namely the three-fingered architecture, where four conserved cysteine pairs are organised as a "disulfide β-cross".[36,37] A third class of postsynaptically active toxins have been isolated from the venom of the mamba snakes (*Dendroaspis* spp). Even though they also have a three-fingered architecture, they show no neurotoxic or cytotoxic activity. Their homology and immunological properties have led to further subdivision into four subgroups[32,39] of which two have been pharmacologically characterised. Toxins of subgroup I are potent inhibitors of acetylcholine esterases,[40] whereas subgroup III contains toxins such as FS2 from *Dendroaspis polyepis polyepis*,[38] C10S2C2 from *D. augusticepceps* (green mamba)[39] and calciseptine from the venom of *D. p. polyepis*. Calciseptine and FS2 differs only in two residue and have similar blocking effect at the L-type VSCC[41] (Fig. 3).

Calciseptine, a 60 residue peptide, has been found to be an antagonist at heart L-type calcium channels, but acts as an agonist at frog and mammalian skeletal muscle L-type calcium currents.[42] Like ω-agatoxin IIIA from the spider venom,[43] calciseptine blocks the L-type VSCC with weak efficacy and low specificity.[44,45] Flanking proline residues have been proposed to bracket the L-type calcium channel binding site of calciseptine and FS2.[46,47] In calciseptine they are spaced four residues apart (P-42 TAMWP-47) to present this series of residues in a well-defined loop in the two proteins. Albrand et al (1995)[48] also proposed that Trp contributes to binding to the L-type channel. In support, an eight residue long peptide containing the P-42 TAMWP-47 motif (L-calchin) retained the parent peptide activity at the L-type calcium channel, but with lower potency.[46]

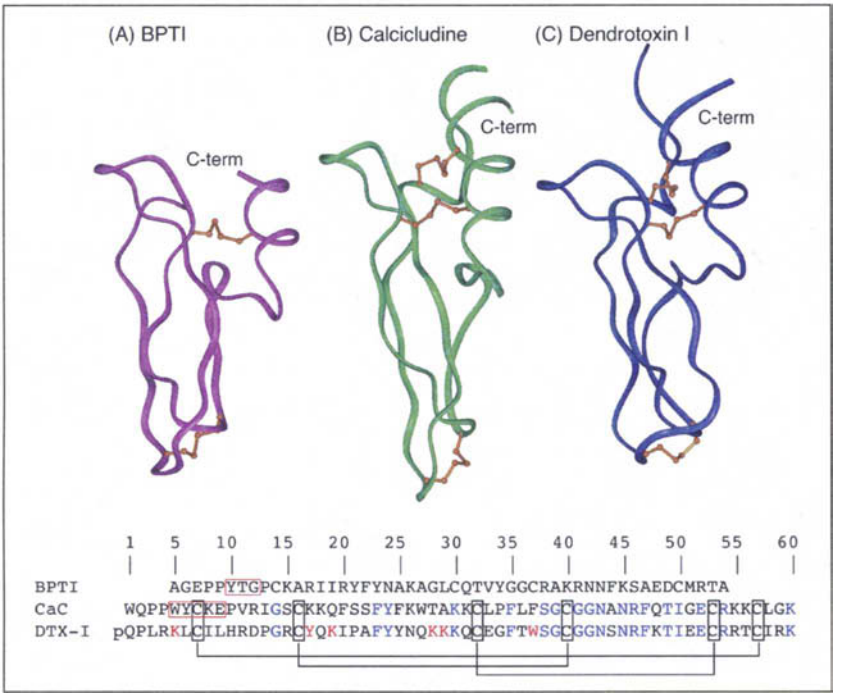



Figure 4. Ribbon diagrams of three venom peptides with low sequence homology but high structural homology. A) Bovine pancreatic trypsine inhibitor (BPTI) in pink, B) Calcicludine (CaC), a L-type calcium channel inhibitor from the green mamba snake, *Dendroaspis augusticeps* (green), and C) Dendrotoxin I (DTX-I) a potassium channel blocker from a snake (blue). Peptides are superimposed across the backbone and disulfide bonds indicated in orange. Disulfide connectivity for CaC and Dendrotoxin I is indicated in the sequences. Residue important for activity boxed for BPTI and CaC.

Calcicludine (CaC), a 60 amino acid peptide isolated from the green mamba venom (*Dendroaspis augusticeps*), irreversibly inhibits L-type calcium channels ($IC_{50}$ ~90 nM), without affecting the voltage-dependence or kinetics of the current. However, even at saturating toxin concentration, block was incomplete (~60%), indicating either a partial occlusion of the pore or an effect on channel gating. Recent data suggest that CaC interacts with multiple transmembrane domains of the L-type VSCC to mediate either a partial occlusion of the conductance pathway or a reduction in the steady-state open probability of the L-type VSCC.[45] CaC block is both tissue and species dependent.[45] Structurally, CaC is unrelated to calcium channel blocking peptides isolated from marine snails, spiders or other snakes. Instead it exhibits structural homology to a number of protease inhibitors such as the bovine pancreatic trypsin inhibitor (BPTI)[49,50] and the dendrotoxin family of snake toxins that inhibit the $K^+$ channel.[51] The secondary structural features shared by these three peptides include a short 3–10 helix, a hairpin β-sheet twisted 180° and a regular α-helix.[51] Closer examination of the secondary structure of dendrotoxin-I and CaC they differ at the N-terminus. N-terminal chimeras between CaC and dendrotoxin indicate that differences in distribution of N-terminal cationic charges underlies their L-type calcium channel or potassium channel preferences[51,52] (Fig. 4). Interestingly, three of the four prolines in the CaC are in the N-terminal region, which also includes a tryptophan previously identified to be involved in TxVII,[17,28] calcispetine and FS2,[47] affinity for the L-type VSCC. However, the precise role for these residues in CaC remains to be elucidated.

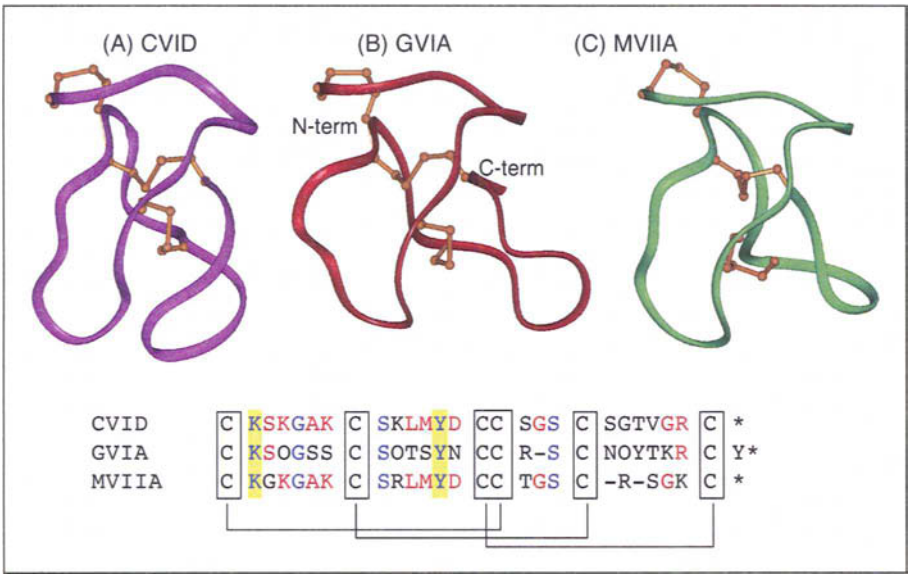



Figure 5. Sequence alignment and ribbon diagrams for selective N-type voltage-sensitive calcium channel blockers from three different cone snail species, A) CVID from *C. catus*, B) GVIA from *C. geographus* and C) MVIIA from *C. magus*. Disulfide bond connectivity (yellow), residues important for activity (yellow boxes) and sequence homology across two peptides (red) and three peptides (blue) are indicated.

DW13.3 is a74 residue peptide isolated from the venom of the spider *Filistata hibernalis* that contains 12 cysteines.[53] It causes a potent and reversible inhibition of $Ca_v2.1$, $Ca_v2.2$, $Ca_v1.2$ and $Ca_v2.3$ expressed in *Xenopus* oocytes with $IC_{50}$ values of 4.3, 14.4, 26.8 and 96.4 nM, respectively. The dose-response data indicate that the peptide binds in a 1:1 manner, producing different extents of block at saturating concentrations, being most effective at $Ca_v1.2$. Structurally, DW13.3 differs from other calcium channel peptide toxins, but it has the same broad specificity across different voltage-sensitive calcium channel subtypes as that of ω-Aga IIIA, a 76 residue peptide isolated from the funnel web spider *A. aperta*.[54-56] Despite the lack sequence homology, they both have an overall positive charge and a hydrophobic core of 12 cysteine residues forming six disulfide bridges. These six disulfide bonds again appear to maintain a conserved three-dimensional fold from which to present amino acids side chains in specific orientations.

## N-Type VSCC Antagonists

Toxins that selectively target the N-type voltage-sensitive calcium channel have mainly been isolated from cone snails (ω-conotoxin), whereas a number of nonselective inhibitors for this channel have been found in both snake and spider venoms. ω-Conotoxin inhibitors have been identified from a number of different fish hunting cone snails, including GVIA from *Conus geographus*,[57,58] MVIIA from *Conus magus*,[59] CVID from *Conus catus*[16] and CnVIIA from *Conus consors*[15] (see Table 2). All ω-conotoxins identified to date are 25–27 amino acid residues long but specific variations in primary sequence can be accommodated without affecting potency, selectivity or the structural core of the peptide (Fig. 5). As found for snake and the spider toxins, the lack of sequence homology among ω-conotoxins suggests that the overall three-dimensional structure and charge distribution underpin their interaction with the N-type VSCC. Interestingly, the two ω-conotoxins that display most sequence homology (MVIIA and MVIIC) (Table 2) target quite different VSCCs ($Ca_v2.2$ and $Ca_v2.1$, respectively). Whereas ω-conotoxins GVIA and MVIIA inhibit the same calcium channel subtype despite remarkably low sequence homology (Table 2).

All the ω-conotoxins are characterised by a common cysteine scaffold that stabilises the four-loop frame work (Fig. 5). This configuration defines the canonical ω-conotoxin fold, which comprises a triple-stranded β-sheet/inhibitory cysteine-knot frame work[60,61] that has also been found in peptides such as PVIIA (a $K^+$-channel blocker from *Conus. purpurascens*,[62,63] and the cyclic cysteine knot peptide Kalata.[64] Interestingly, independent of sequence or structural homology, the affinity of all ω-conotoxins for the N-type ($Ca_v2.2$) calcium channel is reduced by the presence of the $\alpha_2\delta$ auxilary subunit.[65]

## *Structure Activity Studies at the N-Type VSCC*

Only limited information is available on the location of the ω-conotoxin binding site on the N-type calcium channel protein. By using a chimeric approach, Ellinor et al[5] showed that the outer vestivule of the pore region in the third domain of the N-type calcium channel $\alpha_1$ subunit contained amino acid residues that appeared to contribute to conotoxing block. Additional residues in this region were implicated by Feng et al.[66] However, it is likely that other parts of the N-type calcium channel are involved in the docking of the blocking molecules.

Several groups have undertaken extensive studies of the structure-activity relationship (SAR) of ω-conotoxins to identify all residues important for binding to the N-type calcium channel (for review see ref. 67). These SAR investigations have mainly consisted of alanine scans and then further probing of positions found to influence binding. Initially these studies were followed by CD-spectroscopy, but more recently NMR studies have been used to identify structural perturbations that may have accompanied residue replacements.

The first and most important residue identified to be crucial for ω-conotoxin binding to the N-type VSCC was the conserved Tyr13. Replacing the Tyr13 with Phe and the binding drops affinity by 2–3 orders of magnitude in GVIA,[22] MVIIA[26] and CVID,[68] and Ala replacement decreased binding another order of magnitude.[22,25] The orientation of Tyr13 is also important, since D-Tyr13 analogues of MVIIA (N-type specific) and MVIIC (P/Q-type selectivie) had 3–4 orders of magnitude lower affinity than the native conotoxin.[69] In these analogues the shape of loop 2 was severely distorted. The decrease in binding of D-Tyr13-MVIIA for the N-type calcium channel appears to stems from the loss of either (i) a hydrogen bond from the hydroxyl group on Tyr13, (ii) a hydrophobic interaction from Leu11 and/or (iii) a putative electrostatic interaction from Arg10.[69]

Lys2 is another conserved residue in N-type selective ω-conotoxins that contributes to activity. Replacing Lys2 with an alanine in either GVIA,[23-25] or MVIIA[23,26] dramatically reduces affinity. Arginine and ornithine can effectively replace lysine, but bulkier aromatic residues hinder binding.[70] Leucine and norleucine also reduce binding affinity, indicating that side chain length and charge at position 2 contribute to GVIA binding.[70] Lys2 may interact with the VSCC in other ω-conotoxins including MVIIA, CVID and CnVIIA. Alternatively, the positive charge in these latter peptides could interact with Asp14 in loop 2 to provide a structural contribution.

Another residue conserved accross ω-conotoxins that target the N-type calcium channel is glycine 5. One study showed that replacing the glycine with the more sterically hindered alanine resulted in difficulty oxidising the peptide and a nonnative disulfide connectivity dominating the oxidised products. This is not surprising since the glycine is an integral part of the type II β-turn that extends across residue 3–6 in the ω-conotoxins, and fixing the ϕ-angle in a negative position is most likely unfavourable for the native conformation.[25] However, replacing the glycine with a D-alanine, does not hinder folding in either GVIA[25] or CVID (Lewis et al, unpublished data) and these peptides retain activity at the N-type calcium channel.

### Loop Integrity in ω-Conotoxins.

A study by Nielsen et al[71] of 14 loop splice hybrids of MVIIA (N-type inhibitor) and MVIIC (P/Q-type inhibitor) revealed that loop 2 and loop 4 make the greatest contribution to subtype selectivity, whereas loops 1 and 3 contributions are negligible. Peptides with homogeneous combinations of loop 2 and 4 display clear selectivity, while those with heterogeneous

combinations of loops 2 and 4 are less discriminatory.[71] The recently published structure of CVID[16] shows that the orientation of loop 2 in relation to loop 4 in this peptide is quite different from previously published structures of ω-conotoxins. Two additional hydrogen bonds were identified in CVID that position loop 4 closer to loop 2 and introduce a "kink"[16] which is not seen in either GVIA[72-76] or MVIIA.[77-79] GVIA, having a slightly larger loop 4 instead shows preference for a "kink" in the opposite direction.[76]

### *Other N-Type VSCC Inhibitors*

Peptides belonging to the inhibitory cysteine knot family that inhibit N-type calcium channels have been identified in assassin bug venom. Three novel peptides named Ado1, Ptu1 and Iob1 have been isolated[80] and the solution structure of Ptu1 determined by NMR.[81] Despite the fact that these peptides have low sequence homology with the ω-conotoxins, they have a similar four-loop framework. The three peptides have quite high sequence homology, despite coming from different *Reduviiae genera*. Ptu1 and Ado1 are basic peptides, whereas Iob1 is neutral. All the three peptides have one or more aspartic acids in the N-terminal region, which is homologous to PnVIB from *C. pennaceus*.[82] CD spectroscopy indicated that Ptu1 has less β-sheet than ω-conotoxins.[80] The solution structure confirmed this, with the only secondary structure being a β-sheet region comprising two antiparallel β-strands.[81] Ptu1 lacks most of the residues shown to be important for ω-conotoxin binding to the N-type calcium channel, including equivalents of Tyr13 or Lys2.[80] Despite these differences, Ptu1 shares pharmacological specificity reminiscent of MVIIA and GVIA, with 300 nM Ptu1 inhibiting ~50% of $Ba^{2+}$ current through N-type VSCCs expressed in BHK cells. However, neither Ado1 nor Ptu1 displace binding of $^{125}$I-GVIA to N-type calcium channel in rat brain membrane (personal communication).

The spider toxins ω-grammotoxin-SIA[8] isolated from the Chilean tarantula *Grammostola spatulata* or *Phrixotrichus spatulata*, ω-agatoxinIIIA[83] from the funnel web spider *Agelenopsis aperta*[83] and Huwentoxin-I isolated from the chinese spider *Selenocosmia huwena*[84] have also been found to block N-type (and P/Q-type) VSCCs. NMR solution of the two peptides ω-grammotoxin-SIA[85] and Huwentoxin-I,[86] indicates that these toxins share the 'inhibitory cysteine knot' motif characteristic for the ω-conotoxins. Both peptides contain the antiparallel triple stranded β-sheet stabilised by the cysteine knot.

## P/Q-Type VSCC Antagonists

### *ω-Conotoxins*

ω-Conotoxins MVIIC and MVIID from *C. magus*, and SVIB from *Conus striatus* preferentially target the P/Q-type calcium channel.[87] Despite this difference in selectivity, they possess the consensus inhibitory cysteine knot structure found in ω-conotoxins selective for the N-type calcium channel.[71] To start to understand VSCC subtype differentiation among ω-conotoxins, we compared the structure of MVIIA to SVIB and a synthetic hybrid, SNX-202, which has altered specificity for both VSCC subtypes.[79] The secondary structures of the three peptides are almost identical, consisting of a triple-stranded β-sheet and several turns. The three-dimensional structures of SVIB and MVIIA are likewise quite similar, but subtle differences including a change in the relative orientation between loops 2 and 4 are likely to underlie the selectivity differences among the peptides.

From the above structural studies and a large number of other studies of molecules within this family, it is apparent that the ω-conotoxins form a consensus structure despite differences in sequence and VSCC subtype specificity. This indicates that the ω-conotoxin macrosites for the N/P/Q-subfamily of VSCCs are most likely related, with specificity for receptor targets being conferred by the positions of functional sidechains on the surface of the peptides. Structural studies of the type described above are likely to lead to the development of second-generation analogues which may overcome some of the side-effects associated with intrathecal delivery of ω-conotoxins such as MVIIA.

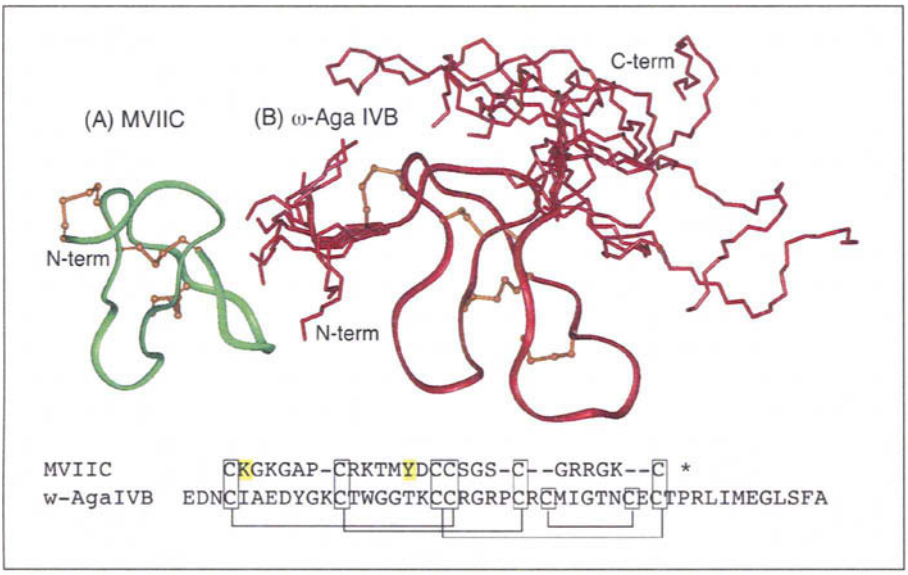



Figure 6. Comparison of two P/Q-type calcium channel inhibitors from a snail and a spider. A) MVIIC from the fish hunting cone snail *C. magus*, and B) ω-Aga IVB from the funnel web spider *Agenelopsis aperta*. Peptides are superimposed over the heavy backbone atoms of residues 5–15 of ω-Aga IVB and residues 1–12 of MVIIC. Disulfide bond connectivity (yellow) and the disordered N- and C-terminal of ω-Aga IVB are indicated. Highlighted residues for MVIIC are believed important for binding to the P/Q-type VSCC.

### *Spider Toxins*

ω-Aga-IVA and ω-Aga-IVB were the first spider peptides identified to specifically inhibit the P-type calcium current in the brain. These toxins block P-type channel activity by antagonizing activation gating and likely act at a region other than the pore.[8] Indeed, Bourinet et al[6] showed that insertion of two amino acid residues (proline and asparagine) through alternate splicing in the extracellular domain IV S4-S4 linker region dramatically reduces ω-Aga-IVA sensitivity of transiently expressed $\alpha_{1A}$ ($Ca_v2.1$) calcium channels. These residues thus act as a molecular switch between P-type and Q-type calcium channel phenotypes with regard to toxin sensitivity, and indicate a site of action outside of the pore region. Unlike the very poorly reversible pore blocking conotoxins, block by ω-Aga-IVA (and ω-grammotoxin SIA) can be reversed by application of strong membrane depolarisations which are thought to dislodge these toxins from their sites of action.[8]

The solution structure of ω-Aga-IVB shows that apart from a long and disordered N- and C-terminal, the peptide has a well-defined core which again is structurally stabilised with a network of disulfide bonds. This configuration produces a structure strikingly similar to the solution structure of MVIIC (Fig. 6).[88] One face of the molecule has a cluster of basic residue that has been suggested to be involved in the binding interaction with the channel.[89,90]

## R-Type VSCCC Antagonists

The R-type calcium channel is the least defined of the six identified calcium channels to date. This has partly been due to the lack of selective inhibitors. However, recently a 41 amino acid peptide (SNX-482) was isolated from the venom of the African tarantula, *Hysteraocrates gigas*.[91] SNX-482 was found to be structurally homologous to the spider toxins grammatoxin SIA[8] and hanatoxin[92] that block calcium and potassium channels, respectively by affecting channel gating. However, SNX-482 was found to completely and irreversibly inhibit expressed

R-type calcium channel current with an $IC_{50}$ ~20 nM[91] and is now being used as a tool[93] to unravel the physiological role of the $\alpha_{1E}$ calcium channel, which is widely distributed in the brain.[94-96] Bourinet et al[97] confirmed that SNX-482 is indeed a gating-modifer reminiscent of grammotoxin SIA, ω-Aga-IVA and hanatoxin, and can be reversed by strong membrane depolarization. They also showed, using chimeric calcium channel $\alpha_1$ subunits, that the toxin needs to interact with both domain III and IV of the $\alpha_{1E}$ subunit to influence channel gating.[97] SNX-482 applied to L-type calcium channels caused an incomplete block (~25%) at 200 nM.[97] Despite the lack of selectivity at the R-type calcium channel, SNX-482 is useful in further characterising the role of an important component of this poorly characterised calcium channel.

## T-Type Antagonists

Since the cloning of the T-type calcium channel[98-100] it has been possible to screen this channel for selective inhibitors. Kuroxin, identified by Chuang and coworkers (1998) in the venom of the South African scorpion *Parabuthus transvaalicus*, inhibits the T-type current by modifying channel gating.[101] This 63 amino acid residues peptide has eight Cys and is most likely a member of the cysteine knot family of peptides. Comparing kurtoxin with other previously described toxins suggests that it is closely related to the α-scorpion toxins, a family of toxins that slow inactivation of sodium channels, but are also known as gating modifiers.[101,102] While kurtoxin acts on the low-threshold $\alpha_{1G}$ and $\alpha_{1H}$ calcium channels, it also partially inhibits the high-threshold N-type, P-type and L-type currents[103] in rat peripheral and central neurones. Despite lack of selectivity, kurtoxin is a promising tool for functional and structural studies of this low-threshold calcium current.

## Conclusions and Future Prospects

Peptide toxins that selectively block voltage sensitive calcium channels have contributed enormously to our understanding of the role of specific calcium channels in normal and pathological conditions. Several members of the ω-conotoxin class of calcium channel blockers are currently in clinical trials for chronic pain. While they are particularly efficacious when delivered intrathecally, side effects may limit their usage. Future advances will come with the discovery of new probes for VSCC subtypes, particularly those that are more selective for pain pathways and disease states.

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# CHAPTER 20

# Calcium Channels in the Heart

**Stéphanie Barrère-Lemaire, Matteo E. Mangoni and Joël Nargeot**

## Introduction

In the sixties, the first $Ca^{2+}$ currents were recorded by voltage-clamp techniques on multi-cellular cardiac strips [1-4] It is only since 1980 that the development of enzymatic dissociation methods allowed the first electrophysiological recordings on isolated cardiac cells.[3] The whole cell patch-clamp technique, developed by E. Neher and B. Sakman in 1976 [5] and awarded in 1991 with the Nobel prize, and the micro-electrode technique [6] initiated the study of voltage-gated $Ca^{2+}$ channels. The improvement of the technique gave the first opportunity to study the activity of single channels owing to the cell-attached[7,8-11] and the inside-out configuration.[12] The nystatin- or amphotericine B-perforated patch recordings prevent the leak of cytoplasmic molecules, nucleotides and diffusing agents in the recording pipette and then, preserves the mechanisms of regulations by second messengers.[13,14]

S. Ringer in 1883 was the first physiologist who evidenced the crucial role of $Ca^{2+}$ in cardiac contraction since $Ca^{2+}$ withdrawal suppresses heart contractility. A century later, A. Fleckenstein reported that "$Ca^{2+}$ antagonists" could mimic $Ca^{2+}$ withdrawal.[15] These drugs selectively block voltage-dependent $Ca^{2+}$ entry through activated L-type $Ca^{2+}$ channels.[16]

Influx of $Ca^{2+}$ through voltage-dependent L-type $Ca^{2+}$ channels plays an essential role in cardiac excitability and in coupling excitation to contraction. $I_{Ca,L}$ contributes to the plateau phase of the action potential as well as to pacemaker activity in nodal cells.

Given the essential role of L-type $Ca^{2+}$ channels in multiple cellular functions, it is not surprising that this protein is extensively regulated by a variety of signaling pathways.

## I-Cardiac $Ca^{2+}$ Channels in Working Myocardial Cells

Two main classes of voltage-dependent $Ca^{2+}$channels coexist in cardiac cells: L-and T-types. L-Type $Ca^{2+}$ currents ($I_{Ca,L}$) are characterized by a Large conductance (25 pS) and Long lasting openings. They are activated at Larger depolarizations and highly sensitive to 1,4-dihydropyridines (DHP).

In contrary to $I_{Ca,L}$ which is largely ubiquitous in cardiac myocytes, $I_{Ca,T}$ is not so widely expressed and is more transient. T-type channels are more specific of atrial cells[17] and Purkinje fibers where $I_{Ca,T}$ is the prominent voltage-dependent $Ca^{2+}$ influx.[18]

### *L-Type $Ca^{2+}$ Channels*

#### Structure of L-Type $Ca^{2+}$ Channels

The molecular biology of $Ca^{2+}$ channels has its origins in the biochemical characterization of the skeletal muscle DHP receptor.[19] The cardiac L-type $Ca^{2+}$ channel is a multi-subunit protein complex composed of $\alpha_1$, the ion-conducting subunit, and auxiliary subunits including $\beta$, and $\alpha_2$-$\delta$. The cardiac $\alpha_{1C}$ subunit was cloned from rabbit heart cDNA libraries using low stringency hybridization with the first cloned $\alpha_1$-subunit, $\alpha_{1S}$, as probe.[20] Like $Na^+$ channels $\alpha_1$-subunit, it contains four internal repeats called Domains I, II, III and IV. Each domain

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is composed of six membrane spanning segments S1 to S6 (α helix) with one particular, the voltage-sensor S4 which contains positively-charged residues every third residue.[21] The SS1-SS2 loops (between S5 and S6) of each repeat coassembles in the membrane to constitute the pore.

Although it is generally accepted that $\alpha_{1C}$ is the predominant L-type $\alpha_1$ in cardiac myocytes, $\alpha_{1D}$-subunit has also been detected at both the mRNA and protein levels.[22,23] $\alpha_{1D}$ was originally cloned from neuro-endocrine cells.[24]

Recently, the nomenclature for voltage-dependent $Ca^{2+}$ channels has changed and the alphabetic descriptor L-type was replaced by $Ca_v1$. The $Ca_v1$ family includes $Ca^{2+}$ channel $\alpha_1$-subunits encoded by four separate genes $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ which correspond to $\alpha_{1S}$, $\alpha_{1C}$, $\alpha_{1D}$ and $\alpha_{1F}$ respectively.[25] The $Ca_v1.2$ gene encodes for cardiovascular $\alpha_{1C}$ subunits. The cardiac $Ca_v1.2a$ and the smooth muscle $Ca_v1.2b$ $\alpha_1$ -subunits are splice variants of the same gene. This may account for the differences of DHP-sensitivity between smooth muscle and cardiac muscle L-type $Ca^{2+}$ channels.[26]

In addition to the pore forming subunit $\alpha_1$, there is an intra-cytoplasmic localized β-subunit. These subunits vary from 53 to 71 kDa and are encoded by four distinct genes: $\beta_1$,[27] $\beta_2$,[28] $\beta_3$,[29] and $\beta_4$.[30] In cardiac muscle the $\beta_2$–subunit predominates.[31,32] The β-subunit binds to AID (Alpha subunit Interacting Domain), a specific sequence located in the cytoplasmic linker between Domains I and II of the $\alpha_1$-subunit.[33] The $\alpha_2$-δ subunit is an extracellularly located complex composed by an $\alpha_2$-subunit linked via a disulfide bond to a membrane-spanning δ-subunit. These two subunits are encoded by the same gene.[34]

## Structure-Function of $Ca_v1.2$ Channels

Functional expression in heterologous systems of $Ca^{2+}$ channels, together with structure-function studies have allowed description of the functional role of the different channels subunits. The loop between Domain II and III of $\alpha_{1C}$ subunit plays a key role in cardiac specific excitation-contraction coupling.[35] $Ca^{2+}$ selectivity is conferred by the presence in the pore region of glutamate residues.[36-38] Numerous studies have also attempted to identify the regions involved in $Ca^{2+}$-dependent inactivation. Based on sequence homology with the $Ca^{2+}$-binding motifs of EF hands, one homologous region was identified previously close to IVS6.[39] However, most of these regions are located in the carboxy terminus.[40,41]

When coexpressed with $Ca_v1.2$ $\alpha_1$-subunit, β-subunits are responsible for an increase in $Ca_v1.2$ -current amplitude,[42] facilitation[43,44] and targeting of the $\alpha_1$-subunit to the plasma membrane.[44,45] $\alpha_2$-δ seems to also increase $Ca^{2+}$ current amplitude and accelerate the current decay kinetics.[46,47] The effects of β and $\alpha_2$-δ subunits on $Ca_v1.2$ currents are cumulative.[48] Interestingly, $\alpha_2$-δ abolishes the prepulse facilitation occurring specifically with $Ca_v1.2$ $Ca^{2+}$currents.[49]

Molecular determinants for $Ca^{2+}$ antagonists have also been determined. The binding sites for DHP have been mapped in IIIS5, IIIS6 and IVS6.[50-52] Residues for phenylalkylamine (PAA) binding are located in IIIS6 and IVS6.[53]

Auxiliary subunits are also important in the formation of high-affinity drug binding sites and their interactions.[54]

## Physiological Role of L-Type $Ca^{2+}$ Channels: Excitation-Contraction Coupling

It is now well established that both the $Ca^{2+}$ from the sarcosplamic reticulum (SR) and the $Ca^{2+}$ entering the cell via L-type $Ca^{2+}$ channels contribute to cardiac contraction. The activation of sarcolemmal L-type $Ca^{2+}$ channels is a prerequisite for the initiation of contraction.[55-57] $Ca^{2+}$ influx through these channels is the trigger of the activation of ryanodine receptors (RyR) and the subsequent release of $Ca^{2+}$ leading to activation of contraction. This mechanism called "$Ca^{2+}$-induced $Ca^{2+}$-release" or CICR was extensively described by the studies of Fabiato on skinned single cardiac myocytes ".[58-68] SR $Ca^{2+}$ release is gated by $Ca^{2+}$ current and not by gating charges.[56] Niggli and Lederer (1990) showed that CICR in cardiac muscle is not dependent on membrane voltage.[69] The amount of $Ca^{2+}$ released is graded with the amount of $Ca^{2+}$

influx. At high concentrations of $Ca^{2+}$, the CICR is inactivated and the SR release terminated.[70] These findings are explained by the existence of two binding sites for $Ca^{2+}$ ions on RyR, an activating site and an inactivating site which turns off the $Ca^{2+}$ release.[66,68] In turn, SR $Ca^{2+}$ release (in addition to $Ca^{2+}$ entering the cell) causes L-type $Ca^{2+}$ channel inactivation as a negative feedback to limit $Ca^{2+}$ entry.[71,72]

The "local control" theory of excitation-contraction was first suggested by Lederer in 1990 who mentioned the importance of the local concentrations gradients of $Ca^{2+}$ in the junctional space.[73] Stern in 1992 suggested several models which could explain the local control and the graded nature of CICR.[74] He considered the colocalization of one DHPR with a cluster of RyR to form a "cluster bomb". The stoichiometry of this association was confirmed later: one single L-type $Ca^{2+}$ channel triggers a cluster of 4 to 10 molecules of $RyR_2$.[75,76]

The whole-cell intracellular $Ca^{2+}$ transient seems to correspond to the temporal and spatial summation of $Ca^{2+}$ "sparks"[77] triggered locally by the $Ca^{2+}$ influx flowing through a single L-type $Ca^{2+}$ channel.[78,79] $Ca^{2+}$ sparks were first described as spontaneous events due to single RyR openings.[78,80] However, they are more likely due to the activation of a cluster of RyR release channels working as a single functional unit. Parker in 1996 has shown that a $Ca^{2+}$ spark can activate a lateral neighbor located along a z-line.[81] This means that gradation of CICR is due to the activation of different numbers of RyR clusters rather than increasing ionic fluxes through each RyR cluster and that CICR is characterized by an intrinsic positive feedback.

CICR seems to be the main mode of excitation-contraction coupling in cardiac myocytes as demonstrated by several studies based on the close correlation between $I_{Ca,L}$ and $Ca^{2+}$ transients as a function of voltage.[55,82-84]

## Regulation of L-Type Channel Activity

### *β-Adrenergic Regulation*

The amplitude and kinetics of $I_{Ca,L}$ depend on the channel properties as well as on voltage and ionic conditions. In addition, $I_{Ca,L}$ is tightly regulated by both physiological and pharmacological agents. To this respect, the β-adrenergic stimulation is a major pathway of regulation of the cardiac function and thereby $Ca^{2+}$ channels. During exercise, release of catecholamines increases cardiac output by increasing the heart rate, by enhancing contractility and accelerating relaxation. β-adrenergic agonists increase cardiac $I_{Ca,L}$[2] by activating the cAMP-dependent channel phosphorylation pathway[85] of cardiac L-type $Ca^{2+}$ channels.[86] Three types of β-adrenoreceptors have been identified on cardiac cells. $\beta_1$ and $\beta_2$-subtypes are colocalized on cardiac myocytes, but $\beta_1$-receptors are more abundant in the normal adult mammalian heart.[87-89] The recently identified $\beta_3$-receptors, are localized on atrial cells and seem to be involved in pathological processes.[90,91]

$\beta_1$ and $\beta_2$ receptors differ in their effect on $Ca^{2+}$ currents, cytosolic $Ca^{2+}$ and contraction.[92] The more specific $\beta_1$-adrenoreceptors stimulation corresponds to the classical notion of β-adrenoreceptors signaling leading to substantial phosphorylation of proteins such as L-type $Ca^{2+}$ channels, phospholamban, I-troponin and C-protein, and responsible for the multiple effects on contraction and relaxation of the cardiac muscle. $\beta_2$-AR stimulation seems to act only on $Ca_v1.2a$ channels. Contrary to $\beta_1$-adrenergic receptors that couple exclusively to the $G_s$ protein,[93] $\beta_2$-adrenergic receptors are coupled to both $G_s$ and $G_i$. This latter unexpected pathway is responsible for the spatial restriction of $\beta_2$-AR signaling.[94] The $\beta_3$-receptor is coupled only to $G_i$ protein and is responsible for a negative inotropic effect.[91]

### *Regulation by the Protein Kinase A*

Stimulation of $I_{Ca,L}$ by β-adrenoreceptors in cardiac cells leads to enhanced single-channel activity by increasing the channel mean open time.[8,95] Stimulation by protein kinase A (PKA) leads to a two to seven fold increase of $I_{Ca,L}$ amplitude and to a negative shift of the current

voltage-dependence of activation and inactivation.[11,96,97] PKA phosphorylates only the 250 kDa form of the $\alpha_1$-subunit but not the truncated 200 kDa form.[98,99] Serine 1928, located in the COOH-terminal tail of the $\alpha_1$-subunit, is essential for the PKA-induced channel phosphorylation.[99] Other studies have shown that the β-subunit is also phosphorylated in vitro[29] and in vivo in cardiac tissue after stimulation of the adenylyl cyclase/PKA pathway.[100]

### *The Missing Link*

Although numerous studies have reported PKA-induced phosphorylation of L-type $Ca^{2+}$ channels in native cardiac cells, it has been a challenge to the reconstitute the PKA-regulation of $Ca_v1.2$ $Ca^{2+}$ currents in heterologous systems. Recombinant $Ca_v1.2$ currents are increased by only 20 to 30% by PKA-stimulation when native currents are amplified two to seven fold.[101] There would be a missing link in the channel complex as suggested by the study of Charnet et al which showed that the coinjection of cardiac mRNA with recombinant $Ca_v1.2$ channels in oocytes allows to recover the regulation properties.[102] More recently, several studies report the need for PKA targeting to the chance of AKAPs (A-Kinase Anchoring Proteins), but it is not sufficient since the coexpression of AKAP79 with the cardiac $\alpha_1$-subunit and $\beta_{2a}$ in HEK293 failed to reproduce the phosphorylation.[103]

In summary, there is clear evidence that PKA can stimulate cardiac $Ca_v1.2a$ channels via serine 1928 on the $\alpha_1$-subunit but probably other regulatory proteins contribute to this process.

### *Acetylcholine and Cardiac $Ca^{2+}$ Currents*

There is a strong interaction between the β-adrenergic signaling cascade and other pathways, particularly with that of the muscarinic receptor stimulation. Different studies have reported cGMP/PKG-mediated inhibition of $Ca_v1.2$ currents.[104-108] Acetylcholine (ACh) has little effect on the basal current but strongly antagonizes the β-adrenergic- or forskolin-stimulated $I_{Ca,L}$.[109,110] Part of this effect is mediated by muscarinic receptors coupled to $G_i$ which inhibits adenylyl cyclase (AC) as confirmed recently in $G_{i2}$ alpha gene knockout mice lacking muscarinic regulation of $Ca^{2+}$ channels in the heart.[111]

Another pathway activated by ACh is the stimulation of guanylate cyclase and the production of cGMP. $Ca_v1.2$ $Ca^{2+}$ currents can be either stimulated or inhibited by the cGMP-pathway. One mechanism accounts for the stimulation of $Ca^{2+}$ currents and reflects the cGMP-dependent inhibition of phosphodiesterase 3 (PDE3) which leads to augmentation of cAMP levels and, consequently, $Ca^{2+}$ current potentiation.[112,113] On the other hand, three mechanisms can explain cGMP-induced inhibition of $I_{Ca,L}$: (1) phosphorylation of the channel by PKG (cGMP-dependent protein kinase);[114] (2) PKG-mediated activation of a phosphatase which dephosphorylates the channel; this mechanism occurs after previous PKA-stimulation;[115,116] (3) cGMP activation of phosphodiesterase 2 (PDE2) leading to decreased cAMP levels.[117,118]

In cardiac cells nitric oxide (NO) stimulates cGMP production by directly activating a cytoplasmic guanylate cyclase and thereby regulates $Ca_v1.2$ currents.[113] ANF, the atrial natriuretic factor, has a membrane located binding site on cardiac cells, which is a guanylate cyclase. ANF also regulates $I_{Ca,L}$ in both positive and negative ways as reported for the cGMP/PKG pathway in cardiac myocytes.[119]

Thus, the final outcome of the regulation of $I_{Ca,L}$ by phosphorylations is the result of the relative balance of protein kinase and phosphatase activity and depends on the cardiac cell type considered.

### *Regulation by Protein Kinase C*

There is no agreement in the current literature concerning the effects of protein kinase C (PKC)-regulation on $Ca_v1.2$ channels.

While several studies in native cardiac cells have reported PKC-induced potentiation of $I_{Ca,L}$[120-122] others have shown an inhibition.[123] In heterologous systems, the same controversy exists concerning the stimulation[124] or inhibition of recombinant $Ca^{2+}$ currents.[125] In vitro, both $\alpha_1$-subunit and $\beta_2$-subunit are phosphorylated by PKC.[126] Recently, recordings of $Ca_v1.2$ currents in tsA-201 cells have shown that PKC produces an inhibition of $I_{Ca,L}$ through phosphorylation of threonine-27 and threonine-31 at the $NH_2$-terminal region of the $\alpha_1$-subunit.[127] However, these data are inconsistent with the model of the tonic inhibition exerted by the $NH_2$-terminal end (first 46 amino acids) in basal condition and relieved by PKC-phosphorylation.[125] Further studies will probably elucidate these discrepancies.

### *Regulation by $Ca^{2+}$ Antagonists*

$Ca_v1$ channels are highly sensitive to DHPs. Indeed, DHPs inhibit $I_{Ca,L}$ by preferentially binding to the inactivated state of the channel.[128,129] DHP binding to $Ca_v1$ channels is voltage dependent.[128] Also, some DHPs stimulating $I_{Ca,L}$ activity are used in academic research. These agonists, BayK8644 (-) and a novel benzoyl pyrrole FPL-64176 dramatically increase $I_{Ca,L}$ by prolonging the channel open time and slightly increasing the single-channel conductance.[130-133] There are two other classes of $Ca^{2+}$ antagonists, phenylalkylamines (PAA, e.g., verapamil) and benzothiazepines (BTZs e.g., diltiazem), that are also widely used as therapeutic agents. Charged compounds (i.e.verapamil) preferentially bind to the open-state of $Ca^{2+}$ channels and are use-dependent. Neutral DHPs molecules (i.e., nitrendipine) may bind and block the channel either in the open or inactivated state and have pronounced voltage-dependent effects. These antagonists are used as vasodilatators since they preferentially act on vascular $Ca_v1$ $Ca^{2+}$ channels and because of the more depolarized voltage of resting smooth muscle myocytes.[134]

### *$Ca^{2+}$-Dependent Facilitation of $I_{Ca,L}$*

$Ca_v1$ $Ca^{2+}$ channels can be positively and negatively regulated by the $Ca^{2+}$ entering the cell and acting as a positive or negative feedback respectively. In addition to membrane depolarization and $Ca^{2+}$ channel phosphorylation, modulation of $I_{Ca,L}$ by frequency of $Ca^{2+}$ channel activation is probably of major importance for heart physiology. Early studies suggested that repetitive stimulations of frog atrial fibers can up-regulate $Ca^{2+}$ channel activity.[135] Similar observations were reported in dissociated mammalian cardiac cells [136-138] and this has been confirmed later at the single channel level.[139] An increase in the rate of stimulation can produce a potentiation of $I_{CaL}$ only when cells are stimulated from negative holding potentials.[135-141] In mammalian cardiomyocytes, the high frequency-induced potentiation of $I_{CaL}$ consists of both a moderate increase of peak current amplitude and a marked slowing of the inactivation kinetics. This "positive staircase", not apparent with $Ba^{2+}$ as the charge carrier, is mediated by CAMKII-dependent phosphorylation and is $Ca^{2+}$ dependent.[142-144]

This frequency-dependent facilitation of $I_{Ca,L}$ occurs in human cardiomyocytes and is modulated by β-adrenergic stimulation. This mechanism could be responsible in part for the "Bowditch staircase" since it disappears in failing myocardium.[145]

### *$Ca^{2+}$-Dependent Inactivation of $I_{Ca,L}$*

Decrease of $I_{CaL}$ occurs when cells are stimulated from depolarized membrane holding potentials, reflecting a reduction of $Ca^{2+}$ channel availability for opening because of incomplete voltage-dependent reactivation.[136,140,146,147] Indeed, full recovery from inactivation requires more negative membrane potentials.

It should be noted that another type of voltage-dependent facilitation can occur, independently of the rate of stimulation.[148,149] Such mechanism was evidenced in human cardiac myocytes. This facilitation by the diastolic membrane potential is favored by intracellular cAMP and reflects removal of $I_{Ca,L}$ inactivation induced by release of $Ca^{2+}$ from the SR.[150]

## *T-Type $Ca^{2+}$ Channels in Myocardial Cells*

### T-Type Cardiac Channels

Two distinct families of $Ca^{2+}$ channels have been identified in cardiac tissue. L-type $Ca^{2+}$ channels which are essential in triggering $Ca^{2+}$ release from internal stores and Low-Voltage-Activated (LVA) T-type $Ca^{2+}$ channels ($I_{Ca,T}$), whose role remains obscure either in physiological or pathophysiological conditions. Functional features of T-type $Ca^{2+}$ channel activity include: low threshold of activation, small unitary conductance, rather slow activation and fast inactivation inducing a typical criss-crossing pattern of current traces for increasing depolarizations, negative steady state inactivation and slow deactivation kinetics. In addition, T-type currents as compared to L-type currents are more sensitive to block by mibefradil and $Ni^{2+}$ ions. An alternative in silico cloning strategy resulted in the identification of full-length cDNAs encoding three distinct $Ca_v$ subunits encoding T-type $Ca^{2+}$ currents: $Ca_v3.1$ in rat,[151] mouse[152] and human,[153,154] $Ca_v3.2$ and $Ca_v3.3$ in rat[155] and human.[156] Because $Ca_v3.1$ was identified by screening a human heart library, it has been originally considered as the cardiac T-type channel isoform. Northern blot analysis has shown the expression of $Ca_v3.2$ in the heart but also the presence of the $Ca_v3.1$ transcript. It has become an interesting question to identify which isoform(s) is coding for $I_{Ca,T}$ in cardiac cells. The molecular basis of the $\alpha_1$ subunit encoding the T-type $Ca^{2+}$ channel in AT-1 cells, an immortalized cell line derived from mouse atrial tissue was investigated. A comparison of the biophysical properties between $I_{Ca,T}$ from this cell line and the $Ca_v3.1/Ca_v3.2$ currents[157] indicated that most of the basic electrophysiological properties are rather similar and not discriminative. However, detailed analysis of the recovery from inactivation as well as PCR experiments from cultured AT-1 cells indicated that $I_{Ca,T}$ in AT1 cells is generated by the $Ca_v3.1$ isoform.[157] The question was to know if this result could be extended to any cardiac cell. When comparing the properties of $I_{Ca,T}$ in native cardiovascular cells with those of recombinant currents, difficulties arise from the fact that many early studies have been either conducted in different experimental conditions or in different species. Cardiac T-type currents have also mostly been studied in cultured embryonic or neonatal myocytes because of their low expression or absence in respectively adult atrial and ventricular myocytes except in several species such as guinea pig. Subsequent studies on freshly dissociated rat cardiomyocytes during development have shown the presence of $ICa_T$ in both atrial and ventricular myocytes during the early neonatal period. $ICa_T$ peaks around 8 days postnatally in atrial myocytes and decreases to reach a very low density after three weeks while it completely disappears in ventricular myocytes.[158] $I_{Ca,T}$ in neonatal rat atrial myocytes has low $Ni^{2+}$ sensitivity ($IC_{50}$ 160 μM), also suggesting a linkage with the $Ca_v3.1$ isoform. A similar result was reported in embryonic mouse and rat ventricular myocytes.[159,160] The expression of a specific $Ca_v3.1$ variant ($Ca_v3.1$-d) was described in embryonic mouse ventricular cells.[159] In some early studies such as in rabbit sino-atrial,[161] in adult guinea pig cells,[162] T-currents were reported to be totally blocked by a lower concentration $Ni^{2+}$ ions (about 40 μM). This may suggest differential expression of $Ca_v3.1$ and $Ca_v3.2$ isoforms among cardiac tissue, differences between species or a possible developmental regulation between isoforms as suggested by Ferron et al.[160] One should also mention another study, using an antisense strategy, which suggested that the cardiac T-type current in three weeks post-natal rats is related to the $Ca_v2.3$ subunit.[163,164] This result remains puzzling since some properties such as the threshold of activation and deactivation properties markedly differ between $I_{Ca,T}$ and $Ca_v2.3$ currents. In addition, $I_{Ca,T}$ from freshly dissociated neonatal rat atrial tissue was found as insensitive to the $Ca_v2.3$ specific toxin SNX 482.[158] It is however worth to note that T-type channel expression in these experiments[163] was induced by insulin-like growth factor 1 (IGF-1) treatment.

It should also be mentioned that no $I_{Ca,T}$ has yet been described in adult human heart.[165] By contrast, a LVA $Ca^{2+}$ current was first reported in human atria[166] sharing some typical properties with sodium channels such as TTX sensitivity and designated as $I_{CaTTX}$. Its descrip-

tion in rat[167] and guinea pig[168] myocytes indicated that it is not exclusively observed in diseased human cells. The coexistence of $I_{CaTTX}$ with $I_{Ca,T}$ was demonstrated in guinea pig ventricular myocytes.[169] The results show that adult rat myocytes lack $I_{Ca,T}$ while adult guinea pig myocytes express both LVA currents which can be isolated by pharmacological dissection using TTX and $Ni^{2+}$ in the absence of external sodium ions. In addition, there are differences between $I_{Ca,T}$ and $I_{CaTTX}$ in terms of biophysical properties. $I_{CaTTX}$ exhibits a run up after the rupture of the patch membrane, a lower voltage for peak current, a more negative steady state inactivation relationship, faster time constants for recovery from inactivation and a faster rate of deactivation. The molecular basis of $I_{CaTTX}$ are unknown. Thus, whether this current is related to modified sodium channels in the absence of external sodium ions[168] or is it related to a new population of channels remains to be determined.

What could be the physiological role of these LVA channels in the heart? T-type channels are assumed to play a role in pacemaking activity because of their presence in sino-atrial node and the negative chronotropic effect induced by their inhibition.[161] An interesting feature of T-type $Ca^{2+}$ currents is related to their slow deactivation kinetics and the existence of a window current in the range of the cell membrane resting potential. In spite of their rapid kinetics of inactivation, the existence of a window current would confer a role of T-type channels in maintaining intracellular $Ca^{2+}$concentration.[170] While T-type currents do not seem to play an important role in cardiac E-C coupling[171] they could contribute to excitability during early development,[159] fine tuning of basal $Ca^{2+}$ levels and control physiological processes such as hormone secretion as previously suggested in adrenal cells.[172] Their expression is also cell cycle dependent[173] and their involvement in cell growth and proliferation was suggested in both cardiac[174] and smooth muscle cells.[175] Future studies will probably look for a role of $I_{Ca,T}$ and $I_{CaTTX}$ in pathological conditions. $I_{Ca,T}$ was shown to be reexpressed during experimental rat cardiac hypertrophy[176,177] and would be expected to generate cardiac arrhythmias. It would be interesting to know if the reexpression of T-type channels is involved in the genesis or a consequence of heart remodeling.

### Pharmacology of T-Type $Ca^{2+}$ Currents

To date, no drugs showing specificity for $I_{Ca,T}$ versus $I_{Ca,L}$ have been described. Some DHPs show moderate block of $I_{Ca,T}$ for micromolar drug concentrations (see for example ref. 178). Recently, mibefradil, a tetralol derivative belonging to a structurally and pharmacologically distinct class of $Ca^{2+}$ antagonists has been proposed for clinical use due to its particular pharmacological and cardiovascular profile. As compared to DHPs, PAAs and BTZs, mibefradil show preferential block for T-type $Ca^{2+}$ channels.[179] In contrast to the other $Ca^{2+}$ channel antagonists, mibefradil decreases heart rate without concomitant negative inotropism.[180] However, mibefradil is not a selective blocker of $I_{Ca,T}$. Indeed, strong voltage- and use-dependent block of $I_{Ca,L}$ in the micromolar range have been reported in cardiac cells.[181] To this respect, mibefradil shows common properties to both DHPs and PAAs, its blocking action being enhanced by both positive membrane voltages and high frequency of current activation.[181] The lack of specificity of mibefradil thus poses a number of interpretative problems when tackling the differential physiological role of $I_{Ca,T}$ and $I_{Ca,L}$ in cardiac physiology.

## Cardiac $Ca^{2+}$ Channels and Pacemaker Activity

### *Ionic Mechanisms of Pacemaking*

Cardiac pacemaker activity is generated in the sino-atrial node (SAN) by a specialized population of cells. Automaticity in SAN cells is due to the presence of the diastolic depolarisation, a slow depolarisation phase which leads the membrane potential from the end of an action potential toward the threshold of the following action potential.[182,183] Pacemaker activity then spreads to other areas of the heart via the conduction system, and finally triggers the contraction of the cardiac muscle. The myogenic heart consists of many cell types capable of pace-

maker activity in the SAN, the His bundle, the atrio-ventricular node (AV node), and the Purkinje fibers. However, the faster intrinsic rate of SAN pacemaker cells determines the heart rate in physiological conditions. Pacemaker cells express a specific set of ionic channels. These channels have been extensively described during the past fifteen years, due to patch clamp studies on isolated rabbit pacemaker cells and more recently in guinea pig.[184] Particularly, five classes of ionic channels are involved in the regulation of pacemaker activity: the hyperpolarization-activated channel ($I_f$), two distinct potassium channels ($I_{Kr}$ and $I_{Ks}$), the G protein-gated potassium channel which is activated by muscarinic agonist ($I_{KACh}$), the sustained inward current ($I_{st}$) and finally, $I_{Ca,T}$ and $I_{Ca,L}$. Consistent with what observed in working cardiac myocytes, $I_{Ca,L}$ is activated by stronger depolarisations than $I_{Ca,T}$ and is sensitive to DHPs. $I_{Ca,T}$ is activated at negative voltages and is sensitive to $Ni^{2+}$ ions.

Another fundamental property of pacemaker cells is the opposite regulation of the spontaneous activity by sympathetic and parasympathetic neurotransmitter receptors agonists. To date, two different pathways underlying autonomic regulation of heart rate have been identified; a fast pathway that is linked to activation of the muscarinic receptor and leads to direct opening of $I_{KACh}$ channels by G-proteins, and a slower pathway which depends from intracellular AC activity and cAMP production. The opening of $I_{KACh}$ channels in pacemaker cells is stimulated by both muscarinic and adenosine receptor agonists and leads to slowing of automaticity by causing membrane hyperpolarization.[185,186] Intracellular cAMP production is stimulated by β-adrenergic receptor agonists and leads to facilitation of $I_f$ channel opening by direct binding of cAMP to the channel[187,188] and activation of PKA which, in turn, stimulates $I_{Ca,L}$ and $I_{st}$ by phosphorylation (for review see refs. 187-189). The chronotropic effect of β-adrenergic stimulation is thus supposed to depend from both the direct activation of $I_f$ by cAMP and channel phosphorylation of $I_{Ca,L}$ and $I_{st}$. Consistently, the negative chronotropic effect following muscarinic receptor activation will depend from both $I_{KACh}$ activation, $I_f$ inhibition and $Ca^{2+}$ channel dephosphorylation. We will now closely discuss the available data on the role of $Ca^{2+}$ channels in the generation and regulation of cardiac pacemaking.

### *Role of $I_{Ca,L}$ in the Generation and Regulation of Pacemaker Activity*

Early studies on the effects of the β-adrenergic agonist adrenaline on pacemaking of SAN tissue strips first suggested that $Ca^{2+}$ currents are involved in the generation of automaticity.[190,191] Action-potential clamp experiments conducted on isolated rabbit pacemaker cells have shown that $I_{Ca,L}$ is present during the diastolic depolarization.[192] Recently, three lines of evidence further support the view that $I_{Ca,L}$ plays an important role in the generation of pacemaker activity. First, pharmacological inhibition of $I_{Ca,L}$ significantly slows pacemaker activity of isolated rabbit SAN cells in vitro.[193] Second, the dihydropyridine $Ca^{2+}$ channel antagonist nicardipine induces bradycardia in anaesthetized mice.[194] Third, it has recently been shown, in rabbit pacemaker cells, that $I_{Ca,L}$ is activated from a threshold of about -50 mV and that, due to this apparently "low" voltage dependency for activation, $I_{Ca,L}$ supplies sizable inward current during the diastolic depolarization.[195]

As it is the case in the working myocardium, $I_{Ca,L}$ in the SAN is sensitive to cAMP-dependent regulation by a mechanism requiring the activity of PKA.[196] Together with that of $I_f$, the stimulation of $I_{Ca}$ by adrenaline has been proposed to play a fundamental role in the β-adrenergic regulation of pacemaking.[191] To this respect, it is interesting to note that $I_{Ca,L}$ and $I_f$ show similar sensitivity to application of Isoproterenol.[197] Also, given that $I_f$ regulation by internal cAMP does not require protein phosphorylation, it has been proposed that fast heart rate regulation by β-adrenergic agonists does not imply $I_{Ca,L}$ rather, it mainly requires $I_f$.[197] However, this hypothesis would need to be experimentally verified.

The intracellular signalling pathway underlying muscarinic regulation of $I_{Ca,L}$ has been recently described into detail.[198,199] This pathway is dependent on NO production and subsequent cAMP down regulation by a cAMP-specific phosphodiesterase, a phenomenon that will inhibit cAMP-dependent phosphorylation of $I_{Ca,L}$ channels. The relative contribution of $I_{Ca,L}$,

$I_f$ and $I_{KACh}$ to the muscarinic regulation of pacemaker activity has been investigated in two different studies.[185,197] Particularly, it has been proposed that, for low concentrations of applied ACh, $I_f$ is the only current involved in rate slowing. For higher doses of ACh, in addition to opening of $I_{KACh}$ channels, $I_{Ca,L}$ inhibition will also be involved. However, this view has been recently challenged in an in vivo study employing a genetically modified mouse strain lacking the cardiac $I_{KACh}$.[200] Indeed, it has been shown that these mice are unable to regulate heart rate in a fast time scale, thus indicating that $I_{KACh}$ plays a fundamental role in the beat-to-beat regulation of cardiac pacemaking.

## *Role of $I_{Ca,T}$ in the Generation of Pacemaker Activity*

Hagiwara, Irisawa & Kameyama provided the first assessment of the role of $I_{Ca,T}$ in pacemaking in their classical paper on rabbit SAN cells.[161] $I_{Ca,T}$ was activated for a negative voltage interval (about -50 mV) thus overlapping most of the diastolic depolarization range. These authors also reported slowing of automaticity by the T-type $Ca^{2+}$ channel blockers tetrametrine and $Ni^{2+}$ at concentrations that did not affect $I_{Ca,L}$. On the basis of these observations, they concluded that $I_{Ca,T}$ plays a significant role in the generation of pacemaker activity. Doerr and coworkers[192] also attempted to measure $I_{Ca,T}$ in action potential clamp experiments by using extracellular 40 μM $Ni^{2+}$. They reported measurable $I_{Ca,T}$ in the diastolic depolarization and suggested that both $I_{Ca,L}$ and $I_{Ca,T}$ participated in the diastolic depolarization. However, we can expect that $I_{Ca,T}$ would be almost completely inactivated by the positive maximum diastolic potentials observed in most primary pacemaker cells (between -50 and -60 mV),[161] thus complicating the interpretation of these experimental observations. One possibility is that $I_{Ca,T}$ participates to the diastolic depolarization by a "window" current component.[162] However, the existence of "window" $I_{Ca,T}$ in primary pacemaker cells has not yet been demonstrated. In summary, the exact role of $I_{Ca,T}$ in primary pacemaking still needs to be elucidated. However, the robust expression of $I_{Ca,T}$ in mouse pacemaker cells[201] suggests that this current may also be involved in pacemaking in physiological situation where the maximum diastolic potential undergoes spontaneous hyperpolarization (e.g., muscarinic receptor activation) or in automaticity of atrial pacemakers. To this respect, Huser and coworkers have recently been able to study the contribution of intracellular $Ca^{2+}$ release to automaticity of cat latent pacemaker cells.[202] They described intracellular $Ca^{2+}$ release during the late phase of diastolic depolarization. $Ca^{2+}$release was abolished by extracellular $Ni^{2+}$ ions, thus indicating that $I_{Ca,T}$ was triggering intracellular $Ca^{2+}$ release in the diastolic depolarization. Such a mechanism, may be also operant in primary pacemaker cells in the SAN.

## *Future Directions*

In order to gain new insights into the generation and regulation of pacemaker activity, it would be extremely important to provide genetic evidence linking the activity of a given ionic channel gene to specific alteration of heart rate. The use of genetically modified mouse strains would thus constitute a new and powerful tool to investigate the role of specific genes coding for ionic channels in the regulation of heart rate. To this respect the recent isolation and electrophysiological description of mouse SAN pacemaker cells opens the way to the possibility of studying cardiac automaticity in genetically modified mice[201] Gene families coding for ionic channels involved in heart rate regulation have been cloned, and expression of some of these genes has been consistently found in cardiac tissue (for review see ref. 203). With respect to $Ca^{2+}$ channels, the $Ca_v1.2$ gene plays a major role in the determination of the cardiac $I_{Ca,L}$. However, the $Ca_v1.3$ gene is also expressed in atrium[204] and in SAN.[205] The $Ca_v3.1$ gene is the major gene expressed in myocardium[154,158] and in SAN tissue.[205] The $Ca_v3.2$ gene has also been detected in mouse SAN.[205] Two different strains of knockout mice lacking $Ca_v1.3$ channels have been developed.[204,206] Both strains present striking SAN dysfunction. Indeed, both bradycardia and sino-atrial arrhythmia have been observed in $Ca_v1.3^{-/-}$ mouse strains in vivo.[204,206] Secondly, slowing of pacemaker activity measured in isolated atria[204] and intact

SAN[206] have been reported. Quantification of $I_{Ca,L}$ in pacemaker cells from one of these knockout strains showed that current activation is shifted positive with respect to wild-type pacemaker cells, an observation which is consistent with the existence of a low-voltage-activated $I_{Ca,L}$ component coded by the $Ca_v1.3$ gene as reported using recombinant $Ca_v1.3$ channels[207,208] A mouse strain lacking $Ca_v3.1$ channels has been recently described.[206] The cardiac phenotype of this strain still needs to be described.

In summary, we can expect that the use of genetically modified mouse strains will allow a better understanding of the ionic mechanisms underlying cardiac pacemaker activity. More generally, understanding the basis of automaticity may yield important indications in the development of new drugs that specifically influence heart rate without affecting cardiac inotropism. Indeed, ionic channels showing preferential expression in pacemaker tissue compared to the myocardium may constitute suitable targets for these drugs. This kind of chronotropic selectivity could be particularly important in the treatment of ischemic heart disease.[209]

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# CHAPTER 21

# Post-Genomic Insights into T-Type Calcium Channel Functions in Neurons

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## Abstract

Genomic mining, the process of sifting through billions of genomic and EST sequences of several different species has led to the molecular identification of a family of low voltage activating channels, more commonly referred to as T-type channels. Historically, these channels were initially identified through the use of the patch-clamp technique on various neuronal preparations. They were characterized by their small conductance, rapid voltage-dependent inactivation, a small window current and slow deactivation kinetics and their remarkable property of being able to open at membrane potentials just above the resting membrane potential of neurons. This property would allow for the entry of $Ca^{2+}$ without the initiation of an action potential triggered by the opening of sodium channels. Thus the activity of these channels would contribute to modifying membrane excitability, allowing $Ca^{2+}$ signaling events to occur at subthreshold potentials, and potentially modulate waveform patterns. Now, with the clones in hand, we have entered an exciting time where the molecular machinery can be dissected, modified and manipulated not only to investigate their biophysical properties, but to appreciate their role in a diverse range of cellular processes, find novel and useful therapeutic and pharmacological reagents, hunt down subunits and modifying proteins, study their localization and trafficking and incorporate these findings into the emerging field of systems biology where their roles can be placed in the context from a single neuron, to the organ, to the organism.

## Introduction

Calcium influx via Low Threshold Spikes (LTS) into neurons near their membrane resting potential has been implicated more than 20 years ago in the rhythmic pacing of inferior olivary neurons.[1,2] These studies established that hyperpolarizing current injection evoked at negative voltages results in a rebound excitation depolarization of a small amplitude, slow kinetics and relatively long duration that is crowned at more positive potentials by fast and large sodium spikes. These particular action potentials or Low Threshold Spikes, where shown to be insensitive to sodium and potassium channel blockers but completely eliminated upon removal of extracellular calcium or in the presence of inorganic calcium channel blockers. Barium ions can substitute to calcium suggesting that the calcium channel underlying this LTS is equally permeable to both ions with similar current kinetics. This LTS and the low voltage gated calcium entry associated with it have been recognized as a widely distributed mechanism of neuronal excitability of the central and peripheral nervous system.

The development of the patch clamp technique in the mid to late 70s helped to isolate and characterize the functional properties of the particular calcium channels gated at these negative

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voltages. Accordingly they have been named Low Voltage Activated (LVA) or T-types in reference to their (T)ransient kinetics and (T)iny conductances. In agreement with the description of LTS, the biophysical characterization of T-type channels showed that their hallmark is a steady state activation and inactivation at negative potentials resulting in a strong channel inactivation at the membrane resting potential, peculiar kinetics (i.e., slow activation kinetics near the membrane resting potential, fast activation and inactivating kinetics at more positive potentials, slow deactivation), and overlapping activation and inactivation curves for a range of potential between –70 and –50 mV generating a steady window current at resting membrane potentials of most excitable cell types. Therefore these biophysical characteristics define two types of calcium influx important to take into consideration to understand T-type channel function. One is transient during action potentials and the other is a small but sustained current at resting potentials. The specific properties of T-type channels suggested their implication in a number of physiological or pathological conditions such as absence seizure epilepsy for example, but two major obstacles to a deeper understanding of their role remained. The first one was the lack of pharmacological tools selective to these channels, and for a long time the lack of molecular substrate for these channels. With all the classical approaches of molecular cloning having proved futile in finding T-type channels, efforts turned to blast and word searching the sequences being deposited at the National Center of Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) databanks, using what is now termed 'in silico' cloning. Thus, in combination of with the near completion of several genomes sequences and millions of ESTs (expressed tag sequences) that exploration of sequence data bases led to the identification of three genes encoding for pore forming alpha1 subunits of T-type channels namely $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ (or $\alpha_{1G}$, $\alpha_{1H}$, and $\alpha_{1I}$ in the former nomenclature).[3-10] This approach pioneered by Ed Perez Reyes's group paved the way for investigating the function and properties of T-type channels in recombinant and native tissues.

## Contributions of Recombinant Channel Studies

One of the first achievements of the T-type channel cloning is an explanation of T-type channel heterogeneity observed in native systems. In the absence of selective pharmacology, functional studies in different models suggested an apparent diversity. These observations were based on differential sensitivity to nickel or amiloride block, diverse inactivation kinetics, and different permeation profiles to divalent ions.[11] Thus, functional expression of recombinant T-type channels has been clearly instrumental to a more clear-cut definition of the characteristics of pure population of channels that can not be distinguished in native cells. For example, high affinity block by Nickel ions is only present in $Ca_v3.2$ channels, while $Ca_v3.1$ and $Ca_v3.3$ are both at least 20 fold less sensitive.[12] Also, $Ca_v3.3$ differs dramatically from $Ca_v3.1$ and $Ca_v3.2$ by slower activation and inactivation kinetics. Isolation and analysis of transcripts by conventional screening of libraries and PCR of specific regions revealed the presence of alternative splicing within each family member.[6,7,14,15] Expression studies of these isoforms revealed important differences in their electrophysiological properties. For example, modulation of steady state inactivation of some $Ca_v3.1$ variants was shown to reduce strongly their potential window current and their availability to be activated during an action potential.[16] In the same line of observations, functional properties of $Ca_v3.3$ were shown to be modulated by exon splicing.[17-19] Transcripts with premature stops or deletion of several exons leading to truncated channels have been reported[20] (Monteil, unpublished observations). Although the effect of these truncated channels remains to be studied, it can be hypothesised that they can function as dominant negatives as reported for some High Voltage Activated (HVA) calcium channels.[21,22]

An other step toward understanding of T-type calcium channel contribution to neuronal excitability has been to stimulate recombinant channels with a variety of neuronal action potential waveforms. These studies highlighted how the three T-type channel forms can induce distinct calcium entry. In terms of kinetics the current generated by the $Ca_v3.3$ channels differs from those associated to $Ca_v3.1$ and $Ca_v3.2$ expression. Using isolated or trains of action po-

tentials, fast or slow EPSPs as stimuli, a number of similar approaches showed that $Ca_v3.3$ can undergo steady and facilitating calcium influx during intense electrical activity. By contrast, $Ca_v3.1$ and $Ca_v3.2$ are more rapidly inactivated but respond better to isolated membrane activities.[23-25] In the absence of specific pharmacology efforts to understand the specific role of a T-type current from of given neuronal type have relied on computer models.[26-28] Properties of recombinant T-type channels have therefore been used to see how they interplay with other conductances to promote rhythmic activity in such neuronal cell models. These studies showed that $Ca_v3.1$ and $Ca_v3.3$ respective properties are compatible with discharge patterns of relay and reticular neurons of the thalamus.[6,25]

Unlike many sodium channels and HVA calcium channels, poor pharmacology has hampered efforts to biochemically characterize the LVA complexes and electrophysiologically tease out the contribution of members of this family to membrane excitability. Here again the use of expressed T-type channels has provided powerful screening assays to identify selective antagonists or agonists for these channels. Conventionally, cone snail, spider and scorpion toxins which contain an array of proteinacious material are isolated, separated by HPLC and then each extract applied onto cells expressing either a single cDNA, as in a stably or transiently transfected cell line or onto a cell expressing several different channel subtypes. Once the interesting fraction has been identified, the isolated peptide fraction is sequenced and resynthesized. The application of extracts purified from the scorpion *Parabuthus transvaalicus* led to the identification of Kurtoxin which was shown to block cloned $Ca_v3.1$ and $Ca_v3.2$ channels at sub-micromolar concentrations.[29] A recent study of two related toxins (KLI and KLII) purified from an other scorpion venom (*Parabuthus granulatus*) showed that $Ca_v3.3$ is much less sensitive suggesting that this toxin can discriminate between members of the $Ca_v3$ subfamily.[30] However Kurtoxin and KLII appeared to also block sodium channel and HVA calcium channels[29-31] rendering them almost useless to dissect selectively the contribution of T-channels to a given physiological function. Efforts in toxin identification using such screens are underway in different laboratories, and in the long term, we can hope to isolate selective toxins for each T-channel isotypes. Although specific toxins have a great interest in fundamental research, therapeutic perspective resulting from functional modification of T-type channels mainly concerns organic chemical compounds. For this aspect, recombinant T-type channels can be instrumental of high throughput screening. These screens mainly based on cytosolic calcium monitoring can certainly exploit the window current of expressed channels at resting membrane potentials that was shown to constitutively increase basal intracellular calcium.[32] Non selective blockade of native T-type channels by a range of neuroactive molecules ranking from anti-epileptics, neuroleptics, anesthetics, or anti-hypertensive molecules have been reported.[11,33] For the moment, recombinant channels have only been used to test the blocking affinities of some of these compounds.[34-36] Two noteworthy clinically relevant molecules are Mibefradil and Ethosuximide. The first one has been developed as an anti-hypertensive therapeutic agent. The mechanism of action proposed was an inhibition of T-type channels.[37] The use of recombinant calcium channels clearly showed the lack of selectivity for T-type channels compared to HVA channels,[38-42] although the preferential affinity for the inactivated state promotes a stronger block of T-type channels.[43] Ethosuximide is an anticonvulsant used in the treatment of absence seizure. Its anti epileptic activity has been proposed to result from T-type channels inhibition in the thalamus,[44,45] although it remains controversial.[46] The use of recombinant T-type channels showed that Ethosuximide and its metabolites are able to block all $Ca_v3$ members at clinically relevant concentrations.[47] In the absence of more selective blockers, these two drugs are still used as tools to explore the role T-type channel in physiologic functions. For example they have been administered in rodents intraperitoneally or intrathecally in studies on the involvement of T-channels in nociception.[48-50] All the results argue for a role of T-type channel in nociception as initially proposed following their identification in isolated dorsal root ganglion neurons.[51-53] More recent studies have reinforced and elaborated on the role of T-type channels in nociception. It as been shown first that antisense transfection in primary

culture of nodose ganglion neuron reduces strongly their T-type currents and their excitability.[54] Northern blots, RT-PCR, and in situ hybridization showed that sensory neurons as well as neurons from the superficial laminae of the dorsal horn express $Ca_v3.2$ almost exclusively.[3,54,55] Therefore recombinant $Ca_v3.2$ was useful to identify new regulatory mechanisms of DRG T-type channels possibly implicated in pain sensing. That was the case for the characterization of their redox modulation,[56] or their modulation by nitric oxide.[57] Other forms of modulation of DRG T-type channels, such as their potential inhibition by the opioid like receptor ligand (ORL) nociceptine,[58] were however impossible to reproduce on expressed $Ca_v3.2$.[59]

Finally, cloned T-type channels have been used to try to identify their possible modulation by intracellular cascades or endogenous circulating biomolecules. Besides redox and nitric oxide modulation, $Ca_v3$ channels where shown to be sensitive to arachidonic acid (AA), an important second messenger.[60] As anandamide, a precursor of AA, has been shown to be a natural ligand of several ion channels including potassium channels and vanilloid receptors, Chemin and coworkers studied its effects on expressed $Ca_v3$ channels.[61] Their results show that $Ca_v3$ channels have a high affinity biding site for anandamide, and that the mechanism of current block by this endocanabinoid is state dependent. The binding of this molecule significantly effects the kinetic properties of the T-type channels, and would alter the contribution of the current to neuronal excitability. Future works will undoubted address issues in recombinant systems on the role of specific residues and regions of T-type channels important in these types of modulation. Other modulators of T-type channels described in isolated neurons will certainly benefit from the availability of T-type channel cDNAs to elucidate the underlying mechanisms.

## T-Type Channels and Neurophysiology

Understanding the role of T-type channels in the global context of physiology is the step forward the cloning of their constitutive genes. Although no selective pharmacology is available yet, identification of the $Ca_v3$ gene family opened the door to their functional elimination using genetic manipulations. This was firstly achieved with $Ca_v3.1$ whose gene (cacna1g) was inactivated in the mouse.[62] The $Ca_v3.1$ (-/-) mice have been used to address the issue of the role of T-type calcium channels in the genesis of spike-and-wave discharges (SWDs), a sign of absence seizures in the thalamocortical network. According to the predominant expression of $Ca_v3.1$ in thalamocortical relay neurons[55] and its increased expression in animal strains genetically predisposed to absence seizures,[63,64] the other T-type channels have been postulated to only play a minor role in this phenomenom.

The results show that thalamic relay neurons are deficient in T-type channels and consequently have profound modifications of their excitability. It was notably demonstrated that low threshold spikes (LTS) are absent in these neurons as well as their burst-mode of firing action potentials. The major consequence of these modifications is a resistance of $Ca_v3.1$ (-/-) mice to the generation of SWDs induced by $GABA_B$ receptor stimulation, a hallmark of absence seizures. Therefore, this study unambiguously demonstrated the epileptogenic role of T-type channels in the genesis of absence seizure, and further validates T-type channels as an antiepileptic drug target. Moreover, in murine models of absence seizure such as the *tottering*, *lethargic*, or *stargazer* strains carrying mutations in various high-voltage-activated calcium channels subunits genes, an increase of T-type channel current in thalamic relay neurons was recently reported.[65] Other forms of epilepsy may be also concerned by plasticity of T-type channel expression. For example after induction of chronic status epilepticus (SE) in the pilocarpine model, hippocampal or subicular pyramidal neurons changes their intrinsic firing properties with an augmented proportion of bursting-mode and after depolarization potentials. This change has been associated with an increase of nickel sensitive T-type currents,[66,67] attributable to a transcriptional regulation of $Ca_v3.2$.[68] Therefore bursting-mode may be controlled by different T-type channel isotypes in different brain regions. Conversely, in other neurons, selective coupling of T-type channels with calcium sensitive potassium channels can hamper bursting by generating after-hyperpolarization potentials.[69,70]

Generation of genetically modified mice has just started with $Ca_v3.1$ and more recently $Ca_v3.2$.[71] We can hope from these models and certainly from the future ones (conditional knock outs, transgenic animals, etc.) to further understand the pertinence of T-type channel expression. These aspects concern an array of neurophysiological functions ranging from neuronal development and differentiation,[72,73] excitability as described for their epileptogenic role but also in long lasting synaptic plasticity,[74-77] neurotransmitter release,[78,79] or multiple intracellular calcium dependant cascades.

In summary, understanding of T-type channel cellular functions tremendously benefited from the identification of their molecular structure, and the exciting challenge is now to unravel their role in the context of integrated physiology.

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# CHAPTER 22

# Voltage-Gated $Ca^{2+}$ Channels of the Vertebrate Retina:

## From the Genetics of Blindness to Encoding the Visual World

**Melanie E.M. Kelly and Steven Barnes**

## Abstract

$Ca^{2+}$ channel-mediated synaptic transmission and calcium signaling in the retina reveals neurobiological solutions to a diversity of signal processing challenges. In rod and cone photoreceptors, graded and sustained transduction signals, generated over a narrow range of membrane potentials, are synaptically transmitted to second order horizontal and bipolar cells. In rods, novel $Ca^{2+}$ channels ($\alpha_{1F}$; $Ca_v1.4$) have been implicated at this synapse through genetic studies of people with night blindness, while in cones, biophysically similar $\alpha_{1D}$ ($Ca_v1.3$) channels appear to be utilized for this purpose. Although the synaptic output of horizontal cells appears to be $Ca^{2+}$-independent, these cells express L-type $Ca^{2+}$ channels. Bipolar cells, which are also graded potential neurons, utilize both L- and T-type $Ca^{2+}$ channels at their output synapse where the transience of the neural signals is enhanced. At the ganglion cell level, network integration of excitatory and inhibitory signals generates the throughput of the optic nerve. T-type $Ca^{2+}$ channels together with several sub-types of high-voltage-activated $Ca^{2+}$ channel subtypes play roles in signal integration and are modulated by neurotransmitters and signaling molecules such as nitric oxide (NO). Pervasive in developing retinal cells and in retinal precursor cells, T-type $Ca^{2+}$ channels ($\alpha_{1G}$ and $\alpha_{1H}$; $Ca_v3.1$ and 3.2) undergo down-regulation as the cells mature into differentiated neurons and glia. Since all neuronal $Ca^{2+}$ channel subtypes appear to be represented in the retina, this accessible and well-understood tissue offers many advantages to aid in our understanding of the physiological role of $Ca^{2+}$ channels in the brain.

## Introduction

The retina, the thin, transparent film of neural tissue at the back of the eye, is composed of a highly organized structure of neurons and glia that express a diversity of voltage-gated calcium channels ($Ca^{2+}$ channels). $Ca^{2+}$ channels provide the principle pathway for $Ca^{2+}$ influx into retinal neurons and play fundamental roles in visual signal processing in the retina. Recent molecular biological and genetic discoveries, together with knowledge garnered from electrophysiological and behavioral experiments, has now provided us with a more complete picture of the $Ca^{2+}$ channel subtypes and their function in specific retinal neurons and glia.

There are at least six major classes of neurons and three types of glia in the retina (Fig.1). Light hyperpolarizes rod and cone photoreceptors by causing the closure of CNG channels located in the outer segments of both cell types. Rods, which mediate vision under low light

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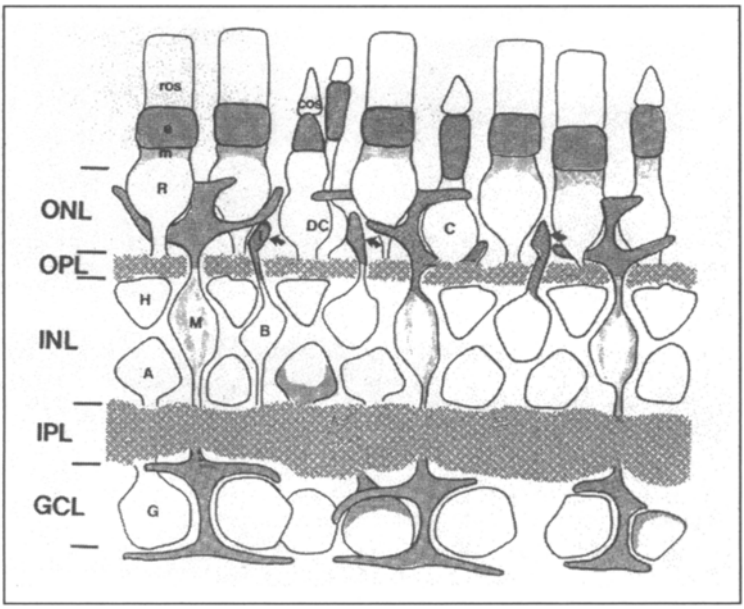



Figure 1. Organization of the retina. This schematic view of a retinal cross section shows the five principle retinal neurons and glia. ONL, outer nuclear layer: Rod photoreceptors (R) are shown with subcellular compartments (*ros*, rod outer segment, the site of visual transduction; *e*, ellipsoid, a mitochondrial rich region; *m*, myoid, a portion of the rod that undergoes circadian extension and retraction). Single cones (C) and double cones (DC) are indicated (cos specifies cone outer segment). Rods and cones synapse with second order cells in the outer plexiform layer (OPL). Within the inner nuclear layer (INL) lie the somata of horizontal cells (H), bipolar cells (B), amacrine cells (A), and Müller glial cells (M; shown with Landolt club – *l*). In the inner plexiform layer (IPL), synapses are made between the neurites of amacrine, bipolar, and ganglion cells (G). Ganglion cell bodies reside in the ganglion cell layer (GCL). The Müller cells span the entire depth of the retinal layers. The shading in this figure reflects the localization of nitric oxide synthase, the nitric oxide producing enzyme, as shown originally in Kurennyi et al, 1995. Nitric oxide was shown to modulate some $Ca^{2+}$ channels in the retina (Reproduced with permission from John Wiley & Sons).

conditions, and cones, used for color vision in medium to brightly-lit conditions, reduce their glutamate release as they hyperpolarize in response to light. Second order horizontal cells, which are involved in the enhancement of contrast in the visual scene by providing lateral and chromatic inhibition, respond to the glutamate released by photoreceptors at sign-conserving synapses. The horizontal cells send signals back to cone photoreceptors and modulate the activity of the synapse. Second order bipolar cells, upon which inputs from many photoreceptors converge, further tune and then transmit the integrated signals to the spiking amacrine and ganglion cells of the inner retina. Müller cells, the radial glia that span the retina, actively maintain the chemical milieu of the retina.

$Ca^{2+}$ channels found in the retina include all known mammalian brain $Ca^{2+}$ channel subtypes.[1] The nine identified pore-forming $\alpha_1$ subunits are usually accompanied by auxiliary or regulatory subunits. $Ca^{2+}$ channel types identified in retina include: the high voltage activated (HVA), dihydropyridine (DHP)-sensitive L-type $Ca^{2+}$ channels formed from $\alpha_{1C}$ ($Ca_v1.2$), $\alpha_{1D}$ ($Ca_v1.3$) and $\alpha_{1F}$ ($Ca_v1.4$) subunits; HVA P/Q-type $\alpha_{1A}$ ($Ca_v2.1$) and HVA N-type $\alpha_{1B}$ ($Ca_v2.2$) subunits; as well as the intermediate voltage-activated R-type $\alpha_{1E}$ ($Ca_v2.3$) and low voltage-activated (LVA) T-type $\alpha_{1G}$ ($Ca_v3.1$), $\alpha_{1H}$ ($Ca_v3.2$), and $\alpha_{1I}$ ($Ca_v3.3$) subunits.

This chapter describes the expression of $Ca^{2+}$ channels in retinal neurons, with emphasis on the properties of the channels in photoreceptors and bipolar cells, the graded potential neurons of the outer retina, as well as ganglion cells, the cells responsible for the neuronal output of the retina. An overview of evidence that describes differential expression of $Ca^{2+}$ channel subtypes during retinal development is included.

## $Ca^{2+}$ Channels in the Graded Potential Neurons of the Outer Retina

The neurons of the outer retina, including rods, cones, and horizontal and bipolar cells, do not generate action potentials and, as in other non-spiking neural networks, signal via graded changes in their membrane potential.[2] Other prominent features of this synaptic regimen include sustained signals, exhibiting little inactivation, and a relatively narrow range of membrane potentials over which rods and cones signal. Photoreceptors and bipolar cells utilize a special type of synapse to support the continuous release of neurotransmitter that accompanies their non-spiking signals. It contains a specialized structure called a ribbon that orchestrates the flow of transmitter filled vesicles to the release site. Here, $Ca^{2+}$ channels mediate the $Ca^{2+}$ influx required for vesicle fusion. In photoreceptors, L-type $Ca^{2+}$ channels (and possibly CNG channels[3,4]) appear to mediate synaptic release, while both T- and L-type $Ca^{2+}$ channels have been shown to contribute to exocytosis from bipolar cell presynaptic terminals. Horizontal cells, on the other hand, do not exhibit presynaptic membrane specializations and release neurotransmitters in a $Ca^{2+}$-independent manner.[5] They express non-classical L-type $Ca^{2+}$ channels,[6] presumably to serve some other calcium signaling roles.

### *A New L-Type $Ca^{2+}$ Channel at the Photoreceptor Synapse*

Neurotransmitter release from vertebrate photoreceptors is mediated by non-inactivating, HVA calcium channels that are considered to be of the L-type due to their dihydropyridine (DHP) sensitivity.[7-12] In both rods and cones, the L-type $Ca^{2+}$ channels have a distinct pharmacological profile comprised of relatively weak sensitivity to DHPs and reversible block by the N-type $Ca^{2+}$ channel blocker, ω-conotoxin GVIA (Fig. 2).[11,12] This profile most closely resembles that of $a_{1D}$ subunit-containing $Ca^{2+}$ channels ($Ca_v1.3$).[13] Antibodies directed against $a_{1D}$ subunits in mammalian retina, however, label only in cones and not in rods,[14,15] although antibody specificity must be considered in non-mammalian studies.[16] In auditory hair cells, the L-type channels have been identified to be formed from splice variants of $a_{1D}$ primary subunits.[17,18] It is not surprising, then, that the phenotype of the $\alpha_{1D}$ knockout mouse includes deafness.[19] Visual defects were not noted in this knockout mouse, although if $\alpha_{1D}$ is the cone $Ca^{2+}$ channel, this would be consistent with the rod-dominated vision present in rodents.

In support of a different L-type $Ca^{2+}$ channel expressed in rods, genetic analysis of people with incomplete X-linked congenital stationary night blindness (CSNB2) has revealed the mutation responsible for the condition: the CACNA1F gene codes for a new L-type $Ca^{2+}$ channel forming subunit, the $\alpha_{1F}$.[20,21] CSNB is a recessive non-progressive retinal disorder characterized by loss of night vision and reduced day vision. Patients with the incomplete version of this disease, CSNB2, have decreased dark adaptation and their electroretinograms show no rod b-waves, indicating a decreased efficacy of synaptic transmission from rod photoreceptors.[22] Genetic analyses revealed that the locus for CSNB2 segregates with markers from Xp11.23, identified as a retina-specific $Ca^{2+}$ channel $a_1$ subunit gene (CACNA1F), the protein product of which showed high homology to L-type $Ca^{2+}$ channel $\alpha_1$ subunits. Analysis of CACNA1F among families with CSNB2 revealed that the loss of function mutations cause premature protein truncation (Fig. 3). Since the defining feature of CSNB2 is night blindness, which is a rod disease, this would suggest that in humans, the gene product of CACNA1F is the previously undefined and novel rod $Ca^{2+}$ channel. In support of $\alpha 1_F$ as the rod photoreceptor $Ca^{2+}$ channel, immunhistochemical staining using antibodies against the human and rat $\alpha_{1F}$ peptide was obtained in the cell bodies and synaptic terminals of photoreceptors in the rat

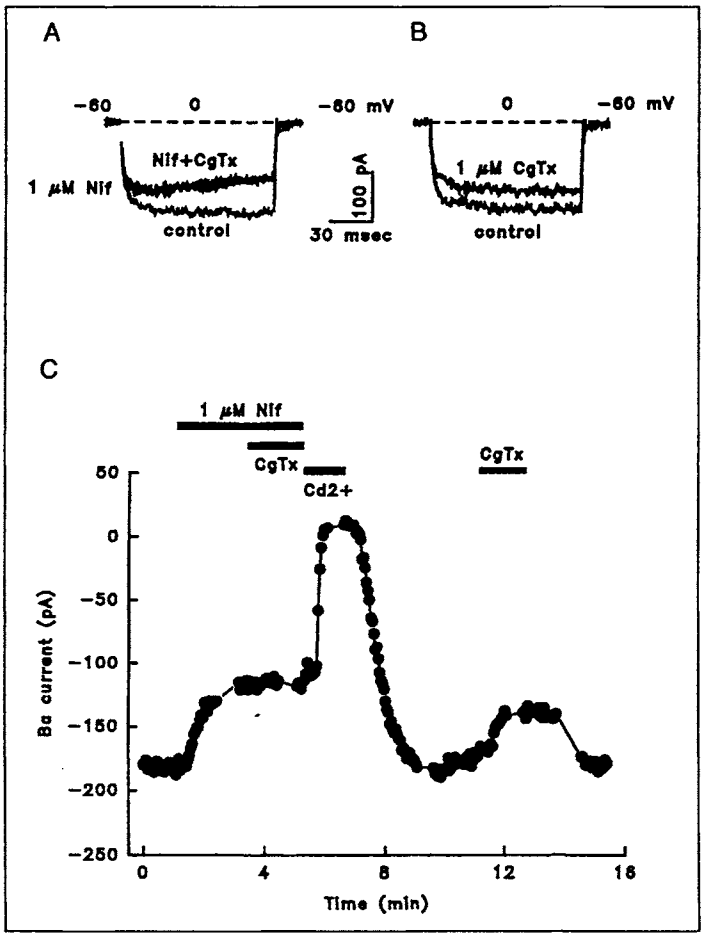



Figure 2. Cone L-type $Ca^{2+}$ channels show reversible block by ω-conotoxin GVIA. Panels A and B show current traces from isolated cone photoreceptors, recorded under voltage clamp during steps from –60 to 0 mV. Block of $Ca^{2+}$ channel current by ω-conotoxin GVIA (1 μM) is occluded by nifedipine. Panel C shows the time course of the entire experiment, with additions of nifedipine, nifedipine plus conotoxin, cadmium and conotoxin alone indicated by the black bars. From Wilkinson & Barnes, 1996 (reproduced with permission from Journal of General Physiology).

retina.[23] In the rat retina, differential staining using both antibodies may have revealed an additional $\alpha_{1F}$ isoform localized to the photoreceptor synaptic terminals.[23]

## *T-Type $Ca^{2+}$ Channels Can Mediate Release of Transmitter from Bipolar Cells*

Bipolar cells, second order retinal neurons that are responsible for transmitting the visual signal from photoreceptors in the outer retina to third order amacrine and ganglion cell neurons of the inner retina, express both L- and T-type $Ca^{2+}$ channels in distinct cellular compartments.[24-27] Bipolar cells have been classified as rod or cone types based on the source of their synaptic input and as ON or OFF types based on the polarity of their response to light.[28]

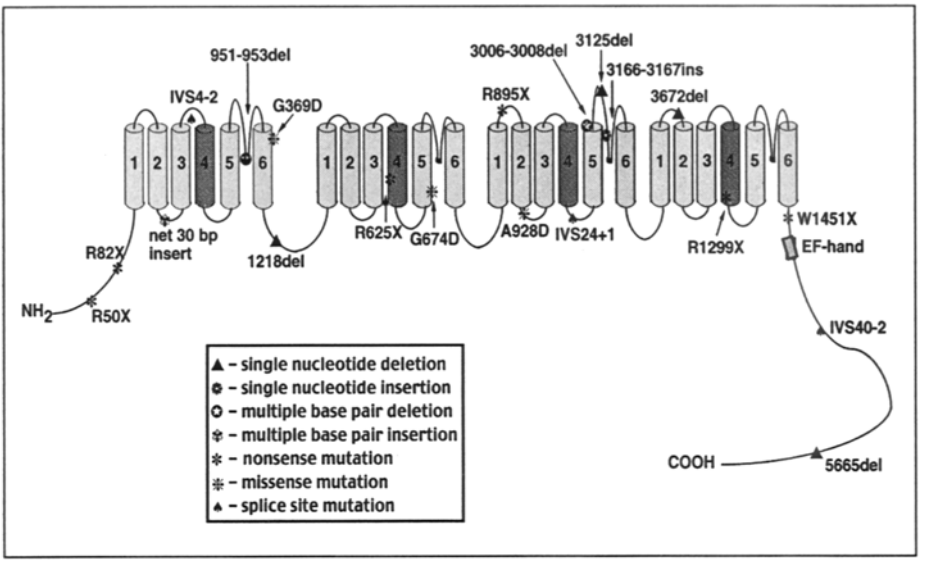



Figure 3. Mutations of the α1F $Ca^{2+}$ channel are responsible for the failed synaptic transmission of rod mediated vision. The four repeating motifs, each with six transmembrane spanning helices, of the predicted membrane topology of the channel subunit encoded by the CACNA1F gene as originally described.[20] Truncated, non-functional channels occur as a result of the deletion, insertion and mutation sites shown. The locations of all 20 mutations identified to date in patients with incomplete CSNB are indicated. Figure is from Boycott et al, 2001 (ref. 85; reproduced with permission of Human Genetics).

While it remains the rule in most neurons, including photoreceptors, that neurotransmitter release appears to be mediated by HVA channels, in bipolar cells, T-type $Ca^{2+}$ channels are also capable of mediating transmitter release.[29] In addition to previous reported L-type $Ca^{2+}$ channels,[30,31] expression of all three T-channel α1 subunits ($\alpha_{1G}$, $\alpha_{1H}$, and $\alpha_{1I}$) has been detected in bipolar cells using single-cell PCR techniques. The presence of T-channels, which activate at hyperpolarized potentials close to rest and undergo fast inactivation, endow these cells with specialized properties. Since the range of membrane potentials over which activation and inactivation occur overlaps, these channels can undergo rapid cycling between open, inactivated, and closed states, giving rise to continuous calcium influx, referred to as window current, in a range of negative membrane potentials where HVA channels do not normally activate.

Pan et al[29] showed that T-type $Ca^{2+}$ channel currents are present in mammalian retinal bipolar cell synaptic terminals and that activation of these channels in isolated axon terminals of rod and cone bipolar cells could trigger transmitter release. Membrane potentials in bipolar cells vary between –70 and –20 mV, well within the normal activation range of T-type $Ca^{2+}$ channels. Neurotransmitter release in response to depolarizations around –40 mV would therefore be expected to be mediated predominantly by T-type $Ca^{2+}$ channel currents, with stronger depolarizations giving rise to the additional activation of L-type $Ca^{2+}$ channels. In some OFF cone bipolar cells, which have dark membrane potentials near –40 and –50 mV, and hyperpolarize in response to light,[32] it is possible that T-channels may be predominantly responsible for presynaptic $Ca^{2+}$ influx and transmitter release. Heterogeneous expression of LVA and HVA $Ca^{2+}$ channels in the terminals of different subtypes of bipolar cells may contribute to the diversity of synaptic transmission observed between bipolar cells and third order amacrine cells and ganglion cells, in which both transient and sustained responses are recorded in response to sustained bipolar cell activity.[33,34]

## $Ca^{2+}$ Channel Subtypes in Spiking Cells of the Inner Retina

Visual information encoded by graded potentials in neurons of the outer retina is distributed to ganglion cells of the inner nuclear layer, which in turn transmit this signal to the various visual centers of the brain. Thus, ganglion cells are the final recipients of visual information in the retina and the receptive field organization of these neurons provides for both contrast enhancement and color opponency in retinal signal processing. Light responses in ganglion cells are shaped principally by glutamatergic synaptic input from bipolar cells,[33,35] the kinetics of which may be further modulated by glutamate receptor-mediated desensitization in ganglion cell dendritic arbors[36] and voltage-gated ion channels.[37,38] The inner retina appears responsible for spatial and temporal processing of the visual signal, and this task places additional requirements on the synaptic interactions, and thus on synaptic (and post-synaptic) $Ca^{2+}$ channels. Spike producing amacrine cells, which carry signals of temporal and spatial change laterally inhibit ganglion cells and feedback negatively onto bipolar cell synaptic terminals, and express L-type $Ca^{2+}$ channels.[39] Amacrine-amacrine synapses have been studied in detail and this model provides an important and insightful tool into the function of L-type $Ca^{2+}$ channels at a spiking synapse.[40]

### *Signal Processing by Multiple $Ca^{2+}$ Channel Subtypes in Ganglion Cells*

Action potential producing ganglion cells in vertebrate retina have been reported to express multiple $Ca^{2+}$ channel subtypes.[41] In salamander ganglion cells, ω-conotoxin GVIA and dihydropyridines act on separate types of $Ca^{2+}$ channels, consistent with expression of both N-type and L-type, respectively.[42,43] This is in contrast to cone photoreceptors of this same species in which a single type of $Ca^{2+}$ channel appears sensitive to both ω-conotoxin GVIA and dihydropyridines.[11] Results obtained in other studies of ganglion cells are also consistent with the notion that L-type $Ca^{2+}$ channels co-express with other types of $Ca^{2+}$ channels in these cells. For example, DHP-sensitive $Ca^{2+}$ channel current coexists with ω-conotoxin GVIA-insensitive current in turtle ganglion cells,[44] but coexists with ω-conotoxin GVIA-sensitive current in rat ganglion cells.[41,45]

Aside from the N- and L-type $Ca^{2+}$ channels, the identities of other $Ca^{2+}$ channel subtypes underlying the whole-cell $Ca^{2+}$ channel current in ganglion cells has not been clarified. However, a study using polyclonal antibodies against $\alpha_{1E}$ (which may mediate some R-type currents) in the tiger salamander retina revealed immunoreactivity for $\alpha_{1E}$ in the ganglion-cell containing inner plexiform layer of the retina.[46] However, this immunoreactivity was not specifically in ganglion cell bodies nor localized to ganglion cells processes and could have been confined to the processes of amacrine, bipolar or Müller cells.[47] In contrast, $\alpha_{1D}$ immunoreactivity was located in both synaptic layers (outer and inner plexiform layers) as well as ganglion cell bodies and optic nerve fibers. These findings are consistent with an in situ hybridization study showing $\alpha_{1D}$ expression in all nuclear layers of the rat retina.[46] The pattern of $\alpha_{1D}$ staining in the inner retina may be consistent with ganglion cell localization, although owing to the close association between Müller cell processes and ganglion cells structures, glial-localization of $\alpha_{1D}$ channels cannot be ruled out. L-type $Ca^{2+}$ currents and $\alpha_{1D}$ expression have been reported in cultured human Müller cells[48] and verapamil-sensitive HVA $Ca^{2+}$ currents have also been described in salamander Müller cells.[49]

### *Regulation of $Ca^{2+}$ Channels in Ganglion Cells: Glutamate, Adenosine and Nitric Oxide in Neuronal Integration and Pathophysiology*

A variety of retinal transmitters and neuromodulators, including glutamate acting via ionotropic and metabotropic receptors,[42,50-52] adenosine acting at A1 receptors,[52,53] as well as NO,[43] have been shown to regulate $Ca^{2+}$ channels in ganglion cells. Alterations in $Ca^{2+}$ channel activation, linked to changes in $Ca^{2+}$ signaling by ganglion cells, can result in changes in retinal output since the integrative activities of ganglion cells depend on a number of $Ca^{2+}$ mediated responses.

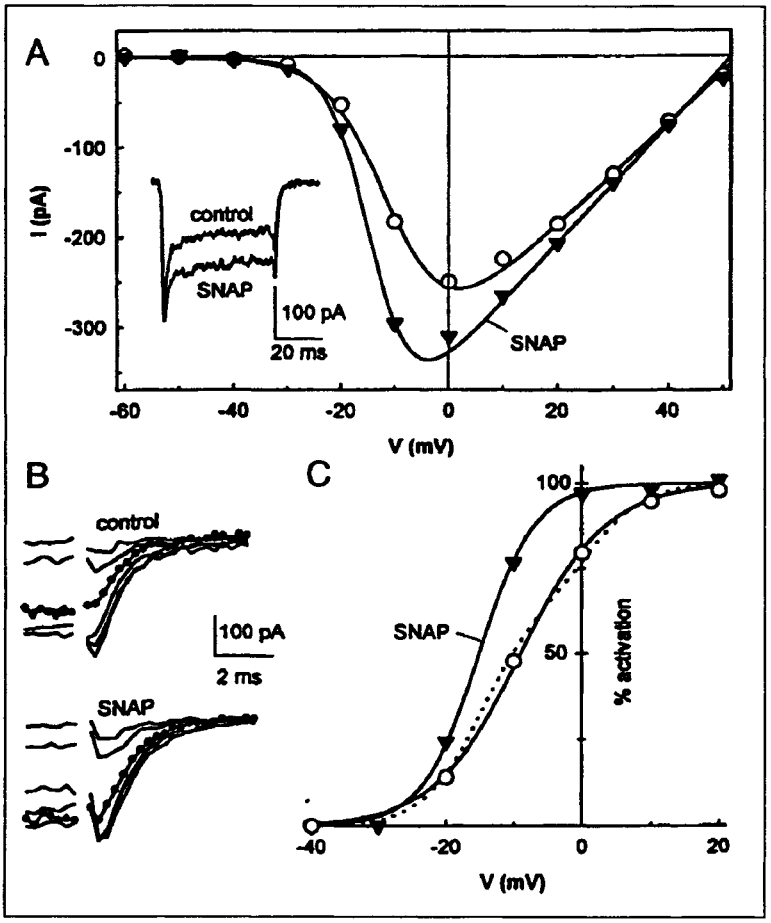



Figure 4. In retinal ganglion cells, the activation of N-type $Ca^{2+}$ channels is facilitated by NO. In panel A, the nitric oxide donor SNAP produces an increase and leftward shift in the current-voltage relation of the cell, an effect mediated by enhancement (facilitation) of approximately 30% of the total HVA current (corresponding to the N-type $Ca^{2+}$ channel population of ganglion cells). Panel C shows activation curves derived from the tail currents shown in panel B. These tail currents were recorded at –60 mV following voltage clamp steps to more positive voltages. The traces illustrated with dots were recorded at –10 mV in control and in the presence of SNAP. The activation curve recorded in the presence of SNAP was steepened and shifted relative to control. In a model where 30% of the channels underwent facilitation of activation with a 16 mV negative shift, such an effect produced by NO reflects an increase in open probability of N-type $Ca^{2+}$ channels. From Hirooka et al, 2000 (reproduced with permission from the American Physiological Society).

There remains considerable interest in the potential roles of $Ca^{2+}$-stimulated production of nitric oxide (NO) as an anterograde and retrograde messenger in retinal signal processing. Neuronal NO synthase (nNOS), the $Ca^{2+}$/calmodulin sensitive enzyme responsible for synthesizing NO from L-arginine, has been localized in specific retinal cells by the histochemical marker NADPH-diaphorase (Fig. 1) and by anti-NOS immunoreactivity.[54-58] Staining for NOS in the inner retina includes a small population of amacrine cells,[59,60] as well as the distal processes of Muller cells that lie in juxtaposition to ganglion cells. Independent of effects on cyclic nucleotide gated (CNG) channels, NO is now known to modulate ganglion cell N-type

$Ca^{2+}$ channels by facilitating their voltage-dependent activation via a soluble-guanylyl cyclase and PKG-dependent phosphorylation.[43] Previous work has detected transcripts for guanylate cyclase in the ganglion cell layer of the rat retina.[61] Thus, it would appear that NO produced in the vicinity of ganglion cells would have the potential to modulate $Ca^{2+}$ signaling via changes to the voltage-dependent gating of $Ca^{2+}$ channels.

Increases in NO that would facilitate the activation of $Ca^{2+}$ channels during depolarizations would in turn be expected to affect all $Ca^{2+}$- mediated pathways, including the activation of $Ca^{2+}$-dependent ion channels, altering the integration of synaptic signals impinging on ganglion cells. It is possible that plasticity of the synapses made onto ganglion cells could be dependent on post-synaptic $Ca^{2+}$ signaling, which appears to be itself NO dependent in these cells.

In addition to the obvious roles in retinal synaptic transmission, NO together with glutamate receptor activation, have also been implicated in retinal diseases such as glaucomatous optic nerve neuropathy and retinal ischemia, in which loss of ganglion cells can lead to blindness.[62-65] It is thought that part of the pathology associated with these diseases may result from chronic increased $[Ca^{2+}]_i$ in ganglion cells. Support for a contribution of $Ca^{2+}$ channels in retinal pathophysiology, has come from both in vitro and in vivo evidence, using ganglion cell culture models[66] and animal models of glaucoma and retinal ischemia, as well as clinical studies of a cohort of patients with open-angle glaucoma.[67] These studies show that $Ca^{2+}$ channel blockers decreased ganglion cell loss and appear to improve long-term outcome.

## Differential Expression of $Ca^{2+}$ Channel Subtypes during Retinal Development

Alterations in $Ca^{2+}$ channel currents and $[Ca^{2+}]_i$ have been demonstrated to occur in the cell cycle and during the embryonic development of a variety of organisms.[68-70] In some cell types, increases in $[Ca^{2+}]_i$ due in part to activation of $Ca^{2+}$ channels are important for the progression of the cell cycle,[71-73] and may thus contribute to the regulation of cell proliferation, migration and neurite outgrowth.[74-76]

### *$Ca^{2+}$ Channels in the Developing Retina*

Alterations in $Ca^{2+}$ channel currents has been reported in developing retina. During embryogenesis, multiple retinal cell types arise from the optic cup, an outcrop of the neural tube composed of what appears to be a homogenous cell population. Ganglion cells, one of the first cell types to differentiate in the vertebrate retina,[77] have been demonstrated to generate spontaneous action potentials at prenatal stages that are associated with the expression of voltage-activated cation channels.[78] Whole-cell patch-clamp analysis of mouse ganglion cells in retinal whole-mounts at embryonic day 15 (E15) revealed that $Ca^{2+}$ channel current from these immature neurons was predominantly due to T-type channels with a small contribution from HVA channels.[79] By E18, the current-voltage relationships of $Ca^{2+}$ currents in embryonic ganglion cells appeared similar to those observed in freshly dissociated postnatal ganglion cells, in which HVA currents were more pronounced. Similar findings were also reported for rat ganglion cells in which the $Ca^{2+}$ channels expressed at E17 to E19 were found to be exclusively of the T-type, with HVA current appearing at E20 and consisting of a conotoxin GVIA-sensitive current, a DHP-sensitive current and a toxin-resistant current type.[80,81] In contrast to the increased density of HVA $Ca^{2+}$ channels observed in ganglion cells during postnatal development, T-type $Ca^{2+}$ channel expression appeared to be down-regulated. These findings are also in keeping with other studies in which developing neurons preferentially express T-type $Ca^{2+}$ channel currents, and suggests that T-type $Ca^{2+}$ channels may be important during neuronal development, possibly by contributing to the generation of spontaneous firing behavior and rhythmic oscillatory activity.[75]

Bringmann et al[82] studied the developmental regulation of $Ca^{2+}$ channel-mediated currents in mammalian retinal Müller cells. They demonstrated differential expression of T-type and HVA $Ca^{2+}$ channel currents during development of immature retinal precursor cells into mature Müller cells. T-type $Ca^{2+}$ channel currents were expressed at high densities in

the developing cells and the current density fell off during differentiation into the mature Müller cell phenotype. HVA currents in contrast were only expressed at later developmental stages. It was suggested that the activity of T-type $Ca^{2+}$ channels may be necessary for: 1) precursor cell proliferation occurring during development, and 2) $Ca^{2+}$ entry in developing Müller cells that leads to differentiation events, including the growth of glial side branches.

### *Retinal Progenitor Cells Express T-Type $Ca^{2+}$ Channels*

A recent examination of the electrophysiological properties and expression of T-type $Ca^{2+}$ channels in retinoblastoma cells, tumor cells derived from retinal precursor cells, reported that these cells expressed mRNA for all three T-channel subtypes ($\alpha_{1G}$, $\alpha_{1H}$ and $\alpha_{1I}$). Expression of $\alpha_{1G}$ and $\alpha_{1H}$ T-type $Ca^{2+}$ channel current was prominent in the undifferentiated mitogenic cells and diminished when retinoblastoma cells differentiated to a neuronal phenotype.[83] Although a small HVA current was present in undifferentiated cells, this became more pronounced in the differentiated neuronal phenotype. These results are consistent with a prominent role for T-type $Ca^{2+}$ channels in proliferating retinal precursor cells, and further suggests that differential expression of $Ca^{2+}$ channel gene transcripts occurs in the developing retina and has a regulatory role in $Ca^{2+}$ channel expression during differentiation.[84]

## Conclusions

A diversity of $Ca^{2+}$ channel subtypes take part in the processing and synaptic transfer of visual information as signals pass from neuron to neuron in the retina. From the graded potential neurons in the outer retina that utilize L-type $Ca^{2+}$ channels to transmit over a narrow range of potentials, to change enhancement and temporal integration mediated by L-, N- and T-type $Ca^{2+}$ channels in spiking neurons that form the output of the retina, $Ca^{2+}$ channels of every type are employed in this neural network. Developmental studies of this tissue reveal changing requirements for the expression of $Ca^{2+}$ channels, with T-type being predominant in the cycling neuronal precursor cells. In this respect, the retina offers useful insights into the neural requirements and types of roles for which the various $Ca^{2+}$ channel subtypes may be best suited.

### *Acknowledgements*

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# CHAPTER 23

# Exploring the Function and Pharmacotherapeutic of Potential Voltage-Gated $Ca^{2+}$ Channels with Gene-Knockout Models

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## Introduction

The importance of the intracellular free $Ca^{2+}$ concentration as a key cellular signal transduction mechanism requires a tight control of $Ca^{2+}$ homeostasis thus enabling the independent regulation of free cytosolic $Ca^{2+}$ levels even within different cellular signaling compartments. In electrically excitable cells voltage-gated $Ca^{2+}$ channels (VGCCs) convert membrane potential changes into an intracellular $Ca^{2+}$ signal by mediating $Ca^{2+}$ ion entry along its electrochemical gradient during depolarization.[1] The resulting intracellular $Ca^{2+}$ transient controls essential $Ca^{2+}$-dependent physiological processes. These include neurotransmitter release from fast chemical synapses in nerve terminals and from sensory cells, hormone release from endocrine cells, excitation-contraction coupling in heart, skeletal and smooth muscle and control of pacemaker activity in heart and neurons.[2] Each of these specific tasks requires adjustment of $Ca^{2+}$ channel function to its specific needs by targeting the channels to specific sites of the plasma membrane and by fine-tuning their opening and closing behaviour (gating). This explains why evolution generated a large number of different voltage-gated $Ca^{2+}$ channel types which can be distinguished based on their biophysical properties and the way they are regulated by second messenger pathways.[2,3] Until now drugs and toxins specifically blocking the activity of individual channel types represented the most important tool to dissect their individual role for specific cellular functions and facilitated their biochemical isolation and cloning.[3,4]

The different $Ca^{2+}$ channel types known to date, their specific modulators and proposed physiological roles are summarized in Table 1. Voltage-gated $Ca^{2+}$ channels form hetero-oligomeric complexes of several subunits (Fig. 1). This subunit structure is reviewed in detail in other articles in this body and therefore is not discussed in much detail here. Figure 1 illustrates that $\alpha_1$ subunits comprise the $Ca^{2+}$-selective ion pore itself. They determine most of the biophysical parameters (i.e. single channel conductance, activation and inactivation) and carry the drug and toxin interaction sites. So far ten different $\alpha_1$ subunit isoforms, each of them undergoing further fine-tuning by extensive alternative splicing, were identified in the human genome. Based on their different biophysical and pharmacological characteristics they give rise to 5 different $Ca^{2+}$ channel types (Table 1). Both $\beta$ and $\alpha 2$-$\delta$ subunits (and to some extent also $\gamma$-subunits, see below) are also required for proper channel targeting to the plasma membrane and fine-tuning of the gating properties of most $\alpha 1$ subunits.[3] At least

*Voltage-Gated Calcium Channels*, edited by Gerald Zamponi.
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***Table 1. $Ca^{2+}$ channel types: Expression, pharmacological properties and proposed physiological function***

| Type | α1 Subunit | Tissue Expression | Function | Deficiency Model | Drugs/Toxins |
|---|---|---|---|---|---|
| L | $Ca_v1.1\alpha1$ (α1S) | skeletal muscle | excitation-contraction coupling | muscular dysgenic mouse (*mdg/mdg*) | Organic L-type channel blockers Dihydropyridines *(Nifedipine)*, Phenylalkylamines *(Verapamil)*, Benzothiazepines *(Diltiazem)* |
| | $Ca_v1.2\alpha1$ (α1C) | cardiac/smooth muscle; neurons; endocrine cells | *excitation-contraction coupling* excitation-transcription coupling *stimulus-secretion coupling* | $Ca_v1.2\alpha1^{-/-}$ (2) | |
| | $Ca_v1.3\alpha1$ (α1D) | heart atria; neurons; endocrine cells; sensory cells | *sinoatrial heart rate control* excitation transcription coupling; modulation of firing pattern stimulus-secretion coupling *stimulus-secretion coupling* | $Ca_v1.2\alpha1^{-/-}$ (2) | |
| | $Ca_v1.4\alpha1$ (α1F) | retina | *stimulus-secretion coupling* | CSNB2 (human) | |
| P/Q | $Ca_v2.1\alpha1$ (α1A) | neurons; endocrine cells | stimulus-secretion coupling | $Ca_v2.1\alpha1^{-/-}$ (2) | ω-Aga-IVA |
| N | $Ca_v2.2\alpha1$ (α1B) | neurons; endocrine cells | stimulus-secretion coupling | $Ca_v2.2\alpha1^{-/-}$ (3) | ω-CgTX-GVIA |
| R | $Ca_v2.3\alpha1$ (α1E) + $Ca_v?\alpha1$ | neurons; endocrine cells cardiac/smooth muscle | stimulus-secretion coupling ? | $Ca_v2.3\alpha1^{-/-}$ (4) | SNX-482 |
| T | $Ca_v3.1\alpha1$ (α1G) | neurons; cardiac muscle | *burst-firing;* automaticity ? | $Ca_v3.1\alpha1^{-/-}$ (1) | (Kurtoxin, Mibefradil) |
| | $Ca_v3.2\alpha1$ (α1H) | smooth muscle, cardiac muscle, kidney, liver | *vasodilation* ? | $Ca_v3.2\alpha1^{-/-}$ (1) | |
| | $Ca_v3.3\alpha1$ (α1I) | neurons | automaticity | not yet available | |

Channel types were classified based on different pharmacological and biophysical properties in cells isolated from different tissues. Functions newly discovered in spontaneous mouse mutants, gene-targeted mice or human genetic diseases are highlighted in italics. *mdg/mdg*= muscular dysgenesis spontaneous mouse mutant; CSNB2= X-linked congenital stationary night blindness type 2 (human disease); the number in parenthesis indicates the number of independently developed knockout mouse strains.

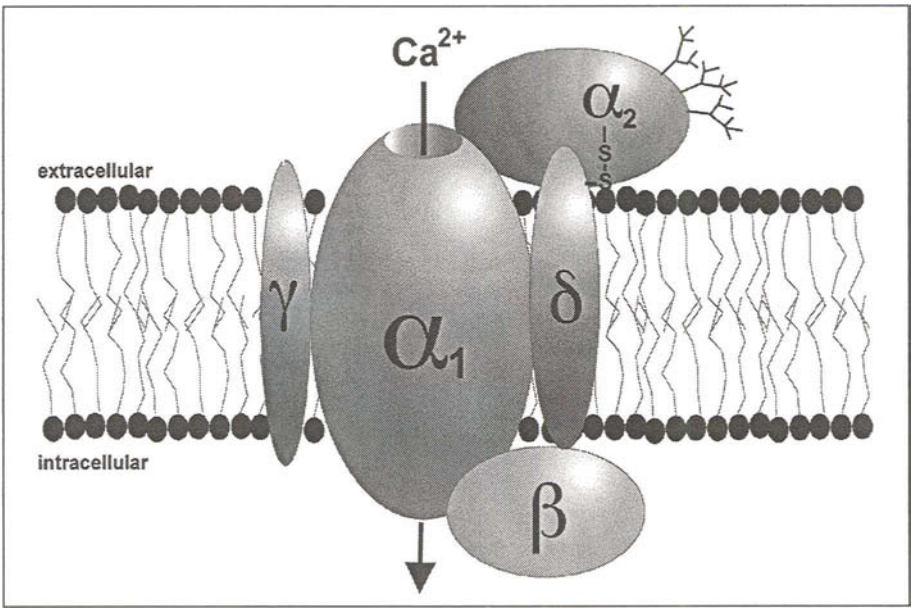



Figure 1. Subunit structure of voltage-gated $Ca^{2+}$ channels. Accessory α2-δ and β subunits are associated with $Ca_v1$ and $Ca_v2$ α1 subunits but their contribution to T-type $Ca^{2+}$ channel complexes ($Ca_v3$ α1 family) is less clear. Biophysical and pharmacological properties are largely defined by the α1 subunits (see Table 1).

four different β-subunits, four α2-δ subunits and 8 γ-subunits exist. More than one isoform of each of these subunits may be expressed in a single cell.

As α1 subunits are required for channel formation, disruption of a particular α1 subunit gene must lead to a complete loss of the corresponding $Ca^{2+}$ currents. To compensate for this defect a cell may upregulate a $Ca^{2+}$ channel type formed by a different $\alpha_1$-subunit isoform. In contrast, disruption of a β or $\alpha_2$-δ gene must not necessarily lead to a complete loss of function of a particular channel type normally associated with this subunit because compensation by other isoforms of these accessory subunits may occur. In this article all so far published $Ca^{2+}$ channel subunit deficient animal models will be discussed. Most of them were generated by gene-targeting in mice. However, researchers also benefit from spontaneous mouse mutants and human diseases with known monogenic genetic defects of $Ca^{2+}$ channel subunits.

## Abbreviations

DHP diyhdropyridine
EPSP excitatory postsynaptic potential
LTCC L-type $Ca^{2+}$ channels
LTS low threshold spikes
TCR thalamocortical relay neuron

## The Cav1 (L-Type) $Ca^{2+}$ Channel Family

L-type $Ca^{2+}$ channels (LTCCs) could be purified from different tissues (skeletal muscle, heart), and their currents isolated in different cells by means of their high affinity for the classical organic $Ca^{2+}$ channel blockers[3] (Table 1). Among those dihydropyridines (DHPs) proved especially helpful due to their very high binding affinity and high selectivity for LTCCs and the availability of both $Ca^{2+}$ channel blockers (e.g., nifedipine, isradipine) and $Ca^{2+}$ channel activators (e.g., BAYK 8644,

FPL 64176 [3,5,6]). All four subunits capable of forming LTCCs ($Ca_v1.1\alpha_1$ – $Ca_v1.4$) possess a high affinity DHP interaction domain whose amino acids have been identified.[5,6] Despite their pharmacological relationship these channels are functionally diverse (Table 1) and should therefore be unable to completely compensate for each other in knockout models.

## *$Ca_v1.1\alpha_1$-Deficiency*

$Ca_v1.1\alpha_1$ is exclusively expressed in the transverse tubules of skeletal muscle where it undergoes a rapid conformational change upon membrane-depolarization. It thereby initiates SR $Ca^{2+}$ release by allosterically activating SR ryanodine receptors.[7] A slower voltage-dependent conformational change also activates the channel's $Ca^{2+}$ conductance, which is not important for immediate signaling but may be involved in the regulation of musle $Ca^{2+}$ homeostasis.[8] Mice with muscular dysgenesis, caused by an autosomal recessive lethal mutation in the $Ca_v1.1\alpha_1$ subunit served as the first $Ca_v1.1\alpha_1$ deficient mouse model (*mdg/mdg* mice[9,10]). *Mdg/mdg* mice enabled the pioneering work revealing the above-mentioned role of $Ca_v1.1\alpha_1$ for skeletal muscle EC-coupling.[11] These mice die at birth from asphyxia due to the expected failure of their skeletal muscle to contract. Differences between $Ca_v1.1\alpha_1$-mediated skeletal muscle – type ECC (independent of $Ca^{2+}$ entry) and $Ca_v1.2\alpha_1$-mediated cardiac muscle – type ECC (dependent on $Ca^{2+}$ entry) and the underlying molecular mechanisms were successfully studied by expressing cloned $Ca_v1.1\alpha_1$ , $Ca_v1.2\alpha_1$ as well as chimeric constructs and mutants thereof in *mdg/mdg* myotubes [7,8] and immortalized *mdg/mdg* cells.[12,13]

## *$Ca_v1.2\alpha_1$-Deficiency*

In contrast to $Ca_v1.1\alpha_1$, $Ca_v1.2\alpha_1$ expression is not restricted to a single tissue. It is widely and densely expressed in the cardiovascular system (heart atria and ventricles, sinoatrial node cells, vascular smooth muscle),[14,15] uterine, tracheal and intestinal smooth muscle[16,17] but also in endocrine and neuroendocrine cells (e.g., pancreatic β-cells, pituitary gland) and neurons.[14,18-20] Vasodilation and cardiac depression, the therapeutically exploited pharmacological effects of $Ca^{2+}$ channel blockers, are believed to be almost exclusively mediated by block of $Ca_v1.2$ channels in the cardiovascular system. Unfortunately, these drugs have only provided limited insight into the role of these channels for neuronal function. Brain LTCCs (80% of which consist of $Ca_v1.2\alpha_1$ and 20% of $Ca_v1.3\alpha_1$;[20]) are not believed to directly control presynaptic $Ca^{2+}$ entry and neurotransmission at fast chemical synapses.[21] Instead, they are located on dendrites and neuronal cell bodies[20] and $Ca^{2+}$ entry through these channels modulates neuronal function by altering gene transcription.[22,23] CNS effects of LTCC modulators are subtle. DHPs alter the sensitivity to cocain reward[24,25] and modulate alcohol withdrawal symptoms.[26] DHP channel activators also produce a (toxic) neurobehavioural syndrome in rodents with severe dystonia and behavioural abnormalities (including self-biting[27]). Upregulation of LTCC activity in aged rats (which may, however, be mainly due to upregulation of $Ca_v1.3\alpha_1$, see reference 28) may contribute to the age-related decrease in cognitive function. Obviously, $Ca_v1.2\alpha_1$-deficient mice are urgently needed to reveal the physiological role of this most abundant LTCC in the mammalian brain.

$Ca_v1.2\alpha_1$-deficient mice have successfully been generated.[29] These animals died at embryonic day 12.5 suggesting that the embronic heart requires $Ca_v1.2$ for normal rhythmic actvity after day 13 p.c. Until day 12.5 p.c. contractions were sustained by an L-type current which could not be attributed to any of the other known L-type $\alpha_1$ subunits. It may therefore comprise a yet unknown $\alpha_1$ isoform or an embryonic, yet unidentified splice-variant of one of the known L-type $\alpha_1$ subunits. These findings emphasize the important role of this subunit for cardiac development but prevented the analysis of $Ca_v1.2\alpha_1$ deficiency after birth and in adult animals. However, by generating a targeting vector exploiting the Cre-loxP recombination technique, mice were constructed in which $Ca_v1.2\alpha_1$ deficiency can be induced after birth by tissue-selective activation of cre recombinase. Selective deletion of $Ca_v1.2a1$ in smooth muscle revealed their important role for blood pressure control. $Ca_v1.2$ not only mediates the depolarization-induced (i.e., voltage-activated) contraction but also contributes to noradrenaline and angiotensin II-induced

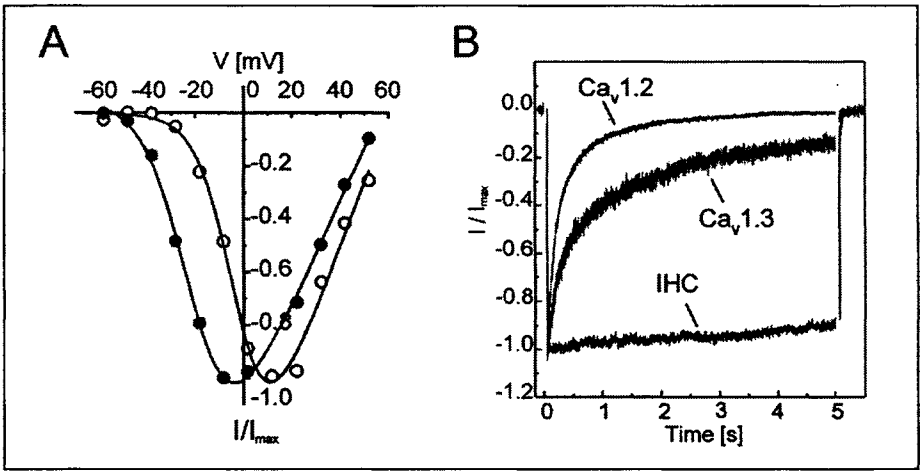



Figure 2. Properties of heterologously expressed Cav1.3 $Ca^{2+}$ channels. $Ca_v1.3\alpha1$ and $Ca_v1.2\alpha1$ subunits were heterologously expressed in human embryonic kidney tsA-201 cells together with $Ca_v\beta3$ and $\alpha2\text{-}\delta1$ subunits. Note that under identical experimental conditions $Ca_v1.3\alpha1$-mediated currents activated at about 15 mV more negative voltages than $Ca_v1.2\alpha1$. Inward currents were recorded using 15 mM $Ba^{2+}$ as charge carrier. A) I-V curves were constructed by depolarizations from a holding potential of -80 mV to the indicated test potentials. Peak current amplitude is plotted against voltage. B) Inactivation time course of $Ba^{2+}$ inward currents during 5-s depolarizing pulses from a holding potential of -80 mV to the peak voltage of the I-V curve. A $Ca^{2+}$ channel current recorded in cochlear inner hair cells (IHC) is shown as a comparison. Using $Ca_v1.3^{-/-}$ mice it was shown[33] that more than 90% of this IHC $Ca^{2+}$ current are $Ca_v1.3$ mediated. Note the even slower inactivation of $Ca_v1.3\alpha1$ currents in the IHCs. Taken from reference 37 with permission.

(i.e., receptor-mediated) contraction.[29a] Selective knockout of $Ca_v1.2a1$ in pancreatic β-cells revealed that only $Ca_v1.2$ channels contribute to glucose-induced insulin secretion in mice.[29b]

## *$Ca_v1.3\alpha_1$-Deficiency*

Until the availability of $Ca_v1.3\alpha_1$ deficient mice the role of this LTCC subtype remained enigmatic. This was in part due to the fact that its unique biophysical properties that can provide important hints about its physiological role were not discovered in early expression studies.[30,31] Based on the high relative abundance of $Ca_v1.3\alpha_1$ mRNA in pancreatic islets it was proposed that this LTCC plays a key role for stimulus-secretion coupling in pancreatic β-cells.[32] $Ca_v1.3\alpha_1$ was found to coexist with $Ca_v1.2\alpha_1$ in many tissues. Due to the lack of $Ca^{2+}$ channel blockers distinguishing between these two isoforms their relative contribution to L-type channel mediated functions could not be assessed.

$Ca_v1.3\alpha_1$ -deficient mice reproduce normally, have no gross anatomical defects and lack deficits in motor function.[33,34] However, these mice show two important phenotypes. They are deaf and suffer from sinoatrial node dysfunction.[33,34] The deafness is due to the complete absence of L-type $Ca^{2+}$ currents in the inner hair cells of the cochlea.[33] $Ca_v1.3\alpha_1$-mediated L-type currents comprise about 90% of the total $I_{Ca,L}$ in these cells. As $I_{Ca,L}$ is closely coupled to tonic neurotransmitter release in IHCs,[35] the absence of $Ca_v1.3$ currents should largely abolish sound-evoked neurotransmitter release. As previously reported for hair cells in the chick cochlea,[36] the activation threshold of $Ca_v1.3$ currents in mouse IHCs was considerably lower (around -60 mV at physiological $Ca^{2+}$ concentrations) than for $Ca_v1.2$ (around -40 mV) and inactivated with a much slower time course.[33] These two biophysical features, negative activation threshold and slow inactivation, distinguish $Ca_v1.3$ from $Ca_v1.2$ channels. The difference is inherent to the $\alpha_1$ subunits as confirmed by heterologous expression studies[37-39] (Fig. 2). Despite normal cochlear

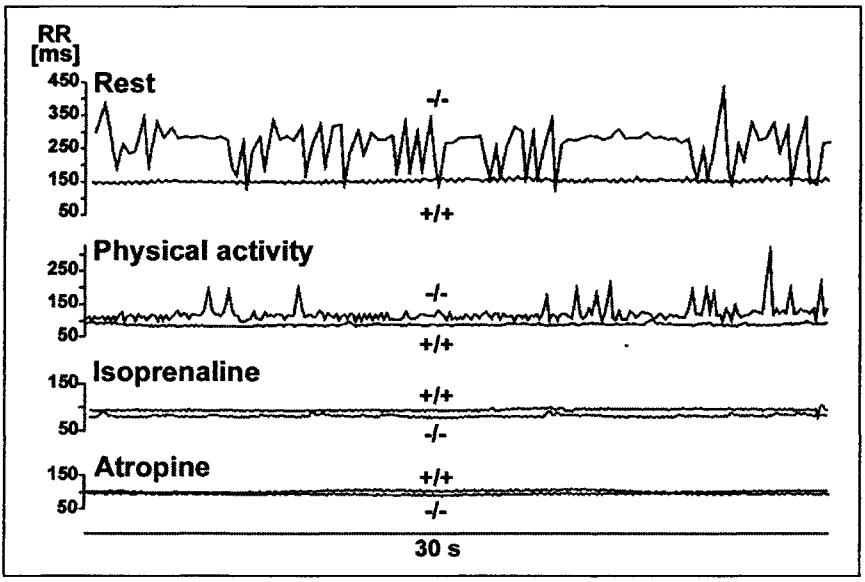



Figure 3. Telemetric electrocardiographic recordings in Cav3.1-deficient mice. $Ca_v1.3\alpha1$-deficiency is associated with severe sinoatrial node arrhythmia and bradycardia. The phenotype disappears under conditions of high heart rates. Heart rates are given as intervals between the R-spikes of ECG-waveforms (RR-intervals) for 30-s episodes under resting conditions, during physical activity and after injection of isoprenaline or atropine (1 mg/kg i.p.) to increase heart rate. 150-ms intervals correspond to a heart rate of 400 beats per minute. Note that the RR variability is a measure for arrhythmia.

development in utero and immediately after birth, a degeneration of the cochlear sensory cells was observed about 2 weeks after birth, starting with degeneration of outer hair cells in apical turns.[33] More recent studies confirmed the role of $Ca_v1.3$-signaling for normal development.[33a] $Cav1.3^{-/-}$ deficiency abolishes postnatal $Ca^{2+}$-spiking and impairs IHC development. $Cav1.3^{-/-}$ IHCs keep their efferent cholinergic innervation and lack high conductance $Ca^{2+}$-activated $K^+$-channels ($BK_{Ca}$).[33b]

$Ca_v1.3\alpha_1$ -deficient mice also exhibit an arrhythmic and bradycardic heart beat (Fig. 3). Telemetric ECG recordings and experiments with spontaneously beating isolated mouse atria clearly demonstrated that this is due to an intrinsic defect in the sinoatrial node.[33] Sinoatrial bradyarrhythmia was present at low heart rates but this pathological phenotype completely dissappeared when heart rate increased (ref. 33, Fig. 3). This finding is in excellent agreement with recent patch-clamp studies of the biophysical properties of L-type currents in mouse and rabbit sinoatrial node cells.[40-42] In these cells L-type $Ca^{2+}$ channels contribute to an inward current throughout the diastolic depolarization phase (DD). DD is the spontaneous depolarization between action potentials responsible for the automaticity of the sinoatrial node. It starts after an action potential at the maximum diastolic potential of about -60 mV. By reaching a threshold voltage at about -45 mV DD triggers the following action potential. It was predicted that two properties of the sinoatrial $I_{Ca,L}$ were important for its contribution to DD: a negative activation threshold[41,42] and a slowly inactivating current component.[41] As expected, $I_{Ca,L}$ with these properties was found in sinoatrial cells isolated from wildtype mice but was absent in cells from knockout mice[43,44] and must therefore be $Ca_v1.3\alpha_1$ mediated. As $Ca_v1.2\alpha_1$ subunits do not possess these special biophysical properties these channels is unlikely to contribute to DD. This explains why $Ca_v1.2$ currents are unable to compensate for regular sinoatrial node function in $Ca_v1.3^{-/-}$ mice.[43]

Conflicting data with respect to the role of $Ca_v1.3$ L-type channels for pancreatic β-cell function have been obtained from the two independently generated $Ca_v1.3\alpha_1$ -deficient mouse models. Whereas one group reported no effect on glucose tolerance, glucose-induced insulin-secretion and β-cell L-type currents[18,33] a decreased glucose-tolerance was reported for the other mouse strain.[34] Although whole cell $I_{Ca,L}$ in β-cells was not decreased in $Ca_v1.3\alpha_1^{-/-}$ mice, the voltage-dependence of activation was shifted by about 10 mV towards more positive voltages.[34] This was interpreted as evidence for the absence of a negatively activating $Ca_v1.3$ L-type current component compensated by enhanced $Ca_v1.2$. Homozygous knockouts also showed a decrease of size and number of pancreatic islets, a reduced β-cell number per islet and evidence for reduced β-cell generation.[34] This suggested that $Ca_v1.3\alpha_1$ sustains a trophic effect on pancreatic β-cells. More recently, a completely different approach has been taken to assess the role of $Ca_v1.3$ channels in vivo and in vitro. By mutation of a single amino acid residue in $Ca_v1.2\alpha1$ subunits (threonine-1066 to tyrosine, see chapter on $Ca^{2+}$ channel drug binding domains in this body) in mice, animals were created with $Ca_v1.2$ channels lacking high affinity for DHP $Ca^{2+}$ channel blockers and activators[34a] but without affecting function and expression of $Ca_v1.2$ ($Ca_v1.2DHP^{-/-}$ mice). This allowed separation of the DHP effects of $Ca_v1.2$ from those of $Ca_v1.3$ and other LTCCs. Using this model, it could be unequivocally demonstrated that $Ca_v1.3$ does not contribute significantly to pancreatic β-cell L-type currents which therefore must be formed by $Ca_v1.2$. These results nicely confirmed the above-mentioned findings with β-cell-specific $Ca_v1.2$ knockout.[29b] $Ca_v1.2DHP^{-/-}$ mice further revealed that $Ca_v1.3$ does not contribute to cardiac inotropy, and arterial smooth muscle contractility. With this model DHPs could also be established as mood-modifying agents: L-type channel blockers revealed antidepressant-like effects in wildtype mice that were abolished in $Ca_v1.2DHP^{-/-}$ mice. The known neurotoxicity of the DHP channel activator BayK 8644 was abolished in these mice (and must therefore be mediated by $Ca_v1.2$) and allowed to disclose a depression-like behavioral effect. BayK 8644 activated only a specific set of brain areas. In the ventral striatum, BayK-induced release of glutamate and 5-HT, but not of dopamine and of noradrenaline was abolished. This animal model should therefore provide a useful tool to elucidate whether $Ca_v1.3$-selective channel modulation represents a novel pharmacological approach to modify CNS function without major peripheral effects.

### *$Ca_v1.4\alpha_1$-Deficiency*

Despite the attractiveness of mouse models, genetic defects in defined human genes provide direct insight into the function of the affected protein for human physiology and pathophysiology. This is the case for $Ca_v1.4\alpha_1$ which was discovered as a retina-specific protein mutated in patients suffering from X-linked incomplete congenital stationary night blindness. Congenital stationary night blindness (CSNB) is a heterogeneous, non-progressive retinal disorder characterized by life-long impairment of night vision and variably reduced day vision without identifiable structural abnormality of the retina.[45,46] Due to the X-linked, recessive inheritance pattern of this disease only males and heterozygous females are affected.[47] Clinical heterogeneity between families has led to the classification into complete CSNB (designated CSNB1) and incomplete CSNB (designated CSNB2) based on abnormalities in the electroretinogram and psychophysical testing.[48] In patients with complete CSNB rod function is completely absent compared to patients with incomplete CSNB where rod function is recordable but decreased. Incomplete CSNB is thought to result from defect neurotransmission mainly from photoreceptors to second-order retinal neurons.[49]

Neurotransmitter release from retinal photoreceptors is mediated by $Ca^{2+}$ entry through DHP-sensitive VGCCs and subsequent glutamate release.[50-53] These VGCCs possess properties resembling $Ca_v1.3$ currents (see above) with activation at negative voltages, slow inactivation and modest DHP sensitivity. Heterologous expression in mammalian cells revealed that $Ca_v1.4$ channels possess similar biophysical properties.[53a-c] From immunohistochemical studies it appears as if $Ca_v1.4\alpha_1$ represented the major synaptically localized $Ca^{2+}$ channel and is therefore crucial for neurotransmitter release from photoreceptors in different species.[52,54]

The discovery of mutations in $Ca_v1.4\alpha_1$ subunits of VGCCs in patients with CSNB2 lead to the conclusion that abnormal $Ca_v1.4\alpha_1$ function results in impairment of $Ca^{2+}$ influx that is required for normal photoreceptor-to-bipolar cell synaptic transmission.[47,55] The majority of mutations reported so far lead to truncations within the transmembrane repeats, their connecting linkers or other severe aberrations in highly conserved regions and are therefore likely to lead to non-functional channels. It is unknown if other ionic currents (e.g., L-type $Ca_v1.3$ channels) undergo compensatory upregulation in retinal cells of CSNB2 patients. Obviously $Ca_v1.4\alpha_1$-deficient mouse models must be produced to answer such questions. However, it must be emphasized that vision predominantly depends on rod function in mice and $Ca_v1.4$ mutations may therefore lead to more pronounced vision disturbances than in humans.

## The $Ca_v2$ (P/Q-, N- and R-Type) $Ca^{2+}$ Channel Family

The $Ca_v2$ family of VGCCs is predominantly expressed in neurons, although expression has also been demonstrated in (neuro-)endocrine cells (e.g., in pancreatic β- and adrenal chromaffin cells ($Ca_v2.1\alpha_1$, $Ca_v2.2\alpha_1$, and $Ca_v2.3$[56-59]), heart muscle ($Ca_v2.3\alpha_1$[60])and testis ($Ca_v2.3\alpha_1$[61]). Unlike LTCCs, $Ca_v2$ channels are not sensitive to DHPs and also much less sensitive to verapamil and diltiazem[62] (Table 1). They are therefore also classified as "non-L-type" $Ca^{2+}$ channels. Also in contrast to LTCCs, $Ca_v2.1$ and $Ca_v2.2$ (and to some extent also $Ca_v2.3$[63]) mediate fast neurotransmitter release from nerve terminals in central synapses and from motor neurons in the neuromuscular junction.[21,64] All three channel types are expressed not only on the presynaptic membrane but also on dendrites and cell bodies.[65,66] The contribution of these different channel types for neuronal non-L-type $Ca^{2+}$ current components and for transmitter release could be quantified by employing specific blockers (Table 1). Given their widespread expression in the mammalian brain and their crucial role for neurotransmitter release one would predict severe disturbances of brain function in knockout mice. From previous pharmacological analysis it was expected that the absence of $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ would exclusively and completely eliminate N-type (ω-CgTx IVA-sensitive) and P/Q-type (ω-AgaIVA- and ω-CgTx MVIIC-sensitive) current components, respectively. In contrast, the role of $Ca_v2.3\alpha_1$ subunits for the so-called R-type current (defined as the $Ca^{2+}$ current component resistant to saturating concentrations of any of the specific inhibitors of the other known $Ca^{2+}$ channel types) was much less clear. This was especially due to the observation that SNX-482, a peptide toxin which selectively blocks recombinant $Ca_v2.3\alpha_1$-mediated currents did not[67] or only partially block R-type currents in different neurons.[67,68] Obviously, $Ca_v2.3\alpha_1$-deficient mice were eagerly awaited to quantify its role for R-type currents in different neurons.

### *$Ca_v2.1\alpha_1$-Deficiency*

At many synapses excitatory neurotransmitter release is more dependent on P/Q-type than N-type $Ca^{2+}$ channels, whereas GABA release is more dependent on N-type than P/Q-type channels[69-73] but control of inhibitory neurotransmitter release by P/Q-type channels has also been reported.[74,75] The preferential contribution of $Ca_v2.1$ to the total $I_{Ca}$ in cerebellar Purkinje cells (about 90%[21]) and acetylcholine release from motor nerve terminals [76] would predict that $Ca_v2.1\alpha_1$ deficiency results in severe myasthenia and cerebellar symptoms. $Ca_v2.1\alpha_1^{-/-}$ mice were independently generated in two laboratories.[21,77] Although embryonic and postnatal development appeared normal they time-dependently developed a severe neurologic syndrome after P10 consisting in ataxia, dystonia, spasms and weakness. Homozygous mice did usually not survive past weaning. They exhibited abnormal and delayed neuronal development evident e.g., as failure of migration and persistence of an external granule cell layer at P21, focal axonal swelling of Purkinje cell axons and increased abundance of TH-positive neurons in deeper cortical layers at P21.[21] In homozygous animals surviving for 15 weeks a decrease in the volume of the cerebellum was associated with loss of Purkinje and granule cells.[77] In heterozygous mice $I_{Ca}$ density was decreased by about 50% only in one of the two models.[77] This decrease did not result in any histological or functional abnormalities, indicating that P/Q-type current must be lowered to more than 50% to cause neurodegeneration in mice.

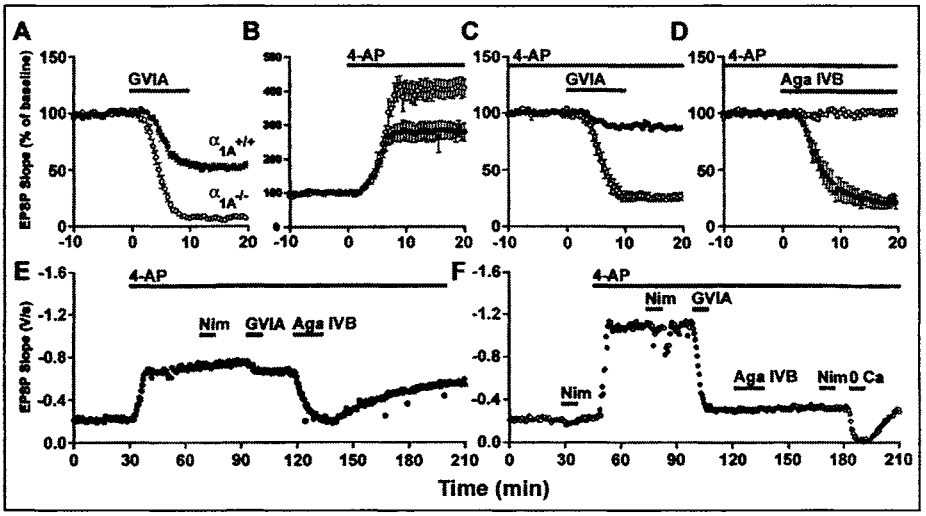



Figure 4. Effect of $Ca_v2.1$-deficiency on neurotransmission in hippocampal synapses. Effects on synaptic transmission of altering $Ca^{2+}$ influx in wildtype (●) and mutant (○) animals. A) Blockade of N-type $Ca^{2+}$ channels with ω-CgTx-GVIA (1 μM) reduced the strength of synaptic transmission by half in wildtype but eliminated the EPSP in mutants. B) Enhancement of $Ca^{2+}$ influx by broadening action potentials with 4-AP (100 μM) increased the strength of synaptic transmission 2-fold in wildtype and 3-fold in mutants. C) After application of 4-AP, blockade of N-type $Ca^{2+}$ channels considerably reduced synaptic strength in $Ca_v2.1\alpha1^{-/-}$ slices, with a much smaller effect in wildtype. D) After application of 4-AP and ω-CgTx-GVIA, blockade of P/Q-type $Ca^{2+}$ channels with ω-Aga-IVB (1 μM) decreased the strength of synaptic transmission in wildtype by more than 75% but had no effect in mutants. E and F) Representative responses to sequential application of $Ca^{2+}$ channel blockers in the presence of 4-AP in wt (E) and $Ca_v2.1\alpha1^{-/-}$ (F) animals. Taken from reference 21 with permission.

In contrast to $Ca_v2.2\alpha_1^{-/-}$ and $Ca_v2.3\alpha_1^{-/-}$ mice, the absence of $Ca_v2.1\alpha_1$ and P/Q-type currents caused alterations in other $Ca^{2+}$ current components. R-type currents (which are unlikely to be carried by $Ca_v2.1\alpha_1$ (see below[78]) decreased in cerebellar granule cells whereas N- and L-type currents were upregulated in cerebellar Purkinje cells.[21,77] Interestingly, an upregulation of L-type currents has also been reported in *tottering* mice, a spontaneous mouse mutant suffering from ataxia, dystonia and absence seizures[79] Upregulated L-type currents seem to enhance the dystonia in these mice.[79] Therefore it is possible that LTCC upregulation also contributes to the dystonic phenotype in $Ca_v2.1\alpha_1^{-/-}$-deficient mice.

The consequences of $Ca_v2.1\alpha_1$-deficiency on synaptic transmission were studied by measuring field excitatory postsynaptic potentials (EPSPs) in the CA1 area of the hippocampus after stimulation of Schaffer collaterals and the commissural pathways[21] (Fig. 4). Blockade of N-type channels with the selective N-type blocker 1μM ω-CgTx-GVIA halved the EPSP slope in wildtype mice but nearly eliminated synaptic transmission in $Ca_v2.1\alpha_1^{-/-}$ (Fig. 4A). This indicated enhanced reliance of neurotransmission on N-type channels in $Ca_v2.1\alpha_1^{-/-}$ mice. If the total number of $Ca^{2+}$ channels available for neurotransmission at individual release sites was reduced in the $Ca_v2.1\alpha_1^{-/-}$ neurons then they should exhibit a stronger relative increase of EPSP slope by stimulation of presynaptic $Ca^{2+}$ influx after broadening of the presynaptic action potential with the $K^+$-channel blocker 4-aminopyridine.[80] Figure 5B showed that this was indeed the case. Compensation by N-type channels was also evident in the presence of 4-AP. Blockade of synaptic strength in wildtype slices depended mainly on P/Q-type current (78% inhibition by the P/Q-type selective blocker ω-Aga-IVB, Fig. 4D) but only marginally on N-type channels (13% inhibition by ω-CgTx-GVIA, Fig. 4C). In contrast, in $Ca_v2.1\alpha_1^{-/-}$ mice

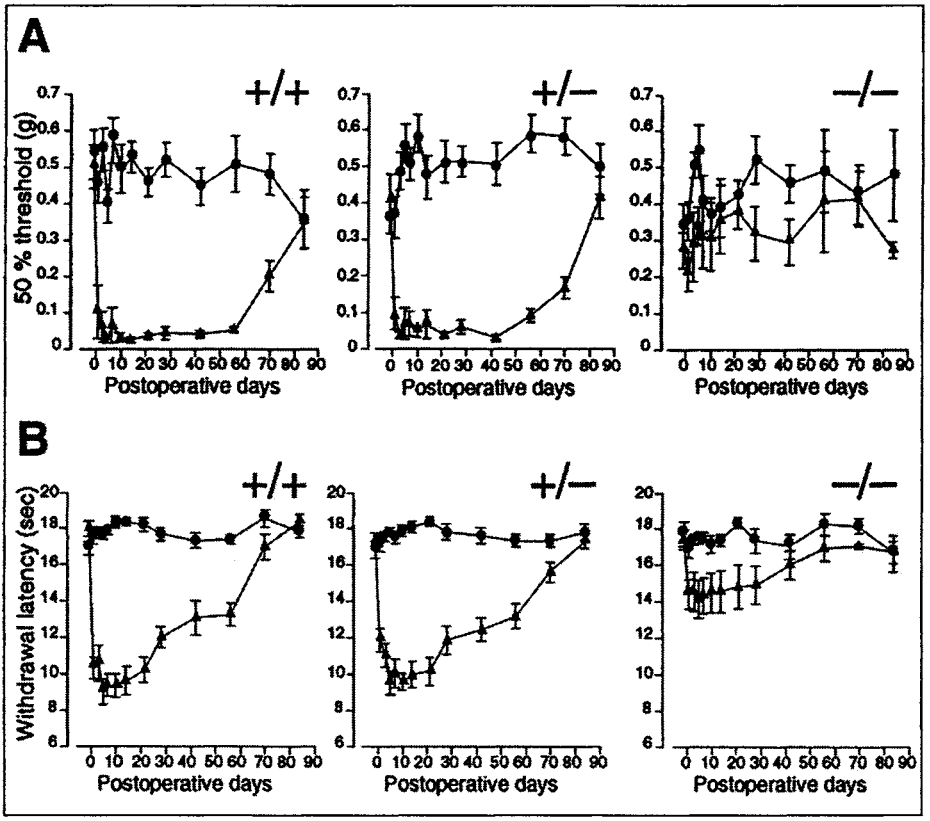



Figure 5. Antinociceptive effects of $Ca_v2.2\alpha1$-deficiency in neuropathic pain. Mechanical allodynia and thermal hyperalgesia induced by spinal nerve ligation. Fifty percent hindpaw withdrawal thresholds to stimulation with von Frey hairs (A) and hindpaw withdrawal latencies to thermal stimuli (B) are plotted against the days after the operation. Circles, uninjured side; triangles, operated side. +/+, wild-type; +/–, heterozygote; –/–, homozygous mutant. Homozygous mutants developed an attenuated post-operative decrease in the threshold and the latency, which are clearly observed in wild-type and heterozygous mutant mice as neuropathic pain symptoms. +/+, $n$ = 9–13; +/–, $n$ = 11–14; –/–, $n$ = 5–8. Taken from reference 87 with permission.

neurotransmission mainly relied on N-type channels evident as a 74% inhibition of synaptic strength by the P/Q-type selective blocker ω-Aga-IVB (Fig. 4D). These data show that N-type channels can, at least in part, compensate for P/Q-type channel presynaptic function. This is also true for the neuromuscular junction where compensation by N- and R-type channels occurs.[21-80a] The lower efficiency of these channels in supporting excitation-secretion coupling is compensated in $Ca_v2.1^{-/-}$ mice by additional presynaptic mechanisms such as enhanced $Ca^{2+}$-sensitivity of the neurotransmitter release machinery.[80b]

Unfortunately, the severity of the $Ca_v2.1\alpha_1{}^{-/-}$ phenotype did not allow a systematic analysis of the role of P/Q-type channels for various brain functions such as cognition, behaviour and pain sensation. Pharmacological block of P/Q-type channels in the brain suggested that they, together with N- and R-type (see below) type channels, are involved in transmission of certain forms of pain.[81,82] Obviously, these questions need to be addressed by brain region specific knockouts preserving P/Q-type channel function in the cerebellum.

The neurological syndrome of $Ca_v2.1^{-/-}$ mice and the pattern of neuronal degeneration resembles the phenotype of spontaneous mouse mutants reported earlier. Spontaneous mutations

(missense and/or C-terminal truncations) in the $Ca_v2.1\alpha_1$ gene (CACNA1A, mouse chr. p19q13) underly the autosomal recessive mutants *tottering*, *leaner*, *rocker*, and *rolling* (for review see ref. 83). Homozygous mutants exhibit cerebellar signs (ataxia, dystonia), absence epilepsy-like seizures and cerebellar degeneration to various extents.[83] These mutations result in a partial loss of P/Q-type channel function.[83] The presence of $Ca_v2.1\alpha_1$ protein and/or of residual P/Q-type currents may explain why these mutants can survive to adulthood unlike $Ca_v2.1^{-/-}$ mice. Interestingly, a 70-80% reduction of P/Q-type current was found in homozygous *leaner* mice. They also show profound cerebellar degeneration unlike the heterozygous $Ca_v2.1\alpha_1^{+/-}$ mice in which current density decreases by about 50% in Purkinje cells (ref. 77, see above). These data suggest that cerebellar degeneration requires reduction of P/Q-type current density below a critical threshold of 50-70%.

Rare inherited human diseases are also caused by $Ca_v2.1\alpha_1$. Episodic Ataxia type 2 (EA-2) is a human disease in which $Ca_v2.1\alpha_1$ mutations cause a complete or almost complete loss of channel function.[84,85] EA-2 is inherited in an autosomal dominant manner allowing synthesis of normal $Ca_v2.1\alpha_1$ subunits from the unaffected allele. In contrast to apparently healthy heterozygous $Ca_v2.1\alpha_1^{+/-}$ mice, disease signs (including cerebellar atrophy) are present despite the existence of an unaffected allele in these patients. This could merely reflect a species difference. An alternative explanation is the presence of dominant negative effects of the human EA-2 mutants, antagonizing the activity of $Ca_v2.1\alpha_1$ derived from the unaffected allele. At present the results about the presence of such dominant negative effects are controversial.[84,85] Moreover, even if dominant-negative effects by truncated constructs could be demonstrated by heterologous expression experiments, their pathophysiological significance is likely to be limited because most of these mutants are likely to undergo Nonsense-Mediated mRNA Decay.[85a]

## *$Ca_v2.2\alpha_1$-Deficiency*

Based on the large contribution of N-type channels to presynaptic $Ca^{2+}$ influx in many synapses, including sympathetic neurons,[86] it was surprising that $Ca_v2.2\alpha_1$-deficiency caused no obvious pathological syndrome. Three independent $Ca_v2.2^{-/-}$ lines have been characterized[87-89] (Table 2). As expected all three lacked ω-CgTx GVIA-sensitive currents. Unlike for $Ca_v2.1\alpha_1$-deficiency, no compensatory upregulation of other VGCCs was found.

The selective block of N-type $Ca^{2+}$ channels is currently developed as a promising pharmacotherapeutic concept to treat severe and opioid-resistant pain syndromes, including neuropathic pain.[82] Intrathecally injected ziconotide (SNX-111), a ω-CgTx GVIA-related peptide, showed considerable efficacy against neuropathic pain in clinical trials. Unfortunately, side effects resulting from N-type block were described. These include increased dizziness, blurred vision, nystagmus, sedation, anxiety, halluzinations and hypotension and limit the clinical use of this peptide.[90-92] Whereas the pathophysiological significance of N-type channels for nociception could be nicely confirmed in $Ca_v2.2^{-/-}$ mice (see below) no other functional disturbances were yet reported that correspond to these side effects of intrathecal ziconotide in humans. Motor function and spontaneous locomotor activity were normal in knockout mice, although a recent study found evidence for higher spontaneous activity during the dark phase and during exposure to a novel environment.[92a] Evidence for a decreased rather than increased level of anxiety was detected in different behavioural paradigms (open field, acoustic startle response, elevated plus-maze[87]). Hypotension was also absent (see below). $Ca_v2.2^{-/-}$ mice had unchanged thresholds for normal and noxious mechanical stimuli[87,88](but see ref. 89). However, antinociceptive effects were evident as delayed responses to thermal noxious stimuli, which involve spinal reflexes (tail and paw flick tests refs. 87,89). $Ca_v2.2\alpha_1$ deficiency consistently delayed inflammatory pain development in the formalin test. In this test a brief period of acute pain (phase 1, lasting about 5 min) is followed by a persistent inflammatory pain response which starts about 15 min after paw injection (phase 2). In $Ca_v2.2^{-/-}$ mice the first half of phase 2 (phase 2A, sensitive to COX-inhibitors[87]), but not phases 2B (insensitive to COX-inhibitors) and 1, was completely

*Table 2. Consequences of $Ca_v2.2\alpha1$ and $Ca_v2.3\alpha1$ deficiency*

| Subunit Defciency | References | Anatomical Abnormalities | Effect on $Ca^{2+}$-Current | Body Functions | Blood Pressure | Heart Rate | Motor Function | Anxiety | Locomotor Activity | Learning and Memory |
|---|---|---|---|---|---|---|---|---|---|---|
| $Ca_v2.2\alpha1$ | 87 | none | N-type absent, no compensatory upregulation (DRG, SCG) | normal body temperature | normal | | normal | decreased | normal | n.e. |
| | 88,93 | none | | n.e. | both elevated; markedly reduced baroreceptor response | | normal | n.e. | n.e. | n.e. |
| | 89 | none | | n.e. | n.e. | | normal | n.e. | n.e. | n.e. |
| Cav2.3α1 | 99 | none | R-type reduced by 81% in CeA | n.e. | n.e. | | normal | increased | normal | n.e. |
| | 98 | none | SNX-482 sensitive component of R-type eliminated in CGC | n.e. | n.e. | | n.e. | n.e. | n.e. | n.e. |
| | 96,97, 140,141 | none | | altered sperm motility; decreased insulin response and glucose tolerance, increased weight | n.e. | | n.e. | increased | reduced | decreased spatial (Morris water maze), unchanged fear memory |
| | 59 | none | | decreased glucose tolerance and insulin secretion | n.e. | | n.e. | n.e. | n.e. | n.e. |

*Continued on next page*

*Table 2. Continued*

| Subunit Defciency | References | Threshold for Mechanical Stimuli | Threshold for Mechanical Noxious Stimuli | Latency to Noxious Thermal Stimuli (Spinal) | Latency to Noxious Thermal Stimuli (Spinal + Supraspinal) | Acute and Inflammatory Somatic Pain (Formalin Writhing) | Visceral Pain (Acetic Acid Writhing Response) | Neuropathic Pain |
|---|---|---|---|---|---|---|---|---|
| Cav2.2α1 | 87 | normal | normal | increased (tail flick, not paw flick) | normal | phase 1 normal phase 2 delayed | normal | allodynia and thermal hyperalgesia decreased |
| | 88,93 | n.e. | normal | | increased | phase 1 normal phase 2 delayed | n.e. | n.e. |
| | 89 | n.e. | increased | increased (tail flick and paw flick) | normal | phase 1 normal phase 2 delayed | decreased | n.e. |
| Cav2.3α1 | 99 | n.e. | n.e. | n.e. | n.e. | n.e. | n.e. | n.e. |
| | 98 | n.e. | n.e. | n.e. | n.e. | n.e. | n.e. | n.e. |
| | 96,97, 140,141 | normal | n.e. | normal | normal | phase 1 normal phase 2 decreased | decreased (but only for heterozygous mutants) | n.e. |
| | 59 | n.e. | n.e. | n.e. | n.e. | n.e. | n.e. | n.e. |

Data obtained with the same mouse line were combined (several references); CeA= central amygdala neurons; DRG= dorsal root ganglion cells; SCG= superior cervical ganglion cells; CGC= cerebellar granule cells; n.e. = no experimental data available at present.

suppressed.[87-89] These mice also showed a marked reduction of neuropathic pain symptoms,[87] measured as decreased thresholds to mechanical (allodynia) and thermal (thermal hyperalgesia) stimuli after nerve ligation. This confirms the important pharmacotherapeutical potential of N-type channels for this pain syndrome.

N-type channels play a major role for noradrenaline release from sympathetic nerve terminals.[86] This explains bradycardic and hypotensive actions of N-type channel blockers in vivo. However, the data on the effect of $Ca_v2.2$ deficiency on the cardiovascular system are conflicting. One study reported normal resting heart rates and blood pressure,[87] whereas another group, surprisingly, found both parameters increased. They could show an abnormal baroreflex response in $Ca_v2.2^{-/-}$ mice resulting in a defect in neurotransmitter release from cardiac sympathetic (but not parasympathetic) nerve endings.[93] It remains to be clarified why this leads to the development of hypertension. Obviously, this is of great interest, because it may shed light on the counterregulatory mechanisms occurring under conditions of long term inhibition of sympathetic tone. Taken together the existing data obtained with $Ca_v2.2^{-/-}$ mice re-emphasize the attractivity of selective N-type channel blockers as analgesics. They show that the complete and permanent block of N-type channel function, at least in mice, does not lead to the side effects observed in humans but mediates potent antinociceptive actions. One could speculate that peptide bockers like ziconotide do not reach all N-type channels in the brain at equally high concentrations due to their low penetration into lipophilic compartments.[94] This could lead to a heterogeneous block of N-type channels which may be the cause for toxicity. This hypothesis can only be tested once more hydrophobic $Ca_v2.2$-selective blockers with high brain penetration become available.

### *$Ca_v2.3\alpha_1$-Deficiency*

Three groups have independently generated $Ca_v2.3^{-/-}$ mice [59] (Table 2). Again no severe pathology has so far been described in these mice. Altered sperm motility, decreased glucose tolerance and insulin secretion were reported indicating that these channels also control physiological processes outside the nervous system.[59,95] Enhanced anxiety[96] and altered spatial (but not fear) memory has also been reported.[97]

Analysis of $Ca^{2+}$ current components in $Ca_v2.3^{-/-}$ mice revealed that $Ca_v2.3\alpha_1$ only partially contributes to the formation of R-type current. Its contribution varied from 30% in cerebellar granule cells[98] to >79% in central amygdala[99] hippocampal CA1 and neocortical neurons.[99a] The molecular entities underlying the residual R-type current in $Ca_v2.3^{-/-}$ neurons remain unidentified.

$Ca_v2.3^{-/-}$ mice have normal mechanosensitivity but altered nociception in the formalin-test with a reduced phase 2 response. Interestingly, by exposing the mice to an episode of visceral inflammatory pain 2-3 weeks before, the phase 2 pain response in the formalin test was reduced in wildtype but increased in $Ca_v2.3^{-/-}$ mice. This suggests activation of a long-lasting descending antinociceptive pathway by the visceral conditional stimulus. $Ca_v2.2\alpha_1$-deficient mice also showed this response. This was interpreted as evidence that $Ca_v2.3\alpha_1$-mediated R-type as well as N-type channels participate in this descending antinociceptive pathway. Expression of these channels in brain regions known to mediate antinociception ($Ca_v2.2\alpha_1$ in the rostral medial medulla including the Nucleus raphe magnus and both $\alpha 1$ subunits in the periaqueductal grey) could be demonstrated.[66,87,96] Effects on neuropathic pain have not yet been assessed in $Ca_v2.3^{-/-}$ mice.

## The $Ca_v3$ (T-Type) $Ca^{2+}$ Channel Family

T-type channels are characterized by their low activation threshold and fast inactivation. This group is therefore also termed "low-voltage activated" $Ca^{2+}$ channels. However, this term may not longer be useful as $Ca_v1.3\alpha_1$ can also give rise to $Ca^{2+}$ currents with activation thresholds close to those of T-type channels (see above). Their low activation thresholds allows T-type channels to mediate $Ca^{2+}$ currents and membrane depolarization even before cells reach their

thresholds for firing action potentials. They can therefore serve pacemaker functions in heart and neurons and underly the $Ca^{2+}$-dependent generator potential triggering burst-firing in thalamic and inferior olive neurons[100] (see below). A specific feature of thalamocortical relay neurons (TCR) is to fire action potentials in response to hyperpolarizing stimuli, such as $GABA_B$-receptor activation. The hyperpolarizing stimulus then triggers a low-threshold $Ca^{2+}$-dependent potential ("low-threshold spikes", LTS) which depolarizes the neurons and triggers brief bursts of $Na^+$-dependent action potentials.[100,101] Hyperpolarization can trigger the LTS because it removes inactivation from the ionic conductance generating this response, presumably T-type $Ca^{2+}$ channels. The resulting burst-firing behaviour contributes to the formation of sustained synchronous network discharges in the thalamus which underlies so-called absence seizures and the typical spike-and-wave discharges in electroencephalograms recorded simultaeously.[101,102] Spike-and-wave discharges are generated when a physiological tonic mode of neuronal firing shifts to a burst-firing mode. LTS is likely to be mediated by T-type channels because succinimide antiepileptics block T-type currents and show efficacy in the treatment of absence epilepsy.[102]

Like L-type channels, T-type channels also possess a broad expression profile not restricted to neurons. They are e.g., expressed in various muscle and endocrine cells as well.[103]

The absence of selective high affinity T-type channel probes has slowed the analysis of their subunit structure, function and pharmacotherapeutic potential. For this reason their further characterization is crucially dependent on the availabilty of knockout mouse models.

$Ca_v3.1\alpha_1$ -deficient mice[101] show no gross anatomical, histological and physiological abnormalities in peripheral tissues and the CNS. As this T-type $Ca^{2+}$ channel isoform is the only one expressed in thalamocortical relay (TCR) neurons it was interesting to look for changes in their neuronal activity and their role for LTS. Low-voltage activated $Ca^{2+}$ currents were completely absent in TCR neurons isolated from $Ca_v3.1^{-/-}$ mice but high-voltage activated channels were expressed at normal densities. Burst firing of TCR neurons was absent but neurons were still able to fire trains of action potentials upon depolarization (Fig. 6). $GABA_B$-receptor activation produced absence seizures evident as spike-and-wave discharges only in wildtype mice but not in homozygous mutants.[101] Thus $Ca_v3.1$ channels could be unequivocally identified as the generators of LTS and important targets for antiepileptic drugs.

The ventroposterolateral (VPL) thalamocortical neurons have also been implicated in central pain pathways (see ref. 101a). Injection of T-type channel blockers into this brain region induce hyperalgesia to visceral pain.[101a] Similarly, such a hyperalgesia is also present in $Ca_v3.1^{-/-}$ mice.[101a] In the knockout animals this could be nicely correlated with characteristic changes in the firing pattern of a subset of single units in the VPL region of the thalamus under basal conditions and after induction of visceral pain. In these "visceral pain–responsive" neurons single spikes as well as burst spikes were observed in wildtype mice but burst spikes were completely absent in homozygous knockouts. An intraperitoneal injection of acetic acid evoked an early-onset surge of mainly unitary single spikes followed by a gradual increase of burst spikes which reflect the activation of T-type channels. This increase in burst activity was associated with a decrease of the firing rate of single spikes. In the $Ca_v3.1\alpha1$ deficient mice VPL neurons showed the early-onset increase of single spikes like in wildtype but, due to the lack of T-type channels, the burst activity did not increase and single spike frequency did not decay with time. This was taken as evidence that T-type $Ca^{2+}$ channels in VPL thalamocortical neurons are activated after a surge of pain signal influx from the viscera and then play an inhibitory role in the processing of those signals, thereby suppressing pain responses.

Cav3.2 channel knockout mice are viable and fertile but are somewhat smaller than wiltype mice. They exhibit limited but progressive cardiac fibrosis.[101b] The mice did not confirm the long-standing hypothesis that T-type channels control diastolic depolarization in the heart. However, homozygous knockouts revealed constricted coronary arteries with irregular shapes. This vasoconstriction could be explained by a decreased NO-mediated vasodilatory response although $Ca_v3.2^{-/-}$ arteries responded normally to vasodilation by L-type channel blockers.

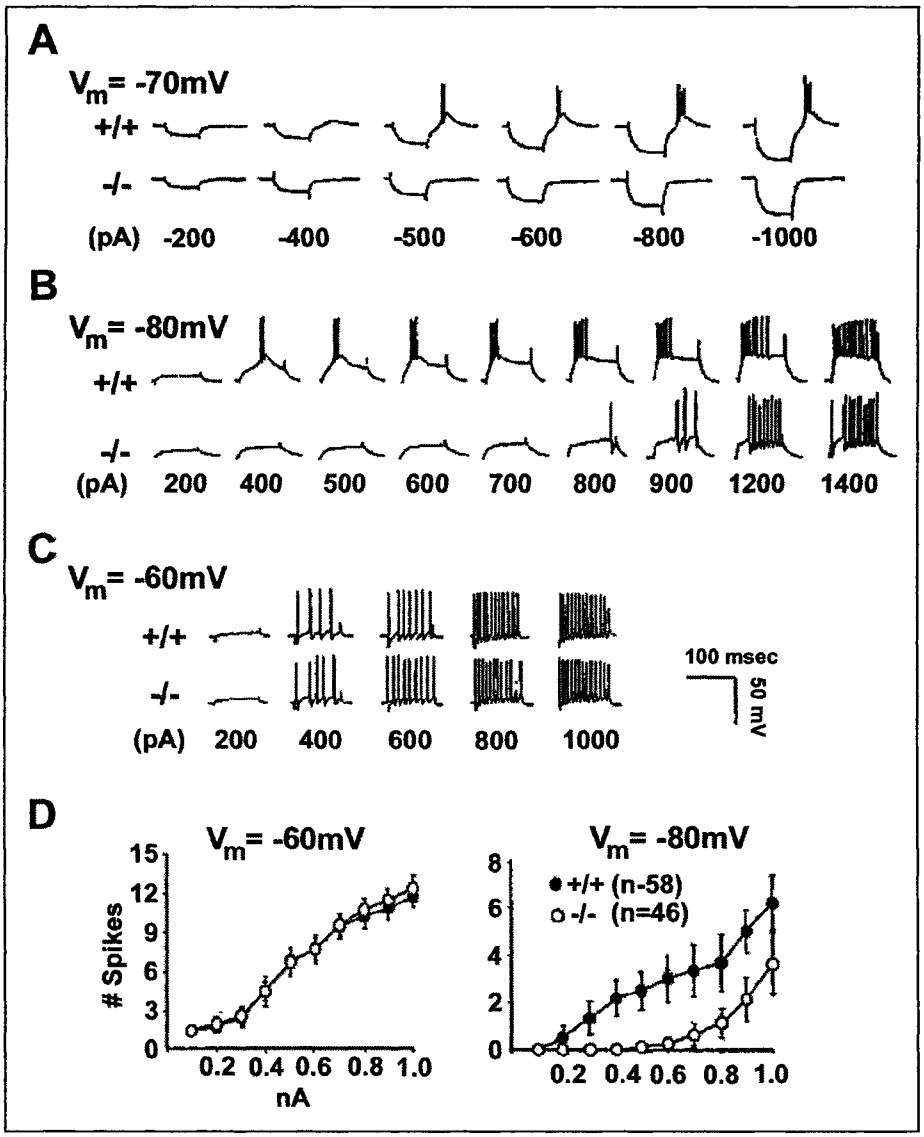



Figure 6. Effect of $Ca_v3.1\alpha1$-deficiency on thalamocortical relay (TCR) neuron firing. Intrinsic firing properties of TCR neurons at the ventrobasal complex. A) Burst firing patterns elicited by hyperpolarization: 100 ms of **negative** step-current inputs at -70 mV elicited a rebound LTS and burst firing. Holding membrane potentials were maintained by DC current input. The amount of current injected is indicated below each trace. Scale bars: horizontal, 100 ms; vertical, 50 ms. B) LTS Burst firing patterns elicited by **positive** step-current inputs at -80 mV. Note the increasing number of lower frequency spikes firing frequency in wild type TCR neurons more than 700 pA of positive current input. These seem to reflect a tonic firing of action potentials. Only the high-frequency, burst-mode firing supported by T-type currents are missing in $Ca_v3.1\alpha1^{-/-}$ neurons. C) Tonic firing patterns elicited by positive step-current inputs at -60 mV. T-type currents are inactivated at this holding potential. The low-frequency spikes are equally elicited in wild-type and $Ca_v3.1\alpha1^{-/-}$ neurons. D) Relation between the number of spikes and the amount of current injected. The number of spikes during 100 ms positive step-current inputs either wehn membrane potentials are held at -60 mV (left) or at -80 mV) (right). Taken from reference 101 with permission.

Interestingly, $Ca_v3.2$ channels were found to biochemically associate with large conductance $BK_{Ca}$ channels after heterologous expression in HEK-293 cells and in mouse brain microsomes.[101b] Taken together these findings suggest that $Ca_v3.2$ T-type channels mediate a $Ca^{2+}$ current in coronary arteries which activates $BK_{Ca}$ channels and allows them to clamp the membrane voltage to hyperpolarized potentials. The impaired activity of this mechanism in $Ca_v3.2^{-/-}$ mice can explain increased vascular tone and the defective response to NO- (cGMP-) mediated vasodilation which requires, at least in part, BKCa channel activation by $Ca^{2+}$. It is likely that the cardiac fibrosis results from reduced myocardial blood flow and myocardial ischemia.

## $Ca^{2+}$ Channel β-Subunits

β-subunits are important for plasma membrane targeting and help fine-tuning $\alpha_1$ function. Deficiency of a particular β-subunit must result in the dysfunction of $\alpha_1$ subunits with which they normally associate. However, compensation by other β-subunit isoforms may occur due to their similar affinity for the β-subunit binding site of $\alpha_1$ subunits and the expression of more than one subunit isoform in neurons or muscle cells.[16,57,104,105]

### *$Ca_v\beta_1$-Deficiency*

$Ca_v\beta_1$ gives rise to two different splice variants. $Ca_v\beta_1b$ represents a neuronal isoform which can associate with different $Ca^{2+}$ channel $\alpha_1$ subunits in mammalian brain.[106-108] In contrast, $Ca_v\beta_1a$ is almost exclusively expressed in skeletal muscle and therefore essential for normal $Ca_v1.1$ function. Unlike in neurons, no other β subunit is abundantly expressed in skeletal muscle which could compensate for $Ca_v\beta_1$. $Ca_v\beta_1$ subunit deficiency results in a dramatic decrease of $Ca_v1.1\alpha_1$ expression.[109] Therefore the phenotype is dominated by skeletal muscle dysfunction indistinguishable from the *mdg/mdg* phenotype (see above).[109] The lethal phenotype has been successfully rescued in $Ca_v\beta_1^{-/-}$ mice carrying a $Ca_v\beta_1a$ transgene under the control of a human skeletal muscle actin-promotor.[110] These mice (produced for studies of β-subunit effects on retinal function) do not express $Ca_v\beta b_1a$ in the retina, suggesting that they also lack $Ca_v\beta1b$ subunits in neurons. They appeared completely normal. Neurophysiological and behavioural tests have not yet been performed with these mice to reveal distinct changes of behaviour or neurophysiological parameters.

### *$Ca_v\beta_2$-Deficiency*

$Ca_v\beta_2$ is the predominant β-subunit isoform expressed in the heart. Only minor amounts of $Ca_v\beta_3$ subunits are present.[16] Like $Ca_v1.2\alpha_1$-deficiency, the absence of $Ca_v\beta_2$ prevents normal embryonic development [110] presumably due to a defect in cardiac function. The lethal phenotype could be rescued in $Ca_v\beta_2^{-/-}$ knockout mice expressing a rat $Ca_v\beta_2$ under a cardiac-specific α-myosin heavy chain promotor.[110] The absence of $Ca_v\beta_2$ in the retina caused visual disturbances similar to those observed in CSNB2 patients with $Ca_v1.4$ mutations (see above). In contrast to wildtype littermates, $Ca_v1.4\alpha_1$ subunits were not present in the outer plexiform layer where they are believed to mediate neurotransmitter release from photoreceptors to bipolar cells. This provides first (albeit very indirect) evidence that $Ca_v\beta_2$ subunits are the major β subunit isoform in photoreceptors and they form complexes with $Ca_v1.4\alpha_1$. Mice deficient in $Ca_v\beta_1$ (see above), $Ca_v\beta_3$ and $Ca_v\beta_4$ [110] (see below) showed no structural and functional abnormalities in the retina.

### *$Ca_v\beta_3$-Deficiency*

$Ca_v\beta3$ and $Ca_v\beta_4$ are the most abundant β-subunits expressed in the mammalian brain. 42% of L-type channels (rabbit cerebral cortex[108]), 56% of N-type channels[106] and 36% of P/Q-type channels (rabbit brain[107]) are associated with $Ca_v\beta3$. Similarly large fractions of channels are associated with $Ca_v\beta4$ (42, 31, and 48%[106-108]). $Ca_v\beta3^{-/-}$ mice did not exhibit a specific pathology, except for an increased blood pressure in response to high-salt diet.

Depolarization-induced vascular smooth muscle contractility was unaffected suggesting a salt-sensitive defect caused by $Ca_v\beta3$ -deficiency in the kidney.[111]

Detailed analysis of sympathetic neurons (superior cervical ganglion cells), which express several $Ca^{2+}$ channel types, revealed a decrease in the density of N-type and L-type $Ca^{2+}$ currents in $Ca_v\beta3^{-/-}$ mice.[112] P/Q-type current density was not affected but the absence of $Ca_v\beta3$ subunits caused a change in the activation kinetics of P/Q-currents. In wildtype cells 61% of the channels activated at negative voltages ($V_{0.5}$' = -21 mV) whereas 39% required stronger depolarizations ($V_{0.5}$ = 20 mV). In $Ca_v\beta3^{-/-}$ cells $V_{0.5}$ was shifted to more negative voltages (-1 mV) which resulted in more P/Q-type current activating by depolarizations positive to -10 mV. These kinetic changes are most likely due to a compensatory association of other β-subunits that are unable to stabilize the more depolarized activation component.

Due to this reduction in N-type currents in $Ca_v\beta_3^{-/-}$ mice it was not surprising that nociception was also found to be altered. Similar to $Ca_v2.2\alpha_1^{-/-}$ mice the phase 2 inflammatory pain (formalin) response was reduced and homozygous mutants showed increased thresholds for acute thermal but not mechanical pain.[113] The density of N-type channels (determined by [$^{125}$I]-ω-CgTx-GVIA binding) was reduced in DRG neuron membranes which seemed to be due to an increase of the proportion of neurons with small N-type current components.[113] In addition, biophysical changes of DRG $Ca^{2+}$ currents were observed which make them less responsive to membrane depolarization and may contribute to the altered pain perception at the spinal level.

At present it is unclear if biophysical changes occur also in other brain neurons and if $Ca_v2.2\alpha_1^{-/-}$ deficiency has any functional consequences on other brain function.

Transient changes in the development of subcortical neuronal pathways were discovered in $Ca_v\beta3^{-/-}$ mice. In anterograde tracer studies a delay in the refinement of visual pathway neurons projecting from the retina to the dorsal lateral geniculate nucleus and superior colliculus was detected.[114] During early postnatal development these neurons project to both appropriate and inappropriate sites. The correct retinotopic representation is then produced by retraction and/or elimination of inappropriately targetet fibers. This process is mediated in part by nitric oxide[115] presumably by affecting long term depression (LTD). The observed delay of the retraction of fibers in the ipsilateral retinocollicular and contralateral retinogeniculate pathways in $Ca_v\beta3^{-/-}$ mice supports the view that LTCCs participate in the generation of a signal inducing fiber retraction. LTCCs also mediate LTD (and long term potentiation) in the developing superior colliculus in a developmentally regulated manner.[115] A likely explanation is therefore that $Ca_v\beta_3$-deficiency decreases L-type currents (like in sympathetic neurons) leading to a decrease of LTD and fiber refinement. However, an alteration of L-type currents in visual pathway neurons has not been shown and no direct correlation between refinement and LTD (or LTD) could yet be established.

## *$Ca_v\beta_4$-Deficiency*

Biochemical studies revelaed that $Ca_v\beta4$ is the major β-subunit associated with P/Q-type channels in the cerebellum.[104,107,108] Spontaneous mouse mutants lacking functional $\beta_4$ subunits (lethargic mice) suffer from ataxia, focal motor seizures and absence epilepsy. It was therefore surprising that P-type $Ca^{2+}$ currents in cerebellar Purkinje cells were not altered in homozygous lethargic mice.[104] This suggested that other β-subunits compensate for $Ca_v\beta_4$-deficiency and was confirmed by coimmunoprecipitation experiments in which enhanced complex formation of N-type ($Ca_v2.2\alpha_1$) and P/Q-type ($Ca_v2.1\alpha_1$) channel mainly with with $Ca_v\beta_1b$ and $Ca_v\beta3$ subunits was detected in lethargic solubilized brain membranes.[104,116] The consequences of $Ca_v\beta_4$-deficiency vary for different neurons. Successful compensation of $\beta_4$ function can explain why the $Ca^{2+}$ dependence and relative contribution of P/Q- and N-type channels to neurotransmitter release at hippocampus Schaffer collateral synapses is unchanged.[117] In contrast, excitatory but not inhibitory neurotransmitter release was reduced in thalamic neurons.[71] This was also observed for *tottering* mouse mutants carrying a $Ca_v2.1\alpha_1$ mutation

disturbing P/Q-type $Ca^{2+}$ channel function and reducing expression density.[72,118] This suggests that P/Q-type channels are mainly affected by the $Ca_v\beta_4$-deficiency and supports the observation that excitatory neurotransmitter release is more dependent on P/Q-type than N-type $Ca^{2+}$ channels (see above). As these thalamic neurons play a role in the generation of absence epilepsy, the imbalance of thalamic neurotransmitter release provides an explanation for the epilepsy phenotype of these mutants.[71]

Mutations in the $Ca_v\beta_4$ subunit have also been found in patients with hereditary forms of episodic ataxia or idiopathic generalized epilepsy.[119] Like in mouse mutants the clinical phenotype resembles another rare human disease with a defect in the $Ca_v2.1\alpha_1$ subunit gene, Episodic Ataxia Type-2. In contrast to the mouse lethargic mutation, the two reported human $Ca_v\beta_4$ mutants lead to a missense mutation in the N-terminal region of $Ca_v\beta_4$ (C104F) or to a C-terminal 38-amino acid truncation (R482X). Both should still bind to $\alpha_1$ subunits and, accordingly, support robust P/Q-type currents after heterologous coexpression because the mutations do not affect the high affinity interaction with $\alpha_1$ subunits. In coexpression experiments only the R482X mutant changed channel function by slightly increasing channel inactivation.[119] It is at present unclear if these slight changes in channel function observed in heterologous expression systems explain the pathology or if other biochemical roles of $Ca_v\beta_4$ are affected, such as channel targeting. It is at present unknown which clinical phenotype would result from a complete absence of $Ca_v\beta_4$ in humans.

## Other Subunits

### *$\alpha_2$-$\delta$-Subunit Deficiency*

Like β-subunits, $\alpha_2$-$\delta$ increases $\alpha_1$-subunit expression at the plasma membrane and can also affect gating when co-expressed in mammalian cells or Xenopus laevis oocytes. In heterologous expression systems effects on gating are usually less pronounced than for β-subunits. At present 4 different $\alpha_2$-$\delta$ subunit isoforms are known. $\alpha_2$-$\delta_1$ was the first to be characterized and was cloned from skeletal muscle.[120] It is not only expressed in skeletal muscle but in many tissues including the brain, heart, lung, spleen, kidney, liver, testis. In the mouse $\alpha_2$-$\delta_2$ is strongly expressed in the brain but also peripheral tissues and $\alpha_2$-$\delta_3$ is brain-specific.[120-123] $\alpha_2$-$\delta_4$ was cloned recently.[124] It is expressed in the pituitary and adrenal gland, the intestine, fetal liver and at low densities in brain. The anticonvulsant drug gabapentin binds with high affinity to $\alpha_2$-$\delta_1$, intermediate affinity to $\alpha_2$-$\delta_2$ but not to $\alpha_2$-$\delta_3$ and $\alpha_2$-$\delta4$.[122,124] As the mechanism and extent of $Ca^{2+}$ channel modulation by gabapentin are still controversial,[125-127] this drug did not prove helpful to assess the (patho)physiological and pharmacotherapeutic role of $\alpha_2$-$\delta$ subunits in vivo.

Two allelic strains of the spontaneous mouse mutant *ducky* (du/du and $du^{2J}/du^{2J}$) exist which lack functional $\alpha_2$-$\delta_2$ subunits.[121] $\alpha_2$-$\delta_2$ is expressed at high densities in Purkinje cells and the molecular layer of the cerebellum consistent with expression on Purkinje cell bodies and its dendritic tree.[121] Homozygous mice suffer from cerebellar ataxia, paroxysmal dyskinesia and absence epilepsy. In the *du/du* strain a genomic rearrangement (including duplications of exons 2 and 3) results in 2 aberrant transcripts, a prematurely truncated N-terminal transcript and a larger aberrant form encoded by exons 2-39.[121] Only the N-terminal transcript yields a low abundance protein product (10 kDa) which is detected in *du/du* cerebellar Purkinje cell bodies.[128] In *du2J/du2J* mice a 2 basepair deletion results in protein truncation (exon 9).[121]

As the absence of full-length $\alpha_2$-$\delta_2$ results in a pathological phenotype which resembles those of spontaneous mutants affecting $Ca_v2.1\alpha_1$ subunits (*tottering, leaner*, see above) it was speculated that $\alpha_2$-$\delta_2$-deficiency could primarily affect P/Q-type $Ca^{2+}$ channel function in the cerebellum. $\alpha_2$-$\delta_2$ enhances $Ca_v2.1$ $Ca^{2+}$ current density upon coexpression in heterologous systems. Accordingly, cerebellar Purkinje cell P-type $Ca^{2+}$ currents were reduced in *du/du* mice due to a decreased density of functional channels in the plasma membrane.[128] These results suggested that $\alpha_2$-$\delta_2$ associates with $Ca_v2.1\alpha_1$ to form P-type currents in cerebellar Purkinje

cells. The 10 kDa *du/du* protein reduced $Ca_v2.1\alpha_1$-mediated $Ca^{2+}$ currents upon heterologous coexpression.[128] Whether this also contributes to P-type current reduction and the *du/du* phenotype remains unclear.

In *du/du* mice the number of cerebellar Purkinje cells was not decreased but they exhibited an abnormal cytoarchitecture. This included atypical initial lateral extensions of the primary dendrite and delayed targeting of the pial surface. The dendritic tree was significantly less complex, reduced in size and frequently did not reach the border of the molecular layer.[128] It must be noted that both allelic *ducky* mutants do not represent true $\alpha_2$-$\delta_2$ null mutations because truncated forms of the protein are synthesized.

## *γ-Subunit Deficiency*

At present at least 8 different γ-subunit genes are known.[129,130] The role of γ-subunits for $Ca^{2+}$ channel function is still enigmatic. In skeletal muscle $Ca_v\gamma_1$-subunits were shown to be stoichiometrically associated with $Ca_v1.1\alpha_1$ in the channel complex. Unlike in the case of $Ca_v\beta_1$, $Ca_v\gamma_1$ deficient mice show no pathology. Detailed analysis revealed a 30% increase in isolated skeletal muscle myocyte $Ca^{2+}$ current density (in mice below 4 weeks of age), a modest slowing of current inactivation kinetics and a shift of the steady-state inactivation curve to more negative potentials (age-independent[131,132]). Intracellular $Ca^{2+}$ release, which is triggered by $Ca_v1.1\alpha_1$ channels independent of $Ca^{2+}$ influx in skeletal muscle myocytes, was also increased.[133] However, $Ca_v\gamma_1$-subunit deficiency had no effect on electrically evoked contraction parameters.[133] $Ca_v\gamma_1$ can therefore not be considered essential for normal excitation-contraction coupling and spontaneous null mutants are likely to remain undetected.

In contrast to $\gamma_1$, an important role for normal brain function could be established for $Ca_v\gamma_2$-subunits but their association with different neuronal VGCCs is controversial. $Ca_v\gamma_2$ was discovered as the protein absent in the spontaneous mouse mutant *stargazer* and therefore termed stargazin.[134] *Stargazer* mice suffer from head-tossing, ataxia and severe absence epilepsy. *Waggler* mutants were identified as an allelic disorder with a very similar phenotype.[134] The *stargazer* allele is associated with the insertion of an early retrotransposon into intron 2 in the same transcriptional direction resulting in a premature transcriptional termination.[134] An association of $Ca_v\gamma_2$ subunits with P/Q- and N-type $Ca^{2+}$ channels has been demonstrated biochemically.[135] Coexpression of $Ca_v\gamma_2$ with $Ca_v2.1$ shifted the steady-state inactivation curve to more negative voltages in some studies[134,136] (but see ref. 135) and decelerated $Ca_v2.2$ current activation kinetics.[135] $Ca_v2.1$ and $Ca_v2.2$-mediated currents were decreased by $Ca_v\gamma_2$ when coexpressed with $\alpha_2$-δ subunit.[135,136] Reduction of $Ca^{2+}$ channel currents seems to be a common feature of many γ-subunits because it was also observed for $Ca_v\gamma_1$ (see above) and $Ca_v\gamma_7$.[130] Both, functional effects and biochemical association, suggest that $Ca_v\gamma_2$ is a neuronal $Ca^{2+}$ channel subunit. However, the interpretation of the *stargazer* phenotype as a $Ca^{2+}$ channel channelopathy is complicated by the finding that *stargazer* mice lack functional AMPA receptors on cerebellar granule cells.[137] Surprisingly, stargazin serves not only as a $Ca^{2+}$ channel subunit but also as an anchoring protein and interacts with both AMPA receptor subunits and synaptic PDZ proteins (e.g.PSD-95). This interaction was found to be essential for delivering functional receptors to the surface membrane and synapses of granule cells.[137] If $Ca_v\gamma_2$ affects $Ca^{2+}$ channel gating and expression then its absence should also affect $Ca^{2+}$ channel gating in intact neurons. However, in cerebellar granule cells $Ca^{2+}$ currents were indistinguishable from wildtype.[137] It is possible that compensation by other γ-subunits can mask $Ca_v\gamma_2$-deficiency. We have learned from the *lethargic* mice (see above) that $Ca^{2+}$ currents (i.e. P-type currents in cerebellar Purkinje cells; ref. 104) must not necessarily be affected in one type of neuron, yet $Ca^{2+}$ channel dysfunction can occur in another (i.e. thalamic neurons; ref. 71). At present it is unclear if AMPA-receptor targeting or altered $Ca^{2+}$ channel function or both are responsible for the *stargazer* and *waggler* phenoptyes.

## Summary

For nine of the ten voltage-gated $Ca^{2+}$ channel $\alpha_1$-subunit genes knockout models are already available. Although many of the associated phenotypes were expected, some of them provided exciting new insight into $Ca^{2+}$ channel physiology and pathophysiology. How can our insight into $Ca^{2+}$ channel function be extended beyond this basic characterization using gene-targeting? Future studies will certainly exploit already available, more sophisticated possibilities of gene-knockout strategies allowing the time-dependent and/or tissue-specific deletion of subunit genes. As evident from the existing data this will be especially useful to study the role of specific $Ca^{2+}$ channel subunits for brain function. In addition targeted mutations within $Ca^{2+}$ channel subunits removing consensus sites for phosphorylation, subunit interaction or drug binding will represent valuable tools to study the impact of altered channel regulation. Such mutations will also help to generate valuable animal models for human diseases. An example is the introduction of single mutations into the gene encoding $Ca_v2.1\alpha_1$,[142] known to cause rare forms of migraine in humans (Familial Hemiplegic Migraine[138,139]).

The described knockout models clearly revealed that voltage-gated $Ca^{2+}$ channel remain an exciting and promising target for drug development. New generations of $Ca^{2+}$ channel modulators may turn out to be useful for the treatment of migraine ($Ca_v2.1\alpha_1$), pain ($Ca_v2.2\alpha_1$, $Ca_v2.3\alpha_1$), hearing disorders ($Ca_v1.3\alpha_1$), cardiovascular diseases ($Ca_v1.3\alpha_1$), cognitive dysfunction ($Ca_v1.3\alpha_1$) and epilepsy ($Ca_v2.2\alpha_1$, $Ca_v3.1\alpha_1$).

## *Note Added in Proof*

This chapter was submitted based on published evidence as of August 2002. Some, but not all, of the more recent literature was included in proof stage (July 2004).

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