CHAPTER 2

STRUCTURAL AND FUNCTIONAL DIVERSITY OF VOLTAGE-ACTIVATED CALCIUM CHANNELS

MICHEL DE WAARD, CHRISTINA A. GURNETT, and KEVIN P. CAMPBELL

1. INTRODUCTION

1.1. Voltage-Dependent Ca^{2+} Channels as Pathways for Intracellular Ca^{2+} Elevation

Action potentials and neurotransmitters are the vehicle of information transfer in the nervous system. The diversity of this information is in part encoded by the quantitative contribution and properties of the ion channels underlying both the action potential and the events that follow. As such, voltage-dependent Ca^{2+} channels represent one class of these ion channels that have both a remarkable ubiquity and importance in a variety of cell types. At rest, there is normally a 10,000-fold concentration difference between intracellular and extracellular Ca^{2+} concentrations. Intracellular Ca^{2+} concentrations are normally in the 10–100 nM range, whereas outside cells, the Ca^{2+} concentration is about 1–2 mM. Elevations of the intracellular Ca^{2+} concentrations can occur not only as a result of the increase in activity of voltage-sensitive Ca^{2+} channels, but also in response to the activation of ligand-gated ion channels (NMDA-sensitive glutamatergic receptors or nicotinic ACh receptors) or through the release of Ca^{2+} from intracellular pools (IP_3- and/or ryanodine-sensitive). During increases in voltage-dependent Ca^{2+} channel activity, the cytosolic Ca^{2+} can reach concentrations up to 100 μM immediately beneath the

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plasma membrane. This rise in intracellular Ca^{2+} is generally not homogeneous but instead seems to be highly organized in space and time. The buffering activity of numerous cellular proteins, which bind Ca^{2+} tightly and rapidly, limits the spatial diffusion of transient cytosolic Ca^{2+} increases and keeps the overall Ca^{2+} concentration to a low free level. Several other mechanisms also ensure an effective buffering of free cytoplasmic Ca^{2+} in the cell. In the plasma membrane, the Ca^{2+}-ATPase and the electrogenic Na^+/Ca^{2+} exchanger can effectively extrude Ca^{2+} from the cell. Several intracellular Ca^{2+} stores (endoplasmic reticulum and mitochondria) will also accumulate Ca^{2+} by activating a Ca^{2+}-ATPase or a $Ca^{2+}/2H^+$ exchanger.

Physiologically, Ca^{2+} acts as an intracellular second messenger by initiating or regulating numerous biochemical and electrical events of the cell. Calcium ions are implicated in the regulation of several enzymes and in the control of gene expression. They are essential for contraction in all types of muscle and for the control of the activity of several other ion channels (i.e., Ca^{2+}-activated K^+ and Cl^- channels). They also control many neuronal events such as neurotransmitter release (Katz, 1969; Augustine *et al.*, 1987), synaptogenesis, and neurite outgrowth.

Thus it is increasingly evident that a diverse number of voltage-dependent Ca^{2+} channels are required to account for the complex spatiotemporal characteristics of Ca^{2+} entry and for the numerous cellular functions of Ca^{2+} ions. In fact, several types of voltage-dependent Ca^{2+} channels have now been identified that differ by their functional properties. In this chapter, we will review the structural features that are the basis of the biophysical, pharmacological, and functional diversity of voltage-dependent Ca^{2+} channels. In particular, we will emphasize the structural determinants that control the subunit composition of Ca^{2+} channels and the role of ancillary subunits in Ca^{2+} channel diversity and function.

1.2. Biophysical Diversity of Voltage-Dependent Ca^{2+} Channels

Five classes of voltage-dependent Ca^{2+} channels have clearly been defined so far based on their biophysical and pharmacological properties (Table I). The brain has the most complex and diverse expression of Ca^{2+} channels as it expresses at least one member of each class. The nomenclature adopted is the one proposed by Nowycky *et al.* (1985) and Fox *et al.* (1987a,b) based on recordings of the peripheral nervous system. This nomenclature was subsequently expanded to include Ca^{2+} current recordings from cells of the central nervous system (Mintz *et al.*, 1992a,b; Usowicz *et al.*, 1992; Ellinor *et al.*, 1993). To date, two main categories of Ca^{2+} channels have been identified in excitable cells: a low voltage–activating (LVA) channel (T-type), which is also rapidly inactivating, and several high voltage–activating (HVA) channels (L-, N-, P-, and R-type). Other than their activation threshold, numerous functional criteria have been used to discriminate among voltage-dependent Ca^{2+} channels. The identification of these channels relies primarily on their conductance, the time- and voltage-dependence of inactivation, the relative permeabilities to several divalent cations, the distribution of open- and close-time durations, and the identification of several gating models. The following sections illustrate the properties of these LVA and HVA channels.

T-type Ca^{2+} channels have been identified in several tissues, including neuronal, smooth muscle, cardiac, and skeletal muscle cells. They are activated by relatively small

TABLE I
Biophysical Classification of LVA and HVA Ca^{2+} Channels[a]

Property	T	L	N	P	R
Conductance	7 to 10 pS	11 to 25 pS	10 to 22 pS	9 to 19 pS	14 pS
Activation threshold	>−70 mV LVA	>−30 mV HVA	>−30 mV HVA	>−40 mV HVA	>−40 mV HVA
Inactivation range	−110 to −50 mV	−60 to −10 mV	−120 to −30 mV	?	−100 to −40 mV
Inactivation rate	14 to 80 msec	>500 msec	50 to >500 msec	>500 msec	20 to 40 msec
Relative conductance	$Ba^{2+} = Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	?	$Ba^{2+} > Ca^{2+}$
Open-time duration	0.5 to 2 msec	0.5 to 10 msec	0.7 to 1.5 msec	<1 msec	?
Gating modes	?	2	3	?	?

[a]The conductance values are estimated from recordings using 90–110 mM Ba^{2+} as the charge carrier. The two gating modes of L-type channels are based on switching of channel activity from a short-lived open-time duration to a long-lived one. The N-type channel has three gating modes that are due to the switching of the channel between low-, medium-, and high-open state probabilities (Delcour and Tsien, 1993).

depolarizations compared to other Ca^{2+} channel types (> −70 mV) and reach peak current amplitude at about −30 mV. Remarkably, these LVA channels inactivate very rapidly with time constants on the order of 10–80 msec and at very negative membrane potentials ($V_{1/2}$ < −50 mV). This set of properties explains the importance of LVA channels in pacemaker activity (Hagiwara *et al.*, 1988). T-type channels also have the lowest conductance of all voltage-dependent Ca^{2+} channels, with values that range between 7 and 10 pS with 100 mM Ba^{2+} as the charge carrier. Unlike HVA channels, LVA channels are equally permeable to Ca^{2+} and Ba^{2+}, suggesting differences in the ion channel structure responsible for ionic selectivity (Nilius *et al.*, 1985).

L-type Ca^{2+} channels probably represent the most heterogeneous group of voltage-dependent Ca^{2+} channels, as several subtypes have been identified in a wide range of tissues from muscle to brain. These HVA channels typically have a conductance between 11 and 25 pS and are long-lasting with little time- or voltage-dependent inactivation. The functional properties of L-type Ca^{2+} channels are most similar in cardiac, secretory, and neuronal cells. However, the skeletal muscle L-type channel is significantly different in that it activates one order of magnitude more slowly than cardiac or smooth muscle L-type channels. This channel is also characterized by a smaller conductance (~11 pS) than most other L-type Ca^{2+} channels. The time course of inactivation can also help in discriminating between the various L-type Ca^{2+} channels. While the kinetics of inactivation of the L-type channels are very slow in neurons and secretory cells, they are more rapid in cardiac and vascular muscle (Kass and Sanguinetti, 1984; Fedulova *et al.*, 1985). There is evidence that functionally different L-type Ca^{2+} channels can coexist in the same cell type. Forti and Pietrobon (1993) have described the presence of no less than three different dihydropyridine (DHP)-sensitive Ca^{2+} channels in rat cerebellar granule neurons. Two of the channels have cardiac-like properties, and another channel was characterized by an unusual increase in gating activity upon membrane repolarization.

N-type Ca^{2+} channels are probably the best characterized of the neuronal HVA channels. They are characterized by a greater sensitivity to holding potential and complex gating behaviors (Delcour and Tsien, 1993; Plummer and Hess, 1991). Their conductances are on average smaller than L-type channels and range between 12 and 20 pS (Fox *et al.*, 1987b; Plummer *et al.*, 1989; Hirning *et al.*, 1988). These channels are completely inactivated at −30 mV, a membrane potential where L-type channels remain largely active. The inactivation time course of N-type Ca^{2+} channels can be both fast (time constants in the tens of milliseconds) or slow (time constants in the hundreds of milliseconds). In fact, individual N-type Ca^{2+} channels can carry two kinetically distinct components, and the switching between both components seems to be under the control of an unidentified cellular factor (Plummer and Hess, 1991).

The existence of a distinct P-type Ca^{2+} channel was recently proposed on the basis of Ca^{2+} current recordings in Purkinje neurons (Mintz *et al.*, 1992a). P-type channels activate for potentials above −40 mV and show no or very little time-dependent inactivation during depolarization (time constant in the range of a second) (Regan *et al.*, 1991; Mintz *et al.*, 1992a,b). The unitary conductance of P-type channels remains a complex issue. Usowicz *et al.* (1992) has recently demonstrated the coexistence of no less than three different conductances (9, 13, and 19 pS) for this channel in Purkinje neurons. It has not yet been resolved whether these conductances are due to the activity of a single Ca^{2+} channel type or of several subtypes of the P channel. Overall, these channels are probably best identified by a different pharmacological sensitivity than N- and L-type Ca^{2+} channels.

In contrast to T-, L- and N-type Ca^{2+} channels, there is more controversy concerning the identity of R-type Ca^{2+} channels that have recently emerged in the biophysical classification (Randall *et al.*, 1993). R-type Ca^{2+} channels are found in cerebellar granule cells and represent another class of HVA channels. Like N- and T-type Ca^{2+} channels, the R-type channel inactivates at fairly hyperpolarized potentials ($V_{1/2}$ = −70 mV). Interestingly, R-type channels also have very fast time-dependent inactivation (time constant between 20 and 40 msec), further enhancing their resemblance to T-type channels.

It is worth mentioning that voltage-sensitive Ca^{2+} channels can be distinguished by their properties of cell regulation, tissue-specific expression, and cell compartmentalization. For instance, LVA Ca^{2+} channels have been successfully isolated after selective run-down of HVA currents (Carbone and Lux, 1987; Nilius *et al.*, 1985). Also, L-type Ca^{2+} channels in smooth or cardiac muscles can be identified by their process of Ca^{2+}-dependent inactivation (Kalman *et al.*, 1988). However, due to overlapping biophysical properties of the various Ca^{2+} channel types, it is now believed that the best discriminating criteria of these channels are their pharmacological susceptibilities to blockage by specific drugs and toxins.

1.3. Pharmacological Diversity of Voltage-Dependent Ca^{2+} Channels

Table II summarizes the pharmacological properties of the various Ca^{2+} channel types. Although the list of compounds that target voltage-dependent Ca^{2+} channels seems to increase exponentially, currently only three classes of drugs efficiently discriminate between functionally different Ca^{2+} channels. First, 1,4-dihydropyridines are synthetic organic compounds that have been useful in identifying a class of Ca^{2+}

TABLE II
Pharmacological Sensitivity of LVA and HVA Ca^{2+} Channels[a]

Drug	T	L	N	P	R
Dihydropyridines	− (10 μM)	+ Kd < 37 nM	− (10 μM)	− (10 μM)	− (10 μM)
Phenylalkylamines	− (50 μM)	+ Kd < 10 μM	− (10 μM)	?	?
Benzothiazepines	− (10 μM)	+ Kd < 1 μM	− (10 μM)	?	?
ω-CgTx GVIA	− (5 μM)	−/+ (R) (1 μM)	+ (I) Kd = 0.7–30 nM	− (5 μM)	− (5 μM)
ω-CgTx MVIIC	− (5 μM)	− (10 μM)	+ Kd = 1–10 μM	+ Kd < 1 μM	− (5 μM)
ω-Aga IVA	− (200 nM)	− (200 nM)	− (800 nM)	+ Kd = 2–10 nM	− (100 nM)
ω-Aga IIIA	− (100 nM)	+ Kd = 1 nM	+ Kd = 1 nM	+ Kd = 0.5 nM	?
Cd^{2+}	+ Kd > 40 μM	+ Kd = 1.5 μM	+ Kd = 1 μM	+ Kd ?	+ Kd = 1 μM
Ni^{2+}	+ Kd < 40 μM	+ Kd = 230 μM	+ Kd = 270 μM	+ Kd ?	+ Kd = 65 μM

[a]The sensitivity of voltage-dependent Ca^{2+} channels to organic (classical Ca^{2+} channel antagonists and various toxins) and inorganic molecules (Ni^{2+} and Cd^{2+}) is shown. DHPs, ω-CgTx GVIA, and ω-Aga IVA are the most specific channel ligands and thus discriminate effectively between L-, N-, and P-type channels, respectively. The highest ineffective drug concentration is shown in parentheses. The effective concentrations of the various drugs are given for Ca^{2+} current recording conditions. When the effect of the molecule depends on the extracellular Ca^{2+} concentration, the effective drug concentrations are given for an extracellular permeant ion concentration of 5–10 mM.

channels found in muscle and several neurons and neuroendocrine cells. For instance, L-type Ca^{2+} currents in rat insulinoma cells are blocked by nimodipine with an apparent K_d less than 37 nM (Pollo *et al.*, 1993). Although L-type Ca^{2+} channels are all sensitive to DHPs, several subtypes of L channels exist that are characterized by small variations in their pharmacological and biophysical properties. For instance, skeletal and cardiac muscle L-type channels have distinctive biophysical properties despite closely related structures and functions. The dissociation constant of DHP for skeletal muscle L-type channel is greater than that for brain and cardiac muscle (Glossmann and Striessnig, 1988). Also, higher concentrations of DHP are required to block HVA channels in neurons than in smooth muscle cells (Triggle and Janis, 1987). Skeletal muscle L-type channels are not only sensitive to DHPs, but also to phenylalkylamines, benzothiazepines, and piperazines, thus defining four pharmacological binding sites that interact allosterically among each other (Catterall and Striessnig, 1992). It is still not well established whether all L-type Ca^{2+} channels, particularly those in brain, are also sensitive to these additional classes of drugs. Also, DHP antagonists are more selective

and potent blockers for smooth muscle than for cardiac muscle L-type Ca^{2+} channels despite a minimum of 95% amino acid identity between the α_1 subunits of both channels (Welling *et al.*, 1993). Subtle pharmacological differences could be very useful to distinguish among subtypes of the L-type family.

Second, ω-conotoxin GVIA (ω-CgTx GVIA), a Ca^{2+} channel antagonist from the venom of the piscivorous marine mollusk *Conus geographus*, specifically blocks N-type channels. In early studies of the N-type Ca^{2+} channels in PC12 cells, ω-CgTx GVIA was shown to irreversibly block a component of the high threshold–activated, DHP-insensitive, Ca^{2+} current (Plummer *et al.*, 1989). Also, the toxin blocks N-type Ca^{2+} channels from sympathetic neurons with high affinity (K_d = 0.7 nM) (Boland *et al.*, 1994). DHP-sensitive Ca^{2+} channels were not affected by the application of ω-CgTx GVIA, demonstrating the specificity of the block. In addition, [^{125}I]-ω-CgTx GVIA binding in the brain is not inhibited by DHPs. However, the toxin specificity is probably not absolute because in rare cases some L-type Ca^{2+} channels can be reversibly blocked by high concentrations of ω-CgTx GVIA (Kasai and Neher, 1992). Thus, irreversible and high-affinity blocking by ω-CgTx GVIA is adopted as the defining characteristic of N-type Ca^{2+} channels.

Third, ω-agatoxin IVA (ω-Aga IVA) is a 48–amino acid peptide antagonist isolated from the spider venom of *Agelenopsis aperta* that is selective for the P-type Ca^{2+} current (Mintz *et al.*, 1992a,b). The venom of this spider contains a second toxin ω-Aga IVB that has 71% amino acid identity with ω-Aga IVA and shares its specificity and potency (K_d = 3 nM) for P-type channels (Adams *et al.*, 1993). Interestingly, the blocking of P-type currents by both ω-Aga IVA and ω-Aga IVB can quickly be reversed by large depolarizations (Mintz *et al.*, 1992a), demonstrating that the toxin binding site is dependent on the conformation of the channel in the same manner that the DHP binding site depends on the L-type channel conformation. Although P-type Ca^{2+} channels were initially characterized by their lack of sensitivity to DHP antagonists and to ω-CgTx GVIA, they are potently inhibited by ω-CgTx MVIIC (or SNX-230). This result was somewhat surprising because ω-CgTx MVIIC, a 26–amino acid peptide toxin from the marine snail *Conus magus*, is structurally related to ω-CgTx GVIA (Hillyard *et al.*, 1992). This toxin seems, however, to lack the specificity required for the unambiguous identification of P-type channels since it also blocks N-type Ca^{2+} channels, albeit with a lower affinity.

In contrast, R-type and T-type Ca^{2+} channels, also present in a variety of neurons, are mainly characterized by their insensitivity to DHPs, ω-CgTx GVIA, and ω-Aga IVA. The lack of selective antagonists has made the identification of these channels more complicated at the unitary level. However, by minimizing the amplitude of membrane depolarizations, T-type channels are easier to isolate than R-type channels. Also, LVA channels are blocked by Ni^{2+} concentrations lower than those required for Cd^{2+}, an order of potency opposite to that displayed by HVA channels. Again, this is consistent with the proposal of a difference in selectivity between these two groups of channels (Nilius *et al.*, 1985; Hagiwara *et al.*, 1988). R-type channels also have a slightly higher sensitivity to Ni^{2+} than other HVA channels, which could aid in their identification. Overall, it is believed that R-type channels may be a heterogeneous population of voltage-sensitive channels mainly characterized by their lack of sensitivity to all three specific Ca^{2+} channel antagonists.

Although many Ca^{2+} channel types are routinely distinguished by their pharmacological properties, not much is known about the structural requirements underlying their pharmacological differences. Clearly, some structural diversity is expected from channels that have differential sensitivities to structurally unrelated drugs. In contrast, it is generally assumed that Ca^{2+} channels, sensitive to one or several related drugs, may differ only by minor structural features. However, several examples seem to contradict these two widely accepted assumptions. For instance, it was found that two peptide toxins from the *Agelenopsis aperta* spider venom (ω-Aga I and ω-Aga IIIA) are potent blockers of both L and N channels despite extensive structural differences between these two channel types (Mintz *et al.*, 1991; Cohen *et al.*, 1992). Thus, these results suggest that very different channels can share some degree of homology restricted to their toxin binding sites, as recently suggested by Adams and Olivera (1994). Also, it seems that only slight differences in channel structure are required to account for marked differences in DHP sensitivities between cardiac or smooth muscle L-type Ca^{2+} channels (Welling *et al.*, 1993) or even between splice variants of the cardiac channel (Soldatov *et al.*, 1995). This suggests that an extensive pharmacological subdivision of voltage-dependent Ca^{2+} channels on the basis of their sensitivity to structurally related drugs may not necessarily translate into a greater functional and biophysical diversity of Ca^{2+} channels.

1.4. Correlation between Ca^{2+} Channel Type and Function in Excitable and Nonexcitable Cells

L-type Ca^{2+} channels play important roles in excitation-contraction (E-C) coupling in skeletal, cardiac, and smooth muscle by triggering Ca^{2+} release from the sarcoplasmic reticulum (SR) through the ryanodine receptor. In cardiac muscle, Ca^{2+} release from the SR is induced by the entry of Ca^{2+} through the L-type channel. In contrast, in skeletal muscle, conformational changes in the L-type channel appear sufficient to trigger Ca^{2+} release from intracellular stores in the absence of any extracellular Ca^{2+} permeation. This has led to the proposal that a direct or indirect molecular coupling exists between the L-type channel in the plasma membrane and the ryanodine receptor in the SR membrane. Finally, neuronal L-type Ca^{2+} channels may play an important role in basal cellular activity and a lesser role in neurotransmission, a proposal that is consistent with their localization in cell bodies and proximal dendrites (Westenbroek *et al.*, 1990).

N-, P-, and R-type Ca^{2+} channels are predominantly neuronal (Tsien *et al.*, 1988). However, N-type Ca^{2+} channels have also been found in endocrine cells such as human small-cell lung carcinoma (Sher *et al.*, 1990a) and rat insulinoma (Sher *et al.*, 1990b) cell lines. Immunocytochemical results demonstrate that both N- and P-type Ca^{2+} channels are located along dendrites as well as at synapses (Westenbroek *et al.*, 1992).

There is a general consensus that DHP-resistant (P-, N-, and R-type) Ca^{2+} channels reside in nerve terminals and are involved in exocytosis in mammalian central neurons (Hirning *et al.*, 1988; Takahashi and Momiyama, 1993; Weeler *et al.*, 1994). For instance, the release of glutamate and GABA from hippocampal neurons can be inhibited by ω-CgTx GVIA, illustrating the role of N-type Ca^{2+} channels in transmitter release (Burke *et al.*, 1993). Also, ω-Aga IVA blocks the GABAergic transmission in cerebellar and spinal cord slices with potencies close to that required for Ca^{2+} current blockade of

the P-type current from Purkinje cells (Mintz *et al.*, 1992a; Takahashi and Momiyama, 1993). Both N- and P-type Ca^{2+} channels have also been found at the neuromuscular junction (Robitaille *et al.*, 1990; Sugiura *et al.*, 1995). In frog neuromuscular junction, the transmitter release is entirely blocked by ω-CgTx GVIA demonstrating the predominant role of N-type Ca^{2+} channels in presynaptic activity (Kerr and Yoshikami, 1984). In contrast, in mammalian neuromuscular junction, synaptic transmission is insensitive to the N-type channel blocker, implicating a role for other voltage-dependent Ca^{2+} channels. Double labeling of mouse neuromuscular junction with biotinylated SNX-260, a structural analog of ω-CgTx MVIIC, and α-bungarotoxin indicates a presynaptic localization of P-type Ca^{2+} channels at the active zone and suggests a role of these channels in mammalian peripheral transmitter release (Sugiura *et al.*, 1995). In many preparations, however, neurotransmission is not completely abolished by the combination of both saturating concentrations of ω-CgTx GVIA and ω-Aga IVA, providing evidence for the involvement of other voltage-dependent Ca^{2+} channels, likely R-type. Although neurosecretion in brain does not appear to be directly dependent on DHP-sensitive L-type Ca^{2+} channels, they have been shown to be intimately involved in secretion in adrenal chromaffin cells. While both P- and N-type Ca^{2+} channels were able to trigger some secretion, activation of L-type Ca^{2+} channels appeared to be even more efficiently coupled to secretion in these cells (Artalejo *et al.*, 1994).

The activity of both N- and P-type Ca^{2+} channels is heavily regulated by several receptor-activated pathways. The N-type channel can be inhibited by norepinephrine in rat sympathetic neurons (Bean, 1989), by GABA in rat hippocampal neurons (Scholz and Miller, 1991), and by glutamate in rat CA3 pyramidal neurons (Swartz and Bean, 1992). P-type Ca^{2+} channels in cerebellar Purkinje neurons are also inhibited by the $GABA_B$ agonist baclofen (Mintz and Bean, 1993). Inhibition of Ca^{2+} channel activity is a reasonable mechanism for regulation of transmitter release by these neuromodulators. Several questions are currently under analysis, in particular which Ca^{2+} channel subtypes control neurosecretion and whether these channels are differently modulated by receptor-activated pathways.

Most nonexcitable cells, including lymphocytes and mast cells, do not contain significant components of voltage-dependent Ca^{2+} channels. While many intracellular signaling events, such as T-cell activation and histamine secretion from mast cells, rely on extracellular Ca^{2+} influx, the Ca^{2+} current is clearly distinguished from voltage-dependent Ca^{2+} current based on its gating, unitary conductance, and ionic selectivity (Tsien *et al.*, 1987).

2. SUBUNIT STRUCTURE OF VOLTAGE-DEPENDENT Ca^{2+} CHANNELS

2.1. Purification of the Skeletal Muscle L-Type Ca^{2+} Channel

The skeletal muscle transverse tubule system represents the richest and most homogeneous source of voltage-dependent L-type Ca^{2+} channels in the body. This channel can also be specifically labeled by a tritiated derivative of PN200-110, a 1,4-DHP

antagonist. This set of properties has helped the first purification of a voltage-dependent Ca^{2+} channel (Flockerzi *et al.*, 1986; Leung *et al.*, 1987; Takahashi *et al.*, 1987). The skeletal muscle L-type Ca^{2+} channel is a complex of four subunits (α_1, $\alpha_2\delta$, β, and γ) which copurify in a stoichiometric ratio (1:1:1:1) with a total molecular mass of about 400 kDa (Fig. 1).

The purified α_1 subunit has a molecular mass of about 170 kDa and is responsible for the process of ionic permeation and the mechanical coupling to the sarcoplasmic reticulum Ca^{2+} release channel. The 170-kDa α_1 subunit appears to be a proteolytic product of a larger and minor 212-kDa protein in which approximately 300 C-terminal amino acids have been posttranslationally removed (De Jongh *et al.*, 1989). The truncated skeletal muscle α_1 subunit, like its full-length counterpart, can function both for Ca^{2+} permeation and E-C coupling (Beam *et al.*, 1992). Although deletions in the C-terminal sequence of the cardiac α_1 subunit resulted in increases in the open probability of the channel (Wei *et al.*, 1994), similar C-terminal deletions in the skeletal α_1 subunit had no effect on Ca^{2+} channel activity. Thus, the physiological relevance of this

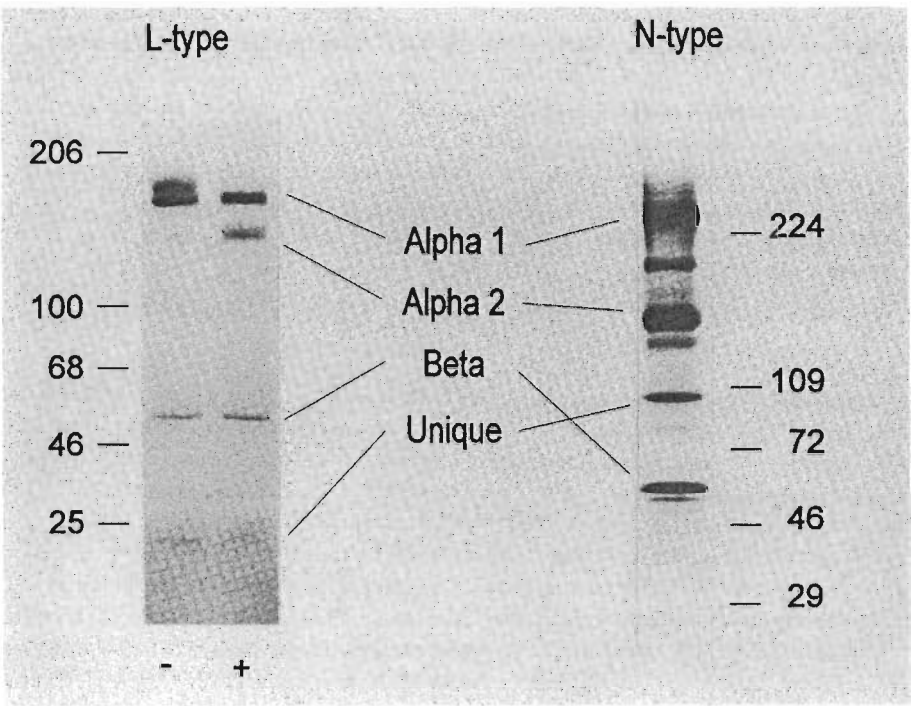


FIGURE 1. Purified L- and N-type Ca^{2+} channel subunits. (A) The four subunits of the voltage-dependent L-type Ca^{2+} channel are shown under reducing (+) and nonreducing conditions (−) (Leung *et al.*, 1987). On reduction, the $\alpha_2\delta$ subunit undergoes a characteristic mobility shift to 150 kDa. The γ subunit is unique to the L-type Ca^{2+} channel complex. (B) The purified subunits of the N-type Ca^{2+} channel complex are shown with the unique 95-kDa subunit (Witcher *et al.*, 1993a).

posttranslational modification is not yet understood. The α_1 subunit also contains the binding sites for DHP (Striessnig *et al.*, 1991), for phenylalkylamines (Striessnig *et al.*, 1990), and for benzothiazepines (Catterall and Striessnig, 1992). These sites have been well characterized by the use of photoaffinity ligands (Striessnig *et al.*, 1990; Nakayama *et al.*, 1991; Regulla *et al.*, 1991; Tang *et al.*, 1993). The phenylalkylamines bind to a cytoplasmic sequence located downstream from the S6 helix of the fourth homologous repeat (refer to the membrane topology of the α_1 subunit in Section 3.3). Instead, the DHP binding site is essentially extracellular and is formed by two extracellular sequences just upstream from the S6 helices of repeats III and IV and the loop between helices S5 and S6 of the third repeat.

The skeletal muscle β subunit has a molecular mass of about 52 kDa. Since it is a nonglycosylated, hydrophilic, and peripheral membrane protein, its localization is completely cytoplasmic (Takahashi *et al.*, 1987). Consistent with this proposal, this subunit is also phosphorylated by cAMP-dependent protein kinase, protein kinase C, and cGMP-dependent protein kinase (Jahn *et al.*, 1988). The $\alpha_2\delta$ subunit is a 170-kDa complex that is cleaved to an α_2 (140 kDa) and a δ (19–32 kDa) peptide under reducing conditions, demonstrating their linkage by disulfide bonds. This subunit is heavily glycosylated, both on α_2 and δ, with 30% of its molecular mass composed of carbohydrates.

The γ subunit has a molecular mass of 32 kDa and, like the $\alpha_2\delta$ subunit, is also a glycosylated transmembrane protein. This subunit is very hydrophobic and is proposed to contain four transmembrane α-helices. Northern blot analyses have demonstrated that this subunit is unique to skeletal muscle (Jay *et al.*, 1990). The specific association of all these subunits within a multisubunit complex was proven, not only by the copurification of $\alpha_2\delta$, β, and γ subunits with the α_1 DHP receptor, but also by coimmunoprecipitation experiments of the complex with antibodies that selectively recognize each subunit (Lazdunski *et al.*, 1987; Takahashi *et al.*, 1987; Leung *et al.*, 1988; Sharp *et al.*, 1988; Sharp and Campbell, 1989).

2.2. Purification of the N-Type Ca^{2+} Channel Complex

There are several structural homologies between L- and N-type Ca^{2+} channels, as demonstrated by the usefulness of several cross-reacting antibodies used in purifying Ca^{2+} channels from brain (Sakamoto and Campbell, 1991; Witcher *et al.*, 1993a). The use of [^{125}I]-ω-CgTx GVIA as a specific marker of N-type channels was critical to its successful purification. Toxin-labeled N-type channels have been purified to homogeneity by successive enrichment steps that include the use of heparin-agarose chromatography, immunoaffinity chromatography with a β-specific monoclonal antibody, and a sucrose density gradient centrifugation (Witcher *et al.*, 1993a). Four proteins from brain of 230, 140, 95, and 57 kDa molecular weight copurify with the toxin binding activity in approximately equivalent stoichiometric ratios (Witcher *et al.*, 1993a) (Fig. 1). These proteins also comigrate on the sucrose gradient with the peak of [^{125}I]-ω-CgTx GVIA activity demonstrating that they all represent tightly interacting subunits of the same Ca^{2+} channel complex.

The N-type α_1 subunit from rabbit brain has a molecular mass of 230 kDa and binds ω-CgTx GVIA, but not DHPs or phenylalkylamines. Two size forms of the α_1 subunit

(235 and 210 kDa) appear in purification of the N-type Ca^{2+} channel (Westenbroek *et al.*, 1992) much as is found in L-type channels of skeletal muscle (De Jongh *et al.*, 1991) or neurons (Hell *et al.*, 1993). Structurally, the lower molecular mass subunit has been shown to result from a C-terminal truncation of the full-length protein. The two size forms have been shown to be differentially phosphorylated by calcium- and calmodulin-dependent protein kinase II (CaM kinase II) *in vitro* (Hell *et al.*, 1994). Differential phosphorylation of the two size forms *in vitro* may indicate the possible relevance of the C-terminal truncations seen *in vivo*. The 140-kDa $\alpha_2\delta$ subunit is biochemically quite similar to the skeletal muscle $\alpha_2\delta$ subunit. It is extensively glycosylated and has a characteristic mobility shift on reducing SDS-PAGE with the appearance of the disulfide-linked δ peptides. The β-subunit, while of another type, shares several immunogenic epitopes with the skeletal muscle β subunit. A unique 95-kDa protein was shown to copurify with the channel complex. Although this protein is less well characterized than the other proteins that had skeletal muscle homologues, it remains possible that the 95-kDa protein is an isoform of the γ subunit. Like the γ subunit, this protein is lightly glycosylated and hydrophobic as suggested by 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine labeling.

In conclusion, the purification of a muscle L-type and a brain N-type Ca^{2+} channel strongly suggests that the minimum subunit composition of most native HVA Ca^{2+} channels is $\alpha_1\alpha_2\delta\beta$ and that some structural variability can be introduced by the association of a fourth subunit whose expression is tissue-specific.

2.3. Characterization of Other Voltage-Dependent Ca^{2+} Channels

There is much biochemical evidence demonstrating that other voltage-dependent Ca^{2+} channels are also multisubunit complexes composed of at least an α_1, an $\alpha_2\delta$, and a β subunit. Two monoclonal antibodies directed against the skeletal muscle $\alpha_2\delta$ subunit immunoprecipitate a very significant fraction of [^{3}H]-PN200-110 from brain homogenates (Ahlijanian *et al.*, 1990), demonstrating that neuronal L-type Ca^{2+} channels also contain the $\alpha_2\delta$ subunit. Moreover, biochemical characteristics of the $\alpha_2\delta$ subunit, such as the shift in molecular mass upon disulfide reduction, were similar to that found in skeletal muscle. In fact, the $\alpha_2\delta$ subunit has been immunologically detected in a variety of tissues suggesting a wide distribution and an important sequence conservation (Morton and Froehner, 1989).

DHP-sensitive Ca^{2+} channels are 30 to 80 times less abundant in cardiac than in skeletal muscle (Perez-Reyes *et al.*, 1989), and the difficulty in obtaining sufficient starting material has made large-scale purification difficult. Nonetheless, several researchers have shown the presence of a 190–200-kDa α_1 subunit and an associated $\alpha_2\delta$ subunit, which is heavily glycosylated and has the characteristic mobility shift with disulfide reduction due to the dissociation of the α_2 and δ peptides (Cooper *et al.*, 1987; Yoshida *et al.*, 190; Haase *et al.*, 1991; Tokumaru *et al.*, 1992). No β subunit has been identified in these cardiac channel purifications, although some candidate proteins of suggestive molecular weight copurify (Kuniyasu *et al.*, 1992). In addition, there is molecular evidence for the expression of several β subunits in cardiac muscles (Perez-Reyes *et al.*, 1992; Hullin *et al.*, 1992).

Characterization of vertebrate Ca^{2+} channels of evolutionary distance from mammals, such as those of *Cyprinus carpio* (carp) has demonstrated striking structural conservation among DHP-sensitive Ca^{2+} channel subunits (Grabner *et al.*, 1991). For instance, the carp DHP-binding α_1 subunit has molecular masses of 211 and 190 kDa, which may indicate a C-terminal truncation. In addition, highly glycosylated $\alpha_2\delta$ subunits copurify with the carp α_1 DHP receptor, although these have higher molecular masses than their skeletal muscle counterparts, and appear as two distinct bands prior to disulfide reduction.

Considering the association of Lambert-Eaton Myasthenic Syndrome (LEMS) with small-cell lung carcinoma, there has been much interest in identifying the Ca^{2+} channel composition of both small carcinoma cells and peripheral nerves. Recent biochemical characterization of Ca^{2+} channel components from small-cell lung carcinoma and neuroblastoma cell lines have revealed shared epitopes in both L-type Ca^{2+} channel α_1 and $\alpha_2\delta$ subunits, suggesting that similarities between the Ca^{2+} channels in these two cells may result in the presynaptic autoimmune disease (Morton *et al.*, 1994). Many other investigations have focused on the ability of LEMS sera to immunoprecipitate ω-CgTx GVIA binding from the small-cell lung carcinoma cell lines (Sher *et al.*, 1990a) and the biophysical and molecular characterization of Ca^{2+} channels in both cell types (Carbone *et al.*, 1990; Oguro-Okano *et al.*, 1992; Condignola *et al.*, 1993).

Finally, it is noteworthy that, in the absence of good ligands, the subunit composition of the T-type Ca^{2+} channel remains totally elusive. The complete lack of structural data on this important Ca^{2+} channel has further hampered its characterization. The channel properties suggest that it may have important differences in sequence that have so far precluded the cloning of its permeable subunit. Alternative cloning strategies (i.e., expression cloning) might be required to finally resolve the identity of LVA channels.

3. MOLECULAR DIVERSITY OF VOLTAGE-DEPENDENT Ca^{2+} CHANNELS

3.1. Cloning of Ca^{2+} Channel Subunits

The skeletal muscle L-type Ca^{2+} channel was the first channel described in which all the subunits (α_{1S}, $\alpha_2\delta_a$, β_{1a}, and γ) were identified and cloned (Tanabe *et al.*, 1987; Ruth *et al.* 1989; Ellis *et al.*, 1988; Jay *et al.*, 1991). Cloning of these subunits followed their purification through peptide sequencing or the development of antibodies to each protein. The full-length clone of the α_{1S} subunit encodes 1873 amino acids with a predicted mass of 212 kDa, consistent with the molecular mass of the purified protein. The proteolytic cleavage of this protein to a lower molecular mass of 170 kDa is believed to occur between residues 1685 and 1699 (De Jongh *et al.*, 1991). Despite the fact that the expression of the α_{1S} subunit failed to produce currents when injected into *Xenopus* oocytes, sequence similarity between the cloned Ca^{2+} channel α_1 subunit (Tanabe *et al.*, 1987) and the previously cloned voltage-dependent Na^+ channel (Noda *et al.*, 1984) suggested that the protein indeed encoded a voltage-dependent channel. Later, experi-

ments in myotubes from muscular dysgenic mice that lack DHP-sensitive currents and E-C coupling proved that the cloned α_{1S} sequence was responsible for these skeletal muscle–specific channel functions (Tanabe *et al.*, 1988). The β subunit cDNA of the DHP receptor encodes a 524–amino acid protein with a predicted mass of about 58 kDa, whereas the $\alpha_2\delta$ subunit cDNA encodes a protein of 1106 amino acids with a predicted size of 125 kDa.

Northern blot analysis carried out with cDNA probes from this L-type Ca^{2+} channel provided the first evidence that similar subunits exist in other tissues (Biel *et al.*, 1991; Ruth *et al.*, 1989; Ellis *et al.*, 1988). For instance, a β_{1a} subunit cDNA probe identified a 1.6- and a 1.9-kb transcript in skeletal muscle and a 3-kb transcript in brain (Ruth et al., 1989). An $\alpha_2\delta$ cDNA probe identified an 8-kb message in skeletal muscle and also in heart, aorta, and brain (Ellis *et al.*, 1988). Degenerate oligonucleotide probes were subsequently used to clone several other Ca^{2+} channel α_1 subunits. The following sections summarize what is known about the structural diversity of Ca^{2+} channel subunits.

3.1.1. Molecular Classification of α_1 Subunits

Mammalian α_1 subunits are encoded by at least six different genes, defined as S, A, B, C, D, and E, of which five (A to E) are expressed in brain (Snutch *et al.*, 1990; Soong *et al.*, 1993). In contrast, the α_{1S} subunit appears to be expressed predominantly, if not exclusively, in skeletal muscle (Ellis *et al.*, 1988; Morton and Froehner, 1989). The different classes of α_1 subunits define two major groups based on sequence homologies (Fig. 2). First, classes S, C, and D are most closely related and different from the other group of α_1 subunits. Sequence analysis of these clones show that the class C and class D gene products share approximately 66–71% amino acid identity with the α_{1S} subunit. Second, classes A, B, and E are highly homologous to each other and form a second separate group of α_1 subunits. Classes A, B, and E, which share as much as 54–64% identity, are more distantly related to the other classes with 33–43%, 34%, and 41–42% identity to the α_{1S} subunit, respectively. It is likely that these Ca^{2+} channel groups have evolved from a single ancestral Ca^{2+} channel subunit and that gene duplication and subsequent genomic divergence form the basis of this structural diversity. In addition to these six classes, alternative splicing of a primary transcript (described by a lower case letter) can also produce distinct Ca^{2+} channels by increasing the structural, functional, and, possibly, pharmacological diversity of this subunit (Snutch *et al.*, 1991; Welling *et al.*, 1993; Hui *et al.*, 1991; Diebold *et al.*, 1992; Williams *et al.*, 1992a). Thus, at present, about 20 structurally distinct α_1 subunits are known. For instance, the cardiac DHP-sensitive α_{1C} subunit encodes a 243-kDa protein of 2171 amino acids that has only 66% homology with the α_{1S} subunit (Mikami *et al.*, 1989). Alternatively spliced forms of the cardiac α_{1C} subunit clone were isolated from rabbit lung (Biel *et al.*, 1990), rat aorta (Koch *et al.*, 1990), and rat brain (Snutch *et al.*, 1991), demonstrating a wide tissue distribution for this channel. Both the cardiac and smooth muscle α_{1C} subunit differ only in the N-terminus, the hydrophobic segments IS6 and IVS3, and by an insertion in the I-II cytoplasmic linker for the smooth muscle α_{1C} subunit (Mikami *et al.*, 1989; Biel *et al.*, 1990; Koch *et al.*, 1990).

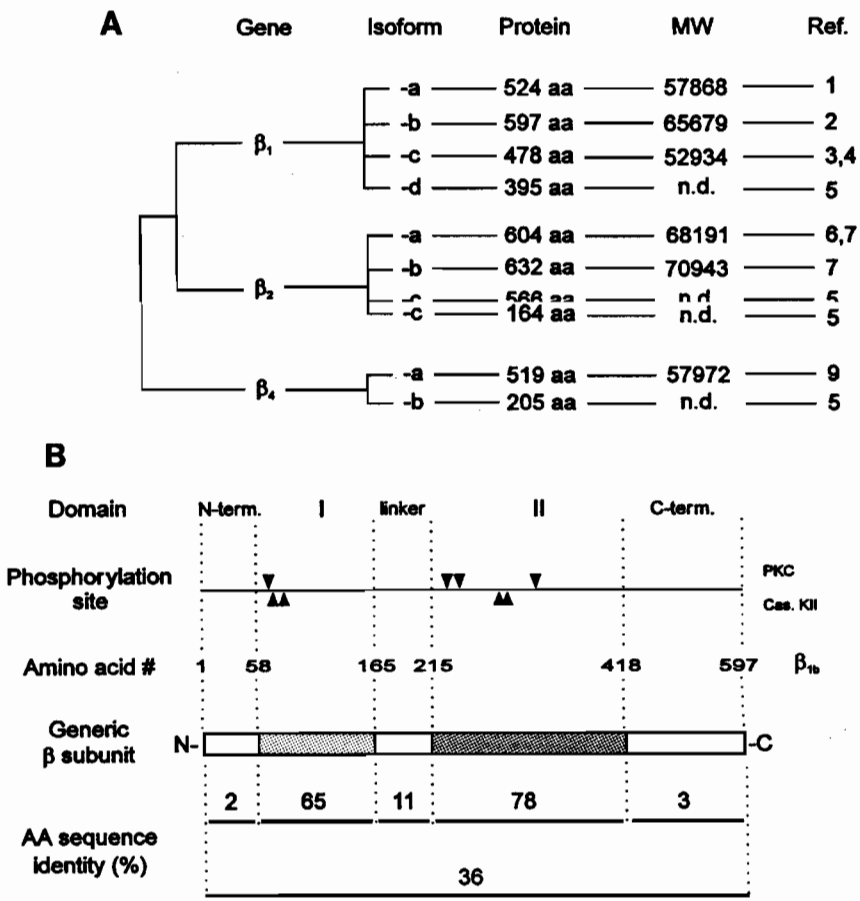


FIGURE 3. Classification of β subunits and amino acid sequence homologies. (A) Gene family tree and protein amino acid length and predicted molecular weight. References: 1, Ruth *et al.* (1989); 2, Pragnell *et al.* (1991); 3, Williams *et al.* (1992a); 4, Collin *et al.* (1993); 5, Castellano and Perez-Reyes (1994); 6, Perez-Reyes *et al.* (1992); 7, Hullin *et al.* (1992); 8, Castellano *et al.* (1993a); 9, Castellano *et al.* (1993b). (B) Schematic representation of β subunits and division in five structural domains. Conserved consensus protein kinase C and casein kinase II phosphorylation sites are shown as arrowheads.

muscle β_{1a} subunit (Ruth *et al.*, 1989), whereas another exon encodes a 7–amino acid sequence for the brain β_{1b} isoform (Pragnell *et al.*, 1991). Second, the β_{1b} subunit has a different and longer C-terminus than the β_{1a} subunit, suggesting that one or several alternative exons are used to construct the 3′ end of the β_{1b} mRNA. Thus overall, the neuronal β_{1b} subunit encodes a protein of predicted molecular mass of 66 kDa which is 8 kDa more than the skeletal muscle β_{1a} subunit. The β_{1c} subunit is another brain splice

variant that expresses the short internal exon of β_{1b} and the exons coding the small C-terminal region of β_{1a} (Williams *et al.*, 1992a). Four splice variants have also been identified for the β_2 gene (β_{2a}, β_{2b}, β_{2c}, and β_{2d}). The β_{2a} (Perez-Reyes *et al.*, 1992), β_{2b} (Hullin *et al.*, 1992), β_{2c}, and β_{2d} subunits (Castellano and Perez-Reyes, 1994) were alternatively spliced at the internal exon ($\beta_{2a} = \beta_{2b} \neq \beta_{2c} \neq \beta_{2d}$), whereas β_{2a} and β_{2b} differed by alternative splicing of the first exon resulting in two different N-terminal sequences. The β_2 gene is expressed abundantly in heart and to a lesser extent in aorta, trachea, and lung (Hullin *et al.*, 1992). Finally, three alternative splice variants were found for β_3, β_{3a} (Hullin *et al.*, 1992), β_{3b} (Castellano *et al.*, 1993b), and β_{3c} (Castellano and Perez-Reyes, 1994), and two for β_4, β_{4a} (Castellano *et al.*, 1993b), and β_{4b} (Castellano and Perez-Reyes, 1994). The β_3 gene is expressed in brain and smooth muscle–containing tissues such as aorta, trachea, and lung (Hullin *et al.*, 1992). Instead, the β_4 gene is almost exclusively expressed in brain and in high levels in cerebellum. The β_{1d}, β_{2d}, β_{3b}, and β_{4b} subunits all contain a reading frame shift resulting in the expression of truncated β subunits that lack the entire second conserved domain and the more variable C-terminus. The physiological significance of these large truncations is not known.

A comparison of the amino acid sequence between all four β subunit gene products and splice variants has defined two high- (conserved domains I and II) and three low-homology domains (the N- and C-terminal domains and the linker between domains I and II). For instance, domains I and II extend from amino acid 58 to 165 and 215 to 418, respectively, in the β_{1b} subunit (Fig. 3B). Overall, these β subunits vary in molecular mass from ~50 to 85 kDa. Figure 3B also locates several conserved consensus phosphorylation sites on β subunits. Four potential phosphorylation sites for protein kinase C (PKC), but not protein kinase A (PKA), are present in all the β subunits. One such PKC site is located at residue 64 in domain I and three others in domain II at residues 228, 238, and 348 in the β_{1b} subunit. The skeletal muscle β_{1a} subunit can indeed be phosphorylated *in vitro* by various kinases (PKC, cAMP- and cGMP-dependent PK, calmodulin-dependent kinase II, and casein kinase II) (Nastainczyk *et al.*, 1987; Jahn *et al.*, 1988). Phosphorylation by cAMP-dependent kinase was shown to occur on threonine 205 and serine 182, although the latter is not a consensus site (Ruth *et al.*, 1989; De Jongh *et al.*, 1989). The functional importance of these phosphorylations are not yet known but such a high convergence of intracellular signaling suggests an important role of β subunits in the regulation of Ca^{2+} channel activity or function.

3.1.3. Primary Structure of the $\alpha_2\delta$ Subunit

Northern blot analysis and antibody cross-reactivity have indicated that $\alpha_2\delta$ is a well-conserved subunit in different tissues (Ellis *et al.*, 1988; Morton and Foehner, 1989). So far, only a single gene has been identified that encodes both the α_2 and δ proteins (Ellis *et al.*, 1988). This gene is expressed in multiple tissues as a glycosylated protein of 175 kDa that represents the disulfide-linked α_2 and δ peptides. It also encodes a 26–amino acid signal sequence that is cleaved after insertion into the plasma membrane. Despite the lack of a recognizable α_2-δ proteolytic cleavage site in the primary sequence, most biochemical analyses have shown that the α_2 protein is formed from the N-terminal sequence and the δ peptide is derived from the remaining C-terminus (De Jongh *et al.*, 1990). There are three hydrophobic potential transmembrane domains, equally spaced

in the protein sequence, although the third lies within five amino acids of the carboxyl terminus. Analysis of the primary sequence indicates that there are 18 potential glycosylation sites and numerous cysteine residues, which may make the elucidation of the extract structure difficult (Ellis *et al.*, 1988).

Five alternative splice products of the $\alpha_2\delta$ gene are now known (Williams *et al.*, 1992a; Kim *et al.*, 1992). The resulting differences in sequence are quite limited since they all occur between the first and second hydrophobic domains and are restricted to short amino acid segments. Although these splice variants are differentially expressed in brain and skeletal muscle, their significance remains unknown.

3.2. Subunit Identification of Native L- and N-Type Ca^{2+} Channels

Correlation of the cloned subunits with those found in native Ca^{2+} channel complexes relies on the use of toxin sensitivities, sequence-specific antibodies, and Northern blot analysis. The α_1 subunit associated with the L-type Ca^{2+} channel in skeletal muscle is molecularly classified as α_{1S}, which was originally cloned from a skeletal muscle library (Tanabe *et al.*, 1987) and appears to be uniquely expressed in skeletal muscle by Northern blot analysis (Ellis *et al.*, 1988). Expression of the α_{1s} subunit in L cells resulted in DHP-sensitive, voltage-gated Ca^{2+} current (Perez-Reyes *et al.*, 1989). The first β subunit cloned was also from a skeletal muscle library (Ruth *et al.*, 1989). Although this cDNA cross-hybridized with a larger mRNA from brain, the smaller hybridizing species in skeletal muscle corresponds to the β_{1a} clone, the small molecular weight β subunit of skeletal muscle, and the larger species is a splice variant of this gene found in brain (Pragnell *et al.*, 1991).

The α_1 subunit associated with [^{125}I]-ω-CgTx GVIA binding activity in purified N-type Ca^{2+} channels in brain has been identified as the α_{1B} subtype, based on reactivity of the 230-kDa protein with antibodies produced to a fusion protein containing the α_{1B} II-III loop (Witcher *et al.*, 1993a). Likewise, when the α_{1B} protein was expressed in HEK 293 cells, ω-CgTx GVIA sensitive currents were produced (Williams *et al.*, 1992b). The β subunit associated with the N-type Ca^{2+} channel has been shown to be the β_3 subtype, based on recognition of the 57-kDa protein by β_3 subunit–specific antibodies (Witcher *et al.*, 1993a). Four peptide sequences from the N-type $\alpha_2\delta$ subunit identified it as the neuronal $\alpha_2\delta_b$ splice variant of the $\alpha_2\delta$ gene (Williams *et al.*, 1992b). It differs from the skeletal muscle $\alpha_2\delta_a$ by a 19–amino acid deletion (residues 507–527 of $\alpha_2\delta_a$) and a 7–amino acid insertion (residues 602–608 of $\alpha_2\delta_b$).

3.3. Membrane Topology of Voltage-Dependent Ca^{2+} Channel Subunits

The three main subunits of voltage-dependent Ca^{2+} channels (α_1, $\alpha_2\delta$, and β) are proposed to be arranged in the membrane as illustrated in Fig. 4. The α_1 subunit bears strong similarities to other voltage-gated channels. This subunit possesses four successive motifs (I to IV) of very similar structure. Each repeat contains six transmembrane segments (S1–S6). There are thus a total of 24 transmembrane segments orderly arranged to define a central ionic pore. The transmembrane segments are highly conserved sequences in Ca^{2+} channel α_1 subunits and also in Na^+ and K^+ channels (Noda *et al.*, 1984). The sequence variability is mostly located in the intracellular loops

connecting the segments and the motifs and in the carboxyl terminus. The fourth segment (S4) in each repeat is 20 amino acids in length and contains repeated motifs of one positively charged amino acid at every third or fourth position followed by several hydrophobic amino acids. Single-point mutations of the positively charged amino acids of the S4 segments suggest that these segments represent portions of the voltage sensors that may initiate channel opening during activation (Stuhmer *et al.*, 1989). In support of this notion, changes in conformation of the S4 segments are held responsible for part of the gating currents that are triggered in response to membrane depolarization (Guy and Conti, 1990). However, it is still not well understood how these changes in S4 conformation, and subsequently elsewhere in the channel, are transduced into modifications of the gating process. It appears that this transduction can be regulated since the association of the β subunit to α_1 does not affect voltage sensing itself but increases the efficiency of the coupling between voltage sensing and pore opening (Olcese *et al.*, 1994). Two short segments, SS1 and SS2 located between S5 and S6 in each of the four repeated motifs, and the α-helical segments S5 and S6 themselves, are proposed to form the permeation pore (Guy and Conti, 1990). Single amino acid mutations within the pore region can alter the ion selectivity of the pore, effectively converting a Ca^{2+} channel into a Na^+ channel (Heinemann *et al.*, 1992). The connecting loops between S5 and S6 of the third repeat and the transmembrane segment S6 of the fourth repeat were identified as binding sites for both tetrodotoxin and DHP, two drugs that block the entry of Na^+ and Ca^{2+} via their respective channels (Catterall and Striessnig, 1992).

In contrast to the complex membrane topology of the α_1 subunit, the β subunit does not contain any putative transmembrane domain. However, it contains seven α-helical domains and four heptad repeats in which most of the first and fourth residues of every seven are hydrophobic. These heptad repeats are thought to be involved in interactions with cytoskeletal proteins (Fuchs and Hanukoglu, 1983).

The membrane topology of the $\alpha_2\delta$ subunit remains a significant controversial issue, as the topology may suggest or refute a direct interaction with the entirely cytoplasmic β subunit. If all three hydrophobic regions are transmembrane domains then a significant fraction of the protein between the first and second transmembrane domains would be intracellular and could possibly interact with β subunits. However, experimental data demonstrate that only a single transmembrane domain is present in the C-terminus of the δ subunit (Jay *et al.*, 1991). Alkaline extraction of skeletal muscle membranes in the presence of reducing agents is able to extract α_2 but not the transmembrane δ peptide. In the single transmembrane model, only five amino acids in the C-terminus would be cytoplasmic.

4. FUNCTIONAL RECONSTITUTION OF PURIFIED VOLTAGE-DEPENDENT Ca^{2+} CHANNELS

4.1. Functional Properties of Purified Ca^{2+} Channels in Bilayers

The functional activity of both L- and N-type Ca^{2+} channels can be reconstituted by insertion of the purified proteins in artificial lipid bilayers (Flockerzi *et al.*, 1986; De

Waard *et al.*, 1994a). Reconstitution of the α_{1S} subunit proves that this protein is sufficient to form a functional channel in the absence of other associated subunits (Pelzer *et al.*, 1989). This simplified channel also retains its sensitivity to numerous drugs (the DHP Bay K 8644 and the phenylalkylamine D600) and to phosphorylation by protein kinase A, unequivocally demonstrating that it is a major target for drug binding and cytoplasmic regulation. Similarly, the activity of the purified N-type channel is blocked by the addition of micromolar concentrations of ω-CgTx GVIA in the presence of 10 mM divalent Ba^{2+} as the charge carrier (Witcher *et al.*, 1993a). However, the channel is insensitive to L-type channel ligands such as the DHP and phenylalkylamine classes of drugs (Witcher *et al.*, 1993a; De Waard *et al.*, 1994a). Figure 5 demonstrates, for instance, that the mean current amplitude and open-time duration of the N-type Ca^{2+} channel in a low-P_0 mode is not affected by the application of 1 μM Bay K 8644. These pharmacological characterizations constitute properties essential for identification of the Ca^{2+} channel type. Once the pharmacological sensitivity of the purified channel is firmly established, further characterization of the biophysical properties of the channel could be undertaken.

Like its native counterpart, the conductance of the purified N-type Ca^{2+} channel is somewhat variable and ranges from 7 to 27 pS (De Waard *et al.*, 1994a). This variability may seem surprising for what appears to be a homogeneous population of channels. These results may suggest the existence of several ω-CgTx GVIA–sensitive α_{1B} isoforms in brain that differ by their unitary conductance. Such a hypothesis is, however, more difficult to sustain in face of the conductance variability of the purified L-type channel from skeletal muscle (Hymel *et al.*, 1988; Ma and Coronado, 1988). This tissue is regarded as the least heterogeneous source of DHP-sensitive channels, and very little sequence variability has been reported for the α_{1S} subunit. Alternatively, it is also possible that differential posttranslational processing (limited proteolysis or specific phosphorylations) is responsible for an alteration of the conductance values. In any case, these results demonstrate that the unitary conductance of a channel cannot be used as a reliable parameter of channel identification until the determinants of conductance variability have been identified. Also similar to native N-type Ca^{2+} channels, purified N-type channels have several gating modes that differ by their probability of opening (Delcour and Tsien, 1993; De Waard *et al.*, 1994a). Purified N-type channels have at least two gating patterns, one characterized by a low opening probability and a second defined by a much higher opening probability. In both cases, however, the distribution of open-time durations reveals short mean values between 1.5 and 8 msec.

Both L- and N-type Ca^{2+} channels are permeable to a large number of inorganic cations including Ba^{2+} and Sr^{2+} in addition to Ca^{2+} ions. However, HVA channels are not equally permeable to these cations. For instance, Ba^{2+} and Sr^{2+} currents are larger than Ca^{2+} currents in native HVA Ca^{2+} channels as demonstrated in rabbit myocytes (Droogmans *et al.*, 1987). Likewise, purified L- and N-type Ca^{2+} channels have a higher permeability to Ba^{2+} than to Ca^{2+} (Fig. 6). For instance, N-type Ca^{2+} channels have a 2.8-fold higher permeability when Ba^{2+} is the charge carrier. These results indicate that the selectivity process is well conserved during the purification and reconstitution procedures (De Waard *et al.* 1994a).

The amplitude of Ca^{2+} currents through voltage-dependent Ca^{2+} channels is a

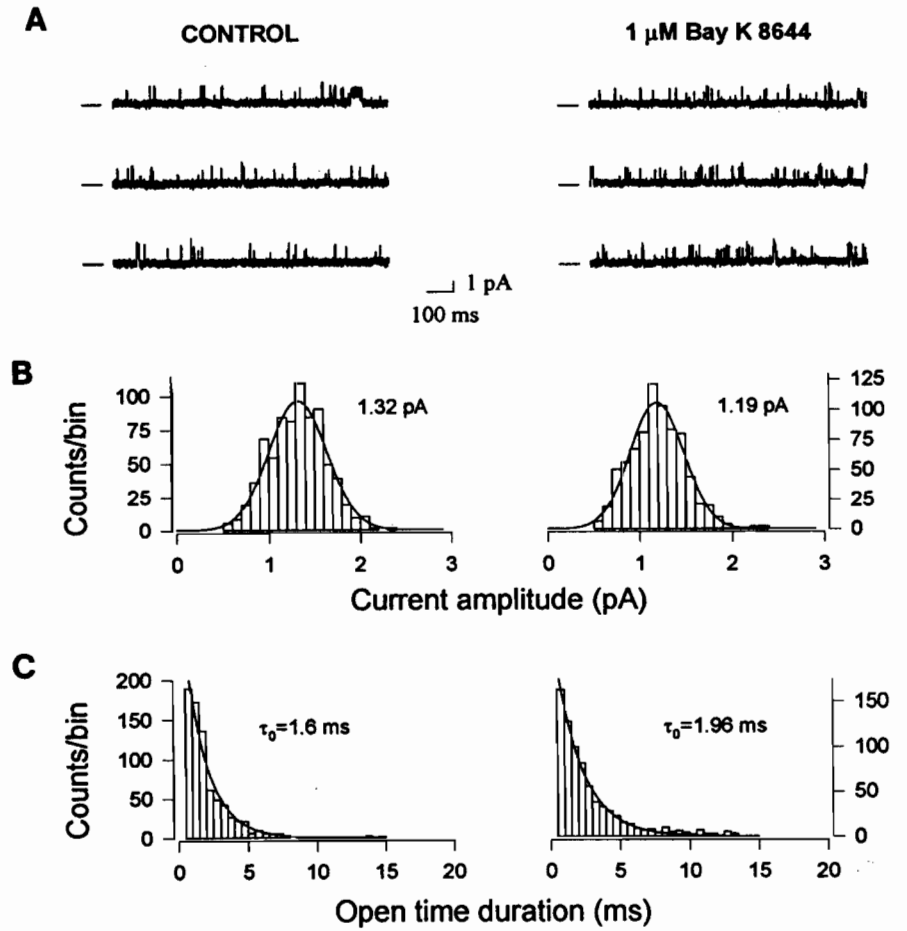


FIGURE 5. The purified N-type Ca^{2+} channel activity is not affected by DHP agonists. (A) Trace examples of purified N-type Ca^{2+} channel activity reconstituted by the tip-dip technique (De Waard *et al.*, 1994a). The holding potential is 100 mV and the permeant cation is 500 nM Ba^{2+}. The channel activity is shown before (left) and after (right) 2 min application of 1 μM Bay K 8644 in the extrapipette medium. Filter frequency is 1.5 kHz and sampling frequency is 10 kHz. (B) Amplitude histograms. Mean ± SD current amplitude is $I_{Ba} = 1.32 \pm 0.32$ pA (control) and $I_{Ba} = 1.19 \pm 0.29$ pA (Bay K 8644). Bin width is 0.1 pA/div. (C) Open-time duration histograms. The mean open-time duration is 1.6 msec (control) and 1.96 msec (Bay K 8644). Bin width is 0.5 msec div.

function of the extracellular Ca^{2+} concentration. In rat skeletal muscle, a twofold increase in Ca^{2+} current occurs in response to a fivefold increase in extracellular Ca^{2+} concentration from 2 to 10 mM (Donaldson and Beam, 1983). Similarly, the mean unitary conductance of purified N-type Ca^{2+} channels increases with elevating symmetrical Ba^{2+} concentrations. Plateau conductances are reached at 60 mM Ba^{2+} and half-maximal conductance occurs at 19 mM Ba^{2+} (De Waard *et al.*, 1994a).

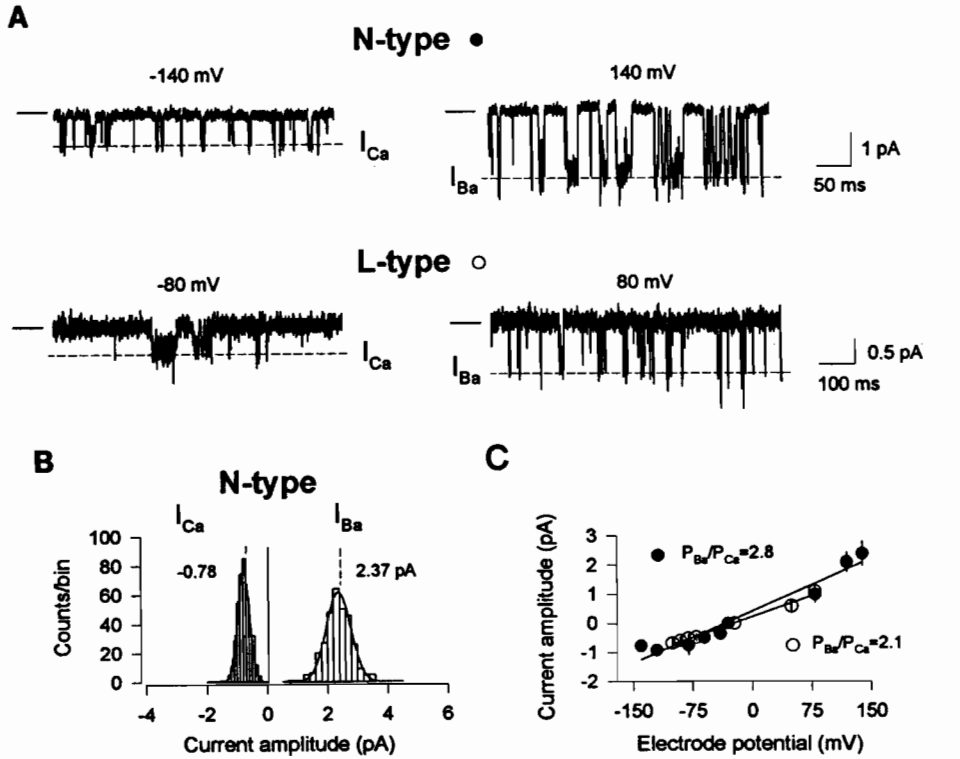


FIGURE 6. Relative Ca^{2+}/Ba^{2+} permeabilities of purified L- and N-type Ca^{2+} channels. (A) Recordings of purified N- and L-type Ca^{2+} channels in asymmetrical ionic conditions (100 mM Ba^{2+} in the electrode and 100 mM Ca^{2+} in the extrapipette solution). At negative driving forces, Ca^{2+} permeability is favored over Ba^{2+} permeability and results in smaller current amplitudes than at positive driving forces. (B) Amplitude histograms of N-type channel at pipette potentials of −140 and 140 mV. Mean current amplitudes are -0.78 ± 0.2 pA (−140 mV) and 2.37 ± 0.41 pA (140 mV). Bin widths are 0.1 and 0.2 pA/div. (C) Unitary conductances of both N- and L-type channels in asymmetrical ionic conditions. The conductances are 12.7 pS (N-type) and 10 pS (L-type) and the reversal potentials are −30 mV (N-type) and −22 mV (L-type). The estimated permeability ratio P_{Ba}/P_{Ca} are 2.8 (N-type) and 2.1 (L-type), assuming a Cl^- permeability of zero. The data for the N-type Ca^{2+} channel are from De Waard *et al.* (1994a).

4.2. Similarities between Purified L- and N-Type Ca^{2+} Channels

Purified channels from both skeletal muscle and brain retain several of the functional and pharmacological characteristics of native Ca^{2+} channels in planar lipid bilayers. They are selective to Ca^{2+} and exhibit open-time durations, conductances, and pharmacological sensitivities to antagonists that are consistent with native channels. Although there may be some cellular regulatory factors that modulate these elementary channel properties, these characteristics seem to be intrinsic to the Ca^{2+} channel multisubunit complexes themselves. The similarities in properties between purified and native voltage-dependent Ca^{2+} channels present strong evidence that the pharmacologi-

cal identity, ionic selectivity, permeability, and gating activity of the channel are intrinsic qualities of the protein complex itself. Unfortunately, several important properties of native Ca^{2+} channels are either lost or difficult to observe after purification and reconstitution. For instance, the purified L-type and N-type Ca^{2+} channels do not inactivate or "run down" and both channels are surprisingly voltage-independent (Flockerzi *et al.*, 1986; De Waard *et al.*, 1994a). These results could be interpreted in either of two ways: first, the consecutive processes of purification and reconstitution could lead to abnormal channel activity due to partial denaturation or aberrant membrane insertion, or, second, the altered properties may instead reflect an absolute requirement for cellular factors that do not biochemically copurify with the channel. The first hypothesis of a conformational abnormality to explain the lack of voltage dependence of the purified channel in lipid bilayers is difficult to reconcile with the observation that purified channels behave normally with regard to complex mechanisms such as the binding of channel ligands and the gating process that should result as a consequence of voltage sensing. Obviously, the alternative hypothesis may prevail suggesting that important cell regulatory factors are required for 1) Ca^{2+}- and voltage-dependent inactivation, 2) run down, and 3) the transduction between voltage sensing *per se* and changes in the gating behavior.

In spite of the technical limitations of Ca^{2+} channel purification and reconstitution and the difficulties in interpreting the data, there is obviously more information to gain from this type of experiment. First, reconstitution of purified channels allows the analysis of the intrinsic properties of voltage-dependent Ca^{2+} channels in the absence of associated cellular factors, which cannot easily be achieved with expression experiments. A renewed interest in channel reconstitution is therefore to be expected for a rigorous analysis of the direct modulatory effects of phosphorylations, second messengers or intracellular proteins such as G proteins, or components of the excitation-contraction or excitation-secretion machinery. Also, valuable data on the mechanisms of action of various toxins (i.e., conotoxins or agatoxins) are to be gained from the analysis of the changes in elementary channel properties. An important controversy remains regarding the mechanism of blockade of ω-CgTx GVIA at the single-channel level, despite its extensive use in functional studies of the cellular role of N-type Ca^{2+} channels. It is not clear whether the toxin blocks Ca^{2+} permeability by simple occlusion of the pore (Ellinor *et al.*, 1994) or by a reduction in opening probability (Witcher *et al.*, 1993b). Likewise, it will be interesting to analyze the mechanisms of P-type current block by ω-Aga IVA when the subunits composing this channel have been identified and reconstituted into lipid bilayers.

5. EXPRESSION OF Ca^{2+} CHANNELS

5.1. Functional Classification of Cloned α_1 Subunits

It is now firmly established that α_1 subunits can form minimum voltage-dependent Ca^{2+} channels with clearly defined functional and pharmacological properties (Lacerda *et al.*, 1991; Mori *et al.*, 1991; Stea *et al.*, 1993; Mikami *et al.*, 1989; Soong *et al.*, 1993). These subunits contain the basic functional elements of voltage-dependent Ca^{2+} chan-

nels such as the voltage sensor and the ionic pore and binding sites for most drugs. These properties were useful to tentatively assign the α_1 subunits to the various classified LVA and HVA Ca^{2+} channels. Table III summarizes the pharmacological and functional properties of the expressed α_1 subunits. On pharmacological grounds, these channels can be divided into two groups based on their sensitivity to DHPs: the L-type (S, C, and D) and the non-L-type (A, B, and E). Surprisingly, this subdivision matches well the division that was made on the basis of molecular α_1 homologies.

Stable expression of the α_{1S} subunit in mouse L cells results in the appearance of DHP-sensitive Ca^{2+} channels (Lacerda *et al.*, 1991). The kinetics of activation of this subunit were excessively slow, suggesting that additional components were required to fully restore the properties of the native channel. The role of the α_{1S} subunit in excitation-contraction coupling was confirmed by expressing its cDNA in mouse dysgenic myotubes (mdg). This manipulation restores both the mechanical contraction and the release of Ca^{2+} from SR, two processes previously lacking in mdg because of a nucleotide deletion in the fourth transmembrane repeat of the endogenous α_1 subunit (Tanabe *et al.*, 1988). Two other α_1 subunits are proposed to be components of L-type Ca^{2+} channels based on their homology to α_{1s} and their sensitivity to DHPs. The α_{1C} subunit is expressed in brain and in cardiac and smooth muscle, where it functions in the cardiac type of E-C coupling (Tanabe *et al.*, 1990b). Similar to the cardiac-type channel that it encodes, the DHP-sensitive currents carried by the α_{1C} subunit are prone to Ca^{2+}-dependent inactivation (Neely *et al.*, 1994) and facilitation by depolarizing prepulses (Kleppisch *et al.*, 1994). No functional differences have yet been observed between the various alternative spliced forms of the α_{1C} transcripts (Itagaki *et al.*, 1992). The α_{1D} subunit constitutes the third kind of L-type Ca^{2+} channel based on its sensitivity to low concentrations of DHP agonists and antagonists (Williams *et al.*, 1992a). Curiously, this channel seems also to be sensitive to micromolar concentrations of the peptide ω-CgTx GVIA, although the affinity of the toxin for the channel is low and the inhibition of the current is reversible. These properties are consistent with previous observations that some native L-type Ca^{2+} channels could be reversibly blocked by ω-CgTx GVIA (Kasai and Neher, 1992). The low affinity and reversibility of α_{1D} block by ω-CgTx GVIA distinguishes this channel from a true N-type Ca^{2+} channel.

Expression of a cDNA that encodes the α_{1A} Ca^{2+} channel produces a current that is insensitive to both DHP antagonists and ω-CgTx GVIA (Mori *et al.*, 1991; Sather *et al.*, 1993). The channel can also be blocked by 200 nM ω-Aga IVA and even more potently by ω-CgTx MVIIC ($IC_{50} < 150$ nM). Figure 7A illustrates, for instance, the rate of current amplitude decay by blockade of the channel activity by an extracellular application of 2 μM ω-CgTx MVIIC. These pharmacological properties are apparently related to those reported for the native P-type channel and seem consistent with the high level of expression of the α_{1A} subunit in cerebellum that parallels well the localization of P-type channels (Mori *et al.*, 1991). There are, however, a number of substantial differences that may indicate that the α_{1A} subunit is not a constituent of the P-type channel: 1) Ca^{2+} currents carried by α_{1A} lack the ~1 nM IC_{50} sensitivity for ω-Aga IVA that is normally displayed by native P-type channels in Purkinje neurons (Tsien *et al.*, 1991; Sather *et al.*, 1993), 2) α_{1A} currents activate in a range of potentials (threshold −30 mV) that are more depolarized than P-type channels (threshold −50 mV), and 3) the time-dependent

TABLE III
Properties of Cloned α_1 Subunits[a]

Property	Gene					
Classification	S	A	B	C	D	E
Splice variants	2	4	2	4	4	3
Primary tissue location	Skeletal	Neuronal Cardiac	Neuronal	Neuronal Aortic-Cardiac	Neuronal Endocrine	Neuronal
Homology	100%	33–44%	34%	66%	71%	41–42%
Pharmacology	DHPs Antagonists/agonists	FTX ω-Aga IV ω-Cx MVIIC	ω-CTx GVIA (irreversible)	DHPs Antagonists/agonists	DHPs ω-CTx GVIA (reversible)	Resistant to all
Component of	Skeletal muscle DHP receptor	?	Brain ω-Cx receptor	?	?	?
Functional type	HVA L	HVA P or Q ?	HVA N	HVA L	HVA L	HVA R
Cell function	E-C coupling	E-S coupling	E-S coupling	Other	Other	E-S coupling

[a]cDNA sequence of the various α_1 subunits are compared to the sequence of the α_{1S} subunit. The α_{1S} and α_{1B} subunits are identified components of the skeletal muscle L-type and brain N-type Ca^{2+} channels, respectively. The pharmacological sensitivity of the other α_1 subunits has aided their identification as L- or non-L-type Ca^{2+} channels. There is still some controversy about the functional correlate of the α_{1A} subunit (P- or Q-type).

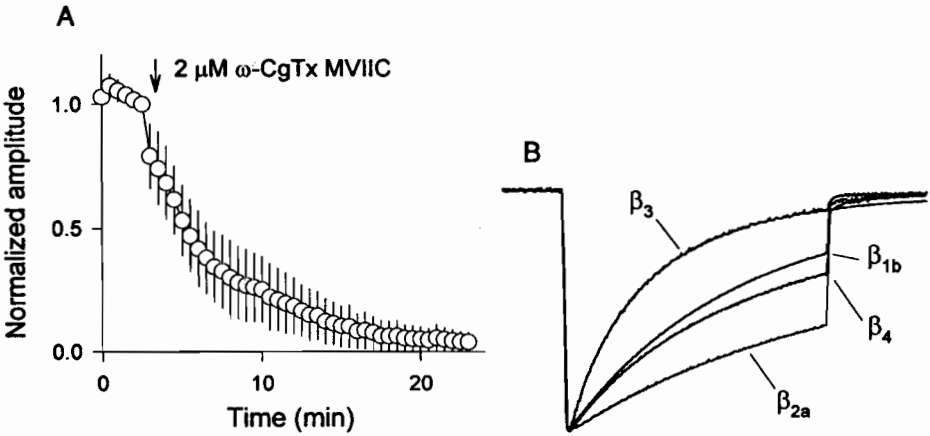


FIGURE 7. Toxin sensitivity and kinetic regulation by β subunits of the HVA α_{1A} Ca^{2+} channel. (A) The currents carried by α_{1A} channels can be totally blocked by 2 μM ω-CgTx MVIIC. The external Ba^{2+} concentration is 2 mM to minimize the antagonism that occurs between divalent cations and the toxin binding. (B) Different β subunits induce variable rates of inactivation with β_3 inducing the fastest rate and β_{2a} the slowest. The holding potential is −90 mV and the test pulses were delivered at 30 mV. The data are from De Waard and Campbell (1995).

inactivation is more rapid (time constant of 150 to 700 msec depending on the β subunit being coexpressed) than P-type currents (little inactivation). This has recently led to the proposal that the α_{1A} channel represents a novel channel type defined as Q-type. However, the precise structural bases for these distinctions remain to be established with more certainty. There is a precedent case for such a discrepancy between cloned and native Ca^{2+} channels. The α_{1C} channel is reportedly 100-fold less sensitive to ω-Aga IIIA than L-type channels in myocytes (Mintz *et al.*, 1991) but is unquestionably a component of these cardiac channels. Clearly, some caution is needed when comparing the properties of native and expressed channels. The subunit composition of the P-type channel is not yet known and the inactivation kinetics of the α_{1A} channel are largely controlled by the association of the ancillary subunits (Fig. 7B).

The α_{1B} subunit has also been characterized pharmacologically, as expression of its cDNA gives rise to [^{125}I]-ω-CgTx GVIA binding and to ω-CgTx GVIA–sensitive currents in HEK 293 cells (Williams *et al.*, 1992b; Fujita *et al.*, 1993; Stea *et al.*, 1993). The high-affinity binding of this toxin to the α_{1B} expressed protein is irreversible, making it a component of N-type Ca^{2+} channels (Williams *et al.*, 1992a,b). These properties distinguish the α_{1B} Ca^{2+} channel from the α_{1D} Ca^{2+} channel that is less specifically blocked by ω-CgTx GVIA. Recently, several α_{1E} Ca^{2+} channels have been cloned (human α_{1E}, rat rbE, rabbit BII, and doe-1 from *Discopyge ommata*) and expressed in *Xenopus* (Soong *et al.*, 1993; Wakamori *et al.*, 1994; Ellinor *et al.*, 1993) or HEK 293 cells (Williams *et al.*, 1994). Initially, this channel was proposed to represent a member of the low voltage–activated Ca^{2+} channel class, possibly a T-type channel (Soong *et al.*, 1993). In low divalent cation concentration, the class E channel activates at negative membrane potentials (threshold at −50 mV in 4 mM [Ba^{2+}]), has a rapid current decay

during inactivation ($\tau \sim 100$ msec), and is blocked by low concentrations of Ni^{2+} (IC_{50} of 28 μM). The conductance of the expressed channel is 12 pS (Wakamori *et al.*, 1994). In fact, it is now clear that the voltage dependence of the α_{1E} channel does not differ from that displayed by the α_{1A} channel (De Waard and Campbell, 1995) and, as all HVA channels, is blocked by lower concentrations of Cd^{2+} (IC_{50} of 1 μM) than Ni^{2+} (Williams *et al.*, 1994). Such a cation sensitivity is thus still opposite to that displayed by T-type channels. The α_{1E} channel is also characterized by its lack of sensitivity to all the discriminating drugs (DHP, ω-CgTx GVIA, and ω-Aga IVA) that block L-, N- and P-type channels. Based on these results, it is therefore proposed that the α_{1E} subunit represents a member of the R-type Ca^{2+} channel family recently identified in cerebellar granule cells (Ellinor *et al.*, 1993).

5.2. Role of Ancillary Subunits

The gating mechanisms of ion channels are often under the control of several biochemical signals including protein–protein interactions and covalent modifications of the channel such as phosphorylation (Kaczmarek and Levitan, 1987; Levitan, 1988). Subunit-subunit interactions have also recently been recognized as a mechanism of gating control by altering the conformation of α_1 subunits. We will review the evidence demonstrating that two of the ancillary subunits (β and $\alpha_2\delta$) control the biophysical and pharmacological properties of the α_1 pore-forming protein.

5.2.1. Effects of β Subunits on Channel Properties

The proliferation of cloned Ca^{2+} channel α_1 and β subunits in the absence of any systematic biochemical characterization has led to the coexpression of numerous α_1-β combinations. Expression of most α_1 subunits alone gives rise to very small current densities, and since β subunits substantially increase the level of current amplitude, there has been a natural tendency to coexpress both subunits in various cells. In fact, current amplitude stimulation by β subunits has been observed for almost any α_1 subunit (Williams *et al.*, 1992a,b; Wei *et al.*, 1991; Mori *et al.*, 1991; Ellinor *et al.*, 1993; Stea *et al.*, 1993). The potency of current amplitude stimulation is somewhat variable and depends on the class of α_1 and β subunits being coexpressed, the cDNA constructs, the cDNA/cRNA transfection/injection technique, and the cell type used to assay the protein expression level. Estimates of current amplitude stimulation by β subunits have been most reliably obtained from injection of cRNAs into *Xenopus laevis* oocytes. In this system, coexpression of β_{1b} with either α_{1A} subunit (De Waard *et al.*, 1994b), α_{1B} (Stea *et al.*, 1993), or α_{1E} (Wakamori *et al.*, 1994) produces, respectively, an 18-fold, a fourfold, or a threefold stimulation in peak current amplitude. Also, β subunits of each of the four genes can produce between a twofold to 19-fold stimulation in current amplitude of the α_{1C} channel (Wei *et al.*, 1991; Hullin *et al.*, 1992; Perez-Reyes *et al.*, 1992; Castellano *et al.*, 1993a,b) or of the α_{1A} channel (Mori *et al.*, 1991; De Waard *et al.*, 1994b). These observations strongly suggest that different β subunits can interact with the same α_1 subunit and that the mechanisms responsible for current stimulation are well conserved among different voltage-dependent Ca^{2+} channels.

Several hypotheses may explain how expression of the β subunit causes an increase in measured current amplitude. First, the direct interaction of the α_1 subunit with the β subunit may result in an increase in the conductance or in the opening probability of the channel by inducing a conformational change in the pore subunit. Conformational effects of the β subunit are likely, as the β subunit has both large effects on DHP binding affinity (Mitterdorfer *et al.*, 1994) and on channel kinetics and voltage dependence. However, estimates of β subunit effects at the unitary level demonstrate that they do not modify the conductance of the expressed Ca^{2+} channels (Wakamori *et al.*, 1994). Second, β subunits may cause a recruitment of new channels from intracellular stores or stabilization of channel complexes at the cell surface. Reports that have evaluated changes in the level of α_1 protein expression in the plasma membrane are contradictory. Perez-Reyes *et al.* (1992) report a fourfold increase in the number of [^{3}H]-PN200-110 binding sites upon coexpression of the α_{1C} with the β_2 subunit, while Nishimura *et al.* (1993) report a similar increase in DHP binding without an increase in the amount of α_1 protein. Mitterdorfer *et al.* (1994) failed to see an increase in DHP binding sites. In any case, DHP binding to L-type Ca^{2+} channels has always been difficult to interpret, as the affinity of these ligands to the channel is very state-dependent in native channels. For instance, it is known that the affinity of DHP antagonists for L-type channels increases upon inactivation, a state that is attained by depolarization. It is not known how the association of a β subunit affects the voltage-dependent behavior of DHP binding. This information would, however, be useful in the interpretation of β subunit effects on DHP binding in heterologous Ca^{2+} channels.

Analysis of the voltage dependence of the recombinant Ca^{2+} channels has revealed that β subunits also have systematic effects on other biophysical parameters encoded by the α_1 channels (De Waard and Campbell, 1995). In all cases studied, it was found that the stimulation of current amplitude by β subunits is greater at more depolarized potentials. This increase in stimulation efficiency by β subunits is due to a hyperpolarizing shift of the threshold and peak of the Ca^{2+} current. In several cases, β subunits also modify the voltage dependence of inactivation by shifting the midpoint of channel inactivation toward hyperpolarized values (De Waard and Campbell, 1995; Soong *et al.*, 1993; Stea *et al.*, 1993). This shift was, however, not consistently observed since β subunits had no effect on the voltage dependence of the α_{1C} subunit (Tomlinson *et al.*, 1993). Finally, kinetic modifications by β subunits have been reported as well (De Waard *et al.*, 1994b; Lacerda *et al.*, 1991; Singer *et al.*, 1991; Varadi *et al.*, 1991). In mouse L cells, β subunits normalize the α_{1S} currents by accelerating the activation kinetics by a factor of 10 (Lacerda *et al.*, 1991; Varadi *et al.*, 1991). These accelerations in activation kinetics are not systematically observed for every α_1 subunit. For instance, β subunits have no effect on the activation kinetics of the α_{1A} subunit (Mori *et al.*, 1991; De Waard and Campbell, 1995) and even slow the activation kinetics of the α_{1E} subunit (Wakamori *et al.*, 1994). In contrast to activation kinetics, β subunits modify the inactivation kinetics of all α_1 subunits (Wakamori *et al.*, 1994; Varadi *et al.*, 1991; Castellano *et al.*, 1993a,b; Lacerda *et al.*, 1991; Sather *et al.*, 1993; Ellinor *et al.*, 1993). Qualitatively, the similarity of β subunits in regulating the inactivation kinetics is not as great as for the current stimulation and the changes in voltage-dependence and kinetics of activation. The β_3 subunit induces the fastest inactivation kinetics, whereas β_2 induces the slowest decay (De Waard and Campbell, 1995; Ellinor *et al.*, 1993; Sather *et al.*, 1993).

In conclusion, it appears that β subunits can potently modulate a fixed set of α_1 channel properties. This extensive cross-reactivity suggests that the various α_1 and β subunits share common interactions. However, the relative differences in extent of current stimulation and modulation of kinetics also suggest that these interactions can be regulated by the structural divergences in the β subunits. Finally it should be mentioned that the importance of β subunit association to the α_1 channel is not limited to the regulation of the biophysical and pharmacological properties of the channel since β subunits also modify the regulation of α_1 subunits by phosphorylation events (Klockner *et al.*, 1992).

5.2.2. Effects of $\alpha_2\delta$ Subunit on Channel Properties

The effects of $\alpha_2\delta$ are not quite as dramatic as those of β subunits and, in most cases, are observed only on current amplitude and inactivation kinetics. Effects of the $\alpha_2\delta$ subunit on current amplitude range from a 1.5-fold increase when expressed with α_{1D} and β_2 in *Xenopus* oocytes (Williams *et al.*, 1992a) to more than a 17-fold increase when expressed with α_{1C} and β_{1a} in the same cells (Singer *et al.*, 1991). The large variability of effects seen may be due to differences in subunit compositions (correct $\alpha_1\alpha_2\delta\beta$ combination) and differences in glycosylation in the various expression systems.

Coexpression of $\alpha_2\delta$ subunit alters the time course of activation and inactivation of the cardiac α_{1C} channel expressed in *Xenopus* oocytes (Singer *et al.*, 1991). However, these results are variable since no effect of $\alpha_2\delta$ was reported on the brain α_{1C} channel, a closely related isoform (Tomlinson *et al.*, 1993). Likewise, no effect of $\alpha_2\delta$ subunit on activation and inactivation time courses was seen in transfected HEK 293 cells (Brust *et al.*, 1993). In only a single case $\alpha_2\delta$ shifted the voltage dependence of inactivation of the α_{1E} channel toward more depolarized values (Wakamori *et al.*, 1994). Once again, environmental factors, such as the appropriateness of subunit combinations and the expression system used, appear to play a large role in determining the effectiveness of the $\alpha_2\delta$ subunit expression on current kinetics.

Recent data demonstrate that the coexpression of the β subunit is required to observe the maximal regulation of the $\alpha_2\delta$ subunit (De Waard and Campbell, 1995). Expression of β and $\alpha_2\delta$ subunits with the α_{1A} subunit produces a current amplitude that is larger than if the effects of both subunits were merely additive (Mori *et al.*, 1991; De Waard and Campbell, 1995). Also, changes in α_{1A} channel kinetics by β subunits are regulated by $\alpha_2\delta$, which increases the rate of inactivation of the current (De Waard and Campbell, 1995). Finally, the maximum number of [^{125}I]-ω-CgTx GVIA binding sites were more than additive when both $\alpha_2\delta$ and β subunits were coexpressed with the α_{1B} subunit in HEK 293 cells than when the subunits were expressed individually (Brust *et al.*, 1993). Thus, there seems to exist a synergistic action between $\alpha_2\delta$ and β subunits. It is likely that α_1-β interactions strengthen the interactions existing between α_1 and $\alpha_2\delta$ and thereby reinforce the effects of $\alpha_2\delta$ on current amplitude and kinetics. Conversely, it is possible that α_1-$\alpha_2\delta$ interactions modulate the conformation of the α_1 subunit and increase the affinity of α_1 for β subunits, which could explain the potentiation in current amplitude increase by β subunits. Coexpression of the $\alpha_2\delta$ subunit has been shown to increase the affinity of recombinant channel complexes 1.5-fold for ω-CgTx GVIA in HEK 293 cells (Brust *et al.*, 1993). This is probably the strongest proof that the

association of the $\alpha_2\delta$ subunit alters the conformation of the α_1 subunit and thereby the toxin binding site.

Overall, expression experiments provide additional evidence that the minimal subunit composition of voltage-dependent Ca^{2+} channels can be described as $\alpha_1\alpha_2\delta\beta$. The data obtained so far suggest that both $\alpha_2\delta$ and β subunits directly interact with the α_1 subunit. These interactions regulate the biophysical properties of the channel, its pharmacological sensitivity, and its regulation by cytoplasmic factors. Overall, these data indicate the complexity that is involved in fully and reliably characterizing native voltage-dependent Ca^{2+} channels without a good understanding of their subunit composition and the regulatory environment provided by the cell. Future investigations should therefore be aimed at identifying the ancillary subunits that are associated with each α_1 subunit in native voltage-dependent Ca^{2+} channels.

6. SUBUNIT INTERACTION SITES IN VOLTAGE-DEPENDENT Ca^{2+} CHANNELS

6.1. Identification of an Alpha 1 Interaction Domain (AID) that Binds β Subunits

There is overwhelming evidence that most voltage-dependent, and probably all HVA channels, contain a cytoplasmic β subunit as an element of the protein channel complex. This ancillary subunit is a constitutive component of both the purified N- and L-type channels and is a strong regulatory component of all cloned α_1 subunits. Moreover, there seems to be an important cross-reactivity among various α_1 and β subunits isoforms, suggesting that the interactions between both α_1 and β subunits are well conserved. For instance, both the α_{1C} and the α_{1A} subunits can be regulated by various isoforms of the β_1, β_2, β_3, and β_4 subunits (Wei *et al.*, 1991; Hullin *et al.*, 1992; Perez-Reyes *et al.*, 1992; Castellano *et al.*, 1993; De Waard and Campbell, 1995). In turn, β_{1b} is reportedly known to regulate several isoforms of α_1 subunits, α_{1A}, $\alpha_{1\beta}$, α_{1C}, and α_{1E} (De Waard *et al.*, 1994b; Stea *et al.*, 1993; Wakamori *et al.*, 1994). As demonstrated by several biochemical reports, the β subunit is entirely cytoplasmic, which confines the α_1-β interaction sites to intracellular loci. Such a localization should avoid the potential structural complexity associated with membrane-spanning segments. It was therefore expected that at least one of the cytoplasmic loops of the α_1 subunit was interacting with conserved sequences on β subunits. *In vitro* translated and [^{35}S] methionine-labeled β subunits were shown to interact with the purified α_{1S} and α_{1B} subunits of the skeletal muscle DHP receptor and the brain N-type Ca^{2+} channel (Pragnell *et al.*, 1994). The same probe could also interact with proteolytic fragments of these α_1 subunits, thereby strongly indicating that the α_1 interaction domain (AID) responsible for the binding of β subunits is poorly conformation-dependent. This unique property of the AID helped the experimental design of the locus determination of the β binding site on the α_1 subunit. The AID site was determined on several α_1 subunits (α_{1S}, α_{1A}, α_{1B}, α_{1C}) by screening epitope libraries of these proteins with [^{35}S]-labeled β subunits. All the epitopes that were obtained using this strategy mapped to the same locus on the α_1 subunit, namely the

cytoplasmic loop separating the first from the second hydrophobic repeats according to the putative transmembrane topology of this subunit. Figure 8 shows a cDNA sequence alignment of representatives of all six α_1 subunit genes that should contain the β binding site AID. The overall sequence identity of this cytoplasmic loop is only 19%, which greatly facilitates the identification of the AID as a minimal conserved sequence, QQ-E-L-GY--WI--E. Thus the AID has a length of 18 amino acids, of which nine are well conserved and expected to participate in the interaction with β subunits. Although there are identical amino acids upstream from the 18–amino acid epitope, these were not required for binding, as demonstrated by the clone detected by overlap method (underlined in Fig. 8). The importance of the AID was demonstrated by single-point mutations that either disrupted or altered the binding of β subunits. These point mutations also altered the β subunit regulation of the α_1 current properties (Fig. 9). Mutations that did not affect the association of β subunits to the α_1 subunit modified only the efficiencies of β current amplitude stimulation, suggesting that the conformation of the AID is essential to this β regulation. Conversely, mutations that affected the ability of α_1 subunits to bind β subunits completely abolished all the regulation by β subunits, demonstrating that the AID is not only a primary attachment site for the β subunit but is also required for all its effects on current properties.

6.2. Identification of a Beta Interaction Domain (BID) that Interacts with AID

The identification of a β subunit sequence involved in an interaction with an α_1 subunit stemmed from our interest in the mechanism of β-induced current stimulation. A

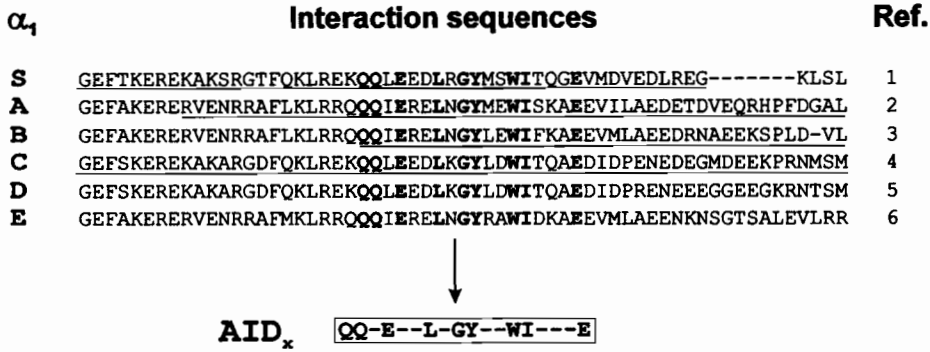


FIGURE 8. AID interaction domain is formed by nine highly conserved amino acids in the I-II cytoplasmic linker of all α_1 subunits. Ca^{2+} channel class A through class E, as well as class S, have been shown to interact with the BID site of the β subunit through this well-defined AID_x epitope (X = S, A, B, C, D, or E). Although there are several conserved amino acids 5′ to the AID_x epitope, these are by definition not required for interaction with the β subunit, as demonstrated by the underlined sequences shown to bind β subunits *in vitro* (Pragnell *et al.*, 1994). References: 1, Tanabe *et al.* (1987); 2, Mori *et al.* (1991); 3, Williams *et al.* (1992b); 4, Mikami *et al.* (1989); 5, Williams *et al.* (1992a); 6, Niidome *et al.* (1992).

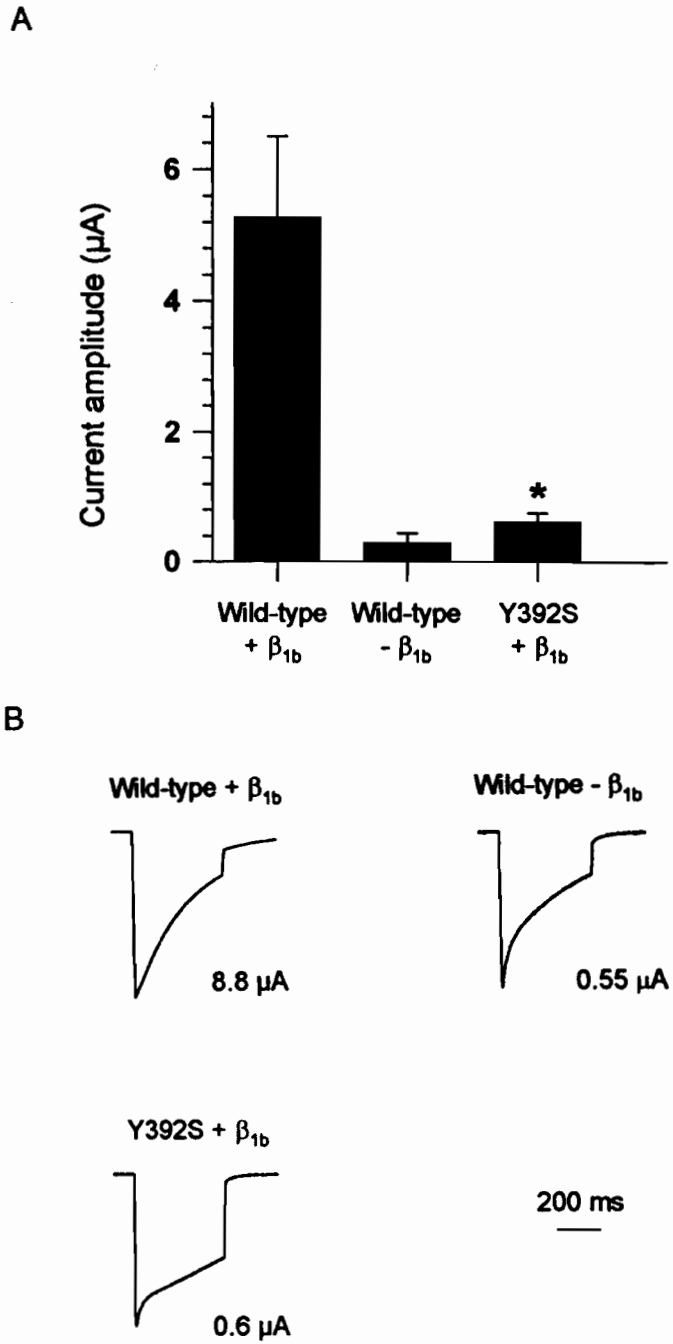
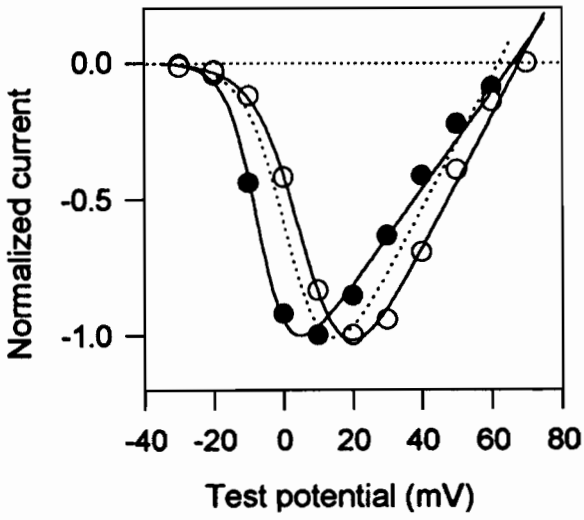
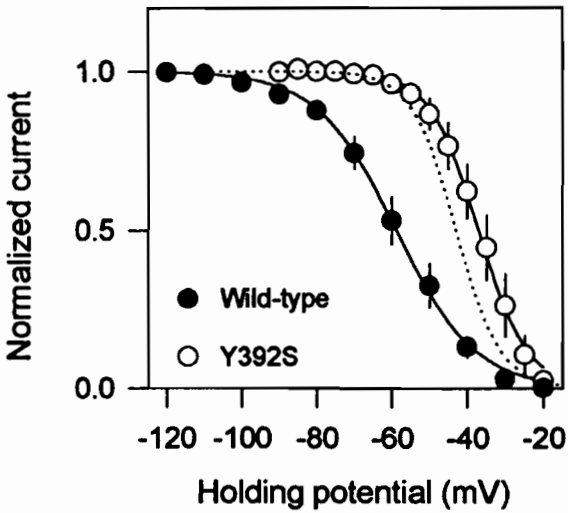


FIGURE 9. The Y392S mutation in AID of the α_{1A} subunit disrupts all the β_{1b} subunit regulation. The data presented in (A), (B), and (C) are from Pragnell *et al.* (1994). The current properties of mutant α_{1A} subunit coexpressed with β_{1b} (filled symbol) were compared to the properties of wild-type α_{1A} subunit in the absence

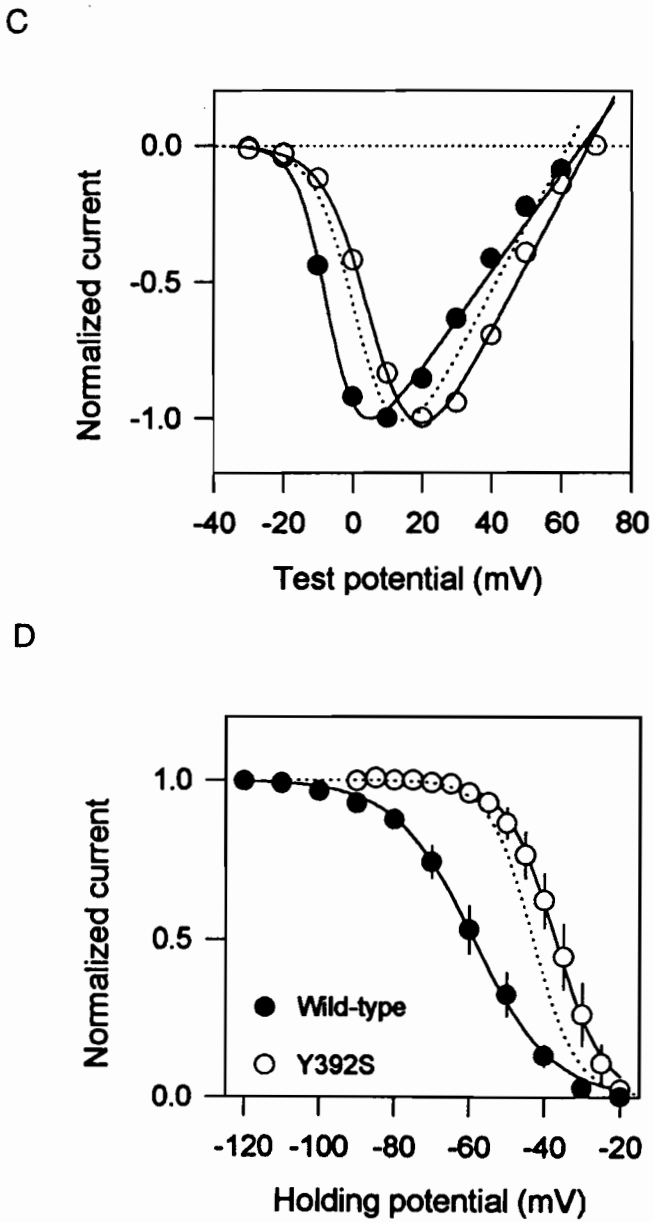
C



D



(dashed line) and presence of β_{1b} subunit (open symbol). (D) The Y392S mutation in AID_A also abolishes the hyperpolarizing shift induced by the β_{1b} subunit.



(dashed line) and presence of β_{1b} subunit (open symbol). (D) The Y392S mutation in AID_A also abolishes the hyperpolarizing shift induced by the β_{1b} subunit.

β interaction domain (BID) was initially identified as the minimum β subunit sequence capable of triggering a stimulation in the current amplitude. It was expected that since all β subunits are almost equally potent with respect to amplitude stimulation, BID probably represented a conserved sequence. As previously demonstrated in Fig. 3B, the β subunit can be divided into five structural regions depending on the level of amino acid homologies among various β subunits. Several cDNA constructs were made that encoded various structural domains of the β_{1b} subunit. The truncated forms of this subunit were then tested for their efficiencies of α_{1A} current stimulation into *Xenopus* oocytes (De Waard *et al.*, 1994b). The results demonstrated that a sequence of 30 amino acids, located at the N-terminus of the second most conserved domain of β subunits, was responsible for a significant fraction of current stimulation. The structural complexity of the BID is demonstrated by the presence of five proline residues that could introduce several β-turns in the interaction site. The BID may potentially be a regulatory target by a protein kinase with the presence of two consensus sites for protein kinase C. Direct interaction of this sequence with the α_1 subunit was proven, not only by current amplitude stimulation, but also by nonspecific changes in the activation and inactivation kinetics of the α_{1A} current (De Waard *et al.*, 1994b). For instance, the rather drastic changes in inactivation kinetics that occur by truncating the β subunit suggest that the BID is strongly influenced by changes in conformation of adjacent sequences in the β subunit. The presence of a splicing region, thus variable for each β subunit isoform, immediately upstream from the BID might be of relevance to this observation. Interestingly, the truncated β_{1b} subunit that encodes the entire second conserved domain (BID + 173 amino acids downstream from BID) was shown to interact directly with the AID on the α_1 subunit in an overlay assay. These data strongly suggested either that there are two independent interaction sites in the second conserved domain of β subunits (one for β stimulation called BID and another for the overlay interaction) or that both the stimulation and the overlay interaction with the AID occurred via the BID. To resolve this dilemma, single-point mutations were performed in BID on the full-length β_{1b} subunit. The abilities of the mutant β subunits to interact with α_1 subunits or AID itself and to regulate α_1 currents were compared to those of the wild-type β subunit. The data obtained were strikingly similar to the results obtained with single-point mutations of the AID site. Again, two categories of mutants could be identified. First, some of the mutations affected the ability of β subunits to bind to the AID and to the full-length α_1 subunit, thereby demonstrating that the AID and BID are directly interacting with each other. These β mutants were unable to regulate the properties of α_1 currents (amplitude, kinetics, and voltage dependence), confirming that the AID-BID association is absolutely required for channel regulation by β subunits. Second, mutations that did not affect the association of β subunits to α_1 subunits only modified the efficiencies of current amplitude stimulation by β subunits, while the properties of kinetic and voltage-dependence modulations were left intact. These results again suggest that the mechanisms that are at the basis of current stimulation are different from those responsible for the changes in current kinetics and voltage dependence. The differences in extent of stimulation by the interacting β subunit mutants support the hypothesis that conformational changes contributed by the β subunit can significantly alter channel activity. This

would then be achieved by either facilitating or hindering the coupling between movement of the voltage sensors and opening of the pore (Neely *et al.*, 1993).

6.3. Perspectives on the Primary Subunit Interaction Sites

Due to substantial molecular diversities of α_1 and β subunits, there is an impressive potential for combinatorial heterogeneity in voltage-sensitive Ca^{2+} channels at the structural level and consequently at the functional level. This possibility is particularly true in brain where five out of six α_1 genes and all four β genes are expressed and is further strengthened by the identification of two conserved structural elements required for the interaction of α_1 and β subunits. It is not known whether such a potential for Ca^{2+} channel diversity is exploited by cells. Preliminary evidence from two purified channels (skeletal muscle L-type and brain N-type Ca^{2+} channel) might indicate that this is not the case. The data gathered so far show that in skeletal muscle, the α_{1S} subunit specifically assembles with the β_{1a} subunit. Also, the α_{1B} subunit associates with the β_3 subunit in brain despite the coexpression of both β_1 and β_4 genes in the same tissue. There may thus exist unidentified mechanisms of specificity in subunit recognition and assembly in native voltage-dependent Ca^{2+} channels. Two hypotheses may explain this selectivity in subunit assembly: 1) It is possible that the affinity between individual α_1 and β subunits governs their interactions. This would then represent a true specificity in subunit assembly. Recent biochemical evidence demonstrates that different β subunits do indeed have different affinities for each α_1 subunit (De Waard *et al.*, 1995). In this respect, it will be important to analyze the importance of nonconserved residues interspersed among the conserved residues of the AID in determining the affinity of various β subunits for each α_1 subunit. 2) The level and timing of expression of each Ca^{2+} channel subunit might determine the composition of the native channels. Recently, Tarroni *et al.* (1994) have suggested that the α_{1B} subunit of IMR 32 human neuroblastoma cell line is not associated with the β_3 subunit, but instead with a β_2 subunit. This is confirmed by expression experiments that demonstrate the functional interchangeability of β subunits in their association to α_1 subunits. Thus, it is possible that the α_{1S} subunit of skeletal muscle L-type channel is associated with a β_{1a} subunit, because these two subunits are predominantly, if not exclusively, expressed in this tissue. Such a mechanism would represent an apparent specificity of subunit assembly. It should be emphasized that both hypotheses of true and apparent specificity in subunit assembly are not mutually exclusive. Future investigations should be aimed at resolving the subunit composition of voltage-dependent Ca^{2+} channels in several other homogeneous cell populations and the effectiveness of β subunit interchangeability in native voltage-dependent Ca^{2+} channels.

7. SUMMARY AND CONCLUSIONS

Data gathered from the expression of cDNAs that encode the subunits of voltage-dependent Ca^{2+} channels have demonstrated important structural and functional similarities among these channels. Despite these convergences, there are also significant

differences in the nature and functional importance of subunit-subunit and protein–Ca^{2+} channel interactions. There is evidence demonstrating that the functional differences between Ca^{2+} channel subtypes is due to several factors, including the expression of distinct α_1 subunit proteins, the selective association of structural subunits and modulatory proteins, and differences in posttranslational processing and cell regulation. We summarize several avenues of research that should provide significant clues about the structural features involved in the biophysical and functional diversity of voltage-dependent Ca^{2+} channels.

7.1. Secondary Subunit Interaction Sites

We have previously demonstrated that the AID and BID are primary interaction sites required for anchoring the β subunit to α_1 in voltage-dependent Ca^{2+} channels. Single-point mutations in either one of these sites proved conclusively that this attachment serves an obligatory role in the regulation of Ca^{2+} channel properties by β subunits. Several research directions remain at this stage. First, it is possible that all β regulation (current amplitude stimulation and changes in kinetics and voltage dependence of channel activity) are transduced via this primary α_1-β attachment site. Two independent observations would be consistent with this possibility. Screening of an α_1 epitope library with [^{35}S]-labeled β subunit failed to identify α_1 sequences other than those containing the AID (Pragnell *et al.*, 1994). Different β subunits induce qualitatively similar regulation in spite of important sequence divergences in the N- and C-terminal regions. Nonconserved β subunit sequences may define their protein conformation and in turn influence the regulatory input of β subunits at the primary interaction site, thereby explaining the quantitative differences seen with different β subunits. Second, there may be functionally important secondary interaction sites operational only upon association of β subunits to the AID in the α_1 subunit. Several observations are in favor of this interpretation: 1) important stretches of conserved amino acid sequence exist in addition to BID itself, 2) truncated β subunits capable of interacting with AID regulate the α_1 inactivation kinetics in an anomalous manner, and 3) mutations in BID on the full-length β subunit that preserve the α_1-β interaction alter the β stimulation efficiency but leave intact the other regulation. This latter observation would not be expected if all β subunit regulation were transduced via the BID and AID sites. Recent experimental evidence suggests that the different β regulation may in fact physically occur at different loci on the β subunit. Olcese *et al.* (1994) have presented evidence that the nonconserved N-termini of β subunits set the rate of channel inactivation. This regulation in inactivation kinetics occurs independently of the effects of the β subunit on activation, suggesting that activation and inactivation gates on the α_1 subunit can be regulated by different parts of β subunits. Future investigations should be aimed at mapping additional interaction sites between α_1 and β subunits.

7.2. Interactions of Ca^{2+} Channels with Other Cellular Proteins

The cytoplasmic loop between repeats II and III of various α_1 subunits has been implicated in several protein-channel interactions. Chimeras in which regions of the α_{1S}

subunit were replaced with equivalent domains of the α_{1C} subunit have demonstrated that the II-III cytoplasmic linker determines the type of E-C coupling. This α_{1S} loop is therefore believed to interact directly, or indirectly via unidentified proteins, with the ryanodine receptor to produce the extracellular Ca^{2+}-independent type of E-C found in skeletal muscle (Catterall, 1991; Tanabe *et al.*, 1990a). Recently, syntaxin, a protein required for transmitter release at the synapse, was shown to interact with the α_{1B} subunit of the N-type channel (Leveque *et al.*, 1994; Sheng *et al.*, 1994). This interaction was mapped to an 87–amino acid sequence also located on the cytoplasmic loop between hydrophobic repeats II and III. This finding demonstrates at a molecular level the colocalization of a Ca^{2+} source (the Ca^{2+} channel) and a Ca^{2+} effector (a protein of the exocytosis complex). This interaction has potential importance for the mechanism of excitation-secretion (E-S) coupling because syntaxin also interacts with αSNAP (Soluble NSF Attachment Protein), SNAP-25, and synaptobrevin by forming a tight exocytotic complex (Sollner *et al.*, 1993). The interaction of this complex with the N-type Ca^{2+} channel is essential not only for the docking of synaptic vesicles at the active zone, but also for an effective coupling between Ca^{2+} entry and transmitter release via mechanisms that are the subject of intense investigations. In both cases, however, it is not yet understood how conformational changes in the α_1 subunit could be transduced to trigger Ca^{2+} release in skeletal muscle or secretion at neuronal synapses. Also, it is not known how these interactions may affect the biophysical or pharmacological properties of the channels. However, the data available clearly indicate that sequence differences in the II-III loop can define the nature of protein–Ca^{2+} channel interactions and thereby the function of each Ca^{2+} channel subtype. Future investigations may prove the existence of additional types of interaction between the II-III loop of other α_1 subunits (A, C, D, and E) and yet unidentified proteins. This may also ultimately lead to a better understanding of the functional significance of alternative splicing in the II-III loop domain of various α_1 subunits (Snutch *et al.*, 1991).

Voltage-dependent Ca^{2+} channels, and N-type channels in particular, are targets of various neuromodulators (Diversé-Pierluissi *et al.*, 1995). Frequently, these modulations involve G proteins acting directly on the channel by a fast and membrane-delimited pathway, although channel activity can also be regulated by indirect mechanisms such as phosphorylation (Hille, 1992). There is also evidence that biochemical modifications of the N-type channel by one pathway (protein kinase C phosphorylation, for example) may alter the susceptibility of the channel to alternative regulations, including G protein inhibition (Swartz, 1993; Diversé-Pierluissi *et al.*, 1995). So far, it is well documented that G proteins can modulate the activity of several types of Ca^{2+} channels by directly interacting with one or more subunits of the protein complex. Direct G protein interaction with N-type Ca^{2+} channels is believed to decrease the current by altering the prevalence of a high-P_o gating mode of the channel activity (Delcour and Tsien, 1993). Purified G_S protein stimulates the activity of the skeletal muscle L-type Ca^{2+} channel (Yatani *et al.*, 1988). Also, the endogenous $G_{o\alpha}$ subunit partially copurifies with the N-type Ca^{2+} channel, indicating the existence of a high-affinity site between both protein complexes (McEnery *et al.*, 1994). Although the subjects of intense investigation, the site of G protein interaction with voltage-dependent Ca^{2+} channels and the precise mechanisms of regulation have not yet been elucidated. It is also not known how the subunit

composition and/or posttranslational modifications of the Ca^{2+} channel complex may affect G protein modulation of the currents.

It is believed that answers to several of these questions may also provide some clues regarding the structural determinants involved in the spatial distribution of calcium channels at the cellular level. Differential distribution of voltage-gated Ca^{2+} channels in cells is required for the compartmentalized activation of Ca^{2+}-dependent processes. For instance, it is not known what factors define the localization and the clustering of L-type Ca^{2+} channels at the branching points of dendrites in hippocampal pyramidal cells (Westenbroek *et al.*, 1990) or similarly the clustering of N-type Ca^{2+} channels at the

structural elements that control the pattern of activity of any given channel and then understand the molecular mechanisms whereby channel gating responds to biochemical changes at their site of regulation. The mapping of several functionally important elements within various α_1 subunits has paved much of the way (Fig. 10). Chimeras between the α_{1S} and α_{1C} subunits have shown that the IS3 segment and the extracellular IS3-S4 linker are critical sequences implicated in the control of the current activation kinetics of both subunits (Nakai *et al.*, 1994). The different inactivation kinetics of α_{1A}, α_{1C}, and α_{1E} seem to be under the control of specific sequences within the IS6

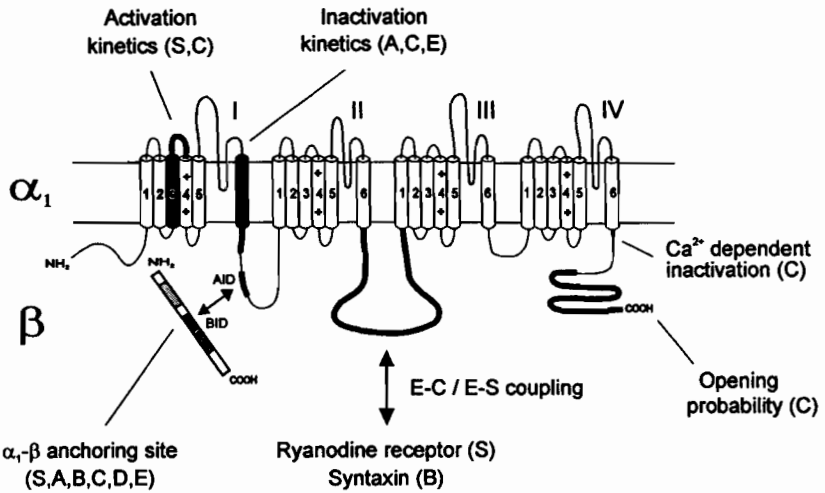


FIGURE 10. Functional domains of the α_1 subunit of voltage-dependent Ca^{2+} channels. Shown are the well-characterized β anchoring site in the I-II cytoplasmic linker, the regions involved in activation kinetics, and inactivation kinetics. The highly variable II-III cytoplasmic linker is important in excitation-contraction secretion in the α_{1S} subtype and in syntaxin binding in the α_{1B} subtype. Carboxyl terminal to IVS6 is a region with slight EF-hand homology that appears to be involved in Ca^{2+}-dependent inactivation. Deletions of the C-terminus result in increased channel open probability, suggesting its importance in channel gating.

segment and of a proximal sequence in the I-II cytoplasmic loop downstream from this IS6 segment (Zhang *et al.*, 1994). Finally, the C-terminal region of α_1 subunits seems to contain important structural elements for the regulation of Ca^{2+} channel activity. The extreme C-terminal portion of the α_{1C} subunit is involved in tonic inhibition of channel opening probability (Wei *et al.*, 1994), whereas a cytoplasmic EF-hand Ca^{2+} binding sequence immediately downstream from the IVS6 segment is essential for Ca^{2+}-dependent inactivation of the α_{1C} subunit (de Leon *et al.*, 1995).

The mapping of subunit-subunit and protein-channel interaction sites has added additional structural elements to the functional complexity of α_1 subunits. We have demonstrated that the primary α_1-β interaction site occurs within the cytoplasmic linker separating the first and second hydrophobic repeats (Pragnell *et al.*, 1994; De Waard *et al.*, 1994b). Also, the intracellular loop between motifs II and III of the α_1 subunit is either involved in skeletal muscle E-C coupling (α_{1S}) or E-S coupling (α_{1B}) via a direct interaction with syntaxin (Sheng *et al.*, 1994). It will be important to determine how conformational changes introduced by β subunits in the α_1 channel may regulate the contribution of the various structural elements of the α_1 subunit to Ca^{2+}-dependent inactivation, to contraction and secretion, or to the control of activation and inactivation kinetics. It is interesting that the implication of the IS6 segment in Ca^{2+} channel inactivation is not consistent with either the N-terminal localization of the "ball-and-chain" inactivation of K^+ channels (Hoshi *et al.*, 1990) or the fast inactivation by the III-IV linker in voltage-dependent Na^+ channels (Catterall, 1993). These differences in the localization of the structural elements may also underlie a different mechanism of inactivation in voltage-dependent Ca^{2+} channels. In this respect, the proximity of AID to the IS6 segment is conspicuous. It will therefore be important to analyze whether the interactions between AID and the β subunit can allosterically modulate the conformation of the IS6 segment and its contribution to the inactivation process.

Another prevalent cellular mechanism for the modulation of ion channel function is phosphorylation. The phosphorylation state of the channel subunits or the proteins associated with the channel influences the amplitude (Yang and Tsien, 1993), voltage dependence (Sculptoreanu *et al.*, 1993), or time course of the current (Werz *et al.*, 1993). Phosphorylation may also affect the effectiveness of several channel regulations (Swartz, 1993). The underlying biophysical and molecular mechanisms of these modulatory processes remain to be analyzed. In particular, it will be necessary to analyze how the interactions between AID and the β subunit may influence the effects of these phosphorylations on channel activity.

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