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STRUCTURE AND REGULATION OF VOLTAGE-GATED Ca^{2+} CHANNELS

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■ **Abstract** Voltage-gated Ca^{2+} channels mediate Ca^{2+} entry into cells in response to membrane depolarization. Electrophysiological studies reveal different Ca^{2+} currents designated L-, N-, P-, Q-, R-, and T-type. The high-voltage-activated Ca^{2+} channels that have been characterized biochemically are complexes of a pore-forming α_1 subunit of ~190–250 kDa; a transmembrane, disulfide-linked complex of α_2 and δ subunits; an intracellular β subunit; and in some cases a transmembrane γ subunit. Ten α_1 subunits, four $\alpha_2\delta$ complexes, four β subunits, and two γ subunits are known. The Ca_v1 family of α_1 subunits conduct L-type Ca^{2+} currents, which initiate muscle contraction, endocrine secretion, and gene transcription, and are regulated primarily by second messenger-activated protein phosphorylation pathways. The Ca_v2 family of α_1 subunits conduct N-type, P/Q-type, and R-type Ca^{2+} currents, which initiate rapid synaptic transmission and are regulated primarily by direct interaction with G proteins and SNARE proteins and secondarily by protein phosphorylation. The Ca_v3 family of α_1 subunits conduct T-type Ca^{2+} currents, which are activated and inactivated more rapidly and at more negative membrane potentials than other Ca^{2+} current types. The distinct structures and patterns of regulation of these three families of Ca^{2+} channels provide a flexible array of Ca^{2+} entry pathways in response to changes in membrane potential and a range of possibilities for regulation of Ca^{2+} entry by second messenger pathways and interacting proteins.

CONTENTS

INTRODUCTION

Ca^{2+} channels in many different cell types activate upon membrane depolarization and mediate Ca^{2+} influx in response to action potentials and sub-threshold depolarizing signals. Ca^{2+} entering the cell through voltage-gated Ca^{2+} channels serves as the second messenger of electrical signaling, initiating intracellular events such as contraction, secretion, synaptic transmission, and gene expression. Multiple types of Ca^{2+} currents have been identified by their physiological and pharmacological properties and have been correlated with cloned Ca^{2+} channel subunits characterized by expression in vitro. In this chapter, I briefly review the structure of Ca^{2+} channels and focus on the different modes of regulation of their functional activity by second messenger pathways and protein-protein interactions. I have restricted consideration to those examples in which Ca^{2+} channel regulation observed at the cellular level has been correlated with analysis of the molecular basis for channel regulation using cloned and expressed Ca^{2+} channels. More broad-ranging reviews of Ca^{2+} channel regulation at the cellular level can be found in Dolphin 1998, Hille 1994, Hosey et al 1996, McDonald et al 1994.

Ca^{2+} CURRENT TYPES DEFINED BY PHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES

Since the first recordings of Ca^{2+} currents in cardiac myocytes (Reuter 1967, 1979), it has become apparent that there are multiple types of Ca^{2+} currents as defined by physiological and pharmacological criteria (Bean 1989a, Hess 1990, Llinas

et al 1992, Tsien et al 1988) (Table 1). In cardiac, smooth, and skeletal muscle, the major Ca^{2+} currents are distinguished by high voltage of activation, large single-channel conductance, slow voltage-dependent inactivation, marked regulation by cAMP-dependent protein phosphorylation pathways, and specific inhibition by Ca^{2+} antagonist drugs including dihydropyridines, phenylalkylamines, and benzothiazepines (Reuter 1983). These Ca^{2+} currents have been designated L-type, as they are long lasting when Ba^{2+} is the current carrier (Nowycky et al 1985). L-type Ca^{2+} currents are also recorded in endocrine cells where they initiate release of hormones (Milani et al 1990) and in neurons where they are important in regulation of gene expression and in integration of synaptic inputs (Bean 1989a). L-type Ca^{2+} currents are subject to regulation by second messenger-activated protein phosphorylation in several cell types as discussed below.

Voltage-clamp studies of Ca^{2+} currents in starfish eggs (Hagiwara et al 1975) and recordings of Ca^{2+} action potentials in cerebellar Purkinje neurons (Llinas & Yarom 1981) first revealed Ca^{2+} currents with different properties from L-type, and these were subsequently characterized in detail in voltage-clamped dorsal root ganglion neurons (Carbone & Lux 1984, Fedulova et al 1985, Nowycky et al 1985, Swandulla & Armstrong 1988). In comparison with L-type currents, these Ca^{2+} currents activate at much more negative membrane potentials, inactivate rapidly, deactivate slowly, have small single-channel conductance, and are insensitive to Ca^{2+} antagonist drugs. They are designated low-voltage-activated Ca^{2+} currents for their negative voltage dependence (Carbone & Lux 1984) or T-type for their transient kinetics (Nowycky et al 1985). Regulation of T-type Ca^{2+} current has not yet been analyzed in detail at the molecular level.

Whole-cell voltage-clamp and single-channel recording from dissociated dorsal root ganglion neurons revealed an additional Ca^{2+} current, N-type (Nowycky et al 1985). In these initial experiments, N-type Ca^{2+} currents were distinguished by their intermediate voltage dependence and rate of inactivation–more negative and faster than L-type but more positive and slower than T-type (Nowycky et al 1985). They are insensitive to organic L-type Ca^{2+} channel blockers but blocked by the cone snail peptide ω-conotoxin GVIA (McCleskey et al 1987, Tsien et al 1988). This pharmacological profile has been the primary method to distinguish N-type Ca^{2+} currents because the voltage dependence and kinetics of N-type Ca^{2+} currents in different neurons vary considerably.

Analysis of the effects of other peptide toxins revealed three additional Ca^{2+} current types. P-type Ca^{2+} currents, first recorded in Purkinje neurons (Llinás et al 1989), are distinguished by high sensitivity to the spider toxin ω-agatoxin IVA (Mintz et al 1992). Q-type Ca^{2+} currents, first recorded in cerebellar granule neurons (Randall & Tsien 1995), are blocked by ω-agatoxin IVA with lower affinity. R-type Ca^{2+} currents in cerebellar granule neurons are resistant to the subtype-specific organic and peptide Ca^{2+} channel blockers (Randall & Tsien 1995) and may include multiple channel subtypes (Tottene et al 1996). Although L-type and T-type Ca^{2+} currents are recorded in a wide range of cell

TABLE 1 Subunit composition and function of Ca^{2+} channel types

Ca^{2+} channel	Ca^{2+} current type	Primary localizations	Previous name of $\alpha\gamma$subunits	Specific blocker	Functions
$Ca_V1.1$	L	Skeletal muscle	α_{1S}	DHPs	Excitation-contraction coupling Calcium homeostasis Gene regulation
$Ca_V1.2$	L	Cardiac muscle Endocrine cells Neurons	α_{1C}	DHPs	Excitation-contraction coupling Hormone secretion Gene regulation
$Ca_V1.3$	L	Endocrine cells Neurons	α_{1D}	DHPs	Hormone secretion Gene regulation
$Ca_V1.4$	L	Retina	α_{1F}		Tonic neurotransmitter release
$Ca_V2.1$	P/Q	Nerve terminals Dendrites	α_{1A}	ω-Agatoxin	Neurotransmitter release Dendritic Ca^{2+} transients
$Ca_V2.2$	N	Nerve terminals Dendrites	α_{1B}	ω-CTx-GVIA	Neurotransmitter release Dendritic Ca^{2+} transients
$Ca_V2.3$	R	Cell bodies Dendrites Nerve Terminals	α_{1E}	None	Ca^{2+}-dependent action potentials Neurotransmitter release
$Ca_V3.1$	T	Cardiac muscle Skeletal muscle Neurons	α_{1G}	None	Repetitive ring
$Ca_V3.2$	T	Cardiac muscle Neurons	α_{1H}	None	Repetitive ring
$Ca_V3.3$	T	Neurons	α_{1I}	None	Repetitive ring

types, N-, P-, Q-, and R-type Ca^{2+} currents are most prominent in neurons. They are regulated by multiple signal transduction pathways, as discussed below.

MOLECULAR PROPERTIES OF Ca^{2+} CHANNELS

Subunit Structure

Ca^{2+} channels were first solubilized and purified from the transverse tubule membranes of skeletal muscle (Curtis & Catterall 1984). The initial purification studies revealed α_1, β, and γ subunits and showed that the α_1 and β subunits are substrates for cAMP-dependent protein phosphorylation (Curtis & Catterall 1984, 1985). More detailed biochemical analyses revealed an additional $\alpha_2\delta$ subunit comigrating with the α_1 subunit (Hosey et al 1987, Leung et al 1987, Striessnig et al 1987, Takahashi et al 1987). Analysis of the biochemical properties, glycosylation and hydrophobicity of these five subunits led to a model comprising a principal transmembrane α_1 subunit of 190 kDa in association with a disulfide-linked $\alpha_2\delta$ dimer of 170 kDa, an intracellular phosphorylated β subunit of 55 kDa, and a transmembrane γ subunit of 33 kDa (Figure 1*A*) (Takahashi et al 1987).

The primary structures of the five Ca^{2+} channel subunits were determined by combination of protein chemistry with cDNA cloning and sequencing. The α_1 subunit is a protein of about 2000 amino acid residues with an amino acid sequence and predicted transmembrane structure like the previously characterized, pore-forming α subunit of Na^+ channels (Tanabe et al 1987) (Figure 1*A*). The amino acid sequence is organized in four repeated domains (I to IV), each of which contains six transmembrane segments (S1 to S6), and a membrane-associated loop between transmembrane segments S5 and S6. As expected from biochemical analysis (Takahashi et al 1987), the intracellular β subunit has predicted α helices but no transmembrane segments (Ruth et al 1989) (Figure 1*A*), whereas the γ subunit is a glycoprotein with four transmembrane segments (Jay et al 1990) (Figure 1*A*). The cloned α_2 subunit has many glycosylation sites and several hydrophobic sequences (Ellis et al 1988), but biosynthesis studies indicate that it is an extracellular, extrinsic membrane protein attached to the membrane through disulfide linkage to the δ subunit (Gurnett et al 1996) (Figure 1*A*). The δ subunit is encoded by the 3′ end of the coding sequence of the same gene as the α_2 subunit, and the mature forms of these two subunits are produced by post-translational proteolytic processing and disulfide linkage (De Jongh et al 1990, Jay et al 1991) (Figure 1*A*).

Purification of the cardiac $Ca_v1.2$ channels revealed subunits of the sizes of the α_1, $\alpha_2\delta$, β, and γ subunits of skeletal muscle Ca^{2+} channels (Chang & Hosey 1988, Kuniyasu et al 1992, Schneider & Hofmann 1988). Immunoprecipitation of L-type Ca^{2+} channels from neurons labeled by dihydropyridine Ca^{2+} antagonists revealed α_1, $\alpha_2\delta$, and β subunits but no γ subunit (Ahlijanian et al

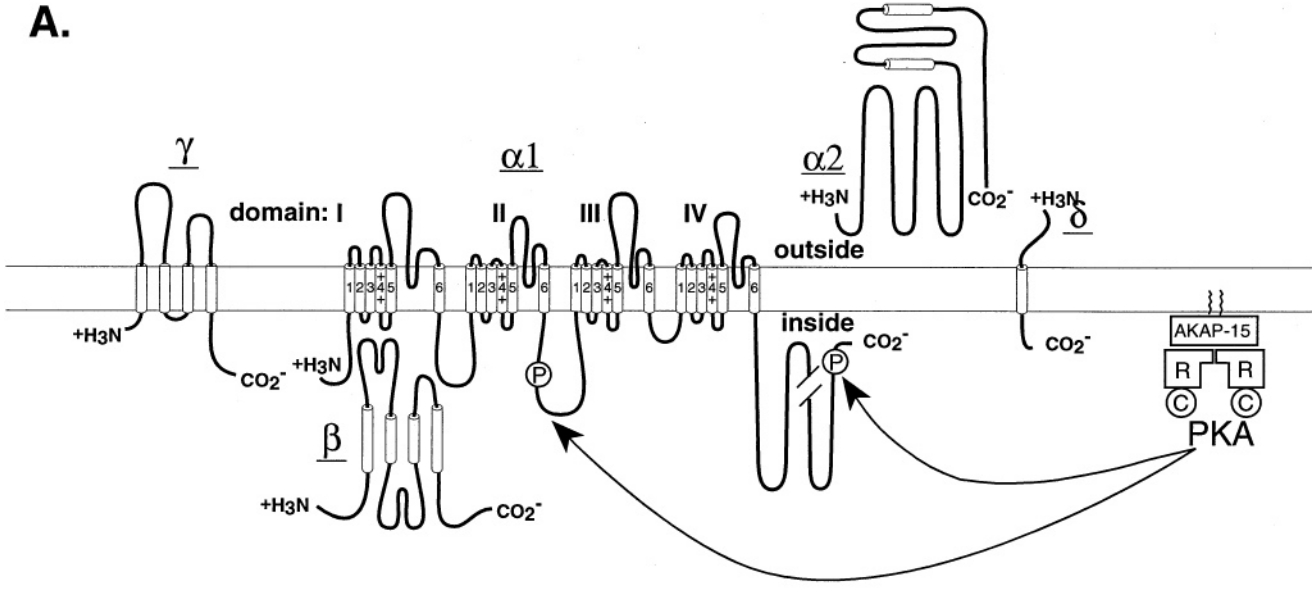

Figure 1 Subunit structure and regulation of Ca_v1 channels. (*A*) The subunit composition and structure of Ca^{2+} channels purified from skeletal muscle are illustrated. The model is updated from the original description of the subunit structure of skeletal muscle Ca^{2+} channels (Takahashi et al 1987). P, sites of phosphorylation by cAMP-dependent protein kinase that have been demonstrated in intact cells.

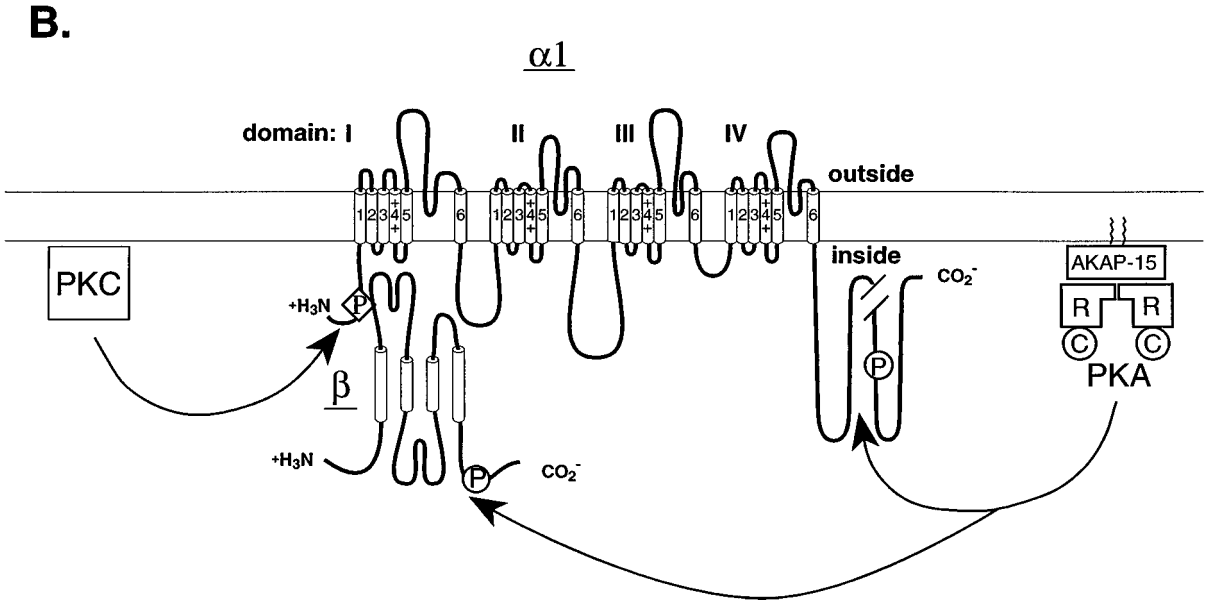

Figure 1 (*B*) Transmembrane-folding model and sites of phosphorylation of the cardiac Ca^{2+} channel subunits. Predicted α helices are depicted as cylinders. The lengths of lines correspond approximately to the lengths of the polypeptide segments represented.

1990). Together, these results suggest a similar subunit composition for L-type Ca^{2+} channels in cardiac and skeletal muscle and neurons, but the results from the purification studies of cardiac and neuronal Ca^{2+} channels are more difficult to interpret because much smaller amounts of protein can be purified for analysis.

Purification and immunoprecipitation of N-type Ca^{2+} channels labeled by ω-conotoxin GVIA from brain membrane preparations revealed α_1, $\alpha_2\delta$, and β subunits (McEnery et al 1991, Witcher et al 1993). Similarly, purified P/Q-type Ca^{2+} channels are composed of α_1, $\alpha_2\delta$, and β subunits (Liu et al 1996; Martin-Moutot et al 1995, 1996). In addition, recent experiments have unexpectedly revealed a novel γ subunit, which is the target of the *stargazer* mutation in mice (Letts et al 1998). This γ subunit-like protein can modulate the voltage dependence of expressed Ca^{2+} channels containing α_{1A} subunits, so it may be associated with these Ca^{2+} channels in vivo (Letts et al 1998). If this new γ subunit is indeed associated with neuronal Ca^{2+} channels, their subunit composition would be identical to that of skeletal muscle Ca^{2+} channels defined in biochemical experiments (Takahashi et al 1987) (Figure 1*A*).

Functions of Ca^{2+} Channel Subunits

The initial analyses of functional expression of Ca^{2+} channel subunits were carried out with skeletal muscle Ca^{2+} channels. Expression of the α_1 subunit is sufficient to produce functional skeletal muscle Ca^{2+} channels, but with low expression level and abnormal kinetics and voltage dependence of the Ca^{2+} current (Perez-Reyes et al 1989). Co-expression of the $\alpha_2\delta$ subunit and especially the β subunit enhances the level of expression and confers more normal gating properties (Lacerda et al 1991, Singer et al 1991). As for skeletal muscle Ca^{2+} channels, co-expression of β subunits has a large effect on the level of expression and the voltage dependence and kinetics of gating of cardiac and neuronal Ca^{2+} channels. In general, the level of expression is increased, the voltage dependence of activation and inactivation is shifted to more negative membrane potentials, and the rate of inactivation is increased. However, these effects are different for individual β subunit isoforms (reviewed in Hofmann et al 1994, Hosey et al 1996). For example, the β_{2a} subunit slows channel inactivation in most subunit combinations. In contrast, co-expression of $\alpha_2\delta$ subunits (Hofmann et al 1994, Hosey et al 1996) and γ subunits (Letts et al 1998) has much smaller functional effects.

Molecular Basis for Ca^{2+} Channel Function

Intensive studies of the structure and function of the related pore-forming subunits of Na^+, Ca^{2+}, and K^+ channels have led to identification of their principal functional domains (reviewed in Catterall 1995, 2000; Hofmann et al 1999; Jan & Jan 1997; Stuhmer & Parekh 1992). Each domain of the principal subunits consists

of six transmembrane α helices (S1 through S6) and a membrane-associated loop between S5 and S6 (Figure 1*A*). The S4 segments of each homologous domain serve as the voltage sensors for activation, moving outward and rotating under the influence of the electric field and initiating a conformational change that opens the pore. The S5 and S6 segments and the membrane-associated pore loop between them form the pore lining of the voltage-gated ion channels. The narrow external pore is lined by the pore loop, which contains a pair of glutamate residues in each domain that are required for Ca^{2+} selectivity. The inner pore is lined by the S6 segments, which form the receptor sites for the pore-blocking Ca^{2+} antagonist drugs specific for L-type Ca^{2+} channels. All Ca^{2+} channels share these general structural features.

Ca^{2+} Channel Diversity

The different types of Ca^{2+} currents are primarily defined by different α_1 subunits. The primary structures of ten distinct Ca^{2+} channel α_1 subunits have been defined by homology screening, and their function has been characterized by expression in mammalian cells or *Xenopus* oocytes (Table 1). These subunits were originally designated classes A through I, and more recently a nomenclature dividing the calcium channels into three structurally and functionally related families (Ca_v1, Ca_v2, and Ca_v3) has been proposed (Ertel et al 2000). L-type Ca^{2+} currents are mediated by the Ca_v1 family of α_1 subunits, which have about 75% amino acid sequence identity with the α_1 subunit of skeletal muscle L-type Ca^{2+} channels (Hui et al 1991, Snutch et al 1991, Strom et al 1998, Williams et al 1992b). The Ca_v2 channels form a distinct family with less than 40% amino acid sequence identity with Ca_v1 α_1 subunits but greater than 70% amino acid sequence identity among themselves (Table 1). Cloned $Ca_v2.1$ subunits (Mori et al 1991, Starr et al 1991) conduct P- or Q-type Ca^{2+} currents, which are inhibited by ω-agatoxin IVA (Sather et al 1993, Stea et al 1994, Bourinet et al 1999). $Ca_v2.2$ subunits conduct N-type Ca^{2+} currents with high affinity for ω-conotoxin GVIA (Dubel et al 1992, Williams et al 1992a). Cloned $Ca_v2.3$ subunits conduct R-type Ca^{2+} currents, which are resistant to both organic Ca^{2+} antagonists specific for L-type Ca^{2+} currents and the peptide toxins specific for N-type or P/Q-type Ca^{2+} currents (Randall & Tsien 1995, Soong et al 1994, Zhang et al 1993). T-type Ca^{2+} currents are mediated by the Ca_v3 channels (Perez-Reyes et al 1998). These α_1 subunits are only distantly related to the other known homologs, with less than 25% amino acid sequence identity. These results reveal a surprising structural dichotomy between the T-type, low-voltage-activated Ca^{2+} channels and the high-voltage-activated Ca^{2+} channels. Evidently, these two lineages of Ca^{2+} channels diverged very early in the evolution of multi-cellular organisms.

The diversity of Ca^{2+} channel structure and function is substantially enhanced by multiple β subunits. Four β subunit genes have been identified; each is subject

to alternative splicing to yield additional isoforms (reviewed in Hofmann et al 1994, Perez-Reyes & Schneider 1995). In Ca^{2+} channel preparations isolated from brain, each Ca^{2+} channel α_1 subunit is associated with multiple β subunits, although there is a different rank order in each case (Pichler et al 1997, Liu et al 1996, Witcher et al 1995). The different β subunit isoforms cause different shifts in the kinetics and voltage dependence of gating, so association with different β subunits can substantially alter the physiological function of an α_1 subunit. Genes encoding four $\alpha_2\delta$ subunits have been described (Klugbauer et al 1999), but the $\alpha_2\delta$ isoforms produced by these different genes have relatively small functional effects on channel gating and expression. A new γ subunit has been recently described (Letts et al 1998), which has small but significant effects on the voltage dependence of Ca^{2+} channel gating.

The striking structural differences among the three classes of α_1 subunits are reflected in marked differences in their regulation. The Ca_v1 family of Ca^{2+} channels is regulated primarily by protein phosphorylation through second messenger-activated kinase pathways. In contrast, the Ca_v2 family of channels is regulated by direct binding of SNARE proteins and G proteins, and that primary mode of regulation is itself regulated by protein phosphorylation pathways. Regulation of the Ca_v3 family of Ca^{2+} channels by protein phosphorylation and G protein pathways is much less prominent and less well studied than for Ca_v1 and Ca_v2 families and is not considered further here.

REGULATION OF THE Ca_v1 FAMILY OF Ca^{2+} CHANNELS BY PROTEIN PHOSPHORYLATION

Modulation of L-type currents by a wide variety of hormones and neurotransmitters has been demonstrated in smooth, skeletal, and cardiac muscle, in endocrine cells, and in neurons (Armstrong et al 1991, McDonald et al 1994). In this review, I have limited consideration to regulation of L-type Ca^{2+} channels in cardiac and skeletal muscle because the expression of a single α_1 subunit type in these tissues allows regulation in dissociated cells from intact tissue to be unambiguously correlated with studies of the cloned Ca^{2+} channels expressed in heterologous cells.

Regulation of the $Ca_v1.1$ Skeletal Muscle Ca^{2+} Channel by cAMP-Dependent Protein Phosphorylation

In skeletal muscle, single twitches do not require entry of extracellular Ca^{2+} (Armstrong et al 1972), and excitation-contraction coupling is thought to proceed via direct protein-protein interactions between the dihydropyridine-senstivie voltage-gated Ca^{2+} channel in the transverse tubules and the ryanodine-sensitive Ca^{2+} release channel (RyR) in the sarcoplasmic reticulum (Adams & Beam 1990, Catterall 1991, Rios & Pizarro 1991). The force of contraction is increased by

high-frequency stimulation by synaptic transmission from motor nerves and by epinephrine and calcitonin gene-related peptide (CGRP) acting through the cAMP signaling pathway (Cairns & Dulhunty 1993, Fleming et al 1993, Gonzalez-Serratos et al 1981, Kernell et al 1983, Ohhashi & Jacobowitz 1988, Uchida et al 1990). These effects require extracellular Ca^{2+} (Oz & Frank 1991) and thus are mediated at least in part by changes in Ca^{2+} entry. Activation of skeletal muscle L-type Ca^{2+} currents is enhanced by phosphorylation by cAMP-dependent protein kinase (Arreola et al 1987, Schmid et al 1985). In cultured skeletal muscle cells, repetitive depolarization causes a dramatic enhancement of Ca^{2+} currents (Fleig & Penner 1996, Sculptoreanu et al 1993b), up to 10-fold in the critical membrane potential range near −20 mV. This potentiation of Ca^{2+} currents is strongly voltage dependent and also is dependent on the activity of cAMP-dependent protein kinase (Sculptoreanu et al 1993b). This may result from interaction between voltage-dependent gating and phosphorylation of the Ca^{2+} channel itself. This novel regulatory mechanism greatly increases Ca^{2+} channel activity during tetanic stimulation of skeletal muscle cells and may play a critical role in the regulation of contractile force of skeletal muscle in response to hormones and to the frequency of stimulation of the motor nerve.

Phosphorylation of the α Subunit of Skeletal Muscle Ca^{2+} Channels The α_1 subunit and the β subunit of skeletal muscle Ca^{2+} channels are substrates for phosphorylation by cAMP-dependent protein kinase and a number of other protein kinases (Curtis & Catterall 1985, Jahn et al 1988, O'Callahan & Hosey 1988, Takahashi et al 1987). Ca^{2+} flux through the purified and reconsituted skeletal muscle Ca^{2+} channel is regulated by cAMP-dependent protein phosphorylation (Flockerzi et al 1986, Hymel et al 1988, Mundiña-Weilenmann et al 1991, Nunoki et al 1989). Ion flux studies in reconstituted phospholipid vesicles show that phosphorylation of the α_1 and β subunits can greatly increase the number of functional Ca^{2+} channels in purified preparations (Mundiña-Weilenmann et al 1991, Nunoki et al 1989). Single-channel recording experiments in planar bilayer membranes detect both increases in the number of functional Ca^{2+} channels and increases in the activity of single Ca^{2+} channels after phosphorylation by cAMP-dependent protein kinase (Flockerzi et al 1986, Hymel et al 1988). Thus, the α_1 and β subunits of the purified Ca^{2+} channel contain the sites at which cAMP-dependent protein phosphorylation modulates channel function in vitro. Two forms of the α_1 subunit, ∼190 and 212 kDa, are present in purified preparations of skeletal muscle Ca^{2+} channels, T-tubule membranes, and intact skeletal muscle cells in culture, and both are phosphorylated by PKA in intact cells (De Jongh et al 1989, 1991; Lai et al 1990). Antibody mapping of the C-terminal region of $\alpha_{1(190)}$ placed the C terminus between residues 1685 and 1699 (De Jongh et al 1991). The most rapidly phosphorylated site in the truncated form of the α_1 subunit in purified Ca^{2+} channel preparations is Ser 687, located in the intracellular loop between domains II and III (Röhrkasten et al 1988, Rotman et al 1992). In contrast, time-course experiments indicated that Ser 1854 near the C-terminal portion of full-length $\alpha_{1(212)}$ is the most intensely and rapidly

phosphorylated (Rotman et al 1992, 1995). To date, the site(s) of phosphorylation that regulates the ion conductance activity skeletal muscle Ca^{2+} channels has not been directly identified.

Phosphorylation of the β Subunit of Skeletal Muscle Ca^{2+} Channels Like the α_1 subunit of the skeletal muscle Ca^{2+} channel, the β subunit is stoichiometrically phosphorylated by cAMP-dependent protein kinase in purified preparations (Curtis & Catterall 1985) and in reconstituted Ca^{2+} channels that are regulated by cAMP-dependent protein kinase (Flockerzi et al 1986, Nunoki et al 1989). Both serine 182 and threonine 205 have been shown to be phosphorylated in vitro (De Jongh et al 1989, Ruth et al 1989). Because phosphorylation of both α_1 and β subunits is correlated with regulation of the ion conductance activity of skeletal muscle Ca^{2+} channels, both are candidates for sites of channel modulation by phosphorylation.

Role of Kinase Anchoring in Voltage-Dependent Modulation of Skeletal Muscle Ca^{2+} Channels cAMP-dependent protein kinase is often anchored to specific subcellular compartments or specific kinase substrates by A kinase anchoring proteins (AKAPs) (Gray et al 1998b, Rubin 1994, Scott & McCartney 1994). These proteins contain a targeting domain that directs the AKAP to a specific cellular site and a kinase-anchoring domain containing an amphipathic α helix that binds the regulatory subunit dimer of cAMP-dependent protein kinase. Peptides containing the amino acid sequence of the kinase-anchoring domain are effective kinase-anchoring inhibitors, which bind to PKA regulatory subunits and thereby prevent their binding to AKAPs. Because regulation of the skeletal muscle Ca^{2+} channel by membrane depolarization and cAMP-dependent protein kinase is very rapid, with observable effects in 50 ms, it was an attractive candidate for regulation through PKA bound to AKAPs. Consistent with this idea, the anchoring inhibitor peptide from a human thyroid AKAP (Ht-31) effectively inhibits rapid, voltage-dependent potentiation of skeletal muscle Ca^{2+} channel activity when perfused inside cultured skeletal muscle cells in whole-cell voltage clamp (Johnson et al 1994). Similarly, voltage-dependent potentiation of the activity of cloned skeletal muscle Ca^{2+} channels expressed in human embryonic kidney cells is prevented by the Ht-31-anchoring inhibitor peptide (Johnson et al 1997). These results clearly implicate anchoring of PKA through interaction with an AKAP in the rapid regulation of skeletal muscle Ca^{2+} channels.

Biochemical studies of skeletal muscle Ca^{2+} channels revealed a novel 15-kDa AKAP (AKAP-15) associated with purified Ca^{2+} channels and with specifically immunoprecipitated Ca^{2+} channels (Gray et al 1997). The primary structure of this AKAP reveals an 81-residue protein with N-terminal palmitoyl and myristoyl moieties that serve as membrane anchors and an amphipathic helix that binds PKA (Figure 1*A*) (Gray et al 1998a). AKAP-15, PKA, and Ca^{2+} channels are co-localized in the specialized junctions formed between sarcoplasmic reticulum and transverse tubule membranes, where excitation-contraction coupling takes

place (Burton et al 1997, Gray et al 1998a). The amphipathic helix peptide from AKAP-15 effectively inhibits regulation of skeletal muscle Ca^{2+} channels (Gray et al 1998a). As no other AKAPs are associated with these Ca^{2+} channels, it is likely that AKAP-15 mediates their regulation by PKA in vivo.

Regulation of $Ca_v1.2$ Cardiac Ca^{2+} Channels

β-adrenergic modulation of the slow inward Ca^{2+} current in cardiac muscle is the first described and most thoroughly studied example of Ca^{2+} current regulation. Voltage-clamp experiments performed on mammalian (Reuter 1967) and amphibian (Vassort et al 1969) cardiac tissue showed that β-adrenergic stimulation enhances Ca^{2+} current. This effect contributes significantly to the increase in cardiac contractility, beat rate, and amplitude of the cardiac action potential caused by β-adrenergic agonists (Reuter 1967, 1974). The potentiation of the slow inward current appears to be mediated by cAMP and consequent activation of cAMP-dependent protein kinase. Incubation of tissue with cAMP derivatives or phosphodiesterase inhibitors to prevent cyclic nucleotide degradation mimics the action of β-adrenergic agonists on cardiac tissue (Morad et al 1981, Reuter 1974, Tsien 1973, Tsien et al 1972). Direct injection of cAMP (Trautwein et al 1982) or the purified catalytic subunit of cAMP-dependent protein kinase (Osterrieder et al 1982) into isolated cardiac myocytes influences the action potential and the Ca^{2+} current in a manner identical to that of β-adrenergic stimulation.

Mechanism of Regulation of Cardiac Ca^{2+} Current by the cAMP-Dependent Protein Kinase Pathway Analysis of β-adrenergic enhancement of Ca^{2+} current under voltage-clamp conditions suggested that the increase was not caused by a change in the sensitivity of channels to voltage but rather by an increase in the maximal Ca^{2+} conductance (Reuter & Scholz 1977). Single-channel recording from cultured rat cardiac myocytes (Cachelin et al 1983, Reuter et al 1982) and from isolated frog ventricular myocytes (Bean et al 1984) showed that the single-channel conductance did not change in the presence of β-adrenergic agonists. Increases in the mean channel open time and probability of channel opening were observed in rat myocytes, whereas the number of functional channels seen within a membrane patch did not increase on treatment with β-adrenergic agonists or 8-bromo cyclic AMP in the membrane potential range studied (Cachelin et al 1983, Reuter et al 1982). In contrast, an increase in the number of functional channels recorded contributes to the enhanced frequency of channel openings and the larger response to adrenergic agents seen in frog myocytes (Bean et al 1984).

Multiple modes of $Ca_v1.2$ channel gating are observed at the single-channel level: a null mode (mode 0) in which channels do not open or open very rarely upon depolarization, a low p mode (mode 1) in which the probability of activation is low and the openings are brief, and a high p mode (mode 2) in which the

probability of opening is much higher and the openings are longer (Hess et al 1984). β-adrenergic stimulation and increases in intracellular cAMP cause a shift of the gating mode away from the null gating mode toward the low p and high p modes for depolarizing stimuli to +20 mV (Yue et al 1990). Thus the increase in inward Ca^{2+} current during the cardiac action potential induced by β-adrenergic agents probably results from a combination of two effects: an increase in the probability of activation of functional Ca^{2+} channels that are in an "activable" pool (i.e. shift from mode 1 to mode 2), and an increase in the number of functional channels by transfer from an "inactivable" pool to the activable pool (shift from mode 0 to mode 1). This results in both an increased probability of opening and an increase in the apparent number of functional Ca^{2+} channels. The definition of activable pool depends on the test potential. Although not all Ca^{2+} channels in unstimulated cells open upon depolarization in the physiological range of membrane potentials, strong positive test potentials can open these channels (Bean 1994). Thus the effect of adrenergic stimulation and cAMP-dependent protein phosphorylation is to shift Ca^{2+} channels from a null mode of gating in which they can be activated only by unphysiological stimulation at very positive membrane potentials to functional modes of gating in which their activation occurs in the physiological range.

Molecular Basis for Modulation of Cardiac Ca^{2+} Channels by PKA Although regulation of the cardiac Ca^{2+} channels by the cAMP pathway was the first example of ion channel regulation through second messenger pathways, the molecular basis for this regulation is still not clearly resolved. Cardiac Ca^{2+} channels consist of $Ca_v1.2$ α_1 subunits in association with an $\alpha_2\delta$ subunit and a β subunit. Early biochemical studies of the cardiac Ca^{2+} channel resulted in purification of a short, 160 to 195 kDa form of the α_1 subunit that was not a substrate for phosphorylation by PKA (Chang & Hosey 1988, Schneider & Hofmann 1988). As for skeletal muscle Ca^{2+} channels, more recent results on cardiac Ca^{2+} channels have revealed a full-length α_1 subunit form with a molecular mass of approximately 220 kDa, as expected from the cDNA sequence (Figure 1*B*) (De Jongh et al 1996). This full-length α_1 subunit is phosphorylated on a single serine residue (Ser 1928) in the C-terminal domain by PKA (De Jongh et al 1996). Similarly, expression of the C-terminal domain in bacteria yields a protein that is phosphorylated primarily on Ser 1928 (Mitterdorfer et al 1996). This site is missing in the truncated form of the α_1 subunit isolated in early biochemical preparations. Primarily the full-length form of $Ca_v1.2$ is present in cardiac tissue (Gao et al 1997a). Both β_1 and β_2 isoforms are expressed in cardiac tissue (Biel et al 1991, Gao et al 1997a, Hullin et al 1992) and likely are both associated in $Ca_v1.2$ channels.

Ca^{2+} channels in a CHO cell line that expresses only the full-length form of the $Ca_v1.2$ α_1 subunit can be regulated by activation of endogenous PKA, by intracellular perfusion of PKA, and by voltage-dependent potentiation, which requires the activity of PKA (Sculptoreanu et al 1993a, Yoshida et al 1992). These

results indicate that at least part of the regulation of Ca^{2+} channel activity is caused by phosphorylation of the α_1 subunit. Because the α_1 subunit is phosphorylated only on Ser 1928 in vitro or in transfected cells (De Jongh et al 1996), regulation likely involves phosphorylation of this site. In support of this conclusion, mutation of this serine residue to alanine reduces the regulation of Ca^{2+} channel activity caused by activation of PKA with 8-Br-cAMP in transiently transfected human embryonic kidney cells from a 35% increase in Ca^{2+} channel current to a 7% increase (Gao et al 1997b). Although these results are promising, the extent of regulation of Ca^{2+} channel activity in these experiments in transfected cells falls well short of the magnitude recorded in native cardiac cells (two- to fourfold increase), and regulation of cloned Ca^{2+} channels analyzed in this way is not observed in some of the major laboratories in the field (e.g. Zong et al 1995). Therefore, it seems likely that additional regulatory influences not yet reproduced and characterized in transfected cells are important for control of the activity of cardiac Ca^{2+} channels in vivo. Two prime candidates are AKAPs and the Ca^{2+} channel β subunits.

Role of AKAPs in Regulation of Cardiac Ca^{2+} Channels As for skeletal muscle Ca^{2+} channels, emerging evidence indicates that AKAPs are important for regulation of cardiac Ca^{2+} channels through the PKA pathway. Reconstitution of PKA regulation of $Ca_v1.2$ channels in transfected cells is enhanced by co-expression of AKAP-79 (Gao et al 1997b), an AKAP expressed primarily in neurons. Similarly, AKAP-15 (also designated AKAP-18) (Fraser et al 1998), the same membrane-targeted AKAP identified in studies of skeletal muscle Ca^{2+} channels (Gray et al 1997, 1998a), also supports a low level of regulation of cardiac Ca^{2+} channels in transfected cells (18% increase in Ca^{2+} channel current with activators of PKA) (Fraser et al 1998). As AKAP-15/18 is expressed in the heart (Fraser et al 1998, Gray et al 1998a), it is likely to be the primary AKAP involved in PKA regulation of $Ca_v1.2$ Ca^{2+} channels in cardiac myocytes.

Phosphorylation of the β Subunit of Cardiac Ca^{2+} Channels by PKA The β subunits of skeletal muscle Ca^{2+} channels were found to be phosphorylated by PKA in the initial studies of purified and reconstituted Ca^{2+} channels (Curtis & Catterall 1985). The β subunits of cardiac Ca^{2+} channels are phosphorylated by PKA in intact hearts treated with β-adrenergic agonists (Haase et al 1996). Recently, Bunemann et al (1999) reported substantial (greater than twofold) regulation of a C-terminal truncated form of $Ca_v1.2$ lacking Ser1928 by intracellular perfusion of activated PKA in human embryonic kidney cells co-transfected with $Ca_v1.2$ and β_{2a} subunits. This regulation required phosphorylation of Ser 478 and/or Ser 479, two serine residues in non-classical PKA phosphorylation sites that are specific to the β_{2a} subunit. These results provide the best evidence to date for an important role of the β subunits in regulation of cardiac Ca^{2+} channels. It will be important to further analyze Ca^{2+} channel regulation when full-length α_1 subunits are co-expressed with β_{2a} and other subunits expressed in cardiac myocytes and

to eventually restore regulation by physiological stimulus procedures rather than intracellular perfusion of activated PKA.

Regulation of Cardiac Ca^{2+} Channels by Protein Kinase C Activation of protein kinase C in cardiac myocytes has biphasic effects on the Ca^{2+} current and on contractility, usually recorded as a transient increase followed by a sustained decrease (Kwan & Qui 1997, Lacerda et al 1988, Satoh 1992, Tseng & Boyden 1991, Woo & Lee 1999). Multiple isoforms of the $Ca_v1.2$ α_1 subunit have been identified by cDNA cloning (Mikami et al 1989) and by genomic sequencing (Biel et al 1991, Diebold et al 1992, Snutch et al 1991, Soldatov 1994). These isoforms differ in the N-terminal domain, the C-terminal domain and in transmembrane segments IS3, IIS6, and IVS3. Expression of $Ca_v1.2$ cDNAs cloned from brain yields L-type Ca^{2+} currents that are not modulated by protein kinase C (Stea et al 1995). In contrast, cardiac Ca^{2+} channels expressed in *Xenopus* oocytes or human embryonic kidney cells from $Ca_v1.2$ cDNA cloned from heart are inhibited by activation of protein kinase C with phorbol esters or oleylacetylglycerol (Bourinet et al 1992). Inspection of the amino acid sequences of the cDNAs encoding $Ca_v1.2$ cloned from heart (Mikami et al 1989) and brain (Snutch et al 1991) reveals a difference in the N-terminal domain: The cardiac isoform has two threonine residues in protein kinase C consensus sequences at positions 27 and 31, whereas the brain isoform does not. Mutation of either of these threonine residues to alanine prevents regulation of the expressed cardiac Ca^{2+} channels by protein kinase C (McHugh et al 2000), indicating that both residues must be phosphorylated to inhibit Ca^{2+} channel activity (Figure 1*B*). Thus the N-terminal domain is a target for tissue-specific regulation of $Ca_v1.2$, resulting in inhibition of the cardiac isoform but not the brain isoform of this Ca^{2+} channel.

REGULATION OF THE Ca_v2 FAMILY OF Ca^{2+} CHANNELS BY G PROTEINS

The Ca_v2 family of Ca^{2+} channel α_1 subunits includes $Ca_v2.1$, which mediates P/Q-type Ca^{2+} currents; $Ca_v2.2$, which mediates N-type Ca^{2+} currents; and $Ca_v2.3$, which mediates R-type Ca^{2+} currents (Table 1). These Ca^{2+} current types are recorded in neurons and neuroendocrine cells and are distinguished by their sensitivity to polypeptide neurotoxins from snails and spiders. They are primarily responsible for Ca^{2+} entry that initiates release of fast neurotransmitters at synapses, and they participate with type Ca_v1 channels in mediating secretion of hormones and neuropeptides. N-type and P/Q-type Ca^{2+} currents are regulated through multiple G protein-coupled pathways (Hille 1994, Ikeda & Dunlap 1999, Jones & Elmslie 1997). For example, in the well-studied rat sympathetic ganglion neuron, five different pathways regulate the N-type Ca^{2+} currents in subtly different ways (Hille 1994). Thus regulation of these Ca^{2+} channels through G protein-coupled pathways is important in control of synaptic transmission and hormone secretion.

Mechanism of G Protein Modulation of Ca^{2+} Currents

Although several G protein signaling pathways regulate these channels, one common pathway, best-studied at both cellular and molecular levels, is voltage dependent and membrane delimited–that is, a pathway without soluble intracellular messengers whose effects can be reversed by strong depolarization (Dolphin 1995, Hille 1994, Wickman & Clapham 1995). Inhibition of Ca^{2+} channel activity is typically caused by a positive shift in the voltage dependence and a slowing of channel activation. These effects are relieved by strong depolarization resulting in facilitation of Ca^{2+} currents (Bean 1989b, Marchetti et al 1986). Synaptic transmission is inhibited by neurotransmitters through this mechanism. The effect of G proteins has been successfully modeled as a shift between two channel states with different gating properties, reluctant and willing (Bean 1989b). The willing state is characterized by a rapid activation during depolarization to voltages within the physiological range, whereas the reluctant state is characterized by slow activation at more positive voltages, usually beyond the normal range of physiological membrane potentials. Activation of G proteins shifts the channel to the reluctant state and strong depolarization reverses that shift in channel state.

G protein α subunits are thought to confer specificity in receptor coupling (Dolphin 1995, Hille 1994, Heschler & Schultz 1993, Wickman & Clapham 1995), but until recently it was not known whether the Gα or G$\beta\gamma$ subunits were responsible for modulation of Ca^{2+} channels. Surprisingly, transfection of G$\beta\gamma$ into cells expressing P/Q-type Ca^{2+} channels induces modulation like that caused by activation of G protein-coupled receptors, but Gα subunits do not (Herlitze et al 1996) (Figure 2). Co-transfection of tsA-201 cells with the Ca^{2+} channel α_1 and β subunits and G$\beta\gamma$ causes a shift in the voltage dependence of Ca^{2+} channel activation to more positive membrane potentials and reduces the steepness of voltage-dependent activation, effects that closely mimic the actions of neurotransmitters and guanyl nucleotides on N-type and P/Q-type Ca^{2+} currents in neurons and neuroendocrine cells. In contrast, transfection with a range of Gα subunits does not have this effect. This voltage shift can be reversed by strong positive prepulses that result in voltage-dependent facilitation of the Ca^{2+} current in the presence of G$\beta\gamma$, again closely mimicking the effects of neurotransmitters and guanyl nucleotides on Ca^{2+} channels. Similarly, injection or expression of G$\beta\gamma$ subunits in sympathetic ganglion neurons induces facilitation and occludes modulation of N-type Ca^{2+} currents by norepinephrine, but Gα subunits do not (Herlitze et al 1996, Ikeda 1996). In both cases, the Gγ subunit is ineffective by itself, but over-expression of exogenous Gβ subunits is sufficient to cause channel modulation. Moreover, the Gβ subunits appear to control the specificity of modulation in sympathetic ganglion neurons, i.e. β_1 and β_2 are more effective than β_3, β_4, or β_5 (Garcia et al 1998). These results surprisingly point to the G$\beta\gamma$ subunits as the primary regulators of presynaptic Ca^{2+} channels in both transfected cells and sympathetic ganglion neurons (Figure 2).

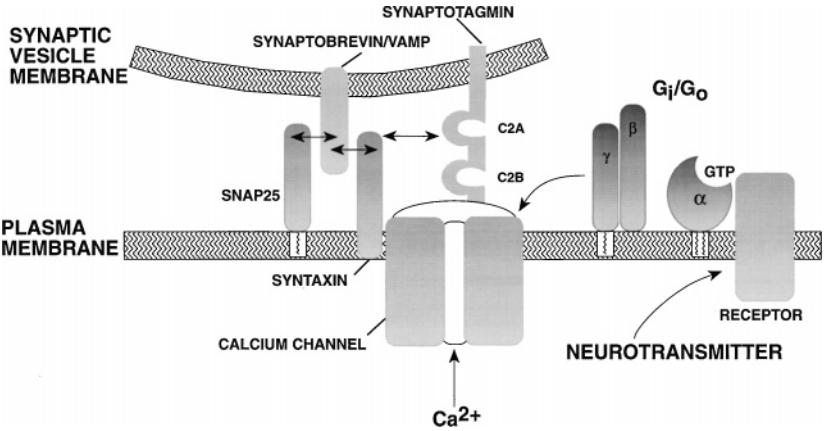

Figure 2 Pathways of regulation of Ca_v2 channels. Interactions of Ca_v2. channels with SNARE proteins, G proteins, and Ca^{2+} all regulate Ca^{2+} channel activity.

Although G protein $\beta\gamma$ subunits may be the primary effectors of Ca^{2+} channel regulation, evidence from isolated nerve terminals suggests that other proteins may be important (Stanley & Mirotznik 1997). Botulinum toxin cleavage of the SNARE protein syntaxin, which interacts with presynaptic Ca^{2+} channels (see below), prevents inhibition of N-type Ca^{2+} currents in the calyx terminals of the chick ciliary ganglion. Thus effective inhibition by G proteins may require additional protein-protein interactions.

Kinetic Basis for G Protein Modulation

The hallmarks of the voltage-dependent inhibition of N-type Ca^{2+} currents by activation of G protein-coupled receptors are slowed rate of activation during a depolarizing test pulse, positively shifted voltage dependence of activation, and less steep voltage dependence of activation. Analyses of the mechanism of regulation by single-channel recording and gating current measurements have given new insight into the underlying kinetic and biophysical mechanisms. As expected from the slowed kinetics of activation of the Ca^{2+} current, there is a prolonged first latency to single-channel opening after G protein activation (Patil et al 1996). In addition, direct measurements of gating currents caused by movement of the voltage sensors of the Ca^{2+} channel show that they are slowed as well (Jones et al 1997). With more positive test pulses, the rate of activation of the channels increases, the first latency to channel opening shortens, and the gating currents are accelerated. Thus the effect of the G protein is to impede the outward gating movement of the S4 voltage sensors of the Ca^{2+} channel, and this effect can be overcome by prolonged depolarization to more positive membrane potential to force voltage sensor movement and subsequent channel activation.

Site(s) of G Protein Modulation

The effects of G protein $\beta\gamma$ subunits might be mediated by binding to one or more sites on the Ca^{2+} channel. This question can be tested functionally by examining the concentration dependence of the rate of G protein action. After the inhibition of N-type Ca^{2+} currents by G protein activation has been reversed by strong depolarizing pulses, re-inhibition is dependent on the concentration of activated G protein and therefore requires re-binding of activated G protein (Lopez & Brown 1991). The concentration dependence of the rate of re-inhibition is consistent with binding of a single $G\beta\gamma$ subunit to re-form an effective complex and re-inhibit channel activation (Zamponi & Snutch 1998). Thus the diverse effects of G protein $\beta\gamma$ subunits on the kinetics and voltage dependence of Ca^{2+} channel activation may be caused by binding to a single site.

Possible sites of G protein $\beta\gamma$ subunit interaction with Ca^{2+} channels have been extensively investigated by construction and analysis of channel chimeras, by G protein–binding experiments, and by site-directed mutagenesis and expression (De Waard et al 1997, Herlitze et al 1997, Page et al 1997, Zamponi et al 1997). Initially, most evidence pointed to the intracellular loop between domains I and II ($L_{I\text{-}II}$) as a crucial site of G protein regulation (Figure 3). G protein-binding and site-directed mutagenesis experiments identified a complex site in $L_{I\text{-}II}$. Peptides from

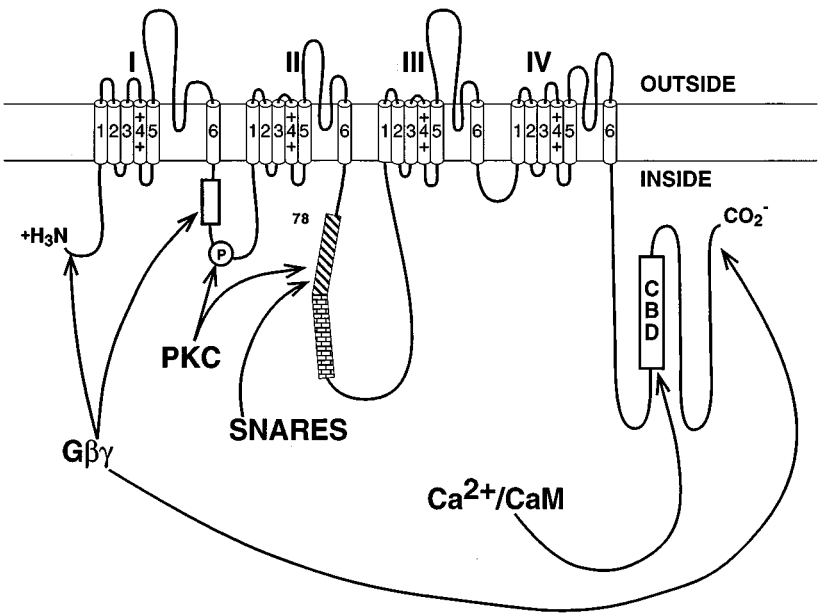

Figure 3 Pathways and sites of regulation of Ca_v2 channels. Transmembrane-folding model of the Ca_v2 channel α_1 subunits. Sites of interaction of regulatory pathways are illustrated.

this region of $Ca_v2.2$ prevent inhibition of channel activity by $G\beta\gamma$, presumably by binding to $G\beta\gamma$ and competitively inhibiting its access to Ca^{2+} channels (Herlitze et al 1997, Zamponi et al 1997). This region of the channel binds $G\beta\gamma$ in vitro (De Waard et al 1997, Zamponi et al 1997) as well as in vivo in the yeast two-hybrid assay (Garcia et al 1998). Site-directed mutations in this channel segment can enhance or reduce the extent of inhibition by $G\beta\gamma$. The specificity of binding of this segment of the Ca^{2+} channel to different G protein β subunits follows the specificity of G protein regulation of N-type Ca^{2+} currents in sympathetic ganglion neurons (Garcia et al 1998). Thus, $L_{I\text{-}II}$ is an important point of interaction of $G\beta\gamma$ with Ca^{2+} channels, and this interaction may determine G protein specificity for modulation.

Increasing evidence also points to segments in the N-terminal and C-terminal domains of Ca^{2+} channels that are also required for G protein regulation (Cantí et al 1999; Furukawa 1998a,b; Page et al 1998; Qin et al 1997; Simen & Miller 1998; Zhang et al 1996). $Ca_v2.2$ channels are strongly regulated by G proteins, whereas the closely related $Ca_v2.1$ and $Ca_v2.3$ channels are less strongly regulated. Formation of chimeric Ca^{2+} channels containing different segments of these channels has been used to define the molecular determinants of these differences in G protein regulation. Transfer of domain I or $L_{I\text{-}II}$ of $Ca_v2.2$ enhanced G protein regulation of $Ca_v2.3$ (Page et al 1997). Transfer of domain I and the C-terminal domain of $Ca_v2.2$ was required to confer full G protein regulation on $Ca_v2.1$ (Zhang et al 1996). Moreover, G protein-binding segments located at the N and C termini are also implicated in $G\beta\gamma$ regulation of $Ca_v2.3$ by mutagenesis and G protein-binding experiments (Cantí et al 1999; Furukawa 1998a,b; Page et al 1998; Qin et al 1997; Simen & Miller 1998). Considered together, the results of experiments on localization of the site(s) of G protein regulation of the Ca_v2 family of Ca^{2+} channels suggest that multiple regions of the Ca^{2+} channel are required for responsiveness. It seems clear that $L_{I\text{-}II}$ is an important site of $G\beta\gamma$ binding. As the N-terminal and C-terminal domains are likely to interact with each other in the folded channel protein, a second site of interaction for G proteins may be formed at their intersection. Other regions of the membrane-associated domains of the channel may be needed to transduce G protein-binding into a change in channel gating. Certainly, more work is needed to give a clear picture of the molecular events that lead to G protein regulation of Ca^{2+} channels.

Reversal of G Protein Modulation by Protein Kinase C

The inhibition of N-type and P/Q-type Ca^{2+} currents in dissociated neurons through binding of $G\beta\gamma$ subunits can be reversed by several neurotransmitters acting through protein kinase C (Swartz 1993, Swartz et al 1993). This reversal of G protein inhibition can be reconstituted by expression of Ca^{2+} channels in *Xenopus* oocytes (Zamponi et al 1997). The mechanism of reversal involves phosphorylation of sites in $L_{I\text{-}II}$, just downstream of a principal site of interaction of $\beta\gamma$ subunits with the Ca^{2+} channel (Figure 2) (Zamponi et al 1997). Thus this intracellular loop of the Ca^{2+} channel integrates G protein, protein kinase C, and voltage signals.

FEEDBACK REGULATION OF Ca^{2+} CHANNELS BY CALCIUM

Calcium-Dependent Inactivation and Facilitation of Cardiac Ca^{2+} Channels

The L-type Ca^{2+} currents conducted by $Ca_v1.2$ channels in the heart are strongly regulated by Ca^{2+} entering the cell through the channels. Accumulation of entering Ca^{2+} inactivates the Ca^{2+} current on the time scale of milliseconds, causing a decay of the current to baseline during long depolarizing stimuli (Lee et al 1985, Nilius & Benndorf 1986). Ca^{2+}-dependent inactivation is more rapid than voltage-dependent inactivation for these channels, and therefore it is a primary determinant of the duration of the Ca^{2+} current. Ca^{2+}-dependent inactivation is observed for cardiac Ca^{2+} channels fused into planar bilayers in the absence of ATP and cellular enzymes, arguing that it is caused by Ca^{2+} binding to the Ca^{2+} channel itself or to an associated protein (Haack & Rosenberg 1994). Recent experiments show that this Ca^{2+}-dependent inactivation of the Ca^{2+} current results from binding of Ca^{2+} and calmodulin to the C-terminal domain of $Ca_v1.2$ channels. Alternately spliced exons in the C-terminal domain confer striking differences in Ca^{2+}-dependent inactivation (Soldatov et al 1997, 1998; Zühlke & Reuter 1998). The exon that allows rapid inactivation includes an IQ domain, a well-known calmodulin-binding motif (Zühlke & Reuter 1998). Calmodulin binds to this C-terminal IQ domain. Both mutations that prevent calmodulin binding and calmodulin mutants that cannot bind Ca^{2+} also prevent Ca^{2+}-dependent inactivation (Peterson et al 1999, Qin et al 1999, Zühlke et al 1999). These experiments clearly show that binding of Ca^{2+}/calmodulin complexes to a site in the C-terminal domain of Ca^{2+} channels causes rapid inactivation. Calmodulin is likely to be constitutively bound to this site, ready to bind Ca^{2+} and initiate a conformational change that inactivates the $Ca_v1.2$ channel in the heart.

$Ca_v1.2$ channels in cardiac and smooth muscle cells are also facilitated by calcium entering through them (Gurney et al 1989, McCarron et al 1992). This effect is thought to be caused by a combination of phosphorylation by Ca^{2+}/calmodulin-regulated protein kinase II (CamKII) (Anderson et al 1994, McCarron et al 1992, Xiao et al 1994, Yuan & Bers 1994) and binding of Ca^{2+}/calmodulin (Zühlke et al 1999). Recent studies show that constitutively activated CamKII can cause facilitation by shifting single Ca^{2+} channels to the high p gating mode (Dzhura et al 2000). These results demonstrate that protein phosphorylation activated by calcium/calmodulin can cause facilitation.

Facilitation and Enhanced Inactivation of $Ca_v2.1$ Channels by Binding of Ca^{2+}/Calmodulin

Ca^{2+} channels in presynaptic nerve terminals are also subject to Ca^{2+}-dependent facilitation and inactivation (Borst & Sakmann 1998, Cuttle et al 1998) (Figure 2). As for cardiac Ca^{2+} channels, P/Q-type Ca^{2+} channels are also regulated by direct

binding of Ca^{2+} and calmodulin (Lee et al 1999). Ca^{2+}-dependent binding of Ca^{2+}/calmodulin to a novel site in the C-terminal domain of the $Ca_v2.1$ α_1 subunit (Figure 3) increases the rate and extent of voltage-dependent inactivation, enhances recovery from inactivation, and causes a Ca^{2+}-dependent facilitation of the Ca^{2+} current up to 200% of control values (Lee et al 1999). The effects of Ca^{2+}/calmodulin binding are greater for $Ca_v2.1$ channels containing β_{2a} subunits, which reduce voltage-dependent inactivation and thereby unmask larger effects of Ca^{2+}-dependent inactivation (Lee et al 2000). During trains of repetitive action-potential-like stimuli, Ca^{2+} entry first causes facilitation and then Ca^{2+}-dependent inactivation (Lee et al 2000). This form of channel regulation observed in transfected cells is likely to be responsible for Ca^{2+}-dependent facilitation of P/Q-type Ca^{2+} currents measured directly in the nerve terminals of the large calyx synapses in the medial nucleus of the trapezoid body in the brain stem (Borst & Sakmann 1998, Cuttle et al 1998).

Ca^{2+} CHANNEL REGULATION BY INTERACTION WITH INTRACELLULAR EFFECTOR PROTEINS

Ca^{2+} entering cells through voltage-gated Ca^{2+} channels initiates many intracellular processes through activation of effector mechanisms. Often these effector mechanisms are highly localized to respond to high local concentrations of Ca^{2+} at the intracellular mouth of the channel. Recent work shows that in two cases the effector proteins themselves are feedback regulators of Ca^{2+} channel function.

Regulation by Ryanodine-Sensitive Ca^{2+}-Release Channels in Skeletal Muscle

In skeletal muscle fibers, excitation-contraction coupling involves direct conformational coupling of the voltage-gated Ca^{2+}channel in the transverse tubule membrane to the ryanodine-sensitive Ca^{2+} release channel (RyR) of the sarcoplasmic reticulum at the transverse tubule/sarcoplasmic reticulum junction (Adams & Beam 1990, Catterall 1991, Rios & Pizarro 1991). Conformational coupling is mediated by the intracellular loop connecting domains II and III of the α_1 subunit (Tanabe et al 1988, 1990). Depolarization of the transverse tubules by the conducted action potential activates $Ca_v1.1$ channels. They rapidly activate the ryanodine-sensitive Ca^{2+} release channel via protein-protein interactions to initiate Ca^{2+} release and muscle contraction, and they slowly activate their own Ca^{2+} conductance activity (Almers & Palade 1981, Sanchez & Stefani 1978), which serves to maintain Ca^{2+} homeostasis by mediating Ca^{2+} entry into the cytoplasm. Remarkably, recent experiments with mice lacking the RyR show that there is a retrograde regulation of the transverse tubule Ca^{2+} channel by the ryanodine-sensitive calcium release channel (Nakai et al 1996). The RyR-deficient mice have greatly reduced Ca^{2+} currents in their skeletal muscle cells, even though nearly

normal levels of voltage-gated Ca^{2+} channels are present based on gating current measurements (Nakai et al 1996). This effect is mediated by the interaction of the intracellular loop connecting domains II and III of the $Ca_v1.1$ α_1 subunit with the RyR, the same region of the α_1 subunit that is required for excitation-contraction coupling between these two proteins (Grabner et al 1999). Thus it appears that the RyR must be present and bind to $L_{II\text{-}III}$ for the transverse tubule $Ca_v1.1$ channel to have its normal level of functional activity.

Regulation by SNARE Proteins

Ca^{2+} entry through voltage-gated Ca^{2+} channels is thought to initiate exocytosis by triggering the fusion of secretory vesicle membranes with the plasma membrane through actions on the SNARE protein complex of syntaxin, SNAP-25, and VAMP/synaptobrevin (reviewed in Bajjalieh & Scheller 1995, Südhof 1995). The function of the SNARE protein complex is regulated by interactions with numerous proteins, including the synaptic vesicle Ca^{2+}-binding protein synaptotagmin. Presynaptic $Ca_v2.1$ and $Ca_v2.2$ channels interact directly with the SNARE proteins and synaptotagmin through a specific synaptic protein interaction (synprint) site in the large intracellular loop connecting domains II and III (Figures 2, 3) (Sheng et al 1994). This interaction is regulated by Ca^{2+} (Sheng et al 1996). Synaptotagmin also binds to the synprint site of Ca_v2 channels (Charvin et al 1997, Sheng et al 1997, Wiser et al 1997). Injection of peptide inhibitors of this interaction into presynaptic neurons inhibits synaptic transmission, consistent with the conclusion that this interaction is required to position docked synaptic vesicles near Ca^{2+} channels for effective fast exocytosis (Mochida et al 1996; Rettig et al 1996, 1997). These results define a second functional activity of the presynaptic Ca^{2+} channel—targeting docked synaptic vesicles to a source of Ca^{2+} for effective transmitter release.

In addition to this functional role of interaction between Ca^{2+} channels and SNARE proteins in the anterograde process of synaptic transmission, these interactions also have retrograde regulatory effects on Ca^{2+} channel function. In *Xenopus* oocytes, co-expression of syntaxin with $Ca_v2.1$ or $Ca_v2.2$ channels reduces the level of channel expression and inhibits Ca^{2+} channel activity by shifting the voltage dependence of steady-state inactivation during long depolarizing prepulses toward more negative membrane potentials (Bezprozvanny et al 1995, Wiser et al 1996). The effects on reduction of peak Ca^{2+} currents may involve changes in synthesis of Ca^{2+} channels or transit to the cell surface when syntaxin is over-expressed. The inhibitory effects of syntaxin are relieved by co-expression of synaptotagmin (Tobi et al 1999, Wiser et al 1997).

The interaction of $Ca_v2.2$ channels with SNARE proteins is also regulated by protein phosphorylation. Both protein kinase C and Ca^{2+}/calmodulin protein kinase II phosphorylate two to three sites in the synprint region of $L_{II\text{-}III}$, and phosphorylation by these kinases inhibits interactions with SNARE proteins (Yokoyama et al 1997). Thus neurons can regulate interactions between

presynaptic Ca^{2+}channels and SNARE proteins through protein kinase C second messenger pathways that are activated by neurotransmitters, just as G protein interactions are regulated by the protein kinase C pathway.

The rbA isoform of $Ca_v2.1$ channels (Sakurai et al 1996, Starr et al 1991) binds SNAP-25 but does not bind syntaxin in vitro (Kim & Catterall 1997, Rettig et al 1996). However, this isoform is still subject to feedback regulation through the SNARE protein SNAP-25 (Zhong et al 1999). Co-expression of SNAP-25 alone with these Ca^{2+} channels in a mammalian cell line causes a negative shift in the voltage dependence of inactivation. However, co-expression of synaptotagmin to mimic the effect of docking a synaptic vesicle nearby relieves the inhibition of channel activity by shifting the voltage dependence of inactivation back to its original position on the voltage axis (Zhong et al 1999). This regulatory mechanism would have the effect of focusing the Ca^{2+} entry on those Ca^{2+} channels having a nearby docked synaptic vesicle and therefore poised for effective exocytosis.

Although the initial experiments on SNARE protein regulation of Ca^{2+} channels only detected regulation of the Ca_v2 channel family (Bezprozvanny et al 1995), subsequent work has indicated that L-type Ca^{2+} currents may be similarly regulated (Wiser et al 1996, 1999; Yang et al 1999). These interactions may be important in secretion of hormones and neuropeptides, which is initiated primarily by L-type Ca^{2+} currents. Ca^{2+} currents mediated by $Ca_v1.2$ and $Ca_v1.3$ channels are inhibited by co-expression of syntaxin, and inhibition of this interaction with synthetic peptides in the pancreatic β cell reduces insulin secretion. Thus it may be a general finding that effectors of Ca^{2+}-dependent secretion mediate feedback inhibition of the Ca^{2+}channel that initiates the exocytosis process.

CONCLUSION

Ca^{2+} channels are the signal transducers that convert electrical signals in the cell membrane into an increase in the intracellular second messenger Ca^{2+} and thereby activate many crucial intracellular processes including contraction, secretion, neurotransmission and regulation of enzymatic activities and gene expression. These channels are complex proteins containing five distinct subunits, each of which is encoded by four to ten separate genes. As might be expected from their central role in signal transduction, Ca^{2+} channels are tightly regulated by a range of signal transduction pathways in addition to regulation by their intrinsic, voltage-dependent gating processes. In this respect, Ca^{2+} channels that have been extensively studied fall into two groups: Ca_v1 channels that conduct L-type Ca^{2+} currents and are primarily regulated by protein phosphorylation and Ca_v2 channels that conduct N-, P/Q-, and R-type Ca^{2+} currents and are primarily regulated by G proteins. The molecular mechanisms underlying these regulatory processes are becoming more completely understood, as outlined in this review, and one can anticipate that this new knowledge will provide new insights into the regulation of many essential cellular functions.

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