7 Calcium Channels

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7.1 Introduction

Ion channels underlie the electrical activity of cells. Calcium channels have a unique functional role, because not only do they participate in this activity, they form the means by which electrical signals are converted to responses within the cell. Calcium concentrations in the cytoplasm of cells are maintained at a low level, and calcium channels activate quickly such that the opening of ion channels can rapidly change the cytoplasmic environment. Once inside the cell, calcium acts as a "second messenger" prompting responses by binding to a variety of calcium sensitive proteins. Calcium channels are known to play an important role in stimulating muscle contraction, in neurotransmitter secretion, gene regulation, activating other ion channels, controlling the shape and duration of action potentials and many other processes. Since calcium plays an integral role in cell function, and since excessive quantities can be toxic, its movement is tightly regulated and controlled through a large variety of mechanisms.

The importance of calcium channels is highlighted by the fact many naturally occurring mutations in voltage-gated calcium channel proteins are known to underlie human disorders including childhood absence epilepsy, familial hemiplegic migraine, spinocerebellar ataxia type 6 (a severe movement disorder), hypokalemic periodic paralysis and X-linked congenital stationary night blindness (French and Zamponi, 2005). Mutations in mice have also been shown to cause a variety of disorders including seizures, atrophy, and lethargy. Knockout mice, deficient in various calcium channel pore-forming subunits (see below) are often not viable as calcium channels play an integral role in muscle function. The knockout mice exhibit abnormalities in cardiac muscle contraction, paralysis of the diaphragm, deafness, and epilepsy. Mutations of Ca^{2+} release channel genes are known to be involved in cardiac arrhythmias and malignant hypothermia (Priori and Napolitano, 2005).

The history of calcium channels dates back to soon after the discovery by Hodgkin and Huxley that Na^+ and K^+ currents were responsible for the action potential in squid giant axon. Fatt and Katz (1953) identified a form of electrical excitability in crab muscle that occurred in the absence of Na^+. In 1958, this phenomenon was correctly explained to be due to a "calcium spike," an influx of calcium during the upstroke of the action potential (Fatt and Ginsborg, 1958). Calcium spikes were also measured using radiolabeled calcium in squid axon (Hodgkin and Keynes, 1957) and in response to lowering intracellular calcium concentrations (Hagiwara

and Naka, 1964). The fact that Ca^{2+} passed through pathways other than Na^+ and K^+ channels was confirmed with the use of Na^+ and K^+ channel blockers. Ca^{2+} currents are generally difficult to measure, as calcium channels are much less densely distributed than Na^+ or K^+ channels. Final verification of the hypothesis that Ca^{2+} passed through independent channels, and detailed investigations to their physiology had to wait until the development of the patch clamp and giga-seal techniques that allowed for recordings to be made from small cells and for single-channel currents to be recorded. Over the past four decades inward Ca^{2+} currents have been observed in every excitable cell, a huge variety of calcium channels have been identified and their essential biological roles are beginning to be well recognized and characterized.

In this chapter we outline the variety of different calcium channels that are utilized in biological organisms, highlighting their differences, nomenclature and function, and recent research. Next we give a more detailed discussion of the role of calcium in stimulating muscle contraction, neurotransmitter secretion and controlling electrical excitability. We then discuss a variety of properties of these channels examining such questions as how calcium channels discriminate between different ion types and how ions migrate through them. Included in this discussion is an examination of recent theoretical investigations into the nature of ion permeation and selectivity. Finally, we discuss the mechanisms by which ion flow is controlled, reviewing our knowledge of channel gating voltage- and calcium-dependent channel inactivation and the regulation of calcium channels by mechanisms such as thiol group oxidation or reduction.

It is the nature of a topic as large as this that we cannot hope to cover all the areas of current investigation. Instead we have highlighted some areas and focused our attention particularly on the family of voltage-gated calcium channels. Calcium-release, mitochondrial or nuclear channels are no less interesting and significant understanding of their structure and function at both a physiological and molecular level has been determined in recent years. We hope that these may form the focus of another review chapter in the near future. The reader is referred to a number of recent reviews for further information into voltage-gated (Jones, 1998, 2003; Catterall, 2000; Sather and McCleskey, 2003) calcium release (Sutko and Airey, 1996; George et al., 2005; Wehrens et al., 2005) and store-operated channels (Parekh and Putney, 2005).

7.2 Types of Ca^{2+} Channels

Calcium channels come in a range of different shapes and sizes, are located in different regions of the cell or different organs, carry out diverse roles and respond to different stimuli. Broadly, calcium channels are distinguished as either voltage-activated or responding to the binding of calcium or other agonists that release calcium from intracellular stores. Here we introduce the different types of Ca^{2+} channels, noting the names applied to them and the functions they fulfil.

7.2.1 Voltage-Gated Channels

Many cell types, notably excitable cells express voltage-gated Ca^{2+} channels that play an integral role in calcium influx and regulate intracellular processes such as contraction, secretion, neurotransmission, and gene expression discussed in more detail below. They are members of a gene superfamily of transmembrane ion channel proteins that includes voltage-gated K^+ and Na^+ channels. Ca^{2+} channels share structural similarities with K^+ and Na^+ channels in that they possess a pore-forming α_1 subunit in four repeats of a domain with six transmembrane-spanning segments that include the voltage sensing S4 segment and the pore forming (P) region. The α_1 subunit is large (190–250 kDa) and incorporates the majority of the known sites regulated by second messengers, toxins, and drugs. This subunit is usually complexed with at least three auxiliary subunits, α_2, δ, β and γ, with the α_2 and δ subunits always linked by a disulfide bond (Fig. 7.1).

Nomenclature for the Ca^{2+} channels has evolved over the decades since the early electrophysiology studies and has had to incorporate diverse biochemical, pharmacological, and physiological properties of the channels with the more recent identification of channel genes. Initially, Ca^{2+} channels were classified according to their ability to be activated by a large or small depolarization and whether the response was rapid or slow and persistent. For example, L-type Ca^{2+} channels are a member of the HVA or high voltage activation type because the channels are activated by strong depolarizations typically to 0 or +10 mV and are long-lasting in that they are slow to inactivate. In addition, they are blocked by the lipid-soluble 1,4-dihydropyridines such as nifedipine and BAY K 8644. T-type Ca^{2+} channels on the other hand are transiently activated at low membrane potentials and are insensitive

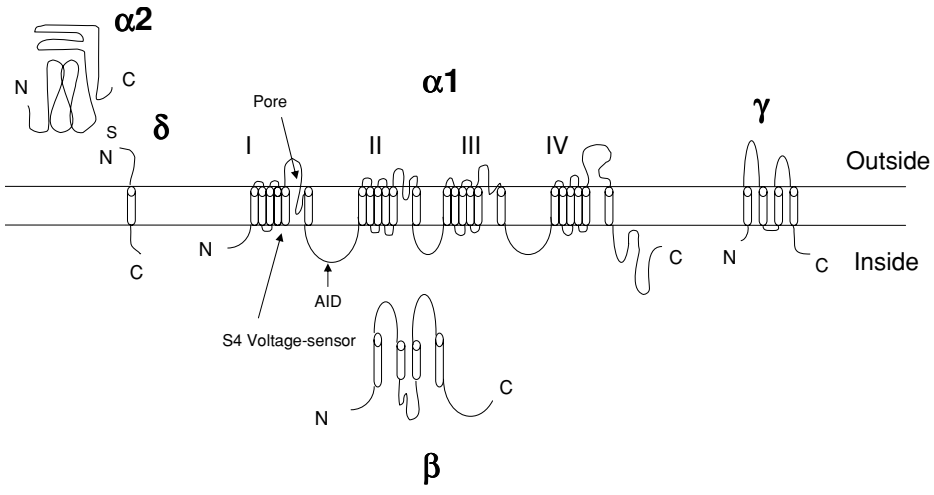

Fig. 7.1 Subunits of Ca_v channels showing the four homologous domains of the α_1 subunit, and auxiliary β, γ, and $\alpha_2\delta$ subunits. Predicted alpha-helices are depicted as cylinders. AID = alpha interacting domain where the β subunit is proposed to interact with the α subunit (see text).

to dihydropyridines and fall into the class of low voltage activated (LVA) channels. Taking into consideration the functional and pharmacological differences, Tsien and colleagues (Tsien et al., 1988) devised a nomenclature based on single letters that continues to be used today to describe the electrophysiological characteristics of expressed channels studied in their native state. In addition to the L-type (long-lasting currents) and T-type (transient currents) channels mentioned above, a number of other channel types were found with single-channel conductances between T- and L-type that were resistant to the dihydropyridines. These channels were predominant in neurons and could be blocked by the ω-Conotoxin GVIA from cone snails. They were named N-type (Nowycky et al., 1985b). A unique Ca^{2+} channel was identified in Purkinje cells by Linás' group that exhibited sensitivity to the venom of funnel web spider (FTX) but was insensitive to ω-Conotoxins and dihydropyridines (Llinas et al., 1989). The channel was named P-type. Another type of channel slightly less sensitive to ω-Conotoxins, termed Q-type, was identified in cerebellar granule neurons that otherwise has very similar physiological characteristics to P-type channels (Randall and Tsien, 1995). An R-type Ca^{2+} channel that is resistant to ω-Conotoxin, ω-Agatoxin IVA, and dihydropyridines but sensitive to ω-Agatoxin IIIA was characterized as a rapid transient current with an activation potential slightly positive to T-type channels (Randall and Tsien, 1997).

This nomenclature required revision as the molecular identities of calcium channel genes were rapidly discovered. Channel types were classified according to distinct hybridization patterns of mRNA on Northern blot analysis and grouped as A, B, C, or D (Snutch et al., 1990). Genes subsequently identified were classified E through I except the skeletal muscle isoform that was referred to as α_{1S} (Birnbaumer et al., 1994). In 2000, Ertel et al. (Ertel et al., 2000) suggested a nomenclature based on that used to classify K^+ channels with Ca as the permeating ion followed by the physiological regulator "v" for voltage in subscript and the gene subfamily in order of discovery. The Ca_v1 subfamily comprises the L-type Ca^{2+} currents, Ca_v2 subfamily comprises the P/Q, N- and R-type Ca^{2+} currents, and Ca_v3 subfamily the T-type Ca^{2+} currents as shown in Fig. 7.2. Detailed listings of voltage-dependent Ca^{2+} channel nomenclature as approved by the Nomenclature Committee of the International Union of Pharmacology including structure–function relationships are available (Catterall et al., 2003, 2005). Table 7.1 lists the nomenclature of Ca^{2+} channels as the classifications have evolved.

Although the main characteristics that define channel subtypes such as ion selectivity, voltage-dependence, and drug binding sites reside in the alpha subunit, the auxiliary subunits also play important regulatory roles. The first β subunit to be identified (now termed $Ca_v\beta1a$) was cloned from skeletal muscle and observed as a 54 kDa protein (Takahashi et al., 1987). To date four genes have been cloned encoding human β subunits ($Ca_v\beta1$–4) and there are several splice variants produced from the genes (for review, see Birnbaumer et al., 1998; Dolphin, 2003). $Ca_v\beta$ subunits are intracellularly located and bind the α subunit with high affinity via the α interaction domain (AID) on the I–II linker (see Fig. 7.1; Pragnell et al., 1994). $Ca_v\beta$ subunits promote functional expression of $Ca_v\alpha$ subunits, localization, and insertion of the

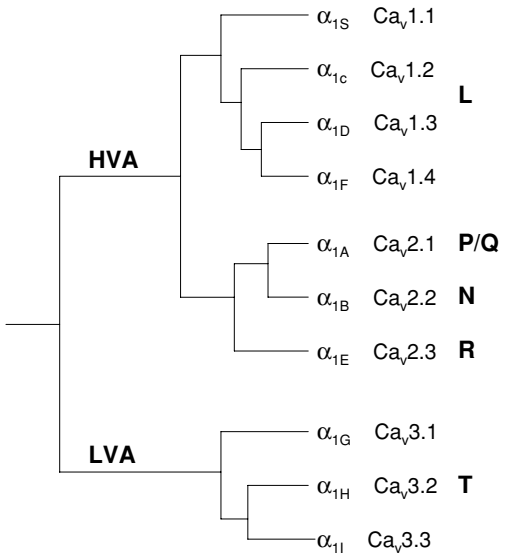

Fig. 7.2 Family tree of mammalian voltage-gated Ca^{2+} channels and nomenclature. Adapted from Jones (2003).

channel complex in the plasma membrane (Chien et al., 1995; Bichet et al., 2000). In addition, $Ca_v\beta$ subunits can modify the kinetics and amplitude of current, including increasing the channel open probability and altering the inactivation rate (Colecraft et al., 2002).

The $\alpha_2\delta$ subunit is highly glycosylated and encoded by a single gene. The δ segment spans the plasma membrane and connects to the extracellular α_2 protein via a disulfide bridge formed between a number of cysteines present on both proteins. To date four genes have been identified that encode $\alpha_2\delta 1$–4 subunits (Ellis et al., 1988; Klugbauer et al., 1999; Qin et al., 2002). The α_2 protein has been shown to influence channel stimulation and the δ protein alters voltage-dependent activation and inactivation kinetics (Felix et al., 1997).

In some tissues a fourth auxiliary subunit is expressed, the γ subunit. At least eight genes are know to encode for γ subunits ($Ca_v\gamma 1$–8), the first known subunit being cloned from skeletal muscle with a mass of 25 kDa (Jay et al., 1990). Co-expression studies reveal the subunit can modulate peak current and activation/inactivation kinetics (Singer et al., 1991).

7.2.2 Ca Release Channels

One of the critical roles served by Ca^{2+} channels in cell function is the maintenance of intracellular calcium levels and the initiation of calcium-dependent cellular processes. This is particularly poignant in cardiac muscle where calcium influx through plasma membrane $Ca_v1.2$ channels is a requirement for the release of calcium from

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Table 7.1

HVA (high voltage activated, large conductance, persistent)				
Tsien class:	L-type			
Snutch/Birnbaumer class:	α_{1S}	α_{1C}	α_{1D}	α_{1F}
IUP classification:	$Ca_v1.1$	$Ca_v1.2$ a,b,c	$Ca_v1.3$	$Ca_v1.4$
Gene:	CACNA1S	CACNA1C	CACNA1D	CACNA1F
Human chromosome:	1q31-32	12p13.3	3p14.3	Xp11.23
Tissue:	skeletal muscle	heart, smooth muscle, brain, adrenal	brain, pancreas, kidney, cochlea, ovary	retina
HVA (high voltage activated, rapid inactivating)				
Tsien class:	P/Q-type			
Snutch/Birnbaumer class:	α_{1A}	α_{1B}	α_{1E}	
IUP class:	$Ca_v2.1$a,b	$Ca_v2.2$ a,b	$Ca_v2.3$ a,b	
Gene:	CACNA1A	CACNA1B	CACNA1E	
Human chromosome:	19p13	9q34	1q25-31	
Tissue:	brain, cochlear, pituitary	brain, NS	brain, cochlea, retina, heart, pituitary	
LVA (low voltage activated)				
Tsien class:	T-type			
Snutch/Birnbaumer class:	α_{1G}	α_{1H}	α_{1I}	
IUP class:	$Ca_v3.1$	$Ca_v3.2$	$Ca_v3.3$	
Gene:	CACNA1G	CACNA1H	CACNA1I	
Human chromosome:	17q22	16p13.3	22q12.3-13-2	
Tissue:	brain, NS	brain, heart, kidney, liver	brain	

Tsien class (Tsien et al., 1988); Snutch/Birnbaumer class (Snutch et al., 1990; Birnbaumer et al., 1994); IUP class (Ertel et al., 2000; Catterall et al., 2003, 2005).

IUP = International Union of Pharmacology; NS = nervous system.

intracellular sarcoplasmic reticulum stores that then initiates contraction. This process is known as excitation–contraction coupling and will be discussed in detail in Section 7.3. There are, broadly speaking, three families of protein, known as Ca-release channels, that are responsible for the release of calcium from intracellular stores. They are inositol triphosphate receptors (IP_3R), ryanodine receptors (RyR), and calcium-release-activated calcium (CRAC) channels. IP_3Rs and RyRs serve to release calcium into the cytoplasm from the sarcoplasmic reticulum and endoplasmic reticulum of muscle but they are also expressed in many other cell types including neurons where they play a role in calcium-dependent neurotransmission. In muscle the activation of IP_3Rs and RyRs is an important step in the process of muscle contraction, allowing intracellular calcium levels to increase and bind to troponin C that then switches on the contractile apparatus. During the course of a single muscle contraction, free intracellular calcium increases from resting concentrations (typically 10–300 nM) to micromolar levels. Equally important to the release of calcium, however, is the reuptake of calcium into intracellular stores to allow relaxation. The sarcoplasmic reticulum is capable of accumulating millimolar concentrations

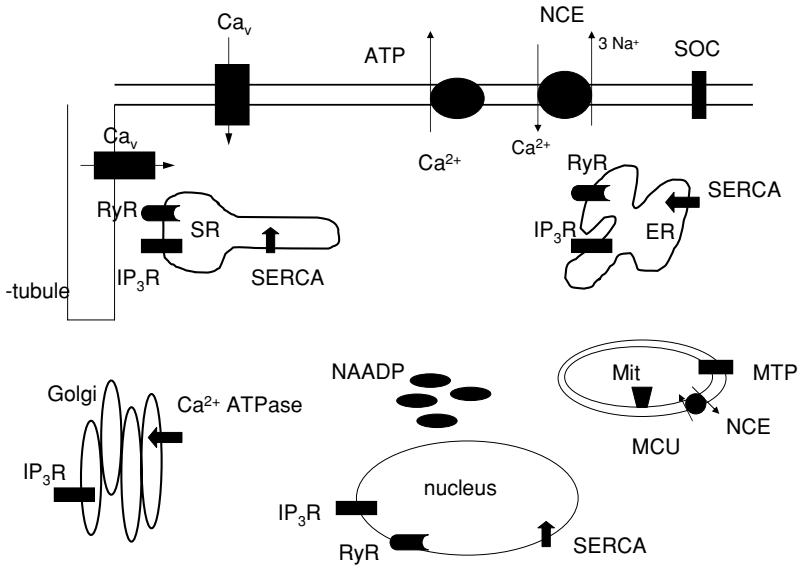

Fig. 7.3 Channels and transporters responsible for regulating calcium in a cell. Mit = mitochondria, ER = endoplasmic reticulum, SR = sarcoplasmic reticulum, SERCA = sarcoplasmic endoplasmic reticulum ATPase, NCE = Na^+/Ca^{2+} exchanger, SOC = store operated channel, MTP = mitochondrial transition pore, MCU = mitochondrial calcium uniporter, NAADP = nicotinic acid adenine dinucleotide phosphate-sensitive calcium channel depicted here on lysosomes.

of calcium and this is returned after release by way of ATP-dependent pumps known as SERCA pumps (sarcoplasmic reticulum, endoplasmic reticulum Ca^{2+} ATPase). The SERCA family of genes encode five isoforms that are all sensitive to the inhibitor thapsigargin (Lytton et al., 1991). Like the plasma membrane Ca^{2+} ATPase and Na^+/K^+ ATPase, SERCA utilize the energy produced from the hydrolysis of ATP to drive the pumping of calcium back into sarcoplasmic reticulum stores. Other means of removing calcium from the cytoplasm and restoring resting intracellular calcium also exist such as sarcolemmal Na^+/Ca^{2+} exchange and mitochondrial calcium uptake (Fig. 7.3).

Three receptor types or isoforms of RyR and IP_3Rs have been isolated and combinations of isoforms are co-expressed in different muscle types (Nakagawa et al., 1991; Fill and Copello, 2002). RyRs are localized within membranes of organelles with stores of calcium (such as the sarcoplasmic reticulum), but IP_3Rs have been identified in the plasma membrane, endoplasmic reticulum, Golgi apparatus, and nuclear membranes (Khan et al., 1992; Leite et al., 2003). Both RyR and IP_3Rs form tetrameric complexes with the channel region located at the C-terminal end of the protein. There is good evidence that they appear to share common calcium stores and can interact with each other (McGeown, 2004). Activation of both receptor types is Ca^{2+}-dependent and varies with isoform. RyRs are activated at micromolar concentrations of calcium while IP_3Rs are generally activated at submicromolar

concentrations (Bezprozvanny et al., 1991). Therefore the local release of calcium by one receptor can be amplified by activating a nearby receptor. The dependence of activation on calcium concentration, however, is often bell-shaped so that high concentrations of calcium also have inhibitory effects on channel function. This is the case for type I IP_3Rs where it has been proposed that the bell-shaped-dependence supports oscillations in cytosolic calcium, but not for type III IP_3Rs where an increase in cytosolic calcium causes a further increase in calcium that is suited to signal initiation (Hagar et al., 1998). In type I IP_3Rs the Ca^{2+}-binding sites lie within the inositol triphosphate ($InsP_3$)-binding core, and it has been proposed that calcium binding is negatively regulated by a conformational constraint imposed by $InsP_3$-binding (Bosanac et al., 2002). Both IP_3Rs and RyRs favor the conductance of divalent cations over monovalent cations exhibiting single-channel conductances of between 30 and 120 pS.

IP_3Rs mediate calcium release in response to activation of G protein or tyrosine kinase-coupled plasma membrane receptors. Agonist binding leads to stimulation of phospholipase C resulting in production of diacylglycerol, an activator of protein kinase C, and IP_3. IP_3 is a highly diffusible second messenger that binds to IP_3Rs on membranes of intracellular organelles. There is evidence, at least in neurons, that IP_3Rs may be directly activated by $G_{\beta\gamma}$ subunit following activation of a G_i protein (Zeng et al., 2003). IP_3Rs can be phosphorylated by many kinases. Protein kinase A phosphorylates IP_3Rs at serine 1589 and 1755 (Ferris et al., 1991a) as does cGMP-dependent protein kinase (Komalavilas and Lincoln, 1994). Protein kinase C and calcium calmodulin-dependent protein kinase also phosphorylate the receptor at distinct sites regulating calcium release in a spatiotemporal manner contributing to formation of calcium microdomains (Ferris et al., 1991b). ATP augments calcium release from IP_3Rs by stabilizing open channel states thereby allowing positive control of calcium release when SERCA have utilized the local ATP during reuptake or when the cell is experiencing metabolic inhibition (Mak et al., 1999). Cyclic ADP-ribose (cADPR) stimulates the association of ryanodine with RyR increasing channel opening (Guse et al., 1999). IP_3 does not bind to RyRs but caffeine and heparin potentiate RyR channel opening.

In nonexcitable cells the predominant mechanism for release of store-operated calcium (SOC) is by CRAC channels. The best characterized SOC influx channel is the CRAC channel expressed in T lymphocytes that is essential to the immune response including the regulation of gene expression and cell proliferation. The increase in intracellular calcium occurs in a biphasic manner typically with an initial transient increase due to binding of an agonist such as IP_3 and release of internal stores. The depletion of intracellular calcium stores then triggers release of calcium via CRAC channels on the plasma membrane and is entirely dependent on extracellular calcium. Recent evidence indicates that a transmembrane protein with an EF hand motif near the amino terminus located in the lumen of the ER termed STIM-1 is the sensor that migrates from the calcium store to the plasma membrane to activate CRAC channels (Zhang et al., 2005). ~~The pore properties and biophysics of the CRAC channel are similar to the calcium transport protein CaT1 (Yue et al., 2001).~~

CRAC channels have a low single-channel conductance ~~(0.5 pS)~~ with high selectivity for divalent cations and typically exhibit inward rectification. They favor opening when the cell is hyperpolarized (Zweifach and Lewis, 1993).

7.2.3 Mitochondrial and Nuclear Ion Channels

Many organelles other than the sarcoplasmic reticulum and endoplasmic reticulum are able to take up calcium and impose strict control over its movement in order to regulate organelle function. As mentioned above, the Golgi complex expresses IP_3Rs. It also contains SERCA-type pumps known as secretory pathway Ca^{2+}-ATPase that are located in close proximity to the nuclear membrane and hence may be involved in calcium signaling with the nucleus (Wootton et al., 2004). Calcium uptake into Golgi may occur by thapsigargin-sensitive and thapsigargin-insensitive mechanisms. Interestingly, only the *cis*-Golgi express IP_3Rs unlike the *trans*-Golgi suggesting that the role of the *trans*-Golgi in vesicle packaging and posttranslational modification has differential requirements for calcium compared with the *cis*-Golgi that appears to play a greater contribution to spatial and temporal calcium signals (Vanoevelen et al., 2004). Calcium release channels that do not involve IP_3 or cADPR have been identified on lysosome-related organelles. A nicotinic acid adenine dinucleotide phosphate (NAADP)-sensitive calcium channel has been located on lysosomes in pancreatic β cells that requires proton exchange to assist with calcium loading (Yamasaki et al., 2004).

It is well established that the mitochondria can accumulate calcium. Control of mitochondrial calcium is necessary for ATP production (McCormack et al., 1990) and the shaping of intracellular calcium signals (Jouaville et al., 1995). Calcium overload is associated with the processes mediating necrosis and apoptosis (programmed cell death) (Halestrap et al., 2000). In addition, several of the mitochondrial enzymes require calcium as a cofactor in higher concentration in the mitochondrial matrix than the mitochondrial cytosol, therefore transport of calcium across the mitochondria must be carefully regulated (Gunter et al., 2004). Calcium accumulation mainly occurs through the mitochondrial calcium uniporter (MCU; see Fig. 7.3) located on the inner mitochondrial membrane. Recently, the MCU was identified as a calcium channel with extremely high calcium affinity ($K_{0.5}$ of 19 mM) and biophysical characteristics that included an inwardly rectifying current suited to calcium uptake into mitochondria and inhibition by ruthenium red (Kirichok et al., 2004). Calcium may exit the mitochondria via the permeability transition pore (MPTP) that is more frequently associated with the collapse of the membrane potential and influx of proteins that lead to cell death (Halestrap et al., 1998, 2000). PTP opening is thought to occur when a protein, cyclophilin D binds to the matrix side of the PTP. Calcium is required for the binding of cyclophilin D. Calcium efflux from the mitochondria also occurs via the Na^+/Ca^{2+} exchanger that, similar to the plasma membrane exchanger transports one calcium ion out of the mitochondria in exchange for three sodium ions.

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Although it is recognized that regulation of calcium within the nucleus is vital for many functions including gene transcription, little is understood about the regulatory processes involved. The nuclear membrane consists of an envelope with a luminal space capable of storing calcium. It also possesses the components necessary to regulate calcium levels such as SERCA, IP_3Rs, and RyRs (Gerasimenko et al., 1996). Both IP_3Rs and RyRs are located on inner and outer nuclear membranes. The nucleus can generate its own IP_3 but it is unclear whether it physically associates with other calcium stores such as ER and SR. However, there is evidence that the nucleus contains its own stores (termed nucleoplasmic reticulum) that may physically associate with the mitochondria suggesting a cooperative role on regulating calcium signals (Lui et al., 2003).

7.3 Roles of Ca^{2+} Channels

Calcium is essential in electrical activity in that it shapes the long plateau phase of the ventricular action potential and the upstroke and duration of the smooth muscle and atrial pacemaker action potentials. However, the influx of calcium through voltage-dependent calcium channels is also an essential component in the initiation of intracellular processes. Here we discuss in detail the role of calcium in muscle contraction, neurotransmitter secretion, and in controlling electrical excitability, highlighting recent research.

7.3.1 Muscle Contraction

Calcium is the switch that initiates the contraction of muscle fibers. These fibers are formed from many overlapping strands, comprising a thick filament made from myosin and a thin filament comprised of actin and tropomyosin. When Ca^{2+} is released from intracellular stores, it binds to troponin present on the thin filaments, and allosterically modulates the tropomyosin to unblock a series of myosin binding sites. The myosin can then be powered by hydrolyzing ATP to move along these binding sites causing muscle contraction until the cytoplasmic Ca^{2+} is removed and the myosin binding sites are once more blocked.

In the heart calcium influx across the plasma membrane through L-type Ca^{2+} channels is essential for triggering calcium release from the sarcoplasmic reticulum, a process known as calcium-induced calcium-release. This occurs as a result of a close physical association between dihydropyridine receptors (L-type Ca^{2+} channels) and transverse or T-tubules that communicate directly with RyRs on the sarcoplasmic reticulum (Fig. 7.3). Because the activation kinetics of L-type Ca^{2+} channels in skeletal muscle is 100-fold slower than in cardiac muscle, the process of calcium influx is too slow to initiate contraction. In skeletal muscle, therefore, a depolarization of the plasma membrane is the trigger for release of calcium from SR stores rather than the influx of calcium itself. Therefore, L-type Ca^{2+} channels serve as the voltage sensor used to initiate the release of Ca^{2+} from intracellular store for initiating

contraction as calcium influx across the plasma membrane is not a requirement (Franzini-Armstrong and Protasi, 1997). In smooth muscle, calcium influx through L-type Ca^{2+} channels can release calcium from the SR to trigger contraction. In addition, a hormone-induced calcium release can initiate contraction through release of calcium from internal stores via binding to IP_3Rs.

The characteristics of the contractile force in muscle can also be controlled by intracellular calcium. The contractile force can be altered either by increasing the duration or amplitude of the calcium transient (the rise in intracellular calcium) or altering the sensitivity of the myofilaments to calcium. Stretch increases myofilament calcium sensitivity as it enhances actin–myosin interaction (Fukuda et al., 2001b) while acidosis reduces calcium sensitivity and enhances the length-dependence of tension (Fukuda et al., 2001a). Cytosolic calcium must be removed to allow for the relaxation of muscle fibers. The majority of cytosolic calcium uptake occurs by the SR Ca^{2+}-ATPase, with the remaining calcium being extruded from the cell either by Na^+/Ca^{2+} exchange or a small amount being taken up by the mitochondria via the MCU. Na^+/Ca^{2+} exchange is voltage-dependent and reversible with high intracellular calcium favoring calcium efflux; while a positive membrane potential and high intracellular Na^+ favors Na^+ efflux (Dipolo and Beauge, 2006). Under physiological conditions, Na^+/Ca^{2+} exchange works mostly to extrude calcium driven by the intracellular calcium transient. It is also a secondary active transport process driven by the Na^+/K^+ ATPase that extrudes three Na^+ ions in exchange for 2 K^+ ions while hydrolysing one molecule of ATP. If the Na^+/K^+ ATPase is partially inhibited, calcium influx through Na^+–Ca^{2+} exchange is increased. This is the basis for the increased force of muscle contractions induced by cardiac glycosides in the heart.

In principle, the Na^+/Ca^{2+} exchanger could be a trigger of intracellular calcium release and muscle contraction, but the influx is too slow and too small to do so (Sipido et al., 1997). A contributing factor to this is the fact that Na^+/Ca^{2+} exchangers are not positioned near the SR junctional cleft (Scriven et al., 2000). Promiscuous calcium entry through tetrodotoxin-sensitive Na^+ channels has also been proposed to trigger contraction, also termed slip-mode conductance (Santana et al., 1998). However, this remains controversial since a number of groups have been unable to trigger contraction by this means (Chandra et al., 1999; DelPrincipe et al., 2000). T-type Ca^{2+} channels are also not located near SR junctions and the current passing through them is less than for L-type channels, therefore any contribution they make to triggering contraction is thought to be small (Sipido et al., 1998; Zhou and January, 1998).

IP_3Rs are the main trigger of calcium release from SR and ER in smooth muscle. Generally, calcium release from IP_3Rs stimulates further calcium release in a cooperative fashion, but with high calcium concentrations inhibiting channel function. This creates the classical oscillatory pattern of calcium release typically seen in innervated smooth muscle such as that resulting in peristalsis in the bowel or agonist mediated calcium release pathways.

RyRs inactivate one of two ways. They either do not reopen until they recover (Schiefer et al., 1995; Sham et al., 1998), or they relax to a lower open probability

but can still be reactivated at high calcium levels, a process known as adaptation and also common to IP_3Rs (Gyorke and Fill, 1993). Adaptation of RyRs enables a graded calcium-induced calcium-release for contraction in muscle.

7.3.2 Neurotransmitter Release and Neuronal Plasticity

Another important physiological process involving calcium is neurotransmission at nerve terminals. Within the presynaptic terminal of neuron hormones, neurotransmitters, or other peptides are stored in vesicles. When an action potential depolarizes the membrane, the vesicles fuse with the plasma membrane and the contents are released into the extracellular space where they can bind with receptors and initiate chemical activity in nearby cells. The process of neurotransmission is calcium-dependent in that secretion requires extracellular calcium and is inhibited by extracellular Mg^{2+} (Douglas, 1968). In addition, intracellular calcium modulates the activity of a number of ion channels involved in this process. Intracellular calcium influences the inactivation rate and inhibition of L-type Ca^{2+} channels (see above) and activates large conductance K^+ channels BK(Ca) that terminate neurotransmitter release in the presynaptic terminal by hyperpolarizing the membrane (Storm, 1990; Sah, 1996). BK(Ca) are expressed in virtually all excitable cells, have long and large unitary conductances typically 100–250 pS and can be blocked by charybdotoxin, iberiotoxin, and tetraethylammonium compounds. The BK(Ca) channels are derived from the *slo* family of genes originally cloned from the Drosophila mutant *slowpoke*. Intermediate (IK) and small (SK) conductance channels of K(Ca) family have also been cloned and characterized (Vergara et al., 1998). Calcium-dependent chloride channels also contribute to membrane potential hyperpolarization along with the BK(Ca) channels in neurons (Mayer, 1985).

The release of neurotransmitters is steeply calcium dependent and a decrease in calcium channel activity contributes to presynaptic inhibition (Dunlap and Fischbach, 1978). An example of presynaptic inhibition is the block of transmission of sensory fibers that occurs with opiates such as morphine. This occurs as a result of rapid coupling of G protein $\beta\gamma$ subunits to Ca_v2 family of calcium channels causing a slowing of activation of the channels (Dolphin, 1998). Several modulatory transmitters such as acetylcholine, norepinephrine, adenosine, and GABA contribute to depression of calcium channels at presynaptic terminals through the G protein-dependent mechanism.

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Both presynaptic and postsynaptic calcium stores are involved in long-term depression in the developing hippocampus (Caillard et al., 2000). However, presynaptic RyRs appear to be important in determining the strength of synaptic transmission induced by NMDA receptors (Unni et al., 2004). Activation of presynaptic NMDA, kainate, and nicotinic acetylcholine receptors can lead to calcium-induced calcium-release from RyR stores similar to that recorded in muscle that acts to inhibit or regulate secretion. Although still controversial, calcineurin may interact with IP_3Rs and RyRs to modulate intracellular calcium release and synaptic plasticity (Bultynck et al., 2003).

Calcium can also play a role in building synaptic connections. Rapid changes in synaptic strength are mediated by posttranslational modifications of preexisting protein but enduring changes are dependent on gene expression and the synthesis of a variety of proteins required for synaptic transmission. One of the key mediators of gene expression involved in synaptic plasticity is the transcription factor cAMP response element binding protein (CREB). This transcription factor plays an important role in behavioral adaptations to changes in the environment and complex processes such as learning and memory. It is also important in proliferation and differentiation in developing vertebrates. Calcium influx through L-type Ca^{2+} channels or NMDA receptors is required for phosphorylation and activation of CREB (Kornhauser et al., 2002). Another transcription factor responsible for shaping long-term changes in neurons is the NFAT family, and similar to regulation of CREB, activation of NFAT is dependent upon calcium influx through L-type Ca^{2+} channels. Interactions of calcium with PDZ domain proteins (PSD-95, Discs-large, ZO-1 domain proteins that often function as scaffolding proteins and have been shown to play important roles in signal transduction) appear to be necessary for the coupling of L-type Ca^{2+} channels to NFAT as does CREB activation (Weick et al., 2003). Neurotrophins, such as the brain derived neurotrophic factor, have also been implicated in the activation of NFAT-dependent transcription. Initiating such transcriptions leads to further increase in brain derived neurotrophic factor mRNA and protein as positive feedback (Groth and Mermelstein, 2003).

7.3.3 Excitability and the Action Potential

Calcium plays an important role in shaping the action potential of muscle and neurons. One example where the shape and duration of the action potential must be carefully controlled is in cardiac pacemaker activity. This originates from either specialized cells located in the right atrium of the heart known as sinoatrial node cells or from secondary pacemaker cells located within the atrioventricular node and also through the Purkinje fibers of the ventricles. Pacemaker action potentials are designed to originate from the sinoatrial node cells so that the resting heartbeat is maintained at 60–80 beats min^{-1}. If the pacemaker fails in its duty, secondary pacemakers in the atrioventricular node or in the purkinje fibers are activated to take over and maintain ventricular function and ultimately cardiac output, albeit at a lower heart rate. The spontaneous activity of pacemaker cells is attributed to a phase of the action potential known as the slow diastolic depolarization. During this phase, the membrane potential slowly depolarizes following termination of an action potential until the threshold for a new action potential is reached. It is well accepted that the electrical activity of cardiac pacemakers is thought to depend exclusively on voltage-dependent ion channels within the plasma membrane of nodal cells. These include the hyperpolarization-activated inward current (I_f), the time-dependent decay of K^+ conductance, inward L-type and T-type Ca^{2+} currents, and the lack of background K^+ conductance. The hyperpolarization-activated channel carries Na^+ and K^+ ions inwards and activates upon hyperpolarization. However, interventions that affect

intracellular Ca^{2+} can also affect pacemaker activity. A rise in intracellular Ca^{2+} results in activation of the Na^+/Ca^{2+} exchanger. This produces an inward current that is sufficient to induce diastolic depolarization and pacemaker activity (Ju and Allen, 1998). The importance of Ca^{2+} in contributing to pacemaker firing is further supported by studies that show that embryonic stem cell-derived cardiomyocytes do not show increased beating rate with differentiation when they lack ryanodine receptors (Yang et al., 2002).

The rate of change in membrane potential during the upstroke is much slower in sinoatrial node cells than in ventricular cells. This is because depolarization of the membrane during phase 0 (the rapid upstroke) of a slow response action potential in pacemaker cells is caused by an increase in the Ca^{2+} conductance due to activation of L-type Ca^{2+} channels. Nodal cells have fewer Na^+ channels than ventricular cells and because the membrane potential in phase 0 is depolarized, changes in Na^+ conductance do not contribute. Pacemaker cells do not have a pronounced plateau phase. Action potential duration is determined by a balance between Ca^{2+} and K^+ conductances.

In ventricular myocytes the prolonged phase 2 (plateau phase) of the action potential is characterized by slow inward current produced by L-type Ca^{2+} channels. This is a distinguishing feature of the cardiac action potential and is the necessary trigger for calcium-induced calcium-release and contraction. Similarly in smooth muscle and skeletal muscle calcium influx through L-type Ca^{2+} channels are important in shaping the duration of the action potential, however, the duration is much shorter than that of ventricular myocytes. In smooth muscle and neurons the calcium-dependent large conductance K^+channels contribute to hyperpolarization of the membrane.

7.4 Ion Selectivity and Permeation

It is clear that to carry out their biological role, calcium channels have to be selective, allowing Ca^{2+} ions to pass while blocking Na^+, K^+, Cl^-, and other ions. A high degree of specificity is important for these channels as Na^+ ions are more than 100-fold more numerous in the extracellular solution than Ca^{2+}. Voltage-gated calcium channels are extremely discriminating, selecting calcium over sodium at a ratio of over 1000:1 (Hess et al., 1986), yet the picoampere currents they carry require over one million ions to pass through a single channel every second (Tsien et al., 1987). A central problem in understanding the function of these channels is to determine how they can be both highly selective while still passing so many ions. A narrow pore can block the passage of large ions, but, Na^+ and Ca^{2+} are similar in size and much larger ions are known to permeate the channel (McCleskey and Almers, 1985). Thus, these proteins must distinguish between ions using more than just size. Instead, it appears that calcium channels utilize the different strengths of interaction between ions and the protein as well as a multi-ion conduction process to obtain ion selectivity. Here

we focus the discussion of selectivity on L-type voltage-gated calcium channels as these have been most widely studied. It is hoped that many of the mechanisms of this channel will apply to a variety of proteins in this family, particularly as the important residues required to obtain calcium specificity are highly conserved.

7.4.1 High-Affinity Binding of Permeant Ions

A surprising characteristic of voltage-gated calcium channels is that monovalent ions conduct through the channel at much higher rates than any divalent ions when no divalent ions are present (Kostyuk et al., 1983; Almers and McCleskey, 1984; Fukushima and Hagiwara, 1985; Hess et al., 1986; Kuo and Hess, 1993a). But, these monovalent currents are blocked when the calcium concentration reaches only 1 μM (Kostyuk at al., 1983; Almers et al., 1984). Indeed, the ability of some ion species to block currents carried by others gave the first clues to the origin of ion selectivity in these channels, suggesting that it relied on high-affinity binding of ions within a single file pore. Those ions that bound most strongly in a single file pore would block the passage of other ions.

A large number of mono- and divalent ion types pass through the pore, but a characteristic sequence in which some ion types block the passage of others has been observed. In addition to Ca^{2+} blocking monovalent cations, Sr^{2+} current is also blocked by Ca^{2+} (Vereecke and Carmeliet, 1971) while Cd^{2+}, Co^{2+}, and $La3^{+}$ block currents carried by Ca^{2+} even though all of these species were known to pass through the channel at some rate (Hagiwara et al., 1974; Lansman et al., 1986; Chow, 1991). Furthermore, Co^{2+} was found to block Ca^{2+} currents less effectively than Ba^{2+} currents, even though Ba^{2+} currents were found to be larger than Ca^{2+} currents (Hagiwara et al., 1974). This property was explained by positing that Ca^{2+} binds more strongly to the pore than Ba^{2+} but not as strongly as Co^{2+}. Together these data suggested a high-affinity binding site in the pore, with an order of affinity estimated from bi-ionic reversal potentials to be $La3^{+} > Cd^{2+} > Co^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} > Li^{+} > Na^{+} > K^{+} > Cs^{+}$ (Reuter and Scholz, 1977; Fenwick et al., 1982; Lee and Tsien, 1984; Hess et al., 1986; Taylor, 1988).

One complication, however, has been observed. Although Ca^{2+} was found to prevent monovalent currents, Na^{+} was also found to attenuate currents carried by Ca^{2+} in N-type channels (Polo-Parada and Korn, 1997). Although this appears to contradict the earlier results, it can easily be explained if these Na^{+} ions compete for entry into the pore rather than permanently occupying the high-affinity binding site (Polo-Parada and Korn, 1997; Corry et al., 2001).

Single-channel conductance measurements on the other hand, have the exact reverse order of conductance values than the binding affinity: $La3^{+} < Cd^{2+} < Co^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+} < Li^{+} < Na^{+} < K^{+} < Cs^{+}$ (Hess et al., 1986; Kuo and Hess, 1993a,b). The inverse relationship between binding affinity and permeation were first explained with the so-called "sticky-pore" hypothesis. In this, ions that are bound with higher affinity pass through the channel more slowly and so have a lower conductance (Bezanilla and Armstrong, 1972). For example, the lower current

carried by Ca^{2+} than by Ba^{2+} can be understood if Ca^{2+} binds more strongly in the pore, and thus takes longer to dissociate once bound. Indeed it has been observed that permeabilities for monovalent and divalent ions are ordered according to an Eisenman sequence that models selectivity between ions as a balance between the dehydration and binding energy of each (Eisenman, 1962). According to Eisenman, the permeability sequence observed in calcium channels can be explained by an electrostatic binding site of strong field strength.

A paradox arises, however, when explaining ion selectivity with high-affinity binding. How can ions conduct through the pore at picoampere rates if they are bound so strongly? This paradox disappears if one considers the implications of evidence that calcium channels are multi-ion pores.

7.4.2 Calcium Channels Have a Multi-Ion Pore

A number of lines of evidence suggest that calcium channels must bind multiple ions. Multi-ion conduction is a natural consequence of the sticky-pore model as otherwise there should not be a difference in conduction rates between ion types. In a single ion pore, ions of all binding affinities conduct equally well. Those that bind more effectively pass more slowly through the pore, but they are also more likely to block the passage of lower affinity ions (Bezanilla and Armstrong, 1972).

Other strands of evidence also suggest a multi-ion pore. In many cases when two permeating species are mixed the current is lower than for either species on its own. This phenomenon is seen clearly for mixtures of Ca^{2+} and Ba^{2+} and also Ca^{2+} and Na^{+} (Almers and McCleskey, 1984; Hess and Tsien, 1984) in calcium channels and has also been observed in gramicidin (Neher, 1975) and potassium channels (Hagiwara et al., 1977). This so-called "anomalous permeability" or "anomalous mole fraction effect" is most easily explained supposing the pore contains multiple ions. If ions moved independently through the pore, then the current in mixed solutions should always lie between the currents observed with either permeating ion on its own. If, however, the channel holds multiple ions then it is possible that unfavorable interactions between the ions in the pore could reduce the current. As noted by Hille (2001), it is possible to explain the anomalous permeability by supposing some form of conformational or chemical alteration of the channel protein caused by binding of ions exterior to the pore, rather than by a multi-ion pore. But, such an explanation lacks the simplicity of the multi-ion model that is also supported by other data.

Another important strand of evidence for multiple ions lies in the measurement of two differing affinities for Ca^{2+} binding. Ca^{2+} blocks Na^{+} currents with an affinity of $\sim$1 μM, while calcium currents saturate with an affinity closer to 14 mM. This can easily be explained by a multi-ion pore if the first affinity represents binding of a calcium ion to either an empty pore or a pore containing one Na^{+}. However, if one Ca^{2+} ion is already present in the pore, then electrostatic repulsion between the two ions means that the second ion is bound more weakly giving rise to the second affinity measured.

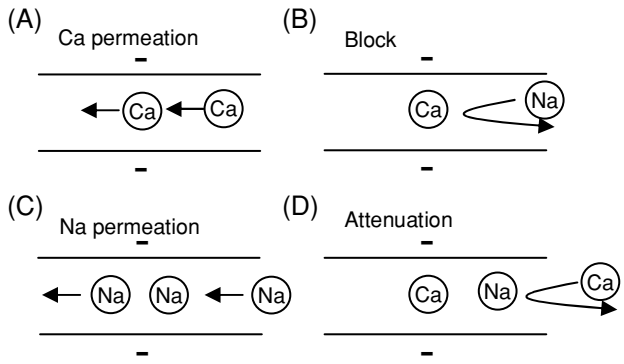

Fig. 7.4 "Electrostatic" model of permeation and selectivity in calcium channels. (A) When one Ca^{2+} ion is electrostatically bound to the negative charge of the EEEE locus, it can only be removed with the aid of Coulomb repulsion from another Ca^{2+} ion. (B) The lesser repulsion from Na^{+} is unable to displace a resident Ca^{2+} ion, thus Ca^{2+} blocks Na^{+} currents. (C) Na^{+} is able to pass through the channel in the absence of Ca^{2+} in a "knock on" mechanism involving three ions. (D) Na^{+} can attenuate Ca^{2+} currents by slowing the entry of the second Ca^{2+} ion required for conduction.

The presence of multiple ions in the pore is also supported by the fact that calcium block of Na^{+} currents is dependent on membrane voltage (Fukushima and Hagiwara, 1985; Lux et al., 1990) and on the direction of ion movement (Kuo and Hess, 1993a,b). Further evidence that two divalent ions can inhabit the pore comes from a detailed study of the blocking of Ba^{2+} currents by Cd^{2+} (Lansman et al., 1986). Raising the concentration of Ba^{2+} was found to increase the unbinding rate of Cd^{2+} as if increasing the probability of Ba^{2+} also being present in the pore enhances the exit of Cd^{2+}.

A multi-ion pore provides a simple explanation of the permeation and selectivity properties of the channel as depicted schematically in Fig. 7.4. The selectivity—conductivity paradox is avoided by utilizing ion–ion interactions inside the selectivity filter. As soon as one Ca^{2+} ion occupies the channel, it binds tightly to the pore and thereby blocks the passage of Na^{+}. However, if the second site in the pore is occupied by a calcium ion then the strong electrostatic repulsion between the two ions allows one to overcome its interaction with the protein and exit the channel at a relatively fast rate (Almers and McCleskey, 1984; Hess and Tsien, 1984). Further details of the mechanisms of selectivity are described below in the context of various theoretical models.

As noted by Hille, the concepts of binding and repulsion described here need not refer to specific details (Hille, 2001). Binding sites need not be specifically shaped or involve stereochemical fit such as in the active sites of enzymes. Rather it is more likely to refer to regions of space that form local energy minima created by broad electrostatic interactions between the ion and the protein. Furthermore, the locations of such minima are likely to change when, for example, other ions are nearby. Thus a multi-ion pore need not contain two or more specific localized sites, but could just

have one or more regions of negative potential. Similarly, the repulsion between ions in the pore may be direct Coulomb repulsion between like charged particles or may involve interaction through the protein, conformational changes or restrictions on the entropic freedom of nearby ions.

7.5 Channel Structure

It has long been recognized that voltage-gated sodium, potassium, and calcium channels are related. Not only do they share many functional similarities, the amino acid sequences indicate many structural similarities as well. This similarity is no longer surprising as we now know that many proteins have evolved in families and that all these channel types most likely evolved from a common ancestor. This means that we can learn much about one member of the family from findings made about other members. As no atomic resolution images of calcium channel structures exist, much has been learnt about their structure since the recent determination of crystal structures of a number of potassium channels (Doyle et al., 1998; Jiang et al., 2003; Long et al., 2005).

7.5.1 Subunit Structure and Function

Voltage-gated calcium channels are formed by the association of separate subunits, which come together to form an active channel. As noted previously the variety of voltage-gated calcium channels arises from differences in the sequences of the individual subunits comprising the channel in particular the $\alpha 1$ subunit. As with voltage-gated sodium channels, but unlike the potassium channels, the pore-forming domain of the calcium channel protein is comprised of four regions of the one subunit rather than by separate subunits.

The first step in determining the structural characteristics of these channels was the purification, cloning, and sequencing of the protein. Purification was first achieved from transverse tubule membranes of skeletal muscle (Curtis and Catterall, 1984). As described previously, this study and further analysis (Hosey et al., 1987, Leung et al., 1987, Striessnig et al., 1987, Takahashi et al., 1987) indicated that this channel was comprised of five subunits: $\alpha 1$ weighing 190 kDa and later shown to form the pore; a disulphide linked $\alpha 2$-δ dimer of 170 kDa; and intracellular phosphorylated β subunit weighing 55 kDa; and a transmembrane γ subunit of 33 kDa as indicated in Fig. 7.5.

Subunits of similar sizes were soon purified form cardiac L-type channels (Chang and Hosey, 1988; Schneider and Hofmann, 1988) whereas neuronal L-type channels revealed $\alpha 1$, $\alpha 2$-δ, and β but no γ subunit (Ahlijanian et al., 1990). The similarity between these various L-type channels also extends to neuronal N-type (McEnery et al., 1991; Witcher et al., 1993) as well as P/Q-type channels (Martin-Moutot et al., 1995, 1996; Liu et al., 1996) that have also been purified and contain the $\alpha 1$, $\alpha 2$-δ, and β subunits. A possible γ subunit has also been found in P/Q

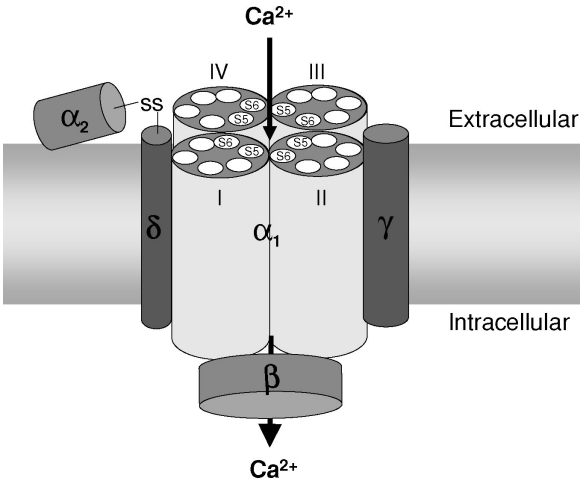

Fig. 7.5 The likely structure and arrangement of the L-type channel subunits. The pore is formed by the S5 and S6 helices and linker of the $\alpha 1$ subunit. The β subunit interacts with the linker between domains I and II of $\alpha 1$ and modifies gating and inactivation kinetics. The δ and γ subunits lie in the membrane while $\alpha 2$ is accessible to the extracellular solution.

channels (Letts et al., 1998) that plays a role in modulating the voltage dependence of the channels, yielding even more similarity with the L-type channels. Cloning and sequencing of the five subunits from L-type channels has since been achieved in skeletal (Tanabe et al., 1987; Ellis et al., 1988) and cardiac muscle (Mikami et al., 1989).

The $\alpha 1$ subunit has a structure similar to the previously cloned pore forming a subunit of the voltage-gated Na^+ channels. It contains four repeated domains (I–IV) each of which includes six transmembrane segments (S1–S6) and a membrane-associated loop (The "P-loop") between segments S5 and S6 as indicated in Fig. 7.5. That the $\alpha 1$ subunit forms the ion-conducting pore is supported by the fact that expression of just this subunit is enough to produce function channels, albeit with unusual kinetics and voltage dependence (Perez-Reyes et al., 1989). The four repeated domains are also remarkably similar to the four subunits known to form the voltage-gated potassium channels. With the aid of the crystal structure of the potassium channel the basic structure of the $\alpha 1$ subunit is clear. However, prior to the existence of this crystal structure, a large number of structural and functional channels in the voltage-gated Na^+, K^+, and Ca^{2+} channels had already characterized the organization and role of many parts of the protein (see the review by Hofmann et al., 1999, for example). Each of the six transmembrane segments most likely form α-helices. The four repeated domains surround the ion-conducting pore, with the S5, S6, and P-loops lining the conduit. Much evidence discussed below, and analogy with the potassium channels implies that ion selectivity takes place in a region surrounded by the four P-loops. The S4 helix contains a number of arginine residues and is believed to act as the voltage sensor whose action is described in more detail below.

7.5.2 Pore Structure

Many lines of reasoning suggest that the pore must be narrow enough to force ions to pass in single file. Firstly, the blockage of currents carried by some ion types by other permeant ions is indicative of single file binding within the pore. The discrimination between different like-charged ion types requires close range interaction between the protein and the permeating ions. Next, the largest known ion to pass through the pore of L-type channels is tetramethylammonium,with a radius of ∼2.8 Å (McCleskey and Almers, 1985), suggesting that the pore acts as a sieve that is too narrow for larger ions to pass. As discussed below, the selectivity properties of the channel can best be explained assuming a single file pore in which ions cannot pass each other. Finally, the similarity to K^+ channels that have a known single file pore structure is persuasive.

Surprisingly, the first evidence for the regions of the pore that determine ion selectivity came from a study of sodium channels. Replacement of particular lysine or alanine residues in the P-loop of a voltage-gated sodium channel with glutamate residues changed the channel from being sodium selective to calcium selective (Heinemann et al., 1992). Not only did this suggest that this region of the pore was critical for ion selectivity, the fact that all calcium channels have conserved glutamate residues at this position suggested that it was these glutamates that are crucial for Ca^{2+} selectivity.

Further, site-directed mutagenesis indeed determined that four glutamate residues, one from each of the P-loop of the channel (often called the "EEEE locus"), were responsible for the high-affinity calcium binding site. Mutation of one or more of these had a significant effect on the channel selectivity (Kim et al., 1993; Yang et al., 1993; Ellinor et al., 1995; Parent and Gopalakrishnan, 1995; Bahinski et al., 1997). Removing any one of these residues was found to decrease the relative permeability and binding affinity of Ca^{2+}. Replacing all of these glutamate residues with either glutamine or alanine residues was found to remove the specificity of the channel for Ca^{2+} over Na^+ altogether and left only weak affinity for Ca^{2+} in the pore as shown in Fig. 7.6 (Ellinor et al., 1995). As no evidence of other high-affinity binding sites was apparent for divalent ions entering either end of the channel (Cibulsky and Sather, 2000) the EEEE locus was posited to be the sole origin of ion selectivity. Mutation of any one or any pair of the glutamate residues affected the binding affinity significantly, indicating that all four residues participated in the binding of even a single ion. Importantly, not all the glutamates are identical as the mutation of each has a slightly different effect on the blockage rate of ions in the pore. Presumably, this is a consequence of structural differences between the four repeated domains, although the functional importance of this nonequivalence is yet to be fully understood.

It is easier to make sense of how all four glutamate residues can contribute to creating a high-affinity binding region that can hold more than one ion by remembering that these binding sites can represent broad regions of local energy minimum. In this way, for example, the negative charge of the glutamate residues can create a

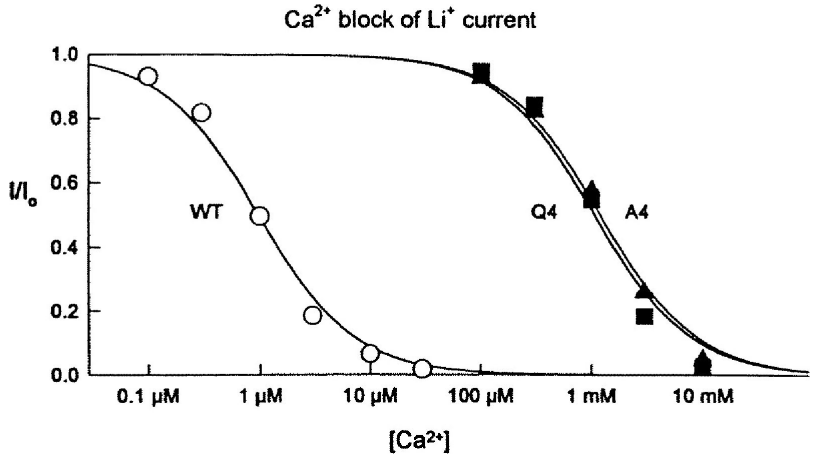

Fig. 7.6 The influence of quadruple mutants of the EEEE locus on high-affinity binding of Ca^{2+}. The fraction of Li^{+} current remaining is plotted against Ca^{2+} concentration for the wild-type (WT), quadruple glutamine (Q4, squares), and quadruple alanine (A4, triangles) mutants.

single attractive region of the pore that can hold multiple ions in a semistable equilibrium. In this model, mutation of any one of the charged residues would influence the binding affinity of both the first and second ion entering the pore.

The determination of the atomic structure of a bacterial potassium channel by X-ray crystallography (Doyle et al., 1998) also aided the understanding of calcium channel structures. The organization of the membrane spanning domains of voltage-gated calcium, potassium, and sodium channels are all quite similar, and the discovery that the P-loop formed the selectivity filter of potassium channels gave credence to the view that this region would also be important in calcium channels.

An important difference is evident in the structures of the potassium and calcium channel selectivity filters. Whereas ions are stabilized by the backbone carbonyl groups in voltage-gated potassium channels, most people believe that Ca^{2+} ions interact with the side chains of the glutamate residues in calcium channels. This belief is supported by a number of tests. Protons are found to be able to block Ca^{2+} currents (Root and MacKinnon, 1994), presumably by weakening electrostatic attraction of the pore. Point mutations within the EEEE locus suggested that multiple glutamate residues interacted with a single proton (Chen et al., 1996; Chen and Tsien, 1997; Klockner et al., 1996), suggesting both that these residues were in close proximity and that their charged side chains were accessible to the interior of the pore. Also, when individual residues in the EEEE locus were replaced with cysteine, the side chains of the cysteine residues appeared to be accessible to the solvent as they could become ionized to react with methanethiosulfonate (Koch et al., 2000; Wu et al., 2000). The bulky methanethiosulfonate groups also blocked the pore upon reaction, supporting the conclusion that the EEEE locus lay inside the pore.

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7.6 Theoretical Models of Permeation and Selectivity

7.6.1 Rate Theory

As the understanding of calcium channel permeation and selectivity was progressing through innovative experimental techniques, a number of theoretical models were also proposed to help elucidate the mechanisms underlying these properties. For a long time, especially when detailed structural information was lacking, the main approach utilized was rate theory. In this, the channel is represented as a series of energy wells and barriers, with each well representing an ion-binding site and its depth being proportional to the binding affinity. Ion conduction is described as the hopping of ions between the wells, with the rate of flux over each barrier related to the exponential of its height. All rate models include at least one binding site for calcium with an energy well depth of about 14 kT to account for the micro molar dissociation constant determined since micro molar concentrations of Ca^{2+} block monovalent currents.

When the multi-ion nature of permeation was determined the first models depicted this with two separate energy wells representing two binding sites, surrounded by two larger barriers as depicted in Fig. 7.7. A single ion in the pore could move between these sites, but not over the barrier to exit the channel (arrow in Fig. 7.7A). When a second ion entered and occupied the second site, however, it was proposed

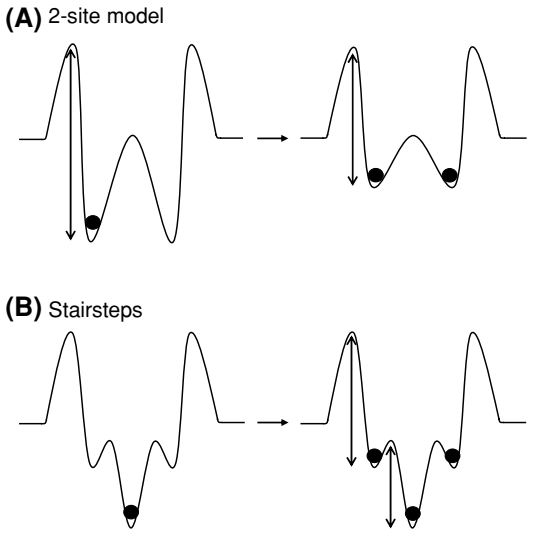

Fig. 7.7 Rate models of ion conduction. The energy landscape in the pore is represented by a series of energy wells and barriers. Ion conduction is slow for a single ion in the pore as it has to overcome a large energy barrier to move out of the channel (arrows). In the two site model (A), ion transit is increased due to interactions between ions in each site altering the energy landscape (B). In a "stair step" model (C and D) the ion can climb out of the deep energy well into low-affinity sites on either side.

that the repulsion between them alters the energy landscape and reduces the barrier for dissociation as shown in Fig. 7.7B (Almers and McCleskey, 1984; Hess and Tsien, 1984). As noted by Sather and McCleskey (2003), the nature of the ion–ion repulsion is ambiguous in this model as it deals only with the shape of the energy landscape not the source.

When it was discovered that there was only one binding region in the channel created by the EEEE locus, the idea of two similar binding sites had to be modified. A new model emerged in which there was only a single high-affinity binding site, but this was flanked by low-affinity sites that helped ions to step out of the large well (Kuo and Hess, 1993a; Dang and McCleskey, 1998) and the role of ion–ion interaction was relegated (Fig. 7.7C and D). Other mechanisms involving single sites have also been developed involving competition between calcium ions for the binding charges (Armstrong and Neyton, 1991; Yang et al., 1993). However, it is difficult within rate theory to accurately model a single binding region that is capable of holding many ions.

The rate theory models are surprisingly simple, yet they capture a number of the salient features of permeation and selectivity. In particular they helped to provide a simple explanation of how a multi-ion pore can utilize repulsion between ions to achieve both specificity and high throughput (cf. Fig. 7.4). However, these models can impart only limited information about the origins of specificity as the energy landscape is usually derived from measurements of binding affinities and is not directly related to the structure of the pore. For example, these models cannot impart information about which residues are responsible for ion binding and as no physical distances are used there is no direct connection between energy minima used in the theory and physical sites within the pore.

7.6.2 Physical Models

More recently there have been a large number of theoretical studies involving physical models of the channel protein. Information about the functional characteristics of the model is then determined by applying either a continuum theory, Brownian dynamics simulations, Monte-Carlo calculations, or molecular dynamics simulations.

In the first of these, Nonner and Eisenberg (1998) modeled the channel as a simple cylinder with conical vestibules and included the charge of the EEEE locus in the channel walls. By adjusting the diffusion coefficients and including an excess chemical potential parameter that helps ions to bind in the pore they are able to use the drift-diffusion equations (or Poisson–Nernst–Planck theory as it is often known) to reproduce the conductance and selectivity of the channel. They find that Ca^{2+} ions bind to a broad region in the interior of the pore due to the Coulomb potential of the glutamate residues as well as the excess chemical potential, and that the multi-ion repulsion may be important in speeding flux. Questions raised about the validity of the continuum description of ions in narrow pores (Moy et al., 2000; Corry et al., 2000a,b), the difficulty in treating ion–ion interactions in this model,

and the arbitrary nature of the adjustable diffusions and chemical parameters limit the impact of these conclusions.

A different explanation of the origins of ion selectivity than that proposed in the rate representation was proposed in two later studies employing different calculation schemes (Monte-Carlo simulations and a Mean Spherical Approximation method; Boda et al., 2000; Nonner et al., 2000). In these models eight partially charged oxygens comprising the glutamate side chains protrude into a cylindrical pore and can freely diffuse within a limited region of the channel. Cation binding is achieved due to the electrostatic attraction of the negatively charged oxygens. Selectivity of Ca^{2+} ions is proposed to be a result of ions competing to achieve charge neutrality in a selectivity filter having finite space. Ca^{2+} ions are preferred to Na^{+} in the model as they have the same charge neutralizing effect as two Na^{+} ions while occupying less of the limited volume of the filter. Essentially, not enough Na^{+} ions can be squeezed in to the filter to achieve charge neutrality. This charge–space competition model was analyzed further by Yang et al. (2003), who conducted nonequilibrium molecular dynamics simulations of a model pore containing half-charged oxygens in an atomistic cylinder. In these simulations a second Ca^{2+} ion is required to release a Ca^{2+} already bound in the channel, but cation binding was found to be nonselective.

A simple version of the single file ion–ion repulsion model of permeation and selectivity has been presented by Corry and coworkers who simulate the trajectories of ions moving in and around models of the channel protein (Corry et al., 2000c, 2001, 2005). For this purpose, Brownian dynamics simulations are used in which the ions pass through the channel in a random walk subject to electrical forces. In these studies a rigid model of the channel is derived from analogy to other channels and a variety of experimental data. The most important aspect of the channel model is that it includes a relatively narrow region in which ions cannot pass each other, surrounded by the four glutamate residues as shown in Fig. 7.8. Unlike the previous models, the glutamate charges do not compete for space within the pore, but the concentration of negative charge does attract cations within this region. Indeed the electrostatic attraction of the protein is all that is required to account for ion permeation and selectivity in this model. The charge of the glutamate residues creates a deep energy well that strongly attracts multiple ions. In a process akin to that suggested by earlier rate models, repulsion between two resident Ca^{2+} ions is found to speed their exit as illustrated in Fig. 7.4. Because the divalent Ca^{2+} ions are more strongly attracted by the channel, they can displace Na^{+} to occupy this region. Once there, the Ca^{2+} can only be moved by the repulsion from another divalent ion and not by the lesser repulsion from Na^{+} (Fig. 7.4). As well as providing a simple mechanism for ion selectivity depicted schematically in Fig. 7.4, this model was also used to calculate the conductance of the channel in a variety of situations and replicate and explain the observed I–V curves, current concentration curves, anomalous mole fraction behavior between Na^{+} and Ca^{2+} (Fig. 7.8B) as well as the attenuation of Ca^{2+} currents by Na^{+}.

All the models described thus far assume simplified pore geometries, but one goal of ion channel modeling is to include all the atomistic detail of the pore in

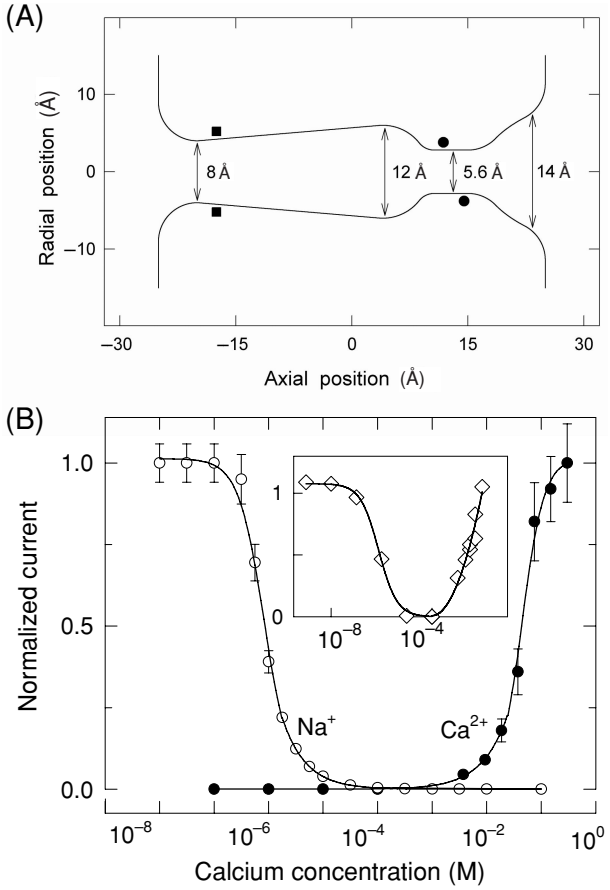

Fig. 7.8 Brownian dynamics model of an L-type calcium channel. (A) The channel shape contains a narrow portion of the pore surrounded by the charge from the four glutamate residues (circles). (B) Results from Brownian dynamics simulations in mixed Na^+ and Ca^{2+} solution reproduce the "anomalous mole fraction" behavior seen in the experimental data (Almers et al., 1984). Figures from Corry et al. (2001).

molecular simulations. Some caution should be applied when taking this approach, however, as the accuracy of an atomistic model cannot be determined until an atomic resolution structure is determined experimentally. A first attempt to make a molecular model of the outer vestibule of an N-type calcium channel was presented by Doughty et al. (1995), who suggested a β-hairpin motif for the P-loop segment of the protein. In contrast, more recent models suggest that the one half of the P-loop actually forms an α-helix (Guy and Durrel, 1995; Lipkind and Fozzard, 2001; Barreiro et al., 2002). Lipkind and Fozzard (2001) created an atomic resolution homology model of the outer vestibule and filter of an L-type channel and determined the likely positions of ions, and the resulting electrostatic fields, within it. The model, based on the KcsA crystal structure, included the P-loops as α-helix-turn-β-strand motifs, with

the glutamate residues of the EEEE motif located at the turns. The glutamate side chains point into the pore and form a 6 Å wide conduit in the absence of Ca^{2+}. With Ca^{2+} present the glutamate residues move inward and form an electrostatic trap that firmly holds a single calcium ion. At millimolar concentrations they find that two additional ions can be bound either side of the first Ca^{2+}, and this enables Ca^{2+} to escape the electrostatic trap and permeate through the channel.

A second model of the P-loop has also been made that also places the important glutamate residues of the EEEE locus at a bend at the end of an α helical segment, but places them asymmetrically in two distinguishable planes to account for the asymmetries seen in site-directed mutagenesis (Barreiro et al., 2002). Molecular dynamics simulations are carried out on this model pore containing either two Ca^{2+} or one Ca^{2+} and one Na^{+}. In a 100-ps simulation they find that the two Ca^{2+} move apart but the Ca^{2+} and Na^{+} do not. It is hypothesized that Na^{+} cannot displace Ca^{2+} but that another Ca^{2+} can, similar to the results seen in the Brownian dynamics model, although the simulations may be too short to asses the significance of these conclusions. This mechanism of permeation and selectivity is supported by a second study into the potential of mean force of Na^{+} or Ca^{2+} in this channel model (Barreiro et al., 2003).

Ramakrishnan et al. (2004) also carry out nonequilibrium molecular dynamics simulations of a much simpler model pore containing glutamate residues in a β-barrel scaffold. Apparent off rates when the pore was preloaded with three Na^{+} ions are much higher than when the channel contained one Ca^{2+} and two Na^{+}, consistent with Ca^{2+} blockage. As in the other models, the presence of multiple Ca^{2+} ions was able to release this block.

The rate theory and physical models all share some common elements. Permeation involves multiple ions, most likely two ions for divalent ion conduction or three ions for monovalent ion conduction. Divalent ions are strongly attracted by the glutamates in the P-loops and this results in divalent blockage of monovalent ion currents.

These models present two similar but distinct physical mechanisms underlying ion selectivity. In both, the electrostatic attraction of the negatively charged glutamate residues is essential for attracting multiple ions into the channel and the size of the pore is such that ions cannot pass each other. In this way, the glutamate residues create an attractive binding region. We avoid using the term "binding site" here as the physical models all suggest a broader electrostatic attraction rather than any kind of chemosteric binding. The difference between the explanations of selectivity lies in whether this electrostatic attraction is of itself enough to create selectivity or whether a specific volume and flexibility of the pore is also necessary.

In the simpler "electrostatic" model of ion selectivity, Ca^{2+} is bound more strongly by the channel due to its greater charge and can only be removed by the repulsion of a similarly charged ion as represented in Fig. 7.4. In the "charge–volume" model, the volume of the pore must be such that competition for space as well as electrostatic attraction is important. Given the limited structural information available it is difficult to say exactly which, if either, of these mechanisms is correct.

An advantage of the simpler model is that less is required; it sets much weaker limits on the size and flexibility of the pore. The only geometric limitation required is that permeation through the selectivity filter occurs in single file. In both cases the charge surrounding this region needs to be appropriate to hold two divalent ions in a semistable equilibrium. The simpler electrostatic model also stresses a common theme that has arisen out of rate theory, continuum and simulation models, some of which are able to elegantly reproduce and explain a wealth of physiological data for a variety of different channel types. It appears that the extra complication of setting limits on the channel volume and flexibility, and appealing to charge–space competition to explain selectivity is not necessary.

It is of interest in this context to compare models of selectivity in calcium channel with explanations of valence selectivity in Na^+ and K^+ channels. As noted in the models of the calcium channel, the discrimination between monovalent and divalent cations cannot just rely on presence of a net negative charge as this acts to attract both kinds of ion. Rather, selectivity between monovalent and divalent ions appears to rely on the exact strength of attraction to the protein (or binding affinity) of the permeating ions. A divalent ion has a stronger electrostatic interaction with any charge on the protein than a monovalent ion due to its larger charge. A recent study suggests that this fact on its own appears to be enough to explain how potassium channels are blocked by Ba^{2+}. When Ba^{2+} enters the selectivity filter it is electrostatically bound such that it is unlikely to leave, even with the aid of repulsion from nearby cations (Corry et al., 2005). Electrostatic calculations in model calcium channels suggest that the binding of divalent ions is not as strong as in the potassium channel (Corry et al., 2000c, 2001, 2005). In this case, once a divalent ion enters it can only be forced out with the aid of the Coulomb repulsion from a second divalent ion, but not with the weaker repulsion from a monovalent ion. Divalent ions are known to block sodium channels, but with a much lower affinity and duration than in potassium channels (Taylor et al., 1976; French et al., 1994). Electrostatic calculations on a model sodium channel (Vora et al., 2004; Corry et al., 2005) find that divalent binding is much weaker than in either the calcium or potassium channels. In this case once a divalent ion enters the channel, additional ions are not attracted into the channel to help relieve the channel blockage through ion–ion repulsion. But also, being less strongly bound, the divalent ion dwell time is much shorter than for potassium channels and so divalent block is less effective.

7.7 Channel Gating

7.7.1 Voltage-Activated Gating

As the name suggest, voltage-dependent calcium channels open in response to depolarization. The open probability rises to a maximum at large depolarizations and is generally faster in muscle than in neuron. Fitting the open probability with a Boltzmann factor, it can be estimated that three to five gating charges move across

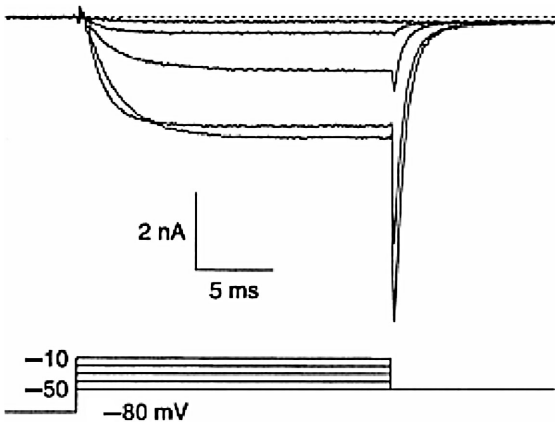

Fig. 7.9 Activation kinetics of whole cell inward calcium currents in frog sympathetic neurons. Measurements are made with 2 mM Ba^{2+}, and the largest currents are at −20 mV and the largest tail currents at −50 mV. Figure reproduced with permission from Jones (1998).

the electric field of the membrane during activation (Kostyuk et al., 1988; Coulter et al., 1989). The opening of calcium channels does not happen instantaneously after depolarization, but rather after a delay of one or more milliseconds depending on the channel type and membrane potential as illustrated in Fig. 7.9. This is generally taken to mean that the channel protein must move through many closed conformations before channel opening occurs.

Open channels are normally deactivated immediately upon repolarization of the membrane. Notably, a transient tail current, observed as a spike in the current immediately after repolarization, occurs before conduction ceases. As noted by Jones (1998), this tail current may serve an important functional role, increasing the calcium intake upon hyperpolarization following an action potential. Measured tail currents typically follow either a single or double exponential time course, however the double exponential behavior has been shown in many cases to be the result of two or more types of Ca channel closing at different rates (Matteson and Armstrong, 1986; Swandulla and Armstrong, 1998).

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There also appear to be a number of means by which calcium currents into cells can be increased in a process known as facilitation. An increase in calcium currents created by depolarizing prepulses was first noticed in bovine chromaffin cells by Fenwick et al. (1982). More recent examinations have demonstrated an almost doubling of Ca^{2+} currents created by such prepulses (Artalejo et al., 1990). These enhanced currents may have a functional role in creating enhanced neurotransmitter secretion or muscle contraction in response to danger or stress. A possible contributor to this effect is delayed channel deactivation that has been noted for some HVA channels previously subjected to large depolarization (Pietrobon and Hess, 1990), in particular for P-type (McFarlane, 1997) and L-type channels (Sculptoreanu et al., 1993) where it is believed to intensify muscle contraction. In extreme cases, continual

calcium channel activity has been seen for many seconds after a series of large depolarizations (Cloues et al., 1997). It has been suggested that such behavior could be a result of voltage-dependent phosphorylation of the channel protein or of changing the kinetics of the voltage sensor, and may be related to the "mode switching" behavior described below.

As described in the chapter by Bezanilla, the S4 segment of the $\alpha 1$ subunit is believed to be the voltage sensor for channel gating, moving across the electric field of the membrane and causing a conformational change of the protein to open the pore. There have been many fewer studies of gating of Ca^{2+} channels than K^+ or Na^+ channels, however, the sequence similarities between the S4 regions means that many of the insights gleaned from studies on the K^+ or Na^+ channels are likely to apply to Ca channels as well. By similarity with K^+ channels it is expected that the channel gate lies at the internal end of the S6 helices, which most likely form a narrowing of the pore in the closed state.

This internal location is also supported by studying blockage of Ca^{2+} currents by Cd^{2+}, which suggest an internal gate as well as a possible gate at the external end of the pore. Cd^{2+} is thought to tightly bind in the selectivity filter in the same high-affinity binding site utilized by Ca^{2+}. Cd^{2+} blockage of the open channel is voltage-dependent: at large hyperpolarizing or depolarizing voltages block becomes alleviated as if Cd^{2+} can be forced out either end of the pore (Thévenod and Jones, 1992; Swandulla and Armstrong, 1999). Block of the resting closed channel is not alleviated at large hyperpolarizing voltages as if Cd^{2+} cannot easily exit the internal end of the pore. But, when Cd^{2+} is applied to closed channels, blockage is much slower than in open channels, and relief of block is much slower if Cd^{2+} is washed out while the channels are closed. Together these data suggest that Cd^{2+} cannot easily enter either end of the pore in the closed state (Thévenod and Jones, 1992). Jones (2003) raises an interesting question in regard to these data. If Cd^{2+} can still exit a closed channel, albeit at a much slower rate than an open channel, is the closed channel really closed? Could for example small Ca^{2+} currents also pass through a closed pore? The question is not as absurd as it sounds, as a small leakage current of only around 1% of the open channel current would be difficult to detect in single channel recordings, but could have important physiological consequences.

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7.7.2 Mode Switching

L-type channels have also been observed to have another interesting gating behavior known as "mode switching." Typically, a single channel will open and close many times during a prolonged depolarization, with each opening lasting less than 1 ms on average. But, on occasion there are times when there is a series of much longer channel openings as if the channel deactivation was 100 times slower. The typical behavior has been termed "mode 1", while the long channel openings is called "mode 2" (Nowycky et al., 1985a; Hess et al., 1986). At other times channel opening appears to cease altogether ("mode 0"). Most likely, there is some physical change taking place to create this mode switching behavior, such as a conformational change or

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chemical modification of important amino acid residues. This is supported by evidence that antagonistic drugs such as dihydropyridine appear to bind more strongly to channels in mode 0, and help stabilize that state, whereas agonists bind most favorably during mode 2. It has been demonstrated that phosphorylation (most likely near the C-terminus of the $\alpha 1$ subunit) can increase the open probability (Bean et al., 1984) and the mean open time of channel opening (Yue et al., 1990) and this may be related to the mode switching behavior (Dolphin, 1996).

7.8 Inactivation of HVA Channels

The influx of calcium is essential for initiating cellular responses; however, these responses must be controlled within a narrow range of calcium concentrations as intracellular calcium is toxic in excessive levels. For this reason calcium entry is tightly controlled in a number of feedback mechanisms. During prolonged periods of depolarization the initial channel activity is lost in a process known as inactivation that prevents the excess buildup of intracellular calcium. Inactivation can be either voltage-dependent, or alternatively calcium influx can also be controlled by the concentrations of internal calcium that can inactivate channels, or create increased currents (facilitation).

7.8.1 Voltage-Dependent Inactivation

Ca channels can be inactivated in a Ca^{2+}-independent, voltage-dependent manner. For L-type calcium channels of muscle the rate of voltage-dependent inactivation is much slower than Ca-dependent inactivation (Kass and Sanguinetti, 1984; Giannattasio et al., 1991), but this is not necessarily the case for other types of HVA channel (Werz et al., 1993; Zhang et al., 1993). Some N-type channels, for example, show no voltage-dependent inactivation (Artalejo et al., 1992), while others convert between inactivating and noninactivating modes (Plummer and Hess, 1991). Most likely these modes are caused by structural or chemical alterations in the protein.

Repeated short depolarizing bursts can also induce rapid inactivation (Patil et al., 1998). It has been proposed that inactivation occurs preferentially from a partially activated state in which some of the voltage sensors have moved to the activated position, but the channel remains closed (Klemic et al., 1998; Patil et al., 1998). While long depolarizing pulses tend to drive all the channels to the fully open state, short repeated pulses might place more channels in this partially activated state and increase the likelihood of inactivation. This possibility could also explain the inactivating and noninactivating modes of the N-type channel and why N-type channel inactivation is maximal at voltages that also produce maximal inward currents (Jones and Marks, 1989).

In many K^+ and Na^+ channels, inactivation has been demonstrated to take place via a "ball and chain" mechanism in which the amino terminus of the α subunit acts as a tethered ball that can enter the channel, occluding the pore and

blocking conduction. Recent evidence suggests that a similar mechanism might form the basis of voltage-dependent inactivation in HVA calcium channels (Stotz et al., 2004). Considerable evidence suggests that both the intracellular ends of the S6 helices (see for example, Kraus et al., 2000; Stotz et al., 2000; Stotz and Zamponi, 2001) and the linker between domains I and II (the AID region; Herlitz et al., 1997; Cens et al., 1999; Berrou et al., 2001) are involved in voltage-dependent inactivation. The simplest way to reconcile these facts is that the S6 helices form a docking site for the I–II linker that when bound partially occludes the pore. Further evidence suggests that the channel β subunit, as well as the N, C, and III–IV linkers can influence inactivation kinetics by interacting with the I–II domain (Stotz et al., 2004). Recent crystal structures of the β subunit–AID complex represent the first detailed pictures of calcium channel components and indicate that binding occurs by the formation of a deep hydrophobic groove in the β subunit that can associate with α1 (Chen et al., 2004; Van Petegem et al., 2004). Furthermore, the structure of the β subunit itself indicates how cellular signaling molecules could interact with the β subunit and therefore influence the kinetics of channel inactivation by altering the interaction with α1.

7.8.2 Ca-Dependent Inactivation and Facilitation

Ca^{2+}-dependent inactivation (CDI) was initially observed in L-type calcium channels from muscle where it was found that inactivation was faster in solutions containing Ca^{2+} than Ba^{2+}. In muscle, CDI is rapid ($\tau \sim 20$ ms). In neurons, CDI of L-type channels can be either fast (Kohr and Mody, 1991) or slow (Heidelberger and Matthews, 1992) depending on the situation. For many years non-L-type HVA channels were believed to not undergo CDI. It recently became apparent, however, that one of the reasons that CDI was not observed was that most recordings were made in solutions containing high levels of the calcium buffer EGTA. When this was dropped to more physiological conditions calcium-dependent regulation has been observed in P/Q- type channels (Lee et al., 1999; DeMaria et al., 2001; Chaudhuri et al., 2005) and more recently N- and R-type channels (Liang et al., 2003). The reasons for EGTA masking CDI in non-L-type channels, but not in L-type channels is due to the differing molecular mechanisms involved as described below. In addition to CDI, it has been shown that non-L-type HVA calcium channels also have a mechanism of calcium-dependent facilitation (CDF), in which calcium influx is amplified by internal calcium concentrations. The study of CDI has progressed significantly in the last few years, and it is beyond the scope of this chapter to cover all the history of the area. For an introduction to the field, the reader is referred to an excellent review (Budde et al., 2002).

Early attempts to understand the mechanisms of CDI revolved around physiological studies of L-type channels. CDI was observed in single channel recordings, suggesting that Ca^{2+} can inactivate the same channel through which it enters the cell (Yue et al., 1990). Buffering of internal Ca^{2+} concentrations by BAPTA reduces, but does not eliminate inactivation (Ginnattasio et al., 1991) as if calcium from the

same or nearby channels (Yue et al., 1990) is inactivating the channels before being chelated by the buffer.

A detailed understanding of the phenomenon of CDI and CDF only took place when calmodulin (CaM) was identified as the Ca^{2+} sensor (Lee et al., 1999; Peterson et al., 1999; Qin et al., 1999; Zühlke et al., 1999). The basic principle of both CDI and CDF appears to involve the specific binding characteristics of CaM in its different Ca^{2+} loaded states. Inactivation and facilitation are inhibited when calmodulin is bound to the channel in its unloaded form. Local or global changes in calcium concentration load the CaM in different ways, which initiate different binding patterns between the CaM and the $\alpha 1$ subunit C-terminus which promote either CDI or CDF.

The location of CaM binding has now been pinpointed to two regions of the C-terminal domain of the $\alpha 1$ subunit: a calmodulin binding domain (CBD) and an isoleucine-glutamine (IQ) or similar motif as mutations of these regions influence CDI and CDF (Qin et al., 1999; Zühlke et al., 2000; DeMaria et al., 2001; Lee et al., 2003; Kobrinsky et al., 2005). The details of CaM binding are beginning to reveal the mechanism of Ca^{2+} regulation and the differences between L- and non-L-type inactivation. CDI and CDF are believed to be regulated independently by the binding of Ca^{2+} to the N-terminal or C-terminal CaM lobes (N-lobe and C-lobe respectively). Binding of Ca^{2+} to the C-lobe takes place at high affinity while the binding affinity of Ca^{2+} to the N-lobe is much weaker (Wang, 1985; Johnson et al., 1996). For this reason, C-lobe binding can reflect local changes in Ca^{2+} concentrations created by local influx through calcium channels, while N-lobe binding is likely to take place only once the global concentration, resulting from the influx through many channels, has increased significantly.

Inactivation is triggered by the binding of Ca^{2+} to the opposite lobes in the two classes of channel. In L-type channels, CDI is governed by binding of Ca^{2+} to the C-lobe (Peterson et al., 1999), whereas in non-L-type channels it is regulated by binding to the N-lobe (DeMaria et al., 2001; Lee et al., 2003; Liang et al., 2003). CDF in non-L-type channels, on the other hand, appears to involve binding to the C-lobe (Lee et al., 2003). Although CDF is not usually detected in L-type channels, a CaM mutation that limits Ca^{2+} binding to the C-lobe promotes clear CDF as if it arises through Ca^{2+} binding via the N-lobe (Van Petegem et al., 2005). The presence of the fast acting Ca^{2+} chelator BAPTA influences both CDI and CDF in all channel types. The slower acting by high-affinity chelator EGTA, on the other hand, prevents CDF in L-type channels and CDI in non-L-type channels. The sensitivities to these chelators support the conclusion that the C-lobe processes are triggered by local Ca^{2+} which can be rapidly intercepted by BAPTA but not by EGTA. N-lobe processes appear to be the result of global Ca^{2+} influx (Lee et al., 2003; Liang et al., 2003). The different responses of CaM to local and global Ca^{2+} concentrations allow the voltage-gated calcium channels to accurately control calcium influx.

The exact details of CaM binding to the $\alpha 1$ subunit and the differences in binding between the unloaded, C-lobe loaded, and fully loaded CaM are still being worked out, but an interesting mechanism for regulating CDI and CDF in P/Q-type

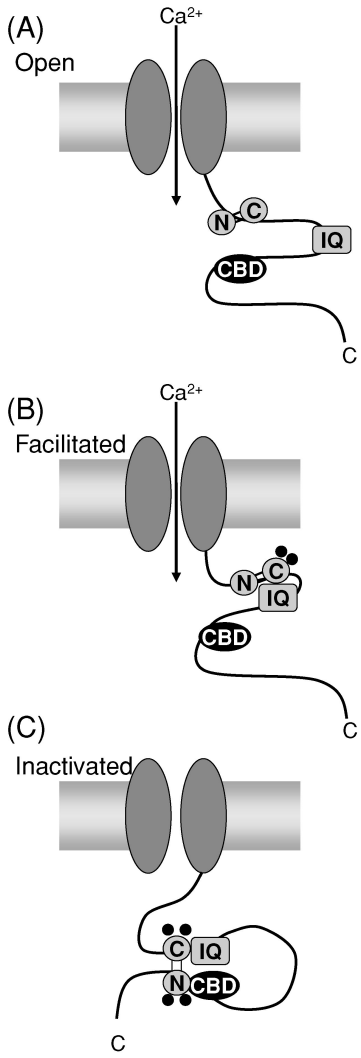

Fig. 7.10 Model of Ca^{2+} binding and the initiation of facilitation and inactivation in $Ca_v2.1$ channels. (A) At rest, no Ca^{2+} is bound to the calmodulin lobes N and C. (B) Low levels of Ca^{2+} prompt binding to the C-lobe which strengthens or initiates the interaction between calmodulin and the IQ domain creating facilitation. (C) High levels of intracellular Ca^{2+} lead to additional binding to the N-lobe and a conformational change enabling interactions of calmodulin with both the IQ and CBD domains and initiates inactivation.

channels has been suggested as depicted in Fig. 7.10 (Lee et al., 2003). In the resting state, with low intracellular calcium, CaM is believed to be preassociated with the α_1 C-terminus (Fig. 7.10A; Erickson et al., 2001). Local influx through the channel promotes Ca^{2+} binding to the C-lobe of the preassociated CaM which either strengthens or initiates binding of CaM to the IQ domain and initiates CDF

(Fig. 7.10B). A global increase in Ca^{2+} encourages Ca^{2+} binding to the CaM N-lobe. The fully loaded CaM then binds to the CBD domain (while possibly remaining bound to the IQ domain) creating a conformational change that promotes inactivation (Fig. 7.10C).

A recent structure of $Ca2^{+}$/CaM bound to the calcium channel IQ domain indicates the details of CaM–α1 interactions (Van Petegem et al., 2005). CaM is seen to bind to the IQ domain primarily through anchoring of a set of aromatic residues in the C-terminus into hydrophobic pockets of CaM. As predicted, C-lobe CaM interactions appeared to be of higher affinity than N-lobe interactions. It is suggested that the difference in CDI and CDF between channel subtypes may be the result of differing CaM anchor positions and the interplay between CDI and CDF appears to arise from competition for binding between Ca^{2+}-loaded N and C-lobes.

One of the big remaining questions in this area is how the binding or unbinding of calmodulin is linked to changes in the channel pore. What are the conformational changes induced by partially or fully loaded CaM? Does CDI utilize the same inactivation gate as voltage-dependent inactivation? If so, what is the link between the C-terminus and the I–II linker of the α1 subunit? What are the differences between L-type and non-L-type channels that are responsible for the opposite effects of Ca^{2+} binding to the CaM C-lobes? How is the calcium influx enhanced by CDF? It should also be noted that in addition to the calmodulin-dependent inactivation mechanisms, it has been suggested that calcium inhibits the channel via activation of protein phosphatase 2B and/or by inhibition of adenylate cyclase (Schuhmann et al., 1997a; You et al., 1997).

7.8.3 Gating of LVA Channels

LVA channels have different activation, deactivation, and inactivation kinetics than HVA channels. As described earlier, activation arises at more negative potentials. Inactivation is very rapid in these channels ($\tau \sim 10$ to 50 ms) and also arises at more negative potentials (Huguenard, 1996). In contrast, LVA channels are slower to activate than HVA channels and are also 10-fold slower to deactivate upon hyperpolarization (Armstrong and Matteson, 1985).

7.9 Regulation of Channel Function

Many factors influence the function of voltage-gated calcium channels so that biochemical pathways can influence intracellular calcium levels. The electrochemical gradient associated with varying concentrations of ions across the plasma membrane, notably calcium ions and H^{+} ions, influences channel gating, activation, and inactivation as described previously. It is well recognized that voltage-gated Ca^{2+} channel function is also regulated by many serine–threonine and tyrosine kinases as a result of direct phosphorylation of the channel protein. However, more recently there is

good evidence that the cell's reduction–oxidation state can also influence channel function. In this section the factors that influence channel function will be discussed with particular emphasis on the effects of oxygen and oxygen metabolites on the redox state of the channel protein and its function.

Acidosis has been reported to inhibit L-type Ca^{2+} channels by reducing channel availability whereas alkalosis increases channel availability (Klockner and Isenberg, 1994). The β subunit of the channel complex may be required for this effect (Schuhmann et al., 1997b), as if chemical modifications in the β subunit may affect its interaction with α1.

The α_1 subunit is phosphorylated by cAMP-dependent protein kinase A at Ser-1928 near the carboxy terminus and this results in an increase in the macroscopic peak inward current while slowing the rate of inactivation (McDonald et al., 1994). It is now recognized that this response requires a direct association of protein kinase A with the channel via a leucine zipper interaction with a kinase anchoring protein (Hulme et al., 2003). Protein kinase G inhibits both basal channel activity and the isoproterenol stimulated current in a voltage-dependent manner that may involve competition with cAMP (Wahler et al., 1990). Tyrosine kinase activation can increase channel activity in smooth muscle (Wijetunge and Hughes, 1995) and mediates α-adrenergic receptor inhibition of β-adrenergically stimulated current at the level of the β-adrenergic receptor in cardiac myocytes probably by phosphorylation of the β-adrenergic receptor (Hool et al., 1998; Belevych et al., 2001).

Activators of protein kinase C (PKC) have been reported to have no effect (Lacerda et al., 1988; Mamas and Terrar, 2001), inhibit (Tseng and Boyden, 1991; Zhang et al., 1997; McHugh et al., 2000), ~~and~~ stimulate (Dosemeci et al., 1988; He et al., 2000; Aiello and Cingolani, 2001; Alden et al., 2002; Blumenstein et al., 2002) basal channel activity while decreasing β-adrenergic receptor sensitivity of the channel (Schwartz and Naff, 1997; Belevych et al., 2004). The variable responses with pharmacological activators of PKC (such as phorbol esters) may be due to nondiscrimatory activation of all PKC isoforms. PKC is a large family of lipid-derived serine/threonine kinases that are classified according to their mode of activation and homology in the regulatory domain. The classical isoforms (cPKC) are activated by Ca^{2+} and diacylglycerol (DAG). This group are the most abundant and comprise the α, β_I, β_{II}, and γ isozymes. β_I and β_{II} are C-terminal splice variants of the same gene that differ by 50 amino acids. The novel PKCs (nPKC) comprise ε, η, δ, and θ PKC and are activated by DAG but not Ca^{2+}. The atypical PKCs (aPKC) are dependent on lipids but do not require DAG or Ca^{2+} for activation. Isoforms appear to be restricted to particular cell sites before stimulation and stimulation results in the localization of isoforms to specific subcellular sites. For example, stimulation of cardiac myocytes with an α-adrenergic receptor agonist causes the translocation of β_I PKC from the cytosol to the nucleus and β_{II} PKC from fibrillar structures outside the nucleus to the membrane (Mochly-Rosen et al., 1990; Disatnik et al., 1994; Mochly-Rosen, 1995). The translocation is then assumed to be associated with specific function at the site. The localization of activated isoforms requires protein–protein interactions between the isoforms and anchoring proteins. These anchoring proteins bind unique sites

on each isoforms and thereby regulate the activity of individual isoforms. It would appear that different isoforms of PKC produce variable responses depending on the level of expression and activity in tissue. The use of specific peptide inhibitors of isoforms has helped to elucidate the role of PKC in physiological and pathological processes. Zhang et al. (1997) exploited the fact that peptides can be delivered into myocytes via the patch-pipette, and recorded the effect of phorbol esters and peptide inhibitors of cPKC isozymes on basal and isoproterenol-stimulated L-type Ca^{2+} channel currents. The authors found that phorbol esters inhibit basal L-type Ca^{2+} current and dialysis of the cell with a peptide inhibitor of cPKC isozymes (βC2-2) partially attenuated the basal inhibition by PMA. In addition, PMA inhibited the isoproterenol-stimulated L-type Ca^{2+} current. Addition of βC2-2 and βC2-4 peptides to the patch pipette resulted in 89% attenuation of the effect of PMA. These results suggest that C2 containing PKC isozymes are involved in the regulation of basal L-type Ca^{2+} channel current and isoproterenol-stimulated L-type Ca^{2+} currents.

Many hormones and neurotransmitters regulate channel function by binding to G proteins that themselves regulate channel function via activation of kinases and second messengers. However, it is well recognized that P/Q-type and N-type Ca^{2+} channels are directly coupled to and regulated by G proteins. This occurs as a result of direct interaction between the G protein βγ complex and the α_1 subunit at the α_1 interaction domain AID that may disturb the interaction with the β subunit (Herlitze et al., 1996; Ikeda, 1996; De Waard et al., 1997). The small G protein kir/Gem inhibits calcium channels by interacting directly with the β subunit and decreasing α_1-subunit expression (Beguin et al., 2001). This occurs with the binding of Ca^{2+}/CaM. The direct coupling of L-type Ca^{2+} channels to G_s proteins has been reported (Yatani et al., 1987). Of the T-type Ca^{2+} channels, only α_{1H} but not α_{1G} can be inhibited by activation of G protein $\beta_2\gamma_2$ subunits that bind to the intracellular loop connecting domains II and III suggesting unique control of channel function by G protein βγ subunits in this class of channel (Wolfe et al., 2003).

More recently, it has been demonstrated that the channel can respond to changes in oxygen (O_2) tension raising the possibility that the channel itself is an O_2 sensor. It is assumed that acute changes in O_2 tension will result in adaptive responses that are designed to restore O_2 to tissue and maintain normal cellular function in mammals. This is true of cardiorespiratory reflexes in the brain that respond rapidly to discharge levels from afferent chemosensory fibers in the carotid body as a result of alterations in blood levels of O_2, CO_2, or pH. However, in the heart, an acute change in cellular oxygen tension represents a trigger for cardiac arrhythmia where an appropriate substrate such as myocardial infarction or a defect in a gene encoding an ion channel exists (Keating and Sanguinetti, 2001; Sanguinetti, 2002). Therefore, it would appear at least for cardiac myocytes that the cellular response to changes in oxygen tension is not always adaptive. Understanding how the myocyte senses changes in oxygen tension is important in determining treatment strategies to prevent life-threatening arrhythmias.

7. Calcium Channels

Ion channels have been considered O_2 sensors because their modulation by hypoxia is rapid and occurs in excised membrane patches where cytosolic variables such as second messengers, ATP, and Ca^{2+} are absent. A number of studies have reported modulations in ion channel activity during hypoxia, the first being the demonstration of O_2-sensitive K^+ channels in glomus or type I cells of the rabbit carotid body (Lopez-Barneo et al., 1988). The closure of K^+ channels by hypoxia results in membrane depolarization and Ca^{2+} influx, transmitter release to the innervated organ and activation of afferent sensory fibers. This is not the case however in some pulmonary resistance vessel myocytes where hypoxia increases Ca^{2+} channel conductance (Franco-Obregon and Lopez-Barneo, 1996a). In fact, ion channel responses to hypoxia appear to vary depending on the cell type and the functional response. In arterial smooth muscle cells, hypoxia appears to inhibit L-type Ca^{2+} channels resulting in relaxation of vessels (Franco-Obregon et al., 1995; Franco-Obregon and Lopez-Barneo, 1996b).

In cardiac myocytes, there is good evidence that hypoxia inhibits the L-type Ca^{2+} channel. This has been demonstrated in the α_{1C} subunit of the human cardiac L-type Ca^{2+} channel recombinant in HEK 293 cells (Fearon et al., 1997) and in native channels in guinea-pig cardiac myocytes (Fig. 7.11; Hool, 2000, 2001) suggesting that there is a requirement for the α_{1C} subunit of the channel in the hypoxia response. In search of the identity of the O_2-sensing site, Fearon et al. (2000) produced splice variants of the α_{1C} subunit of the human cardiac L-type Ca^{2+} channel and examined the O_2 sensitivity of the variants. They were able to isolate a 39 amino acid segment of the C-terminal domain of the subunit as the O_2-sensitive component. However, a direct effect of changes in O_2 tension on the channel protein has not been demonstrated. In addition, the varied responses by ion channels to changes in O_2 tension has made it difficult to assign a universal O_2-sensing component to the channel.

A clue to the mechanism of O_2 sensing arises from the fact that the function of the α_{1C} subunit can be modulated by reduction or oxidation of critical thiol groups (Chiamvimonvat et al., 1995; Hu et al., 1997; Fearon et al., 1999). This has also been demonstrated in native channels (Campbell et al., 1996; Hool, 2000, 2001; Hool and Arthur, 2002). In addition, the reactive oxygen species superoxide and H_2O_2 can modulate ion channel function (Anzai et al., 2000; Hool and Arthur, 2002; Liu and Gutterman, 2002; Lebuffe et al., 2003). Since the channel does not possess an O_2 binding domain it is more likely that the O_2-sensitive region on the C-terminal domain of the α_{1C} subunit contains a number of critical thiol groups that can be modified in response to alterations in cellular redox state occurring during hypoxia. Consistent with this, we have shown that exposure of myocytes to thiol-specific reducing agent dithiothreitol mimics the effect of hypoxia on the channel while the oxidizing agent 5,5'-dithio-bis[2-nitrobenzoic acid](DTNB) attenuates the effect of hypoxia. In addition to decreasing basal channel activity, hypoxia increases the sensitivity of the channel to β-adrenergic receptor stimulation (Hool, 2000, 2001; Hool and Arthur, 2002). In the presence of hypoxia, the $K_{0.5}$ for activation of the channel by the β-adrenergic receptor agonist isoproterenol is decreased from 5.1 nM to 1.6 nM. We used the response of the channel to the β-adrenergic receptor agonist

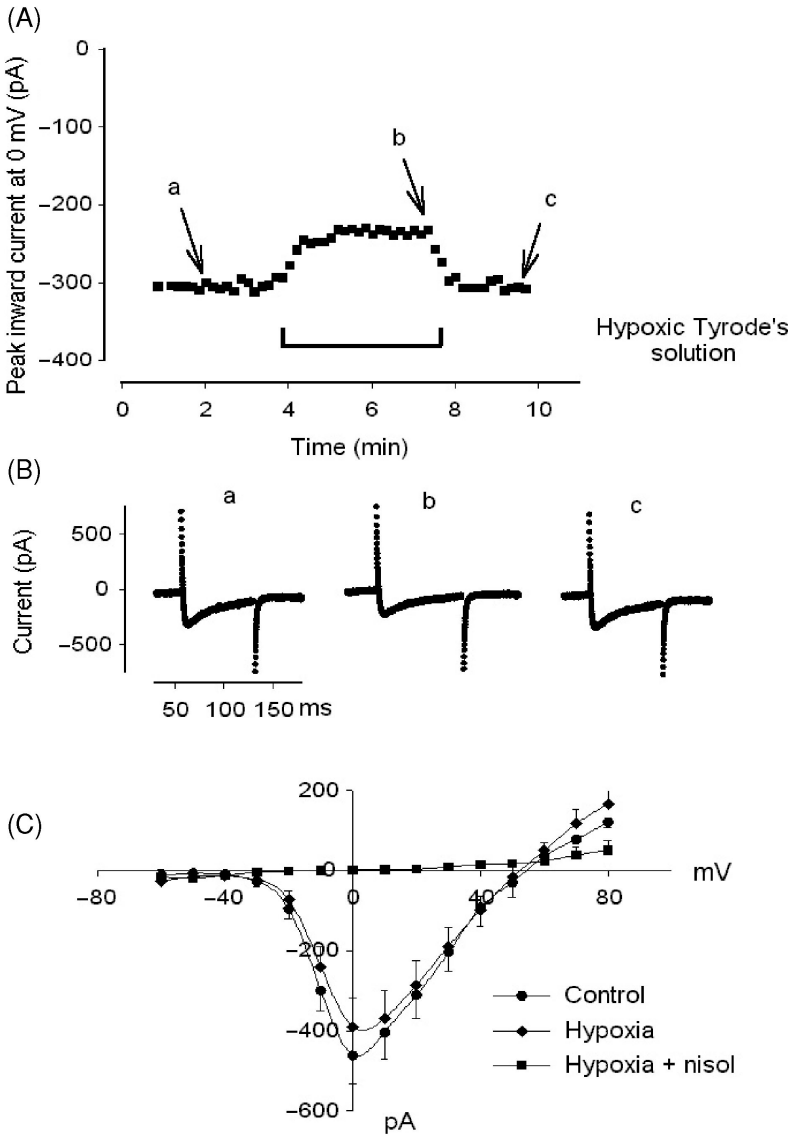

Fig. 7.11 Hypoxia inhibits the basal L-type Ca^{2+}channel in guinea-pig ventricular myocytes. (A) Time course of changes in membrane current recorded during a 75 ms test pulse to 0 mV applied once every 10 s. Basal current recorded during exposure to hypoxic Tyrode's solution that is reversed upon switching the superfusate to Tyrode's solution containing room oxygen tension. (B) Membrane currents recorded at time points in protocol illustrated in (A). (C) Mean ± SE current–voltage (I–V) relationship for five cells exposed to hypoxia and hypoxia + nisoldipine (nisol). Reproduced with permission from Hool (2000).

isoproterenol as a functional reporter of changes in cellular redox state. In cardiac myocytes, hypoxia is associated with a decrease in cellular superoxide (derived from the mitochondria) that then signals a change in the redox state of the channel resulting in altered function. Perfusing myocytes intracellularly with catalase (that specifically converts H_2O_2 to $H_2O$and O_2) mimics the effect of hypoxia on channel function. In addition, preexposing myocytes to H_2O_2 attenuates the effect of catalase and the effect of hypoxia (Fig. 7.12). Given that the channel responds to alterations in cellular redox state, it would appear that O_2-sensing is not intrinsic to ion channels. The challenge now is to determine how a change in channel thiol group redox state alters the conformational state of the channel and ion conduction.

7.10 Conclusions and Outlook

Our knowledge of calcium channels has come a long way since the first calcium currents were recorded in the 1950s. At that time the importance and complexity of the calcium signaling network could not have been appreciated. We now have a much better idea of the role of calcium in translating electrical stimuli into responses and the many functions of Ca^{2+} within the cell such as stimulating neurotransmitter secretion and muscle contraction. Because Ca^{2+} is used to initiate a vast range of responses, a huge variety of biochemical pathways exist to control its movement. Although some of the pathways by which Ca^{2+} can interact with components of the cell, and by which the cell can influence the passage of Ca^{2+} through ion channels are being clarified, many still remain to be elucidated.

Given that there is still much to be learnt about the biochemical properties of Ca^{2+} channels, it is probably not surprising that we know comparatively little about the physical mechanisms underlying their function. Indeed there are many aspects of calcium channel functions that remain to be elucidated. Using channel inactivation as just one example, although the domain I–II linker is implicated as being directly involved in channel inactivation, we still do not have a clear answer as to how this inactivation takes place at a structural level. Does this region physically block the pore along the lines of the ball and chain model? What are the relationships between the mechanisms of calcium- and voltage-dependent inactivation? How do interactions with the β subunits or the calmodulin bound C-terminus affect the process of inactivation, and how does the binding of other regulatory factors to the β subunit get passed on to alter the current passing through the channel pore? Other questions also remain to be answered. How do the structures of calcium channels respond to phosphorylation, binding to G proteins, pharmaceutical agents, or changes in oxidation state? How accurate are our models of ion permeation and selectivity?

Much progress has been made ~~on~~ understanding the mechanisms of voltage sensing and gating in K^+ channels, however, there is still much that is unknown. Indeed one important question that must be asked, given that so much of ~~out~~ our knowledge of Ca^{2+} channel structure and function is derived from the study of bacterial K^+ channels, is whether they really make good models of Ca^{2+} channel pores. It

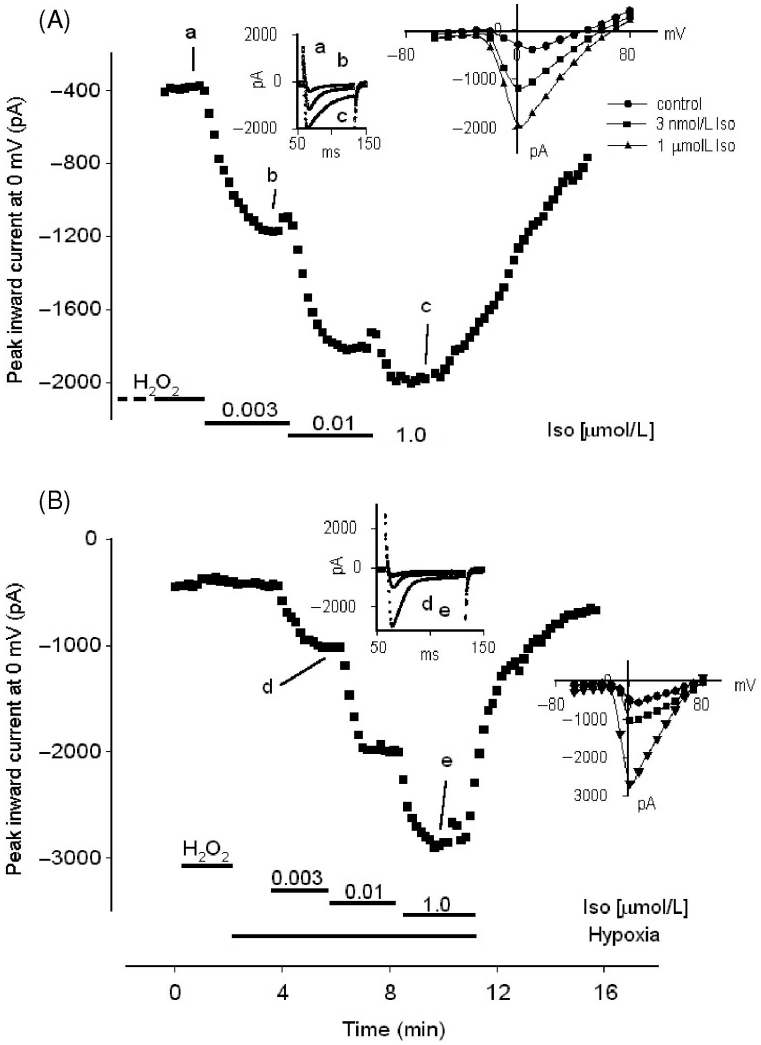

Fig. 7.12 Preexposure of guinea-pig ventricular myocytes to H_2O_2 attenuates the effect of hypoxia and the effect of catalase on the sensitivity of the L-type Ca^{2+} channel to beta-adrenergic receptor stimulation. During hypoxia (or when the cell is perfused intracellularly with catalase) the current produced in response to 3 nM isoproterenol would typically represent approximately 80% of the response elicited by a maximally stimulating concentration of the agonist (1 μM) within the same cell. (A) Time course of changes in membrane current recorded in a cell during exposure to ~~hypoxia and isoproterenol~~. The cell was preexposed to 8.8 μmol L^{-1} H_2O_2 for at least 5 min prior to superfusion with isoproterenol in the absence of H_2O_2. Membrane currents recorded at the time points indicated are shown inset at left. $I–V$ relationship in the same cell shown inset at right. (B) Time course of changes in membrane current recorded in a cell during exposure to isoproterenol ~~while being dialyzed with catalase~~. The cell was also preexposed to 8.8 μmol L^{-1} H_2O_2 for at least 5 min prior to superfusion with isoproterenol in the absence of H_2O_2. Membrane currents recorded at the time points indicated are shown inset at left. $I–V$ relationship in the same cell shown inset at right. Adapted from Hool and Arthur (2002).

is known that the selectivity filter of K^+ channels are lined by the backbone carbonyl oxygen atoms, but it is believed that glutamate side chains perform that role in calcium channels. Whether there are significant differences elsewhere in the structure can only really be determined from an atomic resolution structure of a calcium channel.

Obtaining a detailed structure of a bacterial channel was a major step in K^+ channel studies, and has advanced the field significantly. The study of calcium channels would also benefit significantly from such a structure. This would, for example, enable the details of the selectivity filter to be determined, as well as the likely channel gates. Furthermore, one hopes that the interactions between the pore and the inactivation gate on the $\alpha 1$ I–II linker and calcium sensors on the $\alpha 1$ C-terminus could be deduced from such information. Although crystal structures of the β subunit and of CaM bound to the C-terminus of the $\alpha 1$ subunit have been obtained recently, a complete structure of the entire calcium channel appears a long way off.

Surprisingly, one of the most promising leads in detailed structural studies of calcium channels has come from the successful expression of a bacterial sodium channel NaChBac (Ren et al., 2001). Being more closely related to the family of calcium channels than K^+ channels, deductions on the basic structure of NaChBac will be particularly pertinent to Ca^{2+} channel studies. In particular, NaChBac is found to contain an "EEEE" locus, and using appropriate mutations the NaChBac channel can be made selective for Ca^{2+} (Yue et al., 2002). Although many difficulties and uncertainties will no doubt have to be overcome, if a crystal structure of NaChBac is determined it will undoubtedly provide important information about the selectivity and ion conduction properties of the family of Ca^{2+} channels.

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