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Molecular Elements of Ion Permeation and Selectivity within Calcium Channels

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ABSTRACT: Voltage-dependent calcium channels are located in the plasma membrane and form a highly selective conduit by which Ca^{2+} ions enter all excitable cells and some nonexcitable cells. Extensive characterization studies have revealed the existence of one low (T) and five high-voltage-activated calcium channel types (L, N, P, Q, and R). The high voltage-activated calcium channels have been found to exist as heteromultimers, consisting of an α_1, β, α_2/δ, and γ subunit. Molecular cloning has revealed the existence of 10 channel transcripts, and expression of these cloned calcium channel genes has shown that basic voltage-activated calcium channel function is strictly carried by the corresponding α_1 subunits. In turn, the auxiliary subunits serve to modulate calcium channel function by altering the voltage dependence of channel gating, kinetics, and current amplitude, thereby creating a likelihood for calcium channels with multiple properties. Although for calcium channels to be effective, Ca^{2+} ions must enter selectively through the pore of the α_1-subunit, bypassing competition with other extracellular ions. The structural determinants of this highly selective Ca^{2+} filter reside within the four glutamic acid residues located at homologous positions within each of the four pore-forming segments. Together, these residues form a single or multiple Ca^{2+} affinity site(s) that entrap calcium ions, which are then electrostatically repulsed through the intracellular opening of the pore. This mechanism of high-selectivity calcium filtration, the spatial arrangement of pore glutamic acid residues, and the coordination chemistry of calcium binding are discussed in this review.

KEY WORDS: Ca^{2+} channel, site and mechanism of ion selectivity, Ca^{2+} binding proteins, coordination chemistry in Ca^{2+} binding proteins.

ABBREVIATIONS: VGCC, voltage-gated calcium channel; **HVA,** high-voltage-activated; **LVA,** low-voltage-activated; **TID,** 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine; **ω-Aga-IVA,** ω-Agatoxin IVA, *Agelenopsis aperta* toxin, type IVA; **ω-CTX or ω-CgTx GVIA,** ω-Conotoxin type GVIA from *Conus geographus*; **ω-CgTx MVIIC,** ω-Conotoxin

from *Conus magus*; **DHP,** dihydropyridine; **PAA,** phenylalkylamine; **BTZ,** benzothiazepine; **cDNA,** complementary or *copia*-like DNA; One letter code amino acid abbreviations: **Q,** glutamine; **E,** glutamic acid; **L,** leucine; **G,** glycine; **Y,** tyrosine; **V,** valine; **I,** isoleucine; **K,** lysine; **A,** alanine; **M,** methionine; **W,** tryptophan; **D,** aspartic acid; **F,** phenylalanine; **N,** asparagine; **T,** threonine; **GABA,** γ-amino-butyric acid; **CFTR,** cystic fibrosis transmembrane conductance regulator, a chloride channel; **MTS,** methanethiosulfonate; **G-protein,** trimeric (αβγ) GTP-(guanosine-triphosphate) binding protein; **M,** metal central atom in a coordination compound; **CD,** circular dichroism spectroscopy; **FT-IR,** Fourier-transformed infrared spectroscopy.

I. INTRODUCTION

The major pathway for entry of calcium ions into excitable cells such as muscle, neural, and secretory is through voltage-gated calcium channels (VGCC).[1] Calcium entry across the plasma membrane enables most excitable cells to translate electrical signals into intracellular Ca^{2+} fluxes that ultimately are transduced into biological activity. For example, excitation at the neuromuscular junction is translated into muscle contraction via intracellular Ca^{2+} signals[2] (Plate 1)* and the very complex, yet not fully understood mechanism of excitation-secretion coupling[3,4] is also mediated by the flux of calcium. These functions thereby place a tremendous physiological importance in elucidating the structural determinants of calcium entry and the mechanism by which calcium channels selectively filter Ca^{2+} ions. Studies have shown calcium entry to be based on delicate molecular mechanisms employing specific coordinative interactions of Ca^{2+} ions with a single or multiple Ca^{2+} binding site(s) lining the pore of the calcium channel. The scope of this review is to analyze the biochemical and coordination chemical aspects of this Ca^{2+} binding and the calcium channels subsequent selective permeation of calcium, while biophysical aspects of calcium entry relating to voltage-dependence and kinetics will only be briefly discussed. (For review see Ref. 5.)

II. MOLECULAR BIOLOGY OF CALCIUM CHANNELS

A. Classification and Biochemical Characterization of Ca^{2+} Channels

Electrophysiological and pharmacological studies have defined six subclasses of calcium channels;[5,6] T-, L-, N-, P-, Q-, and R-type. These six classes of calcium channels fall into two major categories: low-voltage- (LVA) and high-voltage-activated (HVA) channels.

T-type channels stand alone, as the only known representative of the low-voltage-activated class of calcium channels. These channels activate and inactivate with small changes of the membrane potential, exhibit small conductances, and small unitary currents (~9 pS).

The L-, N-, P/Q-, and R-type calcium channels have been classified as high-voltage activated. They open on large depolarizations of the membrane potential (≤ -20 mV), inactivate over a wide voltage range (–10 to –120 mV), their decay rate and inactivation rate varies from moderate to very slow and they all exhibit large single channel conductances (13 to 25 pS). Further classification of HVA calcium channels was determined on the basis of sensitivity to various organic calcium channel blockers, peptide toxins, and divalent ions such as Ni^{2+} and Cd^{2+} (Table 1).

* Plate 1 appears following page 184.

TABLE 1
List of Calcium Channel α_1 Subunit Genes

Isoform	Gene name (HUGO/GBD nomenclature)	Chromosomal localization	Tissue distribution	Pharmacological properties	Ref.[a]
			High-voltage activated		
α_{1A}	CACNA1A	19p13.1–2	Brain, neuronal cells, heart	*P/Q-type* ω-Aga-IVA (<100 n*M*) ω-CgTx-MVIIC (>100 n*M*)	17, 18
α_{1B}	CACNA1B	9q34	Brain, neuronal cells	*N-type* ω-CgTx-GVIA (<100 n*M*)	18, 19
α_{1C}	CACNA1C	12p13.3	Ubiquitous (heart, brain, aorta, lung, endocrine tissues, fibroblasts)	*L-type* DHP, PAA, BTZ	20
α_{1D}	CACNA1D	3p14.3	Brain, neuronal cells	*L-type* DHP, PAA, BTZ	21, 22
α_{1E}	CACNA1E	1q25–q31	Brain, neuronal cells	*R-type* Ni^{2+} (<100 μ*M*)	18, 23
α_{1F}	CACNA1F	Xp11.23		*L-type?*	24, 25
α_{1S}	CACNA1S	1q31–q32	Skeletal muscle	*L-type* DHP, PAA, BTZ	26, 27
			Low-voltage activated		
α_{1G}	CACNA1G	17q22	Brain	*T-type* Mibefradil	28
α_{1H}	CACNA1H	16p13.3	Kidney, liver, heart	*T-type* Mibefradil	29
α_{1I}	CACNA1I	22q13	Brain	*T-type* Mibefradil	30

[a] Reference is provided for cloning and identification of chromosomal location of the human gene. Due to extensive research in this field, these findings may not represent the first attempt at identification of these genes.

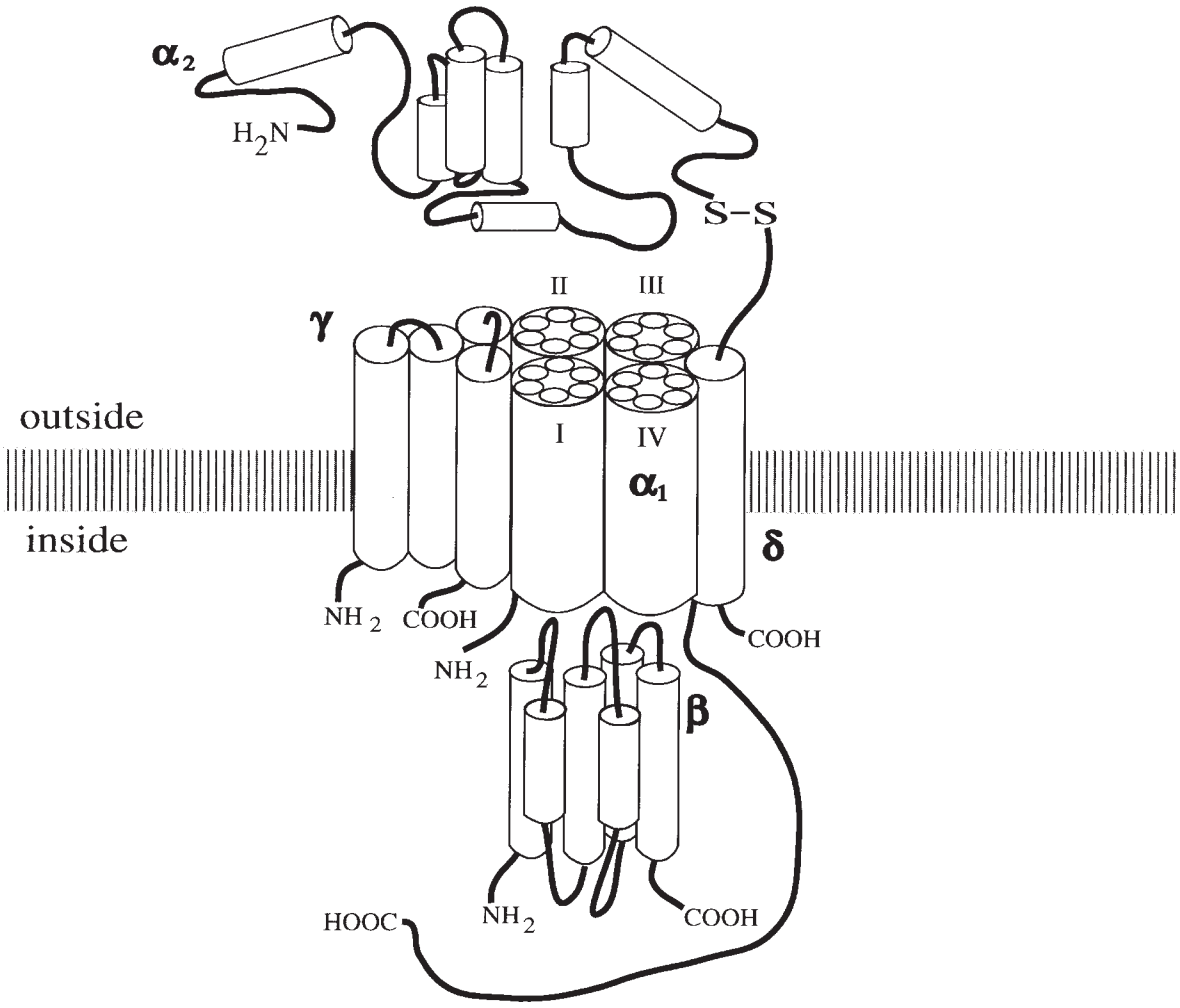


FIGURE 1. Molecular architecture of the voltage-gated calcium channel complex. The central subunit (α_1) is made up of 24 transmembrane helices, connecting loops, and the large cytoplasmic tail. Auxiliary subunits (α_2/δ, β, and γ) surround the pore-forming subunit. The helical structures are represented by tubes. Connecting loops are drawn as solid lines. (Figure 1 reproduced and redrawn with permission from Ref. 31.)

The L-type subfamily of calcium channels from skeletal muscle have been studied extensively by biochemical approaches.[7] These studies revealed the L-type calcium channel complex is a heteropentamer consisting of an α_1, α_2, β, γ, and δ polypeptide (Figure 1). The α_1 subunit is a 165 to 175 kDa polypeptide bearing the receptor sites for three classes of organic calcium channel blockers and based on certain hydrophobic characteristics is believed to be embedded in the membrane. The α_2 and δ subunits are disulfide linked, and the complex is represented by a 155 kDa protein band that changes its electrophoretic mobility after reduction, producing a 125 kDa α_2 and 30 kDa δ polypeptide. In fact, the α_2 and δ subunits are encoded by the same gene and formed by posttranslational processing.[8] The α_2 subunit seems to lie extra-

cellularly, while the δ subunit is believed to reside in the membrane with a single transmembrane segment.[9] The β subunit is a 56 kDa protein in skeletal muscle that has been shown to be phosphorylated *in vitro.* Hydrophobicity analysis has revealed the absence of typical-membrane spanning segments by labeling with [^{125}I] TID,[7] thus this subunit is believed to be located intracellularly. The 35 kDa γ subunit exhibits considerable hydrophobicity, suggesting its localization within the membrane. Both the α_2 and γsubunits have been shown to be heavily glycosylated.

Purification and photoaffinity labeling studies have revealed a similar molecular composition for the cardiac L-type calcium channel.[10] This channel is composed of an α_1 subunit with a molecular mass of 195 kDa, an α_2/δ complex of 175 kDa and a β subunit of 56 to 65 kDa. Further, these purification studies did not reveal the presence of a γ subunit; however, several protein bands in the range of 120 to 130 kDa were found in these preparations. It is not clear whether these latter bands are unrelated contaminants or truly associated auxiliary subunits, as this has been the only comprehensive purification study performed on the cardiac L-type channel. Recently, a γ subunit-like cDNA was isolated from cardiac messenger RNA preparations by PCR cloning.[11]

The neuronal, ω-conotoxin (GVIA)-sensitive calcium channel (N-type) has been purified from the rat brain.[12] A thorough purification and reconstitution study has shown that this calcium channel purifies as a complex of four subunits with molecular weights of 230, 140, 95, and 57 kDa. Antibodies specific to the ω-conotoxin receptor α_1 subunit precipitated a 230 kDa protein that was shown to be derived from the α_{1B} gene. The antibody raised against the α_2 subunit of the ω-conotoxin receptor recognized a 140 kDa band as well as the α_2 subunit of the skeletal muscle DHP receptor. The 57 kDa band was identified as a product of the β_3 gene. The 95 kDa protein is believed to form a close association with the 230 kDa protein and therefore may be an integral component of the ω-conotoxin receptor. Recently, Scott et al. identified this protein component as being a "two motif calcium channel"[13] whose function is not yet identified; however, this type of truncated calcium channel protein has also been found in skeletal muscle.[14,15] Accordingly, these results demonstrate that the purified ω-conotoxin receptor consists of an α_1, α_2/δ, β, and a 95 kDa subunit. The stoichiometric ratio of the subunits was found to be approximately 1:1 and reconstitution of the ω-conotoxin receptor from purified components into lipid bilayers exhibited calcium currents that were sensitive to ω-conotoxin.[12]

Biochemical characterization of the high-affinity ω-conotoxin MVIIC receptor (P/Q-type) was performed using rabbit brain.[16] In these studies, a low concentration of ^{125}I-ω-CTX MVIIC (45 p*M*) was used to label the high-affinity ω-conotoxin MVIIC receptors present in solubilized rabbit brain. The results of this study suggest the high-affinity ω-conotoxin MVIIC receptor is composed of an α_{1A}, α_2/δ, with either β_{1b}, β_2, β_3, and β_4 subunits as determined through immunoblotting. It seems that there exists a rank order for association of β-subunits with the α_{1A} subunit in rabbit brain: $\beta_4 > \beta_3 >> \beta_{1b} \geq \beta_2$. The affiliation of this receptor with different β-subunits may result in calcium channels with distinct electrophysiological profiles.

At this time, experimental limitations have prevented the biochemical characterization of the T- and R-type calcium channel. However, once specific inhibitors have been identified to selectively label these

channels, further biochemical studies can be approached.

B. Calcium Channel Subunit Genes

Molecular cloning has revealed in humans at least seven different genes encoding high voltage-activated calcium channel α_1 subunits and three low-voltage-activated calcium channel genes (Table 1). Analysis of sequence homology has found that among the α_1 subunit cDNAs cloned, there exists considerable base pair divergence. Alternative splicing has been identified in various regions of the α_1 gene and continues to add structural diversity to the multitude of calcium channel α_1 gene products (for review see Refs. 31,32). In addition, the localization of all α_1 subunit genes on different chromosomes in humans (see Table 1 and references therein) provides further evidence that calcium channels are encoded through a multigene family.

The conceptual translation and amino acid sequence analysis of α_1 cDNAs has revealed a rather simple secondary structure (Figure 2). The predicted structure of all α_1 subunits consists of four repeating domains or motifs, each domain being comprised of six hydrophobic segments (S1, S2…S6) that are embedded in the plasma membrane. Thus, calcium channels are believed to maintain the same transmembrane topology as described for Na^+ channels[33,34] and K^+ channels.[33] Both a short amino-terminal and a long carboxy-terminal segment of the α_1 subunit are positioned intracellularly. Further, a single transmembrane segment (S4) in each motif is distinguished by a collection of repeating positively charged amino acids, which sit in every third or fourth position. It is these four positively charged transmembrane segments that are believed to comprise the voltage sensor of voltage-gated calcium channels. A recent report of the X-ray crystallographic structure of a microorganism K^+ channel[35,36] has confirmed the overall predicted topology and structure of voltage-gated ion channels that were previously based on hypothetical models.

The α_2/δ gene has been localized to human chromosome 7q11.23–q21.1 as a single copy gene.[37] Extensive alternative splicing has been established for the 3′-end of the message, which gives rise to the existence of five separate messenger RNA species (Table 2).[21,38–40] However, more recent cloning studies suggest the existence of two additonal α_2/δ genes.[41]

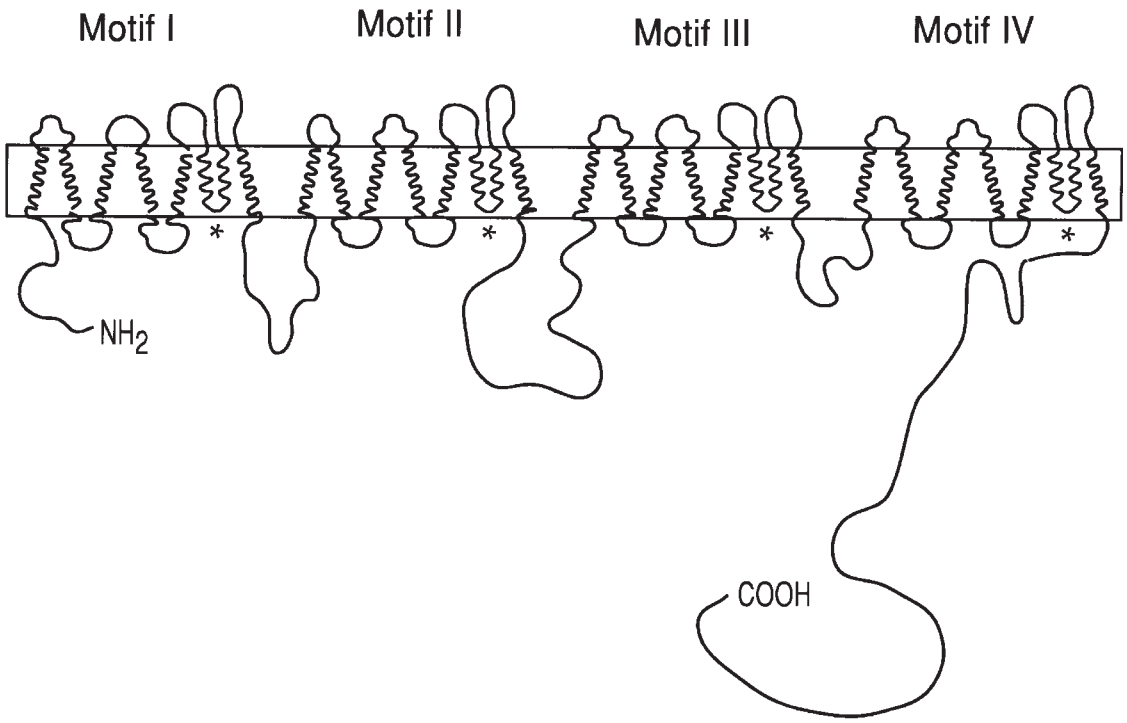
The β genes are represented by a multigene family. cDNA cloning has established the existence of at least four genes. Thus far, alternative splicing has been shown for two β genes; therefore, the combination of a number of mature β messages may coexist in various tissues (Table 2 and references therein).

The γ subunit gene from skeletal muscle[49,50] has been cloned and molecular analysis appears to suggest this transcript is not alternatively spliced. Additionally, a novel gene, Cacng2 (the human counterpart is CACNG2)[51] that encodes a 36 kDa protein primarily expressed in the brain, named stargazin, shows similarity with the skeletal muscle γ subunit. The identification and cloning of γ subunit-related genes is an expanding area of research. However, the functional significance of multiple transcripts as well as the regulatory role of this subunit remain unclear.

C. Functional Characteristics of Expressed Calcium Channel Genes: Regulatory Roles of Auxiliary Subunits

Expression studies employing the recombinant skeletal muscle α_1 subunit cDNA have elucidated a number of calcium chan-

A



B

	Motif I						Motif II						Motif III						Motif IV					
α1C	T	M	E	G	W	T	T	G	E	D	W	N	T	F	E	G	W	P	T	G	E	A	W	Q
α1D	T	M	E	G	W	T	T	G	E	D	W	N	T	F	E	G	W	P	T	G	E	A	W	Q
α1S	T	M	E	G	W	T	T	G	E	D	W	N	T	F	E	G	W	P	T	G	E	A	W	Q
α1A	T	M	E	G	W	T	T	G	E	D	W	N	T	G	E	G	W	P	T	G	E	A	W	H
α1B	T	M	E	G	W	T	T	G	E	D	W	N	T	G	E	G	W	P	T	G	E	A	W	H
α1E	T	M	E	G	W	T	T	G	E	D	W	N	T	G	E	G	W	P	T	G	E	A	W	Q
α1H	T	L	E	G	W	V	T	Q	E	D	W	N	S	K	D	G	W	V	T	G	D	N	W	N
α1G	T	L	E	G	W	V	T	Q	E	D	W	N	S	K	D	G	W	V	T	G	D	N	W	N

FIGURE 2. Formation of the pore in the α_1 subunit. (A) Two-dimensional representation of the α_1 subunit. The SS1-SS2 segments (marked by an asterisk in each motif) line the pore and bear the selectivity filter residues. (B) Sequence of the SS2 segment in HVA and LVA calcium channels in all four motifs. Amino acids are marked by the one letter nomenclature; the constituents of the selectivity filter are boxed.

TABLE 2
List of Calcium Channel Auxiliary Subunit Genes

Isoform	Gene name (HUGO/GDB nomenclature)	Chromosomal location	Tissue distribution	Ref.
		α_2/δ subunit		
α_2/δ-1	CACNA2A	7q21–q22	Ubiquitous	21, 37
α_2/δ-2	CACNA2B		Ubiquitous	41
α_2/δ-3	CACNA2C		Brain	41
		β subunits		
β_1	CACNB1	17q11.2–q22	Skeletal muscle brain	21, 42, 43
β_2	CACNB2	10p12	Brain, heart, lung	21, 44
β_3	CACNB3	12q13	Brain	45, 46, 47
β_4	CACNB4	2q22-q33	Brain	44, 48
		γ subunits		
γ_1	CACNG1	17q23	Skeletal muscle	49, 50
γ_2	CACNG2	22q12–13	Brain	51

nel functional characteristics. First, the α_1 subunit alone is sufficient to direct the permeation of Ca^{2+}-ions in a voltage-dependent manner.[52] Second, this subunit also carries the specific receptor sites for three classes of organic calcium channel blockers and a number of peptide toxins.[19,53] However, when the α_1 subunit is expressed alone in mammalian cells or in *Xenopus* oocytes, the activation and inactivation kinetics of the channel are abnormally slow compared with measurements done in native cell preparations.[52] Recombinant coexpression of the α_1 subunit with the auxillary α_2/δ and β subunits restored normal channel kinetics while increasing current amplitude approximately one order of magnitude.[54,55] Consequently, studies were initiated to determine the relative contributions of both the α_2/δ and β subunits in the reestablishment of normal channel function (for reviews see Refs. 32,56). As shown in these reports, when the α_1 subunit is co-expressed with the β subunit, this auxiliary subunit is capable of regulating channel kinetics, voltage-dependent gating, and channel density.[55] These properties have consistently been noted for cardiac, vascular smooth muscle, and brain calcium channels.[19,21,54,55,57–61] The clear functional impact of the β-subunit on properties of the α_1 led to the hypothesis that the β-subunit confers its modulatory properties by interacting with an intracellular segment of the α_1 subunit.

The putative interaction interface between the adjacent α_1 and β subunits emerges in an allosteric-like control resulting in modulation of voltage sensing and gating. Such an allosteric interface has been localized to the intracellular linker between motif I and II for skeletal, cardiac, neuronal P/Q- and N-type channels.[62] Epitope libraries from different calcium channels were screened with a β_{1b} subunit probe and a conserved binding sequence motif (QQ-E- -L-GY- -VI- - -E) was identified for all six calcium channel α_1 subunits. Mutations within this sequence of the P/Q channel

reduced the stimulation of peak current density by the β subunit and altered the inactivation kinetics and the voltage dependence of activation.[62]

Rigorous gating current measurements[63,64] have revealed a 4- to 6-fold increase in the maximum nonlinear charge movement when α_1 and β subunits were coexpressed in mammalian cells, compared with α_1 alone. Because these experimental approaches directly quantified the channel gating particles on the cell membrane, the finding indicates that the presence of β enhances the expression/translocation of α_1 in the cell membrane. However, additional studies utilizing confocal microscopy and immunovisualization of α_1 on the cell surface have provided further evidence for the chaperone-like function of β subunits in the membrane-targeting of α_1 in both mammalian cell[65–67] and *Xenopus* oocyte[68] expression systems. The gene knock-out of skeletal β_{1a} resulted in about a 10-fold lower density of L-type currents then normal cells and about a three-fold reduction in charge movement,[69] clearly showing that the chaperone function of the β subunit is a general principle even in *in vivo* conditions.

The modulatory role of the α_2/δ subunit has been troublesome to elucidate. Studies have shown the α_2/δ subunit slightly affects current density of the channel and the number of drug receptor sites,[54,55,70] while changes in channel kinetics, shifts of activation threshold, and marked increases in calcium channel density have been attributed to the β subunit.[55,59,60] The large, glycosylated extracellular domain of α_2 has been shown to be required for current stimulation, enhancing receptor density and altering some biophysical properties of the channel.[32] The transmembrane-containing δ subunit seemed to oppose the former effect of the intact α_2/δ, however, fully mimicked the biophysical properties caused by α_2/δ expression.[71]

Taken together, the α_1 subunit of calcium channels possesses the domains necessary for selective permeation of Ca^{2+} ions, however, auxiliary subunits set essential parameters of the channel's biophysical behavior and facilitate cell surface translocation when coexpressed. A model describing the functional architecture of Ca^{2+} channels is depicted in Figure 1.

III. MECHANISM OF ION PERMEATION

A. P Loops and Glutamic Acid Residues: Molecular Determinants of the Pore Lining and Ca^{2+} Selectivity Filter

Selective permeation of calcium ions through voltage-gated calcium channels is the primary feature of the α_1 subunit for each channel type. Studies with native Ca^{2+} channels suggested a multiion theory, or multiple occupancy theory to describe the apparent calcium selectivity found in voltage-gated calcium channels.[72,73] According to this model, the ion-conducting pathway has an extracellular entrance and intracellular exit vestibule, with a narrow middle, where the ion selectivity sites are located. These ion selectivity sites are believed to consist of both a low- and high-affinity ion binding site. The higher affinity site is located closer to the cytoplasmic side of the pore and is believed to be occupied with a Ca^{2+}-ion. When an incoming Ca^{2+}-ion arrives at the entrance of the pore and binds at the second, low-affinity site, cationic repulsive forces repel the previous ion from the high-affinity site, forcing the ion through the channel. The repulsive force between the two divalent cations speeds up the ion flow by a factor of 20,000, such that a 10^6

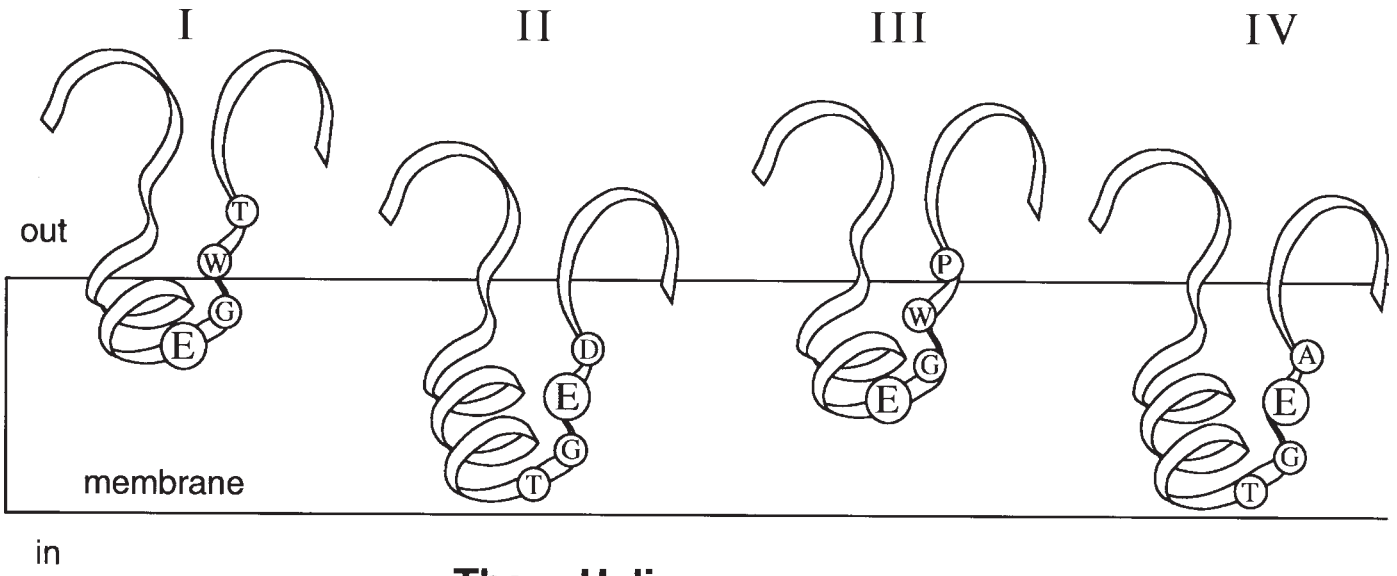
to 10^8 ion/second throughput can be reached. This model impressively unifies almost all selectivity criteria for calcium channels. However, ion filtration and selective permeation that involves a single binding site and repulsive interaction with an incoming calcium ion cannot be excluded and may also satisfy selectivity criteria.[74,75]

B. Mutagenesis of P-Loop Glutamates Alters Selective Permeation of Ions

To determine which molecular domains of the α_1 subunit form the pore and are responsible for calcium selectivity, a thorough review of channel secondary structure was undertaken.[33,34] Initially, the intracellular loops were examined as possible partcipants in the formation of the calcium channel pore. However, the homology between intracellular loops that connect the transmembrane segments is generally low, thus involvement of these regions was unlikely. Nevertheless, this comparison revealed a highly conserved segment connecting the S5 and S6 transmembrane domains in each motif, termed the P loop, also called the "SS1-SS2" region (Figure 2A). A short stretch of amino acids in the P loop of each calcium channel motif exhibits a strong sequence conservation among all known calcium channels (Figure 2B). This segment is believed to be embedded in the membrane, creating the highly calcium-selective pore.[33,34] Computer modeling of voltage-gated calcium channel subunits[33] and experimental success with mutagenesis of ion conduction pathways for both K^+ and Na^+ channels,[76,77] additionally led researchers to focus their attention on the P loops of VGCCs as possible molecular determinants for calcium selectivity.

The presence of four closely aligning glutamic acid residues in the SS2 segment of each motif suggests that these negatively charged side chains might form the Ca^{2+} selectivity filter (Figure 3). Indeed, mutations of individual glutamic acid residues (E) to glutamine (Q), alanine (A), or lysine (K)[78,79] greatly influences the monovalent/divalent cation selectivity of the calcium channel[79,80]. A systematic comparison has shown that in the absence of divalent cations, the mutant channels carried large monovalent cation currents that were blocked by Ca^{2+} in the order of EIIIQ > EIIQ > EIVQ > EIQ. Cd^{2+} block of currents carried either by Ba^{2+} or Li^+, also distinctly showed a nonequivalence of the glutamic acid positions, EIIIQ > EIQ > EIIQ > EIVQ.[81,82] These studies clearly show that glutamic acid residues in the SS2 regions of all four motifs collectively can contribute to the high-affinity binding of calcium.

In order to distinguish between the theories of two independent or interdependent high-affinity Ca^{2+} binding sites[72,73] or the presence of a single site,[74] combined mutagenesis and electrophysiological studies were performed utilizing pairwise replacement or simultaneous replacement of all four P-region glutamates. More specifically, these experiments were set out to examine the possible arrangement of the four glutamates as either being grouped into two subsets forming two Ca^{2+} binding sites or all four contributing to the establishment of one high-affinity binding site. Theoretically, if there is a high enough affinity difference between the assumed divalent cation binding sites, systematic pairwise mutagenesis of the four glutamates, should unravel which glutamate pairs form the two selectivity filters. Indeed, the pairwise replacement of glutamic acid residues with alanines resulted in 150- to 500-fold decrease of the Li^+ current by Ca^{2+}; however, the difference be-



The α-Helix

I.	NFDNFAFAMLTVFQCITMEGWTDVLYWMQDAMGYE
II.	RSTFDNFPQSLLTVFQILTGEDWNSVMYDGIMAYG
III.	DFDNVLAAMMALFTVSTFEGWPELLYRSIDSHTEDKG
IV.	NNNFQTFPQAVLLLFRCATGEAWQDIMLACMPGKKC

FIGURE 3. Arrangement of the pore-lining segments within the plasma membrane. (Top) The SS1 segments are depicted as helical structures,[34] while the SS2 segment in each motif is thought to form a random coil. Note that the selectivity filter glutamates (E) are asymmetrically arranged in the membrane. (Bottom) Alignment of amino acids in the pore-lining region. (Figure 3 reproduced and redrawn with permission from Ref. 31.)

tween glutamate pairs appeared to be marginal (two-fold or less than two-fold). These results demonstrate that the four P-loop glutamates do not form two divalent ion binding sites with markedly different affinities, instead they form a single high-affinity site capable of binding two divalent ions.[80]

Further support of the single high-affinity binding theory was demonstrated in experiments where quadruple mutations of the P-loop glutamates were replaced either by glutamine or alanine. This quadruple replacement brought about a drastic reduction in ion selectivity of the pore, as assessed by Ca^{2+} or Cd^{2+} block of monovalent (Li^{+}) ion currents. The replacement of glutamates by alanine weakened the apparent affinity for Ca^{2+} by about 1000 fold, whereas the sensitivity to Cd^{2+} block was reduced to an even greater degree, 7000- to 210,000-fold. These observations demonstrate that the P-loop glutamates form the major high affinity Ca^{2+} binding site and very likely no other high-affinity binding site exists.[80]

Systematic replacement of P-loop glutamates with aspartate, glutamine, alanine, or positively charged lysine residues in all motifs[80] clearly implies that highly selective divalent ion filtration occurs by coordinative interactions between the four pore glutamates and the calcium ion. Individual substitution of the four glutamates with aspartates resulted in a modest lowering of Ca^{2+} and Cd^{2+} affinity for the pore. More drastic changes were seen with alanine and lysine mutations, where the Ca^{2+} and Cd^{2+} affinity was weakened by a factor of 30 to 1000. Further, an intermediate response was observed with glutamic acid to glutamine substitutions, in which the affinity of divalent ions was decreased from severalfold to 20-fold. The overall patterns of these experiments indicate that the length, charge, and polarity of the amino acid side chains have significant influence on the Ca^{2+} interactions at each of the P-loop glutamate positions.

Furthermore, there is a considerable asymmetry in the functional impact of individual carboxylate side chains from one repeat to the next.[79–85] The interaction of Ca^{2+} with these glutamates must occur in a cooperative manner, such that the binding of the first Ca^{2+}-ion at the higher affinity site, which is located closer to the internal vestibule of the pore, will be weakened by the association of the incoming Ca^{2+}-ion at the second, lower affinity site. This cooperativity can be governed either by repulsion of the positively charged ions or conformational fluctuations of the pairwise-arranged glutamic acid residues, as substantiated by the nonequivalence of these positions.[81] It is possible that the sum of these effects can provide the driving force for this dynamic selectivity. In addition, theoretical calculations lend support to the fact that, in calcium channels, the ion filtering glutamic acid residues are nonsymmetrically arranged.[86,87] This is in contrast to the selectivity filters of K^{+} channels and nicotinic acetylcholine receptor channels, which have symmetrically arranged rings of amino acid residues capable of specific ion filtering.

For calcium channels, the predicted three-dimensional geometry of the SS1-SS2 segment may differ from that established for K^{+} channels.[34,35] First, the SS1 region for calcium channels is composed of amino acids that are predicted to be in an α-helical arrangement. Second, the amino acids that are predicted to form the turn between SS1 and SS2, valine and isoleucine, are predicted to be the least likely amino acids to form turns. Third, and most significantly, is that the strict consensus motifs of β-turns and P-bend structure[86] have been found in the SS2 region of each of the four motifs (MEGW, GEDW, FEGW, and GEAW in motifs I, II, III, and IV, respectively). Accordingly, we assume that the actual ion selectivity filter is located in a P-bend region of the SS2 segment arranged on two

close, but nonequivalent, planes and that they occupy trans positions (Figure 4). This stereochemical model gives an explanation for the nonequivalence of the four glutamic acid residues, agrees with the existence of a selectivity site, provides a plausible explanation for the dynamic ion binding affinity transitions that can occur, and explains how a positive Coulomb potential repulsion might contribute to the selective ion permeation process.

C. Protonation Studies on Recombinant Calcium Channels

Analysis of the interaction between recombinant calcium channels and protons resulted in serendipitous, yet intriguing biophysical findings.[88,89]

The protonation of L-type calcium channels in native cells resulted in a partial block of unitary currents.[90–93] The proton binding

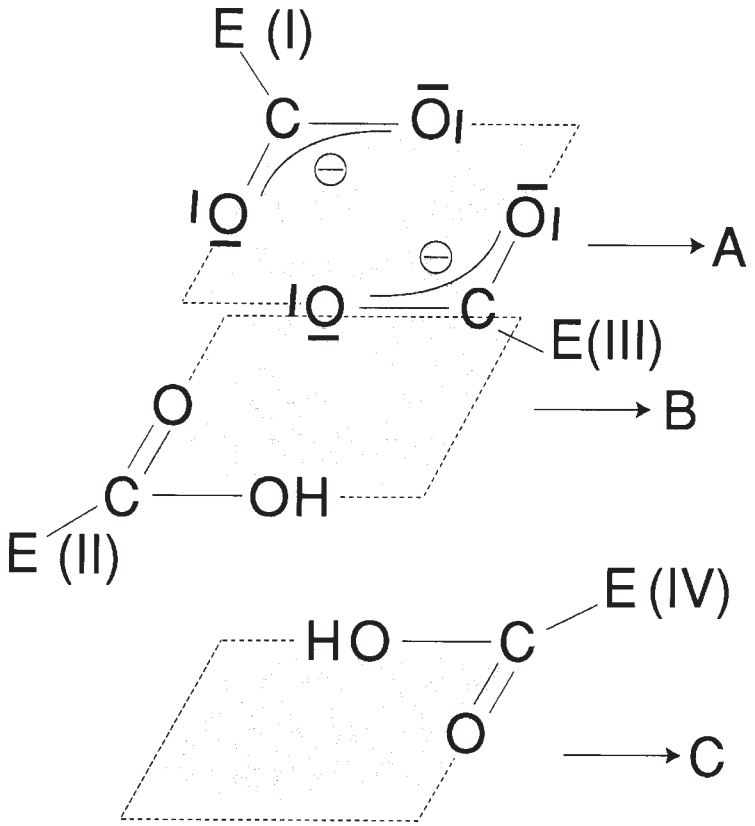


FIGURE 4. Three-dimensional arrangement of selectivity filter glutamates as revealed by mutagenesis and proton binding studies. The selectivity filter glutamates are placed on three different planes (A, B, C) in the membrane electric field. The outermost plane consists of glutamates from motif I and III. These two residues are fully dissociated and can be protonated. Very close to plane A is located plane B that represents the location of a motif II glutamate. Considering the nearby location and the flexibility of the pore-lining segments, this latter residue also can participate in the protonation process under definite conditions (for instance, E $\rightarrow$ Q mutants or double mutants). Plane C, deeper in the membrane field, contains the motif IV glutamate. This residue is located away from plane A, such that, under no circumstances should it participate in the protonation process.

site was shown to be a single binding site with an unusually high affinity for protons (pKa ~ 8.0).[90,92] Root and MacKinnon[94] showed that protonation of the cyclic nucleotide-gated channel occurs at glutamate side chains in the ion-conducting pathway. Klöckner et al.[88] has shown that protonation can be observed on recombinant L-type calcium channels. Analogous to the native L-type channels, the proton blockage resulted in fast transitions between high-conductance and low-conductance states when the current was carried by monovalent ions (K^+ or Na^+).[88,89] The resolution of the two conductance states can be improved by utilizing the kinetic isotope effect, thus substitution of K^+ for Na^+ in a D_2O solution slows down the transitions between the two states. Mutation of P-loop glutamates into alanines showed that the motif III P-loop glutamate is the sole acceptor for extracellular protons, and protonation at this site is responsible for the appearance of the low-conductance state. Mutations of the ion selectivity filter glutamates in motif I, II, and IV had only minor effects on the conductance of the protonated and unprotonated channel, but clearly the two conductance states were retained.[89]

Somewhat different phenomena were observed after protonation of the channel when the P-loop glutamates were replaced with glutamine.[89] Neutralization of the negative charge in the side chain of glutamic acid residues in motif I and III, with the isosteric glutamine, abolished the high-conductance state. This result gives a clear indication that these amino acids are involved in forming the high-conductance protonation site.

Mutation of motif IV glutamate into glutamine did not prevent blockage by external protons, however, the degree of blockage was significantly less than when compared with wild type. Detailed analysis showed that a twofold increase occurs in the rate of deprotonation with no significant change in the protonation rate.

Aspartate substitutions of P-loop glutamates of calcium channels have revealed a further interesting phenomenon of the proton binding site.[95] Glu → Asp substitutions in motif I and III resulted in about half of the proton block compared with that of the wild type or to that of glutamate replacements in motif II and IV. This change corresponded to a twofold reduction in proton affinity in motif I and III. The decrease in pK_a was due to an acceleration of k_{off} of proton, while the k_{on} remained unchanged. Replacement of glutamates with aspartates in motif II and IV showed no or very small changes in the proton block behavior of the channel compared with that of the wild type. Thus, shortening the side chain length of the P-loop glutamate carboxylate with one methylene group (about 1.4 Å difference in length) revealed that EI and EIII form the proton binding site in concert.

These experiments[88,89] provided direct proof that protonation of calcium channels occurs in the ion-conducting pathway, specifically at the P-loop glutamates. In three different approaches[88,89,95] mutations in the P-loop glutamates resulted in three different models (Figure 4). One, where Glu → Ala mutations were utilized proposes the EIII as the sole proton binding site.[88] In this respect, the EIII stands out in the cluster of P-loop glutamates, and this site forms a functionally separate entity. It seems conceivable, however, that glutamic acid to alanine mutations generated more disturbances in the interaction of the P-loop glutamates, and only EIII was identified to have a role in the protonation site, because it stands out with an exceptionally high affinity (pK_a 7.9) for protons.

Replacements of glutamates with glutamine residues[89] showed a drastically altered, almost equivalent behavior in motifs I and III for proton block, an indication

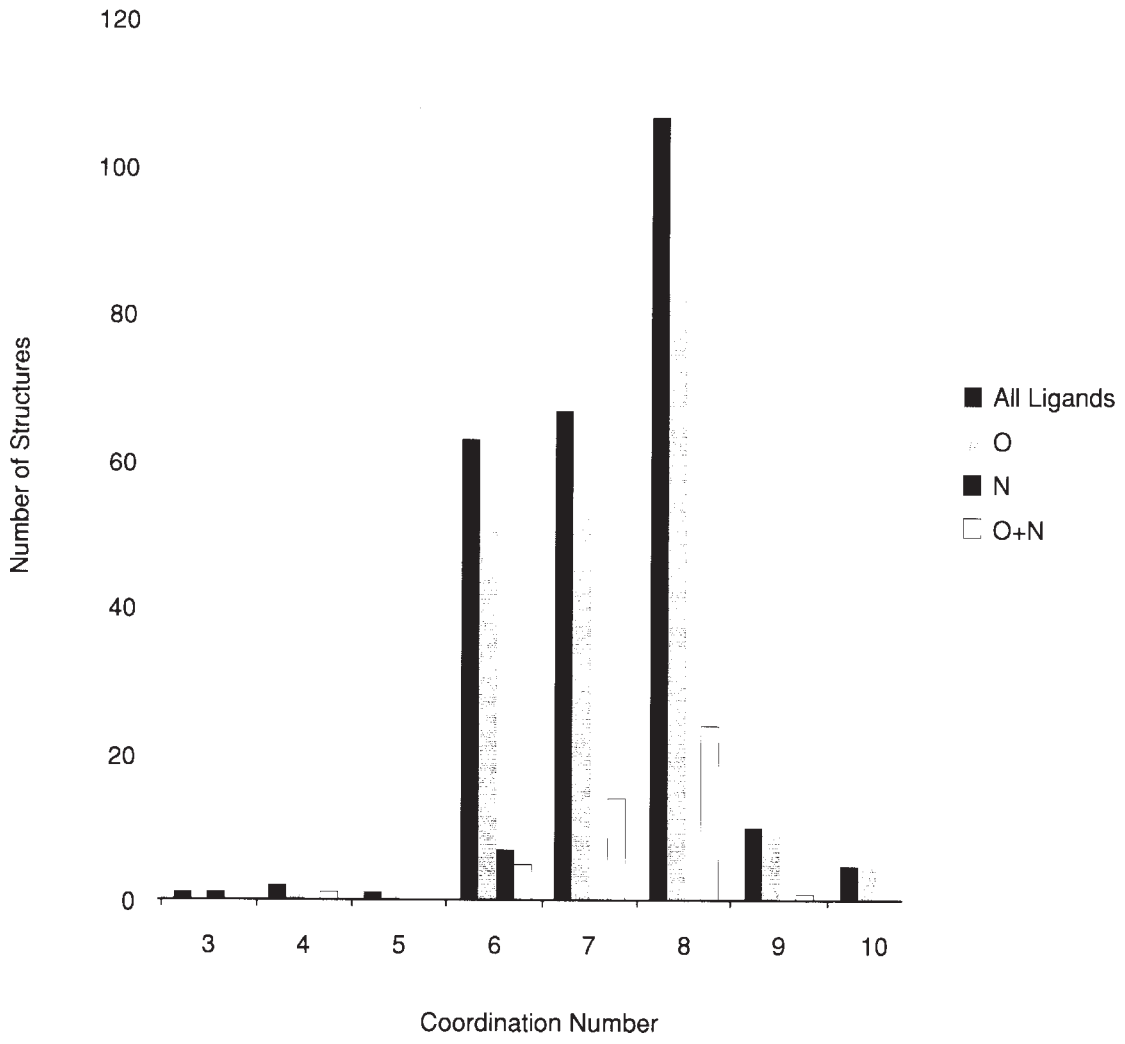


FIGURE 5. Abundance of coordination numbers in calcium complexes of known structure. Histogram analysis detailing the preferred coordination numbers for Ca^{2+} within compounds of known X-ray structures.

that the proton binding site is primarily formed by these two residues. An intermediate change when the same mutation was introduced in motif II prompted these authors[89] to conclude that the proton binding site is made up by the motif I, II, and III carboxylate groups of P-loop glutamates to form a three-centered hydrogen bond with the proton. A further, intriguing characteristic of this model implies that EIV is less externally accessible than the other glutamates and located more downstream along the permeation pathway. In fact, they estimate that the EIV is located >10 Å away from the proton binding site. E-Q mutations seem to have preserved more native interactions of pore glutamates[89,96] and provided compelling evidence that EI and EIII together form the protonation site. To a lesser extent, mutations of EII and EIV also were shown to be involved in the protonation process, which indicates that all four glutamates of the P-loop interact via through-the-space electrostatic interactions. Entrap-

ping the proton in a three-centered H-bond configuration[89] or in a more localized protonation process when an EIIIA mutant was used (Figure 4) shows the chemical and spatial nonequivalence of the P-loop glutamates.

Another distinctive characteristic is the pairwise separation of P-loop glutamates when E → D mutations are used for studying protonation. These three proton binding site models (Figure 4) put forward an important scenario that provides evidence for the asymmetrical arrangement of the pore glutamates. Moreover, these glutamates can be grouped into two sets: one that is involved in proton binding, the other that is not. This arrangement raises the possibility that the two groups of glutamic acid side chains coordinate at least two calcium ions. Now, whether these multiple ion binding sites will correspond to two independent but interacting selectivity filters remains to be seen. Thus far, mutagenesis and permeation studies by electrophysiological methods could not provide enough resolution to see the intricate details of the selectivity filter(s), however, proton blockage of the mutant channels clearly provided compelling evidence for the functional and spatial asymmetry of the P-loop glutamates.

D. Determination of Secondary Structure Within the Pore-Lining Region by the Use of Sulfhydryl-Modifying Reagents

The determination of calcium channel three-dimensional structure is extremely important in understanding the mechanism by which the pore selectively filters and permeates Ca^{2+} ions. Models have been proposed; however, to date no direct experimental evidence exists that descibes calcium channel structure. Recently, a new experimental approach has emerged to explore protein secondary structure. This method has been termed the substituted-cysteine accessibility method (SCAM).

The SCAM method[97] requires that individual amino acids (contained within the protein to be examined) be mutated to cysteine residues. This modification introduces a thiol group into the protein that can then be probed with thiol-modifying compounds.[98,99] Since its development, SCAM has been used to elucidate the secondary structures of many types of channels and receptors, including the muscarinic receptor[97,100,101] nicotinic receptor,[102] cyclic nucleotide-gated channel,[103] sodium channel,[104–106] GABA receptor,[107] CFTR,[108] potassium channels,[109] the ryanodine receptor/channel,[110] and the dopamine receptor.[111]

In using this approach for exploration of calcium channel secondary structure, amino acids lining the pore region are mutated, converting each one individually to a cysteine residue. These mutant channels are then expressed in a recombinant expression system and tested electrophysiologically for block by methanethiosulfonate (MTS) compounds. Current is reduced in channels where the introduced thiol group is exposed within the pore vestibule and can covalently bind the MTS compunds, blocking the channel. Our laboratory[112] has undertaken these studies and the results were quite remarkable. It seems that each of the pore lining segments, not only contributes nonequivocally to the high affinity site as suggested previously, but also contains different structural motifs. Compiling results from each SS1-SS2 segment has determined the calcium channel pore to be composed of two random coils, an α-helix and a β-pleated sheet. These results stand in stark contrast to the studies done on both the Na^+ and K^+ channels, where the the pore lining was arranged in helices and random coils. These differences between the Na^+ channel and Ca^{2+} channel might

serve to explain why mutations in the selectivity filter of the Na^+ channel (converting two positively charged amino acids to glutamic acid) were unable to convert it to a conducting calcium channel. It appears that proper pore structure determines the selective conducting properties of voltage-activated ion channels.

E. G-Protein Regulation of Ion Permeation

Calcium entry through neuronal voltage-dependent calcium channels plays a paramount role in initiating a number of cellular functions such as neurotransmitter release, gene expression, and synaptic plasticity. As such, neurons are required to maintain a tight regulation of calcium channel function. To do this, the neuron recruits a variety of mechanisms, including phosphorylation by protein kinases and direct modulation via intracellular signal transducers, GTP-binding proteins (G-proteins). G-protein modulation of neuronal calcium channels serves as a regulatory mechanism to attenuate neurotransmitter release from synaptic terminals.[113] This point of calcium channel regulation occurs through presynaptic receptor stimulation, subsequent G-protein association, and activation, which as an intracellular signal transducer can modify calcium channel behavior either through activation of second messenger pathways (PKA, PKC) or through a direct membrane-delimited pathway.

The mechanism by which G-proteins inhibit calcium channel properties was first thought to occur from a reduction in the number of functional channels.[114] Bean et al.[115] discovered that agonist-induced modulation biased the equilibrium between modes of gating converting the channel from a 'willing' to a 'reluctant' mode of gating. Furthermore, when a large depolarizing pulse is applied, inhibited channels can be reactivated; therefore, he concluded the overall number of functional channels does not change. Delcour et al.[116] examined the properties of N-type calcium channel gating at the single channel level when compromised by application of norepinephrine. After the application of norepinephrine, the presence of traces in a high P_o mode were almost absent, while the number of traces containing a low P_o increased, resulting in overall reduced macroscopic current. Although this would seem to support earlier observations of a channel interconverting between two distinct modes of gating as suggested by Bean,[115] the population of channels that underwent modulation presented gating behavior that was not homogeneous.

The reduction in calcium channel current resulting from G-protein modulation may also arise from alterations in ion permeation. Divalent Ba^{2+} and monovalent Na^+ currents through N-type calcium channels were measured from bullfrog sympathetic neurons in the presence and absence of luteinizing hormone-releasing hormone (LHRH).[117] Inward monovalent currents were less inhibited than currents carried by Ba^{2+} ions. In fact, prepulse facilitation, a phenomenon associated with G-protein-inhibited channels, where application of a large conditioning pulse amplifies inhibited current, was found to occur less frequently to current carried by Na^+ ions, while Ba^{2+} current was significantly amplified. Moreover, inward tail currents from Ba^{2+} currents were significantly reduced by the hormone, while outward Cs^+ current remained relatively unchanged. Applying a conditioning pulse enhanced the Ba^{2+} current through these channels, while outward Cs^+ current was unaffected. These results indicate that monovalent ions can pass equally well through modified and unmodified channels, while Ba^{2+} is prevented from moving through

modulated channels. Together these results suggest that G-protein modulation results from an alteration in the ion permeation process where Ba^{2+} current is more effected than monovalent current.

F. Coordination Chemistry Aspects of Calcium Permeation

A molecular-level analysis of the elementary steps of Ca^{2+} permeation through calcium channels and the interaction of ions with the selectivity filter meets some inherent, mostly experimental difficulties. These are

1. The Ca^{2+} filtration/permeation process is a very fast dynamic phenomenon;[72–75] however, structural features (obtained mostly by X-ray diffraction[118]) reflect equilibrium states of Ca^{2+}/protein coordination chemistry.[119] Therefore, these data should be applied with the greatest care.
2. Indirect evidence shows that in the Ca^{2+} filtration/permeation process the channel protein undergoes important stereochemical (conformational) changes,[1,72–75,120] therefore, even if the static (crystalline) molecular structure(s) of these proteins would be known, the exact formulation of the coordination (ligand) environment provided by the channel protein(s) would be highly speculative.

Despite these difficulties, the knowledge accumulated on calcium channel protein structure, on the relevant coordination chemistry of Ca^{2+} in channels, and with other Ca^{2+} binding proteins provides a good starting point for a molecular-level description of the Ca^{2+} filtration/permeation phenomena. Aspects of this process, from the viewpoint of the channel protein "ligand" and from that of Ca^{2+} permeation, are analyzed below.

First, it should be pointed out that Ca^{2+} permeation through calcium channels is a very fast process that can be sufficiently explained by the large concentration gradient, and consequently no important protein/Ca^{2+} coordination interaction is to be expected in this part of the Ca^{2+} signal transduction. Coordination interactions between the channel protein and Ca^{2+} ions are expected to occur in the filtration phase of events. Thus, the analysis should be started by exploring coordination phenomena with the pore-lining segment of the channel protein.

Information about the ligand environment within the pore-lining segment of the calcium channel is derived from four major sources:

1. Experiments locating the Ca^{2+} permeation mechanism to the α_1 subunit of the channel protein.[77–89]
2. Mutation experiments (as described earlier in sections of this review) have shown that partial or full substitution of the glutamic acid residues in the SS2 segment[31,77–89] produces radical changes in ion permeation, thus the four glutamic acid residues in the four SS2 segments are responsible for selective filtration of Ca^{2+}.
3. Recent biophysical experiments[88,89,95] have shown that the pK_a values of these glutamic acid residues in the selectivity filter of the channel are dramatically different (pK_a ~ 7.2 to 8.0) from those expected for amino acid carboxyl side chains in biological fluids (pK_a ~ 4.2 to 4.8). This observation indicates that approximately half of the glutamic acid residues in the pore region are not dissociated.
4. Successful computer modeling of the voltage-gated calcium channel α_1 subunit[33,34] complements several biochemical experiments.

These results identify the α_1 subunit protein as a large polypeptide ligand containing four pore-lining regions bearing four glutamic acid residues that form the selectivity filter, with an inner (approximately tubular) cavity of about 6 Å in diameter.[120] These glutamic acid residues are distributed on each SS2 segment, with two of them being deprotonated, while the other two are not ionized (which, however, does not exclude these carboxylic groups from participating in intra helix H-bonding[121,122] — especially in the absence of Ca^{2+} ions in the pore). The cavity of the pore is expected to be "narrowed" to ~ 6 Å in diameter for this glutamic acid-bearing region. Beyond the particularly active and sterically most exposed glutamic acid residues, the polypeptide backbone offers carbonyl-*O* and amide-*N* (weak) donor sites. Concurrently, the peptide bond carbonyl oxygens have been shown to be critically important donor atoms in the formation of the K^+ channel selectivity filter.[35] No other typical donor side chains (e.g., His, Ser, Cys) are in close proximity to the Ca^{2+} selectivity filter.

Another ligand pool is obviously offered by the cellular conditions, simply by the fact that the polypeptide chains are generally highly hydrated. This also means that all coordination sites provided by the protein should compete with water ligands, which are present in considerable excess and are much more mobile than other potential donor groups located on the polypeptide chain.

From the metal ion side, the problem could be formulated as follows: the calcium ions are initially selectively coordinated, then rapidly decoordinated in a direction that is opposite to the direction of their arrival, that is, they should leave the selectivity filter on the opposite side from which they entered. This process would have to be quick in aqueous solutions, even in the presence of a ~ 100-fold molar excess of Na^+.

We believe that neither the observed high degree of selectivity nor the high rate of Ca^{2+} flux can be explained by only one feature of the Ca^{2+} ion or of the ligand. At the present time, a sufficient amount of relevant observations has accumulated for which we have used to construct a reasonable hypothesis for this phenomena. These ideas are now discussed in some detail.

1. Ionic radii. Pauling ionic radii [pm]: Li^+, 60; Na^+, 96; K^+, 133; Mg^{2+}, 65; Ca^{2+}, 99; Zn^{2+}, 74 of alkali metal and other metal ions are substantially different.[123] This difference becomes even more pronounced when effective ionic radii are compared because these values also depend on the coordination number. Comparing only the most frequent coordination numbers and the corresponding radii (coordination numbers are in parentheses): Li^+, (4) 59; Na^+, (6) 102; K^+, (8) 151; Mg^{2+}, (6) 72; Ca^{2+}, (8) 112; Zn^{2+}, (4) 60. Now, comparing all published coordination numbers (in parentheses) and the corresponding radii of Na^+ and Ca^{2+}, respectively: (4) 99, –; (5) 100, –; (6) 102, 100; (7) 112, 106; (8) 118, 112; (9) 124, 118,[124] we should conclude that Ca^{2+} has a markedly different size from that of all but one of its "concurrents", however, it is fairly similar to Na^+ (especially at coordination number 6). This similarity, however, is less pronounced at higher coordination numbers. Calculated values of M–O bond distances for $[M(H_2O)_n]^{2+}$ (M = Mg, Ca, Zn; n = 3–8) complexes[125] are consistent with these tendencies (Table 3). A nonnegligible consequence of these calculations lies in that the $[Ca(H_2O)_6]^{2+}$ or $[Ca(H_2O)_8]^{2+}$ complexes have an external approximate "spherical" diameter of 500 to 600 pm (5 to 6 Å).

TABLE 3
Selected Values of Calculated [125] M–O Bond Distances for $[M(H_2O)_n]^{2+}$ (M = Mg, Ca, Zn) [pm]

n	Mg	Ca	Zn
3	199.0	237.2	195.9
4	201.7	239.2	200.9
5	206.4[a]	241.8[a]	207.5[a]
6	209.7	244.3	212.0
7	[b]	248.2[a]	[b]
8	[b]	252.5	[b]

[a] Average.
[b] Not available.

2. Coordination numbers. Ca^{2+} displays a characteristic preference for higher coordination numbers[119,125–127] (Figure 6), in this respect Ca^{2+} ions share closest similarity to K^+ ions[119] (see also the data given in point 1 in this list).
3. Donor atoms. All cations listed above are of hard character, most of them show preference for charged hard donors.[119,125,128,129] The Ca^{2+} ion, due to its high charge density[128,129] and to the participation of its more diffuse orbitals in bonding,[125,132] shows much less selectivity for donors than other cations (Li^+, K^+, Na^+). Therefore, Ca^{2+} is a candidate for coordination[118,119,128] to ether or alcoholic O, nondissociated carboxylic (OH) oxygens; ketonic, carbonylic or amidic carbonyl O and (a marked difference to the others) N donors (see data in Figure 6 also in this respect). Thus, among the ions discussed, Ca^{2+} displays the most promiscuity when it comes to coordination with various ligands.
4. Kinetics. Unfortunately, no calculations or measurements are available concerning the activation barriers separating the various coordination numbers of Ca^{2+} (especially compared with metals like Na^+, K^+, Mg^{2+}, and Zn^{2+}). A classical set[133] of first-order rate constant data of water substitution in $[M(H_2O)_n]^{x+}$ (M = Na, K, Mg, Ca, Zn; n — not specified, x = 1, 2) shows the following highly interesting trend: Na^+ 8.8×10^7, K^+ 1.5×10^8, Mg^{2+} 10^5, Ca^{2+} 10^8, Zn^{2+} 3×10^7. Although these complexes of alkali metals are of low stability, the high rate observed for Ca^{2+} with respect to (especially) Mg^{2+} or Zn^{2+} might be sufficient to explain the selectivity effects of these metals.
5. Diffusion coefficient. The diffusion coefficients were calculated based on observed Ca^{2+} permeation properties, using measured (reasonable) values for channel geometry and concentration gradient (data shown in Table 4). Limiting values of D in the table are $1.01 \times 10^{-6} \div 1.25 \times 10^{-2}$ $cm^2 \times s^{-1}$ [132] or "expected" $8 \div 10 \times 10^{-6}$ $cm^2 \times s^{-1}$.[135] These values can be compared with the few values published in the literature:[134,135] 0.65×10^{-6} $cm^2 \times s^{-1}$. An important point to note is that calcium ions are diffusing more slowly than should be expected for "naked" or hydrated Ca^{2+} ions in aqueous solutions. The reasons for this "friction" could involve (a) "delay" caused by (at least partial) dehydration at the entrance of the channel, (b) coordination/decoordination reactions in the

TABLE 4
Diffusion coefficients calculated by Fick's law [$cm^2 \times s^{-1}$]

Channel radii $\times 10^{-8}$ (cm)	$Ca^{2+} \times 10^6$ (ions $\times s^{-1}$)	$Dx \times 10^{-8}$ (cm)	$DC \times 10^{-6}$ (mol $\times cm^{-3}$)	$-D \times 10^{-6}$ ($cm^2 \times s^{-1}$)
2.65	1.00	30.0	5.00	45.2
2.65	100.0	23.0	1.80	9620
2.65	100.0	30.0	1.80	12500
5.00	100.0	15.0	1.80	1760
5.00	1.00	23.0	5.00	9.72
7.50	1.00	30.0	5.00	5.64
7.50	100.0	30.0	5.00	564
12.5	1.00	15.0	5.00	1.01
12.5	100.0	23.0	5.00	156

$$D = -J\ Dx/DC$$

Note: D diffusion coefficient [$cm^2 \times s^{-1}$]; J, flux [mol $\times cm^{-2} \times s^{-1}$]; Dx, path [cm]; DC, concentration gradient [mol $\times cm^{-3}$]; Values for calculation; J, $10^6 \div 10^8$ Ca^{2+} ion $\times s^{-1}$ pro channel; channel radius = 1.25×10^{-7}; 2.65×10^{-8}; 5.00 $\times$ 10-8; 7.50×10^{-8} cm; Dx = 15×10^{-8}; 23×10^{-8}; 30×10^{-8} cm; DC = $1.8 \times 10^{-6} \div 5.0 \times 10^{-6}$ mol $\times cm^{-3}$; Limits of D calc. $1.25 \times 10^{-2} \div 1.01 \times 10^{-6}$ $cm^2 \times s^{-1}$

selectitivity filter or along the whole length of the pore-lining region, or (c) formation/dissociation processes of H-bonding interactions between the (partly) hydrated cation and the protein itself or its hydrating molecules. While we know that the hydration/dehydration rates of Ca^{2+} are fairly high (see point (4)), we know little about the extent and rate(s) of these latter reactions.

6. X-ray studies. X-ray single crystal diffraction studies (as already mentioned) provide a considerable body of evidence exploring the coordination of Mg^{2+}, Ca^{2+}, and Zn^{2+} in proteins.[118,125,129] These results contributed heavily to the understanding of the coordination behavior of these metals (as outlined above). One important result from these studies is that Ca^{2+} is coordinated to proteins almost always in a partially hydrated form; therefore, in any interaction with the Ca^{2+} channel or its filter a complete dehydration of Ca^{2+} ions can hardly be expected. On the other hand, it should be pointed out that the structural features observed for Ca^{2+}/protein complexes being in crystalline phase do not necessarily hold for Ca^{2+} ions in an aqueous phase.
7. Solution spectroscopic studies. In the last couple of years considerable efforts have been made to identify the interactions of peptides with alkali and alkaline earth metal ions, using mainly CD and FT-IR spectra of solutions.[121,136] These studies provide two highly interesting qualitative pieces of information: (1) it has been found that spectroscopically significant metal/peptide interactions cannot be detected in aqueous solutions, only in less coordinating solvents, like trifluorethanol or acetonitrile; (2) once interaction takes place, the CD spectra changes and Ca^{2+} was found to cause the most dramatic changes among Li^+, Na^+, K^+, Mg^{2+}, and Ca^{2+}. It should be pointed out that (1) is in good agreement with

our conclusion regarding the strong hydration of Ca^{2+} in point 4. The effect mentioned under (2) can involve the development of chirality on the metal ion, which is now under investigation in our laboratories. The chirality of metals coordinated to proteins may have far-reaching consequences, as demonstrated in Ref. 138.

Summarizing the above features, we can conclude that the published properties of calcium permeation (as described earlier)[72,74,75,78–80] can be essentially confirmed. However, a few caveats still persist.

1. The driving force for "inward" ion flux could be the divalent charge of the incoming Ca^{2+} ion coordinated to nondissociated glutamate residues, pushing the already bound "former" Ca^{2+} ion inward through the channel. This mechanism can be complemented by the reasonable supposition that before the "voltage signal", the glutamates are turned "outward" and fully dissociated and after depolarization are only partly dissociated[88,89,95] and turned "inward" into the less polar medium of the pore.
2. The filtration process is both of a stereochemical and kinetic nature. In the very narrow region (6 Å)[35,120] of the glutamate filter, the larger radius K^+ ion cannot pass; however, smaller cations may but only in a partially dehydrated form. The geometrically similar Na^+ ion bears only one positive charge and this deprives it from contributing to the driving force mentioned in point 1. Moreover, a recent finding suggests that Na^+ channels need (Lys) residues,[139] for generating that excess positive charge for which Ca^{2+} ions can provide themselves. Therefore, this provides evidence supporting the notion that the pore size and coordination phenomena of ions together determine the selectivity of permeation. A recent analysis (published after the completion of this review) provides additional support to the importance of kinetic and not exclusively thermodynamic phenomena in the selection of calcium ions.[75]
3. The molecular features that are believed to support the permeation process in high-voltage-activated channels appear to be similar in T-channels,[28–30] with the only exception that glutamic acid residues are replaced with aspartic acids in the selectivity filter.

G. Coordination Chemistry of Ca^{2+} Ions in Proteins of Known Structure: Lessons from the Structure of a K^+ Channel

A recent X-ray crystallographic determination of the structure of the *Streptomyces lividians* potassium channel (KcsA) has provided a number of breakthrough observations concerning the coordination chemistry and mechanism of ion conduction in voltage-activated K^+ channels.[35] The overall sequence and putative secondary structure of this K^+ channel resembles that of the vertebrate two membrane-spanning segment K^+ channels.[140] However, the sequence in the pore-lining region actually shows closer homology to the *Drosophila* (*Shaker*) K^+ channel and other vertebrate voltage-gated K^+ channels.[141]

The structure of KcsA is in excellent agreement with the topology prediction and functional mutagenesis studies done on *Shaker* and other eukaryotic K^+ channels.[33,76] The channel exists as a tetramer with each monomer arranged symmetrically around a central pore. Each monomer is composed of two membrane-spanning α-helices that are

connected by ~30 amino acids that are believed to line the pore of the channel. The pore-lining region is composed of a turret, pore helix, and selectivity filter (Plates 2A and 2B).* As extensive mutagenesis and functional expression studies revealed, a -V-G-Y-G- signature sequence is a critical element in all K^+ ion-conducting pathways. Some theoretical calculations suggested that perhaps the π-electron cloud of the tyrosine residue aromatic ring would provide the necessary interaction for K^+ ion coordination.[142] However, in the experimental electron density map the (Val) and (Tyr) side chains were directed away from the pore. Thus, the location of the tyrosine side chain precluded (at least in crystalline phase) its participation in the K^+ ion coordination. Simply this fact alone invalidates the previous model established for specific ion filtration. Additionally, the selectivity filter is made up of 20 carbonyl oxygens from the polypeptide chain backbone.[35] The four main chain atoms create a stack of probably five sequential oxygen rings. Analyzing the permeant ion positions in the pore, two ions were found at opposite sides of the selectivity filter, separated by about 7.5 Å. These findings clearly provide an atomic argument for the multiple occupancy required for K^+ channel selectivity.

Unfortunately, no such atomic details are known for Ca^{2+} channels; however, very clear coordination features have been determined for a number of types of calcium binding proteins. The most extensively studied structural domain of proteins that bind Ca^{2+} is the EF-hand motif.[118] Calmodulin, a highly conserved protein throughout evolution contains 148 amino acid residues. This protein belongs to a family of Ca^{2+} binding proteins and performs its function by obtaining a calcium-stabilized configuration that, in turn enables calmodulin to interact with and regulate a wide variety of protein targets. Calmodulin contains four calcium binding sites that are made up of four EF-hand (helix-loop-helix) structural units.[118,126] This calcium binding motif is also commonly found in related proteins, such as troponin C.[143] The calmodulin structure has been determined by X-ray crystallography[144,145] and by multidimensional NMR studies.[146]

The stereochemical arrangement of the four EF hand Ca^{2+} binding sites and the geometry of Ca^{2+} coordination polyhedra are almost identical[144] and all four Ca^{2+} binding sites exhibit sevenfold coordination (Plate 2C). In the -D*/N-x-D*-G-D*/N-G-X*-I/N-X*-x-E*-loops (an asterisk marks those amino acids that coordinate the Ca^{2+} ion) the first ligand for Ca^{2+} is an Asp/Asn side chain, five additional ligand sites are provided by amino acid side chain oxygen atoms. The seventh coordination site for each Ca^{2+} ion is provided by water oxygen.

Some HVA calcium channels show inactivation properties that are different from the voltage-dependent inactivation and are likely due to a local rise in intracellular free Ca^{2+} concentration. This Ca^{2+}-sensitive, biological feedback mechanism is typical for α_{1C}, while other HVA calcium channels like α_{1E}, α_{1A} and all of the LVA channels do not possess these properties. Structural evidence[147] supported the existence of EF-hand-like structures[118] located very close to the inner mouth of the pore. A structural determinant of the Ca^{2+}-sensitive inactivation has been assigned as critical determinant of a relatively conserved, EF-hand-like Ca^{2+} binding motif in α_{1C}.[148] However, further investigations[149,150] have revealed additional sequences in the C-terminal portion of the channel that are located downstream of the

* Plate 2 appears following page 184.

proposed putative EF-hand structure. As shown recently, the mechanism of Ca^{2+}-sensitive inactivation involves the Ca^{2+}/calmodulin complex as a mediator of Ca^{2+} inhibition.[151,152] Thus, it seems likely that calmodulin is constitutively tethered to the C-tail of the calcium channel and inactivation occurs via interaction of Ca^{2+} with the calmodulin component of the complex.[152]

Annexins are a class of widely distributed proteins that bind to phospholipids and membranes in a Ca^{2+}-dependent manner. The crystal structure of human annexin V[153–158] has revealed an almost entirely helical structure for this 320 amino acid long polypeptide that comprises four domains of similar structure, with each domain consisting of five α-helices.[153] This protein possesses five calcium binding sites. Each of these sites are on the convex face of the molecule (Plate 2D) a region proposed to be attached to the membrane. Calcium sites 1 through 3 are on homologous -(M/L)-K-G(A/L)-G-T- segments in repeats I, II, and IV. The ligands for Ca^{2+} are composed of three carbonyl oxygens of the conserved protein backbone and of a sequentially discontinous but spatially nearby Glu or Asp side chain bidentate carboxylate oxygens and also by two solvent water oxygen atoms.[153,154] A somewhat similar but not identical sequence (-G-E-L-K-W-G-T... E) in human annexin V on motif III does not bind Ca^{2+}. However, rat annexin V does bind Ca^{2+} within this domain, a function that is most likely due to a conformational difference.[155] Ca^{2+} binding sites 4 and 5 in human annexin V show lower affinity to Ca^{2+} and have only three proteinaceous sites with two to three coordinating water molecules.[154]

Annexin, when incorporated in phospholipid blayers, forms Ca^{2+}–sensitive ion channels that possess voltage-dependent gating properties.[154–158] The X-ray structure has revealed a hydrophilic four-helix bundle pore-lining arrangement. Although determining the Ca^{2+} coordination site in the pore proved difficult, combined mutagenesis, electrophysiology, and X-ray studies revealed that Glu95 and Glu112 are in the pore and are critical determinants of ion selectivity.

Another well-studied Ca^{2+} binding structural unit in proteins is the C_2 domain. This Ca^{2+}-regulatory domain is present in a variety of proteins. Thus far, more than 50 such structural elements have been identified, including protein kinase C, synaptotagmin, phospholipase A2, phospholipase C, and a large family of GTPase-activating proteins.[159] Typical characteristics of C_2 domains are that they bind Ca^{2+} and phospholipids and exhibit no sequence homology to EF-hands.

The X-ray structure of the synaptotagmin I C_2 domain has revealed an eight-stranded β sandwich constructed around a well-conserved motif called a C_2-key.[160] A novel type of bipartite Ca^{2+} binding site is formed by two loops, connecting strands β2 with β3 and β6 with β7. The ligand field is composed of aspartic acid side chains 172, 178, 230, 232, a peptide backbone carbonyl oxygen from Phe231 and also a water oxygen.[160]

A further refinement of this structure came from the multidimensional NMR spectra of the synaptotagmin C_2-key domain.[161] This structural determination clearly revealed that by arranging the two loops a bipartite Ca^{2+} binding site is formed, with five aspartic acid side chains making up the Ca^{2+} binding site (Asp172, Asp178, Asp230, Asp232, and Asp238), coordinating two Ca^{2+} ions (Plate 2E). Asp 172, Asp230, and Asp232 act as bidentate ligands coordinating two Ca^{2+} ions, while Asp178 and Asp238 are functioning as monodentate ligands. Another important feature of the coordination scheme is that the first Ca^{2+} ion is surrounded by all ligand oxygen atoms trapped in a heptadentate (pentagonal bipyramidal)

structure, while the second Ca^{2+} ion is coordinated by five carboxylate oxygens, thus being partially exposed to oxygen atoms of the solvent. This coordination "asymmetry" at the bipartite Ca^{2+} binding site may explain affinity differences between the two coordination sites.

IV. CONCLUSION

The coordination chemistry of Ca^{2+} in proteins, the protonation studies with mutant calcium channels, and the X-ray and NMR structures of Ca^{2+} binding proteins lend strong support to the notion that the selectivity filter of calcium channels coordinate at least two Ca^{2+} ions. Whether these ions coordinate closely enough to exert strong repulsion that facilitates the continous permeation when the channel is open remains to be seen from extended structural biological studies.

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