Architecture of Ca\textsuperscript{2+} Channel Pore-lining Segments Revealed by Covalent Modification of Substituted Cysteines\textsuperscript{*}

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Sheryl E. Koch, Ilona Bodi, Arnold Schwartz, and Gyula Varadi‡

From the Institute of Molecular Pharmacology and Biophysics, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267-0828

The cysteine accessibility method was used to explore calcium channel pore topology. Cysteine mutations were introduced into the SS1-SS2 segments of Motifs I-IV of the human cardiac L-type calcium channel, expressed in *Xenopus* oocytes and the current block by methanethiosulfonate compounds was measured. Our studies revealed that several consecutive mutants of motifs II and III are accessible to methanethiosulfonates, suggesting that these segments exist as random coils. Motif I cysteine mutants exhibited an intermittent sensitivity to these compounds, providing evidence for a \(\beta\)-sheet secondary structure. Motif IV showed a periodic sensitivity, suggesting the presence of an \(\alpha\)-helix. These studies reveal that the SS1-SS2 segment repeat in each motif have non-uniform secondary structures. Thus, the channel architecture evolves as a highly distorted 4-fold pore symmetry.

Calcium entry via voltage-dependent calcium channels is a key chemical signal responsible for biological events such as E-C coupling, neurotransmitter release, and regulation of gene expression. Although the extracellular space contains high concentrations of sodium, potassium, and calcium ions, the voltage-dependent calcium channel provides a selective means for a high throughput of calcium ions. Despite the large pore size of the calcium channel, the narrowest point being 6 Å (10\(^{-10}\) m) in diameter (1), the channel is specifically permeable to calcium at concentrations as low as 1 \(\mu\text{M}\) (2). For all calcium channels, the selectivity filter consists of four glutamate residues (3–9), one residing in each of the four motifs, with the exception of the newly cloned T-type channel family (10–12), which contains two glutamates (motifs I and II) and two aspartates (motifs III and IV).

There are different models describing calcium movement through the channel at an approximate rate of \(1 \times 10^8\) ions per second. The one-binding site model, first proposed by Almers and McCleskey (8, 13, 14) and recently reevaluated by Dang and McCleskey (15) utilizes the concept of charge repulsion to facilitate the movement of calcium ions through the channel. The multiple-binding site model (16–20), however, suggests the presence of two calcium-binding sites of differing affinity, although it is unclear which, if any, of the four glutamates form the high affinity site(s) and which form the low affinity site(s).

There is general agreement that the four glutamates do not equally contribute to the binding and subsequent movement of the calcium ion into the cell (6, 21), however, voltage-dependent calcium channel topology has not been experimentally determined.

The experimental procedure known as scanning cysteine accessibility method (SCAM)\textsuperscript{3} has been extensively applied to study short regions of the secondary structure of membrane bound proteins to elucidate secondary structure (22). SCAM is based on the fact that known protein secondary structures, such as an \(\alpha\)-helix or \(\beta\)-sheet, contain amino acids that are exposed to the extracellular space. The periodicity of these exposed amino acids, as determined by the secondary structure of the region, provide a means by which overall secondary structure can be determined. Akabas et al. (22) demonstrated that by individually changing each amino acid, in a given segment, to cysteine, then adding sulhydryl-modifying compounds, they could determine which amino acids of the acetylcholine receptor were exposed extracellularly, and subsequently the implied secondary structure of that region.

Since the first description of application of SCAM to the acetylcholine receptor (23, 24), many additional proteins have been investigated, such as the \(\gamma\)-aminobutyric acid type A receptor (25, 26), dopamine D\textsubscript{2} receptor (27–31), cystic fibrosis transmembrane conductance regulator (32–34), cyclic nucleotide-gated channel (35), ryanodine receptor (36), voltage-dependent Cl\textsuperscript{−} channel (37), and \(\beta_{2}\)-adrenergic receptor (38). Among these, the most revealing and detailed studies were done on the potassium channel pore-lining region (39, 40), in which the secondary structure of the voltage-dependent potassium channel pore region was described as random coils. Importantly, these findings were recently confirmed by x-ray crystallographic studies (41, 42), and clearly demonstrated the relevance of this method in determining unknown secondary structure.

To examine the secondary structure of the voltage-dependent calcium channel pore, we systematically introduced cysteine mutations within consecutive positions in all four pore-lining segments of a calcium channel. These mutants were expressed in *Xenopus* oocytes and their sensitivities to methanethiosulfonate compounds were determined. Based on the accessibility to sulphydryl modification, we established a secondary structure for the pore-lining segments and made observations that provide an initial spatial arrangement of the selectivity filter in the pore.

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† To whom correspondence should be addressed: Institute of Molecular Pharmacology and Biophysics, University of Cincinnati, College of Medicine, 231 Bethesda Ave., Cincinnati, OH 45267-0828. Tel.: 513-558-2466; Fax: 513-558-1778; E-mail: varadig@email.uc.edu.

‡ To whom correspondence should be addressed: Institute of Molecular Pharmacology and Biophysics, University of Cincinnati, College of Medicine, 231 Bethesda Ave., Cincinnati, OH 45267-0828. Tel.: 513-558-2466; Fax: 513-558-1778; E-mail: varadig@email.uc.edu.

§ The abbreviations used are: SCAM, scanning cysteine accessibility method; HHT-1, human heart calcium channel; MTS, methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate hydrobromide; DTT, dithiothreitol; BAPTA, 1,2-bis(2-aminoxyethane-N,N,N′,N′′-tetraacetate.

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EXPERIMENTAL PROCEDURES

Scanning Cysteine Mutagenesis of the Human Heart α1C Subunit—
Amino acids that reside within the pore of the human heart calcium channel (HHT-1) (52) were sequentially mutagenized to cysteine residues (Fig. 1). This was accomplished by employing the two-step polymerase chain reaction amplification method, termed “mega-primer” polymerase chain reaction. The polymerase chain reaction primers contained motif I mutations, T232C, V327C, F328C, Q329C, I332C, M333C, E334C, G335C, W336C, and T337C, were subcloned into the Eco47I (1526/ClaI (2663) fragment of the HHT-1 cDNA and sequenced (52). Fragments from these constructs were then cleaved using the unique restriction sites MfeI (1732) and ClaI (2663) and ligated into the full-length HHT-1 cDNA. Substitution by serine for the endogenous pore cysteine residue, Cys, was performed in the same manner as the individual cysteine mutants for motif I. The motif II mutations, T669C, V670C, F671C, Q672C, I673C, L674C, T675C, G676C, E677C, D678C, W679C, and N680C, were subcloned into a BarnHI (2498/BoaI (4550) cassette of the full-length HHT-1 cDNA and sequenced. These fragments were cut and ligated into the full-length HHT-1 ClaI (2663) and ApaI (3920) restriction sites. The motif III, A1075C, L1079C, F1080C, T1081C, V1082C, S1083C, T1084C, F1085C, E1086C, G1087C, W1088C, P1089C, E1090C, and the motif IV, L1379C, L1380C, F1381C, R1382C, A1384C, T1385C, G1386C, E1387C, A1388C, W1389C, Q1390C, and D1391C mutations were subcloned into a fragment of the full-length HHT-1 cDNA clone, called DE7 (3729/BoaI (5635) (52)). The motif III and IV mutations were sequenced and ligated into the full-length HHT-1 BclI (5720) restriction sites. All full-length mutant cDNAs were resequenced at the boundaries and at the mutated site to ensure the desired mutations were present.

In Vitro cRNA Synthesis and Electrophysiological Measurements—
Human heart α1C, and α1D (S5, 54), and human β3 (55–58) were linearized for cRNA synthesis using the XhoI, XhoI, and NheI restriction enzymes, respectively. Complementary RNA synthesis was accomplished by employing the T7 mMessage mMachineTM (Ambion). The resultant transcripts were quantified spectrophotometrically, diluted to a final concentration of 1 μg/ml, and verified by formamide-based agarose gel electrophoresis.

cRNAs were injected into freshly isolated, delipidated stage V-VI Xenopus laevis oocytes in an approximate mass ratio of 2:1 for α1C/β3, respectively. The total concentration of cRNA injected into each oocyte was 1 μg/ml. After 2–4 days of incubation at 19 °C, in physiological solution containing (in mM): 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, 5 HEPES, 2.5 sodium pyruvate, 0.5 theophylline, pH 7.5, supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, whole cell currents were recorded using the two-microelectrode voltage clamp technique. The currents were measured in a solution, containing (in mM): 150 KCl, 5 sodium-methyl-glucamine, 1 niflumic acid, 20 HEPES, titrated to pH 7.4 with methanesulfonic acid. Voltage and current electrodes had a tip resistance of 0.5–1.5 MΩ when filled with 3 M KCl. Oocytes that exhibited a large calcium-activated chloride channel conductance were injected with 50 nl of 40 mM K2BAPTA solution (potassium BAPTA, 10 mM HEPES, pH 7.05) (59). Whole cell leakage and capacitive currents were subtracted using the P4 protocol. Currents were digitized at 1 kHz after being filtered at 1 kHz. The pCLAMP software (Axon Instruments) was used for data acquisition (version 5.6) and analysis (version 6.03). The peak current amplitude of wild-type channels was determined through examining the channels current-voltage relationship (data not shown). The potential which delivered peak current for the wild-type was 20.00 ± 5.14 nA. The voltage pulse was applied every 10 s from a holding potential of −80 mV to a test potential of +20 mV; the duration of the pulse was 80 ms. The current was determined to have reached steady-state when there was less than a 10% increase or decrease in current amplitude after 5 min.

After whole cell Ba2+ currents reached steady-state, methanethiosulfonate (MTS) compounds (Toronto Research Chemicals, Inc.) were perfused into the bath solution surrounding the oocytes for 2 min (4 ml/min). The MTS compounds were freshly diluted from a 100 mM stock solution into the 40 mM Ba2+ solution to a final concentration of 3 mM. The voltage pulse was applied as described previously. The percent difference between the control current amplitude and the current amplitude after the addition of the MTS compound was determined for each individual oocyte. The results have been expressed as an average of the decrease in the peak current potential for each mutant.

RESULTS

To perform SCAM on calcium channels, we made systematic individual mutations converting each amino acid in the pore-lining segments into a cysteine residue (except those that are cysteine residues in the wild-type channel, i.e. Cys230 and Cys1386) as depicted in Fig. 1. Each of the mutated channels, expressed in Xenopus oocytes, produced measurable Ba2+ currents when tested using the two-microelectrode voltage clamp method. However, several of the mutants did express smaller currents when compared with wild-type. This behavior was predominately found in the selectivity filter and in motif II mutants. Current-voltage relationships for each of the pore mutants and HHT-1 demonstrated that the replaced amino acids caused no significant changes in the half-activation of the channel or in the peak current potential (data not shown).

Effect of Endogenous Cysteines on MTS Inhibition—The native L-type calcium channel carries cysteine residues at the outer mouth of the pore, thus treatment of the wild-type channel with methanethiosulfonates may result in inhibition of the current. In order to determine whether the endogenous cysteines in the neighborhood of the pore-lining segments contributed to the inhibition of the calcium channel, we replaced the cysteine residues in the pore region, Cys230, Cys1386, and Cys1383, with serines and tested for sensitivity to each of the three MTS compounds. The HHT-1 channel exhibited mild sensitivity, 20–30% current reduction, to all three of the MTS compounds. However, the current reduction of 2C → S (C1383S and C1386S) and 3C → S (C330S, C1383S, and C1386S), upon addition of MTSET, was 24 and 14%, respectively. While showing a moderate decrease in sensitivity, a two-tailed Student’s t test analysis did not reveal a significant difference in the degree of MTS inhibition for these channels (Fig. 2A). This also means that the above cysteine residues are not located in the narrowest section of the pore. Thus, sulphydryl modification at these residues does not impose a sufficient degree of block to prevent the ion permeation. Testing these mutants with MTSES led to the same pattern of inhibition as that of MTSET (Fig. 2B). However, MTSEA provided consistently higher inhibition of the wild-type and mutant channels than that found for

| Motif III (aa. 1678-1999*) | Motif IV (aa. 1775-1910*) |
|---------------------------|--------------------------|
| α1.1                     | α1.2                     |
| α1.3                     | α1.4                     |
| α1.5                     | α1.6                     |
| α1.7                     | α1.8                     |
| α1.9                     | α1.10                    |

* This number indicates the corresponding amino acid number from the human heart calcium channel.

FIG. 1. Alignment of the pore region amino acid sequences of the different calcium channel α1 subunits. α1.2 corresponds to the amino acid sequence for the human heart clone (HHT-1). The amino acids that are not homologous with the α1.2 are highlighted (red). The gray shaded areas identify which amino acids can be excessively blocked by the MTS compounds.
the other two MTS compounds (Fig. 2C). This effect can be attributed to the promiscuous membrane permeation of this MTS compound (49). As a result, we felt to preserve the integrity of the channel and because the endogenous cysteines did not produce significant block, all subsequent mutations were made in the wild-type HHT-1.

Feasibility of Reducing the MTS Inhibition—In previous SCAM studies, reversible reduction of the disulfide bond formed between the cysteine residue and the MTS compound using dithiothreitol (DTT) (39, 40) has been shown to occur. However, other studies were unable to reverse this reaction despite high concentrations of DTT (38, 44–47). The effect that DTT would have on the modified calcium channel was unknown. To make an attempt to reverse the reaction of the MTS compounds, we used four different concentrations of DTT. Histograms depicting the current remaining after addition of MTSET compound and the subsequent perfusion of four different concentrations of DTT are shown in Fig. 3A. When oocytes expressing the wild-type channel were superfused with a bath solution containing 3 mM MTSET, current reduction of about 20% was observed. Upon exchanging the bath solution with different concentrations of DTT, we observed a slight reversion of current block at 1 mM concentration, however, we were not able to sustain this reduction with increasing DTT concentrations (Fig. 3A). In fact, high concentrations of DTT, 20 and 50 mM, significantly decreased the current remaining, instead of reducing MTS block. Since the overall effects of DTT were not substantial, even at concentrations of 1 mM, we concluded that upon disulfide bond formation the region is sterically hindered, therefore reduction cannot occur.

The test of the E334C mutation, a well exposed site for extracellular attack by all three MTS compounds, confirmed that once the -SH site was reacted with a bulky MTS compound DTT cannot reduce the S-S bond (Fig. 3B). Upon addition of 5 mM and 50 mM DTT, there was a significant decrease in the current remaining (p < 0.05), suggesting that the integrity of the channel has been compromised.

Intermittent Sensitivity of Motif I Cysteine Mutants—Currents expressed by mutants in which amino acids in the SS2 segment of motif I were systematically changed into cysteine residues were tested for block by the MTS compounds. Application of MTSET to mutants in motif I that represent a stretch of six amino acids in the SS2 segment (TMEGWT, which includes the MEGW signature sequence for this motif) resulted in a block of calcium channel currents for every second introduced cysteine residue (Fig. 4A). Treatment of the same mutant channels with MTSES exhibited a similar pattern and percentage of block as that observed for MTSET (data not shown). The pattern of block that begins at T332C and appears substantial for every second amino acid, while the intermittent ones are only slightly blocked, resembles a secondary structure in which every second position is sterically hindered. This type of structure can be best described by a β-pleated sheet-like arrangement of the amino acids in this region. The MTSEA compound shows surprisingly high block with all of the mutants in motif I, again an effect that can best be explained by the high membrane permeability of this compound (data not shown). None of the individual motif I SS1 segment cysteine mutant channels (TVFQCI) exhibited substantial reduction of barium current by MTSET (p > 0.05) (Fig. 4A). However, Q329C showed a significant degree of block by both MTSES (36%, p < 0.04, n = 5) and MTSEA (45%, p < 0.04, n = 7), possibly suggesting the presence of an α-helix. This latter observation is consistent with the simulated structural model for Ca2+ channels (48) and also with that determined by x-ray crystallography for the inner pore-helix in the KcsA channel (42).

Sensitivity of Mutants in Motif II and III to Sulfhydryl Modification—Three of the individual cysteine residue substitutions in the SS2 region of motif II had current inhibited by MTSET (p < 0.001) (Fig. 4B); E677C, D678C, and W679C, a pattern resembling a “random coil,” because it lacks the necessary characteristics of defined structures, such as an α-helix or a β-sheet. Of these residues, only two in the SS2 region had currents reduced by MTSES, D678C (42%, p < 0.02, n = 9) and W679C (52%, p < 0.001, n = 8) (data not shown). All of the
mutants in the SS2 region, with the exception of T675C, had a high degree of current inhibition by MTSEA (data not shown), which is consistent with the high membrane permeability of this compound. From the motif II SS1 mutant channels, only I673C exhibited significant current reduction by MTSES (39%, \( p < 0.004, n = 4 \)) (data not shown), and none of these channels had currents inhibited by MTSET (Fig. 4B) or MTSEA (data not shown).

For the individual cysteine mutant channels in the motif III SS2 region, an increased degree of block by MTSET was obtained after amino acid T1084C. The extent of current block is maximal at W1088C (87%, \( p < 0.001 \)), then quickly decreases (Fig. 4C). The same pattern of block was observed upon addition of MTSES (Fig. 5B). This pattern is typically assigned a random coil structure. All of the mutants between T1084C and E1090C were sensitive to inhibition by MTSEA showing various degrees of block (T1084C, 81% and E1090C, 41%, \( p < 0.001–0.04, n = 6–17 \)) (Fig. 5C) without a definable pattern for secondary structures, again a characteristic that is typical for MTSEA. MTSET application to the motif III SS1 mutant channels was able to block currents from F1080C (51%, \( p < 0.001, n = 8 \)) and T1081C (42%, \( p < 0.01, n = 14 \)) (Fig. 4C). Currents

![Fig. 3. Histograms depicting the effects of DTT on the wild-type (A) and E334C (B) calcium channels following the application of MTSET. Current reduction is after a 2-min perfusion with 3 mM MTSET (black bar) and subsequent 2-min perfusion with various concentrations, 1, 5, 20, and 50 mM DTT (grey bar).](image-url)
from T1081C were also blocked by both MTSES (46%, \(p < 0.001, n = 12\)) and MTSEA (88%, \(p < 0.001, n = 9\)) (Fig. 5, B and C, respectively), again possibly suggesting the presence of an α-helical turn in this region.

Sensitivity of Mutants in Motif IV to Sulfhydryl Modification—The amino acid substitutions from the SS2 region of motif IV resulted in only two neighboring amino acids (G1386C and E1387C) which demonstrated large current block by MTSET (66 and 73%, respectively, \(p < 0.001, n = 7\)) (Fig. 4 D). Interestingly, the two amino acid positions showed little or no block (that is significantly greater than wild-type) by MTSES (data not shown). The mutant channels from positions T1385C to Q1390C all had large current inhibition upon addition of MTSEA (data not shown). Most of the amino acid positions in the SS1 region of motif IV, encompassing a stretch from L1379C to A1384C, were insensitive to the MTS compounds. Only F1381C had a significant current reduction by MTSET (38%, \(p = 0.04, n = 9\)) (Fig. 4 D), while L1380C and F1381C both had currents blocked by MTSES (34 and 36%, respectively, \(p < 0.05, n = 5–6\)) and the current from R1382C was inhibited by MTSEA (50%, \(p < 0.02, n = 5\)) (data not shown).

Differences in Sensitivity Among the Selectivity Filter Mutants—The selectivity filter amino acids demonstrated significant current reduction upon addition of MTSET, 61, 62, 41, and 73%, respectively, to wild-type ( \(p < 0.03\)). The substitutions E334C and E1086C showed currents that were blocked by MTSES, 64 and 50%, respectively ( \(p < 0.001\)). In contrast, barium currents through E677C and E1387C were not significantly sensitive to sulfhydryl modification by MTSES when compared with wild-type (20 and 27%, respectively, \(p > 0.05\)) (Fig. 6). As expected, all four selectivity filter amino acids demonstrated significant current reduction upon addition of MTSEA; 79% (E334C), 88% (E677C), 59% (E1086C), and 84% (E1387C) ( \(p < 0.001\)).

DISCUSSION

Accessibility of Endogenous Cysteines in the Pore-lining Region—The removal of endogenous cysteine amino acids in the pore region of the voltage-dependent calcium channel did not have a significant effect on the inhibition of channel current by the MTS compounds. The three different wild-types tested, HHT-1, 2C3S, and 3C3S all had 15–25% block when the MTS compounds were added to the bath, with the exception of MTSEA which demonstrated a 50% block on 3C3S. This latter observation suggests a conformational change in the channel structure upon removal of the three endogenous pore cysteines. The observed higher degree of block could be a result of an increase in the size of the pore of the channel or potentially exposing another endogenous cysteine residue that is hidden in the native channel conformation.

Sensitivity of Cysteine Mutants to Sulfhydryl Modification—The secondary structure of the pore region was heretofore thought to be uniform for all four motifs. Our present results, however, indicate that they are strikingly distinct. We employed the Scanning Cysteine Accessibility Method to decipher the topology of Ca\(^{2+}\) channel pore-lining segments. The pore region of motif I, based on the intermittent block by the MTS compounds, reveals a secondary structure best described as a β-pleated sheet. The pore region of motifs II and III displayed a sequential block encompassing three and four amino acids, similar to the structural arrangement which is consistent with the presence of a random coil, whereas motif IV, exhibits periodicity in its block by the MTS compounds, strongly suggesting the presence of at least an α-helical turn in this region. Our data supports the findings of Schetz and Anderson (49) who...
determined that the motif IV pore region lacks the amino acids necessary to make a p-bend. Their study indicates that there is a 100% probability of a p-bend in motifs I, II, and III, which is in agreement with the findings of a β-sheet, and two random coils, respectively. These observations establish structural substance for the assumed, impaired 4-fold symmetry of the four selectivity filter glutamates. The high number of conserved amino acids residues in the pore regions of all known calcium channels lend credence to the applicability of this data to all calcium channels, with the possible exception of the low-voltage activated T-type channels.

The size and charge of the MTS compounds are important in analyzing the results of this study. The positively charged MTSET, even though it is the largest of the three compounds utilized, is able to block all four of the selectivity filter amino acids by at least 40%. The negatively charged MTSES has the same effect on the selectivity filter amino acids from motifs I and III, however, the results for motifs II and IV indicate that MTSES does not bind these amino acids. One possible explanation is that the pore regions of motifs II and IV are deeper in the membrane than those of motifs I and III, thus these latter two glutamates may be hindering the MTSES compound from reaching deeper into the membrane by repulsing the negatively charged compound.

The dramatic decrease of channel current upon addition of MTSEA was expected. This compound is freely membrane permeable and has the potential to bind to cysteines from the outside or inside of the membrane (43). This allows MTSEA to
block channel current even if the introduced cysteine residue is hidden from the bath solution surrounding the oocyte.

An important structural implication originating from this SCAM study is the involvement of sequential amino acid residues in high percentage block in each pore-lining segment. In motif I, six residues, in motif II and IV, three and two, respectively, while in motif III a stretch of five amino acids responded to MTS treatment with substantial current reduction. Therefore, our results are similar to those found for the voltage-dependent sodium and potassium channels, having three or more consecutive amino acids demonstrating large block (greater than 50%). Thus, these residues are structurally part of a highly distorted 4-fold symmetry that runs quasi-parallel with the imagined axis of the pore (Fig. 7). Such an arrangement contradicts an earlier model that assumed the narrowest point of the pore is at the selectivity filter glutamates. The model depicted in Fig. 7 does not exclude the possibility that the four glutamates form the high affinity selectivity filter potentially coordinating one or more Ca\(^{2+}\) ions. The model does, however, introduce the possibility that the polypeptide backbone carbonyl groups coordinate additional Ca\(^{2+}\) ions, thus supporting the multiple occupancy theory. In this aspect, perhaps the motif I SS2 segment provides the most important contribution.

**Topography of the Pore-lining Regions in Voltage-gated Channels**—An additional purpose of this study was to investigate the structural relationship between the Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\) voltage-gated channel ions. It is clear that despite the close homology in the pore regions of this family of channels, the selectivity filters of the individual channels are able to differentiate between the ions with high fidelity. Heinemann et al. (50, 51) investigated this principle by conferring Ca\(^{2+}\) ion selectivity onto the sodium channel. However, the obverse of this procedure, conferring Na\(^{+}\) selectivity onto the calcium channel, has been unsuccessful. This indicates that there are more extensive requirements for Ca\(^{2+}\) channel selectivity than just the 4 glutamate residues. The present study revealed that the secondary structures of the pore regions of Na\(^{+}\) and Ca\(^{2+}\) voltage-gated channels are not identical. Furthermore, since a random coil is less strictly structured than an \(\alpha\)-helix or a \(\beta\)-sheet, the presence of these more rigid structures may be necessary for the selectivity observed in Ca\(^{2+}\) channels. Removal of these elements, as in the experiments by Heinemann et al. (50, 51) results in the formation of a non-selective ion channel. Therefore, it is plausible to assume that the secondary structure of the pore regions of Ca\(^{2+}\) channels is critical to the Ca\(^{2+}\) selectivity and dissecting the structure is key to understanding the mechanisms by which calcium moves through the pore.

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