A molecular model of the inner pore of the Ca channel in its open state

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Abbreviations: MTSET, methanethiosulfonate ethyltrimethylammonium; SCAM, substituted cysteine accessibility method; TEA, tetramethylammonium

Introduction

The spacial structure of a Ca channel has not yet been resolved by X-ray crystallography, although extensive mutational data suggest that it has some analogy to the well-studied K channels. We2,3 have previously modeled the closed state of the Ca channel on the basis of the X-ray structure of the closed bacterial KcsA channel.4 Although there are now several X-ray structures of open K channels,5,7 they may not be good templates for the open inner pore of the Ca channel.

The best information about the organization of the Ca channel open pore has been derived from the substituted cysteine accessibility method (SCAM). An extensive SCAM analysis was carried out for each of the four S6 transmembrane α-helices of the P/Q channel (Ca2.1), i.e., single cysteine substituted channels were modified by MTSET.1 For convenience Zhen et al.1 numbered amino acid residues of the S6 segments beginning with the N-terminus, increasing the number in the direction of the C-terminal residues—86% for the residue at position 4,1

Structure of the Ca channel open pore is unlikely to be the same as that of the K channel because Ca channels do not contain the hinge residues Gly or Pro. The Ca channel does not have a wide entry into the inner pore, as is found in K channels. First we sought to simulate the open state of the Ca channel by modeling forced opening of the KcsA channel using a procedure of restrained minimization with distance constraints at the level of the α-helical bundle, corresponding to segments Thr-107-Val-115. This produced an intermediate open state, which was populated by amino acid residues of Ca channels and then successively optimized until the opening of the pore reached a diameter of about 10 Å, large enough to allow verapamil to enter and block the Ca channel from inside. Although this approach produced a sterically plausible structure, it was in significant disagreement with the MTSET accessibility data for single cysteine mutations of S6 segments of the P/Q channel1 that do not fit with an α-helical pattern. Last we explored the idea that the four S6 segments of Ca channels may contain intra-molecular deformations that lead to reorientation of its side chains. After introduction of π-bulges, the model agreed with the MTSET accessibility data. MTSET modification of a cysteine at the C-end of only one S6 could produce physical occlusion and block of the inner pore of the open Ca channel, as observed experimentally, and as expected if the pore opening is narrower than that of K channels.

Table 1

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open pore have been published, but they do not show either the relatively narrow cytoplasmic opening or the specific pattern of MTSET accessibility implied by the available SCAM data. This article explores an alternative approach to modeling of the inner, open Ca channel pore.

Results

The SCAM data suggest that the cytoplasmic end of the Ca channel inner pore is narrower than it is just below the selectivity filter. The diameters of both the closed and open states of the K channels just below the selectivity filter are about 11 Å. If this region is similar in K and Ca channels, then the Ca channel inner pore mouth diameter should be no greater than 11 Å. A relatively narrow cytoplasmic pore mouth is also suggested by the experimental data of T-type Ca channels with verapamil. The width of verapamil at the level of the dimethylsubstituted aromatic rings is ~10 Å, from which we speculate that the cytoplasmic Ca channel pore mouth is about 10 Å in diameter, based on available pharmacological data for charged phenylalkylamines, which are the only calcium channel agents that have been definitively demonstrated to enter the inner pore from the cytoplasm. In the absence of direct information about the Ca channel transition from the closed to the open state, we began by using the closed KcsA channel as a template with the goal of predicting an open pore of the T-type Ca channel, which at the level of the S6 bundle equivalent positions of all four inner α-helices. In contrast for the Ca channel full block of the open state could be produced by MTSET interaction with cysteine residues introduced into single S6 segments.

Not only do crystal structures and SCAM data support the idea that there is a difference between open K channels and open Ca channels, there are also reasons to think that the opening mechanism of Ca channels may be different from that of K channels. The glycine residues of the inner α-helices at the level of the selectivity filter, which are conserved in all K channels, and which appear to be responsible for transformation of K channels from closed to open states ("gating hinges"), are absent in two of the S6 segments of Ca channels. These S6 glycines, Gly-99 in KcsA and Gly-89 in MthK, can be found at similar positions in DI and DII S6 segments of Ca channels, but analogous locations in DIII and DIV have bulky conformationally restricted hydrophobic amino acid residues, e.g., Ile in DIII S6 and Cys in DIV S6 of CaV1.2, (see alignment 3 and in Table 1). Moreover, substitution of Gly-770 in DIII S6 of CaV1.2 by proline did not produce any noticeable effect on voltage-dependent activation or inactivation, implying that even this glycine may not act as a "hinge".

Therefore, the available Ca channel mutagenesis data are not consistent with the predictions of the crystallographic data for the various K channel open channels and suggest that the structural changes that occur to open the Ca channel are different from that of K channels. Attempts to develop such models of the open pore have been published, but they do not show either the relatively narrow cytoplasmic opening or the specific pattern of MTSET accessibility implied by the available SCAM data. This article explores an alternative approach to modeling of the inner, open Ca channel pore.

Table 1. Sequence alignment of KcsA along with the P/Q and T Ca channels for inner pore S6 residues

| Position | 100 | 105 | 110 |
|----------|-----|-----|-----|
| KcsA     |     |     |     |
| Block    | 75% | 99% | 70% |
| P/Q      | 13  | 12  | 11  |
| Block    | 50% | 38% | 65% |
| T channel| 16  | 15  | 14  |
| Block    | 50% | 38% | 65% |
| Adj KcsA Alignment | G I T S F G L V – T A A L A

Arrows below the KcsA sequence show positions that face the pore in the open crystal structures and the corresponding percentage modification by MTSET, observed in Kir2.1. Positions are numbered from the cytoplasmic side as given in Zhen et al. MTSET study. Block is reported as the arithmetic averages of the percentage modification for the four S6 segments. The table also shows the T channel sequence alignment, and the last line presents the KcsA alignment with the open Ca channel, including an insertion that adjusts for the π-bulge. Location of π-bulges for Ca channels (i+4 to i+9) is marked by solid line.
The positions 13, 10, 9, 6, 5 and 1 of the inner S6 of the KcsA channel were optimized. The P/Q-T-type channel alignments were based on the state being populated with residues of the T-type Ca channel and the transmembrane S6 α-helices of Ca channels are likely to be shorter with the C-ends corresponding to Thr-112 of KcsA (Table 1), and so, in general, the S6 helices face the pore lumen do not predict an α-helical structure for the C-ends of the S6 segments, in contrast to K channels. The first challenge was to create an opening at the level of Thr-107 to Thr-112 that would be sterically valid and 10 Å in diameter.

**Forming a KcsA intermediate template for the Ca channel.** The KcsA channel does have intermediate structural and functional states during opening. These depend, however, on the Gly hinges, which are not present in the Ca channel. The K1,2 channel also has the PVP kink, while S6 sequences of Ca channels do not contain Pro residues at all (Table 1). To avoid dependence on K channel opening mechanisms, we instead produced forced opening of the inner pore of KcsA by gradual displacement of the amino acid residues at the level of the α-helical bundle (Thr-104 and Ala-111) from the central axis of the pore. We began with the KcsA M2 teepee structure, introduced distance constraints between the Cα atoms of opposite Thr-107 and Ala-111, and then optimized using the procedure of restrained minimization with an energetic term that included distance constraints along with common energy terms. We next energy minimized all amino acid residues below the selectivity filter (beyond Val-96) and made stepwise gradual increases in distance constraints in 2 Å steps, which avoided artificial conformational solutions that could lead to strained kinks in the α-helices. Energy refinement at each step allowed production of stable structures. The resulting reorientations were distributed throughout the M2 α-helices below the selectivity filter, so that there were small changes in the torsion angles of rotation φ and ψ along the α-helix backbones. The procedure was continued until the mouth opening of an intermediate state was large enough to allow access by tetraethylammonium (TEA). We chose TEA as the caliper because it is a smaller, symmetrical structure, rather than verapamil, which is asymmetrical, but has a similar diameter -10 Å in its extended conformation. The distance between the Cα atoms of opposite Thr-107 residues was increased from 10.8–16 Å, and for Ala-111 atoms it was thus increased from 8.7–15 Å (Fig. 1 and left).

**Conversion of the KcsA model to the T-type channel pore.** To consider the Ca channel pore, the KcsA intermediate open state was populated with residues of the T-type Ca channel and optimized. The P/Q-T-type channel alignments were based on designating identical positions for the perfectly conserved asparagine residues in each S6 segment of all Ca channels (Table 1). The KcsA α-helical structure predicted that the side chains of positions 13, 10, 9, 6, 5 and 1 of the inner S6 α-helices face the pore. These are exactly the positions that, when substituted with cysteine, reacted with MTSET in the Kir 2.1 channel (Fig. 1 and right). However, this structure failed to completely correspond with the predictions of the Ca channel SCAM study. The MTSET accessibility data for the P/Q Ca channel were consistent with the side chains in positions 13 and 9, in that they were modified by MTSET and presumably they face the pore, but closer to the C-end of the S6 segments the orientation based on K channel structures failed to fit the SCAM data. At position 6 in all four S6 segments, MTSET failed to produce any reduction in current for the P/Q channel. Moreover, it is important to note that the native T-type Ca channel contains a cysteine in position 6, and yet MTSET treatment of the native T channel did not produce block. In contrast, cysteine substitution in position 4, which by the K channel template faces away from the pore, produced almost complete block. It, therefore, seems that below position 9 in the Ca channel open organization there must exist a conformation different from the α-helical pattern found in K channels. The failure of position 6 in the T-type Ca channel to react with inside MTSET also is consistent with the idea that the T-type and the P/Q Ca channels have a similar structure in the open state and that the side chains of residues at positions 13, 9, 5, 4 and 1 will face the pore in the open state.

**An alternative proposal for the secondary structure of the open lower pore.** The different pattern for MTSET accessibility data for the C-terminal ends of the Ca channel S6 segments in the open state requires that these segments contain intramolecular deformations that reorient the side chains of residues at positions 6, 5 and 4. Analysis of short intrahelical deformations inside structures from the complete Protein Data Bank (PDB) has shown that a common intrahelical deformation (82%) is a π-bulge. This seems a plausible alternative to the conformation seen in K channels.

π-Bulges or wide turns, contain an additional amino acid residue in the helical turn (4.4 residues) compared with 3.6 residues per turn in the standard α-helix, and as a consequence, the π-bulge has a i-(i-5) main chain hydrogen bond, while the α-helical turn has i-(i-4), where i is a donor of an amino group and i-(i-4) or i-(i-5) are donors of a carbonyl group. In this way an additional amino acid residue in the π-bulge can be considered to be an insertion into the α-helix. There is a preference in π-bulges for poor α-helical residues, His, Tyr and Asn. All Ca channels have at position 8 of their S6 helices in all four domains a very conserved asparagine, which could play a special structural role; Asn itself has a high tendency to be an inner residue in π-bulges.

For example, in the case of IS6, we initiated a π-bulge in the T-type Ca channel at the residue above Asn(8), which is Ile(9). Its main chain carbonyl now forms a hydrogen bond with Val(4) (i-i pattern) instead of Asn(8), the i-(i-4) pattern in a regular α-helical turn (Fig. 2 and left). In this arrangement Cys(6) shifts inside the π-bulge to become the extra residue in the helix, and it is now located at the outer side of the widened turn. As a consequence there is a shift by one residue in the alignment of the eight C-terminal amino acid residues of the S6 helices in the modeled open state. Introduction of the π-bulge also required an insertion.
in the alignment of sequences of the Ca channel with the sequence of KcsA (see the new alignment in Table 1). Thr-107 and Ala-108 of KcsA or Val-91 and Glu-92 of MthK, which face the pore, are now aligned with positions 5 and 4 of the Ca channel. The side chain at position 4 faces the inner pore (Fig. 2 and right), and this is consistent with the observed high level of MTSET induced current reduction by its interaction with a substituted cysteine at this position in the P/Q Ca channel. The location of Cys(6) of IS6 outside the inner pore fits with the failure to show block with MTSET exposure of the naturally occurring Cys in the WT T-type Ca channel\(^1\) or when Cys was introduced into P/Q channels\(^1\) at this position. It also implies that the structure is tightly packed at this level, preventing MTSET access to the back of the S6 segment.

Not only does the introduction of a \(\pi\)-bulge in IS6 reconcile the proposed structure with available experimental data, but introduction of \(\pi\)-bulges similarly in the other S6's now produces a pattern of side chain accessibility for all four S6 segments consistent with the Zhen et al. data.\(^1\) It is reasonable to suggest that in the closed state of Ca channels the S6 helices lack \(\pi\)-bulges and that they are generated by the molecular forces that open the pore, also possibly stabilizing it (reviewed in ref. 11).

**Formation of the open Ca channel with \(\pi\)-bulge modification to the S6 helices.** This preliminary model of the T-type Ca channel with \(\pi\)-bulges at positions 4–9 did not itself represent an open conformation since the cytoplasmic opening was only 6 Å secondary to the fact that Ca channels have bulky hydrophobic residues at their inner pore mouths, e.g., Val, Leu or Ile. In order to produce a 10 Å opening of the inner pore, two molecules of TEA were fixed simultaneously at 45° to each other in the exact center of the inner pore in the plane of the Cα atoms of amino acid residues at the 5, 4 and 1 positions, sequentially. Arranged in this manner, two TEA molecules produced symmetrical perturbation of the inner pore (reviewed in ref. 23). Then, on each step

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**Figure 1.** The KcsA intermediate open structure. Left part shows side view of the intermediate opening of the KcsA structure, which allows TEA (shown by the yellow space-filling image) to pass into the pore. TEA is located at the level of Thr-107 of the M2 \(\alpha\)-helices (shown by green ribbons) of the inner pore (side view). Right part illustrates the directions of the side chains at positions 13, 9, 6 and 4 of IS6 (using numbering from the cytoplasmic side) in the inner pore of the T-type Ca channel when the KcsA structure was used as template.

**Figure 2.** Left part shows the side view of a single \(\pi\)-bulge (IS6) between amino acid residues Ile(9) and Val(4) (i-i-5 pattern) inside the \(\alpha\)-helix in the open state of the T-type Ca channel. Leu9, Cys6 and Val4 are shown as space filled. The \(\pi\)-bulge reorients the side chain at position 6 (Cys) outside the pore. Right part shows the intermediate open state of the T-type Ca channel (top down) that was produced by the introduction of \(\pi\)-bulges between amino acid residues at positions 9 and 4 on the C-ends of the four S6 \(\alpha\)-helices. Shown as balls are amino acids predicted to face the pore, i.e., Val(4), Ile(9) and Ser(13). Also shown by balls is Cys(6), which now faces away from the pore.
energetic optimization of the inner pore was repeated to avoid van der Waals repulsions with the TEA molecules. Adjustment of both amino acid side chains and the helix backbones below the selectivity filter were permitted during this step, resulting in a modeled pore that is cylindrical in shape and ~10 Å in diameter (Fig. 3 and left), and that is large enough to admit verapamil (Fig. 3 and right). Its dimethyl-substituted aromatic ring is located at the level of positions 4 and 5.

This final open pore model predicts the correct side chain locations for modifiable residues found by Zhen et al. but it is also important that it predicts pore occlusion for MTSET modification of cysteines introduced into positions 13, 9, 5, 4 and 1 of S6 α-helices for each of the four domains. In keeping with experimental data, the narrowest point of the inner pore is produced by the side chains of amino acids at positions 4 and 5, and in silico MTSET modification of single cysteine residues at each of these positions is able to produce physical occlusion of the inner pore. This is shown in the model for two positions—IS6 Leu(5)-Cys + MTSET (for both T-type and P/Q-type Ca channels) (Fig. 3 and left), and that is large enough to admit verapamil (Fig. 3 and right). Its dimethyl-substituted aromatic ring is located at the level of positions 4 and 5.

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**Figure 3.** Left part shows top down view of the simulated inner pore of the open T-type Ca channel at the cytoplasmic side, determined by the ring of side chains of residues at positions 4 and 5 of the four S6 α-helices (about 10 Å diameter). Right part shows side view of the pathway of verapamil entering the inner pore of the T-type Ca channel from the cytoplasmic side. The head of verapamil at its widest position, the dimethyl-substituted aromatic ring, is shown at the level of residues at the fourth and fifth positions of four S6 α-helices.

**Figure 4.** Left part shows the location of the side chain created by MTSET modification of the cysteine residue substituted for Leu(5) of IS6 inside the inner pore of the T-type Ca channel. The MTSET treatment also could produce occlusion of the pore. Right part shows the location of the side chain created by MTSET modification of the cysteine residue substituted for Gly(4) of IIIS6 inside the inner pore of the T-type Ca channel. In this case the MTSET reaction can produce physical occlusion of the pore.
Discussion

Direct structural determination of open channels by X-ray crystallography has been achieved only for K channels, so the structure of open Ca and Na channels must be inferred from functional studies. The X-ray crystallographic structure of a prokaryotic Na channel has recently been reported, but its channel is closed. Because of the similarity in primary structure with the well-studied K channels, Na and Ca channel tertiary structures have been modeled by homology with some success. The characteristic feature of channel opening means that conformational changes must take place in ion channels. The original bacterial KcsA channel structure was obtained in its closed form, but subsequently it has been possible to obtain structures of the open K channel pores. Homology models of the open Na and Ca channel pores can be tested by the SCAM method, determining which positions in the pore-lining S6 helices, when substituted by cysteine, can react with membrane-impermeable intracellular MTS reagents, and consequently, determining which positions face the inner pore. This approach has been used successfully for the Na channel. Although homology modeling for the closed Ca channel was reasonable, it has been less successful for the Ca channel in the open state because the model of the C terminal end of S6 does not agree with the SCAM data of Zhen et al. Figure 4 of the report of Bruhova and Zhorov shows that the side chains of positions 6 of the four S6 helices are directed into the inner pore of the open state of their Ca1.2 structure, such that modification of their cysteine substitutions by MTS reagents would be expected to show block, a result that is inconsistent with the experimental data of Zhen et al. That model also does not predict the experimentally determined location of the side chains of position 4. In the model of Ca1.2 proposed by Stary et al. the opening is very wide and the amino acid residues at the C-terminal positions of S6 α-helices are far removed from the pore’s central axis (the Cα-Cα distances between opposite residues exceed 30 Å), so that MTSET modification of only one of the position 4 residues would not be expected to produce any block.

Because the SCAM data rule out a standard α-helical structure for the cytoplasmic end of the inner pore, we sought a model of the S6 segments that would fit the experimental SCAM data. Introduction of π-bulges between positions 9 and 4 in all four S6 α-helices allows the side chains of positions 13, 9, 5, 4 and 1 to face the pore, consistent with the experimental results of Zhen et al. These π-bulges would be expected to stabilize the open state of the Ca channel. Consequently, this conformational change in S6 helices may play a role in the mechanism of Ca channel opening.

A second problem with models of the open Ca channel is that experimentally the inner pore mouth seems to be much narrower than that of the K channel, suggesting a difference in the opening process. Ca channels have potential S6 glycine hinges in only two domains. For the SCAM data, MTSET binding to a single S6 residue could block the pore almost completely; in contrast, for the K channel all four modified cysteines were required to bind MTS reagents for complete block. Furthermore, block was greatest when the cysteine substitution of the Ca channel pore was closest to the cytoplasmic surface, and it was progressively less when the cysteine-substituted position was higher in the pore, in contrast to the results for K channels. The most plausible explanation for these SCAM results is that the open Ca channel inner pore mouth is much narrower than that of the K channel. If the C-terminal ends of the S6 α-helices of Ca channels do not open as widely as those in K channels, then the S6 movement need not be so dramatic. Perhaps instead of a hinge mechanism, Ca channels may use another mechanism of opening, where the desired conformational changes are distributed on long stretches of the S6 segments below the selectivity filter and associated with small changes in the torsion angles φ and ψ along the α-helical backbone.

In the search for a reason why the Ca channel inner pore might have a different open conformation, we explored the possibility that the α-helical structure of the lower S6 segments might be disrupted during the activation process. By analogy with the conformational changes during K channel pore opening, the S4 voltage sensors would move outward, and the S4-S5 segments would draw laterally their associated S5 and S6 segments. If the S6 segments are not sufficiently flexible because of the lack of hinges, then the force exerted by the S4-S5 segments might distort the terminal S6 structure. All Ca channels have highly conserved asparagines in all four S6 segments, and these are known to initiate formation of π-bulges. Stary et al. proposed an alternative kink associated with these asparagines by formation of distorted hydrogen bonds between amino acid residues 13 and 8. However, that construction of a π-bulge reorients residues at the 9 and 10 position outside the pore, which is inconsistent with the SCAM data. It was surprising that the reorientation of both residues and backbone in all four non-identical S6 segments in our proposed open channel model with π-bulges resulted in an energetically stable structure, as if the activation process proceeds along a fairly flat energy profile, as has been suggested for K channels by Cuello et al.

Do all Ca channels have similar open pore structure? The SCAM results were obtained in P/Q channels. The failure of the naturally occurring cysteine on the T channel to be blocked by MTSET suggests that the SCAM data may be applicable to the T channel. No SCAM data are available for the L channels, but their high sequence identity with the T and P/Q channels implies that their mechanism of channel opening is likely to be the same. Available data for Ca1.2 indicate that the similarly sized, but permanently charged verapamil derivative, d888, can access the inner pore of that isoform from the inside. Although that study characterized the inner pore as “widely open” because of the lack of voltage dependence of block, our model is compatible with their data. Ile1781 (at position 3 in Table 1), which did not interact with drug but which did dramatically alter kinetics, is predicted to face away from the pore. Sufficient water would be present to concentrate the voltage drop across the selectivity filter so as to make the blocking rate independent of voltage. Certainly our model predicts limited access to the inner pore for any molecule larger than phenylalkylamines. The model was constructed based on the experimental data indicating this is the only class of drugs...
that can enter the inner pore from the cytoplasm (other, larger calcium channel drugs, e.g., dihydropyridines, are all hydrophobic and not limited to entry via the inner pore), but the usefulness of such models is to provoke experiments, and so it suggests a more aggressive search for larger molecules can enter the pore is warranted. It also predicts SCAM results for other Ca channel isoforms, e.g., T- and L-type Ca channels, which are yet to be carried out and which can speak to the extent to which calcium channels are structurally similar.

Methods
Methods similar to those employed for modeling of anticonvulsant drug binding to the Na channel were used in that modeling was accomplished in the Insight and Discover graphical environment (Accelrys). Energetic calculations utilized the consistent valence force field approximation, and for minimization procedures, conjugate gradients were used. For minimizations with distance constraints the force constant (k) was 100 Kcal/(molÅ²). The T-type calcium channel was used as the representative of the Ca channel family. Alignment is shown in Table 1. As the focus of the model was the inner pore, we therefore, preserved the structure of the selectivity filter that we previously proposed for Na and Ca channels and fragments of the S6’s that are proposed to be in contact with the external P loops.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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