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Models of ion pores in N-type voltage-gated calcium channels

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Two computer models of the outer vestibule of the pore of the N-type voltage-gated Ca^{2+} channel are predicted. The models are constructed from β-hairpin peptide segments in the S5–S6 loops of each of the four domains that produce the channel. These hairpins together are modeled to form a short eight-stranded β barrel. The models contain a ring of glutamates at the base of the barrel, which have been shown by mutagenesis experiments to function as a selectivity filter. These filters are suggested by the models to be of the correct dimensions to allow the permeation of a hydrated calcium ion, where the filter glutamates may substitute for molecules of water from the hydration shell of the ion. The models also suggest that a ring of threonines and an aspartate might be present between the mouth of the pore and the filter, and hence the models may prove useful in suggesting future mutagenesis experiments.

Keywords: calcium channel, conotoxin, molecular model, pore, selectivity filter

INTRODUCTION

Ion channels are enormously important as targets for pharmaceuticals. However, although many gene sequences are known, three-dimensional structures are not readily available. Here we produce alternative models of the all-important pore region of the N-type voltage-gated calcium channel by computer modeling. The resulting structures must necessarily be treated as tentative but they do explain available data and provide hypotheses that are capable of testing by means of mutation or binding studies.

Ion channels are one of the superfamilies of membrane-bound receptors and constitute a major information signaling device of the cell. They have a common organizational pattern, consisting of a "pore" through which ions pass and a "selectivity filter" generating ionic discrimination gates. These open and close to control the flow and type of ion flux. There are also sites or "sensors" on the ion channel that respond to external signals such as neurotransmitters or membrane potentials. Such "sensors" separate ion channels into two major types: the ligand-gated and the voltage-gated channels.

Voltage-gated calcium channels posses four or five distinct subunits. Of these it has been shown that the α_1 subunit contains both the voltage sensor and the Ca^{2+} selective pore.[1] The proposed structure of these α_1 subunits consists of four internal repeated domains (I–IV), each of which is suggested from hydropathy profiles to contain six α-helical transmembrane (TM) regions (S1–S6), including one (S4) that is positively charged and is thought to form part of the voltage sensor. There are two additional regions (SS1 and SS2, collectively labeled H5) between S5 and S6, which are too short to form TM helices and hence are thought to exist as an eight-stranded β barrel, forming the pore region of the channel[2] (Figure 1).

Voltage-gated calcium channels exist in four major classes, currently labeled T, L, N, and P, and are distinguishable by selective ligand binding.[3] All these classes are expressed in neurons, although N-type channels are distinct in that they are primarily observed in neurons.[4]

The N-type calcium channels are involved in the control of neurotransmitter release from neurons and are sensitive to the marine snail (*Conus* genus) peptide neurotoxin ω-conotoxin GVIA (ω-Cgtx GVIA). It is generally thought that the toxin ω-Cgtx GVIA blocks the channel by occluding the pore, although this has not been definitely proven. The ω-Cgtx GVIA has 4 positively charged residues, 14 hydroxyl-containing residues (including hydroxyprolines), and 2 asparagines[5] (see Figure 2). The basic residues of the toxin form a positively charged hydrophilic face and it has been shown that the charged lysines and a tyrosine may be important for ω-Cgtx GVIA binding to Ca^{2+} channels.[6] Ellinor et al. have shown that changes in the loop regions of

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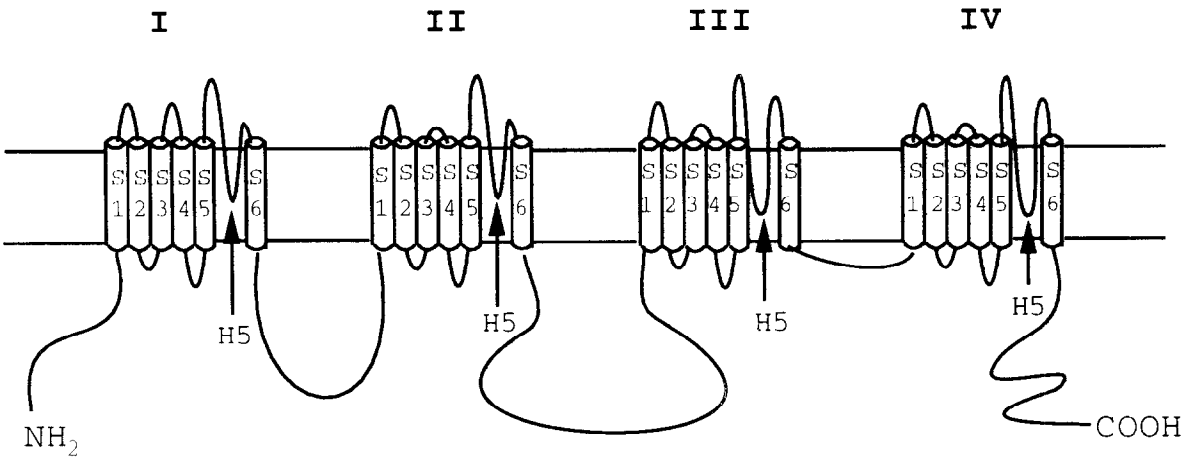

Figure 1. Schematic diagram of the proposed structure of the α_1 subunit, showing the four repeated domains, each with six transmembrane segments (S1–S6) and the pore region (H5). (After Catterall.[4])

the channel connecting the S5 and H5 regions of domains III (III S5–H5) and II (II S5–H5) cause significant slowing of the onset of toxin block.[7] This is especially so for loop residue mutations such as E → K and Q → R, where the change is to a positively charged residue. These mutations are consistent with a hypothesis of the ω-Cgtx GVIA binding to the Ca^{2+} channel with its hydrophilic, positive face toward the channel.

The protein sequence for the human N-type voltage-gated calcium channel has been determined.[8]

Mutagenesis data have shown that the highly conserved H5 regions of the channel are essential for ion selectivity.[9–11] As these regions are too short to form α helices, they are presumed to exist as β strands joined by a tight β turn, although it is conceivable that they may possibly adopt other motifs. The four domains together could therefore produce a pore consisting of an eight-stranded β barrel. The mutagenesis studies located a conserved glutamate residue in each domain that contributes to the ion-selectivity filter.[9–11] The glutamates were found to contribute unequally to Ca^{2+} binding.

Other evidence supporting these studies is that mutating filter residues in the Na^+ voltage-gated channel, corresponding to glutamates in the Ca^{2+} channel, confer calcium channel properties on the sodium channel.[12] This result of transferring calcium channel characteristics to the sodium channel by single mutations was used by molecular modelers to help confirm the validity of a molecular model of the voltage-gated Na^+ channel.[13] The Na^+ model pore was constructed using known and proposed interactions between the channel and the toxins tetrodotoxin and saxitoxin, which bind to the pore of the channel. The pore model constructed was a short β-barrel pore. Theoretical substitutions were made to the pore model, corresponding to mutations carried out experimentally, with the result that calcium channel characteristics were conferred on the Na^+ channel model.[13] Other short β-barrel models have also been proposed.[14]

A number of ion-channel pores modeled on long β barrels have been proposed where the H5 region passes through the membrane to the cytosolic side. These have been suggested on the basis of binding data of tetraethylammonium (TEA) to K^+ channels.[15] However, K^+ channels have extra residues in the H5 region and also possess residues of different hydrophobicity, suggesting that K^+ channel structures may not be obviously related to those of other ion channels. In some models, positions of proposed β turns are not in conformity with any theoretical turn predictions,[16] making the assignment of long β hairpins questionable for channels other than K^+ channels.

MATERIALS AND METHODS

A short, eight-stranded β barrel forming the selectivity filter of the pore, with the remaining intracellular part of the pore being formed by the surrounding helices, was taken as a starting point on which to model the N-type voltage-gated calcium ion channel.

The position of the β turn in the H5 sequence was found by looking for significant local maxima of β-turn propensity (P_{bend} or P_t), using the values of Williams et al.[17]—a modified Chou and Fasman algorithm.[18] Although these values are determined essentially from globular proteins, owing to the accuracy in β-turn prediction they can be equally applied to membrane proteins. As the pore is water filled it may also be reasonable to assume that the protein environment will be relatively similar to that of globular proteins. Also, the successful results of Na^+ pore modeling,[13] where the same technique was used, serve to support the use of β-turn prediction as a valid tool.

To determine the β-turn conformation, P_t values are supplied for single amino acids. If $P_t > 1.0$ for a specific residue then that residue has a high probability of forming a β turn. However, taking the average P_t over four adjacent amino acids gives a more accurate indication. So if $\langle P_t \rangle > 1.0$ and the P_t values for the central two residues are greater than the P_t values for the outer two residues, then this indicates that the four residues are likely to form a β turn. (The latter criterion is less important than the former.) If the four residues are labeled i, $i + 1$, $i + 2$, and $i + 3$, then residues $i + 1$ and $i + 2$ will belong to the β turn, whereas

CKS*Hpro*GSSCS*Hpro*TSYNCCRSCN*Hpro*YTKRCY

Figure 2. Amino acid sequence of the peptide neurotoxin ω-Cgtx GVIA.[5] Hpro, Hydroxyproline.

i and $i + 3$ will belong to both the β turn and the β strand. As neighboring residues of the β strands alternate relative to the plane of the β hairpin, if $i + 2$, $i + 3$, and $i + 5$ are on the outside plane of the β hairpin, then $i + 1$, $i + 4$, and $i + 6$ are found on the opposite side, and vice versa. Therefore if, say, $i + 2$ is important for ion selectivity and accordingly points toward the pore, then so do $i + 3$ and $i + 5$.

Figure 3 shows the β-turn assignments for the H5 region of each domain.

Further support for the positioning of the β turns is the fact that values of propensity for β sheet (P_β) and average P_β values are significantly lower for the proposed β-turn residues than for the surrounding residues.

The conserved glutamates in the proposed β-turn region have been shown to form the ion-selective filter of the pore[9–11] and must therefore project into the pore. In all domains the glutamate is residue $i + 2$ and hence $i + 3$ and $i + 5$ will also project into the pore. Residues $i + 1$, $i + 4$, and $i + 6$ will be on the opposite side of the β hairpin and will project away from the pore.

The β hairpin of each domain was constructed from a decapeptide, with the β turn positioned between two β strands of equal length. The chain length was deduced from hydropathy calculations.

The β barrel was constructed from the following residues, where the β-turn residues are in italics and the glutamate ion-selectivity filter is in boldface.

Domain I	Q	C	I	*T*	*M*	***E***	*G*	W	T	D
Domain II	Q	I	L	*T*	*G*	***E***	*D*	W	N	A
Domain III	T	V	S	*T*	*G*	***E***	*G*	W	P	M
Domain IV	R	S	A	*T*	*G*	***E***	*A*	W	H	E

β Turns are observed in a number of different conformations and so were classified according to the criteria of Wilmot and Thornton,[19] who define a β turn as having $C^\alpha_{(i)} - C^\alpha_{(i+3)} < 7$ Å where the chain is not in a helical conformation. The different β-turn types are defined by their backbone conformation, that is, by values of ϕ_{i+1}, ψ_{i+1}, ϕ_{i+2}, and ψ_{i+2}. For example, Table 1 lists the dihedral angles for three types of β turns.[20]

As a zero approximation for the backbone conformation of a β strand in a standard antiparallel β structure, values of $\phi = -139°$ and $\psi = +135°$ were used.[21]

For each domain, three β hairpins were constructed, each with the zero approximation dihedrals for the β strand and a different β-turn type conformation as specified in Table 1 (i.e., type I, II, and III conformations). These zero approx-

Domain I

	V	F	Q	C	I	T	M	E	G	W	T	D
P_t	0.41	0.59	0.84	0.54	0.47	*0.90*	*0.52*	*1.01*	*1.77*	0.65	0.90	1.24
$\langle P_{ti} \rangle$	0.60	0.61	0.69	0.61	0.73	**1.05**	0.99	1.08	1.14	-	-	-

Domain II

	V	F	Q	I	L	T	G	E	D	W	N	A
P_t	0.41	0.59	0.84	0.47	0.57	*0.90*	*1.77*	*1.01*	*1.24*	0.65	1.34	0.82
$\langle P_{ti} \rangle$	0.58	0.62	0.70	0.93	1.06	**1.23**	1.17	1.06	1.01	-	-	-

Domain III

	L	F	T	V	S	T	G	E	G	W	P	M
P_t	0.57	0.59	0.90	0.41	1.22	*0.90*	*1.77*	*1.01*	*1.77*	0.65	1.32	0.52
$\langle P_{ti} \rangle$	0.62	0.78	0.86	1.08	1.23	**1.36**	1.30	1.19	1.07	-	-	-

Domain IV

	L	F	R	S	A	T	G	E	A	W	H	E
P_t	0.57	0.59	0.90	1.22	0.82	*0.90*	*1.77*	*1.01*	*0.82*	0.65	0.81	1.01
$\langle P_{ti} \rangle$	0.82	0.88	0.96	1.18	1.13	**1.13**	1.06	0.82	0.82	-	-	-

Figure 3. The amino acid sequences and corresponding P_t^{17} and $\langle P_{ti} \rangle$ values for each H5 region, where the $\langle P_{ti} \rangle$ value corresponding to a specific residue is for residue i *of the turn. The values of $\langle P_{ti} \rangle$ in boldface represent the chosen residue* i *of the β turn, and the P_t values in italics correspond to the four residues of the β turn.*

Table 1. Dihedral angles (in degrees) for three different β-turn types[a]

β-Turn type	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}
I	−60	−30	−90	0
II	−60	120	80	0
III	−60	−30	−60	−30

[a]See Ref. 20.

imation β-hairpin models were then energy minimized individually, in vacuo, using the CHARMm22 program.[22] The resulting dihedral angles from each turn type and each domain were then analyzed. The results showed that all type II β turns remained very close to their original conformations, whereas types I and III deviated greatly, and generally in a direction toward a type II conformation. This indicated that given the sequences and β-turn positions, for each domain the best zero approximation conformation was that of the type II β turn.

The position of the filter glutamate residue in the hairpin dictated the orientation of each hairpin (as each glutamate must face inward toward the pore). However, the domains could be orientated in either a clockwise or anticlockwise direction, as shown in Figure 4. There seems to be no experimental evidence to support a preference for either arrangement, so models of both orientations were constructed.

The domains were positioned adjacent to each other and each β hairpin had a right-handed tilt. This follows natural systems of eight-stranded β barrels such as superoxide dismutase and triosephosphate isomerase.

The terminal ends of the domains were capped with acetamide and methylamide groups and the models were solvated before undergoing minimization and molecular dynamics simulations. Energy minimization was performed first on hydrogen atoms alone, then on side chains, and finally on the entire model. A molecular dynamics simulation was carried out at 300 K for 20 ps on side chains alone followed by 100 ps on the entire model.

This leads to our two alternative models of the calcium channel pore.

RESULTS

The domains had hydrogen bond interactions between their backbones, and formed a ring of glutamates at the bottom of the β hairpins, of the dimensions as suggested by Hille[23] to be required by a selectivity filter for Ca^{2+} ions. Hence the model contains the essential features required, that is, a selectivity filter of the correct dimensions that will permit the passing of a Ca^{2+} ion but will prevent the passing of larger ions or smaller ions with their salvation shells, where their energy of desolvation is too great.

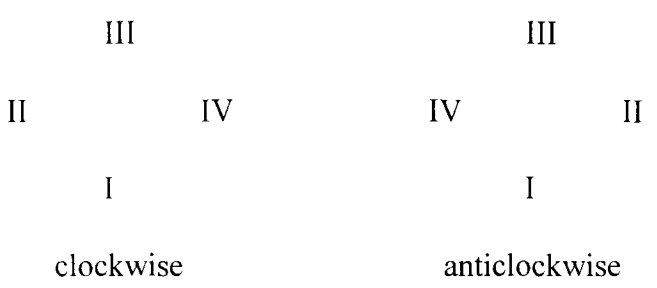

Figure 4. Possible orientation of domains as viewed from the extracellular side.

The models also had a ring of threonines between the filter and the mouth of the pore. The same "intermediate ring" arrangement can be seen in the ion-conducting pore of the nicotinic acetylcholine (AChR) channel.[24] There is also an aspartate residue at this point. It could be postulated that this intermediate region may serve as a "guide" for the ion-conducting pathway, or possibly even a Ca^{2+}-binding site part way down the pore.

Both models were similar in the above respects and had very similar close energy values. Hence it is impossible to determine at this stage whether the domains ought to be arranged in a clockwise or anticlockwise manner.

Color Plates 1 and 2 show ribbon diagrams of the final models.

DISCUSSION

Two computer models of the outer vestibule of the pore region of the N-type voltage-gated Ca^{2+} channel have been produced, differing only in their orientation of the four domains. The models are of the extracellular part of the pore containing the selectivity filter at the bottom of the vestibule. The remainder of the pore is thought to be formed by the surrounding protein, that is, the transmembrane helices, although no attempt has been made to model this. The models were based on a short β-barrel structure rather than the long β barrel preferred in K^+ channel modeling. This was due to the difference in type and number of pore amino acids between Ca^{2+} and K^+ channels, suggesting that the pores may not be homologous with each other. Theoretical calculations indicating the position of the β turn and the length of the pore sequences also favored a short β-barrel model.

The models of the outer vestibules contained selectivity filters that were of the correct size to permit the passage of Ca^{2+} ions.[25] Assuming that these models are stabilized by the surrounding protein, it is suggested that a hydrated Ca^{2+} ion ($Ca \cdot 6H_2O$) will travel through the pore and the interaction of the selectivity filter glutamates will displace three or perhaps four waters from the hydration shell and decrease the energy of hydration of the other shell water molecules (see Color Plate 3). Three waters will probably be displaced, as only three glutamates are required in the mutated Na^+ channel to allow Ca^{2+} ions to pass.[12] Larger ions will be prevented from passing through the selectivity filter owing to their size. An interesting experimental observation is that in the absence of divalent ions the calcium channel becomes unselectively highly permeable to monovalent ions. Under normal physiological conditions a few divalent ions (Ca^{2+}, Sr^{2+}, and Ba^{2+}) permeate Ca^{2+} channels, many block the channel (Mg^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+}), and no monovalent ions pass through (or block) the pore. This is interesting considering the sizes and en-

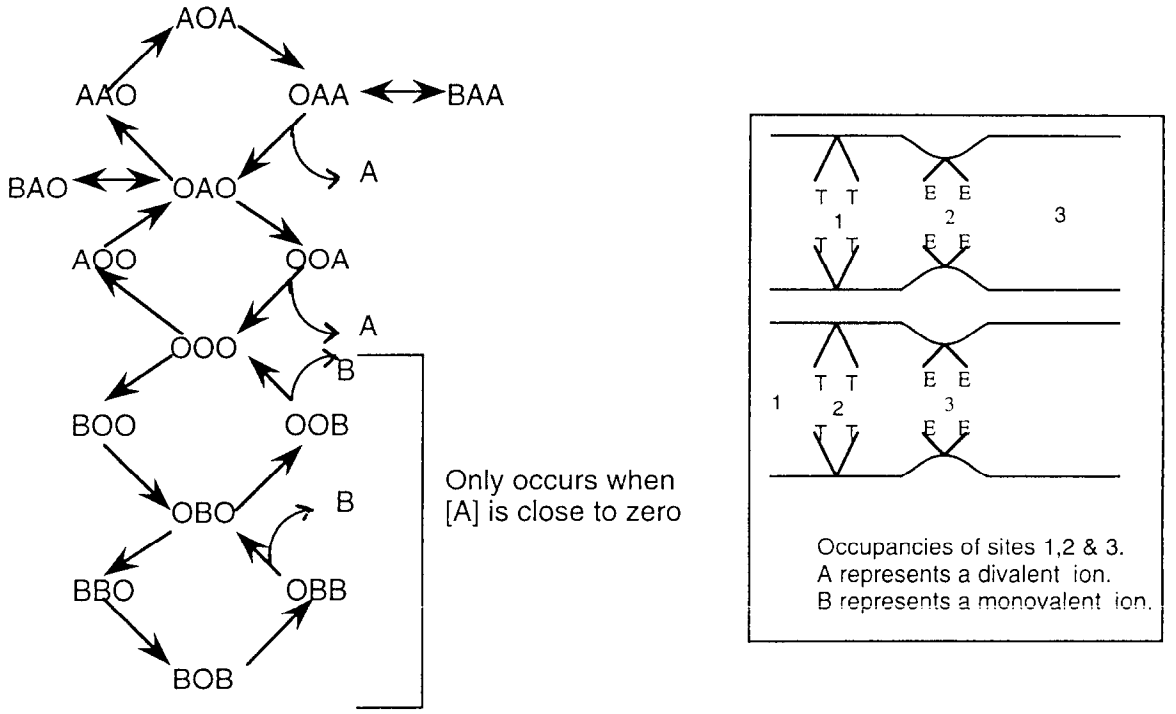

Figure 5. Simplified state diagram of ion occupancies in pore containing a possible three-binding sites. A, a divalent ion; B, a monovalent ion. It is assumed that two ions produce an unstable complex and that ions will move only from left to right. It is also assumed that the energy well is deeper at the filter than it is at the threonine ring and deeper still than at the pore mouth. The two possible situations shown (inset) can both apply to the state diagram.

thalpies of hydration of the ions[26,27] (see Table 2). The blocking of the pore by small divalent ions such as Mg^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} can be explained by considering the enthalpies of hydration. These enthalpies are such greater than the enthalpies of hydration for Ca^{2+}, Sr^{2+}, and Ba^{2+}. In these cases the carboxyl groups of the filter glutamates are unable to offset the hydration energy enough to displace waters from the hydration shell of the ions. Hence these ions are unable to pass through the filter and so block the pore. However, small monovalent ions have low enough enthalpies of hydration to theoretically allow them to pass through the pore, which they actually do when the concentration of Ca^{2+} ions is below the millimolar level. Experimental evidence has led to the hypothesis that the channel contains a multi-ion pore with an ion-binding site present forming part of the selectivity mechanism.[28-30] The models presented here are in agreement with this, whereby under normal physiological conditions the pore contains a Ca^{2+} ion in a deep energy well. If a small monovalent ion (e.g., Na^{+}) enters the pore then it may sit in a smaller well and there will be a mutual repulsion between the ions. Because the pair of ions is unstable, one will dissociate from its binding site. As the Na^{+} ion will have a lower energy requirement for leaving then it is more likely to be repelled to the outside of the pore than the Ca^{2+} ion is to move further into the pore. Hence under normal conditions the small monovalent ions will never reach the selectivity filter. However, when all the Ca^{2+} ions have been removed, the monovalent ions are free to pass through the pore but do so with no selectivity. Figure 5 shows a simplified state diagram for ions passing only one way through the pore, with three possible binding sites present and the assumption that only one ion moves at a time with two ions in a pore producing an unstable system. A ring of threonines with an aspartate at the same level is present in the models between the pore mouth and the filter. This could produce either the smaller energy well with the deep energy well being the

Table 2. Ionic radii[a] and enthalpies of hydration[b] for ions

Ion	Ionic radius	Enthalpy of hydration
Li^{+}	0.60	−131
Na^{+}	0.95	−105
K^{+}	1.33	−85
Rb^{+}	1.48	−79
Cs^{+}	1.69	−71
Ca^{2+}	0.99	−397
Sr^{2+}	1.13	−362
Ba^{2+}	1.35	−328
Mg^{2+}	0.65	−476
Co^{2+}	0.71	−502
Mn^{2+}	0.80	−458
Ni^{2+}	0.72	−517

[a]In angstroms; see Ref. 26.
[b]In kilocalories per mole; see Ref. 27.

selectivity filter, or the Ca^{2+}-binding site distinct from the selectivity filter with the smaller energy well being nearer the pore mouth.

The models appear to fit with a mechanism for selectivity whereby the selectivity filter is able to prevent the passage of large ions simply due to the steric effect of their size and to prevent the passage of small ions with large enthalpies of hydration by the inability of the interacting filter glutamates to offset the hydration energy required to remove waters from the hydration shell, hence causing pore blockage due to steric effects. The models also fit the proposal of a multi-ion pore containing a Ca^{2+} ion-binding site,[28–30] which will repel less well-bound, monovalent ions from the pore mouth, accounting for the impermeability to monovalent ions in the presence of calcium and permeability once calcium ions have been removed.

Both models produced agree with the experimental evidence available, apart from the ω-Cgtx GVIA binding and loop residue mutation data, which cannot be tested without the surrounding protein present. With the transmembrane helices and associated loops in place, mutagenesis data relating to the ω-Cgtx GVIA interactions with loops IIIS5–H5 and IIS5–H5[7] can be used to validate the pore models, along with energy profiles of Ca^{2+} ions moving through the pore. Until this rigorous testing has been performed the models proposed remain speculative.

The models presented may, however, be helpful in suggesting further mutagenesis experiments, especially with regard to the ring of threonines and the aspartate, which are thought to be present between the pore mouth and the filter. These may act as either a Ca^{2+}-binding site or a "guide," and may even possibly have some function in ω-Cgtx GVIA binding. The models proposed may serve to suggest novel mutagenesis experiments while remaining speculative until the surrounding protein has been modeled fully. To facilitate this the atomic co-ordinates for the models are available from the authors.

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