Understanding the structure and functional mechanisms of voltage-gated calcium channels remains a major task in membrane biophysics. In the absence of three dimensional structures, homology modeling techniques are the method of choice, to address questions concerning the structure of these channels. We have developed models of the open CaV1.2 pore, based on the crystal structure of the mammalian voltage-gated potassium channel Kv1.2 and a model of the bacterial sodium channel NaChBac. Our models are developed to be consistent with experimental data and modeling criteria. The models highlight major differences between voltage-gated potassium and calcium channels in the P segments, as well as the inner pore helices. Molecular dynamics simulations support the hypothesis of a clockwise domain arrangement and experimental observations of asymmetric calcium channel behavior. In the accompanying paper these models were used to study structural effects of a channelopathy mutation.

Introduction

The regulation of calcium entry into cells via voltage-gated calcium channels (CaV) plays a fundamental role in controlling synaptic transmission, membrane excitability, muscle contraction, rhythmic activity, gene transcription and signal transduction pathways. Unlike voltage-gated K+ channels (Kv), the pore-forming α1-subunits of voltage-gated calcium channels (CaV) are composed of four distinct domains, each formed by six transmembrane segments (S1–S6) that are linked together on a single polypeptide.1 Channel activation is considered to be triggered by a conformational change in the voltage sensors (formed by multiple charged amino acids located in segment S4 and adjacent structures of each domain) leading to channel opening. Experimental structure information (e.g., crystallographic data) about the molecular architecture of CaV in their different conformational states (closed, open, and closed inactivated) is not available. Evolutionary analyses revealed a common fold of the pore segments for both ion channel families. However, there are important differences at the atomic level. While in the selectivity filter of K+ channels the main carbonyl oxygen atoms project towards the lumen,2 in CaV the side-chains of four negatively charged glutamates are predicted to face the pore.3–6 Another difference is the existence of four non-identical repeats in CaV in contrast to Kv channels, which have four sequentially identical subunits. It is currently not clear if the four repeats in CaV1.2 are arranged clockwise or counter clockwise. To address these fundamental questions, homology models of the open CaV1.2 channel, incorporating available experimental data, as well as modeling criteria,7 were built.

Results

A homology model of the CaV1.2 pore domain. The model of the open conformation is based on the crystal structure of Kv1.2. The alignment of individual CaV1.2 and Kv1.2 sequences is difficult, because the sequence identity is less than 30%. An alignment between CaV1.2 and NaChBac is much less ambiguous (Fig. 1), with sequence identities in the pore S5, P and S6 segments of 50%. Therefore, we used the alignment of NaChBac and K V channels proposed originally by Durell et al.,9 and used by Y Shafrir and HR Guy in developing the NaChBac model (manuscript submitted for publication). This alignment is based on multisequence alignments of many Kv, CaV and NaChBac sequences (Fig. 1), on analyses of the degree of conservation of each residue position in these alignments, and on a modeling criteria that poorly conserved residues tend to be located on the surfaces of proteins. The alignment used to model the closed conformation of NaChBac was the same as proposed by others who have developed models of closed CaV channels to be consistent with effects of mutations on drug binding.26,27 The same alignment was also used in modeling the open conformation for all segments except the C-terminus half of S6, which forms the activation gate. For the open conformation, an insertion of one residue was placed near the middle of the NaChBac relative to K V1.2. This adjustment in the alignment of S6 substantially improves the sequence similarities between NaChBac and K 1.2 and, with modest structural adjustments, is supported by SCAM studies (see below).24

The pore domain is formed by four homologous subunits and consists of helices S5, a selectivity filter P segment re-entrant loop and S6 helices, which line the inner pore cavity. S5 of the pore domain is connected to S4 of the voltage-sensing domain via an amphiphilic L4-5 helix located on the cytoplasmic membrane surface and oriented parallel to the plane of the membrane (Fig. 2). The L4-5 helix is thought to couple movement of the S4 voltage sensor to the

Key words: homology model, CaV1.2 pore structure, domain arrangement, MD simulation, open conformation

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Homology model of CaV1.2 pore region

Experimental basis for modeling the CaV1.2 S6 segments.
Information about the architecture of the pore lining S6 segments was deduced from a surface cysteine accessibility method (SCAM) analysis on a P/Q-type channel. The accessibility results provide first insights into the architecture of the pore lining amino acids in all four S6 segments. Initial models of the position of S6 of NaChBac in the open conformation were developed from a KV1.2 template with an insertion in the region where the S6 helix bends when the channels open. The position of S6 shifted slightly in these NaChBac models during molecular dynamic simulations by different amounts, depending upon how the voltage-sensing domain was modulated. The general tendency was for the inner pore to expand during the simulations and for the C-terminus to swing away from the pore. The NaChBac model used as a template for the CaV1.2 models presented here had S6 in an intermediate position in which the inner pore is larger than in the KV1.2 template. In the adjusted models, all analogous positions where residues were inaccessible in all four repeats (three positions of NaChBac) were oriented away from the pore and buried in the structure; whereas, all analogous positions where residues were accessible in all four repeats (seven NaChBac positions) were on the surface and oriented toward the pore or cytoplasm. For three positions corresponding to NaChBac 223, 226 and 230, analogous residues in the CaV channel were inaccessible in some repeats and accessible in others. These all faced away from the pore and tended to be buried in the NaChBac models. Two of these ‘inconsistent’ accessible residues were in Repeat II, one was in Repeat III, another one in Repeat IV, and none in Repeat I. Zhen et al. interpreted these results to indicate loose packing between IIS6 and the neighboring S5 segment. In our models these residues have little interaction with S5. Our data (see accompanying paper) also show however, that structural disturbances induced by mutations of

Figure 1. Alignment of pore forming S6 segments of CaV1.2, NaChBac (Accession number: Q6U8D0), Catsper (Accession number: Q91ZR5) and KV1.2 (pdb identifier: 2a79). The colouring scheme is according to mutabilities, calculated from a multiple sequence alignment: red < orange < yellow < green < cyan < black (lipid-exposed) = gray (head-group exposed) = blue (water-exposed).

S6 activation gate.

Figure 2. Structural details of CaV1.2 in the open conformation. Sideview of the pore forming domains including L4-5, S5, P and S6 helices. Repeats II and IV are coloured: yellow (L4-5), green (S5), cyan (P) to blue (S6). Repeats I and III are shown in gray.
S6 residues may result in higher flexibility in this area, which may provide an alternative explanation for differences between MTSET accessibility and the orientation of S6 residues described in our models. We thus conclude that these models are consistent with the preponderance of the SCAM data.

The highly conserved asparagine in CaV as well as NaChBac homologues is not conserved in any KV family. We propose that this asparagine stabilizes the open conformation by binding to backbone atoms of the bent region of S6. Differences in this region might indicate different activation mechanisms between CaV and KV channels.

Our study revealed that a clockwise domain arrangement is more stable during molecular dynamics simulations (Fig. 3C and D), supporting this arrangement. Furthermore, comparison of putative binding sites of phenylalkylamines (PAA) for clockwise and counter-clockwise models in the open conformation also support the clockwise orientation (details in Suppl. Fig. 1); i.e., the PAA-sensitive sites in Repeats III and IV form a tighter, more interactive cluster in the clockwise models.

**Structure of the CaV1.2 selectivity filter.** The selectivity filter is the most distinct feature between potassium and calcium channels, with only one highly conserved residue between these two families (Fig. 1). Therefore, we used models of the bacterial sodium
Homology model of CaV1.2 pore region

channel NaChBac (manuscript submitted for publication) as starting point for this region. Both channels contain the highly conserved FxxxTxExW motif, not seen in K+ channels. Further evidence about the calcium channel pore structure was deduced from single site-directed mutagenesis studies, which identified selectivity filter forming residues.4‑6,28 Our models are built in agreement with these experimental data, as can be seen in Figure 4A and B. Furthermore, the proposed structure of the selectivity filter remained relatively stable during 10 ns MD simulations (Fig. 5). This feature of our model is, however, the most uncertain, because the long extracellular loops (36 and 46 residues) of domains I and III have been replaced by the relatively short loops of NaChBac, due to lack of suitable templates and the fact that these regions most likely have coiled conformation. The NaChBac loops were included to reduce disorder that would likely occur in the P segments during MD simulations if no loops were present.

Molecular dynamics simulations of CaV1.2 pore. The final model of CaV1.2 in clockwise orientation was subjected to an extended 10 ns molecular dynamics simulation run, to address its stability. Original models had their domains arranged symmetrically, similar to the Kv1.2 and NaChBac templates. During MD simulations the 4-fold symmetry was not maintained (Fig. 6A and B). This effect was particularly evident in the S6 segments. Changes in these four segments were accompanied by an increase in pore size, compared to the starting structure.

MD simulations were also used to address the stability of different domain arrangements. The stability as measured from calculating the root mean square deviation (RMSD), showed a clear preference towards the clockwise models, as being far more stable. Since the initial counter-clockwise (CCW) model, contained the original S6 conformations (not adjusted according to MTSET data24), we choose to rebuild our final, stable clockwise model, which was refined according to experimental data, as described above, to CCW conformation. This way, we could rule out that the improved model quality of the clockwise conformation was a result of the refinement process only. However, simulations of this second CCW model, termed CCW2, were still less stable, and scored poorly with some structure validation programs (Table 1).

Discussion

The model of the open CaV1.2 pore domain presented here suggests important structural differences between Kv and CaV channels. The most distinct regions are the P segments as well as the S6 helices of the pore forming domain. Substantial effects in the former segments are to be expected, due to the differences in the selectivity filters, designed to provide selectivity for either Ca2+ or K+ ions. Therefore, crystal structures of Kv channels are unsuitable templates for these segments. In order to build this important region, we used the more closely related NaChBac model (Y Shafrir and HR Guy, submitted for publication) as template. Both channels contain the same highly conserved signature sequence FxxxTxExW, which suggests closely related structures at the atomic level.

Differences in the architecture of S6 segments. Differences in
In the lower third of segment IIS6 of CaV1.2 form a critical “hotspot” of accessibility patterns for all four pore helices in the CaV1.2 pore region. The Model of the open conformation of CaV1.2 (Accession number: P15381) is based on the crystal structure of the open conformation of the mammalian voltage dependent potassium channel Kv1.28 and on a homology model of the NaChBac sodium channel built by Y Shafir and HR Guy (manuscript submitted for publication). The NaChBac model was used because it is more closely related to CaV channels (for details see Durell et al., NaChBac = Cav1.2). Large extracellular and intracellular loops are not included in the models, due to lack of structure templates and difficulties in modeling poorly-conserved loops that are likely to have substantial coiled structures. In order to elucidate the arrangement of the homologous repeats, models containing either clockwise or counter clockwise orientations were built. Starting models were generated using the Modeller software followed by energy minimization with the CHARMM27 force field in MOE. Modeling criteria applied are similar to those described earlier by Shrivastava et al. Model quality was analysed using standard structure validation programs (Table 1).

### Methods

#### Molecular modeling of CaV1.2 channels in open conformation.

The Model of the open conformation of CaV1.2 channels was embedded in a lipid bilayer of POPE (palmitoyloleoyl-phosphatidyethanolamine) by superimposing the channel structures onto the lipid and removal of a minimal number of overlapping lipids. The final system contained 311 lipid molecules. Membrane insertion of the channels was guided by patterns of charged, polar and aromatic residues. The system was solvated with 37,000 SPC waters. Calcium ions were manually placed in the selectivity filter near the highly conserved glutamates and cavity, and 11 chloride ions were added to neutralize the system, resulting in an overall system size of 9.6 x 9.1 x 5.3 nm. The starting structures were energy minimized for 10,000 steps using the steepest descent method to reduce close contacts between protein and membrane. After a 500 ps equilibration with harmonic restraints on the backbone, which resulted in close packing of lipids against protein, short 2 ns simulations were performed for models in clockwise and counter clockwise orientation, to analyse the stability of these models. The final model
(clockwise orientation) was subjected to a longer 10 ns simulation that was preceded by a 5 ns equilibration. All simulations were performed with Gromacs 3.3.17 using the 43a2 force field and run on a dual Intel® core (TM) 2 CPU 6600 processor. Electrostatic calculations were done using the particle mesh Ewald method18 under periodic boundary conditions, with a Van der Waals cut-off of 1.0 nm. A time step of 2 fs was applied and the LINCS algorithm21 was used to constrain bond lengths. All simulations were run under NPT conditions (Number of atoms, Pressure and Temperature were kept constant) with the protein, lipid and water each coupled separately to a temperature bath at 310 K with a coupling constant of $\tau_T$ of 0.1 ps, and at a constant pressure of 1 bar in all directions with a pressure constant of $\tau_P = 1.0$ ps. Lipid parameters are based on Berger et al.,20 and the lipid-protein interactions are based on the GROMOS96 parameters.21 Default protonation states, based on calculations with the software Propka 1.0.1,22 were used.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/StaryCHAN2-3-Sup.pdf

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