Structural Model for Phenylalkylamine Binding to L-type Calcium Channels*

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Phenylalkylamines (PAAs), a major class of L-type calcium channel (LTCC) blockers, have two aromatic rings connected by a flexible chain with a nitrile substituent. Structural aspects of ligand-channel interactions remain unclear. We have built a KvAP-based model of LTCC and used Monte Carlo energy minimizations to dock devapamil, verapamil, gallopamil, and other PAAs. The PAA-LTCC models have the following common features: (i) the meta-methoxy group in ring A, which is proximal to the nitrile group, accepts an H-bond from a PAA-sensing Tyr_IIIS6; (ii) the meta-methoxy group in ring B accepts an H-bond from a PAA-sensing Tyr_IVS6; (iii) the ammonium group is stabilized at the focus of P-helices; and (iv) the nitrile group binds to a Ca$^{2+}$ ion coordinated by the selectivity filter glutamates in repeats III and IV. The latter feature can explain Ca$^{2+}$ potentiation of PAA action and the presence of an electronegative atom at a similar position of potent PAA analogs. Tyr substitution of a Thr in IIIS5 is known to enhance action of devapamil and verapamil. Our models predict that the para-methoxy group in ring A of devapamil and verapamil accepts an H-bond from this engineered Tyr. The model explains structure-activity relationships of PAAs, effects of LTCC mutations on PAA potency, data on PAA access to LTCC, and Ca$^{2+}$ potentiation of PAA action. Common and class-specific aspects of action of PAAs, dihydropyridines, and benzo(thi)azepines are discussed in view of the repeat interface concept.

L-type calcium channels (LTCCs) are targets for different drugs. Benzo(thi)azepines (BTZs), dihydropyridines (DHPs), and phenylalkylamines (PAAs) constitute the three major classes of the LTCC ligands (for reviews, see Refs. 1 and 2). All of these ligands bind to overlapping binding sites in the pore-forming domain of the α_{1} subunit, but each class demonstrates unique characteristics of action. Depending on their chemical structure, DHPs act as agonists or antagonists (3). All known PAAs and BTZs are antagonists, but they have different access pathways to their binding sites: external for BTZs (4, 5) and predominantly internal for PAAs (6). Clinical use of verapamil in treatments of hypertension and arrhythmias (7) had stimulated intensive electrophysiological, mutational, and pharmacological studies involving PAAs.

The pore-forming domain of LTCC includes the pore-lining inner helices S6, the outer helices S5, and the P-loops from all four repeats of the α_{1} subunit. According to mutational analyses, the PAA-binding site is located in the interface between repeats III and IV. In particular, residues in transmembrane helices IIIS5, IIIS6, and IVS6 and IVS6 and P-loops of repeats III and IV contribute to binding of PAAs (8–14).

Structure-activity relationships of PAAs were intensively studied (15–17). A common feature of potent PAAs is the presence of two methoxylated aromatic rings (named A and B). The rings are connected by a flexible alkylamine chain with a nitrile and an isopropyl group at the chiral tetrasubstituted carbon atom, which is proximal to ring A. Ring B is proximal to the amino group (see Fig. 1).

Despite the fact that some specific contacts between functional groups of PAAs and PAA-sensing residues (residues that, when mutated, affect action of PAAs) have been proposed (10, 14), the flexibility of the ligands did not allow the characterization of the binding mode and the general pattern of ligand-channel interactions. In the absence of such knowledge, it is hardly possible to provide a molecular basis for structure-activity relationships. The problem is further complicated by the dependence of PAA action on the functional state of the channel, the ionic environment, the transmembrane voltage, and other factors. For example, it is generally believed that PAAs bind to the open/inactivated channels with higher affinities than to the closed state (for review, see Ref 1). However, the molecular basis for this state dependence is unclear.

Lipkind and Fozzard (18) docked devapamil in a KcsA-based homology model of the L-type Ca$^{2+}$ channel. They suggested an angular conformation of the drug, with ring B extended into the III/IV repeat interface and ring A in the central cavity. They also suggested that the protonated amino group of devapamil interacts directly with the selectivity filter glutamates. This model explains the effect of some mutations, particularly those in the P-loops and IVS6. However, other important aspects of PAA action such as the role of the nitrile group, the Ca$^{2+}$ potentiation effect, and the effects of mutations in IIIS6 and IIIS5 remain unexplained.

The gap between the amount of experimental data on PAA action and the level of understanding of the atomic level mechanisms necessitates further studies. In the absence of x-ray structures of Ca$^{2+}$ channels, molecular modeling is the only...
available approach to address the structural aspects of PAA-LTCC interactions. Recently, we proposed molecular models for the action of other classes of L-type channel ligands. In the BTZ-LTCC models (19), the main body of the ligands binds in the repeat interface, whereas the amino group protrudes into the inner pore, where it is stabilized by nucleophilic C-terminal ends of the pore helices. In the DHP-LTCC models (20), the ligands also bind in the interface between repeats III and IV, whereas the moieties that differ between agonist and antagonist extend to the pore. Both models suggest direct interactions between the ligands and a Ca$^{2+}$ ion bound to the selectivity filter glutamates in repeats III and IV.

In this work, we elaborate molecular models for PAA-LTCC complexes that agree with a large body of experimental data. We further discuss common and different aspects of action of different ligands on LTCC and propose that certain aspects of the ligand action may be relevant to other P-loop channels.

MATERIALS AND METHODS

Models were constructed using ZMM software. Energy was expressed as a sum of van der Waals, electrostatic, and torsional components as well as energy of deformation of bond angles of ligands. Bond angles of the channel were kept rigid. AMBER force field was used (21, 22). To take into account that atomic charges may be screened by water molecules, electrostatic interactions were calculated with solvent exposure and distance-dependent dielectric function, $\epsilon = d(8 - 6s)$, where $d$ is the distance between interacting atoms and $s$ is a screening factor calculated using a modified algorithm of Lazaridis and Karplus (23). This screening factor varies from 0 for a pair of water-exposed atoms to 1 for a pair of protein-buried atoms.

We have built four models of Ca$_{1.2}$ that are based on the x-ray structures of K$^+$ channels in the open (24–26) and closed (27) states. The sequence alignment of Ca$_{1.2}$ and K$^+$ channels and the residue labeling scheme are shown in Table 1. The channel models include S5s, P-loops, and S6s from the four repeats. The P-helices and ascending limbs (positions p47–p57) were modeled using our P-loop domain model of Nav1.4 (28). In this model (as well as in all available models of Na$^+$ and Ca$^{2+}$ channels), the side chains of the selectivity filter residues at position p50 face the pore lumen and directly interact with permeant ions. When viewed from the extracellular side, repeats I–IV were arranged clockwise around the pore axis (29). Extracellular linkers, which are far from the PAA-binding site, were not modeled. Ionizable residues except the selectivity filter glutamates (positions p50) were modeled in their neutral forms. Two Ca$^{2+}$ ions were loaded onto the selectivity filter to counterbalance the negative charges of the selectivity filter glutamates. Initial conformations of side chains were assigned using the SCWRL3 program (30). Models were optimized using the Monte Carlo minimization protocol (31). Monte Carlo minimization trajectories were terminated when 10,000 consecutive energy minimizations did not improve the apparent global minimum. During energy optimizations, structural similarity between the model and the template was maintained by “pin” constraints. A pin is a flat-bottom parabolic energy penalty function that allows for penalty-free deviations of $\alpha$-carbons up
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TABLE 1

Sequence alignment used for homology modeling of Cav1.1,2
| Channel | Segment | Residue label prefix | Sequence* |
|---------|---------|----------------------|-----------|
| KcaA   | M1      | ALIKRAAGA TVLLVLVLA GEYGLVLAE | 1-21 |
| MtkK   | M1      | KLKPVATRI LLLLAVIY IAGHGFIE | 1-21 |
| KvAP   | S5      | AAIKIRYFL YAIVMVLYL GAYAPIYVE | 1-2 |
| K,1,2* | S5      | KASQMLLLL IIFLSFVGI FSSAVYFAE | 1-2 |
| Ca,1,2*| S5      | IPRQPHEL ALLLVPTTV YAIQGLLP | 1-2 |
| 2S2     | 2S2     | INSVISIAL LLLLFFII FSLQGKLF | 1-2 |
| 3S5     | 3S5     | PVATRIGNI VVTVLLEQ RMFQCVQLF | 1-2 |
| 4S5     | 4S5     | SXQDALFYT LALLLFFII IYVIMQYLV | 1-2 |

* A universal scheme was used to label residues according to their positions in the sequence alignment (45).

** Sequences are from the Protein Data Bank (codes 1BL8, 1LNQ, 1ORQ, and 2A79, respectively).

* The sequence is from UniProt (accession number P15381).

Experimentaly found PAA-sensing residues are in boldface. Residues whose side chains provided over ~0.6 kcal/mol to the ligand-receptor energy in our models are underlined.

RESULTS

Hands-free docking of highly flexible PAAs (Fig. 1) is not promising because of the limited precision of homology models of LTCC based on x-ray structures of K$^+$ channels. Therefore, we have used available data on ligand-channel interactions to decrease the number of degrees of freedom of the system and thus to guide our calculations to solutions consistent with experimental data.

Developing the PAA-binding Scheme—PAA-sensing residues available from the published mutational data are shown in Table 1, and their positions in the KvAP-based homology model are shown in Fig. 2. Straightforward interpretation of these data is possible only for the IVS6 mutants. In the triple mutant Y4i11F/C4i15S/I4i18A of Ca,1,2, the IC$_{50}$ for devapamil block increases by 100-fold compared with that of the wild type. The single mutation Y4i11F of Ca,1,2 increases the IC$_{50}$ for devapamil block by ~6-fold. However, channel block by verapamil and gallopamil are unchanged between the wild type and the triple mutant (11, 14). Furthermore, a devapamil derivative with a photoreactive moiety at the meta-position of ring B labels a fragment from IVS6 (34). Based on these data and on the fact that devapamil differs from verapamil only by a methoxy group in ring B, the side chain of Y4i11 is proposed to form an H-bond with the m-methoxy group on ring B of devapamil (10).

PAAs also interact with IIIIS residues. In particular, mutation Y3i10F of Ca,1,2 results in an 18-fold increase in the IC$_{50}$ for the resting block by devapamil (10). This suggests an H-bond between devapamil and the side chain hydroxy of Y3i10. However, channel block by verapamil has only one methoxy group, which forms an H-bond with Y4i11, we propose that the side chain of Y3i10 forms a H-bond with a methoxy group in ring A of devapamil. Given the rather close disposition of the tyrosine residues in IIIIS6 and IVS6 (Fig. 2) and the large length of the ligand in the extended conformation, only a folded devapamil molecule can form these two H-bonds simultaneously. It should be noted that alanine substitution of Y3i10 enhances the potency of devapamil, but this mutation strongly increases steady-state inactivation of the channel (10). Taking into account the state-dependent action of devapamil (35), the effect of the Y3i10A mutation on devapamil binding is likely indirect.

The third constraint can be obtained from the study showing that the T3o14Y mutation enhances the potency of devapamil and verapamil but not that of gallopamil (12). In our model, T3o14 is located in the interface between repeats III and IV. The engineered Y3i10 can approach Y3o14 (Fig. 2). Devapamil has two methoxy groups in ring A and only one methoxy group in ring B. On the basis of these data, we suggest that the two methoxy groups in ring A form H-bonds with both Y3i10 and Y3o14.

To form H-bonds with the above-mentioned tyrosines (Y4i11, Y3i10, and the engineered Y3o14), the aromatic rings of devapamil must reach into the III/IV repeat interface. However, the interface does not have enough room to accommodate the entire devapamil molecule. Thus, if both aromatic rings of devapamil bind in the III/IV repeat interface, the central part of the molecule, which contains the amino and nitrile groups, must reside in the inner pore. Indeed, some of the PAA-sensing residues in the P-loops, IIIIS6, and IVS6 face the inner pore rather than the repeat interface.

The model in which the amino group of devapamil is located in the inner pore and interacts with the selectivity filter glutamates directly (18) does not explain the Ca$^{2+}$ potentiation of PAA action and the importance of the nitrile group in PAAs. In the ion-conducting state of the channel, these selectivity filter glutamates are involved in the permeation process and bind the permeating ion directly. If devapamil binds to these glutamates, it must compete with permeating ions. However, PAAs block Ba$^{2+}$ currents less effectively than they block Ca$^{2+}$ currents; this effect is known as Ca$^{2+}$ potentiation (8). Ca$^{2+}$ ions have a higher affinity for the selectivity filter glutamates than Ba$^{2+}$ ions (36). Mutations in the selectivity filter region disrupt the Ca$^{2+}$ potentiation effect (8). Thus, the experimental data allow us to suggest that high affinity binding of PAAs requires the presence of a permeating ion bound to the selectivity filter. In other words, the Ca$^{2+}$ ion in the selectivity filter would enhance the channel-blocking activity of PAAs. Such enhancement seems impossible in the model in which...
the amino group of PAAs binds directly to the selectivity filter glutamates.

How can the binding of a positively charged metal ion to the selectivity filter enhance the binding of a positively charged PAA ligand? There are two possibilities: by an allosteric effect or by a direct PAA-Ca$^{2+}$/H11001 interaction. We cannot rule out the first possibility, but a direct interaction seems more plausible because the ligand-binding site is close to the selectivity filter. Indeed, direct interactions between ions in the selectivity filter and a ligand in the inner pore were demonstrated for K$^{+}$/H11001 channels by a combination of x-ray crystallography and high level free energy calculations (37). If the Ca$^{2+}$/H11001 potentiation effect is due to direct interaction with the ligand, the latter should contain functional group(s) capable of coordinating the Ca$^{2+}$/H11001 ion. Because internally applied PAAs with tertiary and quaternary amino groups exhibit similar channel-blocking potencies (6, 38), the tertiary amino group is likely protonated and hence has no lone electron pair. Methoxy groups have lone pairs, but these groups are involved in specific interactions with PAA-sensing residues of the channel (see above). The only remaining candidate for a direct coordination with a Ca$^{2+}$/H11001 ion is the nitrogen atom in the nitrile group. This group is critically important for PAA action (16), but its role in ligand-channel interactions has not been explained so far. The geometry of the nitrile group with the lone pair extending from the ligand along the triple-bond direction seems ideal for reaching the Ca$^{2+}$/H11001 ion bound to the selectivity filter glutamates. All these considerations suggest that the nitrile group of PAAs interacts directly with the ion at the selectivity filter.

If the amino group of PAAs does not bind to the selectivity filter glutamates, where can it bind? In addition to the selectivity filter, the P-loop channels have another site that attracts cations. This site is located at the focus of P-helices. In K$^{+}$/H11001 channels, the macrodipoles of P-helices create a binding site for permeating ions and positively charged tetraalkylammonium ligands (39, 40).

In summary, the above analysis of experimental data suggests the following scheme of devapamil binding to LTCC: (i) the m-methoxy group in ring B accepts an H-bond from Y4i11, (ii) the two methoxy groups in ring A accept H-bonds from Y3i10 and the engineered Y3o14, (iii) the nitrile nitrogen coordinates a Ca$^{2+}$/H11001 ion bound to the selectivity filter glutamates from repeats III and IV, and (iv) the protonated amino group is located in the nucleophilic region at the focus of P-helices (Fig. 2).

**Modeling the Devapamil-LTCC Complex**—The proposed binding scheme for devapamil is based on the data from mutational, electrophysiological, and ligand binding experiments (8, 10–12, 14, 16). It remains to be explored if this scheme agrees with available structures of P-loop channels, conformational possibilities of PAAs, and experimental data on the interactions of PAAs with the channel residues. We modeled devapamil binding to the T3o14Y mutant of the KvAP-based model of Cav1.2. The mutant contains more “anchors” to dock devapamil than the wild-type channel. Proximity between methoxy groups of devapamil and hydroxy groups of Y3o14, Y3i10, and Y4i11 was established by distance constraints between specific pairs of atoms. Distance constraints were also used to bias coordination of a Ca$^{2+}$/H11001 ion by the nitrile group of devapamil as well as by E3p50 and E4p50. In addition to the six distance constraints, pin constraints (see “Materials and Methods”) were imposed to retain the structural similarity of the channel model to the x-ray
The complex was optimized by the Monte Carlo minimization protocol. The goal of this optimization was to check if all proposed interactions are energetically possible. A ligand-binding pose was considered possible if after Monte Carlo minimization it met the following four criteria: (i) all distance constraints were satisfied; (ii) the complex remained stable upon a subsequent energy minimization with all constraints removed, i.e., the complex corresponded to an energy minimum; (iii) the ligand-receptor energy was negative; and (iv) no amino acid in the ligand-binding site provided repulsive (positive) contributions of van der Waals energy to ligand-receptor interactions.

The proposed binding scheme contains an ambiguity: from the experimental data, it is unclear which of the two methoxy groups in ring A H-bonds with Y$^{3i10}$ and with Y$^{3o14}$. Both possibilities were explored. Computations have shown that just one binding scheme meets the above criteria. In this scheme, $p$- and $m$-methoxy groups accept H-bonds from Y$^{3o14}$ and Y$^{3i10}$, respectively.

The obtained binding mode is shown in Fig. 3. In this complex, the ligand-channel interaction energy is $-41$ kcal/mol. No residue provides unfavorable (positive) energy contribution (Table 2). Despite the fact that the ligand bears a net positive charge of 1 elementary charge unit, the Ca$^{2+}$ ion provides a stabilizing contribution of $-2$ kcal/mol. This is not surprising: the nitrile group is strongly attracted to the Ca$^{2+}$ ion, whereas the ammonium nitrogen is 6.2 Å from the Ca$^{2+}$ ion. The ammonium group of devapamil does not form a direct contact with any residue, but it is stabilized by electrostatic interactions with the nucleophilic C-terminal ends of the four P-loops. The positively charged ammonium group of the ligand at the pore center appears as the major determinant of the ion permeation block. Ring B interacts with Y$^{4i11}$, A$^{4i15}$, and I$^{4i18}$, which were described previously as the PAA-sensing triad (10, 14). Ring A interacts with F$^{3p49}$, I$^{3i11}$, and T$^{3p48}$. The isopropyl group approaches T$^{2p48}$ and F$^{3p49}$. The central part of devapamil is located in the inner pore.

Residues that provide the main contributions to the ligand-binding energy are highlighted in Table 1, and ligand-channel contacts are shown in Fig. 3. Although specific ligand-channel interactions were imposed only for Y$^{4i11}$, Y$^{3i10}$, and Y$^{3o14}$, all experimentally defined PAA-sensing residues, except V$^{3i23}$, contribute to ligand-binding energy (Table 2). The side chains shown in Table 2 provide a total of $-26.6$ kcal/mol to the ligand-channel energy. The remaining $-14.4$ kcal/mol are provided by backbone atoms (contributions that are not possible to estimate via site-directed mutations), by the Ca$^{2+}$ ion bound to E$^{3p50}$ and E$^{4p50}$, and by remote side chains whose absolute energy contributions are $<0.1$ kcal/mol per side chain. A significant difference between the modeling predictions and experimental data is seen for residues at the C-terminal halves of the inner helices. Thus, the experimentally determined PAA-sensing V$^{3i23}$ residue does not specifically interact with devapamil in the model.
(Table 2). By contrast, Phe4i22, which interacts with devapamil in the model, was not identified as a PAA-sensing residue. This probably reflects a difference between the template structure and real LTCC. Notably, available open channel templates demonstrate the most significant structural diversity in this region.

Comparison of Channel Templates and Sequence Alignments—

The model described above was built using the open channel KvAP template. PAAs are know to block LTCC by both use-dependent and resting block mechanisms (e.g. Refs. 35 and 41), indicating that the ligands should be accommodated not only in the open but also in the closed channels. Recent data provide direct support for devapamil trapping in the closed Ca,
1.2 channels (42). Large variations in the pore geometry between the open and closed channels are obvious, but significant geometrical variations can also be found by comparing x-ray structures of open K+ channels (Fig. 4A). In particular, the distance between α-carbons at positions analogous to the PAA-binding tyrosines of our model (Y4i11 and Y3o14) equals 11.6, 11.8, and 14.8 Å in the x-ray structures of K1.2, KvAP, and MthK, respectively.

To explore whether such deviations are critical for our model, we used the same protocol to dock devapamil in MthK-, K1.2-, and KcsA-based models. Fig. 4 shows superimpositions of devapamil in the four models. All four templates appear fully consistent with the proposed binding scheme despite some local peculiarities of individual models. The central cavity of the closed channel accommodates the devapamil molecule and provides contacts for the ligand that satisfy all four criteria formulated above (Fig. 4, B and C). The variations in the open channel templates were not critical for the devapamil-LTCC models. Indeed, despite the above-mentioned variations in the distances between α-carbons at positions 3o14 to 4i11, the two rings of the flexible devapamil molecule readily make H-bonding contacts with mobile tyrosine side chains in all four homology models (Fig. 4). Similarly, other devapamil-LTCC contacts do not change dramatically in different models. Thus, we could not favor a particular template for homology modeling of Ca2+ channels.

Docking of Verapamil and Gallopamil—Verapamil and gallopamil differ from devapamil by having additional methoxy groups in rings A and B (Fig. 1), and some mutations have significantly different effects on the channel-blocking potencies of these three well studied PAAs. Thus, replacements of the YAI triad in IVS6 affect the resting channel block by devapamil but not by verapamil and gallopamil. On the other hand, the same replacements of the YAI triad strongly reduce the use-dependent block by all three PAAs (11, 14). King B of devapamil has a single methoxy group in the meta-position. In our models, ring B interacts mainly with IVS6. The m-methoxy group of devapamil binds to the side chain hydroxyl of Y4i11. Monte Carlo minimization of the LTCC complex with verapamil demonstrated that IVS6 residues do not impose steric restrictions for binding of the additional p-methoxy group at ring B of verapamil and gallopamil. However, the hydrophilic oxygen of the p-methoxy group occurs in the hydrophobic environment of A4i15 and T4i18, an energetically unfavorable situation (Fig. 6A). Thus, in agreement with the above-mentioned data on the resting channel block, ring B of gallopamil and verapamil interacts with the YAI triad in our model less strongly compared with ring B of devapamil. It should be noted that modest conformational changes in the pore domain would allow avoiding unfavorable dehydration of the p-methoxy group in ring B. Diverse geometrical characteristics of the inner pore are expected for different channel states, which prevail in various experimental protocols. This may explain why the YAI mutations reduce the use-dependent block by all three PAAs. Direct modeling sup-

| Residue* | Closest to devapamil side chain heavy atom PDB name Distancea Å | Side chain devapamil energy kcal/mol |
|----------|---------------------------------------------------------------|-------------------------------------|
| M3p48   | CE                                                           | 4.4                                 | −0.9                               |
| L1i12   | CD1                                                          | 4.2                                 | −0.9                               |
| T2p41   | CG2                                                          | 3.6                                 | 0.4                                |
| N2o16   | OD1                                                          | 4.5                                 | 0.8                                |
| L3i19   | CD2                                                          | 4.0                                 | −0.8                               |
| E3p22   | CD1                                                          | 3.5                                 | −2.1                               |
| Y3o14   | OH                                                           | 3.5                                 | −0.3                               |
| T3p43   | CB                                                           | 7.1                                 | −0.1                               |
| F3p49   | CZ                                                           | 3.1                                 | −7.9                               |
| E3p50   | OE                                                           | 4.9                                 | −0.2                               |
| Y4i10   | OH                                                           | 3.0                                 | −2.1                               |
| I3i11   | CG2                                                          | 3.7                                 | −0.7                               |
| I4i14   | CG2                                                          | 3.1                                 | −0.3                               |
| A4i15   | CB                                                           | 3.6                                 | −1.2                               |
| M3i18   | CB                                                           | 3.2                                 | −0.1                               |
| M3i19   | CE                                                           | 4.8                                 | −1.0                               |
| E3i22   | CE1                                                          | 4.3                                 | −1.1                               |
| V3i23   | CG2                                                          | 9.3                                 | 0.0                                |
| T3p48   | CG2                                                          | 5.9                                 | −0.2                               |
| E3p50   | OE                                                           | 5.8                                 | 0.0*                               |
| Y4i11   | OH                                                           | 3.2                                 | −1.4                               |
| A4i15   | CB                                                           | 3.5                                 | −0.2                               |
| I3i18   | CG2                                                          | 3.4                                 | −0.4                               |
| I4i17   | CG1                                                          | 3.3                                 | −1.2                               |
| F3i22   | CD1                                                          | 3.5                                 | −2.0                               |

* Listed are residues that have at least one side chain heavy atom within 5 Å from a heavy atom of devapamil and residues whose mutations were demonstrated to have significant effect on PAA action (8, 10, 11, 43). The latter residues are shown in boldface.

a The distance is between the indicated side chain atom and the nearest heavy atom of devapamil.

b In our model, E3p50 and E3p50 do not interact with devapamil directly, but they coordinate a Ca2+ ion that makes a strong complex with devapamil.
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Mutation T\textsuperscript{3o14}Y increases the activity of devapamil and verapamil but not that of gallopamil (12). In our model, the methoxy groups in ring A interact with Y\textsuperscript{3i10} and the engineered Y\textsuperscript{3o14}. Ring A of gallopamil has an additional m-methoxy group, and it is unclear why the additional H-bonding partner in Y\textsuperscript{3o14} does not improve the gallopamil activity. Fig. 6B shows the Monte Carlo-minimized complex of LTCC with gallopamil. In this complex, the second m-methoxy group of gallopamil approaches the hydrophobic environment of M\textsuperscript{3i18} and M\textsuperscript{3i19}, an energetically unfavorable situation. This explains why the gallopamil potency is insensitive to the T\textsuperscript{3o14}Y mutation.

Docking of Other PAAs—The S-enantiomers of PAAs are more potent than the R-enantiomers (46–52). To explore if the proposed binding model is consistent with these data, we docked (R)-devapamil onto the KvAP-based model. All ligand-channel constraints were readily satisfied, and the ammonium group resided at the focus of P-helices. Understandably, the alkylamine chain between rings A and B adopted a conformation that is different from that in the (S)-devapamil-LTCC complex. The major difference between the two complexes is the orientation of the isopropyl group, which is exposed in the pore in the (R)-devapamil-LTCC complex and does not make specific contacts with the channel (Fig. 6C). This explains the different activity of the R- and S-enantiomers of PAAs.

Structure-activity relationship studies demonstrate the importance of the nitrile group for PAA activity (16). Indeed, PAA analogs with the nitrile group replaced by a primary or tertiary amino group are inactive (compounds D 528 and D 532) (Fig. 1). Our model readily explains these observations: the protonated amino groups would be repelled from the Ca\textsuperscript{2+} ion bound to the selectivity filter glutamates. On the other hand, there are active PAAs, e.g. falipamil, tiapamil, and BRL 32872, that lack the nitrile group. Importantly, these compounds have nucleophilic atoms at positions similar to the nitrile nitrogen of classical PAAs (Fig. 1).

A priori, it was unclear whether such compounds (especially tiapamil with a bulky saturated six-membered ring) could fit our model. Fig. 6D shows the predicted complex with tiapamil. Remarkably, tiapamil readily binds to the channel model, preserving virtually the same ligand-channel contacts as devapamil, whereas two of the four oxygens at the six-membered ring of the ligand simultaneously interact with the Ca\textsuperscript{2+} ion in the selectivity filter.

Conformational flexibility is an important feature of PAAs, which at first glance aided in realizing the proposed scheme of

FIGURE 4. Devapamil-binding LTCC models built with different templates. A, cytoplasmic view on superimposed templates. The templates are arranged to minimize deviations of α-carbons at positions 3o14, 3i10, and 4i11, which correspond to the devapamil H-bonding tyrosines in our model. C=–C= bonds in these positions are shown as sticks colored as follows: KcsA, red; K, 1, 2, orange; KvAP, blue; MthK, purple. The backbones of KvAP are shown as described in the legend to Fig. 2; other backbones are light gray. Note the relatively small variations in devapamil-binding models in the four LTCC homology models based on the four x-ray templates of K\textsuperscript{+} channels. The tyrosines are shown as thin sticks colored as the C=–C= bonds in A. Devapamil is shown by thick sticks with dark-gray carbon atoms. Calcium ions are shown as yellow spheres. The side chains of the engineered Y\textsuperscript{3o14} are hidden in the side view (C) for clarity. Note that the variations in the templates cause only small variations in devapamil binding poses without breaking any specific contact. D, side view of the KcsA-based model of the closed LTCC. Repeats are shown as surfaces with repeat I removed for clarity. Devapamil is shown by sticks and a transparent surface. Note that the folded devapamil molecule matches the size of the central cavity.
interactions. However, there are active PAAs, e.g. compound 2b from Ref. 53 (Fig. 1), in which the flexibility is restricted. Explaining the activity of such compounds is an additional touchstone for the validity of the proposed model. Fig. 6E shows the predicted complex in which the ligand fits the proposed binding scheme. Importantly, the conformation of PAAs in our models agrees with the prediction from a structure-activity study that the biologically active conformation of PAAs is U-shaped (54). Furthermore, the bulky moiety introduced between the ammonium and the tetrasubstituted carbon of compound 2b in Ref. 53 occurs in the inner pore and is readily accommodated in this spacious region. This computational result confirms the location of the PAA ammonium group in the inner pore rather than in a tight pocket, one of the important aspects of our model.

**DISCUSSION**

In this study, we designed a high affinity binding mode of PAAs in the model of LTCC. The majority of available data from mutational studies is explained in our model by specific interactions between PAA-sensing residues and different moieties of the ligands. The key interactions proposed (H-bonding of methoxy groups of devapamil to side chains of Y3i10, Y4i11, and the engineered Y5o12 and interaction of the nitrile group with the Ca\(^{2+}\) ion) were imposed by distance constraints between specific atoms of the ligands and the homology model of the channel. Our calculations demonstrate that all these interactions can be satisfied simultaneously and that the scheme of interactions agrees with the homology model. Certainly, the precision of homology modeling is limited. However, it should be emphasized that we did not modify the template structure to satisfy the proposed interactions. The same model based on the same sequence alignment was used previously for analyses of binding of DHPs and BTZs to LTCC (19, 20). It should be noted that despite the obvious differences in the structure and mechanism of action of these three classes of LTCC ligands, two important features of ligand-channel interactions are common in our models. First, the positively charged amino groups of PAAs, BTZs, and some of the charged DHP derivatives are predicted to bind in the inner pore, at the focus of the P-helices. Second, all three classes of LTCC ligands are proposed to interact directly with a Ca\(^{2+}\) ion chelated by the selectivity filter glutamates from repeats III and IV.

**Structure-Activity Relationships of PAAs**—We used only a small portion of the available structure-activity data to design the PAA-binding scheme. Nevertheless, the resulting model was able to explain interesting aspects of structure-activity relationships (see “Results”). More data can be explained in view of our model even without docking experiments. For instance, the predicted H-bond between the side chain hydroxyl of Y3i10 and the \(m\)-methoxy group of ring A (Fig. 3, A, C, and E) is consistent with experimental data that PAAs without an H-bond acceptor at the \textit{meta}-position of ring A (e.g. compounds D 557 and D 559) in their active conformation, whereas the ligands with H-bond donors in the PAA-binding site. A deficiency of the H-bond acceptors in ligands understandably

**FIGURE 5. Comparison of S6 alignments.** Shown are intracellular views of the KvAP template, with orange sticks indicating C\(^{\alpha}\)–C\(^{\beta}\) bonds at positions that correspond to the H-bonding tyrosines of LTCC according to the different alignments of S6s. For IIIIS5, the alignment proposed in Ref. 43 was used. Orange lines define a triangle with the C\(^{\alpha}\) atoms at the vertices. The triangle indicates the III/IV interface and defines a plane. A vector extending the C\(^{\alpha}\)–C\(^{\beta}\) bond is colored blue or red if its projection on the plane directs into or out of the triangle, respectively. A–D correspond to the alignments proposed in Refs. 44, 18, 43, and 45, respectively. Only the alignment used in this work (D) results in orientation of all three vectors into the III/IV repeat interface and is therefore consistent with the proposed scheme of interactions.
decreases activity, as in the case of completely demethoxylated emopamil (15). An excess of the acceptors also decreases activity because in the confined and predominantly hydrophobic environment of the III/IV repeat interface, dehydration of the methoxy groups not involved in specific interactions (e.g., H-bonding) is costly. This is illustrated by the fact that verapamil and gallopamil are less potent LTCC blockers than devapamil (15).

The ammonium group of PAAs in our models resides near the focal point of the P-helices. In the x-ray structure of K⁺/H11001 channels, the corresponding region (position 5) is occupied by a K⁺ ion, which is stabilized by the nucleophilic C-terminal ends of P-helices. Because the amino group is protonated at physiological pH, the same electrostatic stabilization is expected for PAAs in LTCC. This placement of the ammonium group in the cavity rather than in a tight pocket can explain why large substitutions of the N-methyl group (e.g., an isopropyl group in D 594) (Fig. 1) are tolerated (17). It also explains the LTCC-blocking activity of internally applied quaternary derivatives of devapamil, verapamil, and gallopamil (6).

**Theoretical and Experimental Data on PAA Interactions with Individual Residues of Ca₁.2**—Attempts to correlate the predicted ligand residue energies (Table 2) with energetic characteristics that can be obtained from effects of mutations on PAA would be misleading. Indeed, the latter are free energy changes in ligand-channel interactions upon mutations, whereas our theoretical data are enthalpies of devapamil interactions with the channel that has native residues in all positions except 3o14. Computing the free energy of devapamil-channel interactions in various mutants would be justified if a high resolution x-ray structure of Ca₁.2 were available, but our homology model is an obvious approximation. It should be also noted that a point mutation can change the PAA-binding mode and that the resulting new interactions can partially compensate for the loss of specific interactions with the native residue. Furthermore, some point mutations can affect ligand binding allosterically, and the modeling of such effects is hardly possible.

Despite these caveats, our model generally agrees with the experimental data on involvement of individual LTCC residues in PAA binding. Indeed, almost all residues whose mutations significantly affect the PAA block are within 5 Å from devapamil and provide stabilizing energy contributions to the ligand binding in our model (Table 2). The model also explains the asymmetric effect of mutations of the selectivity filter glutamates on the PAA block (10). The direct contribution of these residues to the devapamil-binding energy is small (Table 2). However, E₃p⁵⁰ and E₅p⁵⁰, whose mutations significantly affect
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Uncharged DHP antagonists inhibit the ion permeation by exposing their hydrophobic moieties to the Ca\(^{2+}\)-binding site.

State Dependence of Ligand Action—The state dependence of PAA action on calcium channels is well known (1, 41, 56), but structural determinants of this important phenomenon are unclear. In this work, we present a single binding mode of ligands that cannot explain per se the state-dependent action of PAs.

An obvious cause of the state-dependent action is the different arrangements of ligand-sensing residues in different states of the channel. However, this is not the only cause because state-dependent action was observed for various ligands, which have different patterns of ligand-sensing residues. A more common cause of the state dependence is the involvement of permeant ions in ligand binding. The population of the selectivity filter by permeant ions should be different in different channel states. Previously, we proposed that the state-dependent action of local anesthetics on the Na\(^{+}\) channel can be explained by their interaction with a permeant cation bound to the selectivity filter (57, 58). For Ca\(^{2+}\) channel ligands, we also suggest a significant interaction between the ligand and the Ca\(^{2+}\) ion bound to the selectivity filter glutamates. Importantly, this interaction requires the coordination of the Ca\(^{2+}\) ion proximal to the inner pore by the selectivity filter glutamates in repeats III and IV. State-dependent changes in the ion occupancy of the selectivity filter or changes in the ion coordination pattern would distort the ligand-ion interaction and thus the ligand-blocking potency. Of course, this hypothesis needs careful experimental verification.

Repeat Interface-binding Mode and Access Pathways of LTCC Ligands—Early photoaffinity labeling experiments identified LTCC segments III/S6 and IV/S6 as components of ligand-binding sites (34, 59, 60). On the basis of these data, Catterall and Striessnig proposed in 1992 (61) the domain (repeat) interface hypothesis for binding of calcium channel ligands. It is well known now that all three classes of LTCC ligands bind to overlapping sites and share several ligand-sensing residues. Ligands of different classes interact with each other allosterically without direct competition (for reviews, see Refs. 1 and 62). The repeat interface hypothesis explains these phenomena, but structural details were not elaborated. Fig. 7A shows the superimposed LTCC models with a DHP (20) and a PAA. The bulky core of the DHP ligand occurs in the repeat interface, and only a small moiety protrudes into the pore. By contrast, the bulkiest part of the PAA ligand, including the nitrile and isopropyl groups, binds in the inner pore, whereas only the aromatic rings partially occupy the III/IV repeat interface (Fig. 7A). Local overlaps seen between the ligands do not exclude their simultane-
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ous binding. On the other hand, the close proximity of the ligands explains interdependence of their binding.

Despite the close proximity of binding sites of the three classes of LTCC ligands, they have different access pathways to their binding sites. The access pathway of PAAs is predominantly intracellular, whereas DHPs and BTZs reach their binding sites from the extracellular side of the membrane (see Ref. 1 for a review). In view of our models, DHPs and BTZs access their binding sites from the extracellular medium along the P-helix of repeat III (19, 20), whereas PAAs access their binding site through the open activation gate (Fig. 7B). Intriguingly, mutation \( T_{339}^{341}Y \) has opposite effects on the action of DHPs and PAAs (12). Our models explain why this mutation enhances PAA action but eliminates high DHP activity through a steric mechanism (12). In Fig. 7B, the engineered \( Y_{34}^{314} \) largely overlaps with the DHP ligand but provides an additional contact for the PAA ligand. Thus, our molecular models not only explain experimental data on different classes of LTCC ligands but also provide a structural basis for understanding interrelationships between these classes. In this regard, our models elaborate the repeat interface hypothesis (61).

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