Molecular Modeling of Benzothiazepine Binding in the L-type Calcium Channel*

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Benz(othi)azepine (BTZ) derivatives constitute one of three major classes of L-type Ca\(^{2+}\) channel ligands. Despite intensive experimental studies, no three-dimensional model of BTZ binding is available. Here we have built KvAP- and hKcsA-based models of the Ca\(_{1.2}\) pore domain in the open and closed states and used multiple Monte Carlo minimizations to dock representative ligands. In our open channel model, key functional groups of BTZs interact with BTZ-sensing residues, which were identified in previous mutational experiments. The bulky tricyclic moiety occupies interface between domains III and IV, while the ammonium group protrudes into the inner pore, where it is stabilized by nucleophilic C-ends of the pore helices. In the closed channel model, contacts with several ligand-sensing residues in the inner helices are lost, which weakens ligand-channel interactions. An important feature of the ligand-binding mode in both open and closed channels is an interaction between the BTZ carbonyl group and a Ca\(^{2+}\) ion chelated by the selectivity filter glutamates in domains III and IV. In the absence of Ca\(^{2+}\), the tricyclic BTZ moiety remains in the domain interface, while the ammonium group directly interacts with a glutamate residue in the selectivity filter. Our model suggests that the Ca\(^{2+}\) potentiation involves a direct electrostatic interaction between a Ca\(^{2+}\) ion and the ligand rather than an allosteric mechanism. Energy profiles indicate that BTZs can reach the binding site from the domain interface, whereas access through the open activation gate is unlikely, because reorientation of the bulky molecule in the pore is hindered.

Benz(othi)azepines (BTZs) represent one of three main classes of ligands of L-type calcium channels (1, 2). Tonic (resting) block of Ca\(^{2+}\) channels is usually measured by applying infrequent depolarization stimuli. Frequency-dependent block is measured during more rapid trains of depolarization stimuli. Increasing the stimulation frequency generally leads to more effective inhibition by BTZs (3–5). Voltage and activation dependence of the block are consistent with the “modulated receptor” hypothesis (6), which was initially proposed to explain the action of local anesthetics on Na\(^{+}\) channels. The effect of BTZs also depends on the ionic environment. Raising concentrations of Ca\(^{2+}\) or Ba\(^{2+}\) antagonize diltiazem block (3). However, the block is more pronounced when Ca\(^{2+}\) rather than Ba\(^{2+}\) is used as a charge carrier (3, 7), and thus the block is considered potentiated by Ca\(^{2+}\) (7). The conductance with Ba\(^{2+}\) is higher than with Ca\(^{2+}\), because Ca\(^{2+}\) binds more tightly to the outer-pore glutamates (8). These experiments suggest that the high affinity binding of BTZs requires a cation binding in the channel.

Extracellular applications of both the tertiary and quaternary BTZ analogs effectively block Ca\(^{2+}\) channels. In contrast, intracellular application of the same compounds does not result in significant block, leading to the conclusion that BTZs block the channels via an extracellular pathway (9–11).

Mutational studies have largely delimited BTZ-sensing residues to the inner helices and P-loops of domains III and IV (see Table 1). However, applying experimental data for mapping the BTZ receptor is complicated because of unequal effects of mutations on different drugs and different characteristics of the block. For example, residue I\(_{4}^{8}\) in position 8 of the inner helix of domain IV (see footnote 3 for residue labels) was reported to affect diltiazem unblocking in the resting state (12). Mutations F\(_{3i23}^{A}\) and V\(_{Y3}^{A}\) reduce diltiazem block, which was considered to slow inactivation kinetics and accelerate the recovery of the V\(_{Y3}^{A}\) mutant from drug blockade (13). The selectivity filter mutations do not alter diltiazem block of closed channels in Ba\(^{2+}\) but disrupt frequency-dependent block and potentiation by Ca\(^{2+}\) (7).

BTZs have a rigid bulky tricyclic core with several substituents. The structure-activity relationships (SARs) of BTZs have been rigorously analyzed in many previous studies (e.g. Refs. 14–18). Blocking potency is affected by the configurations of the chiral centers and any modification of the substituents of the BTZ rings. These SAR data provide a pharmacophore model but do not provide detailed information on how specific BTZ modifications affect the electrophysiological characteristics of block.

Despite numerous studies, the atomic level mechanism of action of BTZs remains unclear. A particularly intriguing ques-

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‡ The abbreviations used are: BTZ, benz(othi)azepine; MCM, Monte Carlo minimization; SAR, structure-activity relationships.

3 We use a residue-labeling scheme, which is universal for P-loop channels. A residue label includes the domain (repeat) number (1–4), segment type (p, P-loop; i, the inner helix; and o, the outer helix), and relative number of the residue in the segment (Table 1).
tion is why BTZs do not block the channel from the cytoplasm despite mutations of many pore-facing residues affect the channel blockade. Molecular determinants of the state dependence of drug action also remain unresolved. The effect of Ca\(^{2+}\) potentiation of the block is another puzzle: divalent cations antagonize BTZ binding (19), but the presence of Ca\(^{2+}\) in the selectivity filter is required for maximal drug effectiveness. Current knowledge about the BTZ action in Ca\(^{2+}\) channels suffers from the lack of structural models that can integrate results of mutational analysis, SAR data, and other experimental observations. There are several reasons for this situation. First, the structure of the L-type Ca\(^{2+}\) channel at the atomic resolution is still unavailable. Second, BTZs are complex molecules with many different functional groups. Third, the BTZ action is also complex: it involves tonic and state-dependent block and potentiation by Ca\(^{2+}\). Combination of these factors precluded elaborating a reliable model of BTZ receptor so far. To our knowledge, this work is the first attempt to obtain insights into the BTZ mechanism of action from the viewpoint of structural analysis.

MATERIALS AND METHODS

The L-type Ca\(^{2+}\) channel model for analysis in this study consists of transmembrane segments S5 and S6 and P-loops contributed by each of four repeats (I, II, III, and IV) of Ca\(_{1,2}\) (Table 1). Among available x-ray structures of the open K\(^{+}\) channels (MthK, KvAP, and Kv1L2), we have chosen KvAP (20) as a template. Like Ca\(^{2+}\) channels, KvAP is a voltage-gated channel whose inner helices lack the PVP motif, which affects the open pore geometry. The closed channel was modeled using the KcsA structure (21) as a template. When viewed from the extracellular side, domains I–IV are arranged clockwise around the pore axis (22). Extracellular linkers are far from the BTZ binding site and, thus, were not included in our model. The sequences of P-loops and inner helices were aligned as in Refs. 23 and 24. The sequences of the outer helices were aligned as proposed by Huber et al. (25).

The conformational energy expression included van der Waals, electrostatic, H-bonding, hydration, and torsion components, as well as the energy of deformation of the ligands’ bond angles. Hydration energy was calculated by the implicit-solvent method (26). Nonbonded interactions were calculated using the AMBER force field version (27, 28), which is consistent with the implicit-solvent approach. More recent versions of the AMBER force field were parameterized for explicit-waters environment. A large number of degrees of freedom associated with explicit waters would reduce efficiency of the Monte Carlo minimization (MCM) method, which we use in this study (see below). To take into account that atomic charges may be screened by water molecules, electrostatic interactions were calculated with a distance- and solvation-dependent dielectric function, \(\varepsilon = d(4 - 3s)\), where \(d\) is the distance between interacting atoms and \(s\) is a screening factor calculated using a modified algorithm of Lazaridis and Karplus (26). The screening factor value varies from 0 for a pair of water-exposed atoms to 1 for a pair of protein-buried atoms.

All ionic residues were modeled in their neutral forms except for the selectivity filter glutamates in position p50 of the P-loops. Nonbonded interactions were truncated at distances >8 Å. This cutoff distance speeds up calculations without noticeable decrease in the precision of energy calculations (29). The cutoff was not applied to electrostatic interactions involving Ca\(^{2+}\) ions and ionized groups; these interactions were computed at all distances. The energy was minimized in the space of generalized coordinates, which include all torsion angles, bond angles of the ligand, positions of ions, and positions and orientations of root atoms of free molecules (30). Energy minimizations were terminated when the energy gradient became <1 kcal mol\(^{-1}\) rad\(^{-1}\).

The channel models were optimized by the MCM method (31). Complexes of the channel with ions and ligands were searched using a multi-MCM method described in our recent report (32). Calculations were performed using the ZMM program.

RESULTS

Model Building—The starting backbone geometry of the open and closed channel models was taken from the KvAP and KcsA K\(^{+}\) channels, respectively. The all-trans conformations were used as starting approximations for side chains of residues that mismatched between the K\(^{+}\) channel templates and Ca\(_{1,2}\). Previous mutations in the selectivity filter DEKA locus rendered Na\(^{+}\) channel Ca\(^{2+}\) selectivity (33). These results indicate that there is likely a similar folding of the selectivity filter region of Ca\(_{1,2}\), channel, which has a ring of four glutamates (EEEE) in place of DEKA in Na\(^{+}\) channels. Given the similarity, we modeled the backbones of the ascending limbs in P-loops of Ca\(_{1,2}\) using our previously reported tetrodotoxin receptor model of Na\(^{+}\) channel (34). This choice of the starting point was not critical for the present study, because BTZs do not bind in the outer pore. The coordination pattern of Ca\(^{2+}\) ions in the EEEE ring is unknown. Asymmetric split (24) and symmetric single-file (35) models were considered. Recent calculations predict that both models are energetically possible (36). However, because substitutions of individual selectivity filter glutamates have unequal effects on BTZ binding (7), we used the split model in this study with the following coordination pattern: E\(^{p350}\),Ca\(^{2+}\)::E\(^{p50}\) and E\(^{p350}\),Ca\(^{2+}\)::E\(^{p50}\). The channel models in the open and closed states were optimized by MCM trajectories, which were terminated when 10,000 consecutive energy minimizations did not improve the energy of the apparent global minimum.

Random Docking—For the ligand-docking computational experiments we have chosen one of the most potent BTZ derivatives, SQ32910 (14, 18). Random docking of the ligand in the open channel was performed using the multi-MCM method as described previously (29, 32). The resulting low energy structures fall into two categories in which the BTZ rings occur either in the inner pore (Fig. 1, A and B) or in the III/IV domain interface (Fig. 1, C and D). The ligand-channel interaction energy is most favorable when the BTZ rings are in the III/IV domain interface. However, the energy difference between the binding modes is not significant enough to favor the domain-interface model based on the energetics alone. More importantly, ligand binding in the III/IV domain interface correlates better with available data on BTZ-sensing residues and with the
proposed access pathway of the drug to the binding site as discussed below. The models with the BTZ rings in the inner pore cannot easily explain why the BTZ binding site is accessible from the outside but not the inside the cell. It should be noted that, in these models, the ligand interacts with all four S6 segments, but some BTZ-sensing residues in the domain interface do not contact the drug molecule (Fig. 1, A and B) (Structure 1).

Energy Profile—The experimental data on the sidedness of BTZ action and on BTZ-sensing residues evidence that BTZs access their binding site through the III/IV domain interface. The inner pore of K⁺ channels has four radial niches between neighboring S6 segments and P-helices. Mutations in these niches affect binding of some K⁺ channel blockers (37, 38). In homology models of Na⁺ and Ca²⁺ channels several residues, which control access of drugs to the inner pore, line a hypothetical access pathway (34, 39) that overlaps with the niche between domains III and IV. The pathway is wide enough to allow drug access to the inner pore (40). We calculated the energy profile for SQ32910 pulled with steps of 1 Å along the proposed pathway parallel to the IIIP helical axis from a starting position outside the channel toward the inner pore (Fig. 2). The energy was Monte Carlo-minimized at each position of the profile. During energy minimizations the ligand retained all internal degrees of freedom and five of the six rigid-body degrees of freedom. The pulled atom Nsp2 was constrained to a plane normal to the IIIP helical axis. To prevent flip-flop of the ligand, the flat-bottomed energy constraints were imposed at each position of the profile to keep atom Nsp3 closer to the pore axis than atom Nsp2, and the angle between line Nsp2–Nsp3 and the IIIP helical axis was retained within 0 ± 90°. In the final point of the profile (at 30 Å), atom Nsp2 is positioned at the pore axis.

van der Waals interaction energy is negative all along the pathway (Fig. 2A) indicating the absence of steric hindrances. The lowest van der Waals energy was observed in the narrowest part of the pathway whose cross-sectional dimensions are similar to the drug width. The electrostatic component of the interaction energy is slightly negative at the beginning of the pathway, and a small electrostatic barrier is present between positions 12 and 20. This barrier is due to repulsion between the ligand ammonium group and the Ca²⁺ ion chelated by E3p50 and E4p50. To reach the inner pore, the ammonium group passes as close as 7 Å from the Ca²⁺ ion. Just after the barrier, the electrostatic interactions have a minimum of -13.2 kcal/
Two types of interactions are responsible for the minimum. First, the ligand carbonyl oxygen approaches the Ca\(^{2+}\)/H\(^{11001}\) ion and binds to it. Second, the ammonium group is located at the focus of P-helices with nucleophilic C-ends. This region in P-loop channels accommodates inorganic (21) and organic (40–42) cations. The hydration component of interaction energy increases as the drug enters a narrow part of the pathway. A sharp maximum at positions 22 and 23 corresponds to the location where the drug replaces water molecules of the Ca\(^{2+}\) inner hydration shell. The dehydration energy cost is partially compensated by the electrostatic attraction between the drug and the Ca\(^{2+}\) ion. Beyond position 23, all interactions weaken because the drug enters the wider inner pore and looses tight contacts with the III/IV domain interface. Notably, the energetically optimal position 22, which is marked on the plot by the vertical line, corresponds to the interface-binding mode obtained by the random docking protocol.

Characteristics of BTZ Binding in the Domain Interface—Intensive multi-MCM docking of the ligand in the III/IV domain interface provided an ensemble of 42 low energy complexes (Fig. 3). The scheme of ligand-channel interactions (Fig. 4A) shows the most conservative features of the ensemble: (i) interaction of the carbonyl oxygen with the Ca\(^{2+}\) ion chelated by E\(^{3p50}\) and E\(^{4p50}\), (ii) location of the ammonium group in the inner pore, at the focus of P-helices, and (iii) location of the BTZ rings in the III/IV domain interface that contains BTZ-sensing residues. Importantly, in our model the Ca\(^{2+}\) ion is the only electrophile that can interact with the carbonyl oxygen of BTZ. Furthermore, in the presence of Ca\(^{2+}\) in the selectivity filter, the only attractive site for the ligand ammonium group is the focus of P-helices. In the majority of structures, the ammonium group is not exactly in the focus but lies close in the vicinity.

Despite individual low energy structures belong to the same ensemble, they differ by details of particular interactions. The methoxyphenyl group interacts with Y\(^{4i11}\) (Fig. 4B). Two types of interaction have been found: (i) face-to-face attraction of the aromatic rings and (ii) an H-bond between the methoxy oxygen and the side-chain hydroxyl of Y\(^{4i11}\) with the aromatic rings approaching each other in the edge-to-face manner. The methyl group in position 3 of the seven-membered ring faces residues A\(^{3i15}\) and I\(^{4i18}\) in the majority of structures. Thus, all three residues of the YAI motif, forming what is known as a fingerprint of the BTZ recognition site (1), significantly interact with the ligand. Ring B of the ligand interacts mainly with F\(^{3i22}\) in the face-to-face (Fig. 4A) or edge-to-face (not shown) manner. The CF\(_3\) group, which has lipophilic properties (43), faces the III/IV domain interface. Interaction of F\(^{3p49}\) at the P-loop turn with the drug varies significantly between low energy structures, because in our model the alpha carbon of F\(^{3p49}\) was not constrained to the template. The aromatic side chain of F\(^{3p49}\) can participate in pi-cation interactions with a hydrated Ca\(^{2+}\) ion or with the ammonium group of the ligand (Fig. 4B). In some structures, F\(^{3p49}\) interacts with ring B of the drug in a face-to-face manner.
Benzothiazepines in L-type Ca$^{2+}$ Channel

FIGURE 3. Ensemble of low energy complexes representing the SQ32910 binding mode in the III/IV domain interface. The ligand is shown as a wireframe with the ammonium nitrogen (blue), carbonyl oxygen (red), and methoxy oxygen (red) shown as small spheres. A, intracellular view; B, side view with domain II removed for clarity. In each complex, the ammonium group is inside the central cavity at the focus of P-helices, carbonyl oxygen binds to the Ca$^{2+}$ ion at the selectivity filter, and the methoxy oxygen is in the domain interface.

Residues providing strong contributions to the ligand-receptor energy are underlined in Table 1. In the proposed binding mode, the drug does not interact directly with residues I$^{3i14}$, I$^{3i11}$, I$^{4i8}$, and I$^{4i8}$, which were experimentally demonstrated to affect the action of BTZ. However, residues I$^{3i14}$ and I$^{4i8}$ contribute significantly to the drug-channel energy along the access pathway (Fig. 2, B and C). Thus, among experimentally defined BTZ-sensing residues, only I$^{3i8}$ and I$^{3i11}$ do not contribute directly to the BTZ binding site or the access pathway. These particular residues could affect BTZ binding allosterically.

In our model, SQ32910 significantly interacts with residues F$^{3p44}$ and T$^{3p48}$ in the domain interface. Mutational studies suggest that F$^{3p44}$ interacts with dihydropyridines (39), and T$^{3p48}$ interacts with verapamil (44). Mutations of F$^{3p44}$ and T$^{3p48}$ are expected to clarify the role of these residues in the binding of BTZs.

In the lowest energy complex, the total ligand-channel interaction energy is $-22.3$ kcal/mol. BTZ-sensing residues contribute $-11.3$ kcal/mol, Ca$^{2+}$ contributes $-9$ kcal/mol, whereas electrostatic repulsion from the EEEE ring provides a "compensation" energy of 4 kcal/mol. Macrodipoles of P-helices contribute $-3$ kcal/mol. Only $-3$ kcal/mol comes from nonspecific interactions. These energetic characteristics are typical for the entire ensemble, in which the BTZ-sensing residues in S6s, the E$^{3p45}$-Ca$^{2+}$:E$^{3p50}$ dipole, and macrodipoles of P-helices provide, respectively, 51 $\pm$ 15%, 16 $\pm$ 6%, and 12 $\pm$ 8% of the ligand attraction energy. Altogether, the energetics of the obtained BTZ binding mode in the III/IV domain interface is consistent with the available experimental data.

In the proposed binding mode, the carbonyl oxygen of BTZ directly interacts with a Ca$^{2+}$ ion in the selectivity filter. We placed a Ca$^{2+}$ ion between E$^{3p45}$ and E$^{4p50}$ in our model to satisfy the experimental data that these glutamates control state-dependent block and the potentiation produced by Ca$^{2+}$ (7). Diltiazem blocks Ca$^{2+}$ currents better than Ba$^{2+}$ currents (7). If the BTZ ammonium group were directly interacting with the selectivity filter glutamates, these interactions would be antagonized by the current-carrying ions in a competitive manner. Because Ca$^{2+}$ binds stronger to glutamates than Ba$^{2+}$ (8), Ca$^{2+}$ would be expected to antagonize BTZ binding more strongly than Ba$^{2+}$. However, experimental data (7) clearly demonstrate the opposite effect, suggesting that, in the presence of Ca$^{2+}$, the ammonium group of BTZ does not bind to the selectivity filter glutamates. In view of our model, a Ca$^{2+}$ ion in the selectivity filter interacts with the BTZ carbonyl oxygen, and the energy of this attraction is stronger than the electrostatic repulsion between the Ca$^{2+}$ ion and the ligand ammonium group located in the focus of P-helices. Ba$^{2+}$ would coordinate with the BTZ carbonyl oxygen less strongly but would have the same electrostatic repulsion with the ammonium group. This explains why BTZ antagonizes Ca$^{2+}$ currents stronger than Ba$^{2+}$ currents.

Ca$^{2+}$-deficient Model—When the negative charges of four selectivity filter glutamates are not compensated by Ca$^{2+}$ ions, the excessive negative charge should attract the ammonium group of BTZ. To explore this possibility, we removed a Ca$^{2+}$ ion from E$^{3p45}$ and E$^{4p50}$ and computed Monte Carlo-minimized energy profile of SQ32910 pulled through the III/IV domain interface (Fig. 5A). Not surprisingly, van der Waals interactions in this Ca$^{2+}$-deficient model are similar to those in the Ca$^{2+}$-saturated model, whereas electrostatic interactions differ significantly between the two models. The energy profile
has a minimum at positions 13–20 where the ammonium group passes near the selectivity filter glutamates in the Ca\(^{2+}\)-deficient model. Further advance of the ligand meets with the energy barrier at position 24, which is attributable to electrostatic repulsion between the BTZ carbonyl group and the EEEE ring. The ligand remains rather far from the Ca\(^{2+}\) ion chelated by E\(^{3p50}\) and E\(^{2p50}\) and does not expel water molecules from it. Therefore, the unfavorable dehydration component of ligand-channel energy in the Ca\(^{2+}\)-deficient model is smaller than in the Ca\(^{2+}\)-saturated model (cf. Figs. 2A and 5A).

In the lowest energy complex, which corresponds to position 21 of the profile, the ammonium group reaches the selectivity filter glutamate E\(^{3p50}\) and interacts with it (Fig. 5, B and C). In this case, BTZ rings do not significantly relocate compared with the Ca\(^{2+}\)-saturated model. The possibility that the BTZ ammonium group can bind either at the focus of P-helices in the presence of Ca\(^{2+}\) or to a selectivity filter glutamate in the absence of Ca\(^{2+}\) may underline the experimentally observed state and ion dependence of the BTZ action (7).

In the Ca\(^{2+}\)-deficient model, the optimal value of electrostatic energy is higher (less preferable) than in the Ca\(^{2+}\)-saturated model. At first glance, this is surprising, because two Ca\(^{2+}\) ions should repel the BTZ ammonium group more strongly than a single Ca\(^{2+}\) ion. The energy analysis provides the following explanation for this paradox. In the Ca\(^{2+}\)-saturated model, two dipoles formed by the four selectivity filter glutamates and the two Ca\(^{2+}\) ions do not strongly repel the BTZ ammonium group located at the focus of P-helices, rather far from the dipoles. At the same time, the dipole of E\(^{3p50}:\text{Ca}^{2+}:E^{4p50}\) significantly attracts the carbonyl oxygen of BTZ, which is coordinated to the Ca\(^{2+}\) ion. There is an odd negative charge in the Ca\(^{2+}\)-deficient model with the cationic BTZ. Repulsion between the selectivity filter glutamates pushes the side chain of E\(^{4p50}\) out of the pore lumen. As a result, only one glutamate significantly attracts the ammonium group of the drug (Fig. 5B).

Other interactions are not markedly different between the Ca\(^{2+}\)-deficient and Ca\(^{2+}\)-saturated models. In the lowest energy complex of the Ca\(^{2+}\)-deficient model, ligand-sensing residues, the selectivity filter glutamates, and dipoles of P-helices provide, respectively, −13, −3, and −2 kcal/mol to the total of −26.2 kcal/mol of the interaction energy.

The resulting model from the above calculations can explain the state-dependent binding of BTZs by means of a state-dependent chelation of Ca\(^{2+}\) ions in the selectivity filter. Slow inactivation may change the selectivity filter configuration in a manner analogous to Na\(^{+}\) channels (32). However, the physical mechanisms of inactivation in Ca\(^{2+}\) channels are unclear (45). S6 residues appear to significantly affect inactivation (46). This suggests rearrangements of the inner pore that cannot be modeled now.

**Ligand Binding in the Closed Channel**—Another potential cause of the state-dependent binding of BTZs may be the dif-
**Benzothiazepines in L-type Ca^{2+} Channel**

**TABLE 1**

Sequence alignment and BTZ-sensing residues

| Channel | Segment | Residue label prefix | Sequence |
|---------|---------|----------------------|----------|
| KcsA    | M1      |                      | IIII     |
| KvAP    | S5      | 1o                   | HVRAGAA  |
| Ca_{a,2} | IS5    | 2o                   | AMVFLILH |
|         | III5    | 3o                   | SLRGIASL |
|         | IVS5    | 4o                   | AIRTIGNI |
| KcsA    | P       | 1p                   | LJZYPRAL |
| KvAP    | P       | 2p                   | IKSVFDAL |
| Ca_{a,2} | IP     | 3p                   | FDNIPQS |
|         | IIIP    | 4p                   | FDNVLAA |
| KcsA    | M2      |                      | IIII     |
| KvAP    | S6      | 1i                   | HIIGVIAA |
| Ca_{a,2} | IS6    | 2i                   | PAVVIFLY |
|         | IIIS6   | 3i                   | FQTFPQAV |

To our knowledge, this study presents the first molecular model of BTZ binding in L-type Ca^{2+} channel. As discussed below, our model provides qualitative explanations of the SAR and mutagenesis data as well as experimental data on the mechanisms of BTZ action, including the access pathway, state-dependent binding, and potentiation of drug binding by Ca^{2+}.

**DISCUSSION**

To our knowledge, this study presents the first molecular model of BTZ binding in L-type Ca^{2+} channel. As discussed below, our model provides qualitative explanations of the SAR and mutagenesis data as well as experimental data on the mechanisms of BTZ action, including the access pathway, state-dependent binding, and potentiation of drug binding by Ca^{2+}.

**SAR of BTZs**—SAR studies (14–18) identified five components of the BTZ pharmacophore: (i) aromatic rings A and B whose spatial disposition is determined by the 7-membered ring, (ii) a tertiary amino group connected to the ring via a flexible alkyl chain, (iii) a para-methoxy group in the aromatic ring attached to the 7-membered ring, (iv) a carbonyl oxygen in position 2, and (v) a hydrophobic group in position 3 of the 7-membered ring. Our receptor model explains how modifications of these groups affect the ligand potency.

The para-methoxy group in ring B is optimal for the activity; other donors and acceptors of H-bond in this position are possible, but the hydrophobic ethyl group dramatically reduces the drug potency (17). This agrees with our model in which the hydroxy group of Y^{411} can donate or accept an H-bond. This interaction is particularly important, because Y^{411} is specific for the L-type Ca^{2+} channel, and its replacement decreases the potency of BTZs.

In our model, aromatic rings of the BTZ ligand interact with the channel aromatic residues Y^{411}, F^{3122}, and F^{2149}. Mutations of these residues are known to affect the potency of BTZs. Substitutions in position 3 of the 7-membered ring have a moderate effect on the BTZ potency, but predominantly hydrophobic groups are preferable (17, 18). This agrees with our model where this substituent faces A^{411} and T^{4118}, which are known as BTZ-sensing residues (1, 2).

The nucleophilic carbonyl group in position 2 should interact with a positively charged receptor site. In our model, the only electrophilic counterpart for this group is a Ca^{2+} ion in the selectivity filter. The ligand-Ca^{2+} coordination can explain the above SAR peculiarity as well as the potentiation of diltiazem binding by Ca^{2+}.

Replacements of the ammonium group by hydrophilic groups significantly decreased the BTZ potency, suggesting a nucleophilic site in the receptor (14). Two nucleophilic sites are...
BTZ Access to the Receptor Site—Some BTZ-sensing residues are localized in the inner pore, but it is well established that BTZs block the channel when applied externally rather than internally (9–11). This fact raises two related questions: (i) how do BTZs reach ligand-sensing residues in the inner pore, and (ii) why don’t internally applied BTZs block the channel despite seemingly non-restricted access through the open pore? In our BTZ-binding model, the BTZ rings are located in the domain interface, whereas the ammonium group protrudes to the inner pore. We suggest that BTZs reach the binding site between inner helices IIIS6s and IVS6 by moving along the IIIP-helix. We do not see steric restrictions for this access pathway for BTZs in our model. The nucleophilic focus of P-helices is an attractive region for a cationic group. The ammonium group of the intracellularly applied BTZ would be attracted to the nucleophilic region, whereas the BTZ rings would bind at the more cytoplasmic end of the inner pore. Such a transient state is possible with our model: a selectivity filter glutamate not bound to a Ca$^{2+}$ ion or the focus of P-helices. Potentiation of diltiazem binding by Ca$^{2+}$ (7) suggests that the ammonium group binds at the focus of P-helices in the presence of Ca$^{2+}$. This model is consistent with the x-ray structures of KcsA-TBA co-crystals (42, 48) and models of Na$^+$ channels with local anesthetics (32, 40).

FIGURE 5. Ca$^{2+}$-deficient model. A, energy profile of the ligand pulled through the domain III/IV interface along the P-helix of domain III. B and C, intracellular and side views at the complex in which the ammonium group of SQ32910 interacts with $E^{3050}$, while the ligand rings are in the domain interface. Side chains of $E^{1950}$ and $E^{4950}$ chelate a Ca$^{2+}$ ion, while side chain of $E^{4950}$, which carries a non-compensated negative charge, is turned away.

FIGURE 6. Binding of SQ32910 to the closed channel model. Significant rearrangement of S6 residues in the closed versus open state results in the loss of some specific contacts and decrease of ligand-receptor interaction energy.
In our model, the access of BTZ through the calcium channel with experimental data. This sidewalk contains BTZ-sensing residues $I_{314}$ and $I_{418}$ that do not make contacts with the ligand in the proposed binding mode (Fig. 1, C and D). Besides the III/IV domain interface, the outer pore could be another access pathway for extracellular BTZ binding, but we are not aware of experimental data that support this hypothesis. BTZs are much wider than the selectivity filter dimensions estimated from the size of the largest permeant organic cation (49). This leaves a sidewalk lined by helices III$S5$, III$S6$, IV$S6$, and III$P$ (34, 39, 40) as a major route of BTZs to their binding site. This sidewalk contains BTZ-sensing residues $I_{3114}$ and $I_{418}$ that do not make contacts with the ligand in the proposed binding mode but contribute to the ligand-channel energy along the calculated pathway (Fig. 2, B and C). Interestingly, mutations of $I_{418}$ affect the channel unblock at the resting state and use-dependent blockade, suggesting that this residue affects access of BTZs to their receptor (12). Thus, sidewalk access is consistent with experimental data.

Kinetics of BTZ binding is temperature-dependent (50, 51). In our model, the access of BTZ through the calcium channel $\alpha_1$ subunit is not restricted. However to approach the sidewalk entrance from the extracellular space, the cationic ligand should first interact with lipids and possibly with an accessory $\beta$ subunit of $Ca^{2+}$ channels. These interactions may govern the temperature dependence of BTZ kinetics.

**Other Ligands**—A critical feature of our BTZ-binding model is that the bulky BTZ rings bind in the domain interface, whereas only the ammonium group protrudes into the inner pore and binds at the focus of the P-helices, a region where cationic groups of other inner-pore ligands of P-loop channels bind (40–42, 48). Balwierczak et al. (52) suggested that bepridil and verapamil regulate $[^3H]d$-cis-diltiazem binding in a negative allosteric fashion through their own distinct binding sites. Flexible ligands such as verapamil may bind in alternative modes and avoid direct competition with the rather small part of the diltiazem molecule, which is exposed to the pore from the domain interface. Such a scenario is less likely for the more bulky and rigid blocker, tetrandrine, which competes with diltiazem (53). Compounds with bulky extensions at the ammonium group occupy most of the inner pore (Fig. 7). Not surprising, such drugs compete directly with the inner-pore ligands (47). The results of our study are consistent with the “domain interface” hypothesis (see Ref. 1), which explains why BTZs and inner-pore blockers like verapamil share some ligand-sensing residues even though they appear to bind to separate regions and have different access pathways to the binding site.

Biochemical experiments demonstrate that dihydropyridine antagonists stimulate diltiazem binding suggesting that these different ligands can bind to the channel simultaneously (50, 51). Published models suggest different locations of dihydropyridines in the channel (24, 55, 56). Further studies are necessary to elaborate a model of simultaneous binding of dihydropyridine and BTZ ligands in the L-type $Ca^{2+}$ channel.

**Potentiation of the BTZ Block by $Ca^{2+}$**—An important prediction of our study is that the carbonyl oxygen in position 2 of the seven-membered ring can directly interact with a $Ca^{2+}$ ion bound between the selectivity filter glutamates E3p50 and E4p50. This model fits experimental data that E3p50 and E4p50 influence the $Ca^{2+}$ potentiation of diltiazem block (7). Lipkind and Fozzard (35) suggest a model in which all four selectivity filter glutamates simultaneously bind up to three $Ca^{2+}$ ions, which align single-file along the pore axis. This model does not explain asymmetric effects of substitutions in the EEEE ring of the $Ca^{2+}$ selectivity filter. The detailed mechanism of $Ca^{2+}$ permeation remains unknown, but it may involve several $Ca^{2+}$-chelating patterns. In K$^+$ channels, permeation through the selectivity filter region involves alternating binding of two $K^+$ ions to sites 1/3 or 2/4 (57). Additionally, some rearrangements in the selectivity filter of K$^+$ channels are coupled with changes of $K^+$ coordination patterns. These rearrangements are proposed for the slow inactivated state (58). Our model represents just one $Ca^{2+}$-chelation pattern, which may correspond to the high affinity binding mode of BTZs.

A previous study suggested that the ammonium group of $Ca^{2+}$ channel blockers directly interacts with the selectivity filter glutamates, whereas potentiation of the block by $Ca^{2+}$ ions has an allosteric nature (55). Our model provides an alternative explanation that involves direct ligand-$Ca^{2+}$ interaction and the ligand ammonium group attracted to the focus of the P-helices. It may seem counterintuitive that diltiazem binds to the channel more tightly when the current-carrying cation is $Na^+$.
rather than divalent cations (19, 47). However, the chelation pattern of Na\(^+\) differs from that of divalent cations. Binding of a single Na\(^+\) ion to two selectivity filter glutamates is unlikely, and thus a glutamate not bound to the Na\(^+\) ion would directly interact with the ammonium group of BTZ.

**Model Limitations**—The proposed BTZ binding mode was selected from other possibilities, because it agrees with a large set of experimental data. Two major limitations of our modeling approach should be noted.

First, we used K\(^+\) channel templates. Despite numerous experiments that imply generally similar architecture of the inner-pore region in K\(^+\) and Ca\(^{2+}\) channels, some structural details are expected to be different. Indeed, there are significant variations of the pore geometry even within the family of K\(^+\) channels. Experimental distance constraints between Ca\(^{2+}\) ions and residues in the inner helices (60) have been used to predict the pore geometry of the Shaker channel (61), but we are not aware of analogous constraints for Ca\(^{2+}\) channels. Without distance constraints, energy calculations cannot predict deviations of the inner pore geometry of the Ca\(^{2+}\) channel from the K\(^+\) channel templates.

The second limitation is energy sampling. Time-consuming free energy calculations are a cutting-edge methodology to explore structures for which high resolution experimental data are available. In the absence of such data, molecular systems can be explored using coarse-grained calculations (see Ref. 62), which have become increasingly popular (54, 59). We have employed the rather simple force field with implicit solvent and Coulomb electrostatics. It was necessary to address the minimima problem by sampling a large number of conformations to predict the BTZ access pathway and binding modes. Taking into account the limited precision of energy calculations with homology models, we considered ensembles of possible structures and did not attempt to discriminate individual structures by energy. We also did not attempt to reproduce the SAR data quantitatively. Our modeling approach filters out structures with overlapping atoms and predicts specific interactions such as electrostatic and H-bond coupling, hydrophobic interactions, and steric complementarity. Despite potential limitations considered above, our model is consistent with experimental data and supports the following conclusions.

**CONCLUSIONS**

We conclude the following. 1) The BTZ rings bind between helices IIIP, IIIS6, and IVS6 of L-type Ca\(^{2+}\) channel, whereas the flexible alkylationammonium chain protrudes in the pore from the domain interface. 2) The position of the BTZ ammonium group depends on the net charge in the selectivity filter. When two Ca\(^{2+}\) ions bind to four glutamates, the ammonium group binds in the central cavity at the focus of P-helices. When the selectivity filter has an excessive negative charge, the ligand ammonium group binds to a glutamate in the selectivity filter. 3) BTZs have unrestricted access to their binding site via the III/IV domain interface. 4) Benziazem and other ligands with large substituents at the ammonium group bind in the same mode as smaller BTZs. 5) BTZ binding in the closed channel is destabilized by the loss of some specific contacts with residues at the C-terminal halves of inner helices. 6) Permeant ions affect BTZ action via direct interaction with the ligand.

**REFERENCES**

1. Hockerman, G. H., Peterson, B. Z., Johnson, B. D., and Catterall, W. A. (1997) *Annu. Rev. Pharmacol. Toxicol.* 37, 361–396
2. Lacinova, L. (2005) *Gen. Physiol. Biophys.* 1, 1–78
3. Lee, K. S., and Tsien, R. W. (1983) *Nature* 302, 790–794
4. Uehara, A., and Hume, J. R. (1985) *J. Gen. Physiol.* 85, 621–647
5. Herzig, S., Lullmann, H., and Sieg, H. (1992) *Pharmacol. Toxicol.* 71, 229–235
6. Hille, B. (1977) *J. Gen. Physiol.* 69, 497–515
7. Dilmac, N., Hillard, N., and Hockerman, J. H. (2003) *Mol. Pharmacol.* 64, 491–501
8. Almers, W., and McCleskey, E. W. (1984) *J. Physiol.* 353, 585–608
9. Seydl, K., Kimball, D., Schindler, H., and Romanin, C. (1993) *Pflugers Arch.* 424, 552–554
10. Kurokawa, J., Adachi-Akahane, S., and Nagao, T. (1997) *Mol. Pharmacol.* 51, 262–268
11. Hering, S., Savchenko, A., Strubing, C., Lakitsch, M., and Striessnig, J. (1993) *Mol. Pharmacol.* 43, 820–826
12. Berjukow, S., Gapp, F., Aczel, S., Sinnegger, M. J., Mitterdorfer, J., Glossmann, H., and Hering, S. (1999) *J. Biol. Chem.* 274, 6154–6160
13. Kraus, L. R., Hering, S., Grabner, M., Ostler, D., and Striessnig, J. (1998) *J. Biol. Chem.* 273, 27205–27212
14. Floyd, D. M., Kimball, S. D., Krapcho, J., Das, J., and Turk, C. F. (1992) *J. Med. Chem.* 35, 756–772
15. Das, J., Floyd, D. M., Kimball, S. D., Duff, K. J., Lago, M. W., Krapcho, J., White, R. E., Ridgwell, R. E., Obermeier, M. T., Moreland, S., McMullen, D., Normandin, D., Hedberg, S. A., and Schaeffer, T. R. (1992) *J. Med. Chem.* 35, 2610–2617
16. Das, J., Floyd, D. M., Kimball, S. D., Duff, K. J., Yu, T. C., Lago, M. W., Moquin, R. V., Lee, V. G., Gougoutas, J. Z., Malley, M. F., Moreland, S., Brittain, R. J., Hedberg, S. A., and Cucinotta, G. G. (1992) *J. Med. Chem.* 35, 773–780
17. Kimball, S. D., Floyd, D. M., Das, J., Hunt, J. T., Krapcho, J., Rovnyak, G., Duff, K. J., Lee, V. G., Moquin, R. V., Turk, C. F., Hedberg, S. A., Moreland, S., Brittain, R. J., McMullen, D. M., Normandin, D. E., and Cucinotta, G. G. (1992) *J. Med. Chem.* 35, 780–793
18. Kimball, S. D., Hunt, J. T., Barrish, J. C., Das, J., Floyd, D. M., Lago, M. W., Lee, V. G., Spergel, S. H., Moreland, S., Hedberg, S. A., and Gougoutas, J. Z., Malley, M. F., and Laud, W. F. (1993) *Bioorg. Med. Chem.* 1, 285–307
19. Galizzi, J. P., Fossett, M., and Lazdunski, M. (1985) *Biochem. Biophys. Res. Commun.* 132, 49–55
20. Jiang, Y., Lee, A., Chen, J., Ruta, V., Cadene, M., Chait, B. T., and MacKinnon, R. (2003) *Nature* 423, 33–41
21. Doyle, D. A., Morais Cabral, J., Pluetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) *Science* 280, 69–77
22. Dudley, S. C., Jr., Chang, N., Hall, J., Lipkind, G., Fozzard, H. A., and French, R. J. (2000) *J. Gen. Physiol.* 116, 679–690
23. Zhorov, B. S., and Tikhonov, D. B. (2004) *J. Neurochem.* 88, 782–799
24. Zhorov, B. S., Folkman, E. V., and Ananthnarayanan, V. S. (2001) *Arch. Biochem. Biophys.* 393, 224–241
25. Huber, I., Wappel, E., Herzog, A., Mitterdorfer, J., Glossmann, H., Langer, T., and Striessnig, J. (2000) *Biochem. J.* 347, 829–836
26. Lazaridis, T., and Karplus, M. (1999) *Proteins* 35, 133–152
27. Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Chio, C., Alagona, G., Profeta, S., and Weiner, P. K. (1998) *J. Am. Chem. Soc.* 106, 756–784
28. Weiner, S. J., Kollman, P. A., Nguyen, D. T., and Case, D. A. (1986) *J. Comput. Chem.* 7, 230–252
Benzothiazepines in L-type Ca\(^{2+}\) Channel

29. Bruhova, I., and Zhorov, B. S. (2007) \textit{BMC Struct. Biol.} 7, 1–13
30. Zhorov, B. S. (1981) \textit{J. Struct. Chem.} 22, 4–8
31. Li, Z., and Scheraga, H. A. (1987) \textit{Proc. Natl. Acad. Sci. U. S. A.} 84, 6611–6615
32. Tikhonov, D. B., and Zhorov, B. S. (2007) \textit{Biophys. J.} 93, 1557–1570
33. Heinemann, S. H., Terlau, H., Stuhmer, W., Imoto, K., and Numa, S. (1992) \textit{Nature} 356, 441–443
34. Tikhonov, D. B., and Zhorov, B. S. (2005) \textit{Biophys. J.} 88, 184–197
35. Lipkind, G. M., and Fozzard, H. A. (2001) \textit{Biochemistry} 40, 6786–6794
36. Cheng, R. C., and Zhorov, B. S. (2007) \textit{Biophys. J.} 93, 1557–1570
37. Dreker, T., and Grissmer, S. (2005) \textit{Mol. Pharmacol.} 68, 966–973
38. Madeja, M., Leicher, T., Friederich, P., Punke, M. A., Haverkamp, W., Musshoff, U., Breithardt, G., and Speckmann, E. J. (2003) \textit{Mol. Pharmacol.} 63, 547–556
39. Yamaguchi, S., Zhorov, B. S., Yoshioka, K., Nagao, T., Ichijo, H., and Adachi-Akahane, S. (2003) \textit{Mol. Pharmacol.} 64, 235–248
40. Tikhonov, D. B., Bruhova, I., and Zhorov, B. S. (2006) \textit{FEBS Lett.} 580, 6027–6032
41. Rossokhin, A., Teodorescu, G., Grissmer, S., and Zhorov, B. S. (2006) \textit{Mol. Pharmacol.} 69, 1356–1365
42. Zhou, M., Morais-Cabral, J. H., Mann, S., and MacKinnon, R. (2001) \textit{Nature} 411, 657–661
43. Yale, H. L. (1959) \textit{J. Med. Pharm. Chem.} 1, 121–133
44. Dilmac, N., Hilliard, N., and Hockerman, G. H. (2004) \textit{Mol. Pharmacol.} 66, 1236–1247
45. Findlay, I. (2004) \textit{J. Physiol.} 554, 275–283
46. Shi, C., and Soldatov, N. M. (2002) \textit{J. Biol. Chem.} 277, 6813–6821
47. Nagiwa, M., Adachi-Akahane, S., and Nagao, T. (2003) \textit{Eur. J. Pharmacol.} 466, 63–71
48. Lenaeus, M., Vamvouka, M., Focia, P. J., and Gross, A. (2005) \textit{Nat. Struct. Biol.} 12, 454–459
49. McCleskey, E. W., and Almers, W. (1985) \textit{Proc. Natl. Acad. Sci. U. S. A.} 82, 7149–7153
50. Glossmann, H., Linn, T., Rombusch, M., and Ferry, D. R. (1983) \textit{FEBS Lett.} 160, 226–232
51. Garcia, M. L., King, V. F., Siegl, P. K., Reuben, J. P., and Kaczorowski, G. J. (1986) \textit{J. Biol. Chem.} 261, 8146–8157
52. Balwierzczak, J. L., Johnson, C. L., and Schwartz, A. (1987) \textit{Mol. Pharmacol.} 31, 175–179
53. King, V. F., Garcia, M. L., Himmel, D., Reuben, J. P., Lam, Y. K., Pan, J. X., Han, G. Q., and Kaczorowski, G. J. (1988) \textit{J. Biol. Chem.} 263, 2238–2244
54. Basdevant, N., Borgis, D., and Ha-Duong, T. (2007) \textit{J. Phys. Chem.} 111, 9990–9999
55. Lipkind, G. M., and Fozzard, H. A. (2003) \textit{Mol. Pharmacol.} 63, 499–511
56. Cosconati, S., Marinelli, L., Lavecchia, A., and Novellino, E. (2007) \textit{J. Med. Chem.} 50, 1504–1513
57. Zhou, M., and MacKinnon, R. (2004) \textit{J. Mol. Biol.} 338, 839–846
58. Berneche, S., and Roux, B. (2005) \textit{Structure} 13, 591–600
59. Periole, X., Huber, T., Marrink, S. J., and Sakmar, T. P. (2007) \textit{J. Am. Chem. Soc.} 129, 10126–10132
60. Webster, S. M., Del Camino, D., Dekker, J. P., and Yellen, G. (2004) \textit{Nature} 428, 864–868
61. Bruhova, I., and Zhorov, B. S. (2005) \textit{Biophys. J.} 89, 1020–1029
62. Saiz, L., and Klein, M. L. (2002) \textit{Acc. Chem. Res.} 35, 482–489