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Modeling of benzocaine analog interactions with the D4S6 segment of $Na_V4.1$ voltage-gated sodium channels

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Abstract

Local anesthetics (LAs) are compounds that inhibit the propagation of action potentials in excitable tissues by blocking voltage-gated Na^+ channels. Mutagenesis studies have demonstrated that several amino acid residues are important sites of LA interaction with the channel, but these studies provide little information regarding the molecular forces that govern drug-binding interactions, including the binding orientation of drugs. We used computational methods to construct a simple model of benzocaine analog binding with the D4S6 segment of rat skeletal muscle ($Na_V4.1$) sodium channels. The model revealed that four hydrophobic residues form a binding cavity for neutral LAs, and docking studies indicated that increasing hydrophobicity among the benzocaine analogs allowed a better fit within the binding cavity. The similarities between our simple model and published experimental data suggested that modeling of LA interactions with sodium channels, along with experimental approaches, could further enhance our understanding of LA interactions with sodium channels.

Keywords: Sodium channel; Local anesthetic receptor; Benzocaine; Computer model

1. Introduction

Voltage-gated Na^+ channels are membrane proteins that are responsible for producing action potentials in excitable tissues. The α-subunit of Na^+ channels consists of four homologous domains (D1–D4), with each domain containing six transmembrane segments that have an α-helical secondary structure [1,2]. The arrangement of the four domains in the membrane forms a selective pore for Na^+ conductance. Specific regions in the sodium channel α-subunit are targets for many toxins and drugs that influence sodium channel function (reviewed by Ref. [3]). For example, local anesthetics (LAs) inhibit the propagation of action potentials in excitable tissues by blocking voltage-gated Na^+ channels [4]. Previous studies have demonstrated that a critical LA binding region on sodium channels is on the sixth segment of domain 4 (D4S6) [5–7]. Ragsdale et al. [6] first showed that point mutations of specific residues to alanine in D4S6 of a rat brain Na^+ channel ($Na_V1.2a$) strongly affected LA block of Na^+ currents. Wang et al. [5] published similar results for comparable point mutations of rat skeletal muscle Na^+ channels ($Na_V4.1$ [8]), and demonstrated that etidocaine, a tertiary amine LA, and benzocaine bind to a common region within D4S6 that lies between residues F1579 and Y1586 (Fig. 1). Most clinically important LAs, including lidocaine and bupivacaine, have a tertiary amine moiety that is protonated at physiological pH (7.2–7.4). Benzocaine is also of clinical importance but is the only LA that is neutral at physiological pH. In contrast to tertiary amine LAs, which block resting and inactivated channels [9–11], benzocaine binds primarily with resting sodium channels to elicit tonic block [12].

Despite the partial localization of the LA binding region on sodium channels, our understanding of the molecular interactions between LAs and the residues within D4S6 is incomplete. Computational methodologies have become

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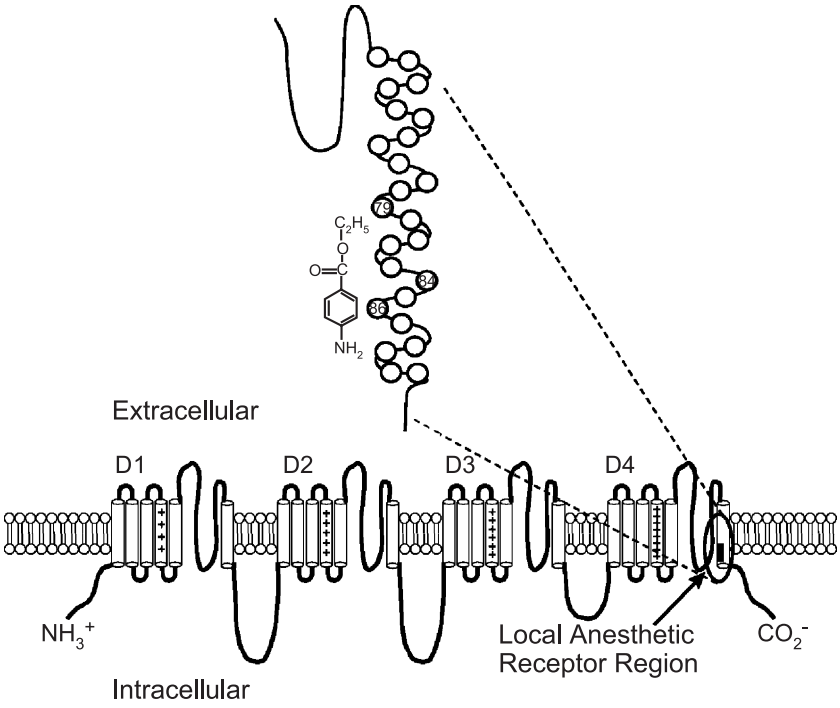

Fig. 1. Structural model of a voltage-gated sodium channel as described by Ragsdale et al. [6]. The four domain regions (D1–D4) are stretched out in the membrane and the enlarged region above depicts the LA receptor region on D4S6 with an associated benzocaine molecule. The residue numbers correspond to F1579, N1584, and Y1586.

extremely valuable for the study of ligand–receptor interactions [13,14], particularly with respect to studying the docking of ligands with their ion channel targets (reviewed by Ref. [15]). For example, computational models have been used to explain experimentally obtained results on ligand interactions with nicotinic acetylcholine receptors [16], glutamate receptors [17], and voltage-gated K^+ channels [18].

In the present study, we used computational methods to examine the molecular properties of benzocaine and three benzocaine analogs (ethyl 4-hydroxybenzoate, ethyl 4-diethylaminobenzoate, and ethyl 4-ethoxybenzoate) and to investigate the relationships between drug chemistry and published experimental estimates of apparent drug affinity for resting sodium channels. We also constructed a model of the D4S6 region of $Na_V4.1$ channels to study the interactions between benzocaine derivatives and the LA binding region. The model of the LA binding region revealed a hydrophobic cavity within which neutral LAs may bind. Furthermore, our model was consistent with experimental observations of sodium channel block by benzocaine analogs [19] and indicated that drug hydrophobicity and size influence the fit of benzocaine derivatives within the hydrophobic cavity in the LA binding region. Computational studies also suggested that introduction of a lysine residue at Y1586 altered the secondary structure of the α helix and changed the binding orientation of benzocaine with the receptor region. These data perhaps explain the published observation that benzocaine affinity for resting $Na_V4.1$ channels increased following lysine point mutation at the same site [7]. Together, these data suggested that computational methods may be useful for elucidating the complex nature of LA interaction with sodium channels and for providing molecular details that are currently inaccessible via experimental methods.

2. Experimental

2.1. Hydrophobicity, volume and surface area of benzocaine and benzocaine analogs

These calculations were performed with the HyperChem 6 molecular modeling program (Hypercube, Gainesville, FL). The volume and surface area grid methods used are those described by Bodor et al. [20], using the atomic radii of Gavezotti [21]. Calculations of log *P* (hydrophobicity) were conducted using atomic parameters derived by Ghose et al. [22].

2.2. Structure of D4S6 helix

The amino acid sequence of the D4S6 helix of $Na_V4.1$ channels (formerly μ1, [8]) was built in the molecular modeling program Molecular Operating Environment (MOE, Chemical Computing Group, Montreal, Canada). The C-terminal end was capped with an amido group while the N-terminal end was capped with an acetyl group. The peptide underwent a geometry optimization protocol ending with the Newton–Raphson algorithm (1000 iterations; RMS

gradient of 0.001 kcal/mol Å). The AMBER94 force field running in the MOE was used in these and all other subsequent calculations [23]. AMBER94 has been parameterized for protein systems and worked well in these studies.

The optimized D4S6 peptide was subjected to quenched molecular dynamics (QMD) to explore the conformational space available to the peptide. QMD techniques have been shown to be useful in the conformational analysis of flexible molecules, such as peptide-based structures [24–27]. Molecular dynamics calculations were carried out using the Verlet LeapFrog Integrator using a time step of 1 fs and an equilibrium time of 0.1 ps under canonical ensemble (NVT) conditions. The total time of the simulation was 1 ns (total of 10^6 iterations) at 500 K with a snapshot of the conformation taken every 1000 iterations. This generated 1000 conformations that were subjected to geometry optimization using the conjugate gradients algorithm to a RMS gradient of 0.001 kcal/mol Å. Of the 1000 structures generated from the QMD analysis, all were α-helical in nature and one particular conformer was 3 kcal/mol more stable than the remaining conformers. A RMSD value for each of the other conformers was calculated with respect to the lowest energy conformer. The average RMSD value for these conformers was 2.72±0.78 Å. Based on these results, the lowest energy conformer of the D4S6 helix was used in subsequent studies to examine interaction between the helix and LAs. The same protocol was used to determine the structure of the D4S6 helix in the Y1586K mutant to compare binding of benzocaine at the mutant LA receptor with binding at the wild-type receptor. In this calculation, the lowest energy conformer was at least 3.5 kcal/mol more stable than the other conformers and the average RMSD value was 2.54±0.84 Å.

2.3. Docking studies of benzocaine and benzocaine analogs

The docking of benzocaine and benzocaine analogs was performed using the docking interface in MOE. Partial charges on the peptide atoms of the D4S6 helix were calculated using AMBER94 and those on the benzocaine and benzocaine analogs were generated in HyperChem using the semi-empirical AM1 method. All docking studies were performed on a Silicon Graphics Octane Workstation (Silicon Graphics, Mountain View, CA) using the AMBER94 force field. The docking regime allowed the LA molecules to explore the space near the D4S6 region critical for LA binding (docking box of 206.5 $Å^3$) with full ligand flexibility. A total of 300 LA-D4S6 complexes were derived from the docking calculations for each LA molecule and placed into structural families. Each drug had a predominant family of related structures that accounted for 75–85% of the 300 complexes. The RMSD values within the predominant families ranged from 0.23 to 0.45 Å. The remaining structures outside these families had energies at least 2.5 kcal/mol higher than the structures in the predominant families. Therefore, a representative structure from each predominant family was chosen for analyses.

3. Results

We first examined drug volume, surface area, and hydrophobicity (log *P*) of benzocaine (ethyl 4-aminobenzoate) and the benzocaine analogs ethyl 4-hydroxybenzoate (OH-benzocaine), ethyl 4-ethoxybenzoate (ETO-benzocaine), and ethyl 4-diethylaminobenzoate (NDE-benzocaine) (Fig. 2). A previous study [19] showed that benzocaine and OH-benzocaine elicited little or no use-dependent block of $Na_V4.1$ channels, whereas the addition of alkyl groups to benzocaine (ETO- and NDE-benzocaine) markedly increased use-dependent block. Use-dependent block by NDE-benzocaine and ETO-benzocaine was attributed to increases in drug hydrophobicity and the authors predicted that the more alkylated LAs dissociated more slowly from the channels during the interval between pulses. Although the main objective of the Quan et al. study [19] was to investigate use-dependent block by benzocaine analogs, their data also suggested that the more alkylated benzocaine derivatives had a higher affinity for resting channels than did the less hydrophobic analogs.

We measured the surface area, volume, and hydrophobicity of benzocaine and the three analogs (Table 1) to

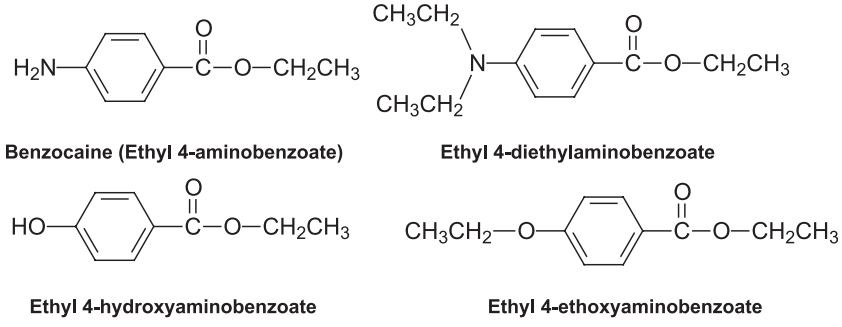

Fig. 2. Structures of benzocaine and benzocaine analogs (ethyl 4-diethylaminobenzoate, ethyl 4-hydroxybenzoate, and ethyl 4-ethoxybenzoate) used in the computational experiments.

Table 1
Parameters associated with the size and hydrophobicity of benzocaine and benzocaine analogs

Molecule	Surface area (Å^2)	Volume (Å^3)	Log P	K_d (mM)[a]
Benzocaine	195.47	157.00	1.34	1.70
Ethyl 4-hydroxyaminobenzoate	190.29	152.68	1.83	1.19
Ethyl 4-ethoxyaminobenzoate	230.58	187.78	2.21	0.463
Ethyl 4-diethylaminobenzoate	269.76	225.92	3.07	0.126

[a] The K_d values were estimated from measures of tonic block of $Na_V4.1$ channels [19].

determine whether the predictions made regarding drug size and hydrophobicity were consistent with experimental measures of drug affinity for resting channels. In general, the addition of alkyl groups increased measures of drug size (surface area and volume) and drug hydrophobicity. The order of increasing hydrophobicity (benzocaine>OH-benzocaine>ETO-benzocaine>NDE-benzocaine) determined in the present study was consistent with the increases in tonic block of $Na_V4.1$ channels by these compounds [19]. Our computational measures of the benzocaine analogs thus supported the suggestion that the addition of alkyl groups increases benzocaine hydrophobicity and could explain the respective increases in affinity of the more alkylated analogs for resting $Na_V4.1$ channels.

3.1. Modeling benzocaine analog interactions with D4S6 of $Na_V4.1$ sodium channels

We constructed a model of the D4S6 region of $Na_V4.1$ channels (Fig. 3) to investigate the docking interactions between the benzocaine analogs and the LA receptor region. As first suggested by Ragsdale et al. [6] for rat brain sodium channels ($Na_V1.2a$), the model placed homologous $Na_V4.1$ residues at positions F1579 and Y1586 (Fig. 3, shown in blue) on the same side of D4S6 and a presumed orientation toward the channel pore. Fig. 3 also shows that four other residues (V1582, M1585, I1581 and I1589, shown in green) are in a region near Y1586 and F1579 and form a hydrophobic cavity along the surface of the D4S6 helix (Fig. 4).

The docking studies indicated that benzocaine and the benzocaine analogs bind in the hydrophobic cavity of the D4S6 helix with the ethyl ester moieties pointing away from Y1586 (Fig. 4). Ethyl 4-hydroxybenzoate (OH-benzocaine), which is most closely related to the size and hydrophobicity of benzocaine (Table 1), binds within the hydrophobic cavity in a manner similar to benzocaine. Although benzocaine is slightly larger than OH-benzocaine, OH-benzocaine is more hydrophobic, suggesting that OH-benzocaine may have more favorable interactions within the hydrophobic cavity on the helix. An increase in hydrophobic interaction between OH-benzocaine and the binding cavity would increase the affinity of OH-benzocaine as compared with benzocaine as described above.

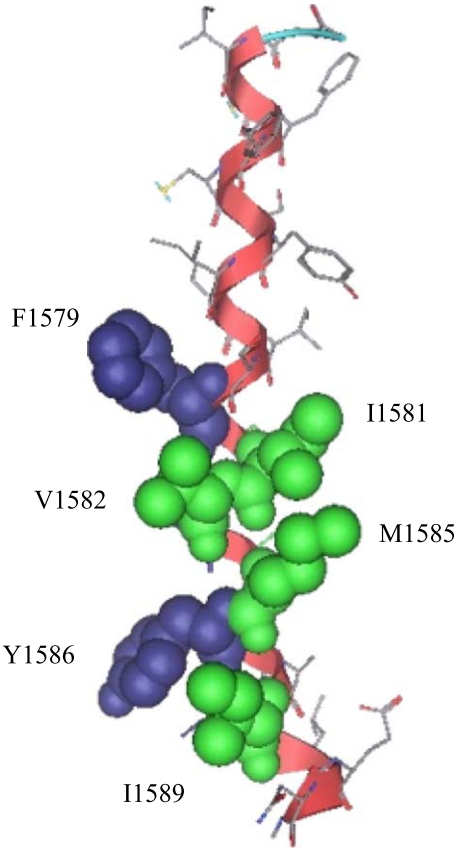

Fig. 3. A model of the D4S6 helix in voltage-gated sodium channels derived from quenched molecular dynamics.

Ethyl 4-ethoxybenzoate (ETO-benzocaine) is more alkylated than either benzocaine or OH-benzocaine. ETO-benzocaine has an ether functional group on the fourth carbon of the aromatic ring, whereas benzocaine has an amino group and OH-benzocaine has a hydroxyl group. The increase in alkylation increases the hydrophobicity of ETO-

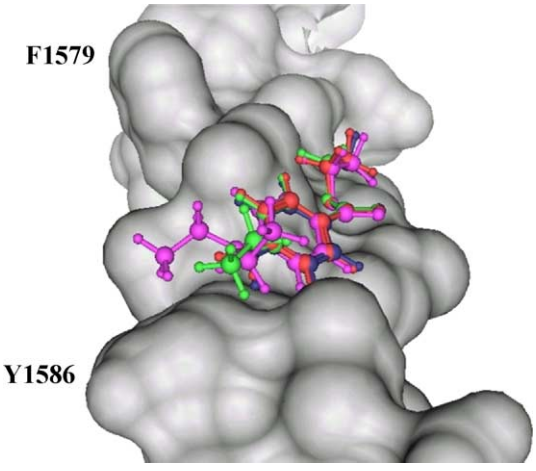

Fig. 4. Docked structures of benzocaine (red), ethyl 4-hydroxybenzoate (blue), ethyl 4-ethoxybenzoate (green), and ethyl 4-diethylaminobenzoate (magenta) with the D4S6 helix (gray surface). The F1579 and Y1586 residues are labeled for orientation purposes. All four drugs bind within the hydrophobic cavity with the same orientation.

benzocaine as compared with benzocaine or OH-benzocaine (Table 1). The docking calculations with ETO-benzocaine and the D4S6 helix suggested that this molecule binds to the receptor region of D4S6 in a comparable manner to benzocaine and OH-benzocaine, but the increase in alkylation permits a larger number of hydrophobic interactions and enhances the fit within the hydrophobic cavity between Y1586 and I1589. Ethyl 4-diethylaminobenzoate (NDE-benzocaine) is the most alkylated molecule of the three benzocaine analogs and subsequently has the largest volume and is the most hydrophobic (Table 1). NDE-benzocaine binds to the D4S6 in a similar manner to that of the other analogs, but the larger size of NDE-benzocaine improves the fit within the binding cavity of the helix and allows the drug to occupy available space between Y1586 and I1589 and between Y1586 and V1582.

3.2. Point mutation in D4S6 alters the helical structure of D4S6 and benzocaine docking

An experimental study [7] suggested that residue Y1586 is critical in tonic block by benzocaine in that a lysine point mutation at this site increased the resting affinity for benzocaine by almost twofold (wild-type K_d, ~1.5 mM; Y1586K K_d, ~0.8 mM). We substituted a lysine residue at position Y1586 of our D4S6 model to determine whether or not the mutation markedly altered benzocaine interaction with the receptor region. Interestingly, our model indicated that substitution of lysine for tyrosine at position 1586 disrupts the α-helical structure of the binding region by partially unwinding the helix at the asparagine residue located at position 1584, suggesting that the mutation partially destabilizes the helix in this region (Fig. 5, panel A). The substitution of lysine for tyrosine at position 1586 could permit the formation of hydrogen-bonding interactions between the lysine side chain and a backbone carbonyl group of V1582 (Fig. 5, Panel B). Although benzocaine does bind within a hydrophobic cavity on the mutant helix (Fig. 6), the orientation of the drug is rotated substantially as compared with the binding of benzocaine with the wild-type helix (Fig. 4). This altered binding orientation may be attributable to the new structural landscape of the mutant helix and the formation of a cation–π interaction between the side chain of K1586 and the benzenoid ring of benzocaine (Figs. 5 and 6). The fact that cation–π interactions are more energetically significant than dispersive-type interactions [28] was consistent with the observed increase in benzocaine affinity of $Na_V4.1$ Y1586K channels [7].

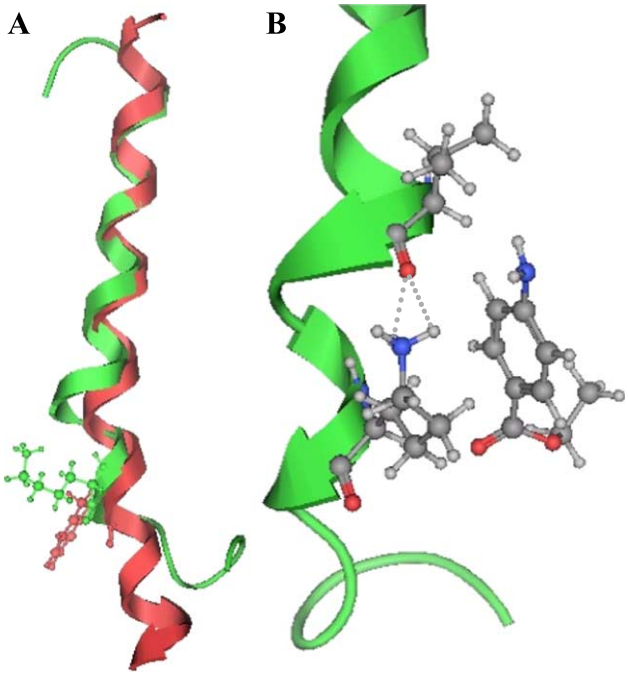

Fig. 5. Panel A: Models of the wild-type (red) and Y1586K mutant (green) D4S6 helices derived from quenched molecular dynamics. The residues at position 1586 are shown in ball-and-stick. Panel B: The Y1586K mutant D4S6 helix with the docked structure of benzocaine. Dashed lines represent H-bonds.

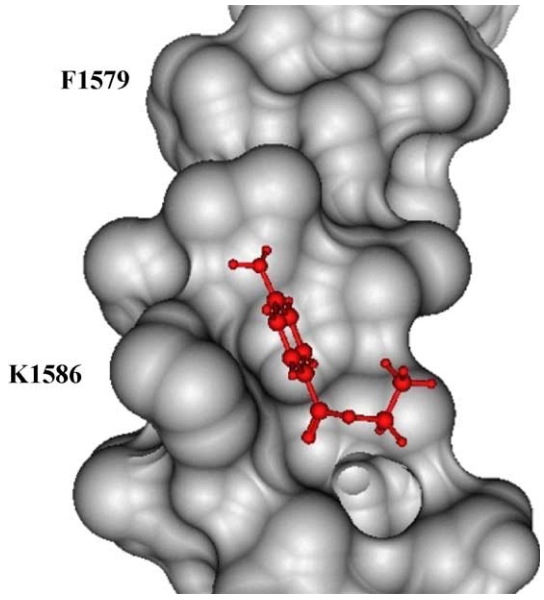

Fig. 6. Docked structure of benzocaine (red) with the Y1586K mutant D4S6 helix. The F1579 and Y1586K residues are labeled for orientation purposes. Note the altered binding orientation of benzocaine with the mutant helix as compared with the wild-type helix in Fig. 4.

4. Discussion

Recent computational investigations that have employed a ligand docking regime have focused on quantum mechanical calculations of binding free energies between ligands and receptors [17,29], and also on the qualitative aspects of ligand–receptor interactions such as ligand binding orientations and estimations of ligand fit within the receptor [16,30]. Obviously, computational investigations of G-protein-coupled receptors [31,32] that use the crystal structure of rhodopsin [33], and studies of ion

channel blockade [15,18] that use the crystal structure of a voltage-gated K^+ channel [34] are promising avenues for advancing our understanding of ligand–receptor interactions. Nevertheless, qualitative computational methods are useful tools for guiding studies of complex ligand–receptor interactions, particularly in cases where there is no crystal structure of the target receptor to accompany a large body of experimental data.

Block of sodium channels by tertiary amine LAs is a complex phenomenon that depends on several factors including pH [35], and the profound changes in channel affinity for LA that accompany the voltage-dependent changes in channel kinetic state [12]. Because of these inherent complexities, we devised a simplistic approach to test the usefulness of computational techniques in investigations of LA–sodium channel interaction. We selected benzocaine as the ligand because it is a relatively small LA that has no net charge, binds to resting sodium channels through hydrophobic interactions, and has a small series of related analogs that block $Na_V4.1$ channels [19]. Because mutagenesis studies have demonstrated that sodium channel D4S6 is a critical target for LAs in general [6] and for benzocaine in particular [5,7], we created a computational model of $Na_V4.1$ channel D4S6 to study the binding interactions between benzocaine analogs and the LA receptor region.

Our docking studies suggested that benzocaine and benzocaine analogs bind preferentially to D4S6 within a hydrophobic cavity located between residues F1579 and Y1586. Increases in hydrophobicity among the benzocaine analogs enhanced the fit of the drugs within the binding cavity on D4S6, suggesting that the more alkylated drugs could participate in more hydrophobic interactions with the binding region. These data generally agreed with the experimental observation that the more hydrophobic benzocaine analogs blocked a greater fraction of resting $Na_V4.1$ channels and increased use-dependent block [19]. Interestingly, lysine substitution at residue Y1586 altered the α-helical structure of D4S6 by partially unwinding the segment. Although benzocaine still fit well within a binding cavity, the altered docking orientation of benzocaine could increase the likelihood that benzocaine and Y1586K interact through a cation–π facial bond which is consistent with the ~2-fold increase in benzocaine affinity of $Na_V4.1$ Y1586K channels [7].

In the present study, we developed a model of benzocaine analog interactions with D4S6 of $Na_V4.1$ channels to compare computationally derived binding interactions with experimentally determined measures of benzocaine block. In these initial studies, we chose to keep the model as simple as possible to determine whether or not the computational interactions were at least qualitatively comparable with experimental results. First, we assumed that the lowest energy conformation of the drugs and the D4S6 helix would reveal information regarding the interactions between neutral LAs and resting sodium channels. Second, we included only the D4S6 region in the analyses with the understanding that the presence of other channel segments would alter the environment for binding and could therefore influence the interactions. Third, we limited our studies to neutral benzocaine analogs, and we acknowledge that the incorporation of tertiary amine LAs and receptor modulation into the model will further enhance these investigations. Despite these limitations, the docking interactions between the benzocaine analogs and the hydrophobic cavity on D4S6 were consistent with published predictions of benzocaine binding interactions.

5. Conclusions

Our data suggested that computational methods could indeed be viable tools for studying the complex interactions between LAs and voltage-gated sodium channels. In particular, the use of computational strategies in conjunction with experimental approaches that include site-directed mutagenesis could further advance our understanding of LA block of voltage-gated sodium channels and could also have potential uses in LA drug development and design.

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