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Structural Basis for Pharmacology of Voltage-Gated Sodium and Calcium Channels

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Drug Receptor Sites on Sodium and Calcium Channels

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ABSTRACT

Voltage-gated sodium channels initiate action potentials in nerve, muscle, and other electrically excitable cells. Voltage-gated calcium channels are activated by depolarization during action potentials, and calcium influx through them is the key second messenger of electrical signaling, initiating secretion, contraction, neurotransmission, gene transcription, and many other intracellular processes. Drugs that block sodium channels are used in local anesthesia and treatment of epilepsy, bipolar disorder, chronic pain, and cardiac arrhythmia. Drugs that block calcium channels are used in treatment of epilepsy, chronic pain, and cardiovascular disorders, including hypertension, angina pectoris, and cardiac arrhythmia. The principal pore-forming subunits of voltage-gated sodium and calcium channels are structurally related and are likely to have evolved from ancestral voltage-gated sodium channels that are widely expressed in prokaryotes. Determination of the structure of a bacterial ancestor of voltage-gated sodium and calcium channels at high resolution now allows a three-dimensional view of the binding sites for drugs acting on sodium and calcium channels. In this MiniReview, we outline the different classes of sodium and calcium channel drugs, review studies that have identified amino acid residues that are required for their binding and therapeutic actions, and illustrate how the analogs those key amino acid residues may form drug binding sites in three-dimensional models derived from bacterial channels.

INTRODUCTION

Introduction to the Pharmacology of Voltage-Gated Sodium and Calcium Channels

Sodium channels. Local anesthetics prevent pain by blocking initiation and propagation of the action potential in sensory nerves through block of voltage-gated sodium channels (Catterall and Mackie, 2011). Some antiepileptic drugs, including diphenylhydantoin, carbamazepine, and lamotrigine, prevent seizures by blocking brain sodium channels (McNamara, 2011). Similarly, some antiarrhythmic drugs including quinidine, procainamide, lidocaine, and flecainide interrupt and prevent cardiac arrhythmias by blocking cardiac sodium channels (Sampson and Kass, 2011). Thus, reduction of sodium channel activity is a major mechanism in pharmacology.

Complete block of sodium channels would have major unwanted side effects including complete loss of sensation in sensory nerves, sedation or coma in the brain, and cardiac arrest in the heart. Moreover, sodium channel blocking drugs in current clinical use are not selective among the nine voltage-gated sodium channels in mammals (Catterall et al., 2005a), so unwanted side effects of complete sodium channel block would be observed in multiple tissues. Sodium channel blockers are only usable in therapy because the frequency- and voltage-dependence of their action allows them to inhibit action potential generation in the depolarized and rapidly firing cells that are responsible for pain, epilepsy, and arrhythmia without complete block of action potential generation in normally functioning cells.

Frequency- and voltage-dependent block of sodium channels is currently understood in terms of the *modulated receptor hypothesis* (Hille, 1977). According to this concept, sodium channel blocking drugs can gain access to their receptor site in the lumen of the pore more rapidly when the intracellular activation gate of the channel is open during an action potential, and these drugs bind with higher affinity to inactivated sodium channels that accumulate during high-frequency firing of healthy cells and/or steady depolarization of damaged cells. Understanding the structural basis for frequency- and voltage-dependent block of sodium channels is an important goal in sodium channel pharmacology and will provide the foundation for future structure-based design of safer and more efficacious drugs.

Calcium channels. Voltage-gated calcium channels are also important drug targets. Phenylalkylamine calcium channel blockers like verapamil are used in treatment of atrial arrhythmias, as are benzothiazepines like diltiazem (Sampson and Kass, 2011). These drugs are thought to act at receptor site(s) in the pore of calcium channels and block them, much like sodium channel-blocking drugs block sodium channels. The action of verapamil and diltiazem is frequency- and voltage-dependent, and these effects are currently understood in terms of the modulated receptor hypothesis (Hondegghem and Katzung, 1984). Effective use of verapamil and diltiazem in treatment of atrial arrhythmias is thought to depend on this state-dependent block.

Calcium antagonist drugs are also used in treatment of hypertension and angina pectoris; however, dihydropyridines are the drugs of choice for these indications (Michel and Hoffmann, 2011). Dihydropyridines can either enhance or inhibit activation of voltage-gated calcium channels in heart and vascular smooth muscle by acting at an allosteric modulatory site located outside of the pore (Hess et al., 1984; Kokubun et al., 1986; Kokubun and Reuter, 1984). Their binding is highly voltage-dependent, and the calcium antagonist drugs used in therapy bind with high affinity to the inactivated state of calcium channels (Bean, 1984; Kokubun et al., 1986; Kokubun and Reuter, 1984). It is thought that their efficacy in hypertension and angina pectoris depends on voltage-dependent block of calcium channels in depolarized, continuously contracting vascular smooth muscle cells. They have lesser effects on calcium channels in the heart because the membrane potential of cardiac myocytes returns to negative values in the range of -85 mV during each cardiac cycle, allowing dissociation of the drugs from the resting state of calcium channels. Understanding the structural basis for frequency- and voltage-dependent block of calcium channels would allow structure-based design and development of safer and more effective drugs for therapy of cardiovascular diseases.

New generations of calcium channel drugs have emerged for treatment of epilepsy and chronic pain. ω -Conotoxins are potent blockers of presynaptic calcium channels in neurons. The synthetic derivative ziconitide (Prialt™) is administered intrathecally for control of severe pain in advanced cancer patients and others with advanced pain conditions (Miljanich and Ramachandran, 1995; Lewis et al.,

2012). Gabapentin (Neurontin™) and pregabalin (Lyrica™) were originally designed as GABA-related drugs (hence the names), but these compounds actually act on calcium channels by altering their membrane trafficking (Davies et al., 2007). These drugs do act on the auxiliary $\alpha 2\delta$ subunits of calcium channels, so recent advances on the structure of prokaryotic channels have not given insight into the sites and mechanisms of action of these drugs.

Structures of Sodium Channels

The sodium channel protein. Voltage-gated sodium channels are complexes of a large, pore-forming α subunit of ~260 kDa and one or two β subunits of 30-40 kDa (Catterall et al., 2005b). The α subunit of ~2000 amino acid residues is organized in four homologous domains that each contains six transmembrane segments (S1-S6) and an additional membrane reentrant segment (Fig. 1A). Segments S1-S4 form the voltage-sensing module, while segment S5, S6, and the P loop between them form the pore. Fast inactivation is mediated by the short intracellular loop between domains III and IV. The receptor site for local anesthetics and related drugs is formed by amino acid residues in the IS6, IIS6, and IVS6 segments, which contribute to a single drug binding site in the pore (Fig. 1B). This two-dimensional map of the sodium channel has provided the template for detailed structure-function studies, but the large mammalian sodium channels have resisted efforts at high-resolution three-dimensional structure determination. The discovery of bacterial sodium channels that are analogous in structure to one domain of mammalian sodium channels and function as homotetramers (Ren et al., 2001) has opened the way to structural biology of sodium channels.

Architecture of the pore. Determination of the crystal structure of the bacterial sodium channel NavAb at high resolution (2.7Å) has given new insights into the structural basis for many aspects of sodium channel function (Fig. 1B-D; (Payandeh et al., 2011)). As viewed from the side, NavAb has a central bundle of four S5 and four S6 segments that form the pore (Fig. 1C, blue). As viewed from the top, the central pore is surrounded by four pore-forming modules composed of S5 and S6 segments

and the intervening P loop (Fig. 1C, blue). Four voltage-sensing modules composed of S1-S4 segments are symmetrically associated with the outer rim of the pore module (Fig. 1C, green), connected by the S4-S5 linkers in each domain (Fig. 1C, red). The transmembrane architecture of NavAb shows that adjacent subunits have swapped functional modules such that each voltage-sensing module is most closely associated with the pore-forming module of its neighbor (Fig. 1C; (Payandeh et al., 2011)). It is likely that this module-swapping arrangement enforces concerted gating of the four subunits of homotetrameric bacterial sodium channels and possibly also has the same function in the four homologous domains of the more complex pseudotetrameric mammalian sodium channels.

Amino acid residues in the short alpha-helical segments between S5 and S6 (Fig. 1A) form the receptor site for the pore blocker tetrodotoxin (Fig. 1A, yellow circles (Noda et al., 1989; Terlau et al., 1991)), and mutations of these amino acid residues control ion selectivity (Heinemann et al., 1992). The crystal structure of the NavAb channel reveals a narrow ion selectivity filter with three ion interaction sites—Site_{HFS} formed by the negatively charge carboxylate groups of Glu177, Site_{CEN} formed by the backbone carbonyls of Leu176, and Site_{IN} formed by the backbone carbonyls of Thr175 (Fig. 1D). This selectivity filter is set in a transmembrane pore with a large external vestibule (Fig. 1E), the narrow ion selectivity filter containing the amino acid residues that determine ion selectivity (Fig. 1E), a large central cavity lined by the S6 segments (Fig. 1E), and an intracellular activation gate formed at the crossing of the S6 segments at the intracellular surface of the membrane (Fig. 1E). The activation gate is tightly closed in the NavAb structure (Payandeh et al., 2011). A high-resolution structure of a pore-only construct of Na_vMs (McCusker et al., 2012) and a cryo-EM structure of Na_vCt at a resolution of 9 Å (Tsai et al., 2013) reveal two possible open-gate conformations.

The NavAb ion selectivity filter has a high-field-strength site at its extracellular end (Fig. 2D), formed by the side chains of four glutamate residues (Payandeh et al., 2011), which are highly conserved and are key determinants of ion selectivity in vertebrate sodium and calcium channels (Heinemann et al., 1992). Considering selectivity-filter dimensions of approximately 4.6 Å square, Na⁺ with two planar waters of hydration could fit in this high-field-strength site. This outer site is followed on its intracellular side by two ion coordination sites formed by backbone carbonyls (Fig. 1D (Payandeh et al., 2011)).

These two carbonyl sites are perfectly designed to bind Na⁺ with four planar waters of hydration but would be much too large to bind Na⁺ directly. Thus, Na⁺ is selected and conducted as a hydrated ion interacting with the pore through its inner shell of bound water molecules.

Structural basis for voltage-dependent gating. Voltage-gated sodium channels are activated in response to depolarization by outward movement of “electrically charged particles” across the cell membrane through the transmembrane electric field (Hodgkin and Huxley, 1952b). The transmembrane movement of these gating charges was detected as a small capacitive gating current in squid giant axon (Armstrong and Bezanilla, 1973). The S4 segments of sodium channels, which contain 4-8 repeated motifs of a positively charged amino acid residue (usually arginine) flanked by two hydrophobic residues, were proposed to span the membrane (Fig. 1A) and to carry the gating charges in the *sliding helix* or *helical screw* model of voltage sensing (Catterall, 1986; Guy and Seetharamulu, 1986; Yarov-Yarovoy et al., 2006; Yarov-Yarovoy et al., 2012). In this model, the S4 segment is in a transmembrane position in both resting and activated states, the gating charges are stabilized by forming ion pairs with neighboring negatively charged and hydrophilic residues in other transmembrane segments, and their outward movement is catalyzed by exchange of ion pair partners (Catterall, 1986; Guy and Seetharamulu, 1986; Yarov-Yarovoy et al., 2006; Yarov-Yarovoy et al., 2012). Extensive structure-function studies now provide strong support for all of the elements of this model (reviewed in (Catterall, 2010)), and a consensus mechanism for voltage sensor function based on the *sliding helix* model has been presented by several leading investigators (Vargas et al., 2012). The voltage-driven outward movement of the gating charges in the S4 segment initiates the conformational changes that lead to pore opening and inactivation of sodium channels.

Sodium channels in vertebrates open in response to depolarization and then inactivate within 1-2 ms (Hodgkin and Huxley, 1952a). This fast inactivation process is required for repetitive firing of action potentials in neural circuits and for control of excitability in nerve and muscle cells. The short intracellular loop connecting homologous domains III and IV of the sodium channel α subunit is responsible for fast inactivation by folding into the intracellular mouth of the pore and blocking it (Fig.

1A), as shown by block of fast inactivation by antibodies directed against this peptide segment (Vassilev et al., 1988)). Fast inactivation is also greatly slowed by cutting this loop by expression of the sodium channel as two pieces, one containing domains I-III and a second containing domain IV (Stuhmer et al., 1989). The key amino acid motif IFM is required to maintain closure of the inactivation gate (West et al., 1992), and peptides containing this inactivation gate sequence motif can restore fast inactivation to mutant sodium channels (Eaholtz et al., 1994). Analysis of the structure of the inactivation gate by NMR showed that it contains a rigid alpha helix preceded by two loops of protein that array the IFM motif and a neighboring Thr residue optimally for interaction with and block of the open pore of the channel (Rohl et al., 1999). The fast inactivation gate is not present in homotetrameric bacterial sodium channels, so further structural analysis must await determination of the three-dimensional structure of a vertebrate sodium channel.

Bacterial sodium channels do not have a fast inactivation process because they lack the equivalent of the linker between domains III and IV of vertebrate sodium channels. However, bacterial sodium channels have a slow inactivation process that does not involve a cytosolic inactivation gate (Pavlov et al., 2005). Like vertebrate sodium channels (Vilin and Ruben, 2001), slow inactivation of bacterial sodium channels involves amino acid residues in the P loop and S6 segment (Pavlov et al., 2005; Zhao et al., 2004a; Zhao et al., 2004b). In crystallographic studies of two different bacterial sodium channels, the nearly square four-fold symmetric structure of the pore in the pre-open state of NavAb is collapsed to a parallelogram in dimer-of-dimers symmetry by movement of two S6 segments toward the central axis of the pore and corresponding movement of the other two S6 segments away from the central axis (Payandeh et al., 2012). This movement is more pronounced in the NavRh than in NavAb suggesting a sequence of conformational changes during the multi-step slow inactivation process (Zhang et al., 2012b).

Drug Receptor Sites in Sodium Channels

Sodium channels are blocked by drugs used clinically as local anesthetics, antiarrhythmics, and antiepileptics as described above. Site-directed mutagenesis studies revealed the receptor site for local

anesthetics and related drugs, which is formed by amino acid residues in the S6 segments in domains I, III, and IV (Fig. 1A, B) (Ragsdale et al., 1996; Ragsdale et al., 1994; Wang et al., 1998; Yarov-Yarovoy et al., 2001; Yarov-Yarovoy et al., 2002). These drugs bind to a common receptor site in the pore and impede ion permeation. The amino acid residues that form the receptor sites for sodium channel blockers line the inner surface of the S6 segments and create a three-dimensional drug receptor site whose occupancy would block the pore (Fig. 1B (Catterall, 2000; Payandeh et al., 2011; Ragsdale et al., 1994; Yarov-Yarovoy et al., 2002)). Access to this receptor site by large or hydrophilic drugs would require opening of the intracellular activation gate, which is tightly closed in the NavAb structure (Fig. 1E; (Payandeh et al., 2011)). This tight closure of the activation gate provides a structural basis for use-dependent block of sodium channels by local anesthetics and related drugs (Hille, 1977), as they would bind much more rapidly when the channel is frequently opened.

Remarkably, as also predicted by the modulated receptor hypothesis (Hille, 1977), fenestrations lead from the lipid phase of the membrane sideways into the drug receptor site, providing a specific hydrophobic access pathway for binding of small hydrophobic drugs in the resting state of the channel (Fig. 2A, B, pore portals; (Payandeh et al., 2011)). Access to the drug binding site in NavAb channels is controlled by the side chain of a single amino acid residue, Phe203 (Fig. 2A, B; (Payandeh et al., 2011)), which is homologous to amino acid residues identified in previous structure-function studies that control drug access and egress from the local anesthetic receptor site in mammalian cardiac and brain sodium channels (Qu et al., 1995; Ragsdale et al., 1994).

In crystallographic studies of two bacterial sodium channels, the central cavity containing the drug receptor site undergoes a major conformational change during slow inactivation (Payandeh et al., 2012; Zhang et al., 2012a). The nearly square four-fold symmetric structure of the pore in the pre-open state of NavAb is collapsed in slow-inactivated states to a parallelogram in dimer-of-dimers symmetry by movement of two S6 segments toward the central axis of the pore and corresponding movement of the other two S6 segments away from the central axis (Payandeh et al., 2012). This movement is more pronounced in the NavRh than in NavAb suggesting a sequence of conformational changes during the multi-step slow-inactivation process (Zhang et al., 2012a). These conformational changes cause

striking rearrangements of the drug binding sites and alter the size and orientation of the fenestrations (Fig. 2C (Payandeh et al., 2012)), which would be expected to alter both drug-binding affinity and drug access to the binding site. State-dependent drug access and drug affinity are both hallmarks of sodium channel pharmacology, whose molecular basis is revealed in preliminary form in these crystal structures of bacterial sodium channels. The conformational changes that take place during slow inactivation likely contribute to the increased affinity for drug binding to inactivated sodium channels.

NavAb and other bacterial sodium channels are blocked by local anesthetics and related drugs in a voltage- and frequency-dependent manner, similar to mammalian Na_v channels but with lower affinity (Bagneris et al., 2014; Lee et al., 2012; Ren et al., 2001; Zhao et al., 2004a). Nevertheless, despite their lower affinity, the amino acid residues in the bacterial channels that are in analogous positions to those that bind local anesthetics and related drugs in mammalian sodium channels form a functional receptor site and mediate drug block. The locations of the side chains of these amino acid residues are illustrated in Fig. 3A, C for NavAb in the pre-open state and in Fig. 3B, D in the inactivated state. When viewed in a transverse section through the pore module, amino acid residues analogous to those in transmembrane segments IIIS6 and IVS6 form a distributed binding site along the walls of the cavity in three of the S6 segments that line the pore (Fig. 3A, C). When viewed from the cytosolic side via a section through the structure above the activation gate, these side chains form a potential drug-binding site on the wall of the cavity with amino acid residues from three domains contributing side chains to the site (Fig. 3C, D). The positions of these amino acid side chains are altered in the slow inactivated state (Fig. 3B, D, orange), potentially contributing to state-dependent binding of local anesthetic drugs. In Fig. 3D, the inner ends of two of the S6 segments have moved away from the pore axis, two have moved closer in the slow inactivated state, and all four S6 segments have twisted with respect to the pore axis. This movement of S6 segments changes the geometry of the receptor site by moving the amino acid side chains in pseudo-domain I (upper left, orange) away from those in pseudo-domains III and IV (lower left and lower right, orange) and by moving the amino acid side chains in pseudo-domains III and IV closer together. Higher resolution structures of NavAb and chimeras with substitutions of amino acid residues from mammalian sodium channels should provide crucial

information on the mechanism of state-dependent block and the design of highly state-dependent sodium channel blockers.

A combination of structural modeling and ligand docking approaches has led to predictions of the location and pose of classical local anesthetics bound in mammalian Na_v channels, whose structures have been modeled based on the crystal structure of Na_vAb (Cheng et al., 2009). Surprisingly, tetracaine is predicted to bind in a pose that lies horizontally across the central cavity, with its positively charged ammonium group in the center of the cavity near the focus of the partially negatively charged ends of the four P helices and its aromatic group in the crevice between domains III and IV (Cheng et al., 2009). In this position, the aromatic group makes pi-pi interactions with highly conserved Phe and Tyr residues, which were previously implicated in drug binding by site-directed mutagenesis with substitutions of natural and unnatural amino acid residues (Pless et al., 2011; Ragsdale et al., 1994). Further structural work with bacterial and mammalian Na_v channels with bound drugs should give a more detailed picture of this important drug-receptor interaction.

Subtype-Selective Sodium Channel Blockers

Sodium channels are encoded by ten different genes, which are differentially expressed in the major excitable tissues, yet all of the sodium channel drugs used clinically are nonselective preventing systemic administration in doses sufficient to saturate their binding sites. The value of tissue selectivity is illustrated by successful systemic administration of a neurospecific peptide snail toxin, μ -conotoxin KIIIA, to prevent pain in a mouse model of inflammatory pain without cardiac and neuromuscular side effects (Zhang et al., 2007). Great interest has arisen in discovery of selective, high-affinity blockers of the Na_v1.7 and Na_v1.8 channels, which are highly expressed in sensory neurons (Krafte and Bannan, 2008; Priest and Kaczorowski, 2007). Pore blockers that are selective for Na_v1.8 channels and gating modifiers that bind selectively and modulate the voltage sensors in Na_v1.7 have been described (Jarvis et al., 2007; McCormack et al., 2013). Moreover, the first structural evidence for the location of a drug receptor site in the sodium channel has come from studies of a novel brominated drug, PI1, that selectively blocks mammalian Na_v1.8 channels and retains binding to a pore-only construct of the

ancestral bacterial sodium channel Na_vMs (Bagneris et al., 2014). The anomalous scattering signal from the Br atom was located in four symmetrically disposed positions, near the fenestrations on the sides of the central cavity in the pore. No electron density was observed for the bound drug itself. However, molecular modeling suggested that the bound drug would extend from the fenestration toward and into the inner mouth of the ion selectivity filter. This pose of the bound drug is generally consistent with previous site-directed mutagenesis studies of classical, nonselective sodium channel blockers in mammalian Na_v channels. Similarly, mutagenesis studies of the amino acid residues in Na_vMs that are required for high-affinity binding of PI1 suggest a similar binding site to the classical channel blockers, despite the selective high affinity of PI1 for Na_v1.8 channels (Bagneris et al., 2014). However, the binding pose of PI1 differs significantly from the predicted binding pose of tetracaine. PI1 extends farther upward into the ion selectivity filter to make interactions with the innermost ion coordination site (Site_{IN}, (Payandeh et al., 2011)) in the ion selectivity filter. Structural studies in which the pose of the bound drug is observed directly in electron density profiles will be needed to determine whether this apparent difference in drug binding inferred from ligand docking algorithms is confirmed.

Structure of Calcium Channels

The calcium channel protein. Like sodium channels, voltage-gated calcium channels have a large, pore-forming subunit (designated $\alpha 1$) that is composed of four homologous domains with six transmembrane segments and a pore loop in each (Catterall et al., 2005b). However, calcium channels have a different set of auxiliary subunits, including Ca_v β , γ , and $\alpha 2\delta$ subunits (Catterall et al., 2005b). The members of the bacterial sodium channel family, including NavAb, are thought to have been the ancestors of both mammalian sodium channels and calcium channels, based on their amino acid sequence similarity to both mammalian channel families (Ren et al., 2001; Yu and Catterall, 2004). Therefore, the general architectures of the voltage-sensing modules and pore modules of Ca_v channels are likely to resemble those of Na_v channels (Fig. 1). The structure of NavAb will give important insights into the structural basis for calcium channel function based on this strong similarity in amino acid sequence.

Structural basis for calcium selectivity. As a first step toward understanding the structural basis for calcium channel function, site-directed mutations were made in the bacterial sodium channels that introduce new negatively charged residues on the extracellular side of the high field-strength site (Shaya et al., 2014; Tang et al., 2014; Yue et al., 2002). Substitution of Asp residues located one and four positions on the extracellular side of the high field strength site plus substitution of Asp for the key Glu at the high field strength site in the selectivity filter confer high calcium selectivity on NaChBac and NavAb, with $P_{Ca}:P_{Na} \sim 400$ similar to mammalian calcium channels (Tang et al., 2014; Yue et al., 2002). Structural and functional analyses revealed that the key substitution of Asp for Ser in the position just on the extracellular side of the Glu at the high field strength site (Glu177 in NavAb) forms a second calcium-binding site in the pore. Remarkably, this single substitution is sufficient to increase calcium selectivity ~ 1000 -fold, from $P_{Ca}:P_{Na} \sim 0.03$ to $P_{Ca}:P_{Na} \sim 30$ (Tang et al., 2014). Structural analysis revealed a series of five binding sites for Ca^{2+} leading from the entry to the outer vestibule through the selectivity filter and into the central cavity (Tang et al., 2014). These results are consistent with a knock-off mechanism in which binding of one calcium ion blocks the pore and prevents monovalent cation entry, whereas binding of a second calcium ion to the newly introduced calcium-binding site at the position on the extracellular side of the high-field-strength site induces electrostatic repulsion that pushes the first bound calcium ion through the selectivity filter and eventually into the cytosol (Tang et al., 2014). This mechanism explains how the calcium channel can achieve high selectivity against monovalent cation permeation and still have high conductance for calcium, even though the atomic diameters of sodium and calcium are nearly identical.

Receptor Sites for Calcium Antagonist Drugs

Calcium channel antagonist drugs used in the therapy of cardiovascular diseases fall into three structural classes that act at separate sites. They all preferentially target the $Ca_v1.2$ channels that initiate excitation-contraction coupling in cardiac and vascular smooth muscle. The polypeptide conotoxins from cone snails used in treatment of chronic extreme pain block presynaptic $Ca_v2.2$

channels by binding at a fourth site, in the outer pore of calcium channels. The gabapentinoid drugs, which are also used in treatment of chronic pain, bind to a separate subunit of neuronal calcium channels and alter their membrane trafficking. We consider the structural basis for binding and action of each of these classes of drugs below.

Phenylalkylamines. The phenylalkylamines have two aromatic moieties connected by a central aliphatic chain containing a tertiary amino group (Sampson and Kass, 2011). Like local anesthetics, they are thought to enter the intracellular mouth of the pore through the open activation gate and bind to a specific receptor site in a protonated, positively charged form. They exhibit both frequency dependence and voltage dependence of channel block (Hondegheem and Katzung, 1984). Frequency-dependent block of calcium channels arises when repeated opening of the pore in rapidly firing cells allows more rapid access of the drugs to their receptor site and increases the fraction of calcium channels that are blocked. Verapamil, the only phenylalkylamine widely used in therapy, is effective in treatment of atrial arrhythmias, particularly those that arise by re-entry of action potentials conducted back from the atrioventricular node. This therapeutic use depends upon rapid frequency-dependent block of calcium channels by verapamil binding to its receptor site in the pore of calcium channels.

Extensive studies by photoaffinity labeling (Striessnig et al., 1990) and site-directed mutagenesis (Hockerman et al., 1997a; Hockerman et al., 1995) have mapped the amino acid residues that are required for high-affinity binding of verapamil and derivatives to the S6 segments in domains III and IV of the cardiac Ca_v1.2 calcium channel. Molecular modeling suggested that these amino acid residues face the lumen of the pore and cooperate to form the drug-binding site (Cheng et al., 2009). In agreement with these prior molecular models, the three-dimensional structure of CavAb places these amino acid residues in positions to cooperate in forming a single drug-binding site in the central cavity of the pore (Fig. 4A, green). Drug access to this site would be controlled by opening and closing of the intracellular inactivation gate as envisioned in the modulated receptor hypothesis (Hille, 1977). Moreover, the dramatic conformational change in the central cavity and the drug binding site during slow inactivation (Fig. 2C) could provide a molecular basis for high affinity binding to inactivated

calcium channels, another hallmark of the actions of phenylalkylamines (Hondegem and Katzung, 1984).

Illustration of the phenylalkylamine binding site by highlighting the side chains of amino acid residues in CavAb that are homologous to those that form this receptor site on Ca_v1.2 channels shows that this set of amino acid residues are located in the pore-lining transmembrane segments (Fig. 4B, side view). These residues are organized to form a coherent drug binding site, as illustrated in top view in Fig. 4C (dark green, Ca_v1.2-specific residues; light green, Ca_v conserved residues). The detailed conformation of this site is changed in the slow-inactivated state of CavAb (Fig. 4D), which is likely to be analogous to voltage-dependent inactivated state of calcium channels. As two of the S6 segments move away from the axis of the pore and two move toward it, the side chains of the amino acid residues that interact with phenylalkylamines in two adjacent S6 segments move farther apart. Thus, these models provide a preliminary view of possible state-dependent binding interactions underlying frequency- and voltage-dependent block of calcium channels by phenylalkylamines.

Molecular modeling and ligand docking methods have been used to predict the binding pose of the phenylalkylamines in their receptor site (Cheng et al., 2009; Lipkind and Fozzard, 2003). In the initial study, based on the structure of the KcsA potassium channel, the bound phenylalkylamine was fit in a half-folded, L-shaped conformation with the positively charged ammonium group projecting toward the negatively charged ion selectivity filter, aromatic ring A in the central cavity and aromatic ring B projecting toward the interface between domains III and IV (Lipkind and Fozzard, 2003). In the second model, based on the KvAP structure, the elongated phenylalkylamine molecule is predicted to lie in a more extended conformation with the conserved nitrile group projecting toward the ion selectivity filter with Ca²⁺ bound in it, the positively charged ammonium group placed at the axis of the pore in the focus of the helical dipole of the four P-helices, and the aromatic rings and methoxy groups making hydrophobic and hydrogen-binding interactions with amino acid side chains and backbone of the S6 segments (Cheng et al., 2009). These models both satisfy many of the requirements derived from structure-function studies; therefore, studies of bound phenylalkylamines in crystal structures of calcium

channels or their homologs will be required to precisely define the position of the bound drug and thereby determine the structural basis for state-dependent binding.

Benzothiazepines. Like verapamil, the benzothiazepine diltiazem is thought to bind in the pore of calcium channels and to derive frequency- and voltage-dependence of its blocking activity according to the modulated receptor hypothesis. The amino acid residues that form the diltiazem-binding site are partially overlapping with the amino acid residues in the verapamil/phenylalkylamine binding site (Hockerman et al., 2000; Kraus et al., 1996), but the binding of these two classes of drugs is not competitive. The conformation of the side chains of these amino acid residues would also be changed in the slow-inactivated state.

Dihydropyridines. The dihydropyridines can function as either activators or inhibitors of calcium channels; therefore, they must act as gating modifiers rather than pore blockers, and they must bind to a site outside of the ion conduction pathway to avoid blocking it (Catterall et al., 2005b; Catterall and Striessnig, 1992). In some cases, enantiomeric pairs are activators or inhibitors, respectively, indicating that very subtle changes in the drug-receptor interaction are sufficient to convert from agonist to antagonist action (Kokubun et al., 1986). The dihydropyridine binding site was initially probed by photoaffinity labeling (Striessnig et al., 1991), which revealed interaction with the S6 segments in domains III and IV and led to proposal of a domain-interface model for drug binding and action (Catterall et al., 2005b; Catterall and Striessnig, 1992). Site-directed mutagenesis studies of these channel segments (Mitterdorfer et al., 1996; Peterson et al., 1997; Peterson et al., 1996; Schuster et al., 1996) identified both conserved amino acid residues that are required for drug binding and Cav1.2-specific residues that create the selective high-affinity binding site for dihydropyridines in cardiac calcium channels. Moreover, nine amino acid residues in the IIS5, IIS6, and IVS6 segments were shown to be sufficient to confer high-affinity binding and selective channel activation and inhibition by dihydropyridines, including enantiomeric pairs of activators and inhibitors (Hockerman et al., 1997b; Ito et al., 1997; Sinnegger et al., 1997). Overall, these reconstitution studies gave a remarkably complete

transfer of dihydropyridine pharmacology to completely dihydropyridine-insensitive $Ca_v2.1$ and $Ca_v2.3$ channels.

The likely structure of the dihydropyridine receptor site is illustrated in Fig. 5A by highlighting the amino acid side chains in CavAb that are analogous to those thought to form this receptor site in $Ca_v1.2$ channels, as viewed from the side. Darker shading represents the two voltage-sensing domains that flank the receptor site and lighter shading represents the membrane-exposed surface of the pore domain containing the binding site. Dihydropyridines bind to a site that is formed by amino acid residues in two adjacent S6 segments plus the S5 segment between them. The same amino acid residues are illustrated in the inactivated state of CavAb in Fig. 5B. The binding site is formed on the “back side” of the S6 segments, oriented away from the pore, plus neighboring amino acid residues in the adjacent S5 segment, as seen in top views of pore domain (Fig. 5C, D). Thus, S6 segments from two adjacent subunits or domains cooperate to form the receptor site, as initially proposed in the original domain-interface model of DHP binding (Catterall and Striessnig, 1992). This site is located very close to the receptor site for phenylalkylamines (Fig. 4A, blue), with some amino acid side chains in position to bind both drugs on the opposite sides of phenyl rings or aliphatic side chains. As expected from the state-dependence of binding of dihydropyridines, the detailed orientation of these amino acid side chains changes significantly in the slow-inactivated state (Fig. 5D). Compared to the pre-open state (Fig. 5C), the amino acid side chains from the two S6 segments that form the binding site move apart in the inactivated state (Fig. 5D). This substantial movement of the drug-binding residues should contribute in an important way to the 100- to 1000-fold differences in binding affinity of dihydropyridines for Ca_v channels.

Molecular models of dihydropyridine binding have been developed based on the structures of the KcsA and KvAP potassium channels (Lipkind and Fozzard, 2003). These models place the bound drug at the interface between domains III and IV, in position to interact with amino acid residues in the IIIS5, IIIS6, and IVS6 segments, as observed in structure-function studies (Tikhonov and Zhorov, 2009). Specific interactions of the hydrophobic groups on the “portside” of the boat-like structure of the dihydropyridine ring are predicted to govern the agonist/antagonist properties of the dihydropyridines

(Tikhonov and Zhorov, 2009). Although the position of the bound dihydropyridine is similar in the available molecular models, the details of these interactions will differ in models based on the Na_vAb structure, which likely resembles calcium channels more closely, and crystal structures of dihydropyridines bound to calcium channels or their homologs are needed to probe structural models for the activation and inhibition of calcium channels by these important drugs.

Conotoxins. The ω -conotoxins from *Conus geographus* and other species of cone snails are potent and selective blockers of $\text{Ca}_v2.2$ channels, which conduct N-type calcium currents that initiate neurotransmitter release at the nerve terminals of primary afferent sensory neurons (Catterall et al., 2005b; Olivera et al., 1994). These toxins are potent analgesics in animal models of neuropathic pain (Miljanich and Ramachandran, 1995). The synthetic version of one of these toxins (ziconitide, Prialt™) is effective in patients with intractable pain from cancer and other causes (Lewis et al., 2012). ω -Conotoxins bind in the outer vestibule of calcium channels, and amino acid residues that are required for high-affinity binding have been identified by site-directed mutagenesis (Ellinor et al., 1994; Feng et al., 2003; Feng et al., 2001). Comparison to the structure of CavAb shows that these amino acid residues in mammalian $\text{Ca}_v2.2$ channels would form a binding site in the vestibule on the extracellular side of the amino acid residues in the ion selectivity filter that determine calcium selectivity. In this position, they would be expected to block ion conductance.

Gabapentinoid drugs. Extensive use of gabapentinoid drugs for treatment of epilepsy, and especially for treatment of chronic pain, has evolved over the past decade. Initially, the molecular target and receptor site at which they exert their therapeutic actions was unknown. However, extensive studies now indicate that they bind to the auxiliary $\alpha2\delta$ subunit of presynaptic calcium channels. In particular, binding of gabapentinoid drugs to the $\text{Ca}_v2.2$ channels that conduct N-type Ca^{2+} current and initiate neurotransmitter release at the central terminals of nociceptive dorsal root ganglion neurons down-regulates channel activity by inhibiting the normal cycle of endocytosis and re-insertion of these channels at the cell surface membrane (Davies et al., 2007). Hopefully, structural studies of $\alpha2\delta$

subunits will give novel insights that will help to guide design of future generations of these surprisingly effective drugs.

Structure and Ion Channel Pharmacology

In drug discovery, high-resolution structures of angiotensin-converting enzymes, HIV proteases, protein kinases, and many other enzyme targets have led to major advances in development of compounds with high selectivity for specific target subtypes and much improved safety and efficacy. The revolution in structures of seven-transmembrane G protein-coupled receptors in the past several years is driving a new wave of drug discovery of therapeutic agents that target these signaling molecules with exquisite selectivity and fine-tuned efficacy. The twenty four-transmembrane voltage-gated ion channels are much more complex, but we look forward to a new era of structure-based drug design for these important therapeutic targets, driven by the rapidly emerging structural information on bacterial homologs, chimeras of bacterial and mammalian channels, and eventually the mammalian sodium and calcium channels themselves.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Structure of voltage-gated sodium channels. **A.** A transmembrane folding diagram of the $\text{Na}_V1.2$ channel. Cylinders represent alpha helical segments. Bold lines represent the polypeptide chains of each subunit with length approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of the $\beta 1$ and $\beta 2$ subunits are shown as immunoglobulin-like folds. Ψ , sites of probable N-linked glycosylation; P in red circles, sites of demonstrated protein phosphorylation by PKA (circles) and PKC (diamonds); blue, pore-lining segments; yellow circles, the outer (EEEE) and inner (DEKA) rings of amino residues that form the tetrodotoxin binding site and the ion selectivity filter; green, S1-S4 voltage sensors; h in blue circle, inactivation particle in the inactivation gate loop; blue circles, sites implicated in forming the inactivation gate receptor. **B.** Model of the local anesthetic receptor site in mammalian $\text{Na}_V1.2$ channels. **C.** Side view of Na_VAb channels colored according to panel A: green, voltage-sensing module; blue, pore module; red, S4-S5 linker. **D.** Side view of the ion selectivity filter. Glu177 (purple) interactions with Gln172, Ser178 and the backbone of Ser180 are shown in the far subunit. F_o-F_c omit map, 4.75σ (blue); putative cations or water molecules (red spheres, Ion_{EX}). Electron-density around Leu176 (grey; F_o-F_c omit map at 1.75σ) and a putative water molecule is shown (gray sphere). Na^+ -coordination sites: Site_{HFS}, Site_{CEN} and Site_{IN}. **E.** Architecture of the Na_VAb pore. Glu177 side-chains, purple; pore volume, grey. The S5 and S6 segments and the P loop from two lateral subunits are shown.

Figure 2. Drug binding sites and fenestrations in NavAb. **A.** Side-view through the pore module illustrating fenestrations (portals) and hydrophobic access to central cavity. Phe203 side-chains, yellow sticks. Surface representations of Na_VAb residues aligning with those implicated in drug binding and block, Thr206, blue; Met209, green; Val213, orange. Membrane boundaries, gray lines. Electron-density from an F_o-F_c omit map is contoured at 2.0σ . **B.** Top-view sectioned below the selectivity filter, colored as in A. **C.** Structure of the drug binding site in the slow-inactivated state in Na_VAb .

Figure 3. Amino acid side chains in the local anesthetic binding site of NavAb. **A.** Amino acid residues identified by mutagenesis of mammalian sodium channels and shown to be important in drug block are illustrated as orange spheres. Helices S5 and S6 and the pore domain are depicted in a side view of the pre-open state of NavAb . **B.** Similar view to panel A showing the slow-inactivated state of

NavAb. **C.** NavAb is shown from the extracellular side. The S5 and S6 segments are shown as cylinders. Amino acid residues identified by mutagenesis of mammalian sodium channels and shown to be important in drug block are illustrated as orange spheres. **D.** Similar view to panel C showing the inactivated state of NavAb.

Figure 4. Amino acid side chains in the phenylalkylamine binding site. A. Top view of the pore module of CavAb in the pre-open state with amino acid side chains analogous to those implicated in phenylalkylamine binding illustrated in green and amino acid side chains specific for dihydropyridine illustrated in blue. **B.** Side view of CavAb in the slow-inactivated state with amino acid side chains analogous to those implicated in phenylalkylamine binding illustrated in ball-and-stick representation. **C.** Top view of CavAb pore module in the pre-open state with the S5 and S6 segments illustrated as cylinders and amino acid side chains analogous to those implicated in phenylalkylamine binding illustrated dark green for Ca_v1.2-specific residues and in light green for Ca_v conserved residues. **D.** Similar view to panel C of the inactivated state.

Figure 5. Amino acid side chains in the dihydropyridine binding site. A. Side view of intact CavAb in the pre-open state with the two voltage-sensing domains illustrated in light gray and the intervening pore domain illustrated in light gray. Amino acid side chains analogous to those implicated in dihydropyridine binding are illustrated in dark blue for Ca_v1.2-specific residues and in light blue for Ca_v conserved residues. **B.** Side view of CavAb in the slow-inactivated state with amino acid side chains analogous to those implicated in dihydropyridine binding illustrated in blue. **C.** Top view of the pore domain of CavAb in the pre-open state with amino acid side chains analogous to those implicated in dihydropyridine binding illustrated in blue. **D.** Top view of the pore domain of CavAb in the slow inactivated state with amino acid side chains analogous to those implicated in dihydropyridine binding illustrated in blue.

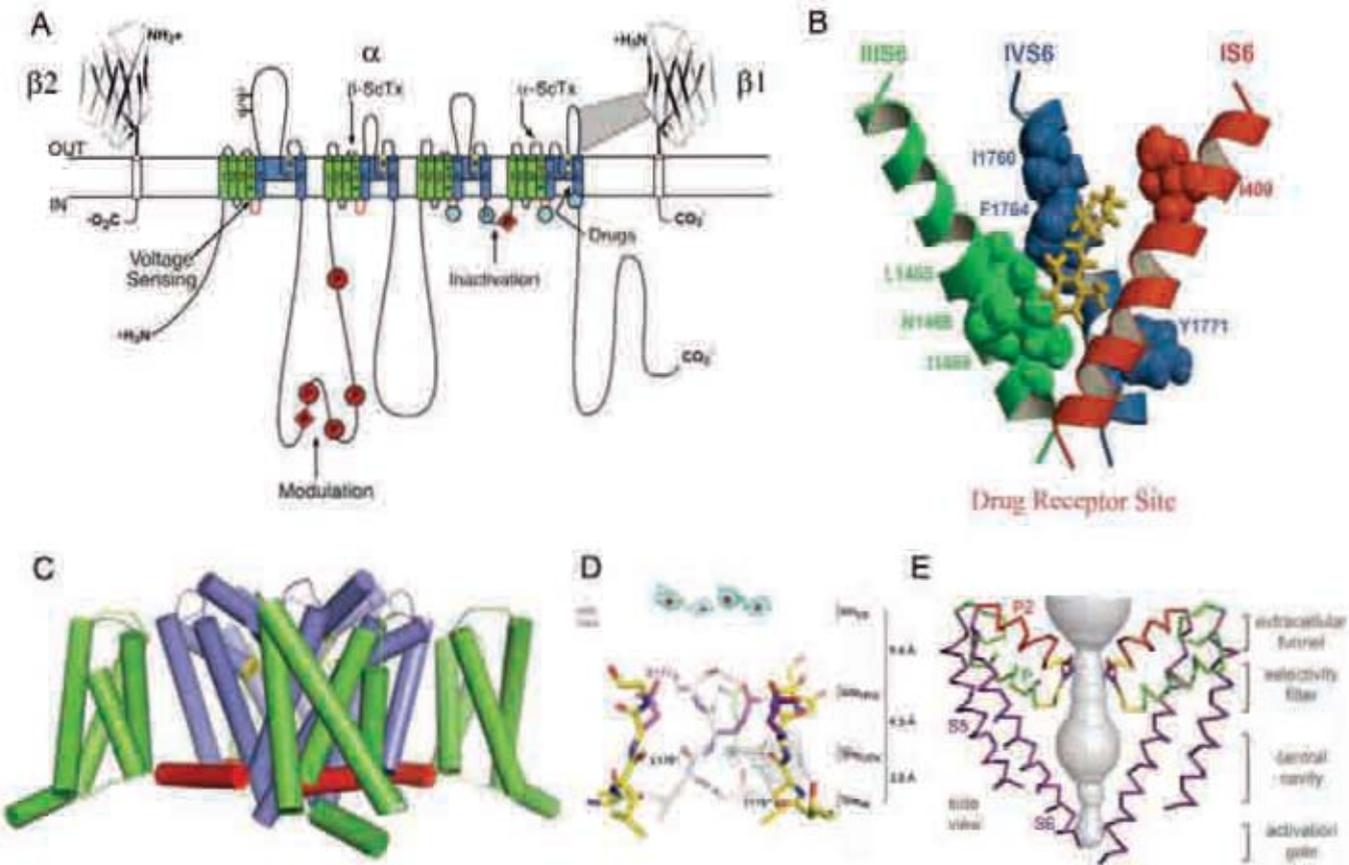


Figure 1

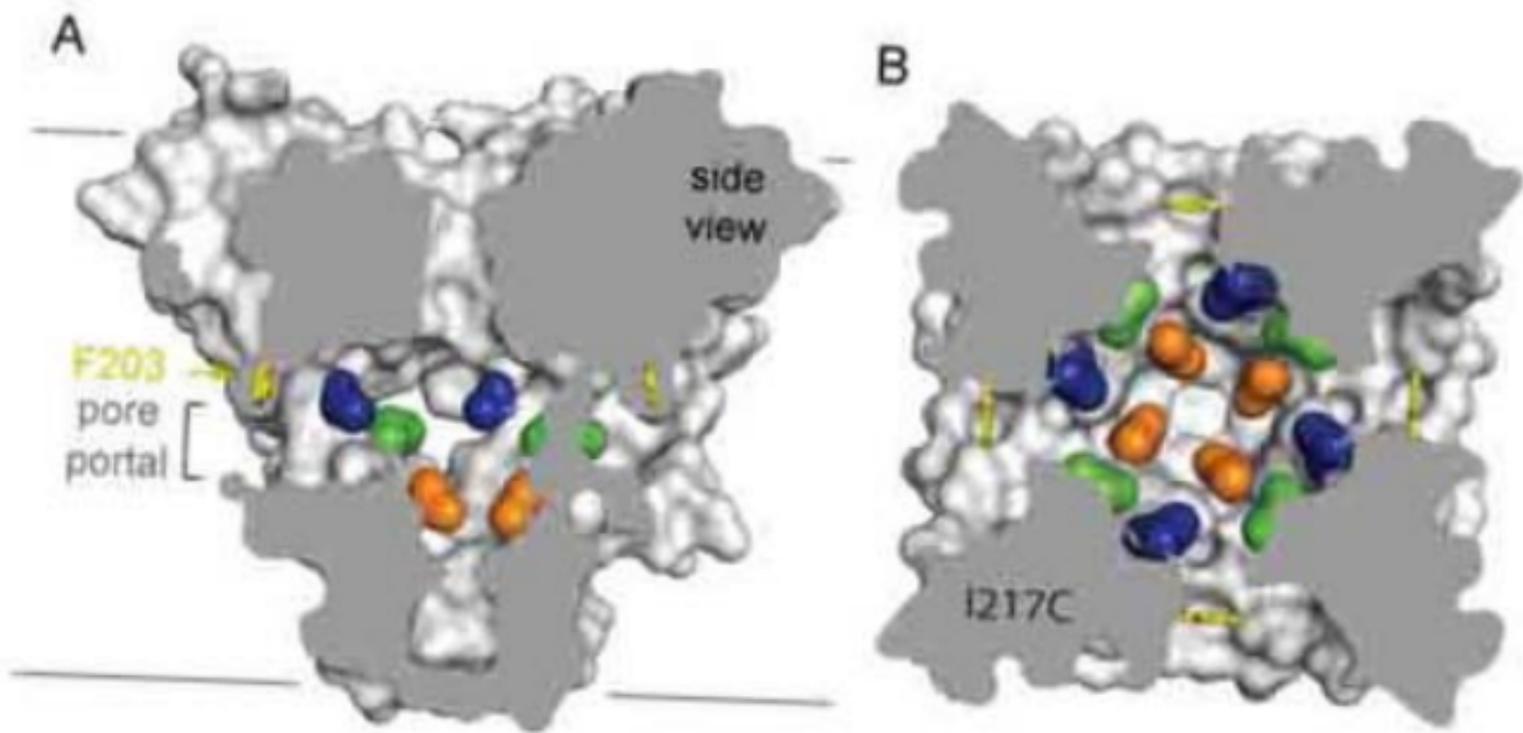


Figure 2

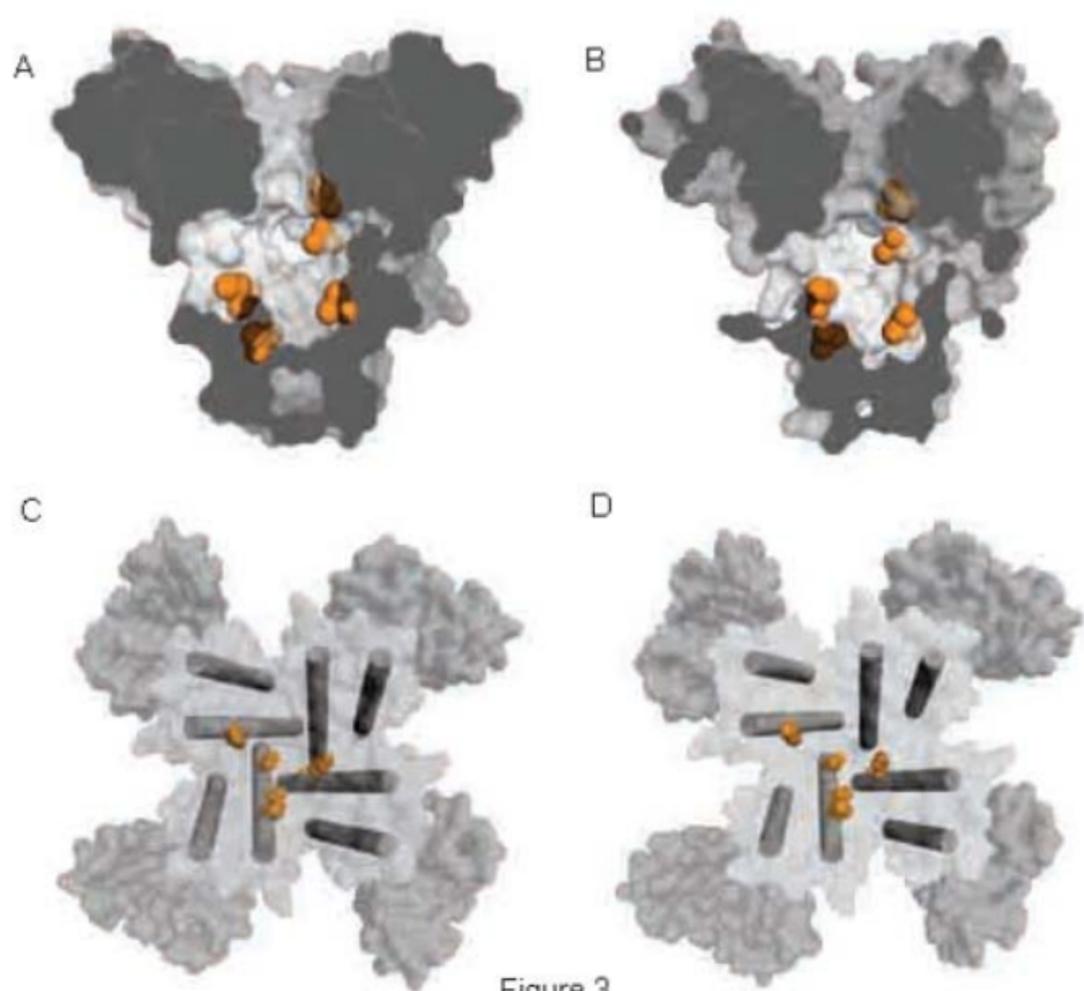
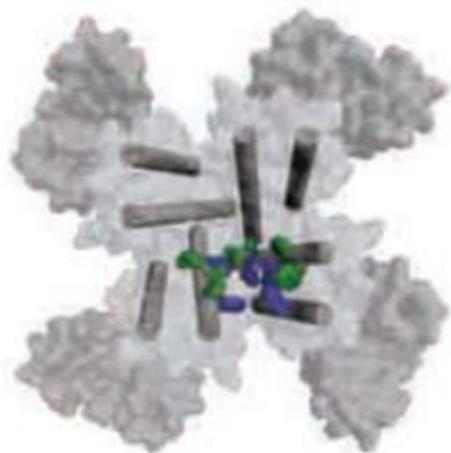
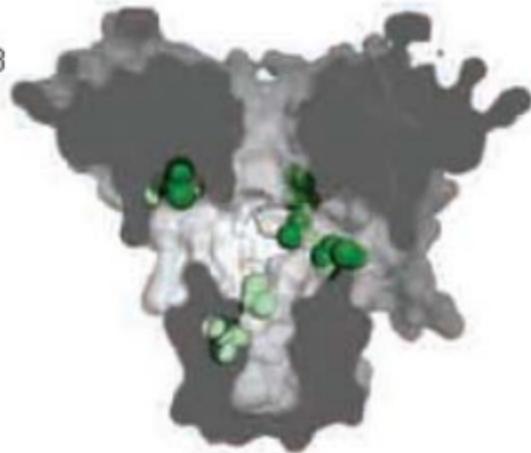


Figure 3

A



B



C



D



Figure 4

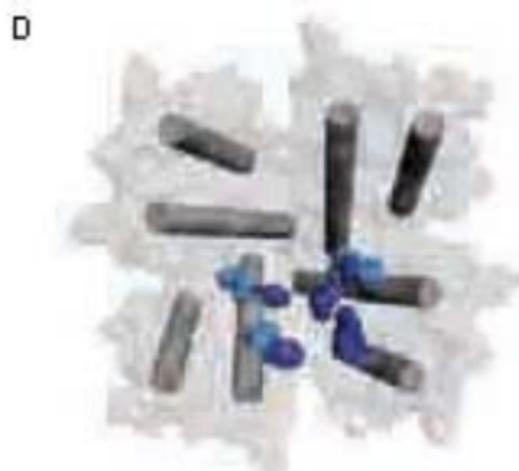
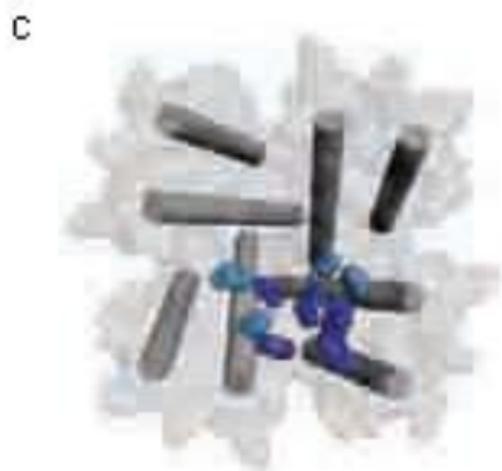
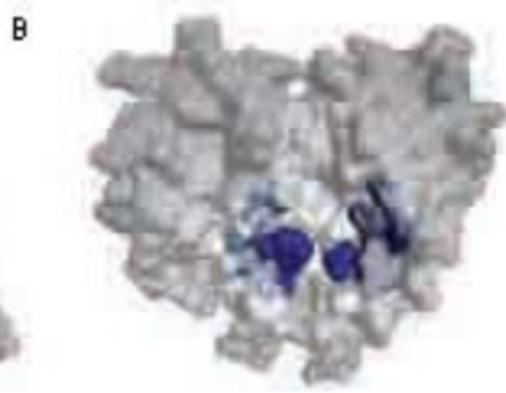


Figure 5