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Verapamil Block of T-type Calcium Channels

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Abbreviations: Cav3.1, the T-type voltage-gated Ca channel isoform; Nav1.5, the heart isoform of the voltage-gated Na channel; PAAs, phenylalkylamines, MTSET, [2-(Trimethylammonium)ethyl]methanethiosulfonate; WT, wild type.

Abstract

Verapamil is a prototypical phenylalkylamine (PAA), and it was the first calcium channel blocker to be used clinically. It tonically blocks L-type channels in the inner pore with micromolar affinity, and its affinity increases at depolarized membrane potentials. In T-type calcium channels, verapamil blocks with micromolar affinity, and has modestly increased affinity at depolarized potentials. We found that a related PAA, D888, is comparable to verapamil both in affinity and in state dependence. Permanently charged verapamil was more effective intracellularly than neutral verapamil. Charged PAAs were able to access their binding site from both inside and outside the cell. Furthermore, membrane impermeant MTSET was able to access the inner pore from outside of the cell. We examined a homology model of the T-type calcium channel to look for possible routes of drug entry. Mutation of L1825W produced a channel that was blocked significantly more slowly by charged verapamil from the outside, with an increase in apparent affinity when the drug was applied from the inside. Data suggest that T-type channels have a back pathway through which charged drugs can access the inner pore of the channel without passing through the plasma membrane.

Introduction

T-type calcium channels (T-type channels) are a subfamily of voltage gated calcium channels that are activated at relatively hyperpolarized membrane potentials (Perez-Reyes, 2003). The three isoforms of T-type calcium channels (Ca_v3.1, 3.2 and 3.3) are distributed throughout the body, with identified roles in the nervous system, the vasculature, and the developing heart. The most widely used T-type channel blocker is mibefradil, which was clinically used as an antihypertensive for a brief period, before being withdrawn due to fatal interactions with other drugs. Mibefradil was initially thought to be selective for T-type channels over other ion channel targets, but more recent data suggest that its selectivity is limited, and its antihypertensive effects are largely absent in animals with a conditional knockout of an isoform of the L-type channel, in whom T-type channels appear to be unaffected (Cribbs, 2006; Moosmang, 2006). More specific T-type channel blockers have recently been developed (Uebele, 2009), but these drugs are not well characterized and are not yet in use clinically. Many classes of drugs modulate or block these channels (Lacinová, 2004). Among these are the phenylalkylamines (PAAs), the best known of which, verapamil, has long been used clinically. Because of its fast pharmacodynamics, verapamil is currently used in hospital settings to terminate cardiac arrhythmias (Kato, 2004). Further, the recent development of a sustained release formulation of verapamil has resulted in verapamil's recognition as a tool for combination treatment of hypertension, particularly in diabetic patients (Reynolds, 2005).

Verapamil blocks both L-type and T-type channels with higher affinity for depolarized channels than for resting channels. This property, known as state dependence, was initially described for local anaesthetics blocking voltage gated sodium channels (Hille, 1977). Conformational changes that occur upon opening and inactivation are thought to produce higher affinity binding sites than those available in closed channels. In L-type channels, the affinity of verapamil for depolarized channels is on the order of ten-fold higher than for closed channels (Johnson, 1996). In T-type channels, the state dependence is less dramatic, with less than two-fold more block of depolarized channels than closed channels in the presence of 10 μ M verapamil (Freeze, 2006).

D888, also known as devapamil or desmethoxyverapamil, is a related PAA lacking one methoxy group. It has affinity in the tens of nanomolar for resting state block of L-type channels (Johnson, 1996), and exhibits a five-fold increase in affinity for depolarized channels. Determinants of D888 binding have been located in the inner pore of L-type channels (Hockerman, 1995, Hockerman, 1997a). Mutation of three residues near the selectivity filter of L-type channels resulted in a 100-fold reduction in the affinity of D888 (Hockerman, 1995). It should be noted, however, that these mutations did not affect the affinity of verapamil (Johnson, 1996).

PAAs are tertiary amines and, therefore, can exist as both neutral and positively charged molecules. Their ability to deprotonate to a neutral form at physiological pH is thought to be critical for their ability to reach a binding site in the inner pore of ion channels. Several studies have attempted to determine the route by which PAAs access their

binding site in L-type channels (Mannhold, 1978; Leblanc, 1989; Hescheler, 1982; Wegener, 1995; Berjukov, 1996). Permanently charged derivatives of several PAAs, including verapamil and D888, have been used to determine whether the binding site is accessible from the intracellular or extracellular side, or from both sides. The preponderance of evidence in L-type channels suggests that PAAs bind in the inner pore. They are presumed to pass through the plasma membrane in their uncharged state to block the channel from inside (Hockerman, 1997b).

Detailed knowledge of drug binding sites and access routes is absent for any T-type channel blockers. Therefore, in this study we sought to use the well-studied interactions between PAAs and L-type channels to extend our understanding of the closely related T-type channel. We examined the relative affinity of verapamil and D888 both for tonic block and for use dependent block. We also examined the sidedness of PAA binding to T-type channels using permanently charged PAAs, to begin to determine where and how PAAs bind to T-type channels.

Methods

Heterologous expression

The cDNA Cav3.1 217, encoding one of the most prevalent splice variants of Cav3.1, was kindly provided by M. C. Emerick and W. S. Agnew (The Johns Hopkins University School of Medicine, Baltimore, MD). It was subcloned into three different vectors: pcDNA3.1/Zeocin, which was stably expressed in HEK 293 cells, pDNA5/FRT, which was stably expressed in HEK 293/FLP cells, and pcDNA5/FRT/TO, which was stably expressed in Flp-In T-REx-293 cells (Invitrogen, Carlsbad, CA). Stable cell lines were created using either 200 µg/ml Zeocin for HEK 293 cells, or 100 µg/ml HygroGold for HEK 293/FLP cells and T-REx-293 cells. Cells were maintained in 100 mm Corning culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, and either 100 µg/ml Zeocin (for HEK 293 cells) or 50 µg/ml HygroGold (for HEK 293/FLP, Invivogen, San Diego, CA), or 15 µg/ml blasticidin, and 50 µg/ml HygroGold (for T-REx-293 cells). Cav3.1 expression in T-REx-293 cells was induced approximately 17 h before use in electrophysiology experiments with 0.05–0.2 µg/ml tetracycline, depending on the desired channel density. The three cell lines were used interchangeably, as the currents produced were indistinguishable. The only difference between the cell lines was the amount of channel expression. Reagents were obtained from Invitrogen unless otherwise indicated.

Site directed mutagenesis

Point mutations were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according the manufacturer's protocols. Silent restriction

enzyme sites were also introduced to allow for identification of positive clones via qualitative restriction enzyme mapping. DNA sequencing was performed to confirm mapping results.

Solutions and Chemicals

The bath solution used for most ionic current experiments contained, in mM: 140 NaCl, 2 CaCl₂, and 10 HEPES, titrated to pH 7.4 with NaOH. For experiments with L1825W channels, the bath solution contained, in mM: 135 NaCl, 5 CaCl₂, and 10 HEPES, titrated to pH 7.4. For experiments with Q1828A cells and some experiments with Q1828C cells, the bath solution contained, in mM: 132 NaCl, 10 CaCl₂, and 10 HEPES, titrated to pH 7.4 with NaOH. The pipette solution contained, in mM: 130 NaCl, 5 MgATP, 1 CaCl₂, 11 EGTA, and 10 HEPES, and was titrated to pH 7.4 with NaOH. The bath solution for experiments with Q1828C in sodium contained, in mM: 140 NaCl, 1 EGTA, 10 HEPES, titrated to pH 7.4 with NaOH. Verapamil hydrochloride (Sigma, St. Louis, MO) and D888 (Abbott Laboratories) were prepared as stocks in DMSO. All PAAs were diluted to the desired concentration in bath or pipette solution daily. MTSET (Toronto Research Chemicals Inc., Toronto, Canada) was diluted into bath solution and used within 60 minutes.

The synthesis of permanently charged PAAs is described in the Supplemental Data. To ensure that our results using permanently charged drugs were not due to contamination with neutral PAAs, we also tested quaternary drugs previously used in other studies. D575 was a kind gift of Abbott Laboratories, and qD888 was generously provided by Dr. Jörg Streissnig (University of Innsbruck, Austria).

Electrophysiological recordings and analysis

For experiments with drugs in the bath solution, drugs were washed on during a train of pulses to -10 mV at a frequency of 0.2 Hz. Typically, block was established over the course of 10 to 15 pulses. All recordings included in this study were made after the peak current elicited at -10 mV was stable for at least three pulses (see supplemental figure 1 for example).

Recordings were made using an Axopatch 200B feedback amplifier (Molecular Devices, Sunnyvale, CA) with a Digidata 1321A digitizer and pClamp 8.1 data acquisition software (Molecular Devices). Patch pipettes were pulled with a P97 micropipette puller (Sutter Instruments, Novato, CA) from TW 150-4 borosilicate glass (World Precision Instruments, Sarasota, FL), and had resistances of 850 k Ω to 1.6 M Ω when filled with pipette solution. Recordings were made using whole-cell voltage clamp of trypsinized cells (0.25% Trypsin-EDTA, Sigma-Aldrich, St. Louis, MO) 3–5 days after plating. Recordings were made at room temperature (20–26°C). Because the extent of state dependent block was highly temperature sensitive, a Sentelek TS-4 temperature controller was used to hold the bath temperature at 21 \pm 1° for most train protocols.

Data were filtered at 5 kHz by an eight-pole low-pass Bessel filter and digitized at 10 kHz. Currents were additionally filtered at 2 kHz offline. Currents were capacity-corrected using 8–16 subthreshold responses (voltage steps of 20 mV), and leak corrected, based on linear leak resistance calculated at potentials negative to -80 mV or by linear interpolation between the current at the holding potential and 0 mV. The data were analyzed using locally written programs in MATLAB (The Math Works, Natick, MA) and Origin software (OriginLab, Northampton, MA). Differences between

parameters were assessed using Student's t test. Current-voltage relationships were fit using a modification of the Boltzmann equation:

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} + A_2 \quad (1)$$

where A_1 is the amplitude, A_2 the baseline, x the voltage, x_0 the half-point of the relationship, and dx the slope factor in mV. A slope factor of ≤ -5 was considered to be evidence of adequate voltage control.

Results

The affinity of verapamil and D888 for tonic block of $Ca_v3.1$ is comparable.

Verapamil produces tonic block, i.e. block at the resting potential, measured with infrequent depolarizations, in both T-type and L-type channels. In heterologous expression systems, the affinity of verapamil is similar, with an ED_{50} for L-type channels of $\sim 10 \mu\text{M}$, versus an ED_{50} of $\sim 20 \mu\text{M}$ for T-type channels (Johnson, 1996; Freeze, 2006). D888 is a much higher affinity ligand for L-type channels than verapamil, with an ED_{50} for closed channel block of $\sim 50 \text{ nM}$ (Johnson, 1996). We examined the ability of verapamil and D888 to tonically block $Ca_v3.1$. Current voltage relationships were measured using sequential depolarizations at 0.2 Hz, a frequency that allows channels to recover fully from inactivation between depolarizations. Using this protocol, the fraction of current blocked at -10 mV by $20 \mu\text{M}$ D888 was $59.6 \pm 6\%$, comparable to $61.1 \pm 0.8\%$ for verapamil (Figure 2A).

The affinity of verapamil and D888 for use dependent block is also similar.

PAAs have demonstrated substantially higher affinity for depolarized L-type channels, resulting in use-dependent block. In particular, D888 has 5-fold higher affinity for depolarized than for resting L-type channels (Johnson, 1996). In T-type channels, block by verapamil is modestly use-dependent (Freeze, 2006). We tested the use-dependence of D888 using a train of 25 ms depolarizing pulses to -10 mV with an interpulse interval of 100 ms. In control cells, accumulation of inactivated channels resulted in a 40% decrease in peak current over the first few pulses of the train, which stabilized as equilibrium was reached between channels entering and leaving the inactivated state (Figure 2B, inset).

The time course of this reduction was 0.8 ± 0.08 pulses. Addition of D888 or verapamil produced an additional reduction in peak current, representing the combination of inactivated and blocked channels. Use dependent drug block was evaluated by subtracting the amplitude of control currents from those recorded in the presence of drug. D888 (20 μ M) produced $63 \pm 2\%$ use dependent block, compared to $59 \pm 6\%$ produced by the same concentration of verapamil. The time course of the subtracted peaks could be fit with a single exponential, yielding a tau of 1.3 ± 0.1 pulses, versus 1.5 ± 0.1 pulses for verapamil. Thus, use dependent block by D888 could not be distinguished from verapamil either in extent or time course, and T-type channels, unlike L-type channels, are not sensitive to the absence of the second methoxy group on D888 (Figure 1).

Verapamil blocks intracellularly at high concentrations.

In the L-type channel, PAAs are thought to bind in the inner pore, and reach their binding site from the intracellular side (Hockerman, 1997b). If this is also the case for the T-type channel, then we would expect that verapamil included in the pipette would be able to reach its binding site and produce block of the T-type channel. One must, when carrying out this experiment, account for the diffusion of PAAs out of the pipette, into the cell, and across the cell membrane. For a charged drug, this might be expected to delay appearance of block, but one would predict that complete block would eventually develop. For a tertiary or uncharged drug, the escape of the drug from the cell by diffusion across the bilayer would result in a lower concentration of drug near the channels, and would be expected to produce a lower apparent affinity. Experiments with lidocaine, a tertiary drug for which binding determinants have been identified in the inner

pore of voltage gated sodium channels, indicate that approximately 10-fold more drug is needed in the pipette than in the bath to produce an equivalent effect for that drug and channel (supplemental figure 2).

We, therefore, tested verapamil in the pipette at 10-fold greater than the ED_{50} for drug dissolved in the bath. Tonic block could not be evaluated in these experiments, as the drug has at least some access to the channels from the moment of break in. However, we could evaluate use dependent block. At 200 μ M, verapamil produced $18 \pm 3\%$ use dependent block, much less than the $59 \pm 6\%$ produced by 20 μ M verapamil applied extracellularly (Figure 3). Successive trains run in the same cell did not demonstrate any further development of use dependent block, even as long as fifteen minutes after break in (supplemental figure 3). Raising the concentration of verapamil to 2 mM, 100-fold greater than the ED_{50} for drug dissolved in the bath, was required to achieve $50 \pm 5\%$ use dependent block, which approached the level seen with 20 μ M verapamil in the bath. At 200 μ M verapamil, the time course of use dependent block had a tau of 4.5 ± 0.4 pulses, significantly slower than for 20 μ M extracellular verapamil ($p < 0.01$). At 2 mM, the concentration at which the extent of block resembled 20 μ M extracellular verapamil, the time constant of block had a tau of 1.6 ± 0.5 pulses, similar to that for 20 μ M extracellular verapamil. Although lidocaine and verapamil are both tertiary amines, verapamil is quite hydrophobic, with two aromatic rings, and short aliphatic chains in the middle linker. These data, combined with the chemical differences between lidocaine and verapamil, strongly suggest that verapamil is able to escape across the membrane much

more readily than lidocaine, resulting in a greater difference between apparent inside and outside affinity for verapamil than for lidocaine.

Permanently charged verapamil blocks intracellularly

If the low affinity of verapamil when placed in the pipette is due to escape across the membrane, then we would expect a membrane impermeant PAA to display higher apparent affinity than verapamil from the intracellular side, although perhaps with a slow onset. We assessed the effect of a quaternary, permanently charged analog of verapamil, D575, in the pipette. 200 μ M D575 in the pipette produced $63 \pm 1\%$ use dependent block when stepped to -10 mV, significantly more than was produced by the same concentration of neutral verapamil ($p < 0.001$, Figure 4). In addition, both the extent and the timecourse of block by D575 showed modest voltage dependence; depolarization to -30 mV produced $47 \pm 1\%$ block, while depolarization to +10 mV produced $75 \pm 2\%$ block. The voltage dependence was most easily seen in the time course of use dependent block, which became more rapid as the cells were depolarized farther (Figure 4, inset). In contrast, neutral verapamil did not display voltage dependence in these parameters (data not shown).

The ability of 200 μ M D575 to produce significantly more block than 200 μ M verapamil is consistent with the idea that neutral verapamil can escape from the cell more easily than the permanently charged D575. However, 10 times more D575 was still required on the inside to produce the level of block seen with 20 μ M verapamil on the outside. This is unlikely to be a consequence of slow diffusion of the drug from the pipette. As was

observed for the neutral verapamil, the amount of use dependent block produced in successive trains did not increase over more than fifteen minutes (supplemental figure 3).

Charged PAAs also block T-type channels from the extracellular surface.

D575, as well as other permanently charged PAAs, are thought to be unable to cross the plasma membrane. In the L-type channel, these drugs have been used to determine which side of the channel contains drug binding sites. Although there are some technical differences between studies, overall the data suggest that most permanently charged PAAs are effective against L-type channels when placed intracellularly, but not extracellularly (Mannhold, 1978; Hescheler, 1982; Leblanc, 1989). The permanently charged quaternary analog of D888 (qD888) has been shown to block L-type channels from both inside and outside, but the characteristics of block differ depending on the route of application (Berjukov, 1996).

D575 and qD888 were applied to T-type channels from the bath. Surprisingly, both charged drugs produced robust block (Figure 5). Tonic block and use dependent block were both present. Most interestingly, block by the quaternary drugs was indistinguishable from block produced by neutral variants. The kinetics of extracellular block by charged drugs were 2- to 3-fold slower than was seen with neutral verapamil (4.4 ± 0.4 pulses for 20 μ M D575 and 5.6 ± 1.3 pulses for 20 μ M qD888 versus 1.5 ± 0.1 pulses for verapamil, $p < 0.01$). Also, in contrast to the effects of intracellular application of the charged drugs, there was no evidence of voltage dependence for use dependent block with charged drugs in the bath over potentials ranging from -30 mV to +50 mV.

A permanently charged MTS reagent can also enter T-type channels from the bath.

It is possible that the ability of charged PAAs to block T-type channels from the extracellular side is due to the presence of a second, extracellular binding site for PAAs. Alternately, it is possible that the charged PAAs are able in some way to penetrate the channel and reach a binding site below the selectivity filter. In order to distinguish between these possibilities, we introduced cysteine residues at two positions in the inner pore of the channel, Gln-1828 and Leu-1831. When these residues are substituted into a homology model of the T-type channel based on the crystal structure of KcsA, they are predicted to face into the inner pore of the channel, below the selectivity filter (Figure 6A; Lipkind, 2003). We applied permanently charged MTSET, which is generally accepted not to cross the plasma membrane, to channels expressing each of these mutants. Application of MTSET to the outside of cells resulted in nearly complete block of inward current in both Q1828C channels ($95 \pm 3\%$ block at -10 mV, Figure 6B) and L1831C channels ($86 \pm 4\%$ block at -20 mV, supplemental figure 4). Block by MTSET occurred within tens of seconds and was irreversible, while WT channels were not blocked over at least seven minutes, suggesting that the effect of MTSET was not due to modification of an endogenous cysteine (data not shown). Also, MTSET had no effect on channels with another small residue (Ala) substituted at this position, suggesting that changes at this residue did not distort the channel, exposing an endogenous cysteine (Q1828A, data not shown). In addition to blocking inward current, MTSET induced a shift in the apparent reversal potential when inward currents were carried by Ca^{2+} and outward currents were carried by Na^{+} . This suggested that Ca^{2+} current was more

completely blocked than Na^+ current. To confirm this, we tested an MTS reagent in the absence of Ca^{2+} . 200 μM MTSEA blocked inward Na^+ currents by $36 \pm 12\%$, significantly less than the 95% block of inward current when carried by Ca^{2+} (supplemental figure 5). We interpret the sensitivity of MTSET to the species of permeant ion to suggest that the modification is in the permeation pathway. These data strongly support the idea that MTSET can modify the introduced cysteine residue in the inner pore of the T-type channel when applied extracellularly. Since MTSET would not be expected to pass either through the membrane or through the selectivity filter, we must suggest that a pathway exists for the entry of charged drugs into the inner pore of the T-type channel.

A back pathway for drug entry may exist in T-type channels.

There is precedent for extracellular entry of charged drugs into ion channels. Quaternary derivatives of local anaesthetics such as QX314 and QX222 (QX) can enter some, but not all, isoforms of the voltage gated sodium channel from the extracellular side (Alpert, 1989; Qu, 1995). In particular, the cardiac sodium channel ($\text{Na}_v1.5$) can be blocked by QX from the extracellular side. Mutational analysis has identified specific residues, predicted to be behind the selectivity filter, that are present in $\text{Na}_v1.5$ but not in other sodium channels, which contribute to this accessibility (Qu, 1995; Sunami, 1997; Sunami, 2000; Sunami, 2001). In addition, in L-type channels, a permanently charged, quaternary form of amlodipine (a dihydropyridine) has been shown to enter L-type channels from the extracellular side (Heath, 1997).

We examined a homology model of Ca_v3.1 to look for possible pathways by which charged drugs might enter Ca_v3.1 (Lipkind, 2003). The model suggests that a crevice may exist at the interface between S6 segments of two neighboring domains, behind the P-loop. Because PAAs, like dihydropyridines, predominantly interact with S6 segments of domains III and IV, we considered a possible pathway between the III-S6, III-S5 and IV-S6 segments (Figure 7). This space is predicted to be large enough to accommodate verapamil. Because the selectivity filter in calcium channels is thought to sit close to the extracellular side of the membrane (Lipkind, 2001), this space would be close to the extracellular surface. It is possible that the charged PAAs might access the inner pore of the channel by passing through the thin, disordered region of membrane separating this crevice from the extracellular milieu.

We sought to test this possibility by introducing bulky residues that would be predicted to protrude into the crevice and might interfere with the movement of drugs through this space. Substitution of an Ile for Ser-1484 of the domain III P-loop, which would form the top of this cavity, had no effect on the ability of D575 to block these channels (20 μ M, data not shown). However, substitution of a tryptophan, the amino acid with the largest indole side chain, for Leu-1825 of domain IV-S6, which is hypothesized to form one of the side walls of the crevice, produced a channel in which the development of use dependent block by 20 μ M D575 was significantly slowed (Figure 8A). The tau for onset of use dependent block in L1825W channels from the outside was 11.4 ± 0.4 pulses, versus 4.4 ± 0.4 pulses for WT channels ($p < 0.001$). In contrast, the time course of block of L1825W channels from the inside was significantly faster than the time course of

block for WT channels for all voltages except the most positive ($p < 0.001$). In further support of the idea that an access pathway was blocked by the tryptophan, application of D575 to L1825W channels from the inside produced more block than the same concentration in the WT, both at 200 μM and at 20 μM (supplemental figure 6). This suggests that D575 may escape from WT channels, even though it cannot cross the membrane, by moving through the extracellular access pathway, thereby accounting for the less than complete block observed. Partially occluding this pathway by introducing a tryptophan restricts movement of drug through the channel, resulting in increased apparent affinity for D575 when applied the inside. The Van der Waals volumes of leucine and tryptophan are 124 \AA^3 and 163 \AA^3 , respectively (Creighton, 1993). Therefore, the tryptophan side chain may restrict the putative pathway for verapamil by approximately 40 \AA^3 . The restriction introduced by the tryptophan also introduces an additional kinetic barrier to the entry of D575 from the extracellular side of the channel to the inner pore. The ability of alteration of one residue to affect both intracellular and extracellular block by D575 supports the interpretation that a pathway, rather than a binding site, is being perturbed.

Discussion

There is extensive literature regarding the sidedness of PAA block of L-type channels. Mannhold et al, in 1978, established the idea that verapamil binds from the intracellular face of the channel by demonstrating, as part of a large structure-function study, that a permanently charged derivative of verapamil (referred to as H1 in their study) was not effective in preventing cardiac muscle contraction when applied in the bath (Mannhold, 1978). Subsequently, the PAA D600 was shown to be effective from both sides, while a permanently charged version, D890, was effective only when injected inside cardiac myocytes (Hescheler, 1982). Permanently charged D888 is unusual among PAAs, as it appears to block L-type channels from either side of the membrane (Berjukov, 1996). However, the effects of permanently charged D888 are not symmetrical — block from the inside is predominantly use dependent, whereas block from the outside is primarily tonic, leading these investigators to suggest that L-type channels may have separate inside and outside binding sites. In general, most studies suggested that PAAs accessed their binding site from inside the cell.

Neutral PAAs, which can pass through the membrane, should be effective regardless of whether they are placed in the pipette or in the bath. However, block of L-type channels by neutral PAAs included in the pipette was not always observed (e.g. LeBlanc and Hume, 1989). Neutral PAAs have been problematic in other channels as well. In $K_v1.3$, for example, intracellular verapamil was thought to be ineffective, until it was tested in the inside-out patch configuration (Rauer, 1996). Concentrations of drug that have no effect in whole cell patch clamp potentially block potassium channels in this configuration,

while permanently charged PAAs are active in both configurations. This strongly supports the idea that the physical properties of verapamil and its analogs allow them to diffuse so readily out of cells into the bath that the concentration at the channel is too low for block to be observed. This may explain why Hescheler et al (1982) were able to see block with neutral D600, as they used a pressure injection rather than passive diffusion. In our hands, very large concentrations (two orders of magnitude higher than the ED_{50} for extracellular block) were required in order to achieve robust inhibition with neutral drug from the inside.

The major finding of this study of T-type channels is that PAAs produce block when applied either intracellularly or extracellularly. Both permanently charged verapamil and permanently charged D888 produced block from the extracellular side, with potencies indistinguishable from their neutral counterparts. When a large amino acid side chain was introduced into a potential extracellular access pathway, use dependent block by charged drugs was significantly slowed, and block by intracellularly applied permanently charged drugs increased.

Since permanently charged drugs are unable to move freely across the membrane, a given concentration of charged drug would be expected to be equally effective from either side. This was not the case in T-type channels – permanently charged drugs were more effective when on the extracellular side. The passage of time did not increase the amount of use dependent block, nor did it accelerate the time course, as one might expect if diffusion out of the pipette were the limiting factor (supplemental figure 3). The ability of

permanently charged drugs to block from the outside, as well as the difference in efficacy between charged drugs placed on the inside and the outside of the cell is consistent with the existence of two routes for drug access to the binding site with differing kinetic characteristics. When charged drugs are placed on the outside, they move into the channel through the extracellular access pathway, but can then exit either back out of the cell or through the pore to the inside of the cell. Charged drugs on the inside enter through the pore, and have the same two possible exit pathways. The greater effectiveness of charged drugs from the outside of the cell could indicate that the on-rate of drugs moving from the outside is faster than from the inside. Alternatively, a diffusional gradient between the outside of the cell and the binding site could slow exit and might allow for greater rebinding when drugs are present in the bath.

Our examination of the L1825W mutant provides support for the existence of an extracellular access pathway. Development of block by charged drugs on the outside was significantly slowed in the mutant, consistent with reduced movement of the drugs through the extracellular access pathway. Further, low concentrations of charged drugs were more effective inside cells expressing L1825W channels than in cells expressing WT channels. This suggests that reducing the ability of drugs to move away from the binding site through the extracellular access pathway reduces the combined off-rate for drug binding. Notably, access of the permanently charged drug to its binding site is impeded, but not eliminated, by this single amino acid exchange. This may indicate that the extracellular access pathway in the T-type channel is affected by more amino acid residues than the pathway that exists for movement of QX314 through the cardiac sodium

channel, which can be completely blocked (or completely recapitulated) by the substitution of a single amino acid (Qu et al, 1995).

The differing efficacy of charged drugs placed inside and outside the cell could also indicate the presence of two different binding sites with differing affinities for the drugs. However, support for the presence of an extracellular access pathway is provided by the ability of MTSET to rapidly and irreversibly block currents in two different mutant channels with a cysteine introduced into the inner pore of the channel. MTSET had no effect on WT channels, nor on channels with an alanine substitution instead of the cysteine substitution. This argues against modification of an endogenous cysteine revealed in the WT channel, or through a distortion in the channel introduced by the mutation. Further, the block created by MTS modification was sensitive to the species of permeant ion; Ca^{2+} current was blocked to a much higher extent than Na^{+} current, indicating that the residue modified was in the permeation pathway. Taken together, these data support the interpretation that MTSET is able to reach cysteines introduced into the inner pore of the T-type channel from the bath, just as permanently charged drugs can reach their binding site.

Interestingly, the time course of the development of use dependent block was voltage dependent when charged drugs were placed on the inside. This was not observed with neutral verapamil, nor when either neutral or charged drugs were placed in the bath. Movement of charged drugs into the channel was facilitated by increasing depolarization, likely because of interactions between the drug and the electric field. Charged drugs on

the outside were not voltage dependent over a range of potentials from -30 mV to +50 mV. This might indicate that when the charged drug travels through the extracellular access pathway to reach its binding site, the substantially lower dielectric of the hydrophobic pathway in contrast to the hydrophilic environment of the direct pathway through the inner pore might shield the drug from alterations in the field that result from channel depolarization.

Several models of PAA binding to L-type channels exist (Lipkind, 2003; Cheng, 2009). While the models differ with respect to details of the conformation of PAAs in the pore, they agree in many respects. Both models depend, in part, on residues involved in the high affinity D888 binding site present in L-type channels. However, a high affinity site for D888 does not exist in T-type channels. Block by D888 is comparable to block by verapamil in extent, in state dependence, and even in the time course for onset of use dependent block. Importantly, both models place part of PAAs in the domain III-IV interface, with the remainder of the molecule in the inner pore. Using a homology model (Lipkind, 2003) to predict the location of an extracellular access pathway that would reach the PAA binding site is, therefore, reasonable, without regard to the specifics of the modeled drug-channel interaction. We have used a model to predict the location of a pathway to the domain III-IV interface. Mutation of Leu-1825, which is postulated to form the back wall of the pathway, to a Trp, with its voluminous side chain, rendered charged drugs less effective from the outside. Not only was the extent of use dependent block reduced, but the time course of block from the outside was three times slower from the outside than the inside, in contrast to WT channels, where the time course of block

did not differ. These data suggest that a novel pathway for drug access may exist in T-type channels.

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Authorship Contribution

Participated in research design: Bergson, Lipkind, Duban, Hanck

Conducted experiments: Bergson, Lipkind, Lee

Contributed new reagents or analytic tools: Lee, Duban

Performed data analysis: Bergson, Hanck

Wrote or contributed to the writing of the manuscript: Bergson, Lipkind, Duban, Hanck

Other: Hanck acquired funding for this research.

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Footnotes

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Figure Legends

Figure 1: Channel active agents studied. Shown are: *verapamil*, **1** (abbrev.; $R_1=H$, $R_2=OCH_3$). *N-methylverapamil*, **1q** ($R_1=CH_3$, $R_2=OCH_3$). *devapamil*, **2** (D888; $R_1=R_2=H$). *N-methyldevapamil*, **2q** ($R_1=CH_3$, $R_2=H$). \ominus OTs represents the *p*-toluenesulfonate counterion dosed with the synthetic compounds, which will exchange with buffer anions. **CI** is a synthetic control substance, (diethyldimethyl)ammonium tosylate, which has in common with the active agents the quaternary ammonium center and the tosylate counterion.

Figure 2: Block by verapamil and D888 is comparable. A: mean current voltage relationship \pm SEM produced in the absence (open squares) and presence of 20 μ M verapamil (filled squares, $n=6$) or D888 (filled triangles, $n=3$). Currents were normalized to peak current at -30 mV in the absence of drug. Inset: representative currents in the absence (upper) and presence (lower) of 20 μ M verapamil (top) or D888 (bottom). B: mean peak current \pm SEM with the mean control current subtracted to separate the accumulation of inactivated channels from the effect of 20 μ M verapamil (filled squares, $n=4$) or D888 (filled triangles, $n=3$). Bottom inset: representative currents produced by repetitive pulses from -110 mV to -10 mV in control (left) or 20 μ M verapamil (middle) and D888 (right). Top inset: mean peak current \pm SEM elicited by repetitive pulses from -110 mV to -10 mV in control cells (open squares, $n=6$) and presence of 20 μ M verapamil (filled squares, $n=4$) or D888 (triangles, $n=3$). Currents are normalized to the first pulse in the train.

Figure 3. PAAs are effective intracellularly at high concentrations. Mean peak currents \pm SEM normalized to the first pulse of the train, and with the mean control peaks subtracted. 200 μ M verapamil (filled triangles, $n=4$) produced $20\pm3\%$ block by the 30th pulse, while 2 mM verapamil (filled squares, $n=5$) produced $69\pm7\%$ block. Inset: representative currents elicited by repetitive depolarizations from -110 mV to -10 mV with an interpulse interval of 100 ms in a control cell (left), a cell with 200 μ M verapamil (middle) and a cell with 2 mM verapamil (right).

Figure 4. Permanently charged D575 produces block when included in the pipette. Mean peak currents \pm SEM, normalized to the first pulse of the train and with mean control values subtracted. 200 μ M D575 produced $47\pm1\%$ block when stepped to -30 mV (black squares), $63\pm1\%$ block when stepped to -10 mV (open squares), and $75\pm2\%$ block when stepped to $+10$ mV (gray squares) ($n=8$). Left inset: representative currents elicited by repetitive depolarizations from -110 mV to -10 mV with an interpulse interval of 100 ms in a cell recorded with 200 μ M D575 included in the pipette. Right inset: taus from exponential fits show voltage dependence for D575 (filled squares, $n=8$).

Figure 5. Charged PAAs block $\text{Ca}_v3.1$ from the outside. A: mean current voltage relationship \pm SEM produced in the absence (open squares) and presence of 20 μ M D575 (filled triangles, $n=5$) or 20 μ M qD888 (filled squares, $n=5$). Currents were normalized to peak current at -30 mV in the absence of drug. Top inset: fractional block at -10 mV produced by 20 μ M D575 or qD888 is not significantly different from 20 μ M verapamil. $N=3$ for verapamil. Bottom inset: Representative currents in the absence (top) and

presence (bottom) of D575 (left) and qD888 (right). B: both 20 μ M D575 and 20 μ M qD888 produced use dependent block. Mean peak currents \pm SEM, normalized to the first pulse of the train and with mean control values subtracted. N=3 for D575, n=4 for qD888. Top inset: fractional block at the 30th pulse is not significantly different from block produced by 20 μ M verapamil. Bottom inset: representative currents in the absence (left) and presence of D575 (middle) and qD888 (right).

Figure 6. MTSET can access the inner pore of $\text{Ca}_v3.1$ from the bath. A. A view of half of the inner pore of the T-type channel (the interface between domain III-S6 and domain IV-S6). Two of the four S6 helices (domains III and IV) are shown as green ribbons. Domain III-S5 is shown as a pink ribbon, and the domain III P-loop is shown in blue. Gln-1828 and Leu-1831, shown as space-filling images, are predicted to project towards the reader and into the inner pore. B: Mean current voltage relationship \pm SEM produced in the absence (open squares) and presence (filled squares) of 2 mM MTSET in Q1828C channels, with 2 mM Ca^{2+} in the bath. Currents were normalized to peak current at -30 mV in the absence of MTSET. N=3. Inset: sample traces recorded in the absence (top) and presence (bottom) of 2 mM MTSET.

Figure 7: An extracellular pathway for verapamil inside the inner pore of the T-type channel. Verapamil (shown in blue space-filling images) can be accommodated in the interface between domain III-S6, IV-S6 (shown by green ribbons), domain III-S5 (pink ribbon) and the domain III P-loop (blue ribbons). Some amino acid residues bordering this crevice are shown as space-filling images.

Figure 8: Insertion of a tryptophan in the back pathway reduces drug entry and exit. A: Outside. Mean peak currents \pm SEM, recorded with 20 μ M D575 in the bath, in WT (open squares, n=3) and L1825W channels (filled squares, n=6). Currents were normalized to the first pulse of the train and mean control values were subtracted. Insets: representative currents elicited by repetitive depolarizations from -110 mV to -10 mV with an interpulse interval of 100 ms in a cell expressing L1825W, recorded with 20 μ M D575 in the bath. Taus from exponential fits show no change with voltage. B: Inside. (i) Mean peak currents \pm SEM, recorded with 200 μ M D575 included in the pipette, in WT (open squares, n=3) and L1825W channels (filled squares, n=4). Currents were normalized to the first pulse of the train and mean control values were subtracted. Inset: representative currents elicited by repetitive depolarizations from -110 mV to -10 mV with an interpulse interval of 100 ms in a cell expressing L1825W, recorded with 200 μ M D575 in the pipette. (ii) Taus from exponential fits show voltage dependence for D575 in both WT (open squares) and L1825W channels (filled squares). (iii) Comparing the extent of block obtained with 200 μ M D575 in WT (dark bars) and L1825W channels (light bars) shows some voltage dependence in each case, with much greater block obtained in L1825W channels at the same drug concentration.

Figure 1

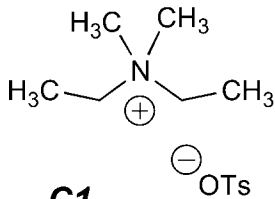
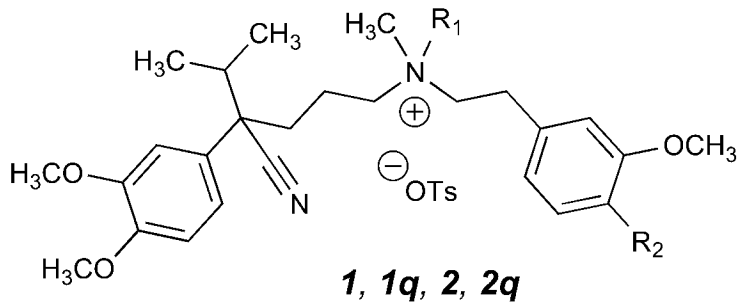


Figure 2

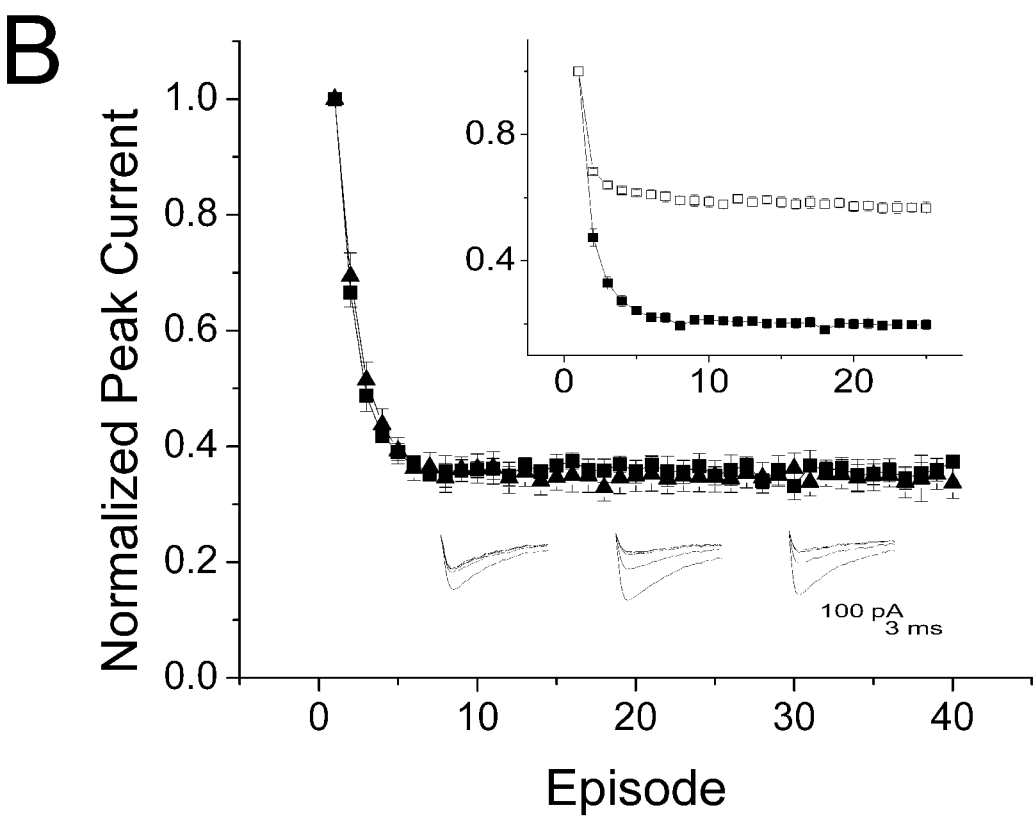
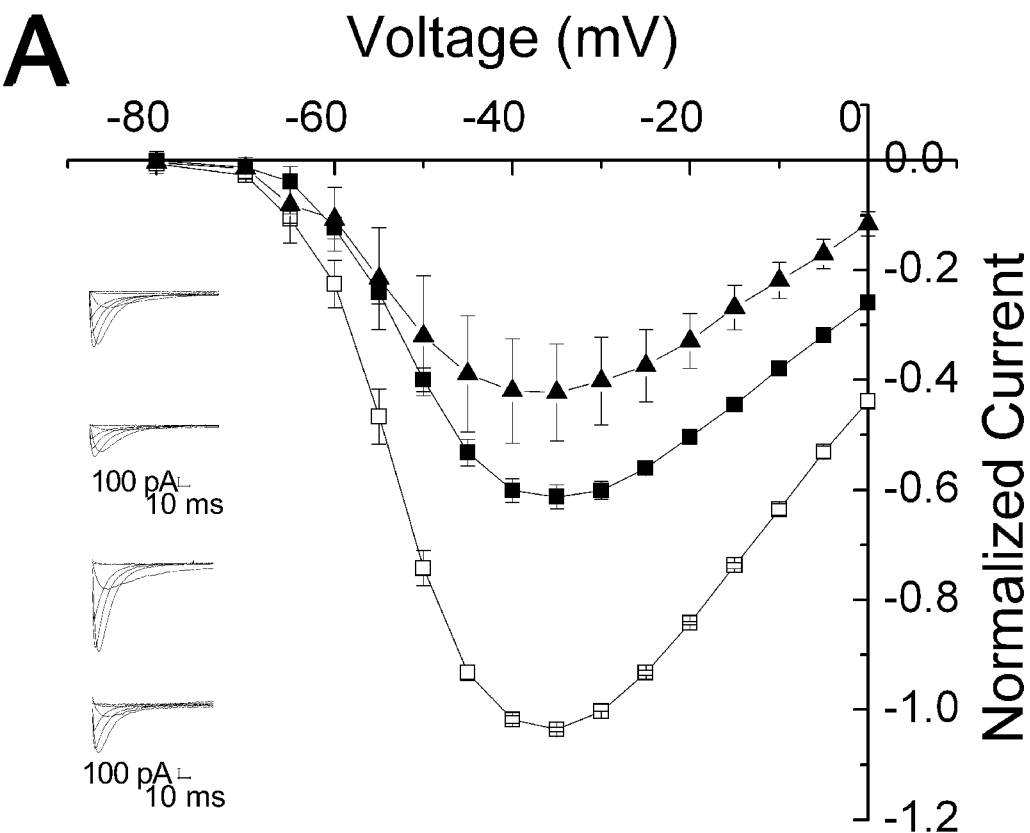


Figure 3

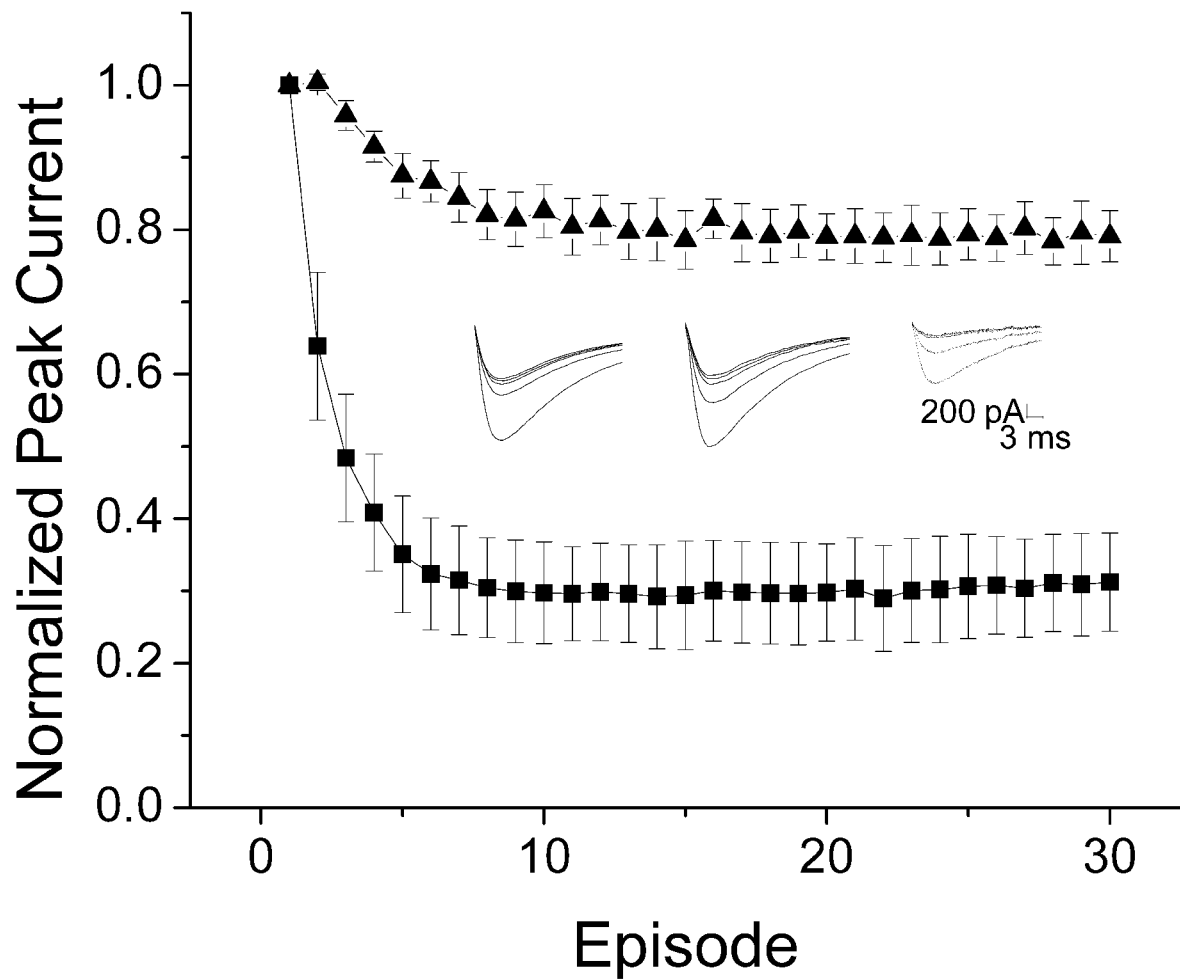


Figure 4

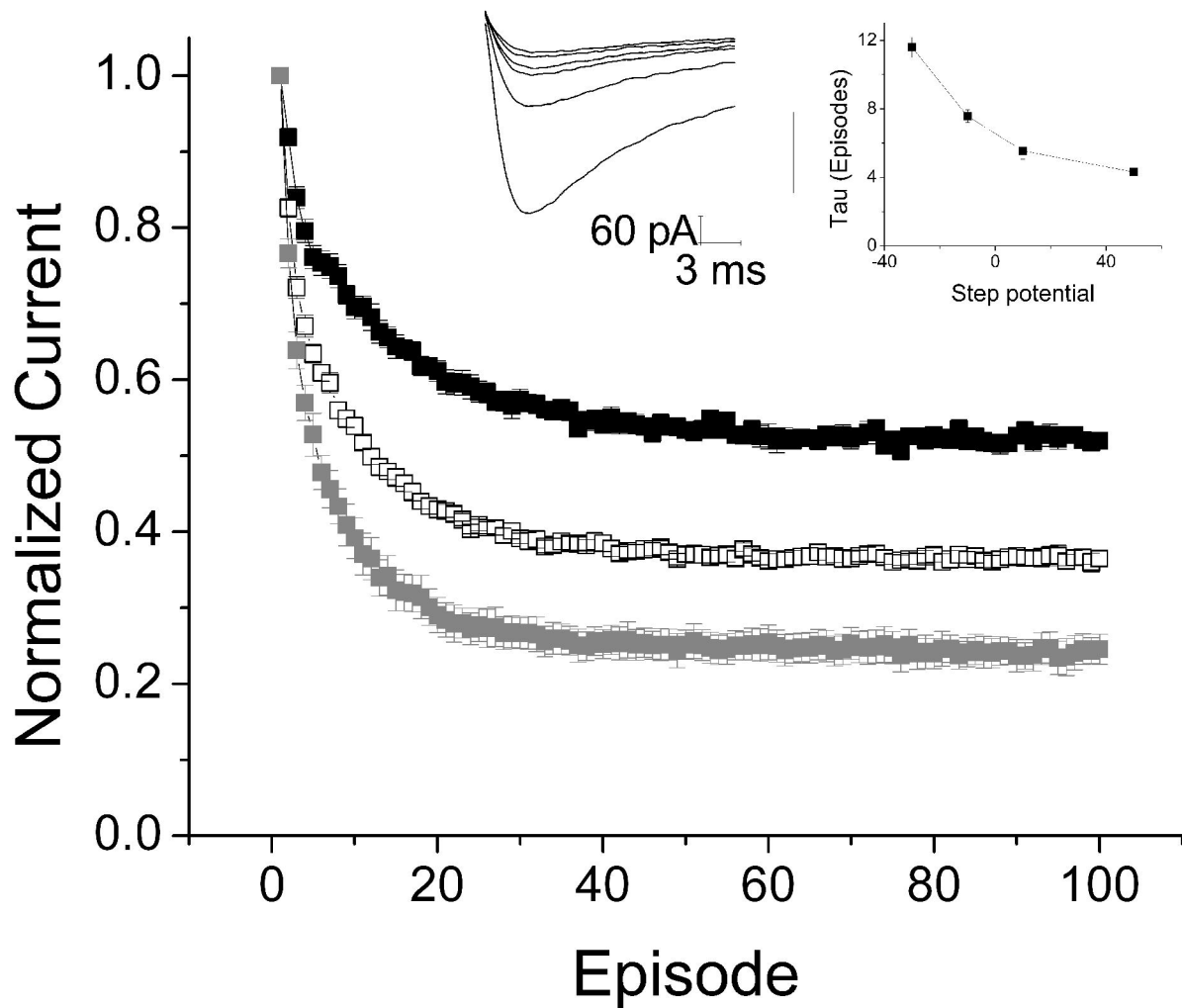
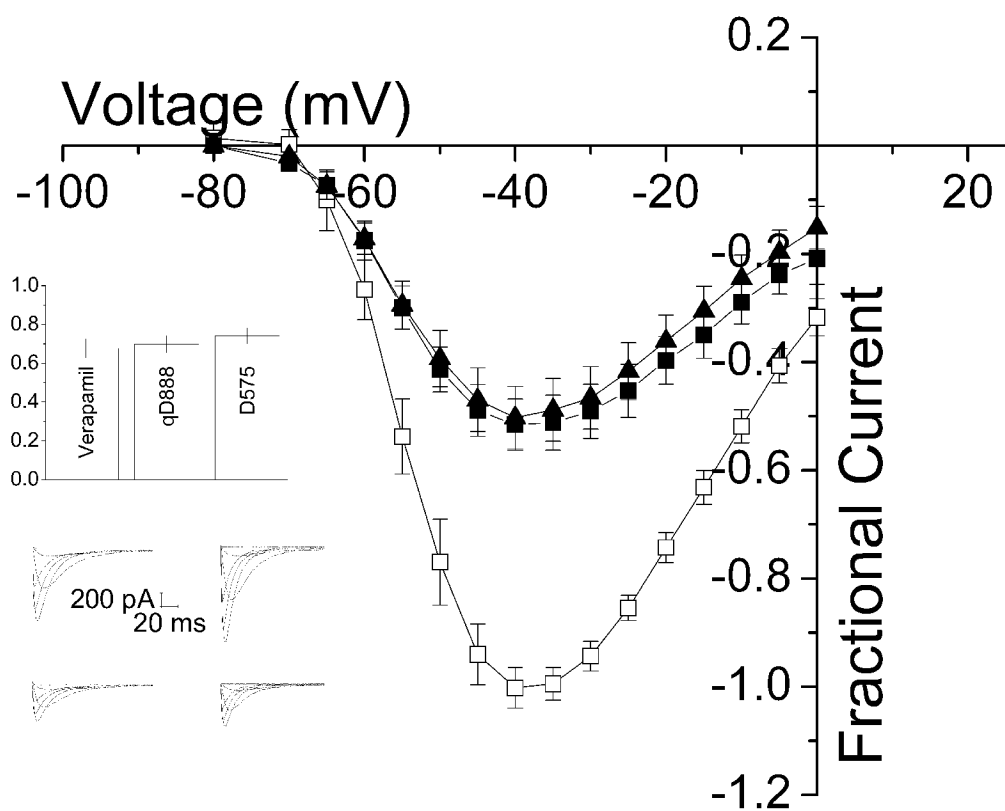


Figure 5

A



B

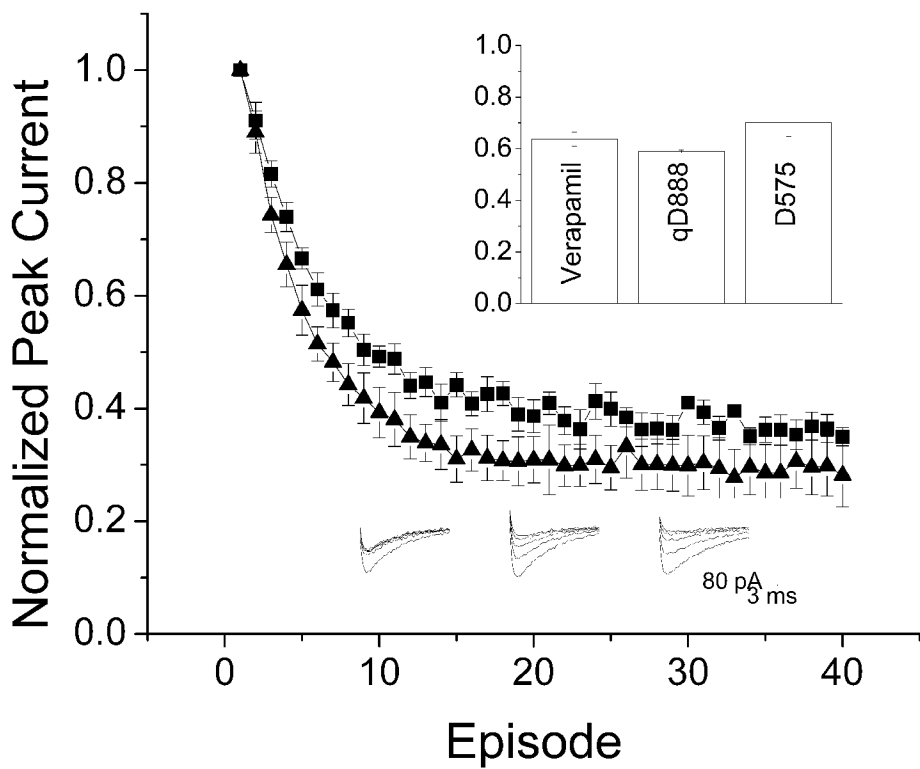
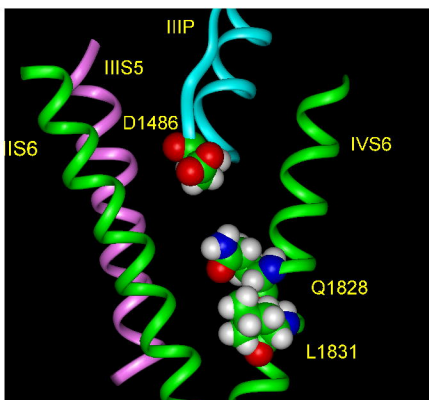


Figure 6

A



B

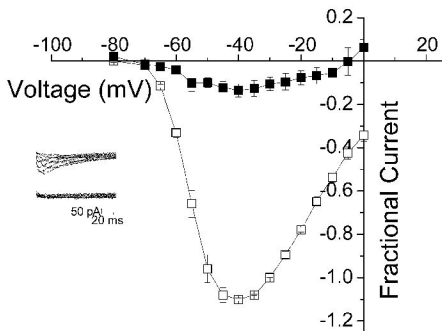


Figure 7

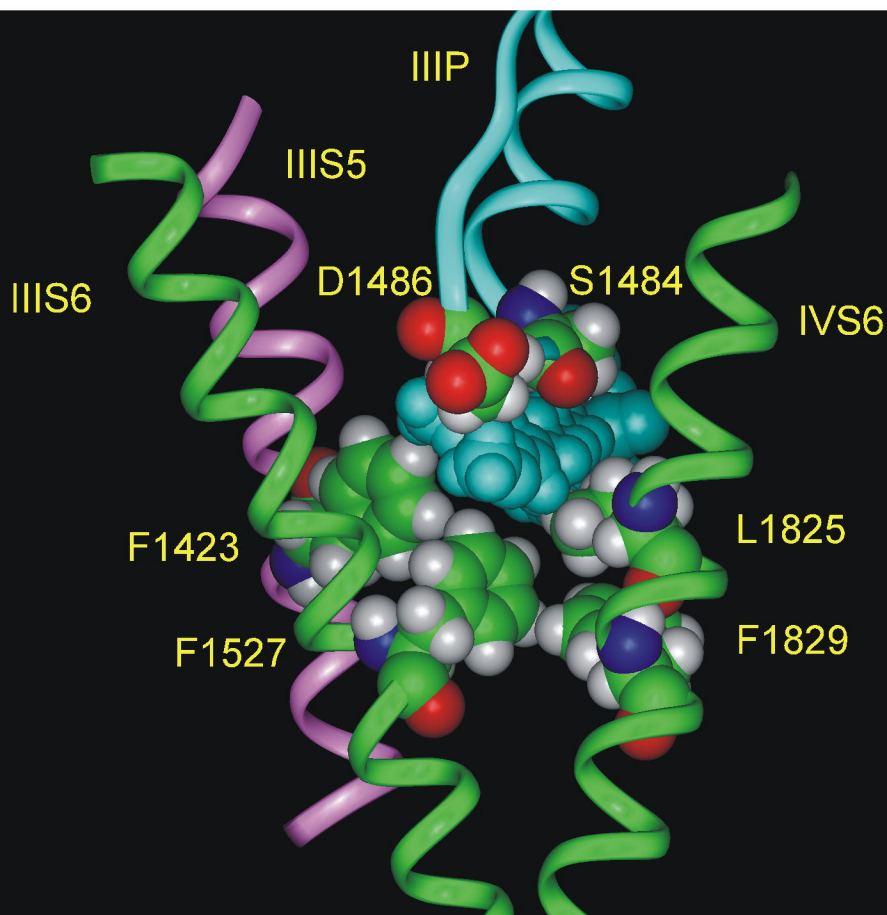


Figure 8

