Exploring the Structure of the Voltage-gated Na\(^+\) Channel by an Engineered Drug Access Pathway to the Receptor Site for Local Anesthetics

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Background: Currently the structure of eukaryotic voltage-gated Na\(^+\) channels (VGSCs) is predicted from available prokaryotic VGSC crystals.

Results: In a mammalian VGSC, the predicted position of a multifunctional tryptophan in the external vestibule enabled design of a second conduction pathway.

Conclusion: External parts of pro- and eukaryotic VGSCs are structurally similar.

Significance: Engineered external drug access pathways may allow development of novel VGSC modulators.

Despite the availability of several crystal structures of bacterial voltage-gated Na\(^+\) channels, the structure of eukaryotic Na\(^+\) channels is still undefined. We used predictions from available homology models and crystal structures to modulate an external access pathway for the membrane-impermeant local anesthetic derivative QX-222 into the internal vestibule of the mammalian rNa\(_V\), 1.4 channel. Potassium channel-based homology models predict amino acid Ile-1575 in domain IV segment 6 to be in close proximity to Lys-1237 of the domain III pore-loop selectivity filter. The mutation K1237E has been shown previously to increase the diameter of the selectivity filter. We found that an access pathway for external QX-222 was created by mutations of Ile-1575 was abolished by the additional mutation K1237E, supporting the notion of a close spatial relationship between sites 1237 and 1575. Crystal structures of bacterial voltage-gated Na\(^+\) channels predict that the side chain of rNa\(_V\), 1.4 Trp-1531 of the domain IV pore-loop projects into the space between domain IV segment 6 and domain III pore-loop and, therefore, should obstruct the putative external access pathway. Indeed, mutations W1531A and W1531G allowed for exceptionally rapid access of QX-222. In addition, W1531G created a second non-selective ion-conducting pore, bypassing the outer vestibule but probably merging into the internal vestibule, allowing for control by the activation gate. These data suggest a strong structural similarity between bacterial and eukaryotic voltage-gated Na\(^+\) channels.

Voltage-gated Na\(^+\) channels (VGSCs)\(^2\) are membrane proteins responsible for initiation of action potentials in nerve, skeletal muscle, and heart. In terms of structure, these proteins are believed to be formed by four homologous domains (DI–DIV), each of them containing six transmembrane helical segments. The first four segments form the voltage sensor. Segment 5 (S5), segment 6 (S6), and the pore-loop (P-loop) between them give rise to the ion-conducting pore. P-loops form an inverted conelike structure referred to as the outer vestibule that contains the selectivity filter. Although these structural features can be found in the recently published crystal structures of several bacterial VGSCs (1–4), the structure of eukaryotic VGSC remains unknown. Unlike the eukaryotic VGSCs, the bacterial channels are homotetramers, which is especially important when structural considerations regarding the region of the selectivity filter are concerned. The eukaryotic selectivity filter is composed of four different amino acids, each contributed by one of the P-loops of domains I–IV (“DEKA motif”; 5–8). By contrast, in the bacterial VGSCs, each monomer contributes the same amino acid to the selectivity filter. In this work, we explored the applicability of the available bacterial VGSC structures for predicting routes of drug access in a region close to the selectivity filter of the mammalian rNa\(_V\), 1.4 channel. Specifically, we examined the modulation of an external access pathway (EAP) for local anesthetic drugs to their binding site in the internal vestibule. Local anesthetic drugs containing a tertiary amino group are considered to reach this binding site either via the membrane or via the inner channel pore (9). Conversely, quaternary amine local anesthetics are membrane-impermeant and have to enter the channel via the inner pore (10, 11). However, in the native cardiac channel (12, 13) and in several mutant VGSCs, EAPs for permanently charged local anesthetic drugs have been found (14–19). These EAPs have provided insights into the three-dimensional arrangement of the channel protein (20).

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\(^2\)The abbreviations used are: VGSC, voltage-gated Na\(^+\) channel; DI–DIV, domains I–IV; P-loop, pore-loop; IIIP, domain III P-loop; S5, segment 5; S6, segment 6; EAP, external access pathway; TTX, tetrodotoxin; IVS6, domain IV S6; TMA, tetramethylammonium; TEA, tetraethylammonium; MD, molecular dynamics; rNa\(_V\), rat Na\(_V\); hNa\(_V\), human Na\(_V\).
In the first part of this study, we explored an EAP created by a mutation of Ile-1575 in rNaV1.4 (18), originally reported in rNaV1,1.2 (11760A; Ref. 14). In the first structural homology models of the mammalian VGSC developed by Lipkind and Fozzard (21) and Tikhonov and Zhorov (22), Ile-1575 of the domain IV S6 (IVS6) is located immediately lateral from the amino acid Lys-1237 of the domain III P-loop (IIIP), which is part of the selectivity filter of the channel (Fig. 1, A and B). The mutation K1237E has been shown to increase the diameter of the selectivity filter, perhaps by a lateral “swing out” of the IIIP (23–25). If the EAP is located between the IIIP and the IVS6, then the lateral movement of Glu-1237 may obstruct the access for QX-222. Here we refer to this idea as the swing out hypothesis.

Fig. 1 shows a comparison of the selectivity filter region predicted by the homology models based on the crystal structures of the bacterial K⁺ channels KcsA (Fig. 1A and Ref. 21) and MthK (Fig. 1B and Ref. 22), the recently published crystal structure of NaVrh (Fig. 1C and Ref. 4), and a revised homology model (26) based on the structure of the bacterial VGSC NaVAb (Fig. 1D and Ref. 1). Interestingly, with the K⁺ channel-based models, the side chains of Lys-1237 of the domain III selectivity filter (green) and of Ile-1575 of the IVS6 (yellow) are in close proximity to each other (Fig. 1, A and B). Conversely, in the NaVAb-based model, Lys-1237 and Ile-1575 are separated by a tryptophan side chain of the domain IV P-loop (homologous to Trp-1531 in rNaV1.4; Fig. 1, C and D, cyan). This difference from the earlier K⁺ channel-based models is due to the fact that in all bacterial VGSCs crystallized so far (1, 3, 4) the side chain of this tryptophan is directed toward the space between the S6 helix and the pore helix of the previous repeat (Fig. 1, C and D). Hence, if the EAP is located at the region between the domain III selectivity filter and the IVS6, then the VGSC-based crystal structures predict that Trp-1531 may obstruct this access pathway. We will refer to this idea as the “Trp-lock” hypothesis. We found that predictions based on the two mentioned hypotheses are consistent with our experimental data. Furthermore, we used a homology model based upon the recently published structure of NaVrh to define the molecular localization of the experimentally explored EAP.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Detailed methods for the mutagenesis have been published previously (16, 27, 28).

**Transfection Procedure**—tsA201 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 20 units/ml penicillin, and 20 units/ml streptomycin (Invitrogen). Cells were maintained at 37 °C in a humid atmosphere containing 5% CO₂. Mixture of plasmids coding 1.5 µg of rNaV1.4 α subunit, 0.2 µg of VGSC β1 subunit, and 0.02 µg of enhanced GFP were transiently transfected into tsA201 cells in a 35-mm dish (Nunc, Roskilde, Denmark) using TurboFect transfection reagent (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) according to the manufacturer’s instructions. 16 h later, cells were dissociated from the dish surface by treatment with a 0.25% trypsin solution (Invitrogen) for ∼2 min, pelleted, resuspended in growth medium, and allowed to settle to the bottom of the recording chamber. Prior to recording, the growth medium was removed and changed to bath solution.

**Whole-cell Patch Clamp Recording**—Experiments were performed using the whole-cell patch clamp recording technique. Na⁺ currents were recorded using an Axopatch 700B patch clamp amplifier (Molecular Devices, Sunnyvale, CA). Pipettes were formed from aluminosilicate glass (AF150-100-10, Science Products, Hofheim, Germany) with a P-97 horizontal puller (Sutter Instruments, Novato, CA) and had resistances between 1.5 and 2.5 MΩ when filled with the intracellular pipette solution consisting of 105 mM CsF, 10 mM NaCl, 10 mM EGTA, 10 mM HEPES. The pH was set to 7.3 with CsOH. For the experiments shown in Fig. 9A, Cs⁺ was replaced by equimolar concentrations of tetramethylammonium (TMA) or tetraethylammonium (TEA). The bath solution consisted of 140 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES. The pH was set to 7.4 with NaOH. For the experiments shown in Fig. 9B, Na⁺ was replaced by equimolar concentrations of K⁺, NH₄⁺, and Cs⁺.

Voltage clamp protocols and data acquisition were performed with pCLAMP 10.2 software (Molecular Devices) through a 16-bit analog-digital/digital-analog interface (Digidata 1440A, Molecular Devices). Data were low pass-filtered at 10 kHz (~3 dB) and digitized at 100 kHz. In some experiments, QX-222 (Alomone Labs, Jerusalem, Israel) and/or tetrodotoxin (TTX; Latoxan, Valance, France) was dissolved in the intracel-
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lunar or extracellular solution. Drugs dissolved in the extracellular solution were applied via an OCTAFLOW drug application device (ALA Scientific Instruments Inc., Farmingdale, NY). This superfusion system permits a complete exchange of the solutions surrounding the cells under investigation within 100 ms (29). Recording was begun about 5 min after whole-cell access was attained to minimize time-dependent shifts in gating.

Voltage Clamp Protocols—Na\(^+\) currents were elicited by 20-ms pulses to \(-10\) mV at a rate of 2 Hz from a holding potential of \(-120\) mV if not stated otherwise.

To assess recovery from intracellular QX-222 block, pipettes were filled with 500 \(\mu\)M QX-222 dissolved in the intracellular pipette solution. After break-in, cells were held at potential of \(-140\) mV to keep channels in a closed state. After a minimum of 10 min of equilibration, a train of pulses (20-ms pulses to \(-10\) mV at 10 Hz) was applied for 5 s to allow binding of QX-222 upon repetitive opening of the channels. Varying time intervals at a holding potential of \(-140\) mV allowed for subsequent recovery of current amplitude. A final pulse to \(-10\) mV tested for the residual current. Current-voltage relationships were determined by 20-ms voltage steps to various potentials between \(-80\) and \(+50\) mV from a holding potential of \(-140\) mV.

Steady-state Inactivation Curves—From a holding potential of \(-140\) mV, inactivation was induced by 50-ms-long prepulses to various potentials from \(-140\) to 0 mV. The current available after the prepulse was tested by a 20-ms test pulse to 0 mV immediately after the prepulse. Plots of current availability as a function of prepulse potential were fitted with Equation 5.

Current-voltage relationships were recorded at a holding potential \(-140\) mV. Currents were elicited by 20-ms-long voltage steps to various potentials from \(-80\) to \(+50\) mV. The currents were normalized to the respective maximum value. The data points were fitted with Equation 3 to calculate the respective reversal potential.

Data Evaluation—The time course of Na\(^+\) current block was fitted with a single exponential function (Equation 1).

\[
y = 1 - A \times (1 - \exp(-t/\tau_1)) \quad \text{(Eq. 1)}
\]

where \(y\) is the normalized peak inward current, \(\tau_1\) is the time constant of block development, \(A\) is the fractional block amplitude, and \(t\) is the time from start of QX-222 perfusion. The time course of Na\(^+\) current unblock was fitted with Equation 2.

\[
y = (1 - A) + B \times (1 - \exp(-t/\tau_2)) \quad \text{(Eq. 2)}
\]

where \(\tau_2\) is the unblock time constant and \(B\) is the fractional amplitude of recovery from QX-222 block with other parameters as stated above.

Current-voltage relationships were fitted with a function (Equation 3),

\[
G_{\text{max}} \times (V - V_{\text{rev}}) \times (1 - (1/(1 + \exp((V - V_{0.5})/k))))
\]

(Eq. 3)

where \(V\) is the step potential, \(G_{\text{max}}\) is the maximum conductance, \(V_{0.5}\) is the voltage at which half-maximum activation occurred, \(V_{\text{rev}}\) is the reversal potential, and \(k\) is the slope factor.

The time course of recovery from intracellular block was fitted with a double exponential function (Equation 4).

\[
y = -A_1 \times (1 - \exp(-t/\tau_1)) - A_2 \times (1 - \exp(-t/\tau_2)) + C
\]

(Eq. 4)

where \(y\) is the normalized peak inward current, \(t\) is the recovery time, \(\tau_1\) and \(\tau_2\) are the time constants of distinct components of recovery, \(A_1\) and \(A_2\) are the respective amplitudes, and \(C\) is the final level of recovery.

Steady-state inactivation data were fitted with a Boltzmann function (Equation 5),

\[
y = 1/(1 + \exp((V - V_{1/2})/k))
\]

(Eq. 5)

where \(V\) is the voltage of the conditioning prepulse, \(V_{1/2}\) is the voltage at which half-maximum inactivation occurred, and \(k\) is the slope factor. Curve fitting was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Statistics—Data are expressed as means \pm S.E. Statistical comparisons were made using the two-tailed unpaired Student’s \(t\) tests or one-way unpaired analysis of variance with Tukey’s post hoc test for comparison of more than two groups. A \(p\) value < 0.05 was considered as being significant. In figures and tables, the levels of significance are indicated in the following manner: * \(p < 0.05\); ** \(p < 0.01\); *** \(p < 0.001\).

Structural Modeling, Molecular Dynamics (MD) Simulations, and Docking—The coordinates of the bacterial VGSC analog Na\(_{\alpha\text{R}}\) (Ref. 4; a proteobacterium HIMB114; Protein Data Bank code 4DXW; resolution, 3.05 Å) with an inactivated pore gate were used.

MD Simulations—100-ns MD simulation were performed with Gromacs (version 4.5.5-dev; Ref. 30) with 250 mM NaCl solution. All charged residues were treated to keep their charge states at physiological pH 7.4. Simulations were carried out with the AMBER99sb (31) all-atom force field. 1-Palmitoyl-2-oleoylphosphatidylcholine (lipids parameters were derived from Berger et al. (32) and Cordomi et al. (33)), and the TIP3P water model was applied (34). The simulation setups were described before (35).

The simulations were performed in wild-type and mutant Na\(_{\alpha\text{R}}\) channels. Mutant Na\(_{\alpha\text{R}}\) channels were generated in the following manner: to mimic c\(\text{Na}_{\text{L}}\)1.4 W1531G, the homologous residue of chain D, Trp-182, was mutated to a glycine. Furthermore, in Na\(_{\alpha\text{R}}\), the amino acid homologous to Ser-1528 in rNav1.4 is Leu-179, which might impose a stronger steric hindrance for permeation of QX-222 through the potential EAP than a serine. Therefore, in the mutant Na\(_{\alpha\text{R}}\), we also introduced the mutation L179S. The mutants were generated by PyMOL (36).

Pathway Visualization—To visualize the dimensions of the putative pathway formed by W182G/L179S mutant compared with wild-type Na\(_{\alpha\text{R}}\), we used the program HOLE (37). The center of mass coordinates of backbone nitrogen of Gin-175 and backbone oxygen of W182G (chain D) were selected to define the entrance of the EAP. The center of mass coordinates of four equivalent positions at the gate from four subunits
were selected to determine the exit of the EAP. Then, the vector (CVECT in HOLE; Ref. 37) representing the EAP was calculated by these two coordinates. A central point (CPOINT in HOLE; Ref. 37) in EAP was defined by the center of mass coordinates of backbone oxygen of positions 179 and 204.

**Model Building**—In MD simulations of the mutant Na_{v}Rh, we observed water influx behind the selectivity filter in the region previously occupied by the tryptophan side chain. Thus, we adopted a snapshot (when this region was solvated with the maximum number of water molecules) from MD as a starting point for modeling rat Na_{v}1.4 channel. Chains C and D were replaced with rat Na_{v}1.4 sequences of domains III and IV. In the model, the EAP was again visualized using the program HOLE.

**Docking of QX-222**—The QX-222 three-dimensional structure was taken from the PubChem home page. Docking along the EAP in the rNa_{v}1.4 model containing the mutation W1531G was performed with the program GOLD v5.2 (Cambridge Crystallographic Data Centre, Cambridge, UK) using the GOLD scoring function as described before (38). In docking, we selected two arbitrary spheres (radius, 6 Å) covering the EAP as visualized by HOLE (axial length, ~9 Å). All side chains within the spheres were treated as flexible. Within these spheres, 30 docking poses were generated.

**RESULTS**

**Mutations at Site 1575 Open an EAP for QX-222**—Using the *Xenopus laevis* expression system, replacement of Ile-1575 by alanine, cysteine, and glutamate has been shown previously to open an EAP for QX-222 (18). Our first aim was to recapitulate these studies using a mammalian expression system. Furthermore, we examined the effect of engineering a positively charged histidine to this site. As shown in Fig. 2, all tested amino acid replacements at site 1575 allowed for external access of QX-222. I_{1575}A and I_{1575}C produced the greatest amount of fractional block, whereas introduction of charged amino acids at this site was associated with less block (Fig. 2; *p* < 0.05 compared with I_{1575}A).

In all constructs, block development was slow with time constants ranging from 12 to 27 s (Table 1). Thus, both charged and uncharged amino acids engineered to site 1575 open an access pathway for QX-222; however, access is very slow, suggesting the presence of a substantial energy barrier or steric hindrance for drug access.

**Drug Access via Site 1575 Leads to the Internal Vestibule**—The data in Fig. 2 suggest that mutations at site 1575 create an EAP for local anesthetics. Alternatively, mutations at this position may allow for current inhibition by external QX-222 by generating an external drug binding site. A phenylalanine in the middle of the IVS6 (Phe-1579; Fig. 1, red) is a major part of the internal binding site of both amphiphilic and permanently charged local anesthetics (14–16). To test whether external block by QX-222 in the mutants at site 1575 is also mediated by this phenylalanine, we generated the double mutant I_{1575}E/F_{1579}A. As shown in Fig. 3A, this construct was insensitive to block of QX-222 than wild type. Asterisks denote levels of significance compared with wild type (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001). Error bars represent S.E.
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The EAP Is Located Close to the Domain III Selectivity Filter—The experiments shown in Fig. 2 suggest that mutations at site 1575 open an access pathway for QX-222. However, the question arises whether this access pathway is also physically located at site 1575. Alternatively, mutations at this site may produce allosteric effects, opening a pathway at a site distant from 1575. Here we made use of the swing out hypothesis (see the Introduction) that predicts that the EAP opened by mutations at site 1575 should be modulated by mutations at the neighboring site 1237. To this end, we combined the mutants that open the EAP, I1575A, I1575C, and I1575E, with the mutant K1237E. The fractional block was 0.03 \(\pm\) 0.05 (n = 7) and 0.18 \(\pm\) 0.01 (n = 4) in K1237E/I1575C and I1575E, respectively, and 0.01 \(\pm\) 0.02 (n = 5) and 0.27 \(\pm\) 0.04 (n = 7) in K1237E/I1575C and I1575E, respectively. Differences between the single S6 and double mutants were significant (p < 0.005) compared with respective data from I1575A.

The binding site for external QX-222 is in the internal vestibule. Modulation of the EAP by an additional mutation in the selectivity filter and by mutations of Trp-1531 is shown. The stimulation protocol and depiction are the same as in Fig. 2A. A, residue Phe-1579 is considered a major determinant of binding of local anesthetics to the internal vestibule. Here we show that the external block by QX-222 in I1575E is abolished by the additional mutation F1579A. In the double mutant I1575E/F1579A channel, QX-222 block was reduced to the wild-type level (by 0.03 \(\pm\) 0.01 (n = 4) versus by 0.18 \(\pm\) 0.01 (n = 5) in I1575E; p < 0.001). B, the mutation K1237E caused a loss of selectivity for Na\textsuperscript{+} ions and allowed for permeation of large organic cations, perhaps by a lateral swing out of the P-loop in domain III. This hypothesis is supported by the fact that block by QX-222 via the EAP in the mutants I1575E/C was abolished by the additional mutation K1237E. The fractional block was 0.03 \(\pm\) 0.05 (n = 7) and 0.18 \(\pm\) 0.01 (n = 4) in K1237E/I1575E and I1575E, respectively, and 0.01 \(\pm\) 0.02 (n = 5) and 0.27 \(\pm\) 0.04 (n = 7) in K1237E/I1575C and I1575C, respectively. Differences between the single S6 and double mutants were significant (p \(\leq\) 0.005). Error bars represent S.E.

The experiments shown in Fig. 2 suggest that mutations at site 1575 open an access pathway for QX-222. However, the question arises whether this access pathway is also physically located at site 1575. Alternatively, mutations at this site may produce allosteric effects, opening a pathway at a site distant from 1575. Here we made use of the swing out hypothesis (see the Introduction) that predicts that the EAP opened by mutations at site 1575 should be modulated by mutations at the neighboring site 1237. To this end, we combined the mutants that open the EAP, I1575A, I1575C, and I1575E, with the mutant K1237E. The fractional block was 0.03 \(\pm\) 0.05 (n = 7) and 0.18 \(\pm\) 0.01 (n = 4) in K1237E/I1575E and I1575E, respectively, and 0.01 \(\pm\) 0.02 (n = 5) and 0.27 \(\pm\) 0.04 (n = 7) in K1237E/I1575C and I1575C, respectively. Differences between the single S6 and double mutants were significant (p \(\leq\) 0.005). Error bars represent S.E.

The EAP Is Located Close to the Domain III Selectivity Filter—Most interestingly, mutations at site 1531 allowed for very rapid interaction of QX-222 with the binding site. Hence, both the time course of onset of block and of relief from block upon washout was significantly faster with mutations at site 1531 than with mutations at site 1575 (Table 1). This suggests that the EAP opened by mutations at site 1531 is substantially more permeable for QX-222 than the pathway opened at site 1575. It is noteworthy that the amount of steady-state block of W1531G at a holding potential of \(-120\) mV was smaller than that with mutations at site 1575 (Table 1). This may be due to the fact that Gly-1531 not only allows quick access to the binding site but also produces a rapid egress pathway, thereby limiting the steady-state block. To determine the time course of egress from the binding site, we tested the time course of escape of QX-222 when applied intracellularly (see “Experimental Procedures”). As shown in Fig. 5, the time course of current increase following a rapid depolarizing pulse train in the absence of the drug was monoeXponential, reflecting the time course of recovery of channels accumulated in the fast inactivated state during the pulse train. During exposure to intracellular QX-222, this time course could be fit by the sum of two exponential functions, reflecting recovery from fast inactivation of unblocked channels and recovery from drug block, respectively. Drug block recovered with a time constant of \(\sim\)100 s in I1575E and with time constants less than 1.5 s in W1531A and W1531G (Fig. 5).
Thus, mutations at site 1531 open a pathway that allows both fast access and fast egress.

**Trp-1531 Allows for Gated Access in 1575 Mutants**—The fast access pathway created by mutations at site 1531 suggests that Trp-1531 constitutes a substantial barrier for the external access into the internal vestibule. This raises the question how QX-222 can travel into the channel with the mutations at site 1575 in which Trp-1531 may still restrict the access to the internal vestibule. Perhaps Trp-1531 acts like a gate and periodically opens or closes the EAP during channel activation and/or inactivation. In that case, block by QX-222 may be use-dependent. To explore this possibility, we held the channels at a potential of −120 mV while applying QX-222 externally for 120 s. During this period, channels are expected to reside in the closed state. Thereafter, a 2-Hz pulse train was applied for 50 s. The amount of use-dependent block was defined as the ratio of current reduction produced by the first versus the last pulse in the train. Fig. 6 shows that I1575E and W1531A but not W1531G were blocked by QX-222 in a use-dependent fashion. This suggests that both tryptophan and alanine side chains at position 1531 act as a gate, perhaps restricting drug egress between pulses. In W1531G, the amino acid side chain is absent, and this appears to allow access during the resting state of the channel with no additional block during repetitive pulsing. This notion is supported by the data presented in Fig. 5 demonstrating a substantially slower egress of QX-222 in W1531A than in W1531G. However, in these experiments, QX-222 was loaded into the channel from the intracellular space. When applied from the extracellular side as with the experiments in Fig. 6, QX-222 may leave the channel not only via the EAP but also via the internal pore when the activation gate is opened by the test pulses. The likelihood of this escape would be greatly determined by the off-rate from the receptor. In this case, the use-dependent block shown in Fig. 6, A and B, may have resulted from differences in the periodic development of a high affinity state during the pulse train. Because the amphiphilic congener of QX-222, lidocaine, is known to bind to the inactivated state (39), we examined the affinity of QX-222 to the fast inactivated state in W1531A and W1531G. 500 μM QX-222 shifted the steady-state inactivation curve to more hyperpolarized potentials in W1531A but not in W1531G, indicating the loss of high affinity binding in W1531G (Fig. 7 A and B). This suggests that the loss of use-dependent development in W1531G may in part be a result of a loss in high affinity binding. (We did not test possible effects of QX-222 in I1575E because block development in this mutant is very slow such that development of steady state during the 50-ms conditioning prepulse is unlikely.) Conversely, if the sole difference between binding of QX-222 to W1531A and W1531G were due to a higher affinity of QX-222 for W1531A then the block time constant should be faster with W1531A. However, the time constant of block development is 3 times slower in W1531A than in W1531G, supporting the notion of a substantial difference in drug access between the constructs.

Mutations that alter the external accessibility of QX-222 may also give rise to alterations in the kinetic behavior of the channel. This has been reported previously for mutations at both sites 1575 and 1531 (14, 40, 41). Because QX-222 is likely to bind to the inactivated state (Fig. 7A), we examined the effect of the mutations at sites 1237, 1531, and 1575 on a number of kinetic parameters of fast inactivation (Table 2). The time constant of entry into inactivation was significantly increased only by K1237E/I1575E. With the exception of I1575E/F1579A, all mutants shifted the half-point of inactivation to more negative potentials, suggesting stabilization of the fast inactivated state. Also, the time course of recovery from inactivation was pro-

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**FIGURE 5. Recovery from block by intracellular QX-222.** A 5-s-long 10-Hz pulse train was applied for repetitive openings of the channels to bind QX-222 to the inner vestibule. After the last pulse in the train, the cell was repolarized to −140 mV for various durations followed by a test pulse to −10 mV. The peak amplitudes of the residual sodium currents evoked by the test pulse were plotted as a function of time and fitted with the sum of two exponential functions. Filled symbols indicate drug-free controls, and open symbols represent data with 500 μM QX-222 in the intracellular solution. In I1575E, intracellular QX-222 gave rise to a slow recovering component, indicating the slow egress of QX-222 via the EAP. The time constants of this second exponential recovery were significantly smaller in W1531G and W1531A than in I1575E (0.7 ± 0.2 s, n = 7; 1.8 ± 0.2 s, n = 5; and 105.2 ± 23.1 s, n = 7, respectively; p < 0.01). Error bars represent S.E.

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**FIGURE 6. Use-dependent block by external QX-222.** A 2-Hz pulse train was applied for 30 s at a holding potential of −120 mV. Thereafter, external perfusion with QX-222 was started while cells were kept at the holding potential for 120 s. Then repetitive pulsing was again applied at 2 Hz for 90 s. During this period, block increased in I1575E (A) and in W1531A (B) but not in W1531G (C). Use-dependent block was quantified as the ratio of block present with the first pulse and the block with the 100th pulse (D). *p < 0.05; **p < 0.01; ns, not significant. Error bars represent S.E.
longed by all constructs except I1575E/F1579A. These data confirm previous reports suggesting that the region at the interface between the P-loops of domains III and IV and the S6 segment of domain IV is a critical determinant of inactivation (23, 42–45).

The Mutation W1531G Opens an Access Pathway for Inorganic Ions—Mutations at site 1531 have been shown previously to shift the $V_{\text{rev}}$ to more negative values, indicating a loss of selectivity for Na$^+$ ions (46). In our hands, the $V_{\text{rev}}$ was unchanged by W1531A but was shifted by $\sim$30 mV in W1531G, suggesting a reduced selectivity for permeation of sodium in this construct. This may be the result of structural alterations in the domain IV P-loop region, perhaps as a consequence of increased backbone flexibility at Gly-1531. Alternatively, the access pathway for QX-222 created by the mutation at site 1531 may be permeable for small inorganic ions. If this additional ionic pathway is nonselective, the $V_{\text{rev}}$ of the inward current would be shifted toward zero. To investigate this possibility, we blocked the current through the selectivity filter in W1531G and in W1531A with TTX. Interestingly, both Trp-1531 mutants had highly reduced TTX sensitivity (the $I_{\text{C50}}$ for reduction of peak inward currents was 9.1 ± 0.7 μM, 0.7 ± 0.2 μM, and 2.0 ± 0.5 μM for wild-type, W1531A, and W1531G, respectively; Fig. 8C, inset). Fig. 8, A and B, show the effect of TTX on the $V_{\text{rev}}$ in wild type and W1531G. TTX had no effect on the $V_{\text{rev}}$ in wild type and W1531A channels (data not shown) but substantially shifted the $V_{\text{rev}}$ of W1531G toward zero (Fig. 8B). This suggests the presence of a nonselective ionic pathway that is not blocked by TTX. Interestingly, with W1531G, even high concentrations of TTX blocked only $\sim$80% of the current (Fig. 8C, inset). This

![FIGURE 7. Effect of QX-222 on inactivation properties of channels carrying mutations at site 1531.](image)

**FIGURE 7.** Effect of QX-222 on inactivation properties of channels carrying mutations at site 1531. A, in W1531A, 500 μM QX-222 significantly shifted the steady-state inactivation curve to hyperpolarized potentials. $V_{1/2}$ was $-74.6 \pm 0.6$ and $-80.0 \pm 0.5$ mV under control conditions and with QX-222, respectively ($n = 9, p < 0.0001$). B, in W1531G, 500 μM QX-222 did not produce a significant shift in the steady-state inactivation curve. $V_{1/2}$ was $-82.7 \pm 0.7$ and $-84.1 \pm 0.8$ mV under control conditions and with QX-222, respectively ($n = 6, p = 0.17$). Error bars represent S.E.

![TABLE 2](image)

**TABLE 2**

Kinetic properties and voltage dependence of inactivation in Na$_{\text{v}1.4}$ channel and its mutants

| Time constant of entry into inactivation | Steady-state inactivation ($V_{\text{1/2}}$) | Steady-state inactivation curve slope (Δ) | Time constant of recovery from inactivation |
|----------------------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| NaV1.4 | 0.34 ± 0.02 | 65.6 ± 0.6 | 5.94 ± 0.55 | 0.87 ± 0.06 |
| I1575A | 0.48 ± 0.19 | 80.1 ± 1.0 | 6.72 ± 0.87 | 1.37 ± 0.12 |
| I1575C | 0.43 ± 0.08 | 76.9 ± 1.1 | 7.64 ± 0.97 | 1.09 ± 0.08 |
| I1575E | 0.30 ± 0.02 | 75.0 ± 0.7 | 7.24 ± 0.61 | 1.61 ± 0.12 |
| I1575H | 0.46 ± 0.03 | 84.3 ± 1.0 | 6.31 ± 0.79 | 0.88 ± 0.07 |
| K1237E/I1575A | 0.40 ± 0.02 | 61.6 ± 1.1 | 6.95 ± 0.98 | 0.94 ± 0.18 |
| K1237E/I1575C | 1.05 ± 0.20 | 76.3 ± 0.8 | 5.41 ± 0.79 | 1.22 ± 0.13 |
| W1531A | 0.42 ± 0.04 | 74.0 ± 1.0 | 7.17 ± 0.92 | 1.66 ± 0.14 |
| W1531G | 0.44 ± 0.03 | 82.7 ± 0.7 | 7.53 ± 0.57 | 1.62 ± 0.10 |

* $p < 0.01$ compared with data from wild type.  
* $p < 0.05$ compared with data from wild type.  
* $p < 0.05$ compared with data from wild type.
could indicate that concentrations of TTX >50 μM block 100% of channels and that the residual current is flowing through the EAP. Fig. 8C shows that in the presence of 50 μM TTX 500 μM QX-222 gives rise to an additional block by ~20%. Under these conditions, QX-222 most likely reaches its binding site only via the EAP in channels whose main permeation pathway is blocked by TTX. Furthermore, in TTX-blocked W1531G channels, addition of QX-222 did not produce any significant shift of the EAP in channels whose main permeation pathway is blocked by TTX. Furthermore, in TTX-blocked W1531G channels, this value is close to the calculated Vrev of a perfectly sodium-selective conductance in this solution (68 mV). B, similar experiment to A but the extracellular 140 mM Na+ was replaced by 140 mM NH4Cl (Vrev = 32 ± 1 mV, n = 6), K+ (Vrev = 18 ± 1 mV, n = 4), and Cs+ (Vrev = 4 ± 1 mV, n = 4). Error bars represent S.E.

A Molecular Model of the EAP—As mentioned above, the access pathway by mutations at site 1531 was predicted by the location of the homologous amino acid in published structures of bacterial VGSCs (1–4). We wondered whether the molecular dimensions of such a pathway would be consistent with the available structures. To this end, we examined the crystal structure of NaVrh (4) and an NaVrh-based homology model of the rNaV1.4 for the presence of such EAP. As mentioned under “Experimental Procedures,” we first simulated the mutation to a glycine at a position homologous to Trp-1531 in the NaVrh crystal structure (W182G of chain D). During the MD simulations, the mutated structure remained stable in the vicinity of Gly-182. The space previously occupied by the side chain of Trp-182 was filled by water molecules (Fig. 10A). Fig. 10D shows that the algorithm HOLE identified a contiguous pathway in this mutated crystal structure but not in the wild-type channel (Fig. 10C). This pathway was parallel to the ion-conducting pore in the outer vestibule at the interface between chains C and D. This snapshot of the MD simulation was used for building a homology model of rNaV1.4. In the mutated homology model (W1531G), the docking procedure (see “Experimental Procedures”) resulted in a homogeneous distribution of docking poses of QX-222 molecules along the EAP in the NaVrh-based homology model. C, in the wild-type NaVrh crystal structure, the HOLE algorithm was unable to identify a continuous tunnel (orange object) from the extracellular space to the inner vestibule between IVS6 and IIIP. The arrow indicates the site of interruption of the pathway. D, after introduction of the mutation W182G, the pathway identified by HOLE (orange object) becomes contiguous.

DISCUSSION

In this work we used the modulation of EAPs for QX-222 to test structural predictions from homology models as well as available crystal structures of bacterial VGSCs.

The EAP Opened by Mutations at Site 1575—Previously, Sunami et al. (18) demonstrated that not only substitutions of the native isoleucine by alanine, (resulting in a shorter side chain at site 1575) opened an EAP but also replacements with larger and more reactive side chains (cysteine) or introduction of a negative charge (glutamate). These latter substitutions produced a stronger block by QX-222 than the replacement by alanine. In our study, however, charged substitutions at site 1575 produced less block than the uncharged mutation I1575A. Perhaps these discrepancies are due to the use of different expression systems, i.e. Xenopus laevis oocytes in Sunami et al. (18) versus mammalian cells in the present study. In addition to this earlier report, we show that a positively charged histidine at site 1575 also allows accessibility by QX-222. In summary, these data suggest that the physiologic closure of the EAP is produced by a subtle “con-
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formational balance” at site 1575 that is easily disrupted by introduction of both steric and electrostatic influences.

Topology of the DIV-S6 EAP—Using a homology model of the Naᵥ1.5 channel, Bruhova et al. (20) proposed that QX-314 enters the internal vestibule through a “sidewalk” between segments IIIP, IIIS6, and IVS6. Tikhonov and Zhorov (22) suggested that this access pathway may be generated by a tunnel formed by the inner helices of repeats III and IV and the IIIP. Thus, we propose that the EAP created by mutations at site 1575 opens a pathway between Lys-1237 of the PIII and Ile-1575 of IVS6. To provide an experimental assessment of this molecular localization of the EAP, we tested the swing out hypothesis, which states that the mutation K1237E should modulate the EAP created by mutations at site 1575 (see the Introduction). Indeed, as shown in Fig. 3B, the double mutants I1575E/K1237E and I1575C/K1237E were impermeable to QX-222, suggesting that the EAP created by I1575E/C is closed by the additional mutation K1237E (Fig. 3B). The simplest interpretation of this phenomenon would assume a lateral motion by Glu-1237, resulting in an interaction with site 1575, thereby causing a local conformational change that obstructs the EAP. In the case of I1575E/K1237E, the fact that both Glu-1575 and Glu-1237 carry negative charges argues against a simple “closing” of the pathway between the IIIP and the IVS6 because electrostatic repulsion would probably prevent a direct contact between these amino acids. Most likely, a complex structural rearrangement at this location is produced by the double mutant, resulting in the closure of the EAP.

As mentioned earlier, the “swing out hypothesis” is based on homology models of the VGSC that were derived from crystal structures of bacterial K⁺ channels. As shown in Fig. 1, the more recent structures of bacterial VGSC predict a substantially different arrangement of the interface between the P-loops of DIII and IV and the S6 segment of DIV: the tryptophan homologous to rNav1.4 Trp-1531 occupies the space between the homologous positions 1237 (selectivity filter of DIII) and 1575 (DIV-S6). In this structure, a swing out of IIIP can only occur if the side chain homologous to Trp-1531 moves away to open a space between DIV-S6 and IIIP. The fact that mutations at site 1531 open a highly permeable EAP for QX-222 suggests that a conformational change at this site may provide substantial space (Trp-lock hypothesis). Perhaps this space could also be provided if Trp-1531 “flipped” into a different direction. Such “flipping” action of Trp-1531 has been suggested previously by Tsang et al. (41) to reconcile the fact that mutation W1531C is both accessible from the outside (47) and interacts with local anesthetics in the internal vestibule. Furthermore, Payandeh et al. (2) recently presented crystallographic evidence for a conformational change involving homologous position Trp-179 associated with inactivation in the bacterial VGSC NaᵥAb. Thus, in the mutants at site 1575, a molecular rearrangement of the interface between the selectivity filter and the IVS6 may allow for access of QX-222 when the entrance into the internal cavity is cleared by a flipping motion of Trp-1531.

The EAP Opened at Site 1531—One motif of the P-loop region that is shared among bacterial and mammalian VGSCs is a ring of tryptophan residues located two positions upstream of the selectivity filter (the only exception is the tryptophan in the IIIP that is located immediately upstream of the glutamate of the selectivity filter in mammalian VGSCs). As mentioned in the Introduction, the Trp-lock hypothesis states that Trp-1531 represents a substantial steric hindrance for external access of QX-222. This idea is supported by the fact that replacements of Trp-1531 by both alanine and glycine resulted in a significant block by externally applied QX-222 (Fig. 4). Moreover, the development of block was extremely fast, having a time constant of <3 s as opposed to time constants >10 s with mutations at site 1575 (Table 1). As a matter of fact, to date there is no report of such rapid access for charged lidocaine analogues when applied from the external solution. This suggests that the pathway opened by mutations at site 1531 is highly permeable for the QX-222 molecule. Such high permeability is also supported by the experiments presented in Fig. 5 examining the egress of QX-222 once loaded into the internal vestibule from the cytoplasmic side. In both mutants at site 1531, QX-222 escaped rapidly with a time constant of <1.5 s (Fig. 5). In W1531G, the combination of rapid drug access and drug egress may also account for the moderate level of external block by QX-222 (Fig. 4 and Table 1). Previously, Tsang et al. (41) investigated the effect of external application of QX-222 to the mutants W1531C, W1531A, and W1531Y. These authors concluded that the tested mutants were insensitive to external QX-222 (although Fig. 6C of Tsang et al. (41) suggests an ~10% block with W1531A). Conversely, these authors observed a very rapid recovery from block by internally applied QX-314 in the mutant W1531C, which might indicate rapid egress from the binding site similar to the data shown in Fig. 5 of the present study. One reason why Tsang et al. (41) might have failed to observe block during continuous application of depolarizing test pulses may be the low pulsing rate in their experiments (0.25 Hz). Given the rapid egress of QX-222 in the mutants at site 1531 (Fig. 5), the drug most likely escapes between pulses at low stimulation frequencies.

Kinetic Changes Imposed by Mutations at Site 1531—The principle kinetic changes imposed by mutations at site 1531 are as follows. 1) Both W1531A and W1531G gave rise to a substantial shift of the voltage dependence of fast inactivation to hyperpolarized potentials. This shift was larger with W1531G than with W1531A (Fig. 7 and Table 2). 2) External application of QX-222 induced a further hyperpolarizing shift in W1531A but not in W1531G. The half-point of fast inactivation with W1531A during application of QX-222 matched the half-point of inactivation of W1531G under drug-free conditions (Fig. 7). 3) Block by QX-222 is use-dependent in W1531A but not in W1531G (Fig. 6). 4) Block by QX-222 is strongly enhanced by depolarized holding potentials in W1531A but not in W1531G (Fig. 4).

Previously, Tsang et al. (41) reported that the mutant W1531C gives rise to a hyperpolarizing shift of the half-point of fast inactivation by ~30 mV, which is consistent with our findings in W1531A/G. These authors argue that “narrow aromatic substitutions of Trp-1531 intrinsically mimic local anesthetic drug action (or normal drug-bound channels) by negatively shifting the steady-state fast inactivation.” In the following, we expand on this suggestion and propose an idea of the molecular...
underpinnings of the kinetic effects produced by mutations at site 1531.

The DIV-S6 is crucial for the development of inactivation (23, 48–51). As mentioned above, the available crystal structures of VGSCs suggest that Trp-1531 is in close spatial relationship to DIV-S6. Perhaps an interaction of Trp-1531 and DIV-S6 intrinsically destabilizes fast inactivation, resulting in the “physiological” V_{1/2} of inactivation (~65 mV; Table 1). This would explain why mutations at site 1531 stabilize inactivation. This stabilization is smaller with W1531A (why mutations at site 1531 stabilize inactivation. This stabilization is smaller with W1531A (Table 2) because the alanine side chain may still interact with DIV-S6, whereas such interaction would be lost with W1531G (V_{1/2} of inactivation = ~82.7 mV; Table 2). Perhaps position 1575 in DIV-S6 is part of this interaction site, which would explain why mutations at this site also stabilize inactivation (Table 2).

In theory, the stabilization of inactivation with mutations at site 1531 should enhance drug binding to the high affinity inactivated state as has been reported for other mutations that stabilize inactivation (52). Paradoxically, however, nonaromatic substitutions at this site reduce high affinity binding of local anesthetic agents (41). In the present study, QX-222 stabilized inactivation in W1531A but not in W1531G. In the context of our working hypothesis, an interaction between QX-222 and Ala-1531 could disturb the destabilizing activity of Ala-1531 on inactivation, thereby shifting the half-point of inactivation to the same potential as with W1531G (V_{1/2} of inactivation = ~83.2 mV; Table 2). The absence of a side chain in this latter construct may result in a complete loss of destabilization of inactivation and consequently of any modulatory activity by QX-222. The fact that inactivation is stabilized by QX-222 in W1531A but not in W1531G may also account for the finding that use-dependent block (Figs. 4 and 6) and enhanced block at depolarized potentials (Fig. 4) were observed with W1531A but not with W1531G.

Hence QX-222 may act as an allosteric modulator of inactivation, a concept that has been proposed previously as a mechanism for local anesthetic drug action (53). However, with the mutations W1531A/G, the situation is more complicated as these constructs also open an access/egress pathway for these constructs also open an access/egress pathway for QX-222. For example, the fact that QX-222 produces more block at a holding potential of ~90 mV in W1531A than in W1531G (Fig. 4) may partially result from slower drug egress via the EAP in W1531A (Fig. 5).

W1531G Gives Rise to a Second Ion Conducting Pathway—To test for the presence of a second non-selective ion-conducting pathway, we sought to block the main ion-conducting pore by application of TTX. To this end, we had to apply substantially higher concentrations of TTX with the mutants at site 1531 than with wild-type channels. A number of mutations in the region of the selectivity filter have been reported previously to reduce affinity to TTX (for a review, see Ref. 54), but site 1531 has not been tested in this regard. Although the insensitivity to TTX by the mutations at site 1531 may indicate a conformational change at the selectivity filter, the V_{rev} is only shifted in W1531G, not in W1531A, despite similar reductions in TTX sensitivity. Also, a number of isoforms of Na^+ channels are naturally insensitive to TTX despite unchanged selectivity (55). Thus, the function of the selectivity filter may be intact in the mutations at site 1531. Fig. 8 shows that the V_{rev} of W1531G is negatively shifted compared with wild-type channels and that this shift is increased in a concentration-dependent fashion with external application of TTX. This suggests the presence of a second nonspecific ion-conducting pathway in this mutant. No shift in the V_{rev} was observed in W1531A, perhaps because of a lower permeability of this EAP as a consequence of steric hindrance by the alanine side chain. Accordingly, the rate of block development by QX-222 was substantially smaller in W1531A than in W1531G. Perhaps QX-222 can still enter the channel during a “flip” of the Ala-1531 side chain (see above). The notion that the EAP is a separate pore is supported by the fact that upon channel block by TTX addition of QX-222 gave rise to a substantial further reduction in the current as if the QX-222 molecules passing through the EAP can still reach their binding site and block the current through the EAP (Fig. 8C).

The fact that QX-222 did not completely block channels via the EAP may be a consequence of a high off-rate from the receptor such that drug molecules may leave the channel into the intracellular compartment during channel opening.

The EAP appears to support size-specific flow of monovalent cations. The largest cation that could pass ionic current was TMA. This suggests that the EAP has a maximum diameter of ~6 Å. Recently, Prütting and Grissmer (56) reported a mutation in the pore helix of the hKv1.3 channel to create an ion-conducting pore parallel to the central potassium-conducting pore. The proposed pathway is lined by the pore helix and the adjacent S5 and S6, i.e. a similar location as the EAP described in this study. Interestingly, this pathway had a permeability for cations that is comparable with the EAP described in this study (Na^+ > NH_4^+ > Cs^+ > K^+). Furthermore, analogous to the EAP, current flow through this parallel current pathway was unaffected by pore blockers like charybdotoxin and tetrathym- ammonium (56). A comparable pathway running in parallel to the central pore was also proposed from molecular dynamics simulations in KcsA channels (57). How large is the conductance of the accessory ion-conducting pathway? If we assume that this pathway is non-selective for cations, then the calculated V_{rev} would be 6 mV under the given experimental conditions. In W1531G, the V_{rev} was shifted from 63 ± 3 (wild type) to 35 ± 1 mV. Assuming that this shift is produced by the addition of a second conductance with a V_{rev} of 6 mV, the conductance of the additional pathway would be about the same as the ionic conductance through the selectivity filter. Thus, the mutation W1531G may have created a substantial second ion-conducting pore.

A Molecular Model of the EAP—As shown in Fig. 10, both the crystal structure of the VGSC Na_V1.4 and a homology model based on this structure support the idea that QX-222 can pass through the interface between the IIIP and the IVS6 once the steric hindrance of Trp-1531 in rNa_V1.4 or its homologue Trp-182 in Na_V1.4 is replaced by glycines. We adopted the Na_V1.4 x-ray structure to construct our homology model because the filter sequence can be aligned without gaps in contrast to other bacterial crystal structures (3, 4). The docking of QX-222 enabled us to confirm the absence of putative steric constraints along the EAP. However, because of the substantial difference
in primary sequences between prokaryotic and eukaryotic VGSCs, no inferences regarding the site of the extracellular entrance to the EAP can be made. With regard to the interface between the selectivity filter and the inner vestibule, our study suggests strong structural similarities between eukaryotic and prokaryotic VGSCs.

Biological Significance of the EAP—The cardiac sodium channel isoform can be blocked by permanently charged local anesthetics in its native state (12), which may be of substantial clinical importance (13). Qu et al. (15) showed that a molecular determinant of the heart-specific access pathway is a threonine located in the upper DIV-S6 at a site homologous to Cys-1572 in rNaV1.4. The location of this site suggests that it may be a part of the DIV-S6 EAP considered in our study. The natural occurrence of an EAP in the cardiac isoform suggests some biological significance of EAPs, although the exact biological function remains to be determined. In addition, EAPs may be relevant in pathological processes. Thus, a naturally occurring mutation in the cardiac isoform, S1710L, has been reported to enhance external block by QX-314 and in reduced use-dependent block by the clinically used antiarrhythmic drug mexiletine (19). Ser-1710 is located next to the putative selectivity filter in domain IV and, thus, is located close to the domain IV-S6 EAP described in our present study. To the best of our knowledge, there are no reports of naturally occurring mutations at the sites in human sodium channel isoforms homologous to site rNaV1.4 Trp-1531. However, a naturally occurring mutation in hNaV1.5 one position N-terminal of the position homologous to rNaV1.4 Trp-1531 has been found to be associated with Brugada syndrome (58). This mutation had no effects on ionic selectivity but resulted in changes in inactivation gating. These findings suggest that mutations in the vicinity of Trp-1531 are compatible with life and may result in significant human pathology.

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