Role of Amino Acid Residues in Transmembrane Segments IS6 and IIS6 of the Na\(^+\) Channel \(\alpha\) Subunit in Voltage-dependent Gating and Drug Block*  

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Alanine-scanning mutagenesis of transmembrane segments IS6 and IIS6 of the rat brain Na\(^{+}\),1.2 channel \(\alpha\) subunit identified mutations N418A in IS6 and L975A in IIS6 as causing strong positive shifts in the voltage dependence of activation. In contrast, mutations V424A in IS6 and L983A in IIS6 caused strong negative shifts. Most IS6 mutations opposed inactivation from closed states, but most IIS6 mutations favored such inactivation. Mutations L421C and L863A near the intracellular ends of IS6 and IIS6, respectively, exhibited significant sustained Na\(^+\) currents at the end of 30-ms depolarizations, indicating a role for these residues in Na\(^+\) channel fast inactivation. These residues, in combination with residues at the intracellular end of IVS6, are well situated to form an inactivation gate receptor. Mutation I409A in IS6 reduced the affinity of the local anesthetic etidocaine for the inactivated state by 6-fold, and mutations I409A and N418A reduced use-dependent block by etidocaine. No IS6 or IIS6 mutations studied affected inactivated-state affinity or use-dependent block by the neuroprotective drug sipatrigine (compound 619C89). These results suggest that the local anesthetic receptor site is formed primarily by residues in segments IIIIS6 and IVS6 with the contribution of a single amino acid in segment IS6.

Voltage-gated Na\(^+\) channels are integral membrane proteins that are responsible for the initiation and propagation of action potentials in nerve and muscle cells (1–5). The rat brain Na\(^{+}\) channel as isolated biochemically consists of \(\alpha\) (260 kDa), \(\beta_1\) (36 kDa), and \(\beta_2\) (33 kDa) subunits (2). The \(\alpha\) subunit is composed of four homologous domains (I–IV), each with six transmembrane-reentrant pore loop (P-loop) between segments S5 and S6 (2, 4, 5). Analogous to the structural topology of pore-forming M2 segments of the K\(^+\) channel from Streptomyces lividans (KcsA) (6), the S6 segments from each domain of the Na\(^+\) channel are thought to be arranged in a square array surrounding the inner pore, whereas P-loops from each domain line the outer pore and form the ion selectivity filter (2, 4, 5).

Voltage-gated Na\(^+\) channel activation is thought to result from a voltage-driven outward movement of gating charges, which initiates a conformational change in the protein that opens the channel (7, 8). Analysis of the primary structure of the Na\(^+\) channel \(\alpha\) subunit led to the prediction that S4 transmembrane segments, which contain repeated motifs of positively charged arginine and lysine residues every three amino acids, might serve as the voltage sensor (9, 10). Depolarization of the membrane was proposed to cause the S4 segments to move outward, inducing a conformational change in the pore of the channel resulting in activation. Site-directed mutagenesis studies have demonstrated that the positively charged residues in all four S4 segments contribute to the voltage-dependent activation of the Na\(^+\) channel (11, 12). The proposed outward movement of the S4 segments has been directly detected using mutagenesis, covalent modification, and fluorescent imaging experiments (13–15). Thus, the S4 segments play a critical role in voltage-dependent activation. Na\(^+\) channel fast inactivation occurs within a few milliseconds of channel opening and is mediated by the intracellular loop connecting domains III and IV (16, 17). Mutagenesis studies of this loop revealed three hydrophobic residues (isoleucine, phenylalanine, and methionine (IFM motif)) that are critical for fast inactivation (18). Scanning mutagenesis experiments have identified multiple amino acid residues that may form the inactivation gate receptor within and near the intracellular mouth of the pore, including a cluster of three hydrophobic residues at the intracellular end of segment S6 in domain IV (segment IVS6) (19, 20) and residues in intracellular loops S4–S5 in domain III (21) and S4–S5 in domain IV (22–25). The voltage dependence of Na\(^+\) channel inactivation derives largely from coupling to the activation process (8). Mutagenesis studies have provided strong evidence that outward movement of the S4 segments in domains III and IV initiates a conformational change that leads to fast inactivation of the Na\(^+\) channel by closure of the intracellular inactivation gate (14, 26–28).

Alanine-scanning mutagenesis was previously used to investigate the role of amino acid residues in the S6 transmembrane segments of domains III and IV of the Na\(^+\) channel in voltage-dependent gating and block by clinically important drugs (19, 20, 29–31). A number of mutations in segments IIIIS6 and IVS6 of the rat brain type IIA Na\(^+\) channel \(\alpha\) subunit, designated the Na\(^{+}\),1.2 channel according to the nomenclature of Goldin et al. (32), produced strong shifts in the voltage dependence of steady-state activation and inactivation, suggesting that the native residues at those positions might play a particularly important role in the voltage-dependent gating of Na\(^+\) channels (20, 30). An \(\alpha\)-helical pattern of the shifts in the voltage dependence of activation and inactivation produced by alanine mutations in the inner two-thirds of segment IIIIS6 suggested rotational movement of segment IIIS6 during channel gating (30). Specific amino acid residues in segments IIIIS6 and IVS6...
were identified that form the receptor sites for Na⁺ channel pore-blocking drugs such as local anesthetics and antiarrhythmic and anticonvulsant drugs (29–31). Mutations F1764A and Y1771A in segment IVS6 of the rat brain Na⁺,1.2 channel reduced the affinity of inactivated Na⁺ channels for the local anesthetic etidocaine by 130- and 35-fold, respectively (29). Mutation of Phe1764 and Tyr1771 in the Na⁺,1.2 channel and their homologs in other Na⁺ channels also substantially reduced block of inactivated Na⁺ channels by other local anesthetics and antiarrhythmic and anticonvulsant drugs (29, 33–39). Less dramatic disruptions in inactivated-state block by etidocaine were observed with mutations L1465A, N1466A, and I1469A in segment IIS6 of the rat brain Na⁺,1.2 channel, resulting in 6-, 8-, and 7-fold reduction in affinity, respectively (30). Mutations L1465A and I1469A also reduced the inactivated-state affinity for the anticonvulsant lamotrigine and its congeners (30). Lysine mutations of rat skeletal muscle Na⁺,1.4 channel Ser1276 and Leu1280 (homologous to Leu1465 in the rat brain Na⁺,1.2 channel) in segment IIS6 reduced the inactivated-state affinity for the local anesthetic bupivacaine by 7–17-fold (40). Lysine mutations of rat skeletal muscle Na⁺,1.4 channel Asn434 and Leu437 in segment IS6 reduced the inactivated-state affinity for etidocaine by 7- and 3-fold, respectively (41).

In this work, we have undertaken a systematic analysis of the role of segments IS6 and IIS6 of the rat brain Na⁺,1.2 channel in channel gating and in block by local anesthetics and anticonvulsants using alanine-scanning site-directed mutagenesis. Our results identify individual residues in segments IS6 and IIS6 that are important for Na⁺ channel activation and inactivation gating and also define novel determinants of the receptor site for the local anesthetics.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis of Rat Brain Na⁺,1.2 Channels**—Mutations were prepared by a two-step PCR protocol using two mutagenic primers and two restriction site primers. The mutagenic fragment and the plasmid pCDM8-Na⁺,1.2 were digested with BstEII and BglI restriction endonucleases. The mutagenic fragment was then subcloned into the pCDM8-Na⁺,1.2 plasmid via those restriction sites. The mutations were confirmed by restriction mapping and DNA sequence analysis.

**Na⁺ Channel Expression in Xenopus Oocytes**—Plasmids encoding wild-type and mutant Na⁺ channel α subunits and wild-type β₂ subunits were linearized, and RNA was transcribed as described previously (20). *Xenopus laevis* oocytes were harvested, maintained, and injected with RNA by standard methods as described previously (20) with the following modifications. Oocytes were separated and defolliculated by shaking gently for 2 h in 1.5 mg/ml collagenase in 82 mM NaCl, 20 mM MgCl₂, 2 mM KCl, and 5 mM HEPES (pH 7.5). After an overnight incubation at 18°C in 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES (pH 7.4) supplemented with 5% horse serum and 50 mM mg/gentamicin, healthy stage V and VI oocytes were pressure-injected with 55 nl of a 10:1 mixture of wild-type β₂ and wild-type or mutant rat brain Na⁺,1.2 channel α subunit mRNA, with the α subunit concentration ranging from 10 to 100 ng/μl of injected solution.

**Two-microelectrode Voltage-clamp Recordings from Oocytes—Na⁺ channels** were obtained from injected oocytes using a Dagan CA-1 voltage clamp as described (20). The bath was continuously perfused with Ringer’s solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES (pH 7.2), adjusted with NaOH. Recording electrodes contained 3 M KCl and had resistances of <0.5 megohms. The stock solution of etidocaine (Astra) was dissolved in dimethyl sulfoxide, and sipatrigine (compound 619C89; a generous gift of Dr. Jeff Clarke, Clovis Pharmaceuticals) was prepared in 25 mM HCl. Drug stocks were then diluted to the desired concentration in Ringer’s solution.

**Multiple Sequence Alignment of the S6 Transmembrane Segments of the Rat Brain Na⁺,1.2 Channel**—Multiple sequence alignment of all four S6 transmembrane segments of the rat brain Na⁺,1.2 channel was performed using ClustalX (42). The BOXSHADE program (Version 3.21, written by K. Hofmann and M. Baron) was used for shading the multiple sequence alignment with the fraction of sequences that must agree for shading set to 0.8. This threshold is the fraction of residues that must be identical (black) or similar (gray) for shading to occur.

**Three-dimensional Modeling of the S6 Transmembrane Segments of the Rat Brain Na⁺,1.2 Channel**—The KsA channel structure (6) was used for homology modeling of the S6 transmembrane segments of the rat brain Na⁺,1.2 channel using Molecular Operating Environment software (Chemical Computing Group). The proposed pore-facing residues of the S6 segments of the Na⁺,1.2 channel (see “Results”) were aligned with the pore-facing residues of segment M2 of KsA. The S6 segments were arranged in a clockwise pattern (43, 44).

**RESULTS**

**Effect of Mutations in Segments IS6 and IIS6 on Voltage-dependent Activation of Na⁺ Channels**—To investigate the functional role of amino acid residues in transmembrane segments IS6 and IIS6 of the rat brain Na⁺,1.2 channel in activation, inactivation, and binding of pore-blocking drugs, we substituted alanine sequentially for the native amino acids at each position from Phe405 to Val424 in segment IS6 and from Phe1465 to Leu1473 in segment IIS6. Alanine substitution changes the size and chemical properties of the residues, but has little or no effect on protein secondary structure (45, 46). We coexpressed the wild-type (WT) or mutant Na⁺,1.2 channel α subunit and the wild-type β₂ subunit in Xenopus oocytes and used two-microelectrode voltage-clamp recording to measure Na⁺ currents (Fig. 1, inset). Twenty-nine mutant Na⁺,1.2 channels conducted sufficient Na⁺ currents for analysis, and 27 of these had rapid kinetics of activation and rapid and complete inactivation like the WT channel (Fig. 1, inset; see Fig. 3 below for analysis of two mutants with altered kinetics of inactivation). Seven alanine mutants in segment IS6 (V408A, S413A, F414A, I417A, I420A, L421A, and V423A) and five alanine mutants in segment IIS6 (V968A, L977A, L979A, L981A, and L983A) did not express sufficient Na⁺ currents for analysis (generally <0.1 μA). Of the seven residues in segment IS6 that did not express...
when substituted with alanine, only L421C expressed sufficient Na⁺ current for analysis when mutated to cysteine.

Only four mutations in segments IS6 and IIS6 caused strong positive or negative shifts in the voltage dependence of activation compared with the wild-type channel. Mutations N418A and L975A shifted the voltage for half-maximal activation positively by +21 and +17 mV, respectively (Fig. 1). Mutations V424A and L983A shifted the voltage for half-maximal activation negatively by −11 and −10 mV, respectively (Fig. 1). The slope factor of the voltage dependence of activation was significantly less steep for N418A \((k = 8.0 \pm 0.2 \text{ mV})\) and L421C \((k = 7.6 \pm 0.4 \text{ mV})\) compared with the wild-type channel \((k = 5.8 \pm 0.2 \text{ mV})\).

Effects of Mutations in Segments IS6 and IIS6 on Steady-state Inactivation of Na⁺ Channels—Mutations throughout segments IS6 and IIS6 caused significant shifts in the voltage dependence of inactivation, which was determined using a test pulse applied after a 100-ms voltage step to the indicated membrane potentials. For example, mutations L416A, L421C, and L983A shifted the voltage for half-maximal inactivation positively by +8, +5, and +3 mV, respectively (Fig. 2). The steady-state inactivation curve for L421C and L983A approached nonzero asymptotes with strong depolarizations (Fig. 2). This residual, non-inactivating component of the Na⁺ current was 6% for L421C and 7% for L983A. Significant positive shifts were also caused by mutations F405A, L411A, Y415A, N438A, and V966A (see Fig. 8, Inactivation Shift). Mutations V424A, M967A, and V974A shifted the voltage for half-maximal inactivation negatively by −7, −11, and −11 mV, respectively (Fig. 2). Significant negative shifts were also caused by mutations I969A, L972A, and N976A (see Fig. 8, Inactivation Shift). Most of the mutations giving significant positive shifts are in segment IS6 (see Fig. 8, red), indicating that native residues at those positions favor closed-state inactivation. In contrast, most of the mutations giving significant negative shifts are in segment IIS6 (see Fig. 8, yellow), so native residues at those positions oppose closed-state inactivation.

Point Mutations in Segments IS6 and IIS6 Affect Open-state Fast Inactivation—Two mutations in segments IS6 and IIS6 caused incomplete inactivation from the open state, resulting in sustained current at the end of a 30 ms depolarization (Fig. 3A). Mutations L421C in segment IS6 and L983A in segment IIS6 exhibited non-inactivating currents, which were −10 and 16% of the peak current, respectively (Fig. 3B). These sustained Na⁺ currents were also observed at the end of 100-ms prepulses in measurements of steady-state inactivation (Fig. 2). Other segment IS6 and IIS6 mutations studied did not affect the inactivation from the open state significantly.

Significant sustained currents produced by mutations L421C and L983A suggest that these two residues might interact either directly or allosterically with the inactivation gate of the Na⁺ channel, which is formed by the intracellular loop between domains III and IV. Leu²¹¹ is positioned near the cytoplasmic end of segment IS6, and Leu²¹³ is positioned at the cytoplasmic end of segment IIS6. Three alanine mutations of residues in the cytoplasmic end of segment IVS6 also caused incomplete inactivation (19, 20). No alanine substitutions disrupted inactivation from the open state in segment IIS6 (30). Taken together, these data suggest an important role of several hydrophobic residues at the cytoplasmic ends of segments IS6, IIS6, and IVS6 in Na⁺ channel fast inactivation gating.

Effects of Mutations in Segments IS6 and IIS6 on the Affinity of Inactivated Na⁺ Channels for Local Anesthetics and Anticonvulsant Drugs—Voltage-gated Na⁺ channel pore-blocking
Role of Na\(^+\) Channel IS6 and IIS6 in Gating and Drug Block

Drugs, including local anesthetics and antiarrhythmic and anticonvulsant drugs, act by inhibiting ionic currents through the channel. The potency of these drugs stems from their ability to selectively block open and inactivated Na\(^+\) channels during abnormal membrane depolarizations and rapid bursts of action potentials that characterize neuronal and cardiac pathologies (47–49). This preferential drug binding to the open and inactivated states rather than the resting channel states can be explained by an allosteric model in which a modulated drug receptor is in a low affinity conformation when the channel is in the resting state and transforms to a high affinity conformation when the channel is opened or inactivated by depolarization (47, 48).

The local anesthetic etidocaine, the anticonvulsant lamotrigine, and its tricyclic congener sipatrigine, formerly termed compound 619C89 (50–52) were previously used to identify specific amino acid residues involved in binding of pore-blocking drugs in transmembrane segments IS6 and IIS6 (29–31). In this study, we used etidocaine and sipatrigine for screening of mutants in segments IS6 and IIS6. Etidocaine is used for epidermal, local, and retrobulbar anesthesia. Sipatrigine is being evaluated in clinical trials for the prevention of neuronal toxicity following stroke (52–55). Both of these compounds have higher affinity for inactivated than for resting Na\(^+\) channels, like other local anesthetics or anticonvulsant drugs, and also are effective frequency-dependent blockers (29–31, 50). Block of inactivated Na\(^+\) channels was determined during a test pulse to 0 mV following a 15-s depolarization to a holding potential at which 70–80% of the Na\(^+\) current was inactivated (Fig. 4A, inset, trace c). At this depolarized holding potential (∼−120 mV for the wild-type Na\(^+\) channel), addition of 5 µM etidocaine reduced the Na\(^+\) current by 70% (Fig. 4A, inset, trace d). Only 5% of the Na\(^+\) current was blocked by the same concentration of etidocaine when the holding potential was −120 mV (Fig. 4A, inset, traces a and b). The apparent dissociation constants for the inactivated state (Kd) for the wild-type and mutant Na\(^+\) channels were determined from the degree of block at the depolarized potential according to Kuo and Bean (56). Wild-type Na\(^+\) channels were inhibited by etidocaine with a Kd of ∼20 µM under our experimental conditions (30). Mutation I409A caused the largest decrease in affinity for etidocaine of all studied mutations (6-fold increase in Kd; p < 0.01 (Fig. 4A)). F410A and V424A in segment IS6 also increased the Kd of etidocaine, but only by ∼2-fold (p < 0.01) (Fig. 4A). Of all the segment IIS6 mutations studied, only F978A and L983A increased the Kd of etidocaine significantly, and their effects were <2-fold (p ≤ 0.02) (Fig. 4B). Interestingly, none of the mutations studied in segments IS6 and IIS6 had significant effects on the affinity of inactivated channels for sipatrigine (data not shown). Likewise, mutations I409A, F410A, N418A, V424A, F978A, and L983A did not significantly affect the affinity of inactivated channels for the anticonvulsant lamotrigine.

Effects of Mutations in Segments IS6 and IIS6 on Resting-state Block by Etidocaine—Block of Na\(^+\) channels at a holding potential of −90 mV mainly reflects drug binding to the resting state of the channel (47, 48, 57). To determine whether segment IS6 and IIS6 mutations affected block of resting Na\(^+\) channels, we applied 15-ms test pulses from a holding potential of −90 mV in the absence and presence of 100 µM etidocaine. No segment IS6 and IIS6 mutations caused significant decreases in resting affinity for etidocaine (data not shown). Mutations Y415A, M967A, N971A, and V974A significantly increased resting block by etidocaine at a holding potential of −90 mV by 3-, 2-, 6-, and 2-fold, respectively (p < 0.01) (Fig. 5). Because of the preferential state-dependent drug binding to inactivated Na\(^+\) channels, it was possible that the apparent increases in affinity were secondary to changes in the voltage dependence of inactivation rather than a reflection of actual changes in resting channel affinity. Mutation Y415A shifted the voltage dependence of inactivation positively by +4 mV, so it opposed inactivation rather than enhancing it; and mutation N971A did not have any effect on the inactivation (see Fig. 8). However, mutations M967A and V974A shifted the voltage dependence of inactivation negatively by −11 mV each (Fig. 2). These negative shifts increased the proportion of inactivated channels at −90 mV, which could have caused the apparent increase in resting-state block for these mutants. Therefore, we examined block at more negative potentials. Consistent with this hypothesis, the affinity of WT channels for etidocaine decreased at more negative holding potentials (Vh) and a greater decrease was observed for V974A and M967A channels. The differences between the WT and mutant channels became insignificant at
The voltage dependence of the equilibrium dissociation constant ($K_r$) for block of resting wild-type and mutant Na$^+$ channels by etidocaine is shown. 15-ms test pulses to 0 mV were applied after stepping to the indicated holding potentials ($V_h$) for 60 s. $K_r$ was calculated according to a single-site binding isotherm: $K_r = [D]/(V/E) - 1$, where $E$ represents the fraction of current remaining at the drug concentration ($[D]$). ○, WT channel; ○, Y415A; ▼, M967A; ▼, N971A; ■, M974A.

the most negative potentials ($p > 0.05$). In contrast, the resting affinities of Y415A and N971A decreased little with further hyperpolarization from −90 mV, and resting affinities for etidocaine were >3-fold greater than that of the WT channel at −120 mV ($p < 0.01$) (Fig. 5), indicating that these mutations increased the affinity of resting Na$^+$ channels for etidocaine.

Effects of Mutations in Segments IS6 and IIS6 on Frequency-dependent Block by Etidocaine and Sipatrigine—Frequency-dependent block of Na$^+$ channels by pore-blocking drugs during rapid trains of depolarizing pulses results from preferential binding of the drug to open and inactivated channels and from slower recovery of drug-bound channels between pulses (47, 48). Etidocaine and sipatrigine are strong frequency-dependent blockers (29–31, 50). To determine whether segment IS6 and IIS6 mutants alter frequency-dependent block by etidocaine, we applied 2-Hz trains of 20-ms pulses to 0 mV from a holding potential of −90 mV. For N418A, activation was shifted positively by +21 mV, and channels activated less steeply with voltage. Therefore, a test pulse of +40 mV was used to compensate for these voltage shifts. Mutation I409A significantly reduced frequency-dependent block by etidocaine, and frequency-dependent block was almost completely abolished by mutation N418A (Fig. 6). No other segment IS6 mutations affected frequency-dependent block by etidocaine, and it was not significantly affected by any segment IIS6 mutation. No segment IS6 or IIS6 mutation had a significant effect on frequency-dependent block by sipatrigine (data not shown).

Effect of Mutations I409A and N418A on Recovery of Na$^+$ Channels from Inactivated-state Etidocaine Block—We studied recovery of etidocaine-blocked inactivated Na$^+$ channels to the resting state for mutations I409A and N418A, which significantly affected voltage- and frequency-dependent block of Na$^+$ channels by etidocaine. We measured the rate of recovery by applying a 500-ms conditioning prepulse to 0 mV (to +40 mV for N418A; see above) to produce drug block of inactivated channels, followed by a recovery interval of variable duration and a test pulse to 0 mV (to +40 mV for N418A). Recovery under control conditions followed a double-exponential time course with fast ($\tau_{fast}$) and slow ($\tau_{slow}$) time constants (Fig. 7, A–C, closed circles). The wild-type channels recovered with $\tau_{fast} = 5.2 \pm 0.8$ ms and $\tau_{slow} = 134 \pm 12$ ms (Fig. 7A). I409A recovered with $\tau_{fast} = 4.5 \pm 0.2$ ms and $\tau_{slow} = 143 \pm 12$ ms (Fig. 7B), which were similar to the wild-type kinetics. In contrast, N418A recovered with $\tau_{fast} = 1.2 \pm 0.2$ ms or ~4-fold faster than the wild-type channels and with $\tau_{slow} = 337 \pm 28$ ms or at least 2.5-fold slower than the wild-type channels (Fig. 7C). This time constant is a minimum estimate because only ~80% of the N418A current had recovered after 4 s (Fig. 7C), whereas 100% of the WT current had recovered after 400 ms (Fig. 7A). These data indicate that mutation N418A is characterized by a slow inactivated state with far slower recovery kinetics than the WT channel. Alanine mutation of the homologous residue in the rat skeletal muscle Na,1.4 channel, Asn134, also enhances slow inactivation (58). Thus, the native asparagine in segment IS6 is critical for setting slow inactivation properties of Na$^+$ channels.

In the presence of 100 μM etidocaine, the fast time constant reflects recovery from inactivation of the small fraction of channels that were not blocked during the conditioning prepulse. The slow time constant reflects slow dissociation of the drug from the channels that were blocked during the conditioning prepulse ($\tau_{drug}$). For the wild-type channels, $\tau_{drug} = 2.2 \pm 0.2$ s (Fig. 7A). For I409A, $\tau_{drug} = 1.7 \pm 0.1$ s, not significantly different from the $\tau_{drug}$ for the wild-type channels ($p > 0.05$) (Fig. 7B). In contrast, N418A recovered with $\tau_{drug} = 0.32 \pm 0.03$ s ($p < 0.01$) (Fig. 7C), 7-fold faster than the $\tau_{drug}$ for the wild-type channels. Furthermore, in the presence of 100 μM etidocaine, N418A reached 100% recovery after ~2 s (Fig. 7C), whereas the control N418A channel had only recovered to ~70% of its initial level by 4 s (Fig. 7A). The dramatically faster recovery of the drug-bound channel indicates that etidocaine impeded entry into the slow inactivated state. The faster recovery compared with the WT channel recovery suggests a lower affinity of the closed inactivated N418A channel for etidocaine compared with the affinity of the WT channels. This faster dissociation of etidocaine from N418A contributes to the reduction in frequency-dependent block of this mutant by etidocaine (Fig. 6). In contrast, the reduction in frequency-dependent block of I409A must be primarily caused by the reduced affinity of this mutant for etidocaine because the mutation has no effect on recovery from etidocaine block.

**DISCUSSION**

Role of Amino Acid Residues in the S6 Transmembrane Segments in Na$^+$ Channel Activation—Our present results on mu-
tions in segments IS6 and IIS6 and previously published data on mutations in segments III6 and IVS6 (20, 30) are summarized in Fig. 8. We used multiple sequence alignment of all four S6 segments to produce a position-dependent plot of shifts in the voltage dependence of activation (Fig. 8, Activation Shift, positions 1–8). Mutations of residues located in the middle part of the S6 segments did not produce significant effects on the voltage dependence of activation (Fig. 8, Activation Shift, positions 9–12). Mutations in the intracellular part of the S6 segments had strong effects on the voltage dependence of activation (Fig. 8, Activation Shift, positions 13–21). Therefore, we suggest that the intracellular part of each of the pore-lining S6 segments plays an important role in the conformational changes leading to the opening of the Na⁺ channel.

Mutations of residues at positions 13 (L975A in segment III6 and V1768A in segment IVS6), 14 (N1418A in segment IS6 and N1769A in segment IVS6), and 17 (I1469A in segment III6 and I1772A in segment IVS6) (Fig. 8, Activation Shift) caused striking positive shifts in the voltage dependence of activation. The positive shifts in activation gating indicate that the native residues at these positions make interactions that stabilize the open state of the channel. Mutations at positions 14 (N1418A in 

![Fig. 7. Recovery of the wild-type and mutant Na⁺ channels from block of inactivated channels by etidocaine. A–C, representative time courses of Na⁺ channel recovery from inactivation under control conditions (○) and in the presence of 100 μM etidocaine (□) for wild-type (A), I409A (B), and N418A (C) channels. Recovery was measured using a 500-ms conditioning pulse to 0 mV (to +40 mV for N418A), followed by a recovery interval of the indicated duration (1.5–4000 ms) at −90 mV, followed by a test pulse to 0 mV (to −40 mV for N418A). The peak test pulse current was divided by the peak conditioning pulse current and plotted against the recovery time interval. The curves are least-squares fits of a two-exponential function to the data.

![Fig. 8. Summary of effect of mutations in all four S6 segments on activation and inactivation gating. Left panel, vertical representation of amino acid sequence alignment of S6 segments in domains I–IV (see "Experimental Procedures"). Segment IS6 residues are from Phe1905 to Ala1935. Segment IIS6 residues are from Phe1936 to Leu1965. Segment III6 residues are from Phe1956 to Ile1973. Segment IVS6 residues are from Phe1976 to Leu1979. Activation Shift, the half-maximal activation voltage (V₀.5) of S6 segment mutants from domains I–IV compared with that of the wild-type Na⁺ channel. The histogram shows the differences in voltage for the half-maximal activation of the wild-type and mutant Na⁺ channels. Mean V₀.5 values were obtained from Boltzmann fits of normalized conductance versus voltage plots as described in the legend to Fig. 1. Bars are colored red for the corresponding mutations in domain I, yellow for mutations in domain II, green for mutations in domain III, and blue for mutations in domain IV. The S6 segments were aligned as described above. Slope Effect, the slope factor of the activation process (k) of the S6 segment mutants from domains I–IV compared with that of the wild-type Na⁺ channel. The histogram shows the differences in k values for the wild-type and mutant Na⁺ channels. Mean k values were obtained from Boltzmann fits of normalized conductance versus voltage plots as described in the legend to Fig. 1. Inactivation Shift, the half-maximal inactivation voltage (V₀.5) of S6 segment mutants from domains I–IV compared with that of the wild-type Na⁺ channel. The histogram shows the differences in voltage for the half-maximal inactivation of the wild-type and mutant Na⁺ channels. Mean V₀.5 values were obtained from Boltzmann fits of normalized current versus voltage plots as described in the legend to Fig. 2. Role of Na⁺ Channel IS6 and IIS6 in Gating and Drug Block]
segment IS6, N1466A in segment IIIS6, and N1769A in segment IVS6), 15 (L1467A in segment IIIS6), and 17 (L421C in segment IS6 and I1772A in segment IVS6) also significantly decreased the steepness of the voltage dependence of the activation process (Fig. 8, Slope Effect). All of the native residues at these positions are proposed to face away from the pore lumen with respect to the local anesthetic etidocaine. Only transmembrane segments IS6 (red), IIIS6 (green), and IVS6 (blue) are shown. Etidocaine (yellow) is shown in stick representation. Residues important in etidocaine binding are shown in space-filling representation. This figure was prepared with MOLSCRIPT and RASTER-3D (70, 71).

A

![Image 1](https://example.com/image1)

B

![Image 2](https://example.com/image2)

FIG. 9. Model of etidocaine binding to transmembrane segments IS6, IIIS6, and IVS6 of the rat brain Na1.2 channel. A, three-dimensional model (see "Experimental Procedures") of the proposed orientation of amino acid residues within the Na+ channel pore with respect to the local anesthetic etidocaine. Only transmembrane segments IS6 (red), IIIS6 (green), and IVS6 (blue) are shown. Etidocaine (yellow) is shown in stick representation. Residues important in etidocaine binding are shown in space-filling representation. This figure was prepared with MOLSCRIPT and RASTER-3D (70, 71). B, α-helical representation showing the axial positions of mutations that caused reduction in the affinity of etidocaine (ETID) for the inactivated Na+ channels.

The position-dependent profile of shifts produced by mutations in the intracellular part of the S6 segments (Fig. 8, Activation Shift, positions 13–20) is also consistent with an α-helical structure of the S6 segments and with a rotational motion of this helix during channel activation. Movements of an intracellular part of the pore-forming S6 segments in the voltage-dependent K+ channels and the corresponding M2 segments in KcsA channels have been proposed to regulate channel opening and closing (59–65). Rotational motion of the pore-forming M2 segments in the KcsA channels about their helical axis during activation gating has been detected using EPR (63, 65). By comparing the structure of KcsA with a newly determined structure of a Ca2+-activated K+ channel from Methanobacterium thermoautotrophicum (MthK), Jiang et al. (60) proposed that the M2 segments rotate and move laterally and outward, resulting in a 12-Å opening at the intracellular aspect of the pore. This movement hinges on a critical glycine residue located approximately halfway through the M2 helix, which is conserved in S6 segments I–III of voltage-gated sodium channels. Our results suggest that the intracellular part of the S6 segments in Na+ channels also may be involved in rotational and translational movements during channel opening.

Role of Amino Acid Residues in the S6 Transmembrane Segments in Na+ Channel Inactivation—The majority of mutations in segment IS6 caused positive shifts in the voltage dependence of inactivation (Fig. 8, Inactivation Shift, red bars), indicating that the native residues at those positions favor inactivation from the closed state. In contrast, mutations in segments IIIS6, IIIS6, and IVS6 produced negative shifts in the voltage dependence of inactivation (Fig. 8, Inactivation Shift, yellow, green, and blue bars), indicating that the native residues at those positions oppose inactivation from the closed state. Large shifts were produced by alanine mutations in the intracellular half of segment IIIS6 (Fig. 8, Inactivation Shift, positions 13–21, green bars), suggesting that the intracellular half of segment IIIS6 plays an important role in a conformational change during the transition from the closed to the open inactivated state. Mutations of several residues in the extracellular half of the S6 segments (Fig. 8, Inactivation Shift, positions 5–7) produced strong negative shifts in the voltage dependence of inactivation.

Role of Amino Acid Residues at the Intracellular End of Segments IS6, IIIS6, and IVS6 in Na+ Channel Open-state Inactivation—Fast inactivation of Na+ channels from the open state is thought to occur by binding of an intracellular inactivation gate formed by the loop between domains III and IV to regions around the channel pore through hydrophobic interactions (8, 66–68). Previous studies have demonstrated that mutation to alanine of three hydrophobic residues at the intracellular end of segment IVS6 strongly disrupts open-state inactivation (19, 20), whereas none of the alanine mutations in segment IIIS6 disrupt open-state inactivation (30). Our present results for transmembrane segments IS6 and IIIS6 demonstrate that mutations L421C in segment IS6 and L983A in segment IIIS6 disrupted inactivation from the open state and caused non-inactivating Na+ currents. Thus, amino acid residues at the intracellular end of segments IS6, IIIS6, and IVS6, but not segment IIIS6, are important for closure of the Na+ channel fast inactivation gate. These are highly specific interactions because >80 other mutations in the S6 segments did not cause non-inactivating Na+ currents.

Alignment of the four S6 segments of the Na+ channel places Leu421, Leu983, and Val1774 the amino acid residues at which alanine substitution greatly impairs open-state inactivation, at positions 17, 21, and 19 of the S6 helices, respectively (Fig. 8). Amino acid residues in equivalent positions are located at the point where the M2 helices cross each other in the closed three-dimensional structure of the KcsA channel (6) and are just beyond the most outward amino acids in the S6 segments that are accessible to methane thiosulfonate reagents applied from the cytoplasmic surface to the closed conformation of the Shaker K+ channel (64). The equivalent amino acid residues in KcsA are thought to move laterally to widen the vestibule of the pore as the channel activates (60, 65, 69). An analogous movement in the sodium channel S6 segments would move the native amino acids near the bundle crossing in the closed state.
sodium channel laterally and reveal newly accessible side chain moieties that form part of the receptor for the inactivation gate and that stabilize the inactivated state. Thus, the specific set of amino acid residues at which alanine substitution disrupts inactivation is likely to become accessible upon channel activation and present a hydrophobic surface to which the IFM motif of the inactivation gate can dock.

Receptor Site for Local Anesthetics and Antiarrhythmic and Anticonvulsant Drugs within Pore-forming S6 Segments of the Na⁺ Channel—Our previous data for transmembrane segments IIIIS6 (30) and IVS6 (29) and our present results on segments IS6 and IIS6 give a more complete view of the molecular determinants of the receptor site for the local anesthetic etidocaine and related pore-blocking drugs. Mutations F1764A and Y1771A in segment IVS6 reduced the affinity of inactivated Na⁺ channels for etidocaine by 130- and 35-fold, respectively (29). Mutation I1760A, also in segment IVS6, did not have a significant effect on etidocaine affinity, but accelerated the off-rate of etidocaine from the closed channels at negative potentials (29). Apparently, mutation I1760A created another pathway for etidocaine to escape from its binding site (29, 33). Mutations L1465A, N1466A, and I1469A in segment IIIIS6 caused 6-, 8-, and 7-fold reductions in the inactivated-state affinity, respectively (30). Of all the mutations studied in segments IS6 and IIS6, only mutation I409A in segment IS6 decreased inactivated-state block by etidocaine by as much as 6-fold. Thus, we propose that Ile⁴⁰⁹ in segment IS6 faces the pore lumen in the inactivated state of the Na⁺ channel, along with the amino acid residues in segments IIIIS6 and IVS6 that bind local anesthetics (Fig. 9). None of the segment IIIS6 mutations studied had any significant effect on the inactivated-state affinity of etidocaine or had an effect on frequency-dependent block by this drug. Therefore, we cannot define any pore-facing residue(s) in segment IIIS6.

Based on the available data for all four S6 segments of the Na⁺ channel, we propose a molecular model for the individual amino acid residues in transmembrane segments IS6, IIIIS6, and IVS6 that form the local anesthetic receptor site (Fig. 9). In addition to our results, site-directed mutagenesis studies of Phe¹⁷¹⁰ and Tyr¹⁷¹⁷ in segment IVS6 of the rat brain Na⁺,1.3 channel (homologous to Phe¹⁷⁶⁴ and Tyr¹⁷⁷¹, respectively, in the rat brain Na⁺,1.2 channel) introduced mutations varying in size, hydrophobicity, and aromaticity and demonstrated in more detail how these residues influence local anesthetic drug binding (38). Block of open and inactivated channels by the local anesthetic tetracaine required an aromatic residue at position 1710 of the Na⁺,1.3 channel. This phenylalanine residue is positioned in the middle of segment IVS6 (Fig. 9) and could stabilize the drug binding to the open or inactivated states by either cation-π or aromatic-aromatic interactions between the aromatic side chain of the amino acid and charged or aromatic moieties on the drug molecule (38). Therefore, Na⁺,1.3 channel Phe¹⁷¹⁰ (homologous to Na⁺,1.2 channel Phe¹⁷⁶⁴), has been proposed to contribute directly to the local anesthetic receptor site (38). In contrast, effects on drug action of mutations of Na⁺,1.3 channel Tyr¹⁷¹⁷ (homologous to Na⁺,1.2 channel Tyr¹⁷⁷¹) were not well correlated with the size, hydrophobicity, or aromaticity of the substituted amino acid. Further studies are necessary to determine whether Ile⁴⁰⁹ in segment IS6 and Leu¹⁴⁶⁵ Asn¹⁴⁶⁶ and Ile¹⁴⁶⁹ in segment IIIS6 contribute directly to the local anesthetic receptor site, but this is the most straightforward conclusion from the experimental results presented here.

None of the segment IS6 or IIS6 mutations studied had any significant effect on inactivated-state affinity or frequency-dependent block by sipatrigine, suggesting that its binding contri-
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