

A Molecular Basis for the Different Local Anesthetic Affinities of Resting Versus Open and Inactivated States of the Sodium Channel

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ABSTRACT

Voltage-gated sodium channels are inhibited by local anesthetic drugs. This inhibition has complex voltage- and frequency-dependent properties, consistent with a model in which the sodium channel has low affinity for local anesthetics when it is in resting states and higher affinity when it is in open or inactivated states. Two residues, a phenylalanine (F1710) and a tyrosine (Y1717), in transmembrane segment IVS6 of the channel α subunit are critical for state-dependent block. We examined how these residues determine channel sensitivity to local anesthetics by introducing mutations that varied their size, hydrophobicity, and aromaticity. Block of resting channels by tetracaine was correlated with hydrophobicity at position 1710, as if hydrophobic drug-receptor interactions stabilize binding to resting states. In contrast, drug action on open or inactivated

channels required an aromatic residue at this position. We propose that the native phenylalanine at position 1710 stabilizes drug binding to 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. We also consider the alternative possibility that mutations at this position affect drug action by either altering access to the receptor or by allosteric changes in receptor conformation. Mutations at position 1717 also altered drug action; however, these effects were not well-correlated with the size, hydrophobicity, or aromaticity of the substituted amino acid. These results suggest that the residue at this position does not contribute directly to the drug receptor.

The voltage-gated sodium channel is responsible for the initiation and propagation of action potentials in neurons (Hille, 1992). Sodium channel function is regulated by voltage-dependent transitions between three sets of functionally distinct conformational states. At resting membrane potentials, most sodium channels are in closed resting states. In response to membrane depolarization, channels first open within a few hundred microseconds, resulting in inward sodium flux, and then convert within a few milliseconds to nonconducting inactivated states. Inactivated channels will not open until they are converted back to resting states by repolarizing the membrane.

In the mammalian brain, the sodium channel consists of a central α subunit (260 kDa) and two smaller auxiliary subunits, designated β 1 (36 kDa) and β 2 (33 kDa) (Hartshorne and Catterall, 1984). The α subunit is the main structural component of the sodium channel and forms the ion-conducting pore, the activation and inactivation gates, and the binding sites for various neurotoxins and therapeutic drugs (Catterall, 1992). The α subunit consists of four quasi-homologous domains

(I–IV), each composed of six hydrophobic segments (S1–S6) that are believed to traverse the membrane as α -helices. The four domains are thought to form a square array in the membrane, with the ion-conducting pore located in the center.

Ionic currents through sodium channels are inhibited by a number of different types of therapeutically important drugs including local anesthetics, such as lidocaine, and anticonvulsants, such as phenytoin (Catterall, 1987). Local anesthetics and related drugs are poor blockers of sodium channels at hyperpolarized membrane potentials, but inhibition is greatly enhanced by prolonged membrane depolarization or high frequency channel activity. According to the modulated receptor hypothesis (Hille, 1977; Hondeghem and Katzung, 1977), inhibition of sodium channels by local anesthetics is voltage- and frequency-dependent because the affinity of the drug receptor on the channel protein depends on whether the channel is resting, open, or inactivated. Resting states, which predominate at hyperpolarized membrane potentials, are thought to bind local anesthetics with low affinity, whereas open and inactivated states, which are more prevalent at depolarized membrane potentials and during trains of channel activity, are proposed to bind local anesthetics with higher affinity.

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ABBREVIATIONS: PCR: polymerase chain reaction; TEA: tetraethylammonium; α III, the type III subtype of the sodium channel α subunit.

The voltage- and frequency-dependent action of local anesthetics has been extensively studied by electrophysiological recording; however, the precise location of the drug receptor and the molecular basis of drug action are less well understood. Biophysical studies suggest that the local anesthetic receptor is located within an inner vestibule of the ion-conducting pore (Strichartz, 1973; Cahalan and Almers, 1979; Gingrich et al., 1993; Zamponi and French, 1994). Site-directed mutagenesis studies (Lopez et al., 1994; Taglialatela et al., 1994; Liu et al., 1997) and recent crystallographic data from a structurally related bacterial potassium channel (Doyle et al., 1998) indicate that S6 transmembrane segments line the inner vestibule of voltage-gated ion channel pores. Thus, it is a plausible hypothesis that the local anesthetic receptor is formed by pore-lining residues within the S6 segments.

A previous study used alanine-scanning mutagenesis to demonstrate that two residues in the S6 segment in domain IV of the sodium channel α subunit are critical determinants of local anesthetic action (Ragsdale et al., 1994). One residue is a phenylalanine located approximately half-way through the transmembrane segment. The other residue is a tyrosine located near the cytoplasmic end of the segment. Mutation of either of these residues to alanine reduced the blocking action of local anesthetics by 1 to 2 orders of magnitude. These two residues were proposed to face toward the channel pore, where they form part of the local anesthetic receptor. In the present study, we have more rigorously examined how these residues influence local anesthetic binding by using site-directed mutagenesis to make a series of amino acid substitutions that vary the size, hydrophobicity, and aromaticity at these two sites. The results of this study support the hypothesis that the critical phenylalanine residue at position 1710 contributes to local anesthetic binding through interactions that depend on hydrophobicity for resting channels or aromaticity for open and inactivated channels.

Materials and Methods

Site-Directed Mutagenesis. Mutations were introduced into the type III subtype of the sodium channel α subunit (α III) (Kayano et al., 1988) using either the Altered States mutagenesis kit (Promega, Madison WI) or polymerase chain reaction (PCR)-based mutagenesis. For mutations using the Altered States kit, a 4.4-kb *Xba*I fragment of α III cDNA containing the region of interest was subcloned into the pAlter vector, and mutations were introduced into this construct following the procedure described in the kit. A 3.3-kb fragment was excised from mutant constructs using *Cla*I and *Spe*I and subcloned back into the full length α III cDNA in the vector pSP64t; the mutated region was then sequenced to confirm the presence of the mutation. PCR-based mutagenesis was performed by the megaprimer method (Barek, 1993). Using α III-pSP64t as a template, a 2.1-kb fragment containing 1.1 kb of α III coding sequence was amplified by two rounds of PCR with *Pfu* Turbo polymerase (Stratagene, La Jolla, CA). The PCR product was cut with *Bst*EII, subcloned into α III-pSP64t, and sequenced to confirm the presence of the mutation.

RNA Preparation. RNA was transcribed from wild type and mutant α III-pSP64t constructs using the mMessage mMachine RNA synthesis kit (Ambion, Austin TX). RNA was resuspended in 0.1 mM EDTA, 5 mM HEPES (pH 7.5); samples of each preparation were analyzed by agarose gel electrophoresis. Total RNA yields for each preparation were estimated by comparing the intensity of ethidium

bromide-stained bands on agarose gels with the intensity of bands corresponding to RNA standards of known concentration.

Isolation and Injection of *Xenopus* Oocytes. Pieces of ovary were surgically removed from female *Xenopus* frogs (*Xenopus* I, Ann Arbor, MI or Boreal, St. Catherine, Ontario), which were anesthetized with 3-aminobenzoic acid ethyl ester. Oocytes were separated and defolliculated by shaking in 1.5 mg/ml collagenase in OR2 (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES pH 7.5). Healthy stage V-VI oocytes were selected and incubated overnight at 18°C in Barth's medium (88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 2.4 mM NaHCO₃, and 10 mM HEPES pH 7.4) supplemented with 50 μ g/ml gentamicin. On the day after isolation, oocytes were microinjected with 50 nl of wild type or mutant α III RNA. The concentration of each RNA was adjusted to give whole cell sodium currents in *Xenopus* oocytes of <10 μ A. Typical sodium currents were between 1 and 5 μ A. Coexpression in *Xenopus* oocytes of α III with auxiliary β subunits results in whole cell sodium currents with highly complex kinetic properties, reflecting multiple gating modes with rates of inactivation and recovery from inactivation that vary over more than three orders of magnitude (Patton et al., 1994; Meadows et al., 1997). In contrast, expression of α III alone results in a functionally more uniform population of channels with intermediate kinetic properties; this allows a more straightforward interpretation of the effects of site-directed mutations. Therefore, in all experiments in this study, wild type and mutant α III subunits were expressed alone, without auxiliary β subunits.

Electrophysiological Recording. After injection, oocytes were incubated for 2 to 3 days at 18°C. They were then examined by two-electrode voltage clamp recording using a Turbo TEC 10C amplifier (Adams & List, Westbury, NY) and pCLAMP software (Axon Instruments, Foster City, CA). Electrodes were filled with 3 M KCl, and had resistances of <0.5 M Ω . Data were filtered at 2.5 kHz and sampled at 20 kHz. Capacity transients were partially compensated using the internal clamp circuitry. Remaining transients, as well as leak currents, were subtracted using the P/4 procedure (Armstrong and Bezanilla, 1974). Oocytes were continuously superfused with frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES pH 7.2). One hundred millimolar stock solutions of tetracaine were prepared in dimethylsulfoxide. Stock solutions were diluted to the appropriate concentration with Ringer's solution, and applied by superfusion. Data were analyzed with pCLAMP. Graphing and curve fitting were performed with SigmaPlot (Jandel Scientific, San Rafael, CA).

Results

Inhibition of Type III Channels by Tetracaine Is Voltage- and Frequency-Dependent. Neurons in the mammalian nervous system express multiple isoforms of the sodium channel α subunit. These isoforms differ in their developmental, regional, and subcellular expression patterns, as well as in their functional properties (Reviewed in Ragsdale and Avoli, 1998). In this study, we examined the type III α subtype (Kayano et al., 1988), which is expressed primarily during embryogenesis (Beckh et al., 1989; Brysch et al., 1991; Black et al., 1994). α III forms functional sodium channels in *Xenopus* oocytes; however, these channels inactivate more than an order of magnitude more slowly than other sodium channel subtypes (Joho et al., 1990; Patton et al., 1994; Meadows et al., 1997). It is not understood why type III channels inactivate so slowly, nor is it known whether this channel inactivates by the same molecular mechanism as other more rapidly inactivating sodium channel isoforms. Because voltage- and frequency-dependent inhibition of sodium channels by local anesthetics depends strongly on chan-

nel inactivation, we began our study by examining how these drugs affect type III channels.

Figure 1A shows typical sodium currents in a *Xenopus* oocyte expressing cloned type III sodium channels. Sodium currents were elicited by depolarizing the membrane to +10 mV from a holding potential of either -100 mV (top two traces) or -40 mV (bottom two traces). In each set of traces, the larger current was elicited in control Ringer's solution and the smaller current was evoked after application of the local anesthetic tetracaine. At -100 mV, virtually all channels were in resting states and current in control was maximal. In contrast, the control trace at -40 mV was approximately 40% smaller than the control trace at -100 mV because 40% of the channels were inactivated at the more depolarized holding potential. Furthermore, it is evident from these traces that tetracaine inhibited a much larger fraction of available current at -40 mV than at -100 mV. This voltage-dependent inhibition is also illustrated in Fig. 1B, which plots current amplitude elicited from a broad

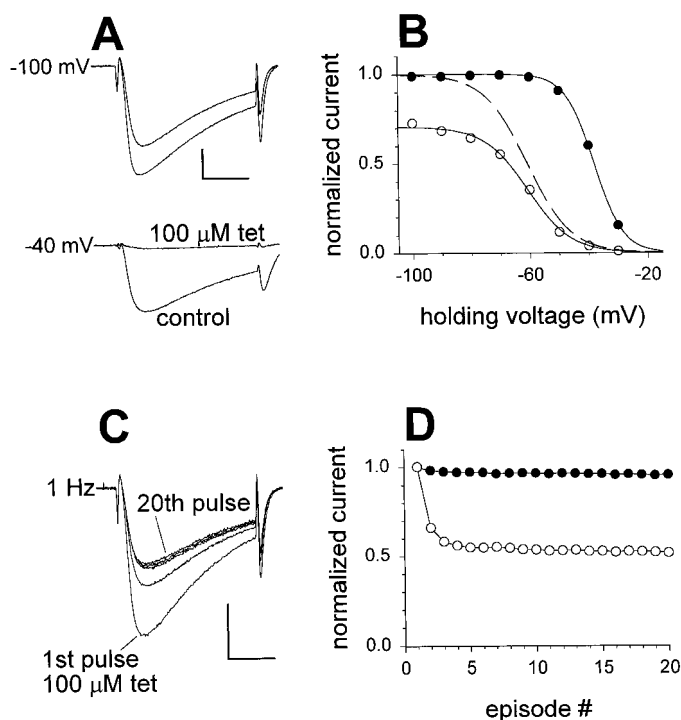


Fig. 1. Voltage- and frequency-dependent block of sodium channels by tetracaine. All four panels show data from the same oocyte. In this and subsequent figures, the concentration of tetracaine was 100 μ M unless otherwise indicated, and all currents were evoked by test pulses to +10 mV. A, inhibition of whole cell sodium currents by tetracaine. Currents were elicited from holding potentials of either -100 mV (top two traces) or -40 mV (bottom two traces). In each set of traces, the larger current was in control conditions and the smaller current was after application of tetracaine. Scale bars are 1 μ A and 10 ms. B, inactivation curves in control (●) and in the presence of tetracaine (○). Current amplitudes were normalized with respect to the largest control currents and plotted as a function of holding potential. The smooth lines are best fits of the equation $a/(1+\exp((V_{\text{hold}} - V_{1/2})/k))$, where V_{hold} is the holding voltage, $V_{1/2}$ is the midpoint of the curve, k is a slope factor, and a is a scaling factor. The dashed line shows the fit of the drug data rescaled to have the same maximum value as the control curve. C, frequency-dependent block by tetracaine. The traces shown were evoked by the 1st, 2nd, 5th, 10th, and 20th pulses in a 1-Hz train, applied from a holding potential of -90 mV. D, amplitudes of currents elicited by 1-Hz pulse trains given in control (●) and in the presence of tetracaine (○). Each set of data points was normalized with respect to the amplitude of the first pulse in the train and plotted as a function of pulse number.

range of holding potentials in control and tetracaine. The control curve shows the voltage dependence of sodium channel steady state inactivation. Comparison of this control inactivation curve with the curve in the presence of tetracaine reveals several important characteristics of local anesthetic inhibition of sodium currents. First, tetracaine block was strongly voltage-dependent in the range of holding potentials over which channels inactivate (-70 to -30 mV), but it approached a voltage-independent asymptote at more hyperpolarized potentials. Second, the midpoint of the curve in the presence of tetracaine was shifted approximately 20 mV negative compared to the control curve, as if the drug enhanced steady state inactivation. This negative shift ($\Delta V_{1/2}$) is easier to see when the drug curve is scaled to the same maximal level as the control curve (dashed line in Fig. 1B). Additional block of type III channels by tetracaine developed during rapid trains of channel activation. For example, tetracaine block increased by approximately 50% over the course of 20 pulses applied at 1 Hz (Fig. 1, C and D). The voltage- and frequency-dependent inhibition of type III channels by tetracaine was virtually indistinguishable from inhibition of rapidly inactivating type IIA sodium channels (data not shown). Thus, despite its slow inactivation time course, the type III channel is blocked by tetracaine in a manner that is characteristic of local anesthetic inhibition of sodium channels.

Voltage- and frequency-dependent inhibition of sodium channels by local anesthetics can be explained by a modulated drug receptor that has a low affinity when the channel is in resting states and a higher affinity when the channel is in open or inactivated states (Hille, 1977; Hondeghem and Katzung, 1977). At strongly hyperpolarized holding potentials (e.g., -100 mV), current inhibition reflects mainly drug binding to low-affinity resting sodium channels. As the membrane is depolarized, the proportion of high-affinity inactivated channels increases, resulting in greater inhibition of sodium currents and a negative shift in the inactivation curve. Frequency-dependent block reflects rapid drug binding to open and inactivated channels that are transiently available during each depolarizing test pulse. Although this modulated receptor model is probably an oversimplification, it accurately describes local anesthetic block; therefore, we use it as the basis for discussion of the effects of mutations on local anesthetic action. Specifically, we assume that block at strongly hyperpolarized holding potentials (e.g., -100 mV) gives a measure of drug action on resting sodium channels, whereas the voltage dependence and frequency dependence of block provide an indication of drug action on open and inactivated channels. The voltage dependence of block was assessed by the magnitude of $\Delta V_{1/2}$ and from dose-effect curves at depolarized holding potentials. Because very few channels opened at the holding potentials used in these experiments, these two parameters mainly reflect preferential drug binding to inactivated states. In contrast, frequency-dependent block of currents elicited by 1 Hz pulses to +10 mV reflects drug action on open and inactivated states.

Mutations of Residues F1710 and Y1717 Alter Tetracaine Inhibition of Type III Channels. It was previously shown that mutation of either a phenylalanine residue or a tyrosine residue in the IVS6 transmembrane segment to alanine reduced sodium channel sensitivity to local anesthetics by 1 to 2 orders of magnitude (Ragsdale et al., 1994, 1996). These studies were performed on the type IIA α subunit,

which is a major isoform in the adult brain. The critical phenylalanine and tyrosine residues in IVS6 are conserved in other α subtypes; however, it is not known whether the importance of these residues in local anesthetic action is also conserved. To investigate this question, we substituted alanine for the homologous phenylalanine (mutation F1710A) or tyrosine (mutation Y1717A) residues of α III, expressed the mutant channels in oocytes, and examined their sensitivity to tetracaine.

For F1710A and Y1717A, $\Delta V_{1/2}$ was greatly reduced (Fig. 2A–D) and frequency-dependent block was almost completely eliminated (Fig. 2, E and F); this suggests that both mutations significantly reduced the affinity of open and inactivated type III channels for tetracaine. For F1710A, resting block was also attenuated (Fig. 2, A and C), indicating that this mutation reduced resting state affinity. Neither mutation dramatically altered current time course (Fig. 2, A and B), the voltage dependence of inactivation (Fig. 2, C and D), or current-voltage relationships (not shown). This indicates that amino acid substitutions at these sites did not result in global changes in protein structure-function. These results are similar to previous findings with type IIA sodium channels (Ragsdale et al., 1994, 1996), and they suggest that the critical role of these two residues in local anesthetic action is conserved at least between the type IIA and type III subtypes of the sodium channel α subunit.

Effects of Different Amino Acid Substitutions at Position 1710. To investigate in more detail how residues at positions 1710 and 1717 determine sodium channel sensitivity to local anesthetics, we introduced a series of amino acid

substitutions that systematically altered the size, hydrophobicity, and aromaticity at these two positions. The effects of substitutions at position 1710 on resting block, $\Delta V_{1/2}$, and frequency-dependent block are summarized in Fig. 3. The amino acids in each panel are arranged in order from the most hydrophilic (serine) to the most hydrophobic (tryptophan) (Hopp and Woods, 1981). Two distinct patterns emerge from this analysis. First, resting block increased steadily with increasing hydrophobicity (Fig. 3A) as if the binding of tetracaine to resting sodium channels involves hydrophobic interactions with the residue at this position. In contrast, $\Delta V_{1/2}$ and frequency-dependent block were large for tyrosine and tryptophan as well as for the wild type phenylalanine, but greatly attenuated for serine, cysteine, alanine, and iso-

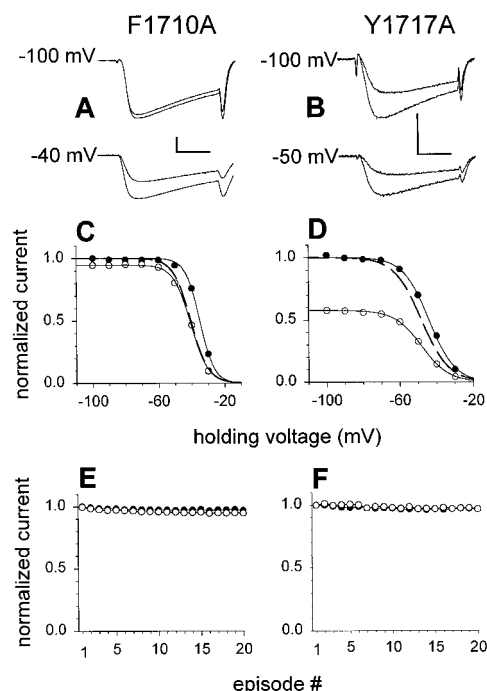


Fig. 2. Mutants F1710A and Y1717A attenuate block of type III channels by tetracaine. A, B, typical currents for mutants F1710A (A) and Y1717A (B) in control and after application of 100 μ M tetracaine. Currents were evoked from the holding potentials shown. Scale bars are 1 μ A and 10 ms. C, D, inactivation curves for F1710A (C) and Y1717 (D) in control (●) and after application of tetracaine (○). The data points and smooth lines were plotted in the same manner as in Fig. 1. E, F, amplitudes of currents for F1710A (E) and Y1717A (F) evoked by 1-Hz pulses given in control (●) and with tetracaine (○). Data were plotted in the same manner as in Fig. 1.

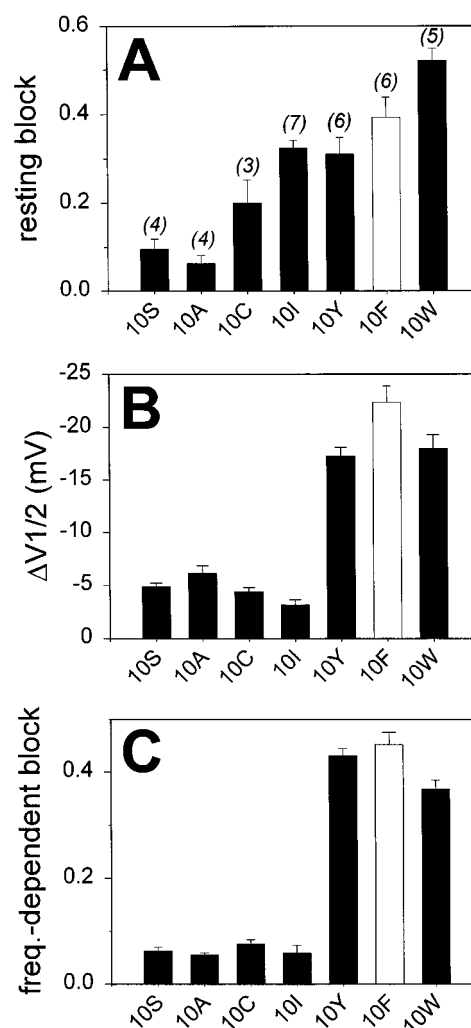


Fig. 3. Mutations at position 1710 alter resting, voltage-dependent, and frequency-dependent block. In this and subsequent figures, columns show means \pm S.E.M.. The number of experiments for each mean are shown above the columns. A, resting block for wild type and 1710 mutants. Columns show fraction of current inhibition at a holding voltage of -100 mV. The residues at position 1710 are arranged in order from the most hydrophilic (serine) to the most hydrophobic (tryptophan). The wild type residue, phenylalanine, is shown as a white column. B, $\Delta V_{1/2}$ values for wild type and 1710 mutants determined from the difference of $V_{1/2}$ values obtained from fits of tetracaine and control inactivation curves. C, fraction of frequency-dependent current inhibition at the end of a train of 20 pulses applied at 1 Hz. Inhibition was determined from the ratio of the currents evoked by the last pulse/first pulse in the train. For wild type and mutant channels, current attenuation in control was $\leq 5\%$.

leucine (Fig. 3, B and C). This observation suggests that high-affinity drug binding to open and inactivated channels requires the presence of an aromatic residue at position 1710. Hydrophobicity did not appear to be important, as substitution with the hydrophobic nonaromatic residue isoleucine at this position resulted in virtually no voltage-dependent or frequency-dependent block by tetracaine.

These results suggest that hydrophobicity at position 1710 is a critical parameter determining tetracaine binding to resting channel states, whereas aromaticity at this position is critical for binding to open and inactivated states. To examine this hypothesis more directly, we determined the affinity constants for resting (K_R) and for inactivated (K_I) channel states for wild-type channels and for two representative mutants, F1710A and F1710I. Values for K_R and K_I were determined, as described below, from complete tetracaine dose-effect relationships at two different holding potentials: -100 mV, and at a second potential, adjusted in each experiment to give 50% inactivation in control. Figure 4A shows a typical experiment in an oocyte expressing wild type channels. At -100 mV, most sodium channels are in resting states. Therefore, the midpoint of the dose-effect curve corresponds to K_R . For the experiment shown in Fig. 4A, this value was 120 μ M. The mean value of K_R for three wild type experiments was approximately 140 μ M (Fig. 4B). K_R for mutant F1710I was 205 μ M, whereas K_R for mutant 1710A was 830 μ M (Fig. 4B). This more quantitative measure of

resting state affinity was consistent with the idea that block of resting channels is well correlated to hydrophobicity at position 1710.

Inactivated state affinity K_I cannot be directly determined from electrophysiological experiments because inactivated channels do not conduct current. According to the modulated receptor hypothesis, the midpoint of the dose-effect relationship at any given holding potential ($B_{1/2}$) is simply the weighted sum of K_R and K_I (Bean et al., 1983; Stocker et al., 1997),

$$B_{1/2}^{-1} = h_{\infty}(V) * K_R^{-1} + [(1 - h_{\infty})(V)] * K_I^{-1} \quad (1)$$

where $h_{\infty}(V)$ is the proportion of resting channels in control. Since this equation predicts that $B_{1/2}$ will approach K_I as inactivation approaches 100%, we could in principle determine K_I from dose-effect curves at potentials where almost all sodium channels are inactivated. However, this is not an experimentally feasible strategy because the remaining current under these conditions would be too small to accurately measure. Nevertheless, a reasonable estimate of K_I can be obtained from equation 1 at intermediate holding potentials, where a significant fraction of channels are inactivated, but whole cell currents are large enough to measure. In each experiment, we used a holding potential that gave 50% inactivation (i.e., $1 - h_{\infty} = 0.5$) and determined $B_{1/2}$ from the midpoint of the dose-effect curve (e.g., open circles in Fig. 4A). Because K_R was already determined from the previously described experiments, it was straightforward to obtain K_I from equation 1. For wild type channels, the mean value of K_I was 6.3 μ M (Fig. 4B). In contrast, K_I for both mutants was almost 10-fold greater (Fig. 4B). Thus, substitution of non-aromatic residues at position 1710 dramatically reduces the affinity of inactivated channels for tetracaine.

Effects of Different Amino Acid Substitutions at Position 1717. Unlike substitutions at position 1710 described in the previous section, the effects of mutations at position 1717 did not follow a pattern that could be well correlated with parameters such as hydrophobicity or aromaticity. Mutant Y1717A greatly attenuated $\Delta V_{1/2}$ and frequency-dependent block (Fig. 5B and C) without altering resting block (Fig. 5A). However, other substitutions at position 1717 had a completely different effect: enhancement of resting block (Fig. 5A) accompanied by a modest to large decrease in $\Delta V_{1/2}$ and frequency-dependent block (Fig. 5, B and C). To more directly assess the effects of mutations at position 1717 on resting state and inactivated state affinity, we determined K_R and K_I values for the representative mutants Y1717C, Y1717F, and Y1717A (Fig. 6). K_R was 34 μ M for Y1717C and 27 μ M for Y1717F; these values represent increases in sensitivity of 4- to 5-fold compared to wt. In contrast, K_I values were 7.0 μ M for Y1717C and 3.1 μ M for Y1717F, similar to wt. Apparently, these mutations selectively increased resting state affinity without altering inactivated state affinity. Conversely, K_R for mutant Y1717A was indistinguishable from wild type but K_I was 17 times larger; this result is virtually the mirror image of the effects of other mutations at this site.

Discussion

Hydrophobicity and Aromaticity Are Critical Determinants at Position 1710. Most therapeutically useful local anesthetic molecules have a characteristic molecular

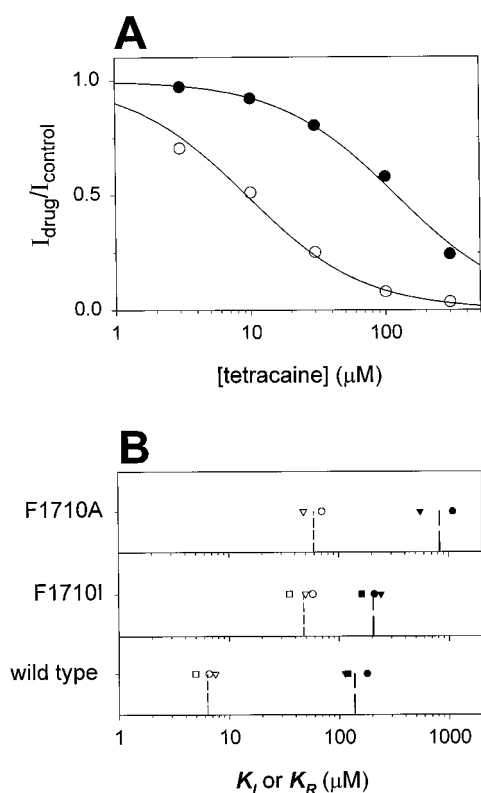


Fig. 4. K_I and K_R for wild type and mutants F1710A and F1710I. A, typical dose-effect relationships for a wild type experiment at holding voltages of -100 mV (●) or -40 mV (○). The smooth lines are according to $1/(1 + [\text{tetracaine}]/B_{1/2})$, where $B_{1/2}$ is the concentration giving 50% block. B, K_I (open symbols) and K_R (filled symbols) for wild type, F1710I, and F1710A, determined as described in the text. Within each row, each pair of symbols (e.g., ○, ●) show K_I and K_R values from a single experiment. The drop lines correspond to mean values.

structure with a tertiary amine at one end and an aromatic ring at the other, separated by a linking alkyl chain. Structure-activity studies suggest that both the tertiary amine and the aromatic moieties are involved in interactions with sodium channels (Courtney, 1980; Bokesch et al., 1986; Sheldon et al., 1991). The correlations observed in this study between local anesthetic block and hydrophobicity or aromaticity at position 1710 suggest several possible mechanisms by which molecular interactions between sodium channels and these drug moieties could occur. In the case of block of resting channels, hydrophobic interactions with F1710 may be involved. Structure-activity studies have shown that increasing the hydrophobicity of the local anesthetic molecule increases resting block (Bokesch et al., 1986). Our results provide the first complementary molecular evidence for an important hydrophobic determinant on the channel protein. F1710 may contribute to a hydrophobic pocket that stabilizes drug binding to resting sodium channels.

The affinity of sodium channels for local anesthetics can be 10 to 100 times higher for open and inactivated states than for resting states (Ragsdale et al., 1994, 1996; Bean et al., 1983; Fig. 4B). Our results suggest that an interaction that

depends on the aromaticity of the residue at position 1710 is one critical factor determining higher affinity drug binding to open and inactivated channels. We suggest two possible ways in which aromaticity could be important. One possibility is that binding to open and inactivated states depends on electrostatic interactions between the positive charge on the protonated tertiary amine of the local anesthetic and the electron rich- π face of the aromatic residue (Dougherty, 1996). These cation- π interactions have been proposed to be important for a number of ligand-protein interactions. For example, Heginbotham and MacKinnon (1992) have proposed that four tyrosines located at the outer opening of the potassium-channel pore form the external binding site for tetraethylammonium (TEA), a positively charged quaternary amine that blocks potassium channels by plugging the external opening of the pore. Cation- π interactions may also be important for the acetylcholine binding sites of the nicotinic acetylcholine receptor (Karlin and Akabas, 1995) and acetylcholinesterase (Sussman et al., 1991). Results presented in this paper suggest that cation- π interactions could stabilize binding of local anesthetics to open and/or inactivated sodium channels as well.

A second possibility is that the aromatic side chain on the phenylalanine molecule could interact with the aromatic group on the local anesthetic molecule. Aromatic-aromatic binding involves favorable interactions between the π face of one ring and the partially positively charged hydrogen atoms on the edge of the other ring (Burley and Petsko, 1985). Aromatic-aromatic interactions have been shown to contribute to the stability of protein folding (Burley and Petsko, 1985) and have been proposed to be involved in binding of ergoline to 5HT_{2A} receptors (Choudhary et al., 1995). Aromatic-aromatic interaction could also be important for stabilizing local anesthetic binding to open and inactivated sodium channels.

How could the F1710 residue interact with the same drug molecule by two different molecular mechanisms? One possibility is that the position of the phenylalanine side chain within the pore changes in response to changes in channel state. Perhaps in resting states the orientation of the side chain permits hydrophobic interactions but precludes more specific aromatic interactions. Channel opening and/or inactivation may reorient the aromatic ring of phenylalanine so that its π -face can directly interact with the local anesthetic.

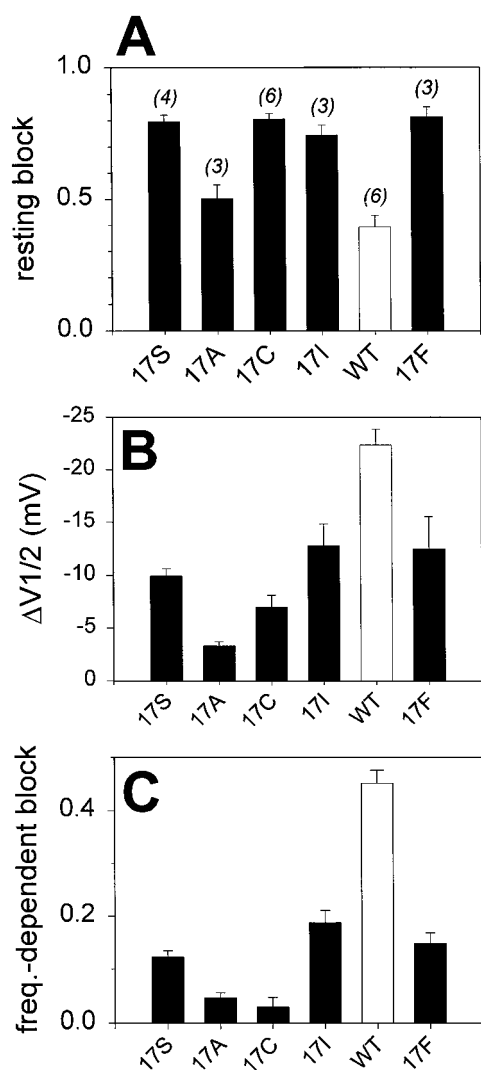


Fig. 5. Mutations at position 1717 alter resting block (A), $\Delta V_{1/2}$ (B), and frequency-dependent block (C). Data presentation is the same as in Fig. 3.

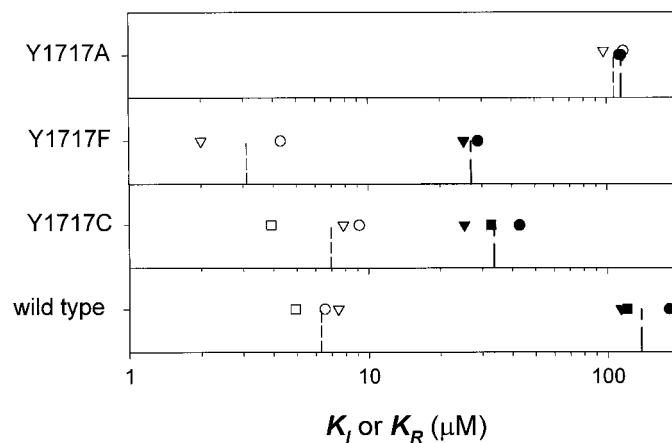


Fig. 6. K_I and K_R values for Y1717C, Y1717F, and Y1717A. Data presentation is the same as in Fig. 4B.

These aromatic-specific interactions may be stronger than the hydrophobic interactions that determine resting block, thus explaining, at least in part, the higher affinity of open and inactivated states.

Crystallographic Data Suggest Residue 1710 Could Lie Within the Pore. Biophysical evidence suggests that the binding site for local anesthetics is within an inner vestibule of the channel pore (Strichartz, 1973; Cahalan and Almers, 1979; Gingrich et al., 1993; Zamponi and French, 1994). An important recent breakthrough in understanding pore structure has been the X-ray crystallographic analysis of a potassium channel from *Streptomyces lividans* (Doyle et al., 1998). This bacterial ion channel contains transmembrane segments that correspond to the S5 and S6 segments of voltage-gated ion channels. These transmembrane segments are separated by a pore loop that dips into the membrane from the extracellular side to form the selectivity filter of the pore. The remainder of the pore is lined by the transmembrane segment corresponding to S6. One unexpected finding of this analysis is a cavity located approximately half-way through the pore. This cavity, which is proposed to be important for ion permeation, contains the intracellular binding site for TEA. (The internal site is distinct from the external TEA site described above.) It is plausible that local anesthetics also bind within this internal cavity. Residue F1710, which is located approximately half-way through the S6 segment, is well situated to contribute to the cavity, although at present it is not known whether this residue is oriented toward the pore, where it could interact with local anesthetics, or away from the pore where it could interact with other residues on the α subunit.

The Residue at 1710 Could Affect Drug Action Without Contributing Directly to the Receptor. Although the results of this study are consistent with the hypothesis that the residue at 1710 contributes directly to the local anesthetic receptor, it is also possible that mutations at this site affect drug binding to the receptor through an indirect mechanism. For example, Hille (1977) proposed that when sodium channels are closed, local anesthetics reach their receptor through a hydrophobic access pathway. Based on dissociation rates of local anesthetics of different size, Courtney (1984) suggested that this hydrophobic access route was a narrow passage, approximately 3.6 Å in radius, through the channel protein. The strong correlation between resting block and hydrophobicity at residue 1710 could be related to the contribution of the residue to this hydrophobic access pathway. Alternatively, mutations at position 1710 could determine sodium channel affinity for local anesthetics through indirect allosteric effects on the conformation of the receptor. For example, the importance of aromaticity in high affinity drug block of open and inactivated sodium channels could reflect the dependence of the high-affinity receptor conformation on an interaction between the aromatic residue at position 1710 and another residue on the channel protein. Clearly, further work will be required to unequivocally determine whether the residue at position 1710 contributes directly to the local anesthetic receptor site.

Mutations at 1717 Increase Resting State Affinity or Decrease Open and Inactivated State Affinity. Although all mutations at position 1717 had strong effects on local anesthetic potency, they did not show a clear pattern that would reveal relationships between side chain structure

and drug action. As has been shown previously (Ragsdale et al., 1994, 1996), substitution of alanine at this position greatly reduced open and inactivated state affinity without altering resting state affinity. In contrast, other mutations at this position, even the conservative mutation Y1717F, increased resting affinity without affecting open and inactivated affinity. The diametrically opposite effects of substitution of alanine on the one hand, or cysteine, serine, isoleucine, or phenylalanine on the other hand, are difficult to explain in terms of direct interaction between the local anesthetic molecule and the residue at position 1717, and suggest that the residue at this position does not contribute directly to the local anesthetic receptor. Although mutations at this position strongly alter drug block, the molecular mechanism for these effects is at present unknown.

Other Determinants of Local Anesthetic Action. The four domains of the sodium channel α subunit are thought to be arranged in a square array with the channel pore at the center. Thus, it is likely that the other S6 segments also contribute to the lining of the pore and may also contribute to the local anesthetic receptor. In addition, other regions of the channel could be involved. For example, a mutation that disables the channel inactivation gate has been shown to attenuate frequency-dependent block by lidocaine (Bennett et al., 1995), suggesting that the inactivation gate may contribute to stabilizing drug binding. Residues in the pore loops that form the pore selectivity filter are also likely candidates for determinants of drug action. Future work will address these various possibilities.

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