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Sodium channel selectivity filter regulates antiarrhythmic drug binding

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ABSTRACT **Local anesthetic antiarrhythmic drugs block Na^+ channels and have important clinical uses. However, the molecular mechanism by which these drugs block the channel has not been established. The family of drugs is characterized by having an ionizable amino group and a hydrophobic tail. We hypothesized that the charged amino group of the drug may interact with charged residues in the channel's selectivity filter. Mutation of the putative domain III selectivity filter residue of the adult rat skeletal muscle Na^+ channel (μ1) K1237E increased resting lidocaine block, but no change was observed in block by neutral analogs of lidocaine. An intermediate effect on the lidocaine block resulted from K1237S and there was no effect from K1237R, implying an electrostatic effect of Lys. Mutation of the other selectivity residues, D400A (domain I), E755A (domain II), and A1529D (domain IV) allowed block by externally applied quaternary membrane-impermeant derivatives of lidocaine (QX314 and QX222) and accelerated recovery from block by internal QX314. Neo-saxitoxin and tetrodotoxin, which occlude the channel pore, reduced the amount of QX314 bound in D400A and A1529D, respectively. Block by outside QX314 in E755A was inhibited by mutation of residues in transmembrane segment S6 of domain IV that are thought to be part of an internal binding site. The results demonstrate that the Na^+ channel selectivity filter is involved in interactions with the hydrophilic part of the drugs, and it normally limits extracellular access to and escape from their binding site just within the selectivity filter. Participation of the selectivity ring in antiarrhythmic drug binding and access locates this structure adjacent to the S6 segment.**

Local anesthetic antiarrhythmic drugs are clinically important for treatment of arrhythmias, control of pain, and suppression of seizures. These drugs work by blocking voltage-gated Na^+ channels (1–3). However, the mechanism by which they accomplish this block at the molecular structural level has not been established. The Na^+ channel α-subunit consists of four homologous domains, each containing six transmembrane segments, S1–S6 (4, 5). Between the S5 and S6, an extracellular pore-forming region invaginates partially across the membrane. The tips of these pore-forming regions come together to form a narrow selectivity filter that contains several charged residues (6, 7).

Clinically useful local anesthetic drugs contain an ionizable amino group and a hydrophobic tail (2, 3) and may have both charged and hydrophobic components to their binding site. Biophysical evidence suggests that the local anesthetic binding site is on the cytoplasmic side of the channel pore, lying between the selectivity filter and the channel gates (8). Recently, Choi *et al.* (9) demonstrated that quaternary ammonium molecules containing aliphatic chains bind and block Shaker K^+ channels by interacting with two parts of the K^+ channel inner pore, the P loop near the channel's selectivity filter and the S6 segment. The S6 residues appeared to contribute to the hydrophobic binding pocket. The Shaker K^+ channel is thought to have a pore structure analogous to that of the Na^+ channel. In the brain IIA Na^+ channel, Ragsdale *et al.* (10) identified several residues in domain IV, S6 affecting local anesthetic binding. Two aromatic residues appear to be directly involved in the local anesthetic binding site. They also suggested that these aromatic residues might be spatially close to the Na^+ channel selectivity filter, which may compose part of the binding site (11).

Another association between local anesthetics and the selectivity filter is the finding that the membrane-impermeant local anesthetic QX314 can block the cardiac Na^+ channel from the outside (12) and that this is probably due to access to the internal binding site through the pore (13). If QX derivatives such as QX314 and QX222 can enter through the pore, mutations of the selectivity filter may affect block by external QX. These possible relationships between local anesthetics and the selectivity filter prompted us to investigate the roles of the selectivity filter in binding and access of the drugs by using mutagenesis of the adult rat skeletal muscle Na^+ channel (μ1).

MATERIALS AND METHODS

Detailed methods for the mutagenesis and the electrophysiological recording from oocytes have been described in our previous report (14). In short, mutagenesis of the adult rat skeletal muscle Na^+ channel (μ1) was carried out by one of several oligonucleotide-directed methods. Messenger cRNA was prepared *in vitro*. Stage V and VI *Xenopus* oocytes were isolated, and approximately 50–100 ng of cRNA was injected into each oocyte. Oocytes were incubated at 16°C for 1–6 days before examination. Recordings were made in the two electrode voltage clamp configuration by using a Dagan CA-1 voltage clamp. All recordings were made at room temperature (20–22°C) in a bathing solution that consisted of 90 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$ or $BaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes (pH 7.2). For experiments with Lys mutants, we used Ba^{2+}-containing solution and compared them to wild-type channel in the same solution because these mutations render the channel permeable to Ca^{2+} (6, 7). Ca^{2+} permeation activated an endogenous Cl^- current in the oocyte (15, 16), but the use of external Ba^{2+} prevented activation of this current. Lidocaine, phenol, and benzocaine were obtained from Sigma. Tetrodotoxin (TTX), neo-saxitoxin (neo-STX), and QX314

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Abbreviations: TTX, tetrodotoxin; neo-STX, neo-saxitoxin.
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were obtained from Calbiochem, National Research Council Canada (Halifax, Canada), and Alomone (Jerusalem, Israel), respectively. QX222 was a gift from Astra Pharmaceuticals (Westboro, MA). Benzocaine was dissolved in dimethyl sulfoxide (DMSO) and added to the perfusing solution. The final concentration of DMSO was ≤1%, but because DMSO at 0.5 and 1% blocked channels expressed in oocytes by 3–12%, the blocking degree by benzocaine was adjusted for this effect. Other chemical solutions were made from stock solutions dissolved in distilled water or 0.1 M acetic acid (for neo-STX). All data are presented as the mean ± SEM. Statistical comparisons were made by using one-way ANOVAs for three or more groups of means or two-way ANOVAs for the dose–response curves of the wild type and its mutants, unless otherwise specified.

RESULTS

To avoid difficulties in interpretation because of accumulated inactivated states or because of shift in gating, rested or tonic block (first-pulse block) by lidocaine was measured at the holding potential of −120 mV for μ1 wild type and its mutants. Mutations of the putative selectivity filter residues were made to change their charge. In wild type, the lidocaine dissociation constant (K_d) was 572 ± 38 μM and no change was observed in domain I mutant D400A (K_d = 552 ± 39 μM) or domain IV mutant A1529D (K_d = 611 ± 35 μM) (Fig. 1*A*). On the other hand, the domain II mutant E755A increased affinity 2-fold (K_d = 284 ± 26 μM) and the domain III mutant K1237E increased affinity 4-fold (K_d = 154 ± 16 μM) ($P < 0.001$).

Because the larger affinity change was observed in K1237E, we further studied the effects of replacement of K1237 with other amino acids. Substitution of Lys with a neutral residue, Ser (K1237S), shifted the curve to an intermediate position (K_d = 281 ± 47 μM) and substitution of Lys with the positively charged Arg (K1237R) resulted in wild-type affinity (K_d = 521 ± 50 μM; $P > 0.05$ by Tukey test following two-way ANOVA) (Fig. 1*B*). These results suggest that Lys at position 1,237 influences lidocaine binding by an electrostatic interaction with the hydrophilic part of lidocaine. To test this idea, we studied the hydrophobic lidocaine analogs phenol (17) and benzocaine (8). Tonic block by phenol was not influenced by the mutant K1237E (wild-type K_d, 6.5 ± 2.7 mM; K1237E K_d, 5.6 ± 1.6 mM; $P = 0.203$; Fig. 1*C*). Consistent with this result, K_d values for tonic block by benzocaine were similar between wild-type (553 ± 82 μM) and K1237E (440 ± 57 μM; $P = 0.619$) values. These results support the proposal that K1237 interacts electrostatically with the charged amine part of lidocaine.

If lidocaine truly binds near the selectivity filter, then this narrow region might influence access and dissociation of the drug. The charged membrane-impermeant quaternary ammonium derivatives of lidocaine such as QX314 and QX222 do not block nerve Na^+ channels when applied to the outside (18, 19). On the other hand, external QX314 blocks the cardiac Na^+ channel (12), perhaps by traveling through the pore to reach the internal binding site (13). μ1 wild-type Na^+ channels showed no block during exposure to 500 μM QX222 in the bath solution when stimulations were applied at 20-s intervals (Fig. 2*A*; 5.4 ± 1.1% peak current reduction, $n = 9$). In contrast, D400A (Fig. 2*A*), E755A (Fig. 2*B*), and A1529D (Fig. 2*C*) allowed reversible block by externally applied QX222. On average, block was 20.5 ± 2.5% ($n = 9$), 38.2 ± 2.5% ($n = 8$), and 29.2 ± 2.6% ($n = 5$) for D400A, E755A, and A1529D, respectively, after 11 min of perfusion. E755A and A1529D also showed 32.1 ± 2.5% ($n = 10$) and 60.8 ± 3.0% ($n = 5$) block 10 min after external application of 500 μM QX314. Interestingly, even though K1237 interacted electrostatically with lidocaine, neither K1237E nor K1237S allowed external QX222 block (Fig. 2*D*; 6.6 ± 1.2% block, $n = 5$ for K1237E, and 4.3 ± 1.4% block, $n = 4$ for K1237S).

If the selectivity filter mutants allow access to the internal site, then they also provide an alternative path for dissociation of the bound antiarrhythmic drug. To compare recovery from QX314 block in wild-type and mutant channels, QX314 was microinjected into oocytes. If we assume an oocyte volume of

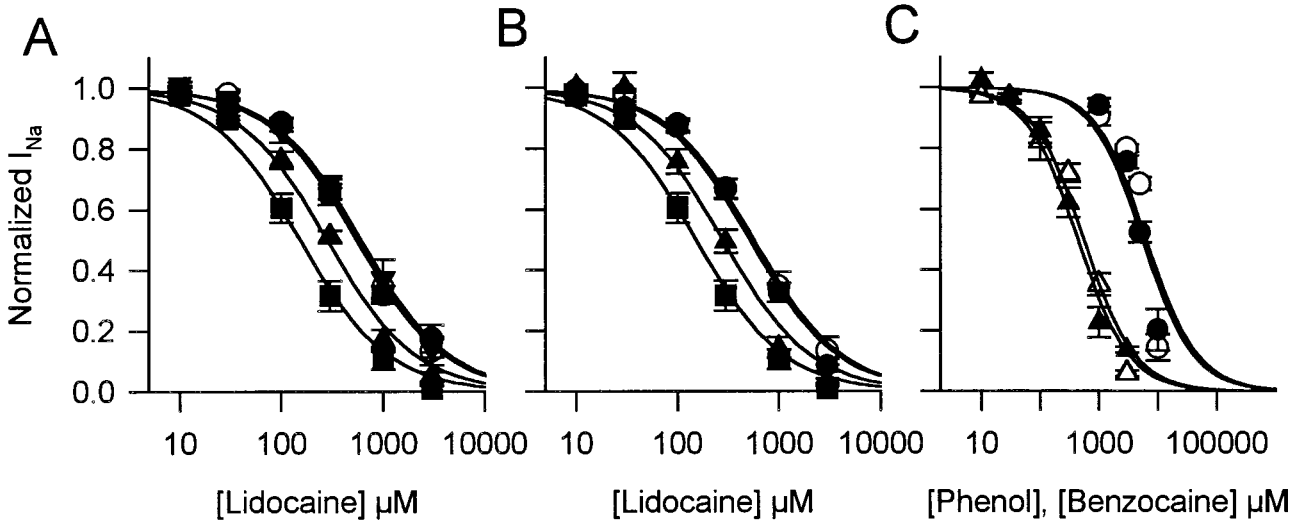

FIG. 1. Selectivity filter mutations alter tonic block by lidocaine. Tonic block was measured by maintaining the holding potential at −120 mV for at least 5 min before applying test pulse to −10 mV. (*A* and *B*) Effects of mutating the putative selectivity filter in domains I–IV on tonic lidocaine block. Peak currents 10–11 min after exposure to indicated concentrations of lidocaine were normalized to that in the control for μ1 wild-type (WT, ○), D400A (domain I, ●), E755A (domain II, ▲), K1237E (domain III, ■) and A1529D (domain IV, ▼) in *A* and for WT (○), K1237E (■), K1237S (▲) and K1237R (●) in *B*. Continuous lines are fits of the data to first-order Hill saturation functions, normalized $I_{Na} = 1/(1 + [\text{lidocaine}]/K_d)$, where K_d is the dissociation constant. K_d values are 572, 552, 284, 154, 281, 521, and 611 μM for WT, D400A, E755A, K1237E, K1237S, K1237R, and A1529D, respectively. (*C*) Effects of K1237E on tonic block by the hydrophobic lidocaine analogs phenol and benzocaine. Peak currents 10–11 min after exposure to the indicated concentrations of phenol (○, ●) or benzocaine (△, ▲) were normalized to that in the control for WT (○, △) and K1237E (●, ▲) and plotted as a function of phenol or benzocaine concentrations. The smooth lines are fits of first-order Hill saturation functions, normalized $I_{Na} = 1/(1 + [\text{drug}]/K_d)$, where K_d is the dissociation constant and [drug] is the indicated concentration of phenol or benzocaine. For phenol block, K_d values are 6.5 and 5.6 mM for WT and K1237E, respectively. For benzocaine block, K_d values are 553 and 440 μM for WT and K1237E, respectively. Data represent the mean ± SEM from three to nine oocytes.

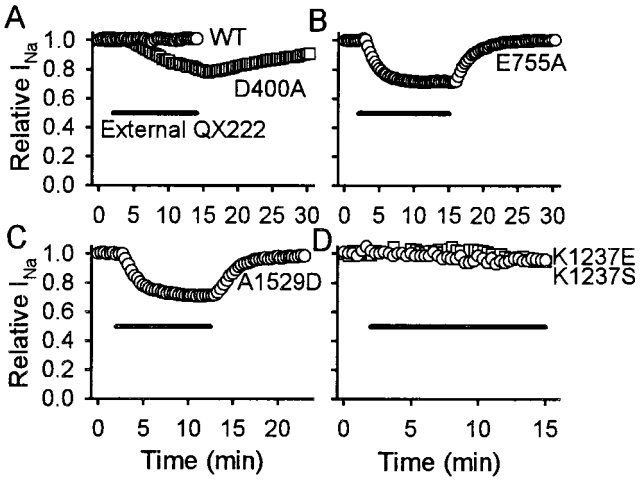

FIG. 2. Some selectivity filter mutations allow block by external QX222. Effects of externally applied 500 μM QX222 were studied for the following channels. (*A*) Wild type (WT, ○) and D400A (□). (*B*) E755A. (*C*) A1529D. (*D*) K1237E (○) and K1237S (□). Peak currents elicited by 35-ms pulses to −10 mV from a holding potential of −100, −110 (for A1529D), or −120 mV (for K1237E) at 20-s intervals were normalized to peak current in control and plotted as relative Na^+ currents (relative I_{Na}). The graphs show typical examples for WT and mutants. The bar in each panel indicates the period of exposure to 500 μM QX222 in the bath solution.

1 μl (10) and an equal distribution of QX314 within the oocyte, then microinjecting 50 nl of a 3 mM QX314 solution corresponded to an intracellular QX314 concentration of 150 μM. For recovery measurement, use-dependent block was initially produced by a 1-Hz stimulation, and then recovery was monitored. Consistent with the previous reports on QX314 recovery in nerve channels (19, 20), recovery in the μ1 wild-type channels was slow, with only 19% of the current recovered during a 30-min period (Fig. 3*A*). In contrast, the selectivity filter mutants that permitted block by external QX222, E755A, and A1529D showed much faster recovery than that of wild type (time constants were 1,263 and 228 s, respectively). D400A allowed moderate external block, and recovery was marginally faster than that of wild type. Although change in voltage dependence of activation can influence the recovery time constant (20), there are several reasons to believe that the observed changes were not the result of alterations in gating. These mutants did not change the voltage dependence of activation (data not shown), the channels were held at a voltage where they were fully available, and the intermittent test pulses were applied to a voltage that fully activated all channels. These results suggested that selectivity filter mutants allowed external QX block by creating an access pathway to the internal binding site and this pathway also provided an escape route for QX to the outside.

If these mutants created an access pathway, rather than a new binding site, the time course of recovery is expected to be independent of the side of application of QX compounds. We compared the time course of recovery from block by internally or externally applied QX compounds. In E755A, the time constant of recovery was 111 s when QX222 was applied internally and 116 s when applied externally (Fig. 3*B*). A1529D also showed similar recovery time constants for internal (228 s) or external application (216 s) of QX314. Although differences in final level between internal and external applications were observed for both mutants, these were probably because of difference in equilibrium concentrations of QX compounds as a result of the two experimental conditions. The similar recovery rates for internal or external QX derivatives suggest a single local anesthetic binding site implying that these

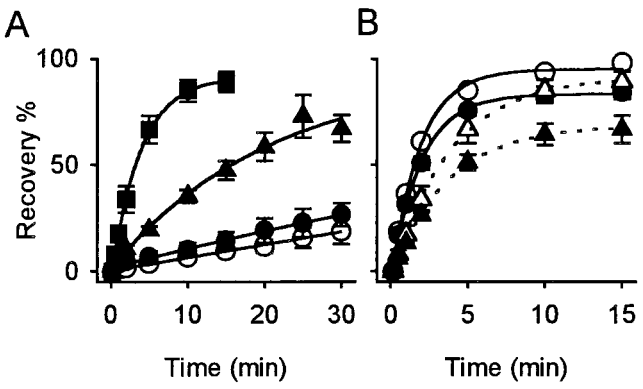

FIG. 3. Selectivity filter mutations create an access pathway for external QX compounds to reach the internal binding site. (*A*) Effects of selectivity filter mutations on recovery from internally applied QX314 block. QX314 was internally applied by microinjecting 50 nl of a 3 mM QX314 solution into oocytes. Twenty to 30 min after microinjection, a 1-Hz train of 20 pulses with a 35-ms duration was applied to −10 mV from a holding potential of −100 or −110 mV (for A1529D) to produce use-dependent block by QX314. Then, recovery from QX314 block was monitored at the indicated intervals after the end of a train for wild type (○), D400A (●), E755A (▲), and A1529D (■). Peak currents during recovery were normalized by the difference between the peak current during the first pulse of a train and the 10-s recovery test pulse and plotted as recovery percent. The 10-s recovery was assumed to allow recovery from inactivation of unblocked channels but be short enough to prevent QX314 dissociation from blocked channels. Degrees of use-dependent block by internal QX314 were 87.5, 78.3, 65.6, and 70.9% for wild type, D400A, E755A, and A1529D, respectively. The smooth lines are single exponential fits, and time constants are 1,264 s for E755A and 228 s for A1529D. Data represent the mean ± SEM from three to nine oocytes. (*B*) Comparison of the time courses of recovery from block by internally or externally applied QX compounds. For internal application, 50 nl of 3 mM QX222 or QX314 was microinjected into oocytes expressing E755A (○) and A1529D (△), respectively. For external application, 500 μM QX222 or 500 μM QX314 was added to the bath solution for E755A (●) and A1529D (▲), respectively and perfused until recovery protocol was complete. Recovery from block was measured by using the same protocol as that in *A*. The smooth lines are single exponential fits for E755A (solid lines) and A1529D (dashed lines) data. For E755A, time constants are 111 s for internal and 116 s for external QX222. Use-dependent block by internal or external QX222 was 67.9 and 36.1%, respectively. For A1529D, time constants are 228 s for internal and 216 s for external QX314. Use-dependent block by internal or external QX314 was 70.9 and 52.7%, respectively. Data represent the mean ± SEM from four to seven oocytes.

mutations created an access pathway but not a second binding site.

If the access pathway created by selectivity filter mutations is through the pore, guanidinium toxin binding in the outer vestibule should inhibit access of QX compounds. To test this prediction, we blocked the channel completely with neo-STX or TTX, before or after exposing to external QX314. Block by toxins or QX314 was distinguished by the different drug dissociation rates. In D400A, 1 μM neo-STX produced 100% block and 1 mM QX314 blocked 21% of the current after 12 min of perfusion (Fig. 4*A*). When the neo-STX and QX314 were applied together, the time course of washout recovery for the mixture (recovery time constant τ = 123 s) was nearly the same as that after neo-STX alone (τ = 111 s) but much faster than that after QX314 alone (τ = 2,096 s) (Fig. 4A). In A1529D, 5 μM TTX and the mixture of 5 μM TTX and 500 μM QX314 produced 100% block during a 5-min perfusion. QX314 alone at 500 μM blocked 73% of the current after 5 min of perfusion. When comparing the time courses of washout recovery, the time constant of the mixture was 106 s, was nearly the same as that of TTX alone (τ = 94 s), but was smaller than that of QX314 alone (τ = 352 s) (Fig. 4*B*). Faster recovery

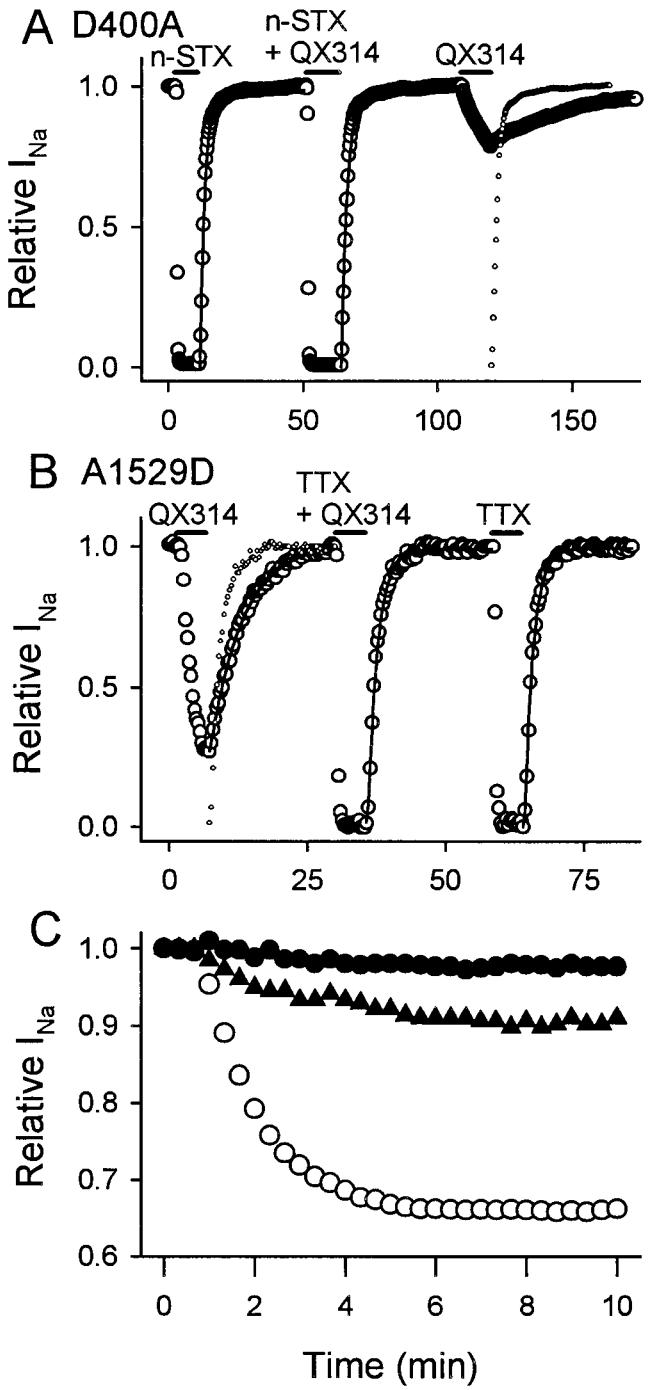

FIG. 4. An access pathway created by selectivity filter mutations is through the pore. (*A* and *B*) Effects of outer vestibule toxins on block by external QX314. The graphs show typical examples of experiments in D400A (*A*) and A1529D (*B*). The protocol was the same as that in Fig. 2, and peak currents were normalized to that in control. The bar shows the period during exposure to 1 μM neo-STX (n-STX), the mixture of 1 μM n-STX plus 1 mM QX314 (n-STX + QX314), or 1 mM QX314 in D400A (*A*); and 500 μM QX314, the mixture of 5 μM TTX plus 500 μM QX314 (TTX + QX314), or 5 μM TTX in A1529D (*B*). The smooth lines are single exponential fits to washout recovery phase. In each panel, recovery upon washout of the mixture was overlapped (small circles) on that of QX314 alone to compare the recovery rates. (*C*) Effects of domain IV, S6 mutations on block by externally applied QX222. Actions of externally applied 500 μM QX222 were studied for E755A (○) and double mutants E755A plus F1579A (●) and E755A plus Y1586A (▲). The protocol was the same as that in Fig. 2, and peak currents were normalized to that in control. The graph shows typical example of block development during exposure to external QX222 for each mutant.

upon washout of the mixture than that of QX314 alone was confirmed in three and six additional oocytes for D400A and A1529D, respectively. These results implied that the number of QX314-bound channels decreased to low levels in the presence of neo-STX or TTX in the outer vestibule. This is similar to the previously reported TTX protection of the heart channel from outside QX314 (13). The E755A channel mutant has a very low toxin affinity, making protection experiments impossible.

If mutations of the selectivity filter are allowing drug access to an internal binding site, then a mutation that disrupts the internal binding site should mitigate the effect of a mutation that creates access. The double mutant composed of E755A and either the mutation F1579 or Y1586 in domain IV, S6 was tested. These S6 mutations are equivalent to F1764 and Y1771 in rat brain IIA channel that appear to form part of the internal binding site for local anesthetic antiarrhythmic drugs (10, 11, 13). If F1579 or Y1586 is involved in local anesthetic binding site and E755A creates an access pathway to the internal binding site from the outside, no block or reduced block by external QX would be expected in a double mutant. When 500 μM QX222 was applied to the bath solution, E755A showed 34% block (Fig. 4*C*). In contrast, the double mutant E755A plus Y1586A showed significantly reduced external block ($10.1 \pm 1.1\%$, $n = 6$) and E755A plus F1579A showed no detectable block ($1.0 \pm 0.5\%$, $n = 5$) during 11- to 12-min exposure to external 500 μM QX222 ($P < 0.001$). These findings demonstrate that mutant E755A creates a second access pathway for QX222 to reach its internal binding site.

DISCUSSION

Our findings show that the selectivity filter of Na^+ channel is close to bound local anesthetic antiarrhythmic drug and that it affects drug access and dissociation. Consequently, local anesthetics interact with the Na^+ channel selectivity filter. In this regard, local anesthetics are similar to tetraethylammonium in internal block of K^+ channel (9).

Mutations of the domain III selectivity filter residue Lys affected drug binding. The most likely explanation is that Lys interacts with the drug electrostatically to reduce its affinity, and mutation of Lys alters the electric field in the critical region of the site. Another possibility is that mutations of Lys alter the gating properties of the channel and indirectly affect affinity through its state dependence. The mutation K1237E did shift the availability curve about -20 mV, requiring these experiments to be made from a holding potential of -120 mV, where channels were fully available. The mutation K1237S changed lidocaine affinity but did not affect the voltage dependence of gating, however. Therefore, affinity change is unlikely to be caused by a gating change. A final unlikely possibility is that mutations of Lys alter the local concentration of ions and these ions in turn affect lidocaine binding. K1237 mutations are known to alter the ion selectivity and increase the permeability of K^+ and Ca^{2+} (6, 7). Nevertheless, K1237R increases the permeation of K^+ (7), but it had no effect on lidocaine block, ruling out any role of K^+ permeation. Also, a role of Ca^{2+} is unlikely because we used Ba^{2+}-containing, instead of Ca^{2+}-containing, bath solution for experiments of Lys mutants and wild type to compare with them. In wild-type channels, no differences in lidocaine affinity were observed between Ba^{2+}- and Ca^{2+}-containing solutions (data not shown). K1237E is permeable to Ba^{2+} (6), but K1237S seems to increase Ba^{2+} block of Na^+ currents because Na^+ currents were dramatically reduced after changing to Ba^{2+} solution (data not shown). The increase in Ba^{2+} block of Na^+ currents is presumably the result of Ba^{2+} binding to the selectivity ring, but the cation's two positive charges would be expected to reduce local anesthetic binding not increase it.

If the sole effect of changing Lys at position 1,237 is on the local electrostatic potential, it follows the following relation

(21): $K_{d\cdot 2u}/K_{d\cdot WT} = (K_{d\cdot 1u}/K_{d\cdot WT})^2$, where $K_{d\cdot WT}$, $K_{d\cdot 1u}$, and $K_{d\cdot 2u}$ are dissociation constants for wild type and mutants changing the residue charge by single unit (that is, Lys to Ser) and by two units (that is, Lys to Glu), respectively. The K_d value for wild type was 572 μM and for the double-charge mutant K1237E was 154 μM. If this affinity change was solely caused by electrostatic effect, the above equation predicts the K_d value of 297 μM in K1237S, which was consistent with the observed K_d value of 281 μM. A further support for an electrostatic effect of Lys at position 1,237 comes from the absence of effects on lidocaine binding affinity with K1237R, where the negative charge at position 1,237 was preserved. Therefore, it appears that Lys has a direct electrostatic effect on antiarrhythmic drug in its binding site. The interaction of lidocaine and K1237 appeared specific because the other selectivity filter residues thought to be near K1237 did not affect binding affinity or resulted in affinity changes that were unexplained by a direct interaction.

The observed 3.7-fold increase in lidocaine binding affinity seen with K1237E requires an increase in binding energy of 0.8 kcal/mol. Estimates of the distance separating the amine portion of lidocaine and K1237 are complicated by uncertainties about the extent of ionization of lidocaine when bound, the polarizability of the protein and solvent in this region, and the extent of counterion shielding. However, this interaction energy is of similar magnitude to the experimentally determined energy in two salt bridges in the ribonuclease barnase (22). At the interface of barnase with its inhibitor barstar, experiments using mutant cycle analysis determined the dependence of interaction energies on the distance of separation between residues. If the barstar–barnase interface is roughly analogous to the environment containing the K1237–lidocaine interaction, then the predicted distance of separation is about 5 Å (23).

Presumably, K1237 has a negative impact on lidocaine affinity in the wild-type channel. This negative impact does not invalidate the idea that lidocaine binds near the selectivity filter, but it does suggest that the other residues of the selectivity filter ring may also affect antiarrhythmic drug binding. E755A, the negative charge reducing mutation in domain II, increased lidocaine affinity, but D400A, a similar charge reducing mutation of domain I, and A1529D, the negative residue replacement of domain IV, had no effect on affinity. Increased affinity by E755A does not fit an electrostatic effect, and its mechanism is unclear. As mentioned below, it is possible that E755A mutation increases the rested channel affinity for lidocaine by opening a second access pathway to the site, but this is unlikely because lidocaine block was judged at steady state. Consistent with this, D400A and A1529D create an access pathway but do not affect drug binding. E755A is the only mutant affecting both drug access and binding. Perhaps, the charged portion of lidocaine is attracted by E755, but this attraction pulls the drug's hydrophobic portion away from its binding site. Loss of the attraction with E755A might improve binding by allowing a better hydrophobic interaction and results in a paradoxical improvement in lidocaine affinity. This idea is similar to the proposal of Choi *et al.* (9) to explain observations of the effect of K^+ channel mutations on block by internally applied tetraethylammonium derivatives.

Mutations of the selectivity filter allowed access of external QX to its binding site on the cytoplasmic side of the selectivity ring, and the mechanism appears to be direct permeation of QX through the pore. These conclusions are derived from the following findings in selectivity filter mutants: (*i*) accelerated recovery from internal QX block; (*ii*) a similar time course of recovery from QX block when QX was applied from the inside or the outside; (*iii*) no block or reduced block by external QX in double mutant (E755A·F1579A, E755A·Y1586A); and (*iv*) outside QX access impeded by the presence of outer vestibule toxin. The selectivity filter normally acts as a barrier, preventing drug access to and escape from its binding site.

How QX compounds pass through the mutated selectivity filter is unclear. E755A increases K^+ permeability over Na^+ (7) and A1529D is predicted to increase K^+ permeability from A1529E data (6). Because K^+ (radius = 1.33 Å) is bigger than Na^+ (radius = 0.95 Å), the selectivity filter region in E755A and A1529D may have a larger diameter, and consequently, QX compounds might directly pass through it. However, K1237E also increases K^+ permeability (6) but shows no detectable block by external QX, and D400A allows external QX block without an increase of K^+ permeability (7). Therefore, QX permeation cannot be explained by a simple change in selectivity filter pore size.

Because both the selectivity filter residues and the extracellular end of domain IV, S6 (10, 13) influence QX314 access to its internal binding site from the outside in a similar manner, the amino-terminal end of S6 and the P loop in each domain are probably close to each other and may both contribute to the structure of the outer vestibule. This possibility is compatible with the radially arranged P loops in the Lipkind–Fozzard model of the outer vestibule (24), which provides space for the S6 structures between the P loops. Perhaps, the S6 segments and the selectivity filter might constitute a single potential access pathway for local anesthetics where the hydrophobic tail of the drug interacts with the generally hydrophobic S6 segment while the charged portion of the drug interacts at or near the selectivity filter. Mutations that sufficiently favor interaction with either half of the drug lower the activation energy enough to make passage possible.

In conclusion, residues in the Na^+ channel selectivity filter both influence the affinity for antiarrhythmic drugs and control their access to and dissociation from their binding site on the cytoplasmic side of the selectivity filter. The approximation of bound drug to the selectivity filter suggests that the drugs block the channel by interacting at this level to prevent Na^+ flux. The involvement of residues of both the selectivity filter and S6 segment in interactions with the drugs implies that these structures are contiguous in the channel's pore.

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