

# Structural and Gating Changes of the Sodium Channel Induced by Mutation of a Residue in the Upper Third of IVS6, Creating an External Access Path for Local Anesthetics

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## ABSTRACT

Membrane-impermeant quaternary amine local anesthetics QX314 and QX222 can access their binding site on the cytoplasmic side of the selectivity filter from the outside in native cardiac Na<sup>+</sup> channels. Mutation of domain IV S6 Ile-1760 of rat brain IIA Na<sup>+</sup> channel or the equivalent (Ile-1575) in the adult rat skeletal muscle isoform ( $\mu$ 1) creates an artificial access path for QX. We examined the characteristics of mutation of  $\mu$ 1-I1575 and the resulting QX path. In addition to allowing external QX222 access, I1575A accelerated decay of Na<sup>+</sup> current and shifted steady-state availability by -27 mV. I1575A had negligible effects on inorganic or organic cation selectivity and block by tetrodotoxin (TTX), saxitoxin (STX), or  $\mu$ -conotoxin ( $\mu$ -CTX). It exposed a site within the protein that binds membrane-permeant methanethiosulfonate ethylammonium (MTSEA), but not membrane-impermeant methanethiosulfonate ethyltrimeth-

ylammonium (MTSET) and methanethiosulfonate ethylsulfonate (MTSES). MTSEA binding abolished the QX path created by this mutation, without effects on toxin binding. The  $\mu$ -CTX derivative R13N, which partially occluded the pore, had no effect on QX access. I1575A exposed two Cys residues because a disulfide bond was formed under oxidative conditions, but the exposed Cys residues are not those in domain IV S6, adjacent to Ile-1575. The Cys mutant I1575C was insensitive to external Cd<sup>2+</sup> and MTS compounds (MTSEA, MTSET, MTSES), and substitution of Ile with a negatively charged residue (I1575E) did not affect toxin binding. Ile-1575 seems to be buried in the protein, and its mutation disrupts the protein structure to create the QX path without disturbing the outer vestibule and its selectivity function.

Local anesthetics are clinically important drugs that act by blocking voltage-gated Na<sup>+</sup> channels. Drug efficacy is dependent on membrane potential and electrical activity (use dependence). This is explained by the "modulated receptor" hypothesis (Hille, 1977; Hondeghem and Katzung, 1977) and/or the "guarded receptor" hypothesis (Starmer et al., 1984) and reflects a dependence of drug binding or access to its intramembrane site with gating. Detailed studies of the normal mechanism of drug action in nerve or muscle using permanently charged quaternary ammonium analogs, such as QX314 or QX222, have shown that the drug binding site is within the Na<sup>+</sup> channel pore between the selectivity filter

and the channel gates within the membrane electrical field (Strichartz, 1973; Schwarz et al., 1977; Hille, 1992). Two aromatic residues in the middle of domain IV S6 have been proposed as an important part of the local anesthetic binding site for permanently charged, partially charged or neutral forms of the local anesthetics (Ragsdale et al., 1994; Qu et al., 1995; Ragsdale et al., 1996; Wang et al., 1998). Commonly used tertiary amine drugs such as lidocaine can traverse the membrane to reach the binding site through the inner pore mouth, but membrane-impermeant quaternary amines, such as the QX compounds, normally cannot reach the binding site from the outside in neuronal and skeletal muscle channels (Hille, 1992).

The cardiac Na<sup>+</sup> channel is different. Alpert et al. (1989) reported that QX could block the cardiac Na<sup>+</sup> channel from the outside and suggested that either the drug could pass through the pore or that a second external binding site existed. This cardiac-specific QX block was confirmed by Qu et al. (1995), and they suggested that it was by QX permeation

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**ABBREVIATIONS:** TTX, tetrodotoxin;  $\mu$ 1, adult rat skeletal muscle Na<sup>+</sup> channel  $\alpha$ -subunit; MTSEA, methanethiosulfonate ethylammonium; MTSET, methanethiosulfonate ethyltrimethylammonium; MTSES, methanethiosulfonate ethylsulfonate; CTX, conotoxin; STX, saxitoxin; I-V, current-voltage; DTT, dithiothreitol; Cu(phe)<sub>3</sub>, Cu(II)(1, 10-phenanthroline)<sub>3</sub>; WT, wild-type.

through the external pore vestibule because occlusion of the pore with tetrodotoxin (TTX) prevented QX permeation. They could reduce this QX access by a mutation in the upper part of domain IV S6, where the cardiac specific residue was replaced with the corresponding residue in brain IIA. Subsequently, we found that unnatural mutation of the selectivity filter also resulted in an access path for QX to its inner pore site (Sunami et al., 1997). The effects of selectivity filter mutants on local anesthetic binding implied that the selectivity ring is located in the pore at a level just above the binding site of the drug.

Another unnatural mutation reported by Ragsdale et al. (1994) to create an access path for QX was rat brain IIA I1760A, which was located four residues above (N-terminal to) one of the aromatic residues of the putative local anesthetic binding site (Phe-1764) on domain IV S6. The equivalent mutation in the adult rat skeletal muscle isoform ( $\mu$ 1) (I1575A) was shown by Wang et al. (1998) to produce QX permeation, as seen in the brain IIA channel. We find that the mutation I1575A exposes a Cys residue that is recognized by methanethiosulfonate ethylammonium (MTSEA) modification and that participates in disulfide bond formation under oxidizing conditions. MTSEA modification prevents QX access, but neither of the membrane-impermeant methanethiosulfonate derivatives MTSET or MTSES has any effect on I1575A. Partial occlusion of the outer pore by  $\mu$ -conotoxin ( $\mu$ -CTX) derivative does not affect QX access in I1575A. Neither the mutation to Ala or Glu (I1575A, I1575E) nor MTSEA-modified I1575A alters the blocking efficacy of TTX, saxitoxin (STX), or  $\mu$ -CTX, and I1575A does not alter ion selectivity, emphasizing that the outer vestibule and Na<sup>+</sup> permeation path is not significantly distorted by mutation of Ile-1575. The Cys mutant I1575C is insensitive to external cysteinyl ligands. We conclude that this Ile is buried in the protein and that its replacement with Ala creates a hydrophobic path for QX without significant alteration of the selectivity ring.

## Materials and Methods

**Site-Directed Mutagenesis.** Site-directed mutagenesis was carried out on two different rat skeletal muscle ( $\mu$ 1) cDNA constructs. One is the  $\mu$ 1 cDNA flanked by the *Xenopus laevis* globulin 5' and 3' untranslated regions in pAlter (Promega, Madison, WI) (provided by J. R. Moorman, University of Virginia, Charlottesville, VA) and another is in the Bluescript SK vector (Stratagene, La Jolla, CA). In the pAlter construct, the D400A mutation was introduced using the Unique Site Elimination Kit according to the manufacturer's protocol (Amersham Pharmacia Biotech, Piscataway, NJ). Also in the pAlter construct, the E755A, K1237E, and A1529D mutations were made using polymerase chain reaction in a four-primer strategy. In the Bluescript SK construct, the K1237A, I1575A, I1575C, I1575E, C1521A/I1575A, C1569L/I1575A, and C1572T/I1575A mutations were made using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene). The double mutant C1521A/I1575A was made with the I1575A construct as the template using the QuikChange Kit. Mutagenic oligonucleotide primers included changes in silent restriction sites as well as the desired mutation. Mutations identified as positive through qualitative restriction mapping were confirmed by DNA sequencing of the regions contained between unique restriction sites used in the subsequent reconstruction of the full-length plasmids.

All  $\mu$ 1 mutations in the pAlter construct (D400A, E755A, and A1529D) were linearized with *Sa*I and templates were transcribed

with SP6 RNA polymerase using the mCAP mRNA Capping Kit (Stratagene) according to the manufacturer's protocols. Mutations in the Bluescript construct (K1237A, K1237E, I1575A, I1575C, I1575E, C1521A/I1575A, C1569L/I1575A, and C1572T/I1575A) were linearized with *Spe*I and templates were transcribed with T7 RNA Polymerase using the T7 Message Machine Kit according to the manufacturer's protocols (Ambion, Austin, TX).

**Electrophysiological Recordings.** Stage V and VI *X. laevis* oocytes were isolated, and approximately 50 to 100 ng of cRNA was injected into each oocyte. Oocytes were incubated at 16°C for 1 to 5 days before examination. Recordings were made in the two-electrode voltage clamp configuration using a CA-1 voltage clamp (Dagan, Minneapolis, MN), as reported previously (Sunami et al., 1997). 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<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES, pH 7.2. For most of the experiments except the following gating and selectivity experiments, 35-ms test pulses were applied to -10 mV from a holding potential of -90 to -120 mV every 20 s. To determine the activation parameters, the current-voltage (I-V) relationship was fitted to a transform of a Boltzmann distribution:  $I = (V - V_{rev})G_{max} / \{1 + \exp[(V_{1/2} - V)/k]\}$ , where  $I$  is the peak Na<sup>+</sup> current during the test pulse of voltage,  $V$ . The parameters estimated by the fitting were  $V_{1/2}$  (the voltage for half-activation),  $k$  (slope factor),  $V_{rev}$  (the reversal potential), and  $G_{max}$  (the maximum peak conductance). After induction of steady-state inactivation by 20-s depolarized prepulses from a holding potential of -100 mV, Na<sup>+</sup> currents were measured during the test pulses to -10 mV applied every 45 s. Depolarized prepulses with 20-s duration will produce both fast and slow inactivation. Availability was described by the following Boltzmann equation:  $I / I_{max} = 1 / \{1 + \exp[(V - V_{1/2})/k]\}$ , where  $I$  is the peak current,  $I_{max}$  is the maximum peak current,  $V$  is the prepulse voltage,  $V_{1/2}$  is the voltage for half-inactivation, and  $k$  is the slope factor. The recovery from inactivation induced by 1-s depolarization to -10 mV was monitored and the time course of recovery was fitted by a single exponential.

For ionic selectivity experiments, standard bath solution contained 94 mM NaCl, 0.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES, pH 7.2, and NaCl was replaced with equimolar test cations that were adjusted to pH 7.2 with the corresponding hydroxide salt or Tris base. In case of K1237 mutants, 500  $\mu$ M niflumic acid was added to the bath solution to prevent endogenous Ca<sup>2+</sup>-activated Cl<sup>-</sup> currents. For the I-V relationship, a  $P/4$  method was used for leak and capacitance subtraction. Permeability ratios ( $P_X/P_{Na}$ ) for a given test cation were calculated using the following equation (Hille, 1992):  $P_X / P_{Na} = ([Na^+]_o/[X^+]_o) \exp[(E_X - E_{Na})zF/RT]$ , where  $E_X$  and  $E_{Na}$  are the reversal potentials for the test cation (X) and Na<sup>+</sup>, respectively,  $z$  is the valence of the test cation,  $R$  is the gas constant,  $T$  is absolute temperature, and  $F$  is the Faraday constant. Reversal potentials were calculated by fitting the I-V relationship to a Boltzmann distribution function as described in the above.

**Chemicals.** TTX and STX were obtained from Calbiochem (La Jolla, CA).  $\mu$ -Conotoxin GIIIA and methanethiosulfonate compounds (MTSEA, MTSET, MTSES) were obtained from Research Biochemicals International (Natick, MA) and Toronto Research Chemicals (North York, ON, Canada), respectively. R13N, a  $\mu$ -CTX analog, was a gift of Dr. R. J. French (University of Calgary, Canada). Dithiothreitol (DTT) was from Sigma (St. Louis, MO) and Cu(II)(1,10-phenanthroline)<sub>3</sub> [Cu(phe)<sub>3</sub>] was prepared by dissolving Cu(II)SO<sub>4</sub> and 1,10-phenanthroline in a 4:1 water/ethanol solution (Careaga and Falke, 1992; Bénitah et al., 1997). QX222 was a generous gift from Astra Pharmaceuticals (Westboro, MA).

Pooled data are presented as the mean  $\pm$  S.E.M. Statistical comparisons were made by using Student's  $t$  test or one- or two-way analyses of variance followed by Tukey test, and  $p < 0.05$  was considered significant.

## Results

**Mutations of Ile-1575 Create an Access Path for External QX.** We first examined the effects of externally applied 500  $\mu\text{M}$  QX222 on  $\mu\text{1}$  wild-type (WT) and Ala mutant of Ile-1575 (I1575A), which is equivalent to I1760A in the rat brain IIA channel. When stimulations were applied at 20-s intervals with 35-ms pulses to  $-10$  mV from a holding potential of  $-100$  (for WT) or  $-120$  mV (for I1575A), WT showed little block, but I1575A allowed obvious block during exposure to 500  $\mu\text{M}$  QX222 in the bath solution (Fig. 1A, B). Twelve minutes after external application of 500  $\mu\text{M}$  QX222, I1575A showed significant block compared with WT (WT,  $14.2 \pm 1.6\%$  block,  $n = 8$ ; I1575A,  $22.2 \pm 0.4\%$  block,  $n = 5$ ;  $p < 0.01$ ), which is consistent with the previous reports on external QX314 block (Ragsdale et al., 1994; Wang et al., 1998). Substitution of Ile with Cys (I1575C) or with a negatively charged Glu (I1575E) also showed  $66.4 \pm 5.4\%$  ( $n = 5$ ) and  $63.1 \pm 2.5\%$  ( $n = 4$ ) block by 500  $\mu\text{M}$  QX222, respectively (Fig. 1, C and D), which is greater block than that of I1575A ( $p < 0.001$ ). The time course of block also differed between these mutants. I1575A showed the fastest onset rate ( $\tau = 76 \pm 16$  s,  $n = 5$ ,  $p < 0.05$  versus I1575C or I1575E) and those of I1575C and I1575E were similar to the each other (I1575C,  $\tau = 122 \pm 9$  s,  $n = 5$ ; I1575E,  $\tau = 121 \pm 7$  s,  $n = 4$ ).

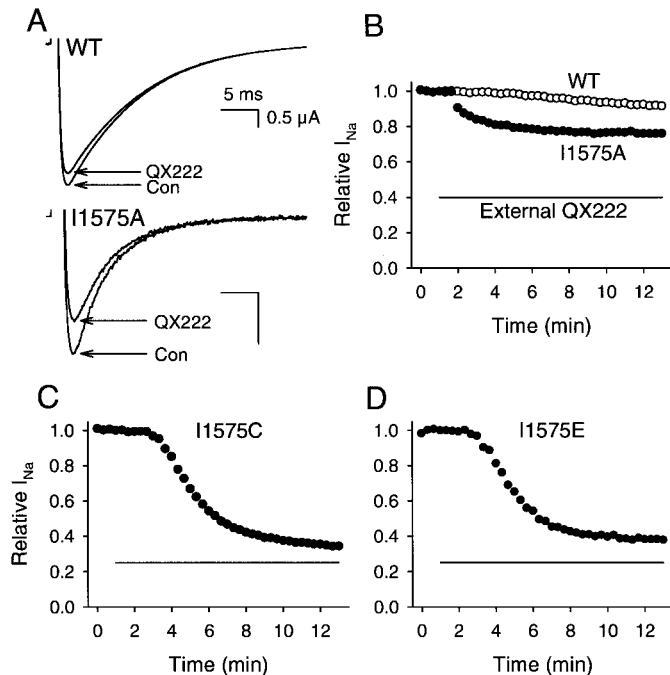
**Gating Changes by Ile-1575 Mutations.** Although the three mutations of Ile-1575 all allowed external QX block, they affected the gating properties of the channel differently (Table 1). In general, Cys and Glu substitutions behaved

similarly, but differently from Ala substitution. I1575A had little effect on the voltage dependence of activation, but it was shifted by  $-9$  and  $-7$  mV in I1575C and I1575E, respectively. I1575A reduced the slope of the activation curve by 2 mV, but the others had no effect on the slope. All three mutations shifted the steady-state availability curve in the negative direction,  $-27$  mV for I1575A,  $-16$  mV for I1575C, and  $-12$  mV for I1575E. Recovery from inactivation was unchanged for I1575A, but accelerated for I1575C and I1575E. Decay of the activated current was accelerated by all three mutants, with the most dramatic effect by I1575A and intermediate effects by I1575C and I1575E.

**I1575A Has Minimal Effects on Ion Selectivity.** Because selectivity filter mutations created an access path for external QX (Sunami et al., 1997), the possibility that Ile-1575 mutant changed the ionic selectivity and consequently allowed QX permeation by a similar mechanism was investigated by determining the permeability ratio ( $P_{\text{X}}/P_{\text{Na}}$ ) for a series of monovalent alkali cations and organic cations (Fig. 2). As in previous reports on ion selectivity (Heinemann et al., 1992b; Chiamvimonvat et al., 1996; Favre et al., 1996; Sun et al., 1997; Tsushima et al., 1997a), mutations of the putative selectivity filter changed the permeability of various cations, but I1575A produced minimal changes in selectivity compared with WT. This was also confirmed by calculation of the current ratio of peak inward current in the presence of the test cations to that in the presence of  $\text{Na}^+$  (not shown). Consistent with this, I1575A changed the reversal potential minimally with normal outside solutions containing 94 mM  $\text{Na}^+$ , 0.5 mM  $\text{Ca}^{2+}$ , 1 mM  $\text{Mg}^{2+}$  (WT,  $49.8 \pm 0.8$  mV,  $n = 28$ ; I1575A,  $45.8 \pm 1.1$  mV,  $n = 24$ ). On the other hand, negative shifts of the reversal potential were observed in mutations of the putative selectivity filter (E755A,  $34.7 \pm 0.6$  mV,  $n = 24$ ; K1237A,  $-2.0 \pm 0.4$  mV,  $n = 29$ ; K1237E,  $3.7 \pm 0.7$  mV,  $n = 19$ ; A1529D,  $40.7 \pm 1.1$  mV,  $n = 24$ ). Dramatic changes in selectivity were observed in Lys-1237 mutants, but these mutants never allowed external QX permeation (Sunami et al., 1997). On the other hand, E755A and A1529D revealed intermediate effects on ionic selectivity, but showed apparent external QX block (Sunami et al., 1997). This means that QX permeation does not correlate with a change in ionic selectivity for mutations of the selectivity filter residues or Ile-1575.

**Ile-1575 Mutants Do Not Affect Binding of Outer Vestibule Toxin.** To investigate the structural change in the outer vestibule with mutants of Ile-1575 or the possible contribution of Ile-1575 to the outer vestibule, we examined the effects of Ile-1575 mutants on binding of TTX, STX, and  $\mu\text{-CTX}$ . I1575A did not affect binding of any of the toxins (Table 2). From this result and the lack of effect on ion selectivity, it seems that the molecular structure of the outer vestibule in I1575A was preserved, despite the large change in gating. Substitution of Ile-1575 with a negatively charged residue, Glu (I1575E), also had no effect on the affinity for TTX, STX, and  $\mu\text{-CTX}$  (Table 2). This supports the idea that Ile-1575 is not exposed in the outer vestibule.

**I1575A Exposes Cys Residue.** Wang et al. (1998) reported an increase of  $\text{Cd}^{2+}$  sensitivity by I1575A using a mammalian cell expression system. Here we also examined the  $\text{Cd}^{2+}$  sensitivity of Ile-1575 mutants using an oocyte expression system.  $\text{Cd}^{2+}$  block was measured by applying 35-ms pulses to  $-10$  mV from a holding potential of  $-100$



**Fig. 1.** Mutation of  $\mu\text{1}$  Ile-1575 allows block by external QX222. A, typical current traces before (con) and 12 min after external application of 500  $\mu\text{M}$  QX222 recorded from  $\mu\text{1}$  WT (upper panel) and I1575A (lower panel). B to D, effects of externally applied 500  $\mu\text{M}$  QX222 on WT (○) and I1575A (●) (B), I1575C (C), and I1575E (D). The bar indicates the period of exposure to 500  $\mu\text{M}$  QX222 in the bath solution. Currents were elicited by 35-ms pulses to  $-10$  mV from a holding potential of  $-100$  (WT),  $-110$  (I1575C, I1575E) or  $-120$  mV (I1575A) at 20-s intervals, and peak currents were normalized to peak current in control and plotted as relative  $\text{Na}^+$  currents (relative  $I_{\text{Na}}$ ). Representative data were shown in B to D, and data of A and B were from the same oocytes.



(WT),  $-110$  (I1575C), and  $-120$  mV (I1575A) at 20-s intervals. In WT, the  $\text{Cd}^{2+}$  dissociation constant ( $K_d$ ) was  $430 \pm 34 \mu\text{M}$  ( $n = 16$ ) and little change was observed with I1575C ( $K_d = 580 \pm 52 \mu\text{M}$ ,  $n = 4$ ,  $p > 0.05$ ) (Fig. 3A). I1575A showed a small but significant increase of  $\text{Cd}^{2+}$  sensitivity ( $K_d = 359 \pm 22 \mu\text{M}$ ,  $n = 12$ ,  $p < 0.05$ ) (Fig. 3A). Instead, a more obvious change in sensitivity to cysteinyl ligands was observed in MTSEA modification: 2.5 mM MTSEA blocked the WT channel slightly ( $9.0 \pm 1.2\%$  block,  $n = 5$ ) but blocked I1575A current by  $40.9 \pm 3.0\%$  ( $n = 10$ ,  $p < 0.001$ ) after a 4-min exposure and 5-min washout (Fig. 3, B and C). The concentration of 2.5 mM seemed to be sufficient to modify all of the  $\text{Na}^+$  channels because exposure to 5 mM MTSEA blocked I1575A current to the same extent [ $39.1 \pm 7.6\%$  ( $n = 4$ )] after a 4-min exposure. Reduction of I1575A current by 2.5 mM MTSEA was accompanied by a slower component of recovery from inactivation ( $\tau = 8340 \pm 1183$  s,  $n = 4$ ,  $p < 0.01$  versus I1575A), but MTSEA modification had negligible effects on activation, availability, and current decay (not shown). On the other hand, 2.5 mM MTSEA had little effect on I1575C channel ( $6.5 \pm 0.1\%$  block,  $n = 2$ ) (Fig. 3, B and C). We also examined the effects of other charged MTS reagents (MTSET, MTSES) on these channels because MTSEA can cross the membrane quite easily (Holmgren et al., 1996). In contrast to the effects of MTSEA, saturated concentrations of membrane-impermeant positively charged MTSET (1 mM) and negatively charged MTSES (10 mM) had no effects on I1575A (Fig. 3C). In addition, failure of block by MTSET and MTSES was observed in I1575C (Fig. 3C). The fact that none of these three MTS reagents and  $\text{Cd}^{2+}$  from the outside affected the I1575C current suggests that Ile-1575 is not on the surface of the outer vestibule. It is possible that binding to Cys occurred without measurable functional effect, but if the residue were in the narrow vestibule, some block would have been expected.

#### QX Access in I1575A Is Not through the Outer Pore.

To investigate the possible location of Cys exposed in I1575A, we compared the binding affinity of outer vestibule toxins before and after MTSEA modification (Table 2). The residual currents after exposure to 2.5 mM MTSEA for 4 min and subsequent washout for 5 min were further examined for toxin block. MTSEA-modified I1575A channel had similar affinities for TTX, STX and  $\mu$ -CTX to those of WT and I1575A. As noted earlier, this seemed to be a saturated concentration of MTSEA, implying that the residual currents after MTSEA exposure were from channels with incomplete block, rather than from unaffected channels. The mechanism of block of current by MTSEA in the I1575A mutant channel

is not clear, but we did find that recovery from inactivation was slowed substantially after MTSEA modification. Based on this result, the Cys exposed in I1575A and its complex with MTSEA seemed not to reside in the outer vestibule. We further studied the effects of MTSEA modification on external QX block. After a 12-min perfusion with 500  $\mu\text{M}$  QX222, the MTSEA-modified I1575A channel showed  $5.8 \pm 1.3\%$  block ( $n = 3$ ), which is significantly less than that of I1575A ( $p < 0.001$ ) (Fig. 4A). On the other hand, 2.5 mM MTSEA had little effects on external QX222 block in I1575C ( $65.8 \pm 5.7\%$  block,  $n = 3$ ) and I1575E ( $61.5 \pm 4.9\%$  block,  $n = 5$ ).

To investigate whether QX access in I1575A is through the outer vestibule, we studied the effects of binding the  $\mu$ -CTX derivative R13N on external QX block.  $\mu$ -CTX binds superficially to the outer pore (Dudley et al., 1995; Chang et al., 1998) and R13N blocks the channel with a small residual single channel current. When a saturating concentration (10  $\mu\text{M}$ ) of R13N was applied to the I1575A channel, the currents were blocked by  $82.0 \pm 2.8\%$  ( $n = 4$ ) (Fig. 4B). The remaining residual currents after R13N treatment were further examined for QX222 block. In the example of Fig. 4B, when 500  $\mu\text{M}$  QX222 was applied externally to R13N-bound I1575A channel, the current was further blocked by 28.5%. On average, R13N-bound channels showed  $25.7 \pm 1.5\%$  ( $n = 3$ ) block by 500  $\mu\text{M}$  QX222 with onset rates of  $79 \pm 13$  s, which is similar to that of I1575A (Fig. 4, C and D). This failure of R13N to interfere with QX222 block in I1575A is different from the results with the selectivity filter mutants (D400A, A1529D) and Y401C, where guanidinium toxins or R13 analog reduced outside QX block substantially (Sunami et al., 1997, 2000). These results support the suggestion that the external QX path in I1575A is not through the outer pore/vestibule and that the space in which MTSEA forms a disulfide bridge with an exposed Cys may be part of that external path for QX.

**Two Cys Residues Are Exposed in I1575A.** To our surprise, in the presence of a redox catalyst,  $\text{Cu}(\text{phe})_3$ , I1575A current was almost completely blocked ( $93.8 \pm 0.8\%$  block,  $n = 5$ ) (Fig. 5A). Washout of  $\text{Cu}(\text{phe})_3$  had minimal effect, but exposure to the reducing agent DTT rapidly reversed the current up to the level of 75% of the control. Initial exposure to DTT did not affect the I1575A current (not shown), so it seems that no disulfide bond had developed spontaneously. Consequently, it seems that I1575A reveals two Cys residues close enough to form a current-inhibiting disulfide linkage (distance of  $\beta$  carbons of two Cys  $< 4.6 \text{ \AA}$ ) (Srinivasan et al., 1990; Careaga and Falke, 1992). On the other hand,  $\text{Cu}(\text{phe})_3$  had little effect on WT current ( $3.9 \pm$

TABLE 1

Gating parameters of  $\mu 1$  WT and Ile-1575 mutants

Parameters of activation and availability ( $V_{1/2}$ ,  $k$ ) were estimated according to the Boltzmann equations, and time constant ( $\tau$ ) of recovery from inactivation was determined by single exponential fit as described under *Materials and Methods*.  $T_{1/2\text{decay}}$  and  $n$  represent the time to half decay after peak of the maximum current ( $I_{\text{max}}$ ) and number of experiments, respectively.

Channels	Activation			Availability			Recovery		Current decay of $I_{\text{max}}$	
	$V_{1/2}$	$k$	$n$	$V_{1/2}$	$k$	$n$	$\tau$	$n$	$T_{1/2\text{decay}}$	$n$
		mV			mV		ms		ms	
WT	$-16.2 \pm 0.9$	$4.2 \pm 0.2$	9	$-62.5 \pm 1.0$	$4.6 \pm 0.1$	12	$3451 \pm 286$	10	$11.6 \pm 0.9$	11
I1575A	$-13.9 \pm 2.1$	$6.0 \pm 0.4^{***}$	8	$-89.3 \pm 1.3^{***}$	$6.4 \pm 0.3^{***}$	11	$3094 \pm 283$	5	$3.3 \pm 0.4^{***}$	6
I1575C	$-25.5 \pm 0.9^{***}$	$4.2 \pm 0.2$	6	$-78.6 \pm 1.0^{***}$	$5.6 \pm 0.1^{***}$	6	$1027 \pm 104^{***}$	3	$7.0 \pm 0.5^{**}$	6
I1575E	$-23.0 \pm 1.1^{***}$	$4.3 \pm 0.3$	5	$-74.7 \pm 1.8^{***}$	$6.3 \pm 0.2^{***}$	7	$1377 \pm 196^{**}$	3	$6.9 \pm 0.4^{**}$	5

\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  compared with WT.

1.3% block,  $n = 4$ ) (Fig. 5A). Which Cys residues contribute to the disulfide bond formation? If domain IV S6 is an  $\alpha$ -helical structure, Cys-1569 and Cys-1572 are good candidates because these and Ile-1575 are on the same face of  $\alpha$ -helical structure, and these are one or two turns above Ile-1575 (Wang et al., 1998). To test this idea, we constructed the double mutants composed of I1575A plus C1569L (C1569L/I1575A) or C1572T (C1572T/I1575A). Similar to the I1575A channel, 100  $\mu$ M Cu(phe)<sub>3</sub> blocked C1569L/I1575A and C1572T/I1575A channels almost completely, and their irreversibility was verified (Fig. 5, C and D). This excludes the possibility of contribution of Cys-1569 and Cys-1572 to the disulfide bond formation. We also tested the effects on another double mutant containing C1521A, which is eight residues amino-terminal to the selectivity filter residue, Ala-1529 in P-loop of domain IV, because of the possibility that P-loops are flexible (Bénitah et al., 1997; Tsushima et al., 1997b). However, C1521A/I1575A also allowed disulfide bond

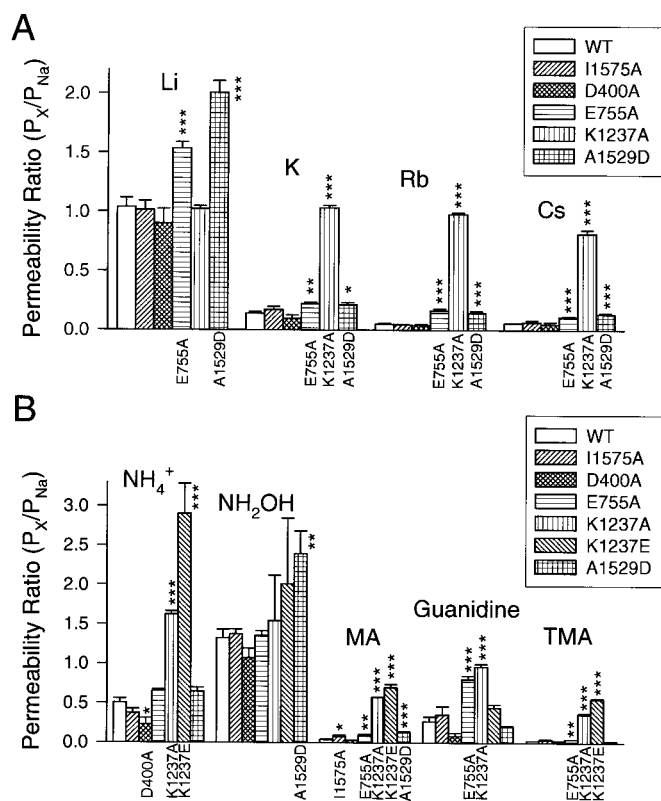
formation in the presence of Cu(phe)<sub>3</sub> (Fig. 5B). When 2.5 mM MTSEA was applied externally to these three double mutants, the blocking amount was similar between these double mutants and I1575A (not shown). These results suggest that Cys-1521, Cys-1569, and Cys-1572 are not the Cys residues exposed by I1575A to MTSEA modification and that these Cys residues are not involved in the external QX path.

## Discussion

Channels with the  $\mu$ 1-I1575A, I1575C and I1575E mutations expressed ample currents in *X. laevis* oocytes, implying that the mutation did not seriously interfere with protein folding, stability in the membrane, and channel function. This was also true for the analogous I1760A mutation in rat brain IIA (Ragsdale et al., 1994), and the  $\mu$ 1-I1575A expressed in human embryonic kidney 293t cells (Wang et al., 1998). However, the corresponding mutant in rat heart (I1758A) did not express (Qu et al., 1995), and Wang and Wang (1999) did indicate that mutations  $\mu$ 1-I1575K and I1575D expressed poorly in human embryonic kidney 293t cells.

**Kinetic Effects.** The three mutations of Ile-1575 produced two types of gating changes. Ala substitution had little effects on voltage dependence of activation or recovery from inactivation but it speeded current decay and shifted the voltage dependence of inactivation 27 mV in the negative direction. Cys or Glu substitution shifted activation in the negative direction and accelerated recovery from inactivation. They also affected current decay and voltage dependence of inactivation, but to a lesser degree than Ala substitution. The only observations available for comparison are those of Ragsdale et al. (1994) and McPhee et al. (1995), who reported that the analogous mutation in rat brain IIA (I1760A) expressed with the  $\beta$ 1-subunit in oocytes showed no change in activation or current decay. Mutation of other residues in domain IV S6 also affects channel gating, and McPhee et al. (1995) have suggested that domain IV S6 plays an important role in fast inactivation of the Na<sup>+</sup> channel. Recently, voltage sensing for inactivation has been associated with domains III and IV (Cha et al., 1999), so that mutations in domain IV S6 could possibly affect the voltage sensor of domain IV. Our demonstration that mutations of Ile-1575 also affect activation raises the possibility that this residue contributes to interdomain interaction, perhaps with domain I, as suggested for batrachotoxin (Linford et al., 1998; Wang and Wang, 1998; Wang and Wang, 1999).

**External QX Block.** All three mutations of Ile-1575 permitted block of the current by external QX222 with time constants of 1 to 2 min. I1575A showed less block than the other mutations, but block developed faster. Two kinds of structural differences have been previously associated with



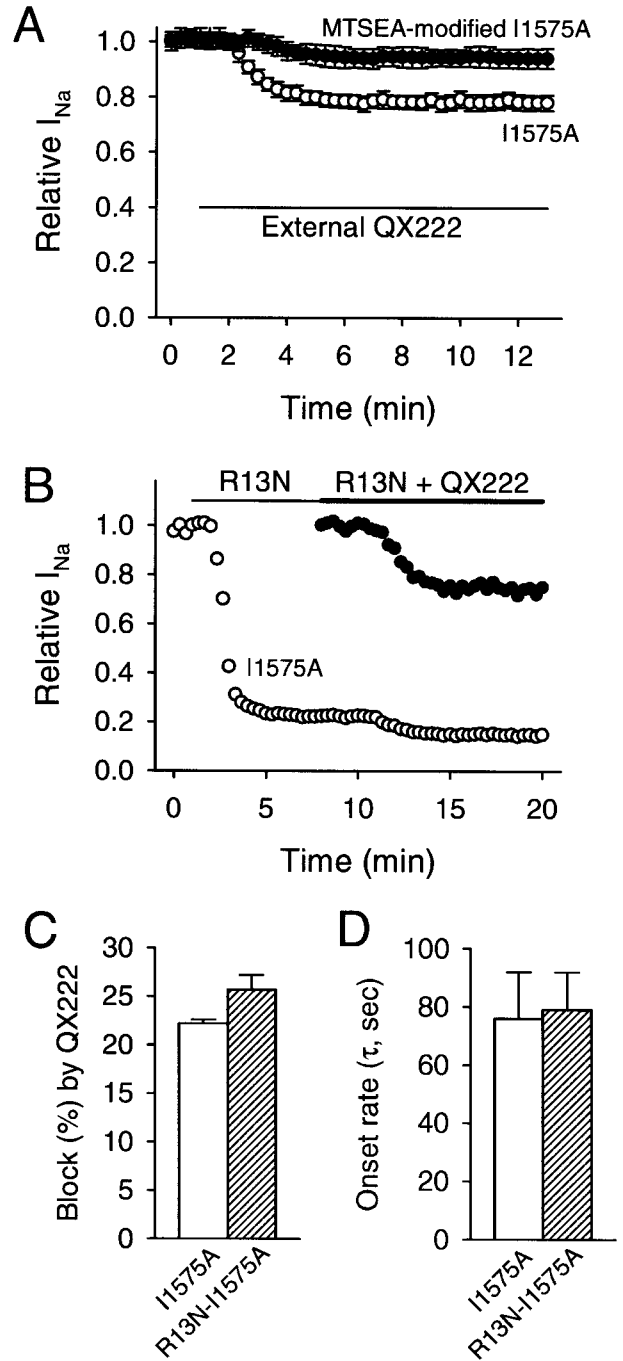
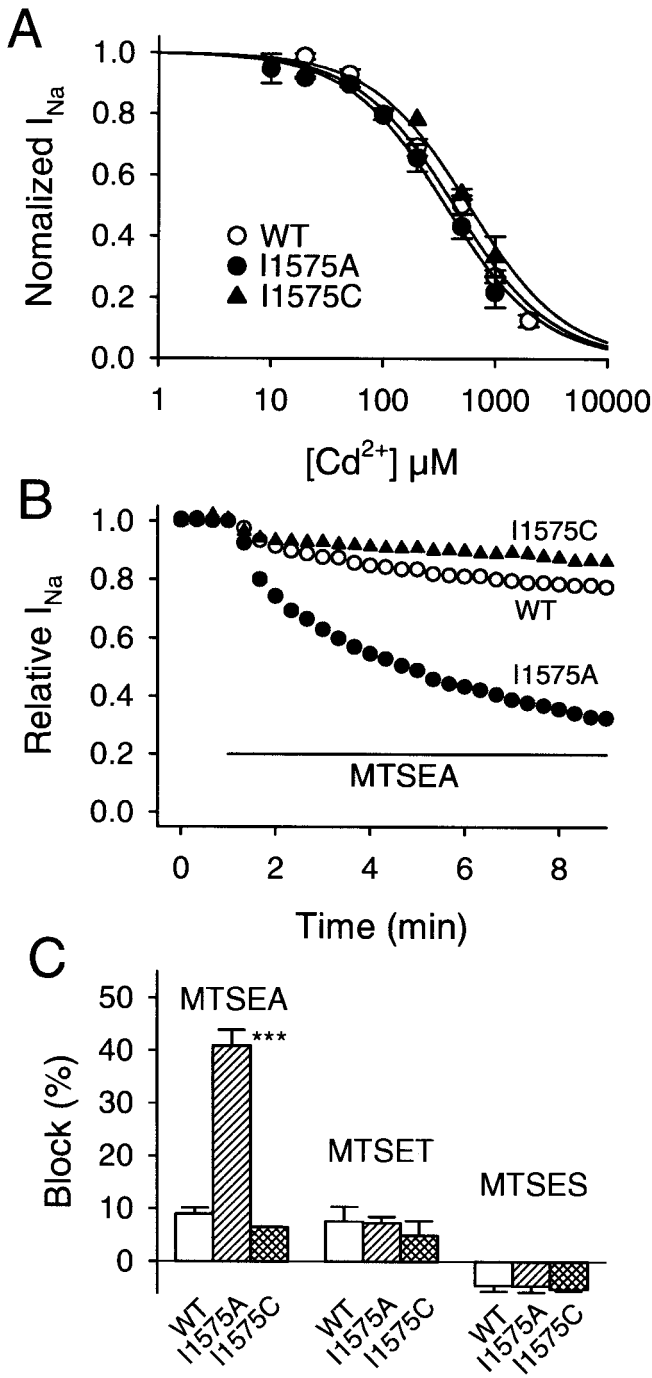
**Fig. 2.** Permeability ratios for alkali (A) and organic cations (B) of I1575A and putative selectivity filter mutants. For determination of permeability ratios ( $P_X/P_{Na}$ ), see under *Materials and Methods*. Data represent the mean  $\pm$  S.E.M. from three to eight oocytes. Mutants' names are labeled below columns for those showing significant change in permeability. MA, methylamine; TMA, tetramethylammonium. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared with WT.

**TABLE 2**

Effects of Ile-1575 mutants on outer vestibule toxin binding

$IC_{50}$  values were calculated from the equation,  $IC_{50} = [TXN](I_{TXN}/I_{Con})/(1 - I_{TXN}/I_{Con})$ , where [TXN] is toxin concentration, and  $I_{TXN}$  and  $I_{Con}$  are the peak currents in the presence and absence of toxin, respectively. Values are reported as nanomolar concentrations.

	WT		I1575A		MTSEA-modified I1575A		I1575E	
	$IC_{50}$	$n$	$IC_{50}$	$n$	$IC_{50}$	$n$	$IC_{50}$	$n$
TTX	36.6 $\pm$ 3.7	5	39.1 $\pm$ 6.5	5	40.2 $\pm$ 5.6	3	41.0 $\pm$ 9.9	3
STX	3.1 $\pm$ 0.4	4	3.2 $\pm$ 0.5	4	3.5 $\pm$ 0.7	3	3.9 $\pm$ 0.8	4
$\mu$ -CTX	15.1 $\pm$ 2.8	5	14.9 $\pm$ 4.7	5	12.1 $\pm$ 2.2	3	14.5 $\pm$ 4.5	3





the existence of an access path for QX from the outside in the cardiac isoform and its creation in other isoforms—isoform sequence differences and selectivity filter changes. The outer third of domain IV S6 has a Thr in position 1755 of the rat heart channel, a Cys in the analogous position in  $\mu 1$ , and a Val in rat brain IIA. Qu et al. (1995) found that substitution of Val for the Thr in rat heart isoform reduced the outside QX block seen in the wild-type cardiac channel. We found that substitution of Thr in the analogous position of  $\mu 1$  created an outside access path for QX in that isoform (Sunami et al., 2000). Another well known isoform difference that produces the different guanidinium toxin affinity is in the domain I P-loop (Backx et al., 1992; Chen et al., 1992; Heinemann et al., 1992a; Satin et al., 1992). Just above the selectivity filter residue in heart is a Cys, with a Tyr in an analogous position in  $\mu 1$  and a Phe in brain IIA. We found that this residue in the cardiac isoform also contributes to outside QX block and that it is additive to the domain IV S6 isoform difference (Sunami et al., 2000). These two isoform differences may fully explain the sensitivity of the native cardiac  $\text{Na}^+$  channel to outside QX block.

The second type of structural change producing outside QX block is in the selectivity filter. QX is larger than organic cations that just have a finite permeability in the  $\text{Na}^+$  channel, but mutation of three of the four putative selectivity filter residues in the  $\mu 1$  isoform permitted outside QX block (Sunami et al., 1997). Our recent molecular model of the  $\text{Na}^+$  channel based on the structure of the KcsA channel crystal structure (Doyle et al., 1998) suggested that Ile-1575 is immediately adjacent to the selectivity filter (Lipkind and Fozzard, 2000). Although this raised the possibility that the mechanism of creation of a QX pathway by the Ile-1575 mutations might be dislocation of the selectivity filter, we found no difference at all in selectivity for the mutation

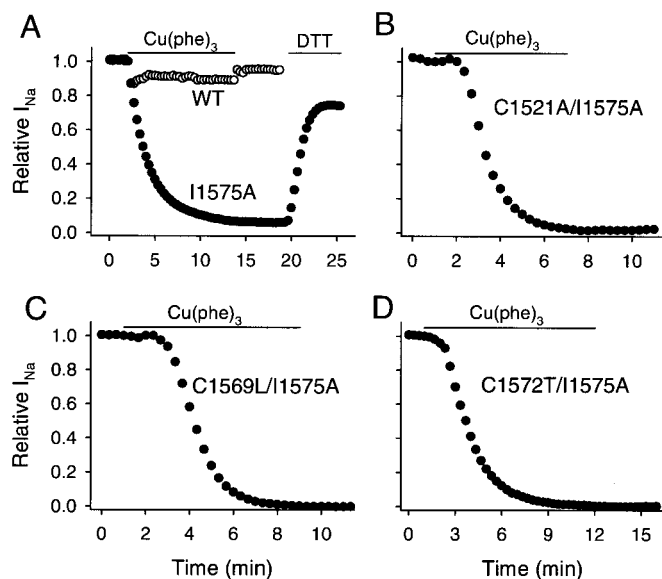
I1575A, in contrast to the changes from selectivity filter mutants. A second test of the integrity of the outer vestibule and selectivity ring is binding of multivalent site-1 toxins, TTX, STX, and  $\mu$ -CTX. Any distortion of these sites would be expected to reduce the toxin binding affinity, but no change was found with the Ile-1575 mutations. We must conclude that Ile-1575 is not important in maintaining the precise structure of the selectivity ring and outer vestibule.

The Cys/Tyr/Phe residue in the site 1 toxin site and the selectivity filter residues are unquestionably in the  $\text{Na}^+$  permeation path, and it is plausible to assume that in that case QX reaches its binding site by traversing the pore itself. However, the path created by the S6 isoform-specific residues seems to be additive and probably does not lead directly through the pore.

**Location of Ile-1575.** Wang et al. (1998) found the surprising result that the current of the  $\mu 1$ -I1575A mutation was strikingly blocked by outside  $\text{Cd}^{2+}$ , leading them to suggest that this domain IV S6 residue is close to the permeation path. Indeed, there is ample evidence that the inner halves of the S6 segments line the inner pore of the Shaker  $\text{K}^+$  channel (Liu et al., 1997) and this will be the case for the  $\text{Na}^+$  channel, as suggested from local anesthetic studies (Ragsdale et al., 1994; Nau et al., 1999). Wang et al. (1998) suggested that block by  $\text{Cd}^{2+}$  in the mutant I1575A channel means that the mutation has exposed a  $\text{Cd}^{2+}$  binding site, presumably a Cys, to the permeation path. They reported that  $\text{Cd}^{2+}$  blocked the I1575A current very slowly and the block could not be reversed, implying to us that the  $\text{Cd}^{2+}$  site is not freely accessible but instead that it is buried in the protein. We used the methanethiosulfonates (MTS) as alternative ligands for detection of an exposed Cys. MTSEA, which can enter the membrane phase (Holmgren et al., 1996), had no effect on the wild-type  $\text{Na}^+$  current, but it blocked the I1575A current. However, the membrane-impermeant MTSET and MTSES failed to block the I1575A current. Neither  $\text{Cd}^{2+}$ , MTSEA, MTSET, nor MTSES produced block of the mutant I1575C. Also, substitution with the negatively charged residue I1575E did not change the toxin affinity. These results support the conclusion that Ile-1575 and the sulfhydryl site exposed by its replacement with Ala are not exposed on the surface of the protein facing the aqueous pore.

**Location of the Outside QX Access Path.** If Ile-1575 is buried in the protein and its mutations fail to affect the outer vestibule or selectivity ring structure, then the QX access path created by mutation of Ile-1575 is unlikely to lead directly through the normal permeation path. Furthermore, MTSEA interaction with the exposed Cys in the I1575A mutation blocked the QX access path without affecting the binding of site 1 toxins in the permeation path. The inverse question is block of QX permeation by pore-blocking toxins. We could block about 80% of the current with the  $\mu$ -CTX analog R13N without affecting QX access in I1575A. This further supports the idea that the path for QX in I1575A does not lead through the pore.

DTT had no effect on wild-type or I1575A mutant currents, but the redox agent  $\text{Cu}(\text{phe})_3$  did block the current of I1575A. The mutation must have exposed not one, but two Cys residues, which are close enough to each other to form a disulfide bond under oxidizing conditions. Because the reactive Cys residues are not in the permeation path, the mechanism of



**Fig. 5.** Disulfide bond formation in I1575A in the presence of a redox catalyst,  $\text{Cu}(\text{phe})_3$ . A-D, representative data showing the effects of externally applied  $\text{Cu}(\text{phe})_3$  on WT (○) and I1575A (●) (A), double mutants, C1521A/I1575A (B), C1569L/I1575A (C), and C1572T/I1575A (D). Currents were elicited by 35-ms pulses to  $-10$  mV from a holding potential of  $-100$  or  $-120$  mV every 20 s, and peak currents were normalized to that in the control. The bar indicates the period during exposure to  $100 \mu\text{M}$   $\text{Cu}(\text{phe})_3$  or  $10$  mM DTT in the bath solution.

this block is probably by disabling the gating of the channel. The block was almost complete, so that the effect of Cu(phe)<sub>3</sub>-induced block on QX access could not be made. Wang et al. (1998) made the logical suggestion that the involved Cys was located on domain IV S6, one or two turns above Ile-1575. However, neither these nor the Cys in the N-terminal end of domain IV P-loop are involved. Other choices for Cys to participate in the disulfide linkage are two conserved Cys in the linker between the P-loop and S6 of domain IV.

#### Role of QX Access Path in Antiarrhythmic Therapy.

An access path from the outside to the internal binding site for local anesthetic drugs could have important implications for the pharmacokinetics of this class of drugs (Lee et al., 2000). Clinical actions depend in part on drug off-rates because they underlie the phenomenon of use dependence. The opportunity for the drugs to dissociate to both the inside and the outside of the membrane means that use dependence will be influenced by the pathway.

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