Batrachotoxin-Resistant Na^+ Channels Derived from Point Mutations in Transmembrane Segment D4-S6

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ABSTRACT Local anesthetics (LAs) block voltage-gated Na^+ channels in excitable cells, whereas batrachotoxin (BTX) keeps these channels open persistently. Previous work delimited the LA receptor within the D4-S6 segment of the Na^+ channel α-subunit, whereas the putative BTX receptor was found within the D1-S6. We mutated residues at D4-S6 critical for LA binding to determine whether such mutations modulate the BTX phenotype in rat skeletal muscle Na^+ channels (μ1/rSkm1). We show that μ1-F1579K and μ1-N1584K channels become completely resistant to 5 μM BTX. In contrast, μ1-Y1586K channels remain BTX-sensitive; their fast and slow inactivation is eliminated by BTX after repetitive depolarization. Furthermore, we demonstrate that cocaine elicits a profound time-dependent block after channel activation, consistent with preferential LA binding to BTX-modified open channels. We propose that channel opening promotes better exposure of receptor sites for binding with BTX and LAs, possibly by widening the bordering area around D1-S6, D4-S6, and the pore region. The BTX receptor is probably located at the interface of D1-S6 and D4-S6 segments adjacent to the LA receptor. These two S6 segments may appose too closely to bind BTX and LAs simultaneously when the channel is in its resting closed state.

INTRODUCTION

Batrachotoxin (BTX) is an alkaloid neurotoxin found most abundantly in the skin of South American poisonous frogs, *Phyllobates terribilis* (Daly et al., 1980). This toxin contains a complex steroidal moiety and is highly hydrophobic. The primary target of BTX is the voltage-gated Na^+ channel, which is essential for the propagation of action potentials in excitable cells (Catterall, 1980; Hille, 1992b). Classified as a Na^+ channel activator, BTX binds specifically to Na^+ channel isoforms with a dissociation constant of ~10 nM (Creveling et al., 1983). Upon binding, BTX drastically alters the gating properties of voltage-gated Na^+ channels (Khodorov, 1978; Khodorov and Revenko, 1979). The activation threshold of Na^+ channels is shifted by BTX to the hyperpolarizing direction by 30–50 mV, whereas the fast and slow inactivations are nearly eliminated. As a result of these gating changes, BTX-modified Na^+ channels open persistently, even at the resting membrane potential. Aside from a decrease in single-channel conductance by BTX, the ion selectivity of Na^+ channels is also reduced; BTX-modified Na^+ channels display increased permeability for NH_4^+, K^+, and Cs^+ ions.

The Na^+ channel α-subunit consists of four homologous domains (D1 to D4, Fig. 1), each with six transmembrane segments (S1–S6). Using a photoreactive BTX probe, Trainer et al. (1996) first reported that the site of covalent labeling is located at or near the transmembrane segment D1–S6 of the brain Na^+ channel α-subunit. Subsequent studies showed that three lysine point mutations at this region render the rat skeletal muscle α-subunit Na^+ channels completely resistant to BTX (Wang and Wang, 1998). The putative BTX receptor is probably located near the middle of the D1–S6 segment, which includes the residues of μ1-I433, N434, and L437 in the rat skeletal muscle Na^+ channel α-subunit.

Local anesthetics (LAs) are clinical drugs that block the voltage-gated Na^+ channels and hence stop the propagation of action potentials (Hille, 1992a). LAs are well known to be noncompetitive antagonists of BTX (Postma and Catterall, 1984). The LA binding site has been delineated near the middle of segment D4–S6 of the voltage-gated α-subunit Na^+ channels (Ragsdale et al., 1994; Fig. 1). Three residues, μ1-F1579 (homologous to brain channel RBIIA-F1764), μ1-N1584 (RBIIA-N1769), and μ1-Y1586 (RBIIA-Y1771), were found critical for LA binding (Ragsdale et al., 1994; Wright et al., 1998). The quaternary ammonium derivative of lidocaine QX-314 is able to reach the binding site and to block the channel only through internal application (Ragsdale et al., 1994; Strichartz, 1973). Therefore, the LA binding site is probably within the pore region between the selectivity filter and the internal vestibule of Na^+ channels. The P-region (located at the external loop between S5 and S6 segments; Fig. 1), which controls the ion selectivity of Na^+ channels, may also be directly involved in LA binding (Sunami et al., 1997).

The binding of LAs and BTX with the Na^+ channel is highly state dependent. LAs bind preferentially to the inactivated state of Na^+ channels, whereas BTX binds favorably to its receptor site when the channel is in its open state (Hille, 1992a,b). The binding sites for LAs and for BTX have been believed to be distinct and separated from each

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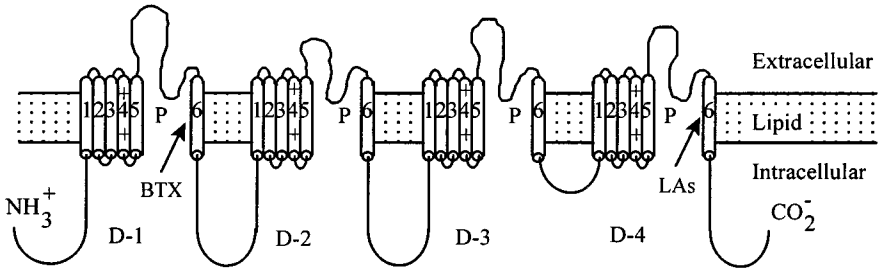

FIGURE 1 The transmembrane organization of the Na^+ channel α-subunit. Arrows indicate the receptor sites for BTX and for LAs. D-1 to D-4 domains, segments 1–6, and the P region are labeled.

other, and the antagonistic reaction between BTX and LA binding within Na^+ channels has been presumed to be due to an indirect allosteric interaction of these two types of ligands (Postma and Catterall, 1984). In a recent report, we demonstrated that BTX-resistant channels with mutations at the D1–S6 segment are less sensitive to LA block than are their wild-type counterparts (Wang et al., 1998a). Based on this finding, we suggested that the binding sites for LAs and for BTX at the D4–S6 and D1–S6 segments of Na^+ channels are aligned in close proximity. Such an alignment, if true, predicts that some of the point mutations at the LA binding site (i.e., at D4–S6) will also affect the binding of BTX (i.e., at D1–S6). In this report we show that the lysine point mutations of μ1-F1579K and μ1-N1584K at and near the LA binding site, respectively, can indeed affect the binding of BTX. Both mutants display an apparent BTX-resistant phenotype. The third lysine point mutation located at the LA binding site, Y1586K, remains BTX sensitive. We also demonstrate the time-dependent block of the open BTX-modified μ1 Na^+ channels by cocaine and present a model for the antagonistic reaction between BTX and LAs in Na^+ channels. Finally, before the submission of this manuscript, we learned about a closely related paper published by Linford et al. (1998). These authors likewise reported the involvement of segment D4–S6 of rat brain RBIIA in BTX binding. However, some of their results based on alanine point mutations are significantly different from those of our homologous mutations. These differences will be reviewed in the Results and Discussion.

MATERIALS AND METHODS

Site-directed mutagenesis

A transformer site-directed mutagenesis kit (Clontech) was used to create μ1 Na^+ channel mutant clones as described (Wright et al., 1998). Two primers (a mutagenesis primer and a restriction primer) were used to generate the desired mutant. The mutation was confirmed by DNA sequencing, using appropriate primers near the mutated region. Mutants of μ1-F1579K, μ1-F1579A, μ1-N1584K, μ1-N1584A, μ1-N1584D, μ1-Y1586K, μ1-Y1586A, and hH1-N1765A were used in this study. Mutants of μ1-I1575K and I1575D were prepared, but they failed to express sufficient Na^+ currents for the determination of the BTX phenotype.

Transient transfection

Human embryonic kidney (Hek293t) cells were grown to ~50% confluence in Dulbecco's minimum essential medium (Gibco) containing 10% fetal bovine serum (HyClone), 1% penicillin and streptomycin solution (Sigma), 3 mM taurine, and 25 mM HEPES (Gibco) and then transfected by a calcium phosphate precipitation method in a Ti25 flask. Transfection of μ1-pcDNA1/Amp (10 μg) and reporter plasmid CD8-pih3m (1 μg) was adequate for later current recording. Cells were replated 15 h after transfection in 35-mm culture dishes, maintained at 37°C in a 5% CO_2 incubator, and used for experiments after 1–4 days. Transfection-positive cells were identified by immunobeads (CD8-Dynabeads, Lake Success, NY).

Whole-cell voltage clamp

Whole-cell configuration was used to record Na^+ currents in cells coated with CD-8 beads (Hamill et al., 1981; Cannon and Strittmatter, 1993). Pipette electrodes contained 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES adjusted to pH 7.2 with CsOH. The tips of the electrodes had a resistance of 0.5–1.0 MΩ. All experiments were performed at room temperature (22–24°C) under a Na^+-free bath solution containing 150 mM choline Cl, 2 mM $CaCl_2$, and 10 mM HEPES adjusted to pH 7.4 with tetramethylammonium hydroxide. The benefit of using a Na^+-free external solution was described by Cota and Armstrong (1989) and was adapted here to further minimize Na^+ loading due to persistent opening of BTX-modified Na^+ channels. Residual outward currents were evident in some cells at voltages $\geq$ +30 mV (Wang and Wang, 1998); these currents were present in untransfected cells and were insensitive to tetrodotoxin (TTX). Stock solutions of BTX (0.5 mM) and TTX (1 mM) were dissolved in dimethylsulfoxide and distilled water, respectively. BTX was a generous gift from John Daly (National Institutes of Health, Bethesda, MD); TTX was purchased from Calbiochem. To conserve the drug we applied BTX at a final concentration of 5 μM in the internal pipette solution when needed. This BTX concentration is ~500 times the known dissociation constant value. For practical reasons, we could not increase the BTX concentration to 100 μM because such experiments would quickly deplete our stock solution. Whole-cell currents were measured by Axopatch 200B, filtered at 2–5 kHz, collected, and analyzed using pClamp7.0 software (Axon Instruments, Foster City, CA). Leak and capacitance were first subtracted by the Axopatch device and further by the pClamp leak subtraction protocol (P/−4). Cells were always held at −140 mV to avoid cumulative fast and slow inactivations in mutant channels during repetitive pulses. This procedure was particularly important for cells transfected with μ1-Y1586K, in which a significant number of channels were inactivated at −100 mV (Wright et al., 1998). An unpaired Student's *t*-test was used to evaluate estimated parameters (means ± SEM); p values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

BTX-resistant phenotype derived from lysine point mutations at residues critical for LA binding

The rat muscle α-subunit Na^+ channels (μ1 or SkM1) (Trimmer et al., 1989), when transiently expressed in Hek293 cells, display normal current kinetics comparable to

those of their native counterparts (Ukomadu et al., 1992). Fig. 2 *A* shows the μ1 Na^+ current family at various voltages under reversed Na^+ gradient conditions. The wild-type μ1 channels are activated between -50 and -40 mV. After a brief opening, Na^+ currents declined quickly with a time constant of $\sim$0.3 ms at voltages $\geq +30$ mV. The mutant μ1-F1579K channels exhibit current kinetics similar to those of μ1 wild type (Fig. 2 *B*). The activation and fast inactivation kinetics of several lysine mutants at the LA receptor site have been reported recently (Wright et al., 1998). The μ1-F1579 residue was thought to be involved in binding with the tertiary amine group of LA drugs, probably through a positive charge and π-electron interaction (Ragsdale et al., 1994; Wright et al., 1998). In the presence of internal 5 μM BTX, μ1 Na^+ channels were readily modified when the cell was depolarized repetitively (at $+50$ mV for 24 ms at 2 Hz). A large portion of the μ1 Na^+ currents became noninactivating, as shown in Fig. 2 *C*, after 1000 pulses. In contrast, BTX failed to modify the μ1-F1579K channels in all cells tested ($n > 10$) after 1000 repetitive pulses under identical conditions. Nor did it alter the mutant channel kinetics, such as the current-voltage relationship and h_∞. For this BTX-resistant phenotype, we increased repetitive pulses up to 3000 pulses in three cells and still found no modification of the mutant channels by BTX. This result demonstrated clearly that a lysine substitution at this LA binding site renders the Na^+ channel completely resistant to BTX.

Another lysine mutant, μ1-N1584K, also expresses Na^+ currents that are completely resistant to 5 μM BTX. Fig. 3 *A* shows the current family of μ1-N1584K at various voltages in the absence of BTX. We have noticed that a maintained Na^+ current becomes apparent in this mutant during depolarization, particularly at large potentials (see *arrow* at $+50$ mV). This mutant phenotype has been reported recently (Wright et al., 1998). The residue of μ1-N1584 was proposed to affect the LA binding through an allosteric action on the LA receptor. BTX at 5 μM again failed to modify the current kinetics of mutant channels after 1000 pulses (Fig. 3 *C*). There was no evidence indicating that any portion of the maintained current was induced by BTX modification. Nor did an increase in pulse number to 3000 alter this BTX-resistant phenotype in μ1-N1584K. Like wild-type transient μ1 Na^+ current, most of the maintained current from this mutant can be blocked by TTX (Fig. 3 *C*) or by LAs (Wright et al., 1998) and is therefore not derived from leak or other unspecified ion channels.

The third lysine mutant that affects the LA binding is μ1-Y1586K, which expresses Na^+ currents with normal kinetics (Fig. 3 *B*) comparable to those of wild type. The μ1-Y1586 residue was proposed to interact directly with the phenyl ring of LA drugs, probably through hydrophobic interaction (Ragsdale et al., 1994). Internal BTX at 5 μM readily modifies the current of μ1-Y1586K (Fig. 3 *D*), demonstrating that this residue with a positive charge does not affect the BTX phenotype. In the presence of BTX, the

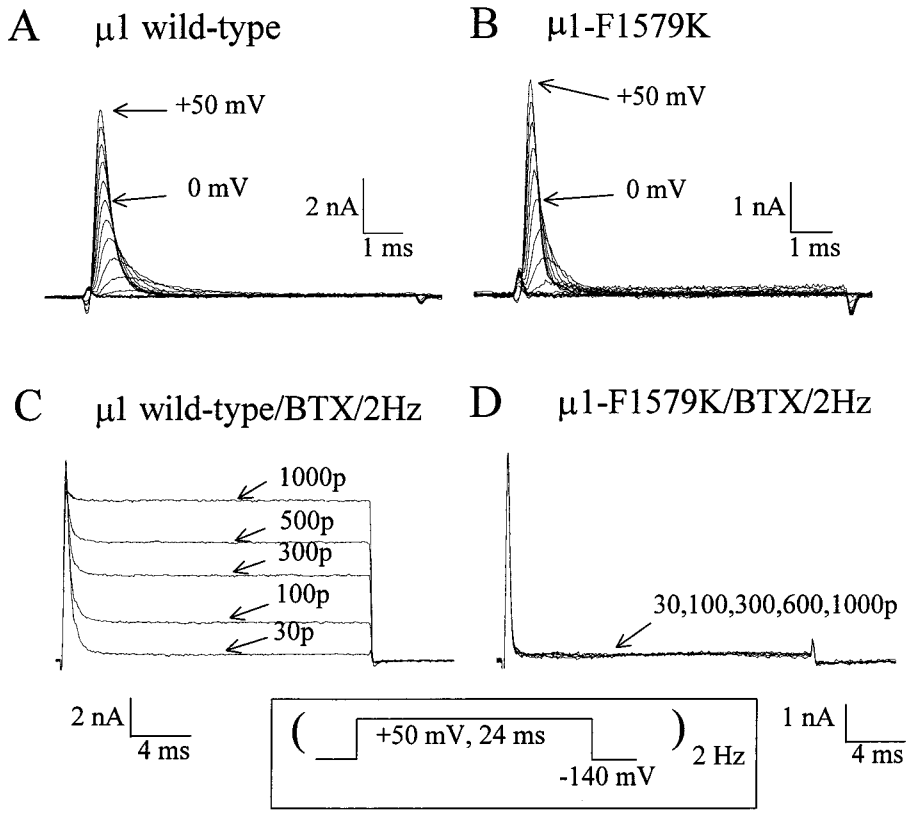

FIGURE 2 Families of Na^+ currents were recorded from cells expressing μ1-wild-type (*A*) and μ1-F1579K mutant (*B*) channels under reversed Na^+ gradient conditions. Current traces were superimposed from -70 to $+50$ mV in 10-mV steps. Cells were dialyzed by internal solution for at least 20 min before recording. In separate cells, BTX at 5 μM was included in the pipette solution, and repetitive pulses of 24 ms at $+50$ mV were applied at 2 Hz in cells expressing μ1-wild-type (*C*) and μ1-F1579K (*D*) mutant channels. Current traces were superimposed; the numbers given correspond to the pulses. 30p represents the 30 pulses applied to monitor Na^+ currents during cell dialysis.

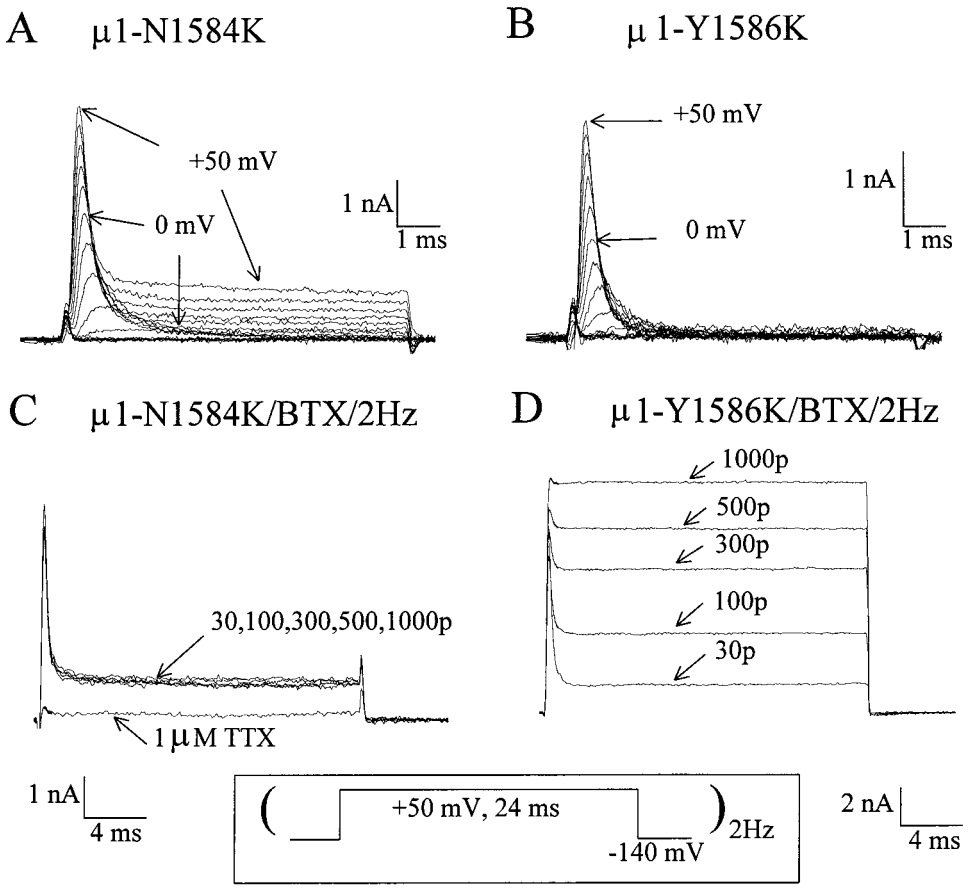

FIGURE 3 Families of Na^+ currents from cells expressing μ1-N1584K (*A*) and μ1-Y1586K mutant (*B*) channels were superimposed. Current traces were recorded from −70 to +50 mV in 10-mV steps. Notice that there are substantial late currents remaining at the end of depolarization in μ1-N1584K mutant channels. In the presence of internal 5 μM BTX, repetitive pulses were applied as shown in the inset. Current traces of μ1-N1584K (*C*) and μ1-Y1586K (*D*) were superimposed along with the numbers corresponding to the number of pulses applied. External TTX at 1 μM (*C*) effectively blocked the late current.

amplitude of μ1-Y1586K BTX-modified Na^+ current during repetitive pulses grows larger than the original peak current amplitude. This phenomenon may be due to the removal of an enhanced inactivation process (Wright et al., 1998) in this particular mutant by BTX. BTX also shifts the activation threshold of μ1-Y1586K significantly, by ~40 mV toward the hyperpolarizing direction. Thus two out of three residues critical for LA binding (μ1-F1579K and μ1-N1584K but not μ1-Y1586K) display a complete BTX-resistant phenotype after the lysine substitution. We surmise that this BTX-resistant phenotype is due to a decrease or a lack of BTX binding in these mutant channels. An adjacent residue μ1-I1575, which was known to regulate the access of external LAs to their binding site, could not be studied because of the lack of expression when substituted with a lysine residue.

Restoration of BTX-sensitive phenotype by different point mutations

In the original mapping of the LA receptor, Ragsdale et al. (1994) used the alanine-scanning method to delineate the LA binding site. As in brain Na^+ channels for homologous mutations, the point mutation at μ1-F1579A reduces LA binding, whereas the point mutation at μ1-N1584A drastically enhances LA binding (Wang et al., 1998b). We therefore determined the effects of BTX in these two mutants. The μ1-F1579A mutant channels are readily modified by BTX, whereas the μ1-N1584A channels remain relatively BTX resistant (Fig. 4, *A* and *B*). Notice that there is little BTX-induced steady-state current in the μ1-N1584A after repetitive pulses, although a slower decaying phase becomes evident in the presence of BTX. Hence, μ1-N1584A is not completely resistant to BTX modification under our assay conditions. Despite the presence of the slow decaying phase in Na^+ currents, we observed no significant change in steady-state maintained current, even after 3000 repetitive pulses. The expression of μ1-N1584A is generally poor, and detailed studies of this mutant are difficult (Wang et al., 1998b). For comparison, μ1-Y1586A mutant channels, like μ1-Y1586K, are again BTX-sensitive (Fig. 4 *C*). These results suggest that the positive charge brought in by the lysine mutation at the μ1-F1579 position may be crucial for the BTX-resistant phenotype, but less so at the μ1-N1584

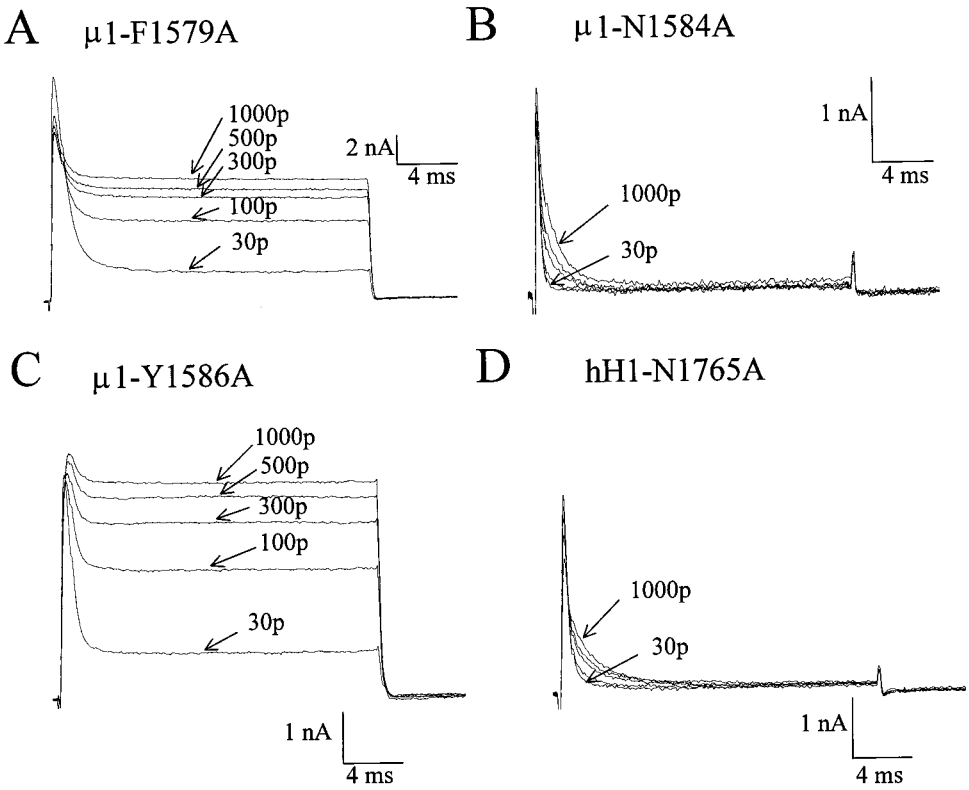

FIGURE 4 Superimposed current traces were recorded from cells expressing μ1-F1579A (*A*), μ1-N1584A (*B*), μ1-Y1586A (*C*), and hH1-N1765A (*D*). BTX at 5 μM was applied internally, and repetitive pulses were applied at 2 Hz for 24 ms at +50 mV in each cell. Numbers given correspond to the pulses. Notice that little or no BTX-induced current was present in *B* or *D*. Peak current amplitudes in μ1-F1579A, μ1-N1584A, and hH1-N1765A were significantly reduced during repetitive pulses. Expression of μ1-N1584A and hH1-N1765A was generally poor in most cells (<1 nA).

position. Residue μ1-Y1586, when replaced by either lysine or alanine, appears to have no clear involvement in the BTX-resistant phenotype.

Because μ1-N1584A is the only nonlysine mutant that displays a partial BTX-resistant phenotype thus far in this series of mutants, we tested whether the equivalent mutant in the human heart Na^+ channel isoform hH1 (Gellens et al., 1992) is also partially BTX-resistant. Fig. 4 *D* shows that the hH1-N1765A current indeed displays a partially BTX-resistant phenotype similar to that of the μ1-N1584A current; a slower decaying phase is again evident after repetitive pulses. Thus this partial BTX-resistant phenotype is obviously not isoform specific.

Why μ1-N1584A become partially BTX-resistant is unclear. One possible explanation is that BTX may interact directly with the μ1-N1584 residue during binding. We have found that μ1-N1584D channels (substituted with the negatively charged residue aspartate) display an intermediate BTX-resistant phenotype (Fig. 5 *A* versus Fig. 5 *B*). The apparent fast decaying phase for μ1-N1584D current has a

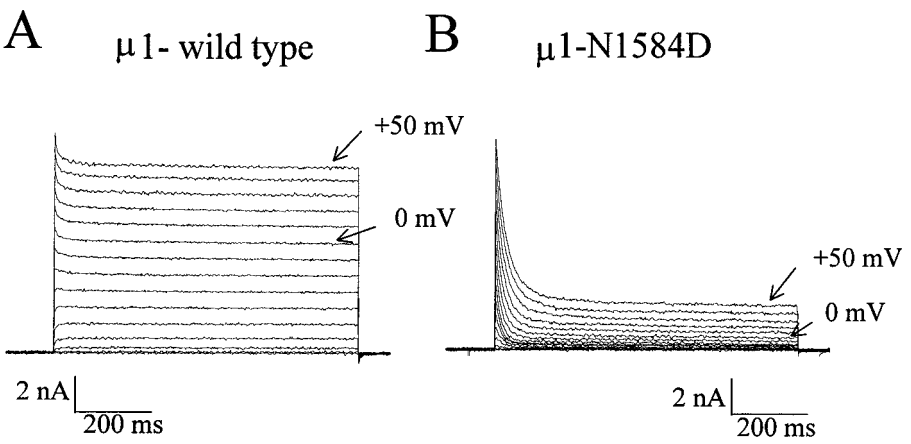

FIGURE 5 Superimposed current traces of μ1-wild-type (*A*) and μ1-N1584D (*B*) were recorded in a slow time frame under reversed Na^+ gradient conditions. Currents were recorded from −90 to +50 mV in 10-mV steps. One thousand pulses of 24 ms at +50 mV at 2 Hz were applied to enhance BTX binding before recording. The pipette solution contained 5 μM BTX. The threshold for channel activation is about −70 mV for μ1-wild-type (*A*) and μ1-N1584D mutant (*B*). Notice that a large fraction of BTX-modified μ1-N1584D current was inactivated during prolonged depolarization.

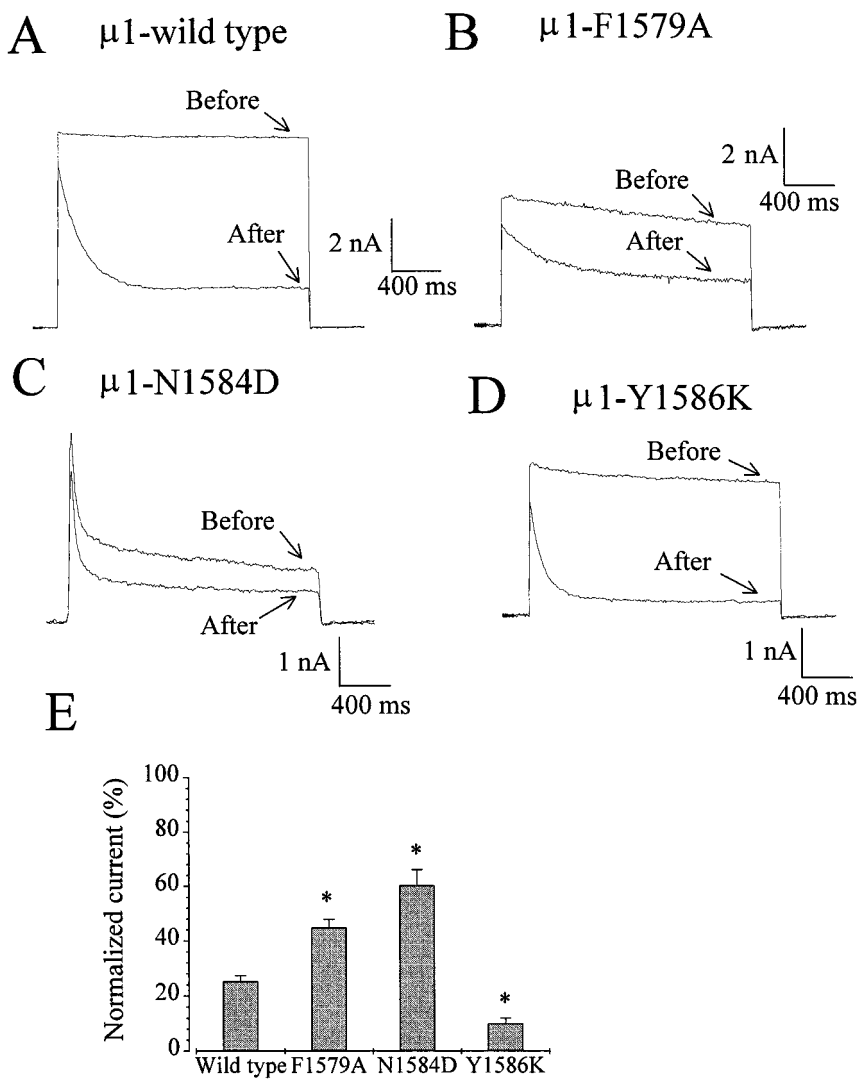

FIGURE 6 Time-dependent block of Na^+ currents after 300 μM cocaine application was recorded from cells expressing μ1-wild-type (*A*), μ1-F1579A (*B*), μ1-N1584D (*C*), and μ1-Y1586K currents. BTX was applied in the pipette solution in each cell, and 1000 repetitive pulses (+50 mV for 24 ms at 2 Hz) were applied before the control current was recorded. After the control current was recorded, cocaine at 300 μM was applied externally for 2 min. The current was then recorded (*After*) and superimposed on the control (*Before*). The late steady-state currents after cocaine application were measured at the end of the depolarization, normalized with respect to the control currents, and plotted against the mutant type (*E*). The numbers of experiments for wild-type, F1579A, N1584A, and Y1586K are 7, 6, 8, and 5, respectively. $*p < 0.05$ compared with the wild type.

time constant (τ_s) of 32.6 ms at +30 mV. Steady-state BTX-modified Na^+ currents with an even slower decaying phase are present during prolonged depolarization (up to 2 s). The fast decaying phase is probably not due to the fast dissociation of BTX from its binding site, because the same current appears at the following pulse without further repetitive pulses. If it were caused by the BTX dissociation, many repetitive pulses would be required to obtain the same type of current traces. Wild-type BTX-modified μ1 currents generally have a small or no inactivating current phase (<10%; Fig. 5 *A*). By this argument we may tentatively equate the BTX binding affinity with the BTX-resistant phenotype in the following rank order: wild type $\geq$ μ1-N1584D $\gg$ μ1-N1584A > μ1-N1584K. Direct BTX binding studies will be required to confirm this inference. In any event, these results strongly suggest a possible linkage of the μ1-N1584 residue in BTX binding and its subsequent signal transduction. In other words, BTX may bind to D1–S6 (residues I433, N434, and L437) and D4–S6 (residue N1584).

It is noteworthy that RBIIA-F1764A of the rat brain Na^+ channel was found to exhibit a complete BTX-resistant phenotype under voltage-clamp conditions (Linford et al., 1998). This phenotype is very different from that of homologous μ1-F1579A (Fig. 4 *A*). In fact, the current of this RBIIA-F1764A mutant is the only alanine mutant current that shows a complete resistance to 10 μM BTX. The RBIIA-Y1771A mutant channel, like μ1-Y1586A (Fig. 4 *C*), remains sensitive to BTX. Linford et al. (1998) did not report the BTX phenotype of RBIIA-N1769A in their study. Why does μ1-F1579A mutant, unlike RBIIA-F1764A, display a BTX-sensitive phenotype? Preliminary results showed that cardiac homologous mutant channel hH1-F1760A is also BTX sensitive ($n = 5$). However, its phenotype is more similar to that of μ1-N1584D (Fig. 5 *B*) than that of μ1-F1579A (Fig. 4 *A*). Our results thus suggest that these variable BTX phenotypes of homologous RBIIA-F1764A, μ1-F1579A, and hH1-F1760A mutants are likely isoform specific. Whether BTX interacts directly with the μ1-F1579 residue will be discussed later.

State-dependent cocaine block of BTX-modified μ1 wild-type channels

Cocaine, a naturally occurring LA, is an open channel blocker of native BTX-modified Na^+ channels (Wang and Wang, 1992) and appears to interact minimally with the resting state of BTX-modified Na^+ channels. To determine whether such a state-dependent cocaine block occurs in μ1 BTX-modified Na^+ channels, we first modified the wild-type μ1 channels with BTX, using 1000 repetitive pulses (Fig. 6 *A*, *Before*). In the presence of 300 μM cocaine, a strong time-dependent block could be readily detected (Fig. 6 *A*, *After*). This time-dependent decaying phase of BTX-modified currents induced by cocaine is not due to the dissociation of BTX from its binding site (Wang and Wang, 1992). The initial current, however, was inhibited far less than the late current, as if cocaine does not interact strongly with the resting BTX-modified μ1 Na^+ channels, but interacts preferentially with their open state. Fig. 6 *E* shows the average remaining late currents at the end of the test pulse (25 $\pm$ 2%) after 300 μM cocaine ($n = 7$). The average initial current is blocked less at the beginning of the test pulse (83 $\pm$ 2% remaining, $n = 7$). The inhibitory action of cocaine is completely reversible upon washing. Thus the state-dependent block by cocaine is preserved in μ1 α-subunit Na^+ channels without the β-subunit present. This result also demonstrates that cocaine and BTX can simultaneously occupy their respective binding sites in the open form of BTX-modified α-subunit Na^+ channels. The simplified kinetic scheme that describes these interactions is as follows:

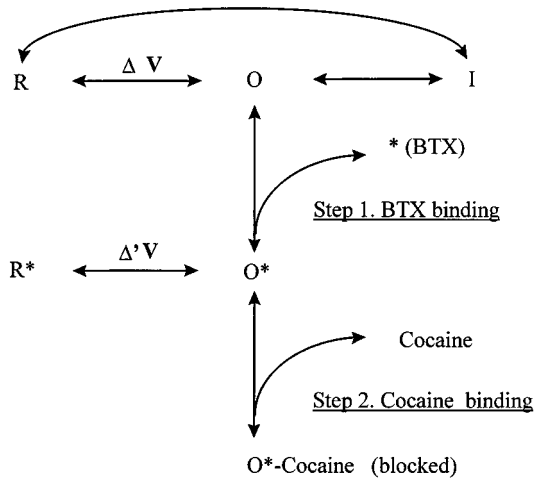

FIGURE 7 A simplified kinetic scheme for BTX binding, followed by cocaine binding. *BTX. ΔV is the depolarization required for channel activation.

R and R* are resting and BTX-modified resting states, respectively; O and O* are open and BTX-modified open states, respectively; and I is an inactivated state. BTX preferentially binds to the open state of Na^+ channels during repetitive pulses, and cocaine preferentially binds to the open state of BTX-modified Na^+ channels. Upon binding, BTX nearly eliminates both the fast and the slow inactivation of the α-subunit Na^+ channels. In addition, the leftward shift of the channel activation threshold is shown as $\Delta' V$ in Fig. 7. A similar kinetic scheme was proposed for the native Na^+ channels (Wang and Wang, 1992). Although cocaine does bind strongly to the inactivated state of Na^+ channels (Wright et al., 1998), such binding interaction is ignored in this simplified scheme, which focuses first on BTX binding. If the LA drug is applied first, BTX will not be able to bind and modify the Na^+ channels effectively.

State-dependent cocaine block of the BTX-modified mutants μ1-F1579A, μ1-N1584D, and μ1-Y1586K

As expected, the cocaine affinity for the open channel of BTX-modified μ1-F1579A is diminished significantly (Fig. 6, *B* and *E*). Such a reduction in LA affinity is found in resting and inactivated μ1-F1579A Na^+ channels in the absence of BTX (Wang et al., 1998b). Based on the complete BTX-resistant phenotype of homologous RBII-F1760A, this residue was suggested to interact directly with BTX (Linford et al., 1998). Such a suggestion seems to be supported by the result for the mutant μ1-F1579K, which also displays a complete BTX-resistant phenotype. Unfortunately, the alanine substitution at this residue yields the BTX-resistant phenotype in RBII channels but not in μ1 channels. Because cocaine still elicits a time-dependent block in the open form of BTX-modified μ1-F1579A mutant channels (Fig. 6 *B*), a direct contact of this μ1-F1579 residue with BTX (and then with cocaine) may therefore be state dependent in μ1 channels.

As in BTX-modified μ1-F1579A, cocaine block is substantially reduced in BTX-modified μ1-N1584D channels (Fig. 6, *C* and *E*). This phenotype is the opposite of the enhanced LA block in μ1-N1584A, possibly due to residue-specific allosteric effects. Unexpectedly, we found that the state-dependent block of BTX-modified μ1-Y1586K channels by 300 μM cocaine was stronger than that of their wild-type counterparts (Fig. 6, *D* and *E*). Without BTX, the cocaine binding affinity in μ1-Y1586K channels was recently reported to be identical to that in the wild-type channels in their resting state, whereas the benzocaine affinity was increased (Wright et al., 1998). Although the phenyl group of LAs is supposed to bind to the μ1-Y1586 residue through hydrophobic interactions, it is possible that a lysine substitution establishes new interactions between this positive charge from lysine and the π-electron from the phenyl group of LAs, as suggested previously (Wright et al., 1998). This cation-π interaction may be facilitated by channel opening, because the cocaine-induced time-dependent block is significantly faster in μ1-Y1586K mutant channels ($\tau = 71.2 \pm 12.2$ ms, $n = 5$) than in wild-type channels ($\tau = 177.5 \pm 18.2$ ms, $n = 7$, $p < 0.05$). Accordingly, the initial current is also inhibited more than that in wild type

(remaining current, 75.4 ± 2.2%, $n = 5$ versus 83.1 ± 2.1%, $n = 7$; respectively, $p < 0.05$). Together, these results support the conclusion that μ1-F1579, N1584, and Y1586 residues are indeed critical for LA binding in μ1 Na^+ channels with or without BTX (Wright et al., 1998; Wang and Wang, 1998b).

Molecular basis of antagonistic binding reactions between BTX and LAs in μ1 Na^+ channels

What is the underlying mechanism by which BTX and LAs antagonize each other noncompetitively in binding? The findings that cocaine binds preferentially to the open conformation of BTX-modified μ1 Na^+ channels (Fig. 6) and that point mutations at μ1-F1579K and μ1-N1584K render channels resistant to BTX (Figs. 2 and 3) may provide insights into this issue. At the resting state of Na^+ channels, it is likely that the two receptors cannot simultaneously accommodate both BTX (538 Da) and cocaine (303 Da). The weakness in cocaine binding with the resting state of BTX-modified Na^+ channels may indicate that 1) the resting receptor is not fully exposed for binding interactions and that 2) the steric hindrance from the BTX molecule, if bound, prevents LA binding. Fig. 8 shows a simplified model that accounts for our findings. When the BTX-modified Na^+ channels are in their open conformation (*right side* in Fig. 8), the D1–S6 and D4–S6 segments may accordingly change their configuration and result in an exposure of the LA receptor by widening the permeation pathway, by expanding the interface between these two segments, and/or by segment crossing/tilting. After such conformational changes, internal cocaine can now bind to its receptor at the middle position of segment D4–S6 and elicit a strong time-dependent block of the open BTX-modified Na^+ channels (Fig. 8, *right side lower panel*). The observation that simultaneous occupancy of BTX and LAs occurs preferentially at their distinct receptor sites during channel opening would easily explain the noncompetitive BTX binding inhibition by LAs under the assay conditions in which various channel states were present (Postma and Catterall, 1984). Our model thus suggests strongly that the antagonistic action is due to closer and more direct interactions between LAs and BTX.

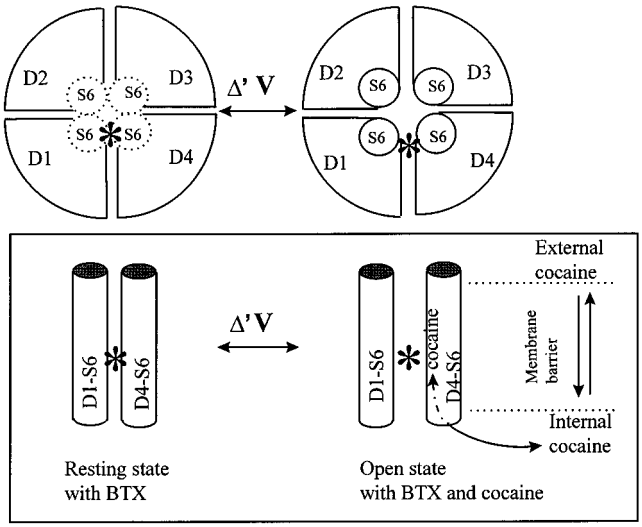

FIGURE 8 Upper panel illustrates the cut-open view of four domains of the Na^+ channel α-subunit. Lower panel shows a model for the binding of BTX and cocaine with D1–S6 and D4–S6 α-helices. *BTX. $\Delta' V$ is the depolarization required for channel opening.

Evidence for the proposed model?

Although overly simplified, the model shown in Fig. 8 is in agreement with reported observations and hypotheses. First, conformational changes at the LA receptor site during state transitions have long been suspected. According to Hille's modulated receptor hypothesis (Hille, 1977), the conformational changes at the receptor site would explain the state-dependent block of Na^+ channels by LAs. Second, it is likely that the BTX receptor and the LA receptor are in close proximity. This close alignment between segments D1–S6 and D4–S6 will explain the reduction of LA binding by point mutations at the BTX receptor site (Wang et al., 1998a). A similar close alignment for these two receptors was suggested by Linford et al. (1998). Third, at the resting closed state, the spaces occupied by bound BTX and by bound LA partially overlap, as implied in Fig. 8 (*left side*). Even a slight overlap in the binding spaces for BTX and cocaine will effectively reduce BTX binding when the LA receptor is first occupied by cocaine, and vice versa. In contrast to this view, Linford et al. (1998) suggested that, regardless of state transitions, these two binding sites are distinct, although binding of the two ligands may involve interactions with opposite faces of the side chains of the same amino acid residues in the D4–S6 segment (i.e., RBIIA-F1760). Fourth, BTX binds preferentially to the open state of Na^+ channels during repetitive pulses. This preferential access of BTX to its binding site at the open state can be explained by a transient expansion in the interface of the D1–S6 and the D4–S6 segments during channel opening. Finally, upon binding, BTX stabilizes the open state of Na^+ channels and shifts the activation threshold leftward by 30–50 mV ($\Delta' V$ in Fig. 8). These phenomena are consistent with the bound BTX acting as a stabilizer at the domain interface, because of the close alignment of D1–S6 and D4–S6 segments. To date, there is strong evidence that portions of the S6 segment (or equivalent) contribute to the pore-forming region and the activation gate for tetrameric K^+ channels (e.g., Lopez et al., 1994; Doyle et al., 1998; Liu et al., 1997; Holmgren et al., 1998; Ogielska and Aldrich, 1998). A close alignment among S6 segments along the permeation pathway may be important for the proper function of voltage-gated channels in general and may be well conserved in voltage-gated Na^+ channels.

In summary, we have created several D4–S6 mutants of muscle μ1 Na^+ channels that show a complete (μ1-F1579K

and μ1-N1584K) or a partial BTX-resistant phenotype (μ1-N1584A and μ1-N1584D). These mutations at D4–S6 also strongly affect LA binding. As described before for native BTX-modified Na^+ currents, cocaine at 300 μM likewise elicits a strong time-dependent block of BTX-modified μ1 Na^+ currents. We propose a model that invokes conformational changes in receptor sites for BTX and for LAs during state transitions. Whether the two receptor sites overlap slightly or not may depend on the channel states. Determination of the relative distance of these two binding sites during state transitions will be needed to validate this model.

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