Irreversible Block of Cardiac Mutant Na\textsuperscript{+} Channels by Batrachotoxin

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\begin{abstract}
Batrachotoxin (BTX) not only keeps the voltage-gated Na\textsuperscript{+} channel open persistently but also reduces its single-channel conductance. Although a BTX receptor has been delimited within the inner cavity of Na\textsuperscript{+} channels, how Na\textsuperscript{+} ions flow through the BTX-bound permeation pathway remains unclear. In this report we tested a hypothesis that Na\textsuperscript{+} ions traverse a narrow gap between bound BTX and residue N927 at D2S6 of cardiac hNav1.5 Na\textsuperscript{+} channels. We found that BTX at 5 \textmu M indeed elicited a strong block of hNa\textsubscript{v}1.5-N927K currents (~70\%) after 1000 repetitive pulses (+50 mV/20 ms at 2 Hz) without any effects on Na\textsuperscript{+} channel gating. Once occurred, this unique use-dependent block of hNa\textsubscript{v}1.5-N927K Na\textsuperscript{+} channels albeit with a faster on-rate; ~90\% of peak Na\textsuperscript{+} currents were abolished by BTX after 200 repetitive pulses (+50 mV/20 ms). This use-dependent block of fast inactivation-deficient hNa\textsubscript{v}1.5-N927K Na\textsuperscript{+} channels was duration dependent. The longer the pulse duration the larger the block developed. Among N927K/R Na\textsuperscript{+} currents were highly sensitive to BTX block. We conclude that (a) BTX binds within the inner cavity and partly occludes the permeation pathway and (b) residue hNa\textsubscript{v}1.5-N927 is critical for ion permeation between bound BTX and D2S6, probably because the side-chain of N927 helps coordinate permeating Na\textsuperscript{+} ions.
\end{abstract}

\begin{keywords}
Na\textsuperscript{+} channels, BTX, inner cavity, S6 model
\end{keywords}

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\end{acknowledgements}

\begin{abbreviations}
BTX, batrachotoxin; LA, local anesthetic; MC, Monte Carlo; DEKA, the four residues forming the putative selectivity filter- aspartate, glutamate, lysine, and alanine; KvAP, voltage-activated K\textsuperscript{+} channel from Aeropyrum pernix; MthK, Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel from Methanobacterium thermoautotrophicum; hNa\textsubscript{v}1.5, human cardiac voltage-gated Na\textsuperscript{+} channel; hNa\textsubscript{v}1.5-CW, hNa\textsubscript{v}1.5-L409C/A410W mutant channel; rNa\textsubscript{v}1.4, rat skeletal muscle voltage-gated Na\textsuperscript{+} channel; rNa\textsubscript{v}1.4-WCW, rNa\textsubscript{v}1.4-L435W/L437C/A438W mutant channel.
\end{abbreviations}

\begin{introduction}
Voltage-gated Na\textsuperscript{+} channels are transmembrane proteins responsible for the generation and propagation of action potentials in excitable membranes. Mammalian Na\textsuperscript{+} channels contain a large \(\alpha\)-subunit (Na\textsubscript{v}1.1–1.9) and one or two small \(\beta\)-subunit (\(\beta\)1–\(\beta\)4).\textsuperscript{1} The primary structure of the \(\alpha\)-subunit isoform consists of four homologous domains (D1–D4) each with six transmembrane segments (S1–S6). When expressed in mammalian cells, the \(\alpha\)-subunit can alone form a functional channel with gating characteristics comparable to its Native counterpart.

Various potent neurotoxins have been used as tools to study the voltage-gated Na\textsuperscript{+} channels.\textsuperscript{2,3} One of them is an activator, batrachotoxin (BTX), which specifically binds to the site 2 receptor of voltage-gated Na\textsuperscript{+} channels. BTX was first isolated from poisonous neotropical frogs (\textit{Phyllobates})\textsuperscript{4} but more recently was also found in New Guinea passerine birds (\textit{Pitohui} and \textit{Ifrita}).\textsuperscript{5} Figure 1A shows the chemical structure of BTX, which is a rigid steroidal alkaloid with a tertiary amine.\textsuperscript{6} BTX also contains an oxygen triad as revealed in its 3-D structure,\textsuperscript{7} which can chelate a cation ion\textsuperscript{8} (Fig. 1B). Opposite to the
only about half of BTX (MW 538 for BTX vs 234 for lidocaine). Lidocaine, blocks Na+ currents in an all-or-none manner but its size is large enough to enter the Na+ channel inner cavity near a D2S6-asparagine residue directly by Linford et al.17 Unlike BTX, the traditional LA, Tl+ ions. Finally, BTX actions were considered irreversible at room temperature, since the dissociation of BTX from BTX-modified Na+ channels is minimal.12-15

The BTX-sensing residues have been delimited to multiple S6 segments of the Na+ channel α-subunit.16,17 A number of residues clustered in all four S6 segments seem critical for BTX actions.9 Interestingly, BTX interacts with the local anesthetic (LA) receptor site directly by Linford et al.17 Unlike BTX, the traditional LA, lidocaine, blocks Na+ currents in an all-or-none manner but its size is only about half of BTX (MW 538 for BTX vs 234 for lidocaine).

Recently, we hypothesized that Na+ ions flow through the BTX-bound Na+ channel inner cavity near a D2S6-asparagine residue, which is adjacent to the gating hinge, D2S6-glycine.8,18 Consistent with this hypothesis, we noticed that, after lysine substitution of this asparagine residue, BTX appeared to reduce substitution of this asparagine residue, BTX appeared to reduce the voltage dependence of activation by 30–50 mV toward the hyperpolarizing direction. Second, BTX abolishes fast inactivation of Na+ channels, which normally have a brief open time of ~0.5 ms. Third, BTX removes slow inactivation of Na+ channels. Fourth, BTX reduces single channel conduction to ~60% of the normal channel.11 Fifth, BTX alters ion selectivity of the Na+ channel, probably by widening the selectivity filter, which becomes more permeable for NH4+ and Tl+ ions. Finally, BTX actions were considered irreversible at room temperature, since the dissociation of BTX from BTX-modified Na+ channels is minimal.12-15

The M MATERIALS AND METHODS

Site-directed mutagenesis. We used the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to create hNa1,5-N927 mutants within the pcDNA1/Amp vector.20 The hNa1,5 clone was kindly provided by Dr. Kallen (University of Pennsylvania) and was originally Named hH1.21 The residue in hNa1,5 is numbered according to the hH1 amino acid sequence. DNA sequencing around the mutated region confirmed the mutations. Fast inactivation-deficient N927 mutants were created in hNa1,5-L409C/A410W background channels (hNa1,5-CW).22 A rat skeletal muscle counterpart, rNa1,4-N784K, was also created in the fast inactivation-deficient rNa1,4-L435W/L437C/A438W background channel (rNa1,4-WCW/N784K).

Transient transfection. Human embryonic kidney cells (HEK293t) were grown to ~50% confluence in DMEM (Life Technologies, Inc., Rockville, MD) containing 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin and streptomycin solution (Sigma, St. Louis, MO), 3 mM taurine and 25 mM HEPES (Life Technologies, Inc.), and then transfected by calcium phosphate precipitation. Transfection of wild-type or hNa1,5-N927 mutants (10 µg) along with rat β1-pcDNA1/Amp (10 µg) and reporter CD8-pih3m (1 µg) was adequate for current recording. Cells were replated 15 hr after transfection in 35-mm dishes, maintained at 37°C in a 5% CO2 incubator and used after 1–4 days. Transfection-positive cells were identified with immunobeads (CD8-DyNabeads, Lake Success, NY).

Whole-cell voltage clamp. Whole-cell configuration was used to record Na+ current.23 Borosilicate micropipettes (Drummond Scientific Company, Broomall, PA) were pulled (P-87, Sutter Instrument Company, Novato, CA) and heat-polished. Pipette electrodes contained 100 mM Naf, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES adjusted to pH 7.2 with CaOH. The pipette electrodes had a tip resistance of 0.5 to 1.0 MO. Access resistance was generally 1 to 3 MO. We minimized the series resistance (> 90%) and corrected the leak and capacitance using the Axopatch 200B device. Holding potential was set at -140 mV. Further correction was achieved by the P/4 protocol.

Experiments were performed at room temperature (23 ± 2°C) under a Na+-containing bath solution with 65 mM NaCl, 85 mM choline Cl, 2 mM CaCl2 and 10 mM HEPES adjusted to pH 7.4 with tetramethylammonium hydroxide. Liquid junction potential was not corrected from voltage clamp experiments. An unpaired Student’s t test was used to evaluate estimated parameters (mean ± s.e.m. or fitted value ± SE of the fit); p values of < 0.05 were considered statistically significant.

Drug solutions. BTX was supplied by Dr. John Daly (National Institutes of Health), dissolved in DMSO at 0.5 mM and stored in -20°C. The final concentration of BTX was 5 µM in the pipette solution.

Computer modeling. KvAP models of the open Na+ channel and its N927K mutant were constructed and optimized with the Monte Carlo (MC) energy minimization method as described elsewhere.8 The KvAP channel29 was used as the template because it contains no Pro-Val-Pro motif in S6 and, like the Na+ channel has a voltage-sensor domain, whereas the MthK channel lacks such a domain. The selectivity filter region (DEKA locus) in the P-loop domain was taken from Tikhonov and Zhorov.25 United-atom residues were used. Only the D372, E898 and K1419 residues at the selectivity filter (DEKA locus) were considered ionized. The outer carboxylates in the P-loop domain were treated as neutral because in real channels the negative charges at these carboxylates should be compensated by counter-ions.
Docking of BTX within the Na+ channel inner cavity. BTX was manually placed within the Na+ channel inner cavity in the orientation proposed by Tikhonov and Zhorov. The complex was then MC-optimized. Despite some quantitative differences with the previous MthK-based model, major results from the KvAP-based model are the same. The resulting structure included the BTX molecule, S5, P-loop and S6 regions of the hNa\textsubscript{1.5} channel. To explain the N927K phenotype, we further explored three models regarding: (a) the structure of the wild-type hNa\textsubscript{1.5} open channel with BTX and Na+ ion bound to the N927 side chain and (b) the structures of N927K and N927W mutants with BTX. Coordinates of these models are available upon request.

RESULTS

Expression of hNa\textsubscript{1.5} wild-type, N927K and various fast inactivation-deficient N927 mutant Na+ channels in Hek293t cells. Figure 2A and B show families of superimposed wild-type and hNa\textsubscript{1.5}-N927K mutant currents at various voltages in Hek293t cells after transfection. Inward Na+ currents appeared at voltages larger than −70 mV, but turned outward around voltages larger than −10 mV. Fast inactivation appeared rapid at +50 mV and reached its completion within 1–2 ms for wild-type and hNa\textsubscript{1.5}-N927K mutant currents. Mutant N927K and 18 other N927-mutant channels (N927W,R,H,D,S,Q,E,G,C,F,T,L,I,V,M,A,Y,P) were then created in the hNa\textsubscript{1.5}-CW fast inactivation-deficient background channels. Figure 2C and D show families of superimposed hNa\textsubscript{1.5}-CW and hNa\textsubscript{1.5}-CW/N927K fast inactivation-deficient mutant currents, respectively. Currents were again activated at voltage near −70 mV but with minimal fast inactivation at +50 mV. Expression of N927W/R/H/D/S/Q/G/E mutant channels varied considerably as listed in Table 1. The voltage dependence of activation of these mutants also varied (Table 1, E\textsubscript{1/2}). In general, slow inactivation in hNa\textsubscript{1.5}-CW fast inactivation-deficient background mutant channels was enhanced drastically, whereas in wild-type hNa\textsubscript{1.5} Na+ channels slow inactivation was fairly modest. Table 1 lists the relative slow inactivation defined as a normalized peak current reduction after a prolonged 10-s depolarization at 0 mV for wild type (12%) and for hNa\textsubscript{1.5}-CW mutant (89%). A similar enhanced slow inactivation phenotype was also found in pronase-treated inactivation-deficient squid Na+ channels. Substitutions of N927 also affected the slow inactivation with varying degrees. Such phenomenon appeared common for other S6 mutants. There was still no accepted model for the mechanism that led to slow inactivation but gating movement of the S6 segments was implicated along with the constriction of the P-loop domain.

The degree of enhanced slow inactivation for various mutant channels is listed in Table 1.

Apparent inhibition of hNa\textsubscript{1.5}-N927K mutant currents by BTX. In the presence of 5 μM BTX within the pipette solution, repetitive pulses at +50 mV for 20 ms induced a large fraction of wild-type Na\textsubscript{1.5} Na+ currents that maintained at the end of the pulses (Fig. 3A). Although the use of the peak current with intact fast inactivation might underestimate the real peak amplitude by -60%, we found that the amount of maintained currents in wild-type hNa\textsubscript{1.5} channels reached 90.4 ± 2.9% (n = 7) of the peak amplitude at pulse #1000 (P1000 at 2 Hz). Furthermore, inward tail currents appeared at the end of the pulse and their peak amplitudes increased proportionally during repetitive pulses. Under identical conditions, however, the results from hNa\textsubscript{1.5}-N927K channels were quite different from those found in wild-type. First, we found no increase in maintained currents after 1,000 repetitive pulses at 2 Hz (Fig. 3B vs A). Second, no increase in tail currents was found at the end of the pulse. Third, repetitive pulses continued to reduce the peak current amplitude. This peak reduction varied from cell to cell ranging from 46.9% to 93.7% with a mean value of 71.5 ± 5.1% (n = 10). This variation in the peak current reduction by BTX was probably due to size variations in cells and in pipette-tips. Larger cells or smaller pipette-tips would take longer for BTX to diffuse toward Na+ channels located in the cell membrane and would therefore decrease the magnitude of use-dependent block by BTX during repetitive pulses. Once occurred, most of Na+ currents (> 95%) that were blocked during repetitive pulses did not recover even after 30 min of rest at the holding potential, −140 mV. When BTX was omitted in the pipette, repetitive pulses alone did reduce the peak current amplitude of hNa\textsubscript{1.5}-N927K channels, but this reduction was relative limited (< 20%) and was reversible as found in hNa\textsubscript{1.5} wild-type counterparts.
Role of Residue hNav1.5-N927 in BTX Action

Figure 3. Distinct BTX phenotypes in wild-type hNav1.5 (A) and hNav1.5-N927K (B) Na⁺ channels. Repetitive pulses at +50 mV for 20 ms were applied at 2 Hz to facilitate BTX binding. Pulse numbers 1, 100, 300, 600 and 1000P are labeled. Notice that maintained Na⁺ currents increased during repetitive pulses in wild-type channels with a corresponding rise in peak tail current amplitudes (A), whereas no such phenotypes were observed in hNav1.5-N927K mutant channels (B). Instead, the peak hNav1.5-N927K continued to decline during repetitive pulses.

Table 1  Expression and relative slow inactivation in hNav1.5 wild-type, N927K and various N927 mutants in fast inactivation-deficient background channels

| Channel Type          | Expression (pA/pF) | Activation E0.5 (mV) | Relative Slow Inactivation (Peak INa Reduction) |
|-----------------------|--------------------|----------------------|-----------------------------------------------|
| hNav1.5               | 183.2 ± 38.9 (n = 5) | -46.0 ± 3.1 (n = 5)  | 0.12 ± 0.04 (n = 5)                           |
| hNav1.5-1.5-N927K     | 315.5 ± 101.5 (n = 6) | -53.1 ± 2.7 (n = 6)  | 0.31 ± 0.09 (n = 5)                           |
| hNav1.5-CW            | 259.8 ± 40.0 (n = 5)  | -30.0 ± 2.7 (n = 5)  | 0.89 ± 0.07 (n = 5)                           |
| hNav1.5-CW/N927K      | 262.8 ± 56.1 (n = 5)  | -25.5 ± 1.3 (n = 5)  | 0.97 ± 0.04 (n = 5)                           |
| hNav1.5-CW/N927R      | 530.8 ± 87.5 (n = 5)  | -42.3 ± 2.5 (n = 5)  | 0.96 ± 0.02 (n = 6)                           |
| hNav1.5-CW/N927W      | 142.3 ± 32.6 (n = 6)  | -31.2 ± 2.2 (n = 6)  | 0.93 ± 0.02 (n = 5)                           |
| hNav1.5-CW/N927H      | 199.9 ± 58.0 (n = 6)  | -21.7 ± 2.6 (n = 6)  | 0.93 ± 0.02 (n = 5)                           |
| hNav1.5-CW/N927S      | 58.9 ± 16.4 (n = 6)   | -33.4 ± 3.4 (n = 5)  | 0.78 ± 0.13 (n = 6)                           |
| hNav1.5-CW/N927Q      | 530.7 ± 111.3 (n = 5) | -26.9 ± 1.1 (n = 5)  | 0.93 ± 0.01 (n = 5)                           |
| hNav1.5-CW/N927D      | 320.1 ± 97.5 (n = 6)  | -37.6 ± 2.0 (n = 6)  | 0.94 ± 0.02 (n = 5)                           |
| hNav1.5-CW/N927R      | 168.9 ± 26.6 (n = 6)  | -28.5 ± 2.1 (n = 5)  | 0.97 ± 0.04 (n = 5)                           |
| hNav1.5-CW/N927E      | 90.7 ± 13.9 (n = 6)   | -21.8 ± 3.6 (n = 6)  | 0.94 ± 0.08 (n = 5)                           |

The level of expression was determined as the ratio of peak currents (in pA) at +30 mV over the capacitance (in pF) of individual cells. The voltage dependence of activation (E0.5) was estimated from the data as shown in Figure 2. Peak currents were measured and the conductance was determined by an equation gm = Ina/(E_m - E_rev), where the Ina is the peak current, E_m is the corresponding voltage and E_rev is the reversal potential. The data were least-squares fitted with the Boltzmann equation g_m/g_max = 1/(1 + exp[(E0.5 - E_m)/kE]), where E0.5 is the voltage at which g_m/g_max = 0.5, and kE is the slope factor. For the relative slow inactivation, peak currents before and after a prolonged 10-s conditioning pulse at 0 mV was determined by a brief test pulse at +30 mV after a 100-ms interpulse at -140 mV to allow recovery from fast inactivation (detailed in ref. 20).

The amount of reduction in peak currents after the conditioning pulse was measured and normalized with the peak amplitude before the conditioning pulse.

Figure 4. Use-dependent block of fast inactivation-deficient hNav1.5-CW/ N927K (A) and CW/N927R Na⁺ currents (B) by BTX. Repetitive pulses at +50 mV for 20 ms were applied at 2 Hz to facilitate BTX binding. Pulse numbers 1, 10, 20, 40, 100 and 200P are labeled. Notice that both maintained currents at the end of the pulse and tail currents after the pulse were blocked by 5 µM BTX when applied internally.
Role of Residue hNav1.5-N927 in BTX Action

Use-dependent block of \textit{h}Na\textsubscript{v}1.5-CW/N927K/R mutant channels by BTX. The inhibition of \textit{h}Na\textsubscript{v}1.5-N927K mutant currents by BTX indicated that the activator acted as a Na\textsuperscript{+} channel blocker within the inner cavity in a manner similar to local anesthetics. Most of studies on BTX action suggested that BTX interacted preferentially with the open state of Na\textsuperscript{+} channels.\textsuperscript{15} We asked whether this was also the case for the BTX block of hH1-N927K mutant channel. To address this question, we used fast inactivation-deficient mutant channels, hH1-CW and hH1-CW/N927K. We found that during a 20-ms pulse, the hH1-CW/N927K currents were maintained without fast inactivation-gating when the initial whole-cell configuration was established (Fig. 4A). Repetitive pulses again reduced the mutant currents continuously although with a more rapid rate than that found in hH1-N927K channels. The reduction of hH1-CW/N927K currents measured as the peak current ratio of \( \frac{P_{200}}{P_1} \) ranged from 88.9% to 97.1% with an averaged value of 92.1% ± 2.5% (mean ± SE, \( n = 6 \)). Further application of 1000 repetitive pulses resulted in a total loss of Na\textsuperscript{+} currents. The use-dependent reduction of hH1-CW/N927K currents was again irreversible; little or no recovery of the currents was found after 10–30 min of waiting when cells were held at -140 mV. Control experiments showed that in the absence of 5 nM BTX the use-dependent reduction of hH1-CW/N927K currents was limited in amplitude (19.8 ± 2.3%, \( n = 7 \); \( \frac{P_{200}}{P_1} \) and was reversible.

A similar irreversible use-dependent block by BTX was found for \textit{h}Na\textsubscript{v}1.5-CW/N927R mutant channels. Figure 4B shows the expected decrease of currents during repetitive pulses at 1 Hz. A total of 20 pulses were applied for each of duration in a given cell. The averaged results were shown in Figure 7 (\( n = 11 \)). Control experiments without BTX showed that the use-dependent block was minimal even with 20-ms duration of repetitive pulses. The reduction of \textit{h}Na\textsubscript{v}1.5-CW/N927R currents as the peak current ratio of \( \frac{P_{200}}{P_1} \) ranged from 91.9% to 98.8% with an averaged value of 96.2 ± 1.6% (mean ± SE, \( n = 4 \)). The use-dependent block in \textit{h}Na\textsubscript{v}1.5-CW/N927R mutant channels was not significantly different from that in \textit{h}Na\textsubscript{v}1.5-CW/N927K mutant channels (\( P = 0.104 \)). These results thus supported the conclusion that BTX preferentially bound with the open state of \textit{h}Na\textsubscript{v}1.5-CW/N927K/R mutant channels and then blocked their conductance.

Could the irreversible use-dependent block by BTX be unique to \textit{h}Na\textsubscript{v}1.5-CW/N927K/R mutant channels? We used seven additional fast inactivation-deficient mutant channels (W, H, D, E, S, Q, G) that expressed sufficient Na\textsuperscript{+} currents to determine whether the irreversible use-dependent block by BTX was unique to CW-N927K/R mutant channels only. Mutant \textit{h}Na\textsubscript{v}1.5-CW/N927C/F/T/L/I/V/M/A/Y/P Na\textsuperscript{+} channels did not express sufficient Na\textsuperscript{+} currents and were not used in this study. None of the mutant currents (\textit{h}Na\textsubscript{v}1.5-CW and \textit{h}Na\textsubscript{v}1.5-CW/N927W, H, D, S, Q, E, G), however, were irreversibly blocked by BTX during repetitive pulses. Each mutant was tested in 5–10 cells with similar results. Figure 5A–D shows the families of \textit{h}Na\textsubscript{v}1.5-CW, CW/N927W, CW/N927H, and CW/N927D current traces, respectively, at voltages ranging from -120 to +50 mV. In contrast to \textit{h}Na\textsubscript{v}1.5-CW/N927K/R mutants, BTX elicited non-inactivating maintained currents and shifted the activation gating to the hyperpolarizing direction in all remaining mutants. Currents were activated near the activation threshold of ~ -100 mV (Fig. 5A–D). These results demonstrated that the irreversible use-dependent block by BTX occurred only in \textit{h}Na\textsubscript{v}1.5-CW/N927K/R mutant channels and not in mutants with other \textit{h}Na\textsubscript{v}1.5-CW/N927W/H/D/S/Q/E/G substitutions.

Could the irreversible use-dependent block of CW-N927K mutant channels by BTX occur with the pre-open states? One of familiar characteristics of open-channel blockers was their ability to elicit a time-dependent block of fast inactivation-deficient Na\textsuperscript{+} currents during a prolonged depolarization. Since we observed no apparent time-dependent block of fast inactivation-deficient \textit{h}Na\textsubscript{v}1.5-N927K/R by BTX (Fig. 4), could the BTX block occur with the pre-open states? Such a possibility has been previously suggested.\textsuperscript{20} Figure 6A–D shows that the amount of use-dependent blocking phenotype after repetitive pulses. The reduction of \textit{h}Na\textsubscript{v}1.5-CW/N927R currents as the peak current ratio of \( \frac{P_{200}}{P_1} \) ranged from 91.9% to 98.8% with an averaged value of 96.2 ± 1.6% (mean ± SE, \( n = 4 \)). The use-dependent block in \textit{h}Na\textsubscript{v}1.5-CW/N927R mutant channels was not significantly different from that in \textit{h}Na\textsubscript{v}1.5-CW/N927K mutant channels (\( P = 0.104 \)). These results thus supported the conclusion that BTX preferentially bound with the open state of \textit{h}Na\textsubscript{v}1.5-CW/N927K/R mutant channels and then blocked their conductance.

![Figure 5. BTX modifications of various fast inactivation-deficient \textit{h}Na\textsubscript{v}1.5-CW mutant channels.](image-url)

The following is a summary of the figure:

- **A**: \textit{h}Na\textsubscript{v}1.5-CW + BTX
- **B**: \textit{h}Na\textsubscript{v}1.5-CW/N927W + BTX
- **C**: \textit{h}Na\textsubscript{v}1.5-CW/N927H + BTX
- **D**: \textit{h}Na\textsubscript{v}1.5-CW/N927D + BTX

Each figure shows current traces from different mutant channels before and after exposure to BTX. The traces demonstrate the irreversible use-dependent block by BTX.
(Fig. 7; -10%, gray color; n = 5) and was reversible. With a duration of 1 ms, we found that the use-dependent block was small after 20 pulses, suggesting that the block did not occur significantly before channel opening (Fig. 6A). With a duration of 20 ms, in contrast, the block reached 63% of the peak amplitude after 20 pulses. A similar duration-dependent block was found in hNa_{1.5-CW/N927R} currents (unpublished observation). Our results therefore indicated that the on-rate for BTX action was relatively slow during each pulse in hNa_{1.5-CW/N927R/K} mutant channels and as a result, no apparent time-dependent block was observed in a 20-ms duration (Fig. 6D).

**Block of hNa_{1.5-CW/N927K} currents by BTX required Na^+ channel opening.** To confirm that the irreversible BTX block occurred only when the channel opened, we examined its voltage dependence. Repetitive conditioning pulses (100P at 2 Hz with 20-ms duration) were applied at various voltages. A brief test pulse was then given to measure the use-dependent block occurred by these conditioning pulses. The pulse protocol was shown in inset Figure 8A. The use-dependent block from the same cell was cumulative in this assay since the block was nearly irreversible. Results from seven separate cells were averaged and plotted against the conditioning voltage (Fig. 7B; open squares). This plot closely followed the voltage dependence thus indicated that Na^+ channel opening was required for the irreversible use-dependent block. With the same pulse protocol, only a small reversible reduction of peak currents was observed when BTX was not included in the pipette solution (Fig. 8B; open circles). At +20 mV the reduction in currents was 15.5 ± 2.1% (n = 6), whereas the reduction was 96.0 ± 3.3% (n = 7) with BTX present. This small reversible reduction in currents without BTX was likely due to slow inactivation accumulated during repetitive pulses.

**Irreversible block of rNa_{1.4-WCW/N784K} muscle Na^+ channels by BTX.** As mentioned in the introduction, the role of this homologous asparagine N927 residue was studied previously in the rNa_{v1.4} muscle isoform. It was noted in the report that BTX reduced rNa_{1.4-N784K} mutant currents significantly during repetitive pulses. In one recording, the reduction in the peak amplitude was shown to reach ~50% after 1000 pulses. Since muscle Na^+ channels had faster current decay than cardiac counterparts, such fast inactivation in rNa_{1.4-N784K} mutant channels could limit BTX binding. We therefore examined this phenotype using fast inactivation-deficient rNa_{1.4-WCW/N784K} mutant channels. Figure 9 shows that the use-dependent block of fast inactivation-deficient rNa_{1.4-WCW/N784K} currents is also strongly duration dependent (Fig. 9A–C). Furthermore, this use-dependent block recovered little after 10 min of rest at the holding potential -140 mV (Fig. 9D). These results were comparable to those shown in Figure 6 for the cardiac isof orm. We concluded that the BTX-induced irreversible block occurred in Na^+ channels with homologous mutations from both muscle (N784K) and cardiac (N927K) isoforms.

**BTX block based on the ligand docking model.** To better understand the open-channel block by BTX in structural terms, we constructed a KvAP-based open Na^+ channel inner cavity structure consisting of S5/P-loop/S6 regions of hNa_{1.5} and docked BTX in the inner cavity with the MC energy minimization method as described previously. The resulting open Na^+ channel model revealed two interesting features near the gating hinge region. First, residue N927 formed a part of the S_{401}N_{927}S_{1458}F_{1760} structure (space filled) that faced the inner cavity. This pore-facing SNSF structure was adjacent to the putative gating hinge, G_{400}G_{926}G_{1457}S_{1759}. Within this S_{401}N_{927}S_{1458}F_{1760} structure, residue S1458 at D3S6 and F1760 at D4S6 were known critical for BTX actions. Figure 10A and B shows cytoplasmic and side views of residue N927, respectively. Although residue S401 at D1S6 was designated as a gating hinge in the MthK-based model proposed by Lipkind and Fozzard, both models agreed on the alignment of the gating hinge at G_{926}G_{1457}S_{1759}. Second, the SNSF structure was near a putative intermediate...
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Figure 8. Voltage vs. the use-dependent block of hNav1.5-CW/N927K mutant Na⁺ channels by BTX. Repetitive conditioning pulses with 20-ms duration at various voltages were applied for a total of 100 times at 2 Hz. A brief test pulse at +50 mV was given 400 ms later to measure the peak current amplitude (A, inset). BTX was included in the pipette solution at 5 μM. Current traces were recorded during the test pulse, superimposed, and labeled with the corresponding voltages of the conditioning pulses. Peak currents were measured, normalized with the peak amplitude corresponding to the conditioning voltage at −140 mV, and plotted against the conditioning voltage (B; open squares, n = 7). The data was fitted by a Boltzmann equation \(y = C_\infty \frac{1}{1 + \exp\left(\frac{E - E_{0.5}}{k}\right)}\), solid line) where \(E\) was the conditioning voltage applied, \(E_{0.5}\) was the estimated voltage where 50% of use-dependent block occurred, \(C_\infty\) was a constant, 0.05 (the current remaining at \(E = +20 \text{ mV}\)), and \(k\) was the slope factor. The estimated \(E_{0.5}\) and \(k\) values were −47.8 mV and 8.5 mV, respectively. Without BTX, the conditioning pulse reduced the peak current minimally by comparison (open circles, n = 6).

Figure 9. Pulse duration vs. the use-dependent block of rNav1.4-WCW/N784K mutant Na⁺ channels by BTX. Repetitive pulses at +50 mV with duration of 1 ms (A), 5 ms (B) and 20 ms (C) were applied at 2 Hz. Current traces corresponding to 1 and 20P were superimposed. BTX was included in the pipette solution at 5 μM. After applying additional 100 pulses (+50 mV for 20 ms at 2 Hz), currents were recorded from the same cell and superimposed before and after 10 min without stimulation (D). A small increase in currents at the holding potential (−140 mV) was likely due to recovery from slow inactivation induced by repetitive pulses.
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cation-binding site for a Na+ ion on its way from the selectivity filter to the cytoplasm. This intermediate Na+ site (yellow sphere; Fig. 10A and B) was situated at the focus of macrodipoles of the pore helices (in green) within the inner cavity as predicted by the electrostatic field of K+ channels.32 When BTX was docked within the inner cavity, a small hydrophilic gap was found between BTX and residue hNav1.5-N927 while the intermediate cation-binding site was now occupied by BTX (Fig. 10A vs C). Residues F1760 at D4S6, Y1767 at D4S6 and L1462 at D3S6 (space filled) likely provided the major contributions to the free energy for ligand-receptor interactions. The pyrrole ring of BTX formed a stacking contact with F1418 at D3/P-loop (Fig. 11A).18 The nitrogen atom within the pyrrole ring was not protonated and hence would not block the Na+ permeation. The protonated amino group of BTX was facing away from the narrow hydrophilic permeation pathway between the BTX polar surface and N927 and likely interacted with the π electrons from the aromatic group of F1760. This docking configuration of BTX within the inner cavity provided possible explanations for the leftward shift in the voltage dependence of activation of BTX-bound Na+ channels. Because BTX bound multiple pore-facing residues adjacent to the gating hinge position and because it extended longitudinally to the S6 C-termini, these attributes could stabilize the open conformation of the Na+ channels and favor channel opening energetically.

By occupying a significant part of the inner cavity and the focus site of the macrodipoles, BTX thus redirected the flow of Na+ ions toward N927 residue. Figure 11A shows the possible coordination of a Na+ ion at the site 1 between the outer and the inner pore. The Na+ ion was bound to the ester group of BTX, the sidechain of N927 and the main-chain oxygen of C896, located at the C-end of the P-loop. The oxygen triad of BTX faced the lumen against the inner cavity provided possible explanations for the leftward shift in the voltage dependence of activation of BTX-bound Na+ channels. Because BTX bound multiple pore-facing residues adjacent to the gating hinge position and because it extended longitudinally to the S6 C-termini, these attributes could stabilize the open conformation of the Na+ channels and favor channel opening energetically.

1 position and created charge-charge repulsion between the Na+ ion and the protonated amino group of lysine (Fig. 11B). Contrary to this, tryptophan substitution of N927 did not block the ion permeation through BTX-bound channel (Fig. 5B). The aromatic side chain of W927 lined the lumen and contributed its π electrons to the permeating ion at site 1 (Fig. 11D).

DISCUSSION

We have demonstrated a strong use-dependent phenotype of BTX block in hNa1.5-N927K mutant Na+ channels. Besides block of Na+ currents, BTX did not alter gating properties of hNav1.5-N927K and fast inactivation-deficient hNav1.5-CW-N927K Na+ channels during repetitive pulses. However, we observed little or no recovery from the block induced by BTX after repetitive pulses and no apparent time-dependent block in fast inactivation-deficient Na+ channels during the pulse (Fig. 4). All hNa1.5-CW-N927K Na+ currents can be blocked by BTX if enough repetitive pulses are given. These results strongly suggest that BTX blocks hNav1.5-CW-N927K/R Na+ channels in an all-or-none manner as a full open-channel blocker. A robust use-dependent block by BTX was also found in rat skeletal muscle homologous mutant channels, rNa1.4-WCW-N784K. Possible mechanisms underlying this unique BTX blocking phenotype are discussed next.

BTX binding occurs irreversibly in hNa1.5-N927 mutant channels. Little or no recovery from the BTX-induced block of N927K mutant channels was found after repetitive pulses. This irreversible use-dependent block by BTX suggests that binding of BTX with the hNav1.5-N927K mutant channel occurs during repetitive pulses, and upon binding, BTX blocks the Na+ channel and does not dissociate from its receptor during our experiments. Except N927K/R mutants, other N927-substituted mutants (hNa1.5-N9-7W,H,D,S,Q,E,G) display BTX phenotypes similar to those of wild-type hNa1.5 Na+ channels (Fig. 5). These BTX phenotypes
with respect to gating modifications after repetitive pulses also appear irreversible. Thus, none of the N927 substitutions appeared to reduce the BTX affinity based on the irreversibility of their phenotypes. We therefore conclude that mutations at residue hNav1.5-N927, including K/R substitutions, do not significantly affect BTX binding within the inner cavity.

It is noteworthy that BTX binding with pre-open states in fast inactivation-deficient hNav1.5-CW/N927K Na⁺ channels is likely minimal since short repetitive pulses elicited much smaller use-dependent block than longer repetitive pulses (Fig. 6). If BTX acts as a full open-channel blocker in fast inactivation-deficient hNav1.5-CW/N927K Na⁺ channels, why is there no apparent time-dependent block during depolarization (Fig. 4)? This phenomenon could be understood if BTX binding with the open state has a relative slow on-rate. The slow interaction between BTX and the open Na⁺ channel in squid axons yielded a forward rate constant ~0.2 μM⁻¹s⁻¹ for BTX binding with the open channel. With this on-rate, BTX binding will have a τ value of 1 s at 5 μM (k_on = 0.2 μM⁻¹s⁻¹ = 1/[τ × 5 μM]). This slow BTX on-rate reaction explains why we observed no apparent time-dependent block in Figure 4 during each 20-ms pulse.

How Na⁺ ions pass through the inner cavity of BTX-bound hNav1.5 Na⁺ channels. BTX is much larger in size than local anesthetics, which block Na⁺ currents in an all-or-none manner. It is therefore imperative to explain why a molecule within the inner cavity as large as BTX would allow Na⁺ ions to flow through. One approach to demonstrate that BTX partially occludes the inner cavity is to find residues critical for ion permeation in the BTX-bound Na⁺ channel and to substitute these residues so that the conductance is blocked. To our knowledge, this study provides the first demonstration on the irreversible block of hNav1.5-N927K mutant Na⁺ currents by BTX. This subtle change of BTX from a partial blocker to a full open-channel blocker is similar in concept to the direct conversion of μ-conotoxin from a full blocker to a partial blocker reported previously. In the case of μ-conotoxin, the direct conversion of a full to a partial blocker comes from the substitution of arginine-13 within μ-conotoxin, which occludes the Na⁺ channel via a receptor located at the external surface. In the case of BTX, the phenotype of a full open-channel blocker comes from a point mutation of residue N927K/R near the BTX receptor within the inner cavity of the hNav1.5 Na⁺ channel. Because of this unique use-dependent block by BTX, we conclude that residue N927 is critical for Na⁺ permeation in BTX-bound hNav1.5-N927K/R mutant channels.

Our S6 open-channel model suggests that N927 faces the narrowest hydrophilic lumen when BTX is bound within the inner cavity (Fig. 11A). Introduction of a positive charge at N927 with lysine or arginine results in the irreversible block of Na⁺ currents by bound BTX since permeating Na⁺ ions would be repelled by this charged lysine residue (Fig. 11B; site 1). Facing this narrow lumen, residue N927 may also involve in the reduction of the single-channel conductance of BTX-modified hNav1.5 Na⁺ channels. In the absence of BTX, the N927K mutant channels and their fast inactivation-deficient counterparts are permeable (Figs. 2 and 4), perhaps because the inner cavity is wide enough and because the long K/R side chain are mobile enough to avoid strong electrostatic repulsion between the residue N927K/R and a permeating ion. Attempts to occlude the BTX-bound permeation pathway near the N927 site by hydrophobic amino acid substitutions were unsuccessful as many such mutants (N927L/I/V/M/A) failed to express sufficient Na⁺ currents.

Our S6 open-channel model with BTX docked within the inner cavity also indicates that the oxygen triad of BTX may function to chelate a permeating Na⁺ ion at the site 2 as shown in Figure 11A. The presence of the site 1 and site 2 therefore allows permeating
Na⁺ ions to flow through a narrow crevice between D2S6 and BTX. Similar but not identical oxygen triads are present in the structures of veratridine, grayanotoxin, and aconitine. A recent report showed that lappaconitine elicits an irreversible block of hNa,1.5 Na⁺ channels. The oxygen triad in aconitine is different from that in lappaconitine and could also involve in the reduction of the single-channel conductance in activator-modified Na⁺ channels.

CONCLUSION

Based on BTX-resistant phenotypes in various S6 mutants, the irreversible use-dependent block of hNa,1.5-N927K channels by BTX, and computer modeling, we conclude that BTX is bound within the inner cavity of the open Na⁺ channel and leaves a narrow gap between BTX and residue N927 at D2S6. Significantly, the location of bound BTX is at or adjacent to three loci critical for Na⁺ channel functions: (a) the G/S gating hinge (G,400,G,929,G,1455-S,1759), (b) the selectivity filter (D,37,E,989,K,1419-A,1711 locus), and (c) the focus of the macrodipoles derived from the pore helices. The proximity of BTX with these functional loci explains how BTX may directly affect Na⁺ channel activation gating, ion selectivity, as well as single-channel conductance simultaneously. The knowledge on how Na⁺ channel activators reduce the single-channel conductance in terms of partial occlusion of the open Na⁺ channel may help design potent full open-channel blockers based on the diverse structures of various activators. Such high-affinity open-channel blockers could be beneficial for patients with defective-defective Na⁺ channels.

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