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Batrachotoxin and α-Scorpion Toxin Stabilize the Open State of Single Voltage-Gated Sodium Channels

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SUMMARY

The combined effects of batrachotoxin (BTX) and either scorpion (*Leiurus quinquestriatus quinquestriatus*) venom (*Lqq*V) or α-scorpion toxin (α-*Lqq*TX) purified from *Lqq*V on single voltage-gated Na channels were studied in planar lipid bilayers. In the presence of BTX, *Lqq*V caused the channels to remain open at membrane potentials at least 50 mV more hyperpolarized than with BTX alone. α-*Lqq*TX mimicked the effect of *Lqq*V, suggesting that this toxin is the active component of the venom. *Lqq*V did not significantly alter single-channel conductance, voltage-dependent block by saxitoxin, or voltage-dependent block by Ca^{2+}, indicating that the venom preferentially affects gating rather than ion permeation. The results indicate that a cooperative interaction between α-*Lqq*TX and BTX strongly favors the open state of the Na channel by causing a large hyperpolarizing shift in the voltage dependence of activation. This effect on activation gating is not predicted from the individual effects of the toxins.

Voltage-gated Na channels can be modulated by several classes of neurotoxins acting at distinct binding sites (1). In addition to tetrodotoxin and STX, which block ion flux through the open channels, other toxins from a variety of animal and plant sources alter channel gating, ion permeation, or both. BTX eliminates both fast and slow inactivation, slows activation, causes a hyperpolarizing shift in channel activation (2–4), and decreases channel selectivity (5, 6). Thus, BTX modification has profound effects on Na channel properties, affecting both gating and permeation. Many of these properties are shared by other toxins such as aconitine, veratridine, and grayanotoxin, which act at the same binding site (1). Overall, BTX causes a persistent activation of the channels at the normal resting potential, resulting in depolarization, increased internal Na^+, and action potential failure.

Polypeptide toxins from scorpions and sea anemones have quite different effects on Na channels. α-Scorpion toxins (from *Leiurus, Centruroides,* and *Androctonus*) interfere with Na channel inactivation, as indicated by the slowing of macroscopic Na current decay (7–9) and prolongation of the mean single channel open time (10, 11). BTX and α-scorpion toxin act synergistically in ^{22}Na influx (12, 13) and toxin binding (14) experiments, suggesting a cooperative interaction between the binding sites. This synergism is reflected in an α-scorpion toxin-induced increase in the apparent affinity for BTX (12–14).

We have studied the synergism between BTX and *Lqq*V or α-*Lqq*TX by examining the combined effects of these toxins on the voltage-dependent gating and permeation properties of single Na channels incorporated into planar lipid bilayers. Our results revealed an unexpected effect of the polypeptide toxin on the activation gating of BTX-activated Na channels, with the channels remaining open at membrane potentials where they would normally be closed. This effect would not be predicted from the individual actions of the two toxins.

Experimental Procedures

Materials. *Lqq*V was obtained from Latoxan (Rosans, France). BTX was a generous gift of Dr. John Daly (National Institutes of Health, Bethesda, MD). [^{3}H]-Batrachotoxinin A 20-α-benzoate was purchased from New England Nuclear (Boston, MA). Purified lipids were obtained from Avanti Polar Lipids (Pelham, AL).

Na channel incorporation into planar bilayers. Vesicles containing rat brain Na channels were prepared as previously described (15). Bilayers were formed across a 0.25-mm Lexan partition from a solution of 36 mg/ml 1-palmitoyl-2-oleoyl-phosphatidylethanolamine and 24 mg/ml 1-palmitoyl-2-oleoyl-phosphatidylserine in decane (16). Both electrolyte solutions contained 250 mM NaCl, 10 mM HEPES, 0.05 mM $CaCl_2$, pH 7.0. Vesicles and BTX (120 nM) were added to the *cis* side. Voltage across the bilayer was controlled by a voltage-clamp circuit with the *trans* side held at virtual ground (17). Single-channel currents were stored on video tape, low pass filtered (100–250 Hz), digitized, and analyzed using pClamp (Axon Instruments, Foster City,

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ABBREVIATIONS: STX, saxitoxin; BTX, batrachotoxin; *Lqq*V, *Leiurus quinquestriatus quinquestriatus* venom; α-*Lqq*TX, *Leiurus quinquestriatus quinquestriatus* α-toxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

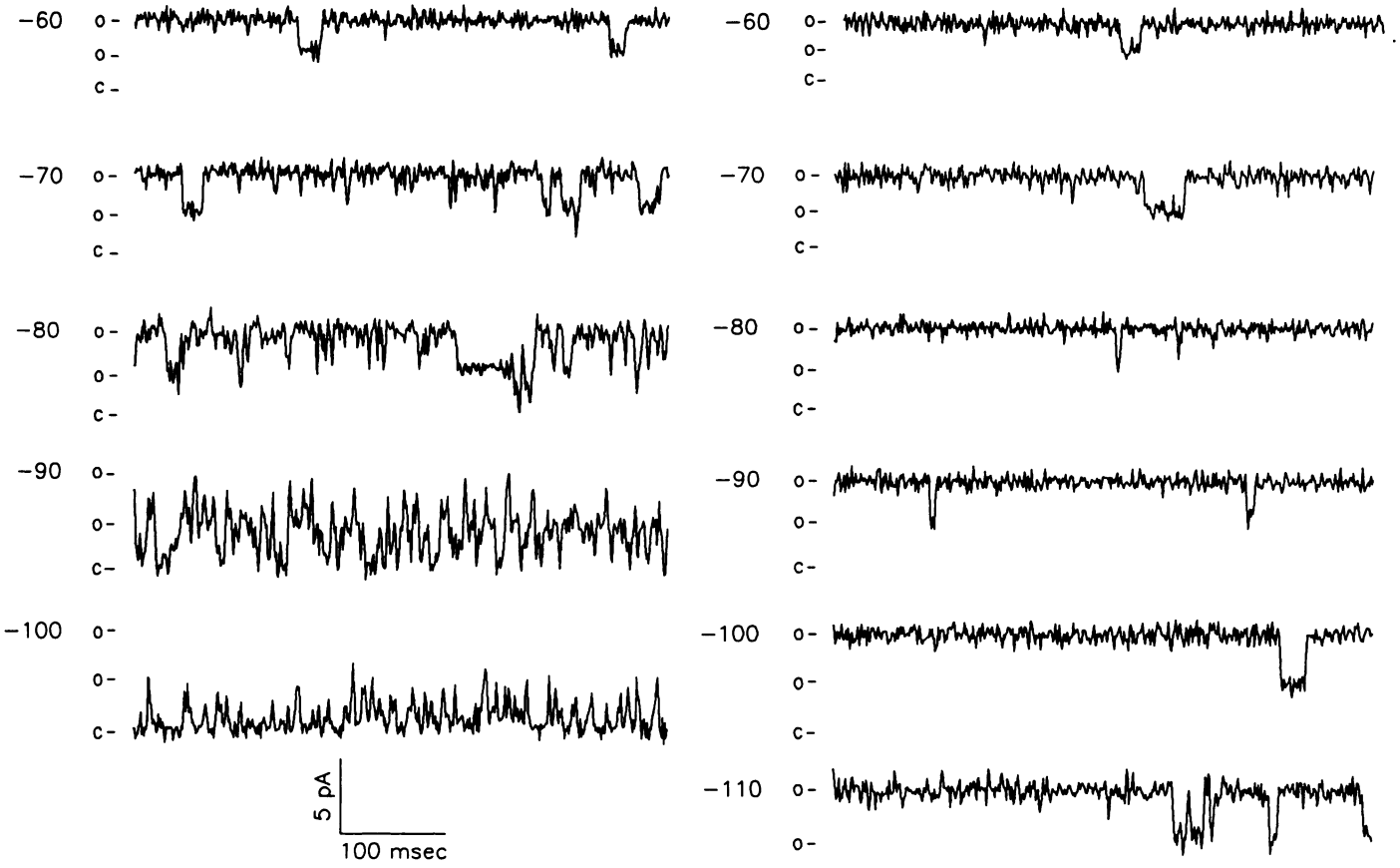


Fig. 1. Effects of *Lqq*V on BTX-activated Na channels in planar bilayers. Vesicles containing Na channels were incorporated into 1-palmitoyl-2-oleoyl-phosphatidylethanolamine-1-palmitoyl-2-oleoyl phosphatidylserine bilayers separating symmetrical solutions containing 250 mM NaCl. *Left*, Single-channel current records from two Na channels in the presence of BTX (110 nM). *Right*, The same channels after the addition of external *Lqq*V (1.5 μg/ml). Records were low-pass filtered (250 Hz). Channel openings are shown as *upward deflections*. *o*, Current levels for one or two open channels; *c*, the level for both channels closed. Records in B at −120 mV and −130 mV were selected to illustrate clearly resolved closing events during which both channels were simultaneously closed and do not reflect the average P_o of the entire records.

CA) running on a 80286 microcomputer with a Labmaster (Scientific Solutions, Solon, OH) A/D board.

Single-channel data current analysis. The probability of channels being open (P_o) was calculated from the average single-channel current (18):

$$P_o = \frac{\langle i \rangle_{Na} - i_{leak}}{i_{max} - i_{leak}} \quad (1)$$

where $\langle i \rangle_{Na}$ is the mean single channel current, i_{leak} is the current level with all channels closed, and i_{max} is the current level with all channels fully open.

Data were fit to the Boltzmann relation:

$$P_o/1 - P_o = \exp[qF(V - V_{0.5})/RT] \quad (2)$$

where R, T, and F have their usual meanings and $V_{0.5}$, the midpoint of the P_o versus V_m relation, and q, the apparent gating charge, were determined from a nonlinear least squares fit to Eq. 2.

Voltage-dependent block by STX (19) was analyzed by determining the apparent K_i from the fraction of current blocked (f_b):

$$K_i = (1/f_b - 1)[STX] \quad (3)$$

where $f_b = 1 - P_o(+STX)/P_o(-STX)$ and [STX] is the concentration of STX.

Purification of α-scorpion toxin. *Lqq*V was reconstituted in water (1 mg/ml) and insoluble material was removed by centrifugation (12,000 × *g*, 10 min). The active polypeptide toxin component (α-*Lqq*TX) was purified on a cation exchange column (Amberlite CG-50) as described (20). Active column fractions were identified by their ability to stimulate [^{3}H]BTX binding to rat brain synaptosomes (14). Crude synaptosomes (P_2) were prepared from rat brains (21) and suspended in Na-free incubation solution containing 140 mM choline-Cl, 5 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4. Aliquots of synaptosomes (0.5 mg of protein) were incubated with 10 nM [^{3}H]BTX and 1–5 μl of column fractions in a total volume of 0.4 ml for 30 min at 30°. Bovine serum albumin (1 mg/ml) was included to reduce the nonspecific binding. Aliquots of the synaptosomes were filtered on glass fiber filters and [^{3}H]BTX binding was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 300 μM veratridine.

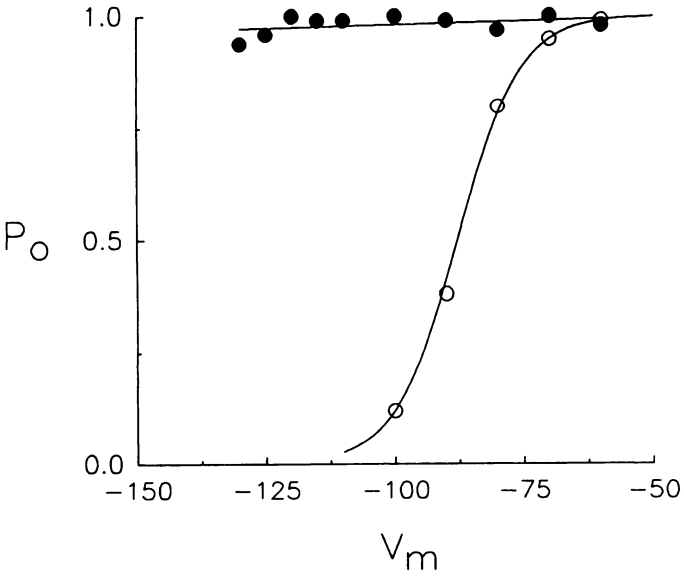


Fig. 2. *Lqq*V prevents channel closing. The probability of channel opening (P_o) was calculated for BTX- and BTX+*Lqq*V-modified channels according to Eq. 1 (same experiment as in Fig. 1). The P_o of BTX-activated Na channels (○) decreased at hyperpolarizing potentials. The *smooth curve* was fit with a Boltzmann function (Eq. 2) with a midpoint ($V_{0.5}$) of −88 mV and an apparent gating charge, q, of 4.2. After addition of *Lqq*V (●), channels did not close and the P_o remained high (>0.95) at large hyperpolarizing potentials.

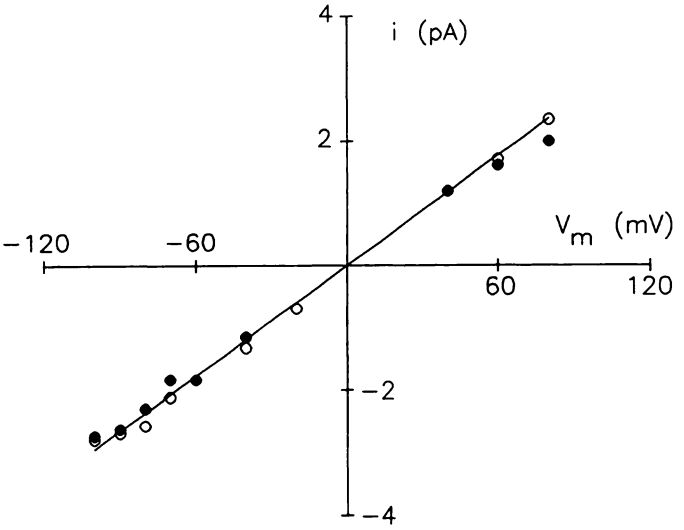


Fig. 3. Single-channel *i/V* relations of BTX- and BTX+*Lqq*V-modified channels. The current-voltage relationship of a single BTX-activated Na channel before (○) and after (●) addition of *Lqq*V was determined in symmetrical 250 mM NaCl. The conductance of the channel (28 pS) was not altered by the addition of *Lqq*V (1.5 μg/ml) to the external side of the channel.

Whole-cell patch clamp. Macroscopic Na currents were measured in GH_3 cells by the patch-clamp method in the whole-cell configuration (22), using an Axopatch 1B patch clamp (Axon Instruments). GH_3 cells were grown under standard conditions (23) and switched to external solution consisting of 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4, for 30 min before recording. Recording electrodes were fashioned from thin-walled borosilicate glass (TW150; World Precision Instruments, New Haven, CT) and had a resistance of 1–2 MΩ after polishing. The pipette solution contained 130 mM Cs-aspartate, 20 mM CsCl, 10 mM HEPES, and 10 mM glucose, pH 7.4. After the membrane was ruptured, cells were held at −70 mV and allowed to stabilize for 5–10 min before the experiments were begun. Pulses were generated and data were recorded on an microcomputer equipped with a Labmaster A/D board (Scientific Solutions) and controlled by pClamp (Axon Instruments). Current records were filtered at 10 kHz with an eight-pole Bessel filter and sampled at 35-μsec intervals.

Results

Effects of *Lqq*V on the Gating of BTX-activated Na Channels

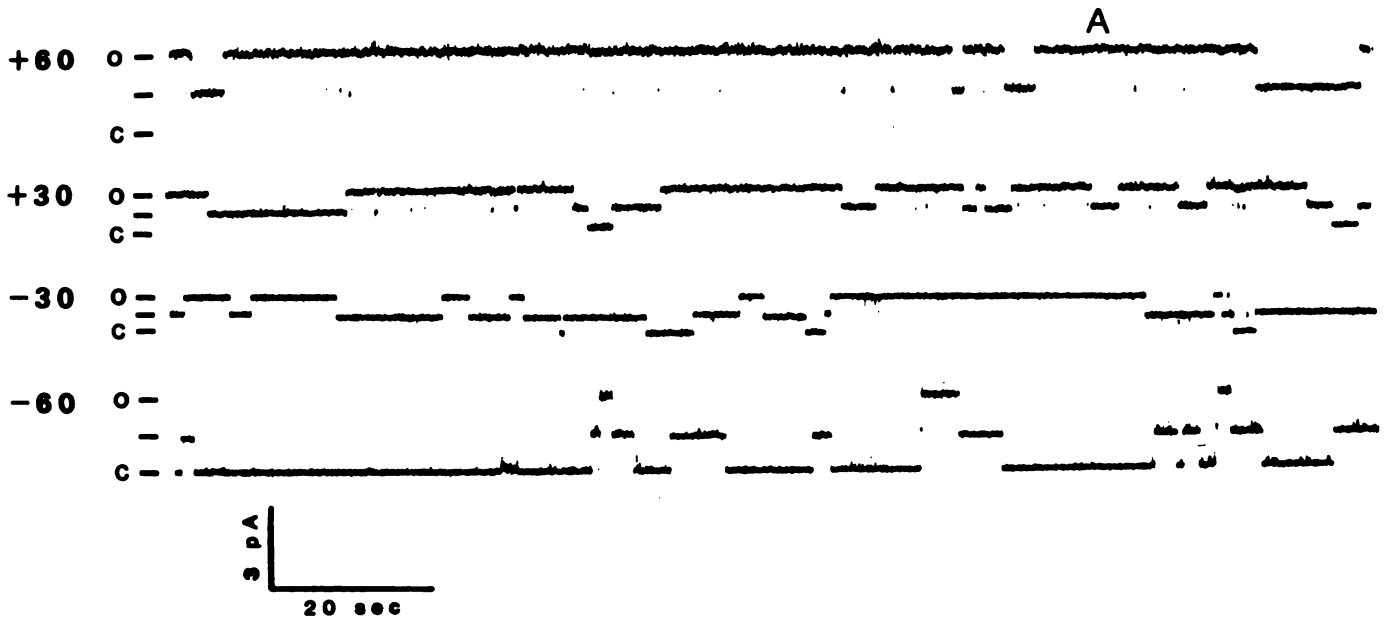
Fig. 1 shows single-channel current records from two BTX-activated Na channels in the absence (Fig. 1A) and presence (Fig. 1B) of *Lqq*V. BTX-activated Na channels are open most of the time at potentials greater than −70 mV and spend less time in the open state as the membrane is hyperpolarized (Fig. 1A). Fig. 1B shows records from the same two channels following addition of *Lqq*V (1.5 μg/ml). In the presence of *Lqq*V, BTX-activated channels remained open at hyperpolarizing potentials where they are normally closed. The mean probability of being open (P_o), calculated according to Eq. 1, is plotted as a function of membrane potential in Fig. 2. The P_o versus V_m relation was fit to a Boltzmann function (Eq. 2) with a $V_{0.5}$ of −88 mV and an apparent gating charge, q, of about 4.2. In the presence of both BTX and *Lqq*V, channel closings were brief and no significant reduction in P_o was observed even at hyperpolarizations to −135 mV. We could not determine whether, in the presence of both BTX and *Lqq*V, the channels were incapable of closing or whether $V_{0.5}$ was much more negative than −135 mV, because the bilayers are not stable at more negative potentials.

Effects of *Lqq*V on Na Channel Permeation and Block

Single-channel conductance. The single-channel current-voltage (i/V) relationships for BTX and BTX+*Lqq*V-modified Na channels are illustrated in Fig. 3. In symmetrical 250 mM NaCl, the single-channel conductances of both BTX-activated and BTX+*Lqq*V-activated channels were similar (about 28 pS). In other experiments (not shown), the i/V relation of BTX+*Lqq*V-activated channels was linear to −135 mV and to +120 mV.

Voltage-dependent block by STX and Ca^{2+}. BTX-activated Na channels display voltage-dependent block by STX and TTX (18, 19, 24–27). The block of BTX+*Lqq*V-modified channels by STX was examined to determine whether *Lqq*V altered the affinity or voltage dependence of the block. In the experiment shown in Fig. 4A, the bilayer contained two BTX+*Lqq*V-activated channels and 1 nM STX was present on the extracellular side. The channels spent significantly more time in the blocked state (downward deflections) at negative than at positive membrane potentials. The potency of STX block (K_i) was calculated from the fractional block (Eq. 3) at each membrane potential (Fig. 4B). The slope of the ln K_i versus V_m relationship for the same experiment (Fig. 4) indicates that K_i changes e-fold/37 mV. Overall, in four experiments the mean slope was e-fold/43 ± 5 (mean ± SE) mV and the mean K_i (0 mV) was 1.6 ± 0.4 (mean ± SE) nM. These are similar to previously published values for BTX-activated Na channels from a variety of sources in planar bilayers (18, 19, 24, 27).

Because Na channels are blocked by Ca^{2+} in a voltage-dependent manner at a site in the permeation pathway (28), it was of interest to determine whether the Ca^{2+} block was altered



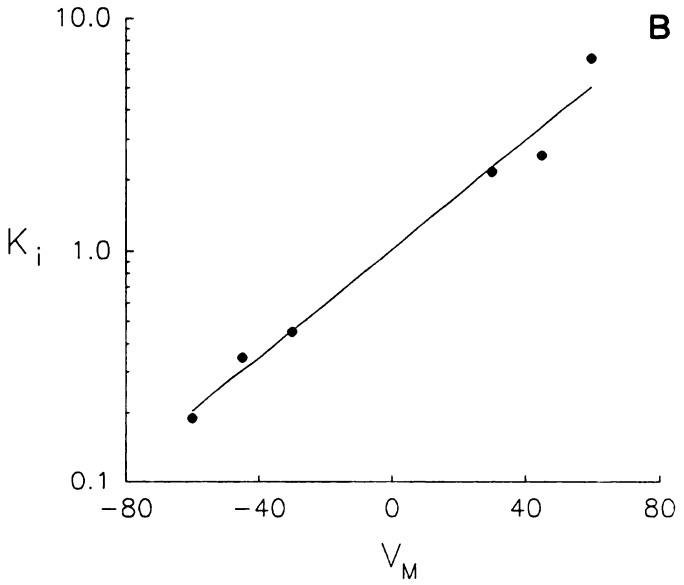


Fig. 4. Voltage-dependent block by STX. A, Single-channel current records from a bilayer containing two BTX+*Lqq*V-modified channels are shown in the presence of 1 nM external STX. Block was greater at hyperpolarized potentials (*bottom*) than at depolarized potentials (*top*). Current levels with both channels unblocked (*o*) or blocked (*c*) are indicated. B, The fractional block (f_b) at each membrane potential was determined from 5-min recordings similar to those shown in A. The apparent affinity of STX block (K_i) was calculated from Eq. 3 at each potential. The *straight line* through the data indicates an e-fold change in K_i/37 mV.

in the presence of *Lqq*V. Fig. 5 shows the block of BTX+*Lqq*V-activated Na channels by 5 mM external Ca^{2+} (symmetrical 250 mM NaCl). Single-channel currents were blocked by hyperpolarizing but not at depolarizing potentials. The voltage dependence of the block was consistent with Ca^{2+} binding at a site about 25% of the electrical distance across the channel pore (28). This result is virtually identical to that obtained with BTX-activated Na channels from a variety of sources (28–31). In separate experiments, *Lqq*V added after Ca^{2+} did not change the characteristic curvature of the i/V relation reflecting voltage-dependent block (data not shown).

In the Absence of BTX, α-*Lqq*TX Slows Inactivation with Little Effect on Activation

α-*Lqq*TX was purified from *Lqq*V by cation exchange chromatography (20) using stimulation of [^{3}H]BTX binding to synaptosomes (14) to identify the active fractions. The $K_{0.5}$ for activation of [^{3}H]BTX binding by *Lqq*TX was 16 nM (Fig. 6), in reasonable agreement with previous estimates (14). The effects of purified α-*Lqq*TX on Na currents in the absence of other neurotoxins was examined in GH_3 cells (Fig. 7). As has been reported previously (7), the polypeptide toxin slowed Na current inactivation (Fig. 7A), increased peak inward current (27%), and caused a slight (−7 mV) hyperpolarizing shift in the peak I_{Na} versus V_m relation (Fig. 7B). Other investigators have reported either no shift in the voltage dependence of activation (32–34) or a small hyperpolarizing shift (11, 35) in the presence of α-scorpion toxins. The increase in peak inward current and the apparent hyperpolarizing shift in activation reported in this study may result from a reduced rate of inactivation (11, 36). Overall, based on the high potency of stimulation of [^{3}H]BTX binding (Fig. 6) and the slowing of inactivation with little or no effect on activation (Fig. 7), this purified toxin has actions similar to those of other α-scorpion toxins (7).

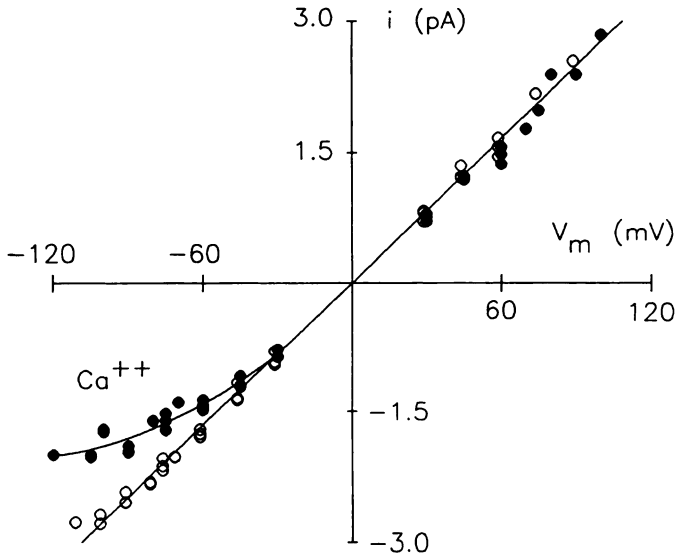


Fig. 5. Voltage-dependent block of Na channels by external Ca^{2+}. The single-channel current-voltage relation of BTX+*Lqq*V-modified Na channels was determined before (○) and after (●) the addition of 5 mM external $CaCl_2$. Inward current is blocked to a greater extent than outward current. Data from five experiments are shown.

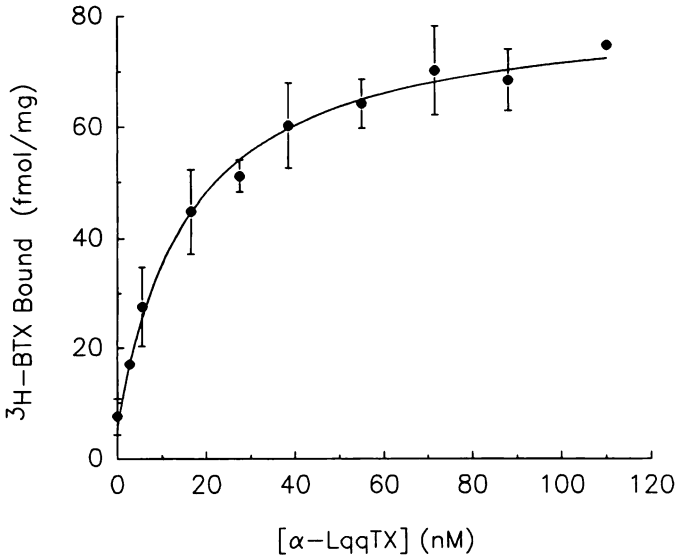


Fig. 6. Stimulation of [^{3}H]BTX binding by *α-LqqTX*. The amount of BTX bound to rat brain synaptosomes was determined after 30-min incubation with 10 nM [^{3}H]BTX and increasing doses of *α-LqqTX*. The *smooth curve* is a fit to a single-site binding model with $K_{0.5}$ of 16 nM and B_{max} of 76 fmol/mg of protein.

Effects of *α-LqqTX* on BTX-Activated Na Channels in Planar Bilayers

As illustrated in Fig. 8, *α-LqqTX* has effects on BTX-activated Na channels that are similar to those of *LqqV* (Fig. 1). Addition of purified polypeptide toxin (350 nM) to the extracellular side of a single BTX-activated Na channel suppressed closing so that the channel was nearly always open (Fig. 8B) at potentials (e.g., −120 mV) where it would normally be closed (Fig. 8A). The alteration in the P_o versus V_m relation caused by *α-LqqTX* (Fig. 9) was similar to that observed with *LqqV* (Fig. 2). Thus, *α-LqqTX* appears to be the active component of *LqqV* responsible for the effects of the crude venom on BTX-activated Na channels.

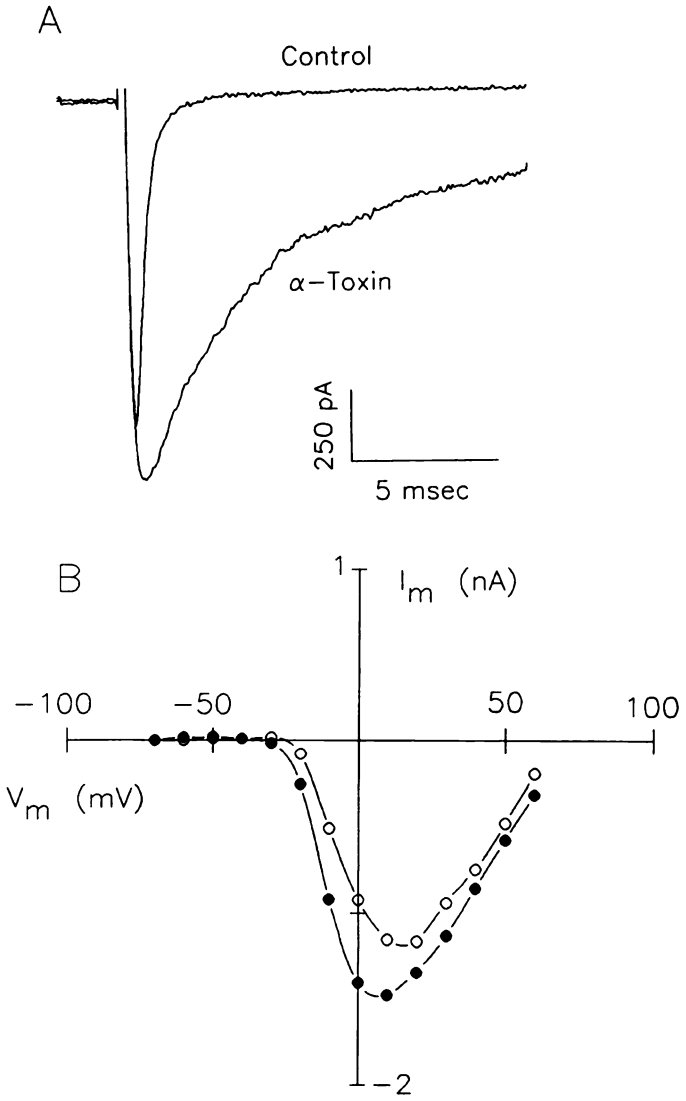


Fig. 7. Effects of *α-LqqTX* on macroscopic Na currents in GH_3 cells. A, Whole-cell Na currents of GH_3 cells following a voltage step from V_h = −70 mV to +20 mV before and 5 min after the addition of *α-LqqTX* to the bath near the cells. The nominal concentration of *α-LqqTX* was 440 nM. Na current inactivation was dramatically slowed after the addition of *α-LqqTX*. B, Current-voltage relationship of the currents shown in A before (○) and after (●) addition of *α-LqqTX*. Peak current is 27% larger in the presence of toxin.

Discussion

Unique effects of BTX+*α-LqqTX* on Na channel gating. Our results clearly show that *LqqV* (Fig. 1) and *α-LqqTX* (Fig. 8) alter the activation gating of BTX-activated Na channels in planar bilayers. In the presence of both toxins, voltage-dependent single-channel closing events were eliminated, even at very hyperpolarized potentials. Many studies have identified a synergistic interaction of α-scorpion toxins with BTX. For example, in tracer flux experiments, α-scorpion toxins such as *LqqTX* (12, 13) increase the apparent potency of BTX. This appears to result from an increased binding affinity of BTX, as indicated by a decrease in the K_a for potentiation of BTX-stimulated ^{22}Na influx (12) and the K_d for [^{3}H]BTX binding (14). BTX-activated Na channels in planar bilayers appear to be irreversibly modified (16). This is consistent with the very slow rate reported for BTX dissociation (14, 37). Thus, under

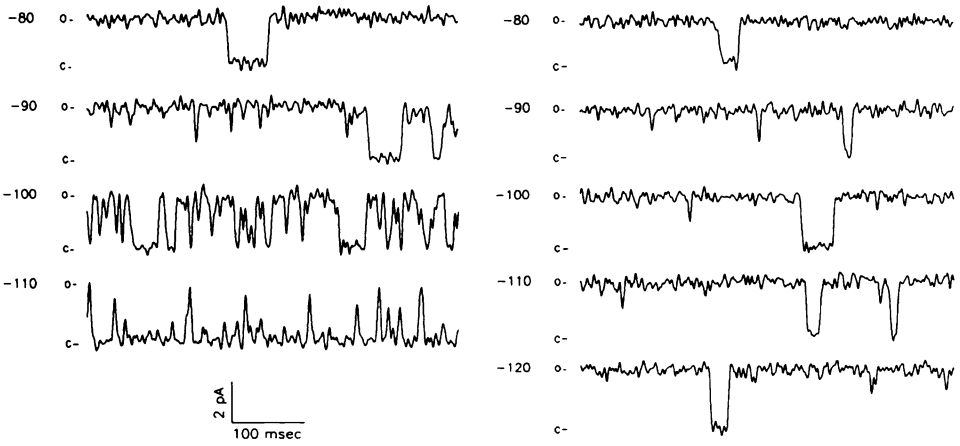


Fig. 8. Effects of α-*Lqq*TX on BTX-activated Na channels in planar bilayers. *Left*, Single-channel currents from one BTX-activated Na channel. The channel was open ($P_o > 0.95$) at potentials more positive than −70 mV and was closed at −110 mV. *Right*, After addition of α-*Lqq*TX (350 nM), the channel remained open despite large hyperpolarizations. Open (*o*) and closed (*c*) current levels are shown.

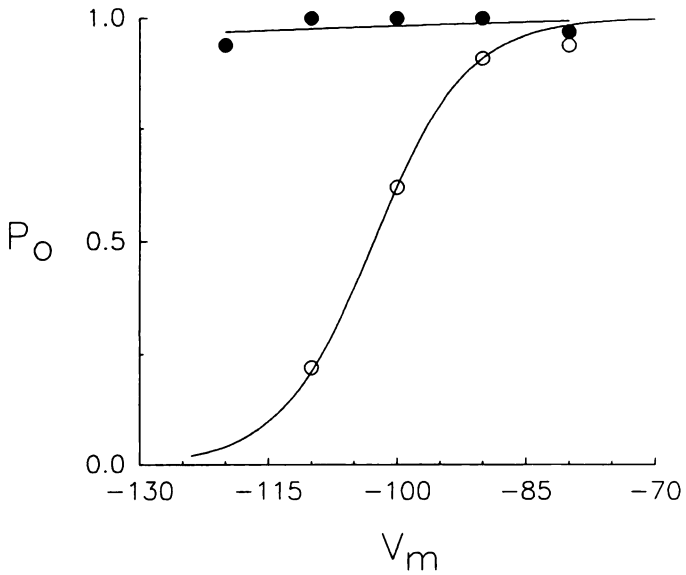


Fig. 9. α-*Lqq*TX prevents channel closing. The P_o versus V_m relation for the same channel shown in Fig. 8 before (○) and after (●) the addition of external α-*Lqq*TX. The *smooth curve* is a plot of a Boltzmann function with $V_{0.5} = -100$ mV and an apparent gating charge $q = 4.6$.

the experimental conditions employed here, a simple change in BTX affinity would not be expected to cause the dramatic change we observed in the single-channel gating of BTX-activated channels.

Due to large capacitative currents and limited recording bandwidth, it has not been possible to study Na channels in planar bilayers in the absence of BTX. However, we have used the whole-cell configuration of the patch-clamp technique (22) to examine the effects of purified α-*Lqq*TX on Na channels in GH_3 cells in the absence of BTX. We found that the purified scorpion toxin behaved like a typical α-toxin (7), having its principal effect on inactivation (Fig. 7A) with only a minimal effect on the voltage dependence of activation (Fig. 7B), and showed no indication of decreased peak I_{Na} or slowed deactivation, as is observed with β-scorpion toxins (8, 38–40). Thus, in the absence of other toxins, α-*Lqq*TX has only a minimal effect on the voltage dependence of activation. This is in sharp contrast to the novel effect of α-*Lqq*TX on noninactivating BTX-activated channels (Fig. 9), which is to cause a dramatic hyperpolarizing shift in the voltage dependence of channel activation.

Based on the individual actions of BTX and α-*Lqq*TX and their synergistic effects determined in tracer influx and [^{3}H] BTX binding experiments, it might be expected that the polypeptide toxin would have little or no effect on Na channels already fully activated by BTX. In contrast, the voltage-dependent closings of BTX+*Lqq*TX-modified channels were suppressed even at −140 mV, nearly 50 mV more negative than would normally cause closing. Due to the instability of the bilayers at potentials less than −140 mV, we have not been able to determine whether the channels undergo voltage-dependent closing at more negative potentials; however, the midpoint ($V_{0.5}$) of the P_o versus V_m relation must be shifted at least 50 mV in the hyperpolarizing direction to account for our results. Clearly, the effects of the BTX+α-*Lqq*TX are dramatically different from the sum of the effects of each toxin alone.

α-*Lqq*TX increases the efficacy of BTX. BTX has a wide variety of effects on Na channels, including modification of both the gating and permeation processes (2, 5, 41) presumably resulting from BTX-induced conformational changes in the channel structure. Although the molecular mechanisms of these effects are not understood, it is likely that BTX binding alters the properties of the channel's voltage sensor (41). The very negative (−90 to −100 mV) $V_{0.5}$ for BTX-activated channels reported here reflects the well documented hyperpolarizing shift in steady state activation induced by BTX (2, 5, 41). Our results demonstrate that BTX+α-*Lqq*TX either causes a much larger hyperpolarizing shift in the P_o versus V_m relation or prevents voltage-dependent closing completely. Because the Na channels we are studying are virtually irreversibly activated by BTX, and α-*Lqq*TX alone does not substantially alter the voltage dependence of activation (Fig. 7B), the novel effect of polypeptide toxin we observe is not due to a simple increase in the affinity of BTX for its receptor on the channel and must occur at a step after BTX binding.

We suggest that α-*Lqq*TX enhances the BTX-induced hyperpolarizing shift in Na channel activation rather than directly affecting channel activation. Thus, the potentiation of BTX action by α-scorpion toxins can be attributed to two distinct effects on the Na channels, 1) an increase in the affinity for BTX as previously concluded from tracer flux and BTX binding experiments (13, 14, 42) and 2) an increase in the efficacy of BTX once it is bound to the Na channels.

α-*Lqq*TX does not alter permeation through BTX-activated Na channels. We have examined the effect of α-

*Lqq*TX on several properties of BTX-activated channels related to the channel pore rather than to the voltage-dependent gating mechanism. The polypeptide toxin does not affect single-channel conductance (Fig. 3), affinity and voltage dependence of STX block (Fig. 4), or voltage dependence of block by external Ca^{2+} (Fig. 5), leading to the conclusion that the *α-Lqq*TX alters channel gating without having a significant effect on ion permeation of BTX-activated Na channels. The ability to study permeation in a voltage range where the channels are normally closed may enable more extensive studies of such properties as voltage-dependent block by guanidinium toxins (19) and local anesthetics (43, 44), voltage-dependent block by divalent cations (19, 28), and permeation by marginally permeant ions such as K^+, Ca^{2+}, and guanidinium (45–47). Thus, in addition to being a potentially useful probe of the voltage-dependent gating mechanism of Na channels, *α-Lqq*TX may provide the means to study some aspects of the channel's permeation pathway that have previously been inaccessible.

Acknowledgments

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