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Actions of a Polypeptide Toxin from the Marine Snail *Conus striatus* on Voltage-Sensitive Sodium Channels

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SUMMARY

The effects of a polypeptide toxin of 25,000 Da from the marine snail *Conus striatus* (CsTx) on sodium channels in mouse neuroblastoma cells and rat brain synaptosomes were studied. CsTx slowed sodium channel inactivation without altering the time course of activation of the channels. The voltage dependence of sodium channel inactivation was shifted to more negative membrane potentials and made less steep. Peak sodium currents were increased, and the voltage dependence of activation was shifted to more negative membrane potentials. The action of the toxin was voltage-dependent. Maximum toxin effects were observed at membrane potentials in the range of −100 to −60 mV. Apparent K_D values were calculated assuming a one-to-one binding interaction. At more positive membrane potentials, the apparent K_D for toxin action increased e-fold for each 19-mV depolarization. Apparent K_D also increased at membrane potentials more negative than −100 mV. CsTx did not have significant effects on the binding of saxitoxin or *Leiurus* α-scorpion toxin to their receptor sites on sodium channels. CsTx enhanced the binding of batrachotoxinin A 20-α-benzoate to sodium channels in the same concentration range as its physiological effects. It is concluded that CsTx interacts with a new receptor site on the extracellular surface of the sodium channel at which specific effects on channel inactivation can occur.

The piscovorous marine snails of the genus *Conus* produce various polypeptide toxins that are used in the capture of prey (1, 2). The primary structures and mechanisms of action of low molecular weight toxins of 12 to 26 amino acid residues that block nicotinic acetylcholine receptors (3), sodium channels (4, 5), and calcium channels (2, 6) have been described. The μ conotoxins, Geographutoxin I and II, from *Conus geographus* inhibit muscle sodium channels specifically (2, 7) by binding at the same receptor site as tetrodotoxin and saxitoxin (8–10). The venom of *Conus striatus* causes contracture of skeletal and smooth muscles (11–13), positive inotropic effects in the heart (14, 15), and repetitive firing and prolonged action potentials in myelinated nerve (16, 17). A purified glycoprotein with a molecular weight of 25,000 retains the positive inotropic activity of the whole venom, suggesting that it is a major toxic component (15). In this report, we describe the actions of this purified protein on sodium currents in mouse neuroblastoma cells and on binding of specific neurotoxins to their sites of action on sodium channels in rat brain synaptosomes.

Experimental Procedures

Materials. *C. striatus* toxin was purified as previously described (15). The α-scorpion toxin from LqTx was purified, and the mono [^{125}I]iodo derivative was prepared by lactoperoxidase-catalyzed iodination and repurification (18). [^{3}H]saxitoxin was radiolabeled by specific tritium exchange (19), repurified, and characterized as previously described (20). BTX-B was obtained from New England Nuclear (Boston, MA). Batrachotoxin was provided by Dr. John Daly (National Institutes of Health). Other materials were obtained from the following sources: tetrodotoxin from Calbiochem (La Jolla, CA); fetal calf serum and DMEM were from Grand Island Biological Co. (Grand Island, NY).

Cell culture. N18 mouse neuroblastoma cells were grown in 100-mm diameter Petri dishes (Falcon) in DMEM containing 5% fetal calf serum for 5–8 days until the cells became confluent, essentially as described previously (21). One day before use in the experiments, the growth medium was changed to DMEM without fetal calf serum to increase the size of the sodium currents. The kinetics and voltage dependence of sodium channel function are unaffected by growth in serum-free medium (22). Before recordings, the cells were harvested by trituration in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, and 2×10^5 cells were then reseeded in 35-mm diameter Petri dishes (Falcon) containing 1 ml of growth medium. Recordings were made 2–6 hr after reseeding. This procedure allowed us to record from spherical cells as required for precise voltage clamp measurements (23).

ABBREVIATIONS: LqTx, *Leiurus quinquestriatus* toxin; DMEM, Dulbecco-Vogt modified Eagle's medium; BTX-B, batrachotoxinin A 20-α-benzoate; CsTx, *Conus striatus* toxin.

Voltage clamp recording. At the beginning of each experiment, the growth medium was replaced with 0.7 ml of recording medium consisting of (in mM): 150 NaCl, 5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 5 glucose, 5 Na^+ HEPES (pH 7.4), and the culture dish was mounted on the stage of an inverted phase contrast microscope at room temperature (22–23°). The voltage clamp recording method was based on the one-pipette, tight-seal whole cell recording method of Hamill *et al.* (24). Glass microtubes (Curtis Matheson, Houston, TX; 1.5-mm outside diameter) were pulled in two steps and fire polished. The tip resistances after fire polishing were between 200 and 400 kohms in recording medium. Pipettes were filled with pipette medium consisting of (in mM): 85 CsF, 60 CsCl, 10 NaF, and 5 EGTA. In some experiments, all the NaCl in the extracellular recording medium was replaced with 150 mM choline chloride, and in these experiments the intrapipet medium consisted of 135 mM NaF, 20 mM CsCl, and 5 mM EGTA. The pH of the pipet media was adjusted to 7.2 with CsOH. Cs^+ in the intrapipet medium effectively blocked K^+ currents in N18 cells (23).

A List EPC-7 patch clamp system was used for voltage clamp recordings. Series resistance of the pipets was compensated by an internal feedback circuit. Junction potentials between the two Ag/AgCl electrodes were compensated electrically. Linear leakage and capacitive currents were subtracted with an analog subtractor. Currents were displayed on an oscilloscope and photographed. More details of the recordings from N18 neuroblastoma cells were described previously (23).

Recordings were made from round cells with diameters of 18–25 μm and no processes as observed in the phase contrast microscope. Cells were maintained at a holding potential of −80 mV and stimulated with prepulses and test pulses as described in the figure legends. Sodium conductance (g_{Na}) was calculated from the relation $g_{Na} = I_{Na}/(E-E_{Na})$ where I_{Na} is the Na^+ current, E is the membrane potential, and E_{Na} is the reversal potential for current in the sodium channels as measured in each experiment. Averages of pooled data are given as means ± standard deviation.

Neurotoxin binding to sodium channels in synaptosomes. Specific binding of [^{3}H]saxitoxin to neurotoxin receptor site 1 on sodium channels in synaptosomes was measured as previously described (25). Synaptosomes (75 μg) in 25 μl of standard binding medium consisting of 130 mM choline chloride, 50 mM HEPES (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and 1 mg/ml bovine serum albumin were added to a reaction mixture containing [^{3}H]saxitoxin and other toxins as noted in the figure legends in 175 μl standard binding medium. Samples were mixed and incubated 30 min at 37°. The reactions were stopped by addition of 3.0 ml wash medium at 0° consisting of (in mM) 163 choline chloride, 5 HEPES (adjusted to pH 7.4 with Tris base), 1.8 $CaCl_2$, and 0.8 $MgSO_4$, and the membranes were collected by filtration on Whatman GF/C filters and washed twice within 10 sec. The filters were then placed in counting vials, and radioactivity was determined. Nonspecific binding was measured in the presence of a saturating concentration of tetrodotoxin (1 μM) and was subtracted from all results (25).

Specific binding of [^{3}H]BTX-B to neurotoxin receptor site 2 on sodium channels was measured as described previously (26). Synaptosomes (200 μg protein) in 100 μl of standard binding medium containing 1 μM tetrodotoxin were mixed with 7–10 nM [^{3}H]BTX-B and other neurotoxins as noted in the figure legends in 150 μl of standard binding medium at 37°. After incubation for 30 min, reactions were stopped by addition of 3 ml wash medium containing 1 mg/ml bovine serum albumin, and synaptosomes were collected by filtration of GF/C filters and washed rapidly three times with wash medium at 0°. The filters were placed in counting vials, and bound radioactivity was determined. Nonspecific binding was determined in the presence of 300 μM veratridine and was subtracted from all results.

Specific binding of [^{125}I]$_1$LqTx to neurotoxin receptor site 3 on sodium channels was measured as described previously in the presence of 0.1 nM [^{125}I]$_1$LqTx (27). The assay is exactly as described for [^{3}H]BTX-B except that washes were performed at 37°, and nonspecific binding was determined in the presence of 200 nM LqTX.

Other methods. Protein concentrations were determined by the method of Peterson (28). Except where specifically mentioned, the data presented are the results of a single experiment that is representative of two or more similar experiments. All data are the mean of two to five replicate determinations. Smooth curves describing data points were drawn by eye. Straight lines were computed by linear regression.

Results

Modification of sodium channel kinetics by CsTx. Sodium currents mediated by voltage-sensitive sodium channels were measured by the whole cell voltage clamp technique as described under Experimental Procedures. Fig. 1A illustrates a family of sodium currents elicited by depolarizations to test potentials of −50 to +80 mV at intervals of 1 sec. Sodium channels are activated within 1 msec and are inactivated within a few msec depending on the test potentials applied. This recording was made 20 min after making a high resistance seal between the cell membrane and a micropipet. By this time the exchange of ions between the cell and the micropipet was complete so that the sodium reversal potential and the sodium

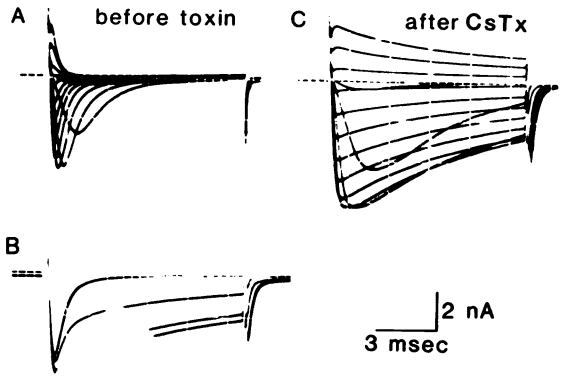

Fig. 1. Effect of CsTx on the time course of sodium currents. A, a high resistance seal was formed on an N18 cell. After 20 min at a holding potential of −80 mV, the cell was hyperpolarized to −120 mV for 100 msec and depolarized for 10 msec to test potentials of −50 mV to +80 mV, in intervals of 10 mV, once per second to elicit the sodium currents illustrated. B, 18 μl of 4 × 10^{-6} M CsTx were added to the recording medium approximately 7 mm from the cell to give a final concentration in the recording bath of 1 × 10^{-7} M. Sodium currents were elicited every 30 sec by hyperpolarizing to −120 mV for 100 msec and depolarizing to +10 mV for 10 msec. Superimposed traces from a storage oscilloscope are shown. C, 6 min after addition of CsTx, a family of sodium currents was recorded as in A.

currents were no longer increasing with time. CsTx (18 μl of 4 × 10^{-6} M) was added to the bathing medium approximately 7 mm from the cell to give a final bath concentration of 1 × 10^{-7} M. Changes in the time course of the sodium currents were measured during 10-msec test pulses to +10 mV delivered every 30 sec (Fig. 1B). Inactivation of sodium channels was progressively slowed, reaching a new steady state rate of inactivation after 5 min. Fig. 1C shows a family of sodium currents elicited by depolarizations to test potentials of −50 to +80 mV 6 min after addition of CsTx. Inactivation of the sodium channels was fully modified by the toxin, and the sodium channels did not inactivate completely at the end of the 10-msec test pulse period. Similar slowing of inactivation was observed in all four cells tested at this concentration of toxin. The toxin increased the peak sodium currents elicited by a test pulse to +10 mV in three of these cells to 120 ± 18% (SD) of the control level as illustrated in Fig. 1 (compare panels A and C). In contrast to the results with these and all other cells studied, the fourth cell in this group was exceptional and showed a decrease in peak sodium current. The reversal potential of the sodium current was not significantly affected by the toxin (+59.9 ± 4.4 mV before toxin, +58.4 ± 8.1 mV after toxin), and the toxin-modified currents were completely blocked by 1 × 10^{-6} M tetrodotoxin. These results show that CsTx slows the inactivation of sodium channels and increases peak sodium conductance through the channels.

When CsTx was applied at a lower final concentration of 3 × 10^{-8} M, the time course of sodium channel inactivation had two components, a rapid one corresponding to inactivation of unmodified channels and a slower one corresponding to toxin-modified channels (for examples, see Figs. 4 and 5). Peak sodium currents were also increased at the lower toxin concentration to a mean of 136% of control for two cells studied.

Fig. 2 compares time courses of decay of sodium currents during test pulses to +10 mV for 70 msec in the presence or absence of 1 × 10^{-7} CsTx on semilogarithmic coordinates. The decay of the sodium currents in the absence of toxin was described by a single exponential with a decay constant of 0.7 msec. In contrast, in the presence of CsTx the sodium current decayed more slowly in a multiexponential time course. The limiting slope of the first phase of decay was consistent with a time constant of 15.8 msec. Thereafter, the current decayed progressively more slowly, with 12% of the peak sodium current remaining at the end of the 70-msec test pulse period.

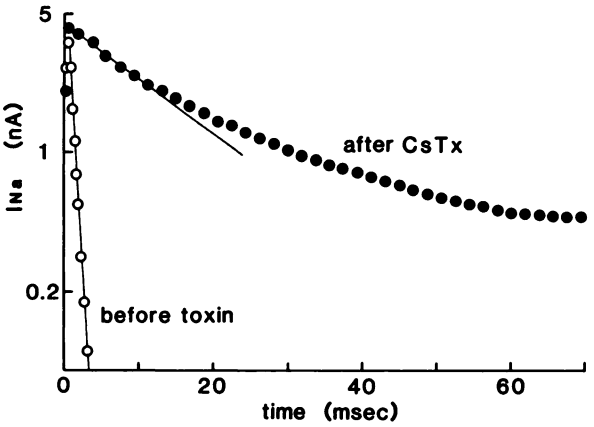

Fig. 2. Time course of inactivation of sodium current (I_{Na}) in the presence and absence of CsTx. A high resistance seal was formed on an N18 cell. After 20 min at a holding potential of −80 mV, a sodium current was recorded by hyperpolarizing to −120 mV for 100 msec and depolarizing to a test potential of +10 mV for 70 msec. CsTx was added at a final concentration of 1 × 10^{-7} M; the cell was incubated for 10 min at 37°C; and a sodium current was elicited by hyperpolarization to −120 mV for 100 msec and depolarization to +10 mV for 70 msec. The exponential decay of the currents is illustrated on semilogarithmic coordinates. The limiting straight lines correspond to time constants of 0.7 and 15.8 msec, respectively.

Fig. 3 illustrates the relationship between normalized peak sodium conductance and the test pulse potential before and after treatment with 1 × 10^{-7} M CsTx. The potential for half-maximal activation was shifted to more negative membrane potentials by 10.8 ± 1.9 mV in four cells. Similar shifts in the conductance-activation curve were observed when the inactivation of sodium currents of N18 neuroblastoma cells was inhibited by treatment with *Leiurus* scorpion toxin (23), *Goniopora* coral toxin (29), or proteolytic enzymes (30).

The apparent voltage dependence of inactivation is also altered by CsTx (Fig. 3). In the presence of the toxin, the test pulse potential required for half-maximal inactivation in a 100-msec pulse was shifted to more negative membrane potentials by 13.7 ± 6.4 mV in eight cells. Since the time course of inactivation is greatly slowed by CsTx, the inactivation curve determined at 100 msec may not represent the true steady state. Long prepulses were not used because slow inactivation becomes important. In all cells tested, the voltage dependence of inactivation measured at 100 msec was less steep in the presence of toxin, and inactivation was incomplete, even after a 100-msec prepulse at −20 mV (Fig. 3).

Voltage dependence of CsTx action. The binding and action of several other polypeptide neurotoxins that slow sodium channel inactivation are voltage-dependent including α-scorpion toxins (18, 23, 31–33), sea anemone toxins (34–37), and *Goniopora* coral toxin (29). In each case, the affinity of these toxins for their receptor sites on the sodium channel is reduced by membrane depolarization. We examined the mem-

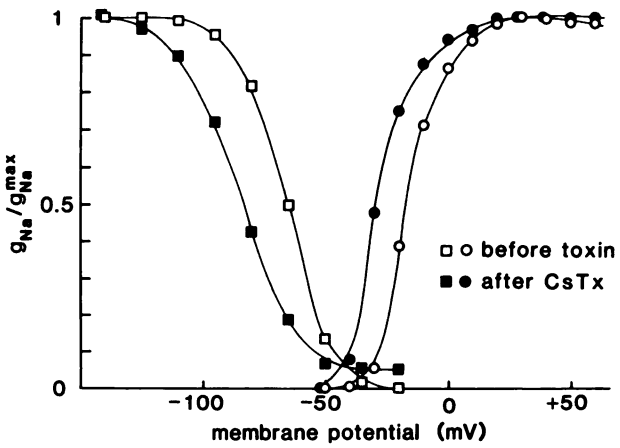

Fig. 3. Effect of CsTx on the voltage dependence of sodium channel activation and inactivation. Activation: families of sodium currents were recorded in the presence and absence of 1 × 10^{-7} M CsTx as described in the legend to Fig. 1. Peak conductance values were calculated as described under Experimental Procedures and plotted as the ratio of the measured value to g_{Na} observed in a pulse to +30 mV (before toxin ○; after toxin ●). Inactivation: N18 cells were hyperpolarized for 100 msec to the indicated membrane potentials, and then sodium currents were elicited by a test pulse to +10 mV for 10 msec. Conductance values were calculated as described under Experimental Procedures and plotted relative to the sodium conductance observed after a prepulse to −140 mV (before toxin □; after toxin ■).

brane potential dependence of the action of CsTx by varying the holding potential of the cell as described previously for scorpion and coral toxins (23, 29). Before beginning the recordings, cells were incubated in recording medium containing 3×10^{-8} M CsTx for 30 min at 37° to allow equilibrium binding of the toxin. After forming a seal on a cell, the holding potential was maintained at −80 mV for 20 min, and a family of sodium currents was elicited by 10 msec depolarizing test pulses to potentials of −50 to +80 mV after 200 msec hyperpolarizing prepulses to −120 mV (Fig. 4A). The sodium currents decayed with a biphasic time course as expected for a mixture of modified and unmodified channels. After changing the holding potential to −40 mV and incubating the cells for 5 min to allow equilibration of the toxin, the extent of modification of the sodium currents was reduced (Fig. 4B). After further depolarization of the holding potential to 0 mV, the time course of the sodium currents returned to essentially that of unmodified channels (Fig. 4C). Hyperpolarization of the membrane potential to −80 mV increased the degree of modification of sodium currents by CsTx to the original level (data not shown). These results demonstrate that the binding and/or action of CsTx is reduced by membrane depolarization in the range of −80 to 0 mV.

Similar experiments were performed over the membrane potential range from −160 to −80 mV (Fig. 5). Surprisingly, we found that hyperpolarization of the holding potential beyond −80 mV decreased the fraction of sodium channels that inactivated slowly. To examine this effect more carefully, we conducted experiments starting from a holding potential of −160 mV so that the direction of membrane potential change was the same as in Fig. 4. After incubating cells with 3×10^{-8} M CsTx for 30 min at 37° and hyperpolarizing to −160 mV for 10 min (Fig. 5A), the extent of modification of sodium currents

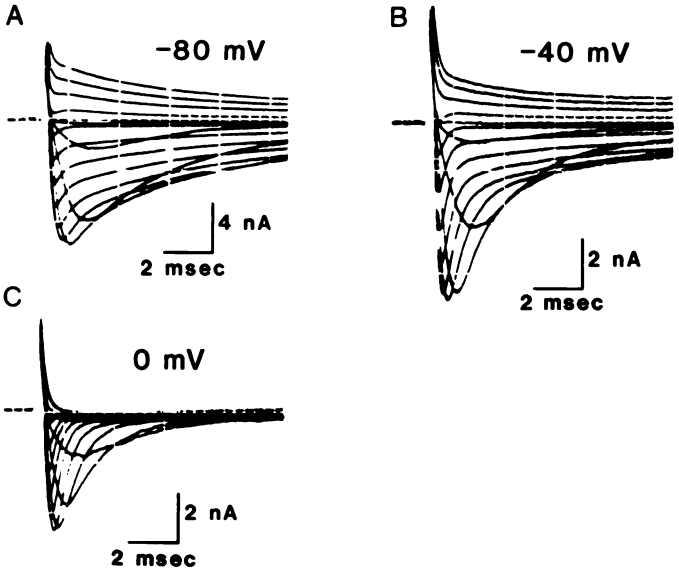

Fig. 4. Voltage dependence of CsTx action between −80 and 0 mV. A, N18 cells were incubated in the presence of 3×10^{-8} M CsTx at 37°C for 30 min. An individual cell at a holding potential of −80 mV was hyperpolarized to −120 mV for 200 msec, and a family of sodium currents was elicited by 10-msec test pulses to potentials of −50 to +80 mV in intervals of 10 mV. B, the holding potential was changed to −40 mV for 5 min, and a family of sodium currents was recorded as in A. C, the holding potential was then changed to 0 mV for 5 min, and a family of sodium currents was recorded as in A.

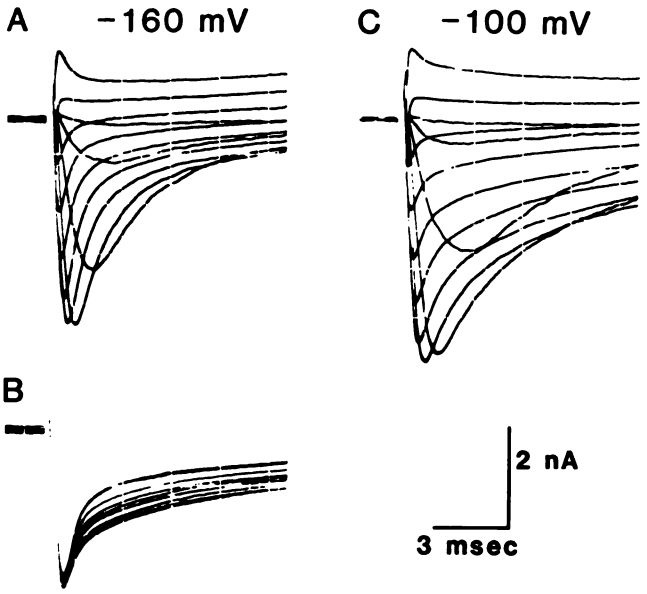

Fig. 5. Voltage dependence of CsTx action between −160 and −80 mV. A, N18 cells were incubated in the presence of 3×10^{-8} M CsTx at 37°C for 30 min. An individual cell at a holding potential of −80 mV was hyperpolarized to −160 mV for 10 min, and a family of sodium currents was elicited by 10-msec test pulses to potentials of −50 mV to +60 mV in intervals of 10 mV. B, the holding potential was changed to −100 mV; sodium currents were elicited every 30 sec by 10-msec test pulses to +10 mV, and the resulting traces were stored in a storage oscilloscope. Note the increase in slowly inactivated sodium current due to the depolarization. C, after 10 min at −100 mV, a family of sodium currents was elicited as in A except that the test pulse potentials ranged from −60 mV to +60 mV in intervals of 10 mV.

appeared less than in the corresponding experiment at −80 mV (compare Fig. 4A). Depolarization of the holding potential to −100 mV followed by measurement of the sodium current elicited by a single 10-msec test pulse to +10 mV revealed a progressive increase in the slowly inactivating fraction of the sodium current on depolarization in this membrane potential range (Fig. 5B). The new steady state time course of the sodium current was reached in 5 min. A family of sodium currents elicited at this time showed that, in comparison to the records at a holding potential of −160 mV, the inactivation of the sodium current was slowed at all test potentials, the peak sodium current was increased, and the sodium current was activated at more negative test potentials (Fig. 5C). Thus, the binding and action of CsTx is increased by depolarization of the membrane potential in the range of −160 to −80 mV.

Since unmodified sodium channels inactivate nearly completely by 3 msec after the beginning of a test pulse to +10 mV (see Fig. 2), the fraction of sodium conductance that remains at 3 msec provides an estimate of the fraction of sodium channels with slowed inactivation (23). The fraction of sodium conductance remaining at 3 msec is plotted as a function of the holding potential in Fig. 6A. The results at the nine holding potentials tested define a biphasic dependence of the binding and action of CsTx on membrane potential with maximum effect in the range of −100 to −60 mV. If we assume that the voltage dependence of the toxin effect is due to voltage dependence of the affinity for CsTx binding at a single receptor site on the sodium channel protein, as has been shown for α-scorpion toxins and sea anemone toxins (18, 23, 31–37), then apparent K_D values for toxin binding at each membrane poten-

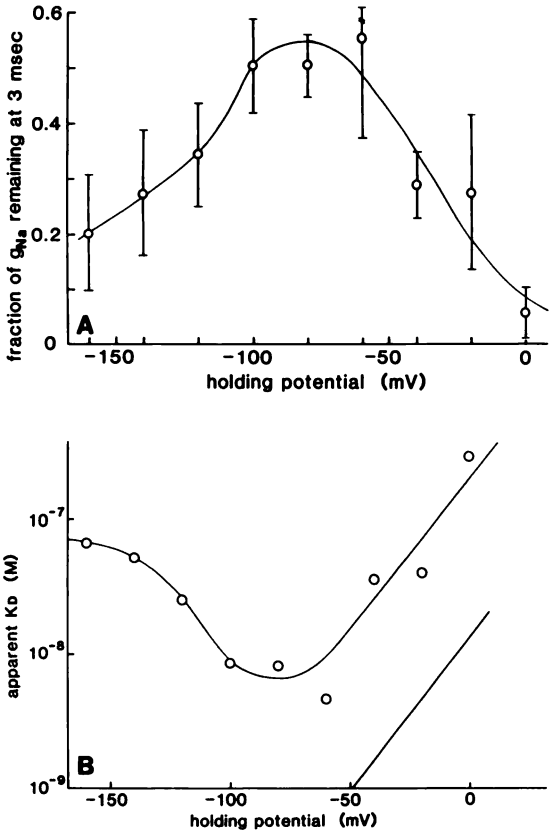

Fig. 6. Voltage dependence of the apparent K_D for CsTx. A, sodium currents were recorded in the presence of 3×10^{-8} M CsTx as described in the legends to Figs. 4 and 5. The fraction of sodium conductance (g_{Na}) remaining 3 msec after the peak was measured and plotted as mean ± S.D. for three to nine cells at each membrane potential. The smooth curve connecting the data points was drawn by eye. B, the results of A were converted to values of apparent K_D assuming one-to-one binding of CsTx to sodium channels according to the relationship $K_D = [\text{CsTx}]([\bar{F}g/Fg]-1)$, where Fg is the fraction of sodium conductance remaining 3 msec after the peak and $\bar{F}g$ is the fraction of sodium conductance remaining 3 msec after the peak in the presence of a saturating concentration of CsTx (1×10^{-7} M) at a holding potential of −80 mV (23, 29). The straight line illustrates the voltage dependence of binding LqTx derived from Refs. 18 and 23. The smooth curve is drawn by eye.

tial can be calculated from the data of Fig. 6A. As illustrated in Fig. 6B, a plot of the log of the apparent K_D versus membrane potential is also biphasic. At membrane potentials more positive than −60 mV, the apparent K_D increases e-fold for each 19-mV depolarization. The slope is similar to that observed for *Leiurus* scorpion toxin in the same cells (23) as indicated by the straight line in Fig. 6B. At membrane potentials more negative than −100, the apparent K_D is increased 10-fold. A similar increase in the apparent K_D for *Leiurus* toxin at negative membrane potentials was not observed in our previous experiments (23), although we cannot exclude the possibility that it may occur at even more negative membrane potentials than those tested.

Effect of external Na^+ on CsTx action. In previous work, we found that the inhibition of sodium channel inactivation by a polypeptide toxin from the coral *Goniopora* required Na^+ or another alkali metal cation in the extracellular medium. No effect of the toxin was observed in Na^+-free, choline-substituted medium (29). To examine the requirement for extracellular Na^+ for CsTx action, neuroblastoma cells were incubated for 30 min at 37° in sodium-free medium with or without 3×10^{-8} M toxin, and outward sodium currents were measured with micropipets containing 135 mM Na^+ as described under Experimental Procedures. Outward sodium currents were markedly prolonged by incubation with CsTx under these conditions (Fig. 7), indicating that extracellular Na^+ is not required for the action of CsTx on sodium channel inactivation.

Site of action of CsTx. Sodium channels have five receptor sites for neurotoxins that have been defined in previous neurotoxin binding studies (reviewed in Refs. 31, 38, and 39). We have examined the effects of CsTx on specific binding of neurotoxins at three of these sites as an initial step in determination of its site of action.

Neurotoxin receptor site 1 on the sodium channel binds the heterocyclic guanidines tetrodotoxin and saxitoxin that block the sodium conductance of the channel (31, 40). The polypeptides geographutoxin I and II, μ conotoxins from *C. geographus*, specifically block muscle sodium channels by interaction with this same receptor site (8–10). It was of interest therefore to determine whether CsTx also binds at this site. Specific binding of [^{3}H]saxitoxin to this receptor site on sodium channels in rat brain synaptosomes was measured as described under Experimental Procedures. No effect of CsTx on saxitoxin binding was observed at concentrations up to 1×10^{-7} M (Fig. 8), indicating that CsTx does not bind to neurotoxin receptor site 1 on sodium channels.

Neurotoxin receptor site 2 on the sodium channel binds lipid soluble neurotoxins, including batrachotoxin, that cause persistent activation of sodium channels (reviewed in Refs. 31 and 41). Binding and activation of sodium channels by these toxins is enhanced by polypeptide toxins that inhibit inactivation of sodium channels through an allosteric mechanism (31, 39, 42). Since CsTx inhibits inactivation of sodium channels, we expected that it would also enhance binding of neurotoxins to neurotoxin receptor site 2. Specific binding of 10 nM [^{3}H]BTX-B was measured as described under Experimental Procedures in a Na^+-free, choline-substituted medium. In the absence of other toxins, CsTx increased specific binding of [^{3}H]BTX-B by 50% with half-maximal effect at a concentration of approximately 1.5×10^{-8} M (Fig. 9). At the membrane potential of synaptosomes (−55 mV, Ref. 43), the apparent K_D for inhibition of sodium channel inactivation by CsTx is 1.1×10^{-8} M (Fig. 6B). Thus the enhancement of specific binding of [^{3}H]BTX-B

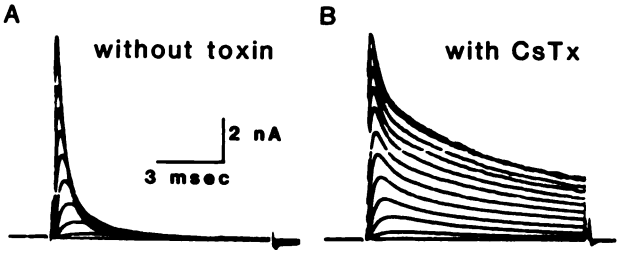

Fig. 7. Effect of CsTx in sodium-free medium. A, N18 cells were incubated in choline-substituted, sodium-free recording medium in the absence of CsTx at 37°C for 30 min. Cells were maintained at a holding potential of −80 mV, and families of outward sodium currents were recorded by hyperpolarizing to −120 mV for 100 msec followed by depolarizing to test potentials of −50 to +80 mV for 10 msec at intervals of 1 sec as described under Experimental Procedures. B, the experiment in panel A was repeated after incubation for 30 min at 37°C in the presence of 3×10^{-8} M CsTx. The outward sodium currents were completely blocked by addition of tetrodotoxin to a final concentration of 1×10^{-6} M.

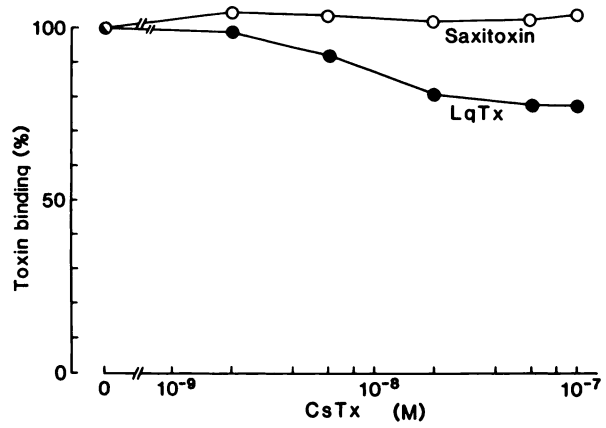

Fig. 8. Effect of CsTx on binding of saxitoxin and *Leiurus* scorpion toxin to sodium channels. Specific binding of [^{3}H]saxitoxin (○) and [^{125}I]LqTx (●) to sodium channels in rat brain synaptosomes was measured as described under Experimental Procedures in the presence of the indicated concentrations of CsTx.

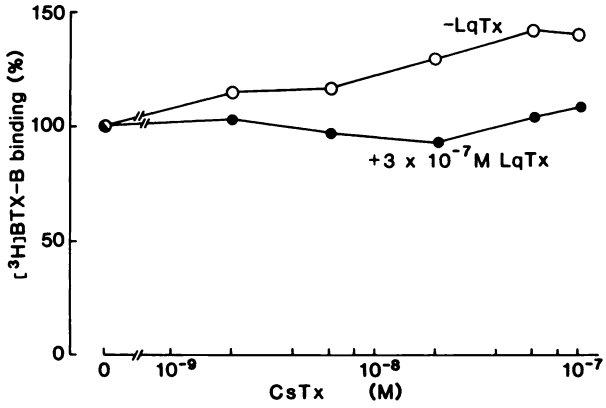

Fig. 9. Effect of CsTx on binding of [^{3}H]BTX-B to sodium channels. Specific binding of [^{3}H]BTX-B to sodium channels in rat brain synaptosomes was measured as described under Experimental Procedures without (○) or with (●) 3×10^{-7} M LqTx in the presence of the indicated concentrations of CsTx.

is observed in the same range of CsTx concentrations that inhibits inactivation of sodium channels. *Leiurus* α-scorpion toxin enhances specific binding of [^{3}H]BTX-B by as much as 10-fold (26). In the presence of 3×10^{-7} M *Leiurus* toxin, CsTx did not cause a detectable further enhancement of [^{3}H]BTX-B binding (Fig. 9). These results suggest a limited allosteric interaction between CsTx and neurotoxins binding at site 2 on the sodium channel.

Neurotoxin receptor site 3 on the sodium channel binds the polypeptides α-scorpion toxin and sea anemone toxin that inhibit sodium channel inactivation and enhance persistent activation of sodium channels by neurotoxins acting at receptor site 2 (reviewed in Ref. 31). To determine whether CsTx exerts similar effects on the sodium channel by interaction with neurotoxin receptor site 3 as well, we examined the effects of CsTx on specific binding of [^{125}I]-labeled *Leiurus* scorpion toxin as described under Experimental Procedures (Fig. 8). A saturating concentration of CsTx (1×10^{-7} M) reduced specific scorpion toxin binding by only 17%. These results show that CsTx does not occupy neurotoxin receptor site 3 in causing its effects on inactivation of sodium channels, although its binding may reduce the affinity of neurotoxin receptor site 3 for *Leiurus* scorpion toxin slightly. CsTx must affect sodium channel inactivation by interaction with a site other than neurotoxin receptor sites 1 through 3.

Discussion

Our results establish a fourth class of polypeptide neurotoxins that specifically inhibit inactivation of sodium channels. Early voltage clamp experiments showed that scorpion venoms, and basic polypeptide toxins of approximately 7,000 Da isolated from them, preferentially slow and block sodium channel inactivation (44, reviewed in Ref. 31). These toxins bind in a voltage-dependent manner to a single receptor site (receptor site 3) on the sodium channel (18, 31, 32) and enhance, through an allosteric mechanism, the activation of sodium channels by lipid soluble neurotoxins like batrachotoxin that act at neurotoxin receptor site 2. Basic polypeptides of 3,000–5,000 Da from sea anemone nematocysts also preferentially slow or block sodium channel inactivation (reviewed in Ref. 31) and bind to neurotoxin receptor site 3 in a voltage-dependent manner (34–37). In contrast to these two classes of toxins that exert their effects on sodium channel inactivation by binding at receptor site 3, recent results show that a polypeptide of 10,000 Da from the coral *Goniopora* inhibits sodium channel inactivation by voltage-dependent interaction with a different receptor site (29), and the results presented in this report demonstrate that a polypeptide of approximately 25,000 Da isolated from the venom of the marine snail *C. striatus* has a similar action. It is of interest to compare the physiological actions of these four structurally distinct classes of toxins.

The common denominator of the action of all four classes of toxins is their ability to slow sodium channel inactivation markedly without altering the time course of channel activation. This action is usually accompanied by three other effects: a reduction in the steepness of the voltage dependence of steady state inactivation for α-scorpion toxins, sea anemone toxins, and CsTx, but not for *Goniopora* toxin (23, 29, 33, 34, 36, 37, 44, 45); incomplete inactivation after long depolarizing pulses to positive membrane potentials for all four classes of toxin (23, 29, 33, 44, 45); and a shift in the voltage dependence of steady state inactivation to more negative membrane potentials for CsTx (this paper) or more positive membrane potentials for *Goniopora* toxin (29).

These toxins alter the voltage dependence of activation and increase the peak sodium current in N18 neuroblastoma cells (23, 29, 31). These effects are also evident when inactivation is blocked by treatment with proteolytic enzymes or chemical reagents (30). Thus, these effects should be considered secondary consequences of the primary effect of the toxins to slow inactivation. Inactivation of sodium channels in N18 cells is very rapid and is not strongly voltage-dependent (30). Inactivation therefore abbreviates the rise of the sodium current before maximum activation is achieved. The peak sodium current increases, and the voltage dependence of activation shifts to more negative membrane potentials when inactivation is slowed or blocked because the increase in sodium current after depolarization attains the full level allowed by the voltage dependence of sodium channel activation without attenuation by inactivation. Kinetic models that account for this behavior have been discussed previously by Gonoi and Hille (30).

Voltage-dependent binding and/or action is also a common feature of the mechanism of these four classes of toxins. However, the voltage dependence differs quantitatively among the

toxins that have been studied. The K_D for binding of *Leiurus* toxin increases *e*-fold for each 15- to 21-mV depolarization, and there is a linear relationship between the log of K_D and membrane potential over a wide range (18, 23, 32, 33). The voltage dependence of sea anemone toxin action is similar with an *e*-fold increase in apparent K_D for each 15-mV depolarization over the range that has been examined (37). *Goniopora* coral toxin has a shallower voltage dependence with an *e*-fold increase in apparent K_D for each 48-mV depolarization (29). However, the change in apparent K_D with hyperpolarization appears to level off at potentials more negative than −100 mV. The voltage dependence of CsTx action differs even more markedly from a log-linear relationship between apparent K_D and membrane potential (Fig. 6). Between −60 and 0 mV, K_D increases *e*-fold per 19-mV depolarization as for *Leiurus* scorpion toxin. However, at more negative membrane potentials, K_D decreases to a minimum value and then begins to increase again. What mechanisms might explain the widely differing voltage dependence of the binding and action of these toxins that all inhibit sodium channel inactivation? The voltage dependence of *Leiurus* toxin action results from preferential binding to activated states of sodium channels (18, 32). Current models of sodium channel gating indicate that the channel protein must undergo several voltage-dependent transitions connecting discrete nonconducting states before activation of the channel can occur (46, 47). Inactivation of the channel may occur from one or more of these nonconducting states as well as from the activated state of the channel. The different voltage dependence of binding and action of the toxins that inhibit inactivation may result from preferential binding of these different toxins to different states along the pathway toward the activated and inactivated states of the channel. Since the fraction of sodium channels in each of these states is expected to have different voltage dependence for each state, toxins that have preferential affinity for different channel states would be expected to have different voltage dependence of binding.

Sea anemone toxins and α-scorpion toxins share a common receptor site, neurotoxin receptor site 3, on the sodium channel protein (31, 34). In contrast, *Goniopora* toxin and CsTx act at one or more different receptor sites. The sites of *Goniopora* toxin action and CsTx action may be separate from one another since extracellular Na^+ is required for *Goniopora* toxin action but not for CsTx action, and the voltage dependence of toxin action at the two sites is distinctly different. Evidently, there are multiple sites on the extracellular surface of the sodium channel at which inactivation can be altered, and each of these receptor sites can undergo voltage dependent conformational changes during the transition from resting to activated sodium channels. Identification of these receptor sites at the molecular level may provide insight into the structural changes that occur during channel activation and inactivation.

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