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Mechanism of Action of a Polypeptide Neurotoxin from the Coral *Goniopora* on Sodium Channels in Mouse Neuroblastoma Cells

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SUMMARY

Goniopora toxin (GPT), a polypeptide toxin of 9700 Da isolated from coral, markedly slows inactivation of sodium currents recorded under voltage clamp in mouse neuroblastoma cells. The voltage dependence of sodium channel activation is shifted to more negative membrane potentials by 9.8 ± 2.1 mV, and the voltage dependence of channel inactivation is shifted to more positive membrane potential by 6.0 ± 2.5 mV. These actions of GPT are voltage dependent with an *e*-fold increase in $K_{0.5}$ for toxin action for each 48.3-mV depolarization between −80 and +40 mV. GPT requires Na^+ or another alkali metal cation in the extracellular medium for its effect on sodium channels. The relative effectiveness of the different cations tested is $Na^+ > K^+ > Rb^+ > Li^+ > Cs^+ \gg$ choline$^+$. Like other polypeptide neurotoxins that slow inactivation of sodium channels, GPT enhances persistent activation of sodium channels by veratridine. However, GPT does not block the binding of ^{125}I-labeled *Leiurus* scorpion toxin to neurotoxin receptor site 3 on sodium channels at concentrations which effectively slow channel inactivation. Therefore, our results define a new site on the sodium channel at which specific effects on inactivation can occur.

GPT is a polypeptide of 9700 Da isolated from the coral *Goniopora* spp. (1).[1] The toxin enhances neurally mediated contraction of blood vessels and taenia coli of the guinea pig (2) and has a positive inotropic action on the myocardium (3). The myocardial effects of GPT are due to a prolongation of the action potential caused by a sustained inward current which is reversed by low sodium medium, and by tetrodoxin, a specific inhibitor of voltage-sensitive sodium channels (4). These results suggest that GPT may alter sodium channel gating as part of its mechanism of action. Voltage clamp analysis of the effects of GPT on sodium currents in crayfish giant axons shows that inactivation of Na^+ channels is markedly slowed (5).

Mouse neuroblastoma cells have provided a valuable experimental preparation for analysis of neurotoxin action on voltage-sensitive sodium channels using both biochemical and biophysical methods. In this report, we describe the actions of GPT on sodium currents in neuroblastoma cells using the giga-ohm seal voltage clamp technique in the whole cell configuration (6, 7), and we examine the interaction of this toxin with previously defined neurotoxin receptor sites on the sodium channel using radioligand binding and ion flux methods (8–11).

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[1] K. Ashida, H. Toda, M. Fujiwara, and F. Sakiyama, unpublished experiments.

Experimental Procedures

Materials. GPT was purified as described previously (1) from *Goniopora* spp. obtained from the Sea of Japan. Lqtx was purified as described previously (8) from venom purchased from Sigma Chemical Co. Other materials were obtained from the following sources: TTX, Calbiochem; fetal calf serum, Gibco; Dulbecco-Vogt modified Eagle's minimum essential medium, Grand Island Biological Co.

Cell culture. Mouse neuroblastoma cells (clone N18) were cultured in 100-mm-diameter Petri dishes (Falcon) for 5–8 days before recordings until cells reached 80% of confluence. Details of the culture techniques were described previously (7–9). Culture medium consisted of 5% fetal calf serum and 95% Dulbecco-Vogt modified Eagle's minimum essential medium. Before recordings, cells were harvested by trituration in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline and then reseeded in 35-mm-diameter plastic dishes (Falcon) containing 1 ml of the culture medium at a cell density of 2×10^5 cells/ml. The recordings were performed with cells attached to the bottom of the dishes 2–6 hr after reseeding. The reseeding procedure enabled us to record from isolated round cells as required for accurate voltage clamp measurements. Cell diameters were 18–25 μm.

Voltage clamp recording. The recording medium consisted of 150 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM glucose, 5mM Hepes or an identical solution with NaCl replaced by equimolar choline chloride, CsCl, RbCl, LiCl, or KCl, leaving the other compo-

ABBREVIATIONS: GPT, *Goniopora* toxin; Lqtx, the α-scorpion toxin from *Leiurus quinquestriatus*; TTX, tetrodotoxin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′N′*-tetraacetic acid.

nents unchanged. The pH of the medium was adjusted to 7.4 with NaOH solution for normal recording medium and by KOH for Na^+-free solutions. For recordings in the presence of a toxin, GPT or Lqtx was dissolved in recording medium, and cells were incubated in the recording medium at 37° for 30 min before starting recordings, unless otherwise noted. The plastic dish was mounted on a stage of an inverted phase-contrast microscope. Determination of voltage dependence of the toxin action was performed at room temperature (20° ± 1°). The other experiments were performed at 15° by cooling the dish with a water cooler.

The voltage clamp was based on the one-pipette giga-ohm seal whole cell recording technique of Hamill *et al.* (6). Glass microtubes (Curtin Matheson Scientific Inc., 1.5 mm o.d.) were pulled in two steps, and tips of the pipettes were fire-polished. The tip resistances were between 200 and 400 kohms in recording medium. Internal pipette medium was 90 mM CsF, 60 mM CsCl, and 10 mM NaF for measurement of inward current, or 140 mM NaF and 20 mM CsCl for measurement of outward sodium current. The EPC-7 patch clamp system (Medical Systems Corp.) was used for voltage clamp recordings. The voltage difference between the two Ag-AgCl electrodes was canceled by an internal compensatory circuit. Linear leakage and capacitive currents were subtracted both with an analog subtractor and by computer. The series resistance of the pipette was compensated by an internal feedback circuit. Currents were sampled at 25-μsec intervals for 1.25 msec during the rising phase of the sodium current or at 125-μsec intervals for 7.5 msec during the falling phase of the sodium current and stored on a magnetic tape through an LM2 computer. Details of the recordings from the neuroblastoma cells were described previously (7).

The cells were maintained at a holding potential of −80 mV and were subjected to the voltage steps described in the figure legends. Sodium conductance was calculated by the relation:

$$g_{Na} = I_{Na}/(E - E_{Na})$$

where E is membrane potential and E_{Na} is the reversal potential for current in sodium channels as measured in each experiment.

Scorpion toxin binding. The Lqtx was purified and radiolabeled with ^{125}I as previously described (8). Specific binding of ^{125}I-Lqtx to sodium channels in intact neuroblastoma cells (8) or synaptosomes (10, 11) was measured by established procedures. Nonspecific binding was determined in the presence of 200 nM Lqtx and subtracted from the results.

$^{22}Na^+$ influx. $^{22}Na^+$ influx into intact N18 cells mediated by veratridine-activated sodium channels was measured as previously described. (9). Influx in the absence of veratridine and other neurotoxins was subtracted from the results.

Results

Modification of sodium channels by GPT. Fig. 1A shows a family of sodium currents in an N18 neuroblastoma cell before applying GPT. The sodium channels are activated within 1 msec and are inactivated before the end of the 8.75-msec test pulse period. This recording was obtained 50 min after making a seal between the cell membrane and the micropipette. At this time, the exchange of ions between the cell membrane and the micropipette was complete and the amplitude of the sodium currents and the sodium equilibrium potential were constant. Then, 50 μl of 10 μM GPT were added to the bathing medium approximately 5 mm from the cell. Fig. 1B shows a family of sodium currents from the same cell 3 min after adding GPT to the dish. The decay of the sodium currents was markedly slowed, so that the currents were no longer inactivated fully at the end of the test pulse period. The maximum sodium currents elicited by all test pulse potentials examined were increased after the addition of the toxin. The increases in current amplitude are shown more clearly in Fig. 1, C and D, in which the sodium currents elicited by the same test pulse before and after the addition of the toxin are superimposed. The peak currents increased 2.7- and 1.3-fold at test pulse potentials of −20 mV and 0 mV, respectively. The mean increase in three cells tested was 2.31 ± 0.34 (SD) at −20 mV and 1.39 ± 0.15-fold at 0 mV. Reversal potentials of the currents calculated from the above measurements were unaffected by GPT (+72.3 mV versus +71.5 mV). The decay of the sodium currents in the absence of the toxin was accurately described by a single exponential with a decay constant of 0.76 msec. In contrast, the sodium current decays more slowly and follows a multiphasic time course in the presence of 500 nM GPT. The time constant for the first phase of current decay was about 10 msec at +10 mV. Thereafter, the current decayed progressively more slowly, with 13% of the peak sodium current remaining at the end of the 52-msec test pulse period.

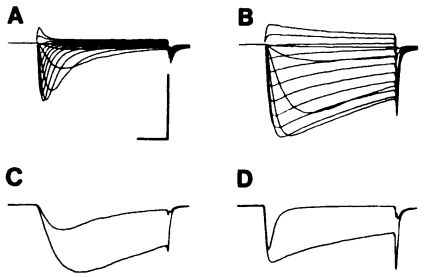

Fig. 1. GPT prolongs sodium currents in N18 cells. An N18 cell was maintained at a holding potential of −80 mV, hyperpolarized once per second to −120 mV for 100 msec, and then depolarized to potentials from −40 mV to +90 mV in 10-mV intervals to elicit the sodium currents. A. Recordings of sodium currents 50 min after a seal was made between the cell and a recording micropipette and 2 min before applying the toxin. B. Three min after adding 50 μl of 10 μM GPT at a distance of 5 mm from the cell. The 14 traces were superimposed by computer. C. Sodium currents elicited by test pulses to −20 mV before (C) and after application of the toxin (GPT) are superimposed from panels A and B. D. Sodium currents elicited by test pulses to 0 mV before and after application of the toxin are superimposed from panels A and B. Calibration: 10 namp, 2 msec for each panel.

The voltage dependence of sodium channel inactivation during 100-msec prepulses is illustrated in Fig. 2. In the absence of GPT (Fig. 2, *open circles*), half-maximal inactivation is observed at 62.7 mV. A saturating concentration of GPT (500 nM) shifts the inactivation curve by 6.0 ± 2.5 mV (n = 3) in the positive direction with little decrease in the steepness of the curve (Fig. 2, *solid circles*). In addition, GPT causes inactivation to be incomplete. Even after 100 msec at −20 mV, more than 5% of the sodium current remains (Fig. 2, *solid circles*).

The voltage dependence of activation of sodium channels is also altered by GPT. The toxin increases sodium current more at small depolarizations than at large depolarizations (Fig. 1, C and D). Therefore, when normalized conductance-voltage curves for activation are plotted, GPT (500 nM) shifts the potential for half-maximal activation from −10.7 mV to −19.9 mV (Fig. 2, *solid* and *open squares*). The mean of the negative voltage shift in three cells examined was −9.8 ± 2.1 mV. A similar effect is observed with other toxins and treatments that slow or block inactivation of sodium channels in N18 cells (7).

Voltage dependence of GPT action. Polypeptide neurotoxins from the scorpions *Leiurus quinquestriatus* or *Buthus eupeus* and the sea anemones *Homostichanthus duerdemi* or *Anemonia sulcata* slow the inactivation process of sodium chan-

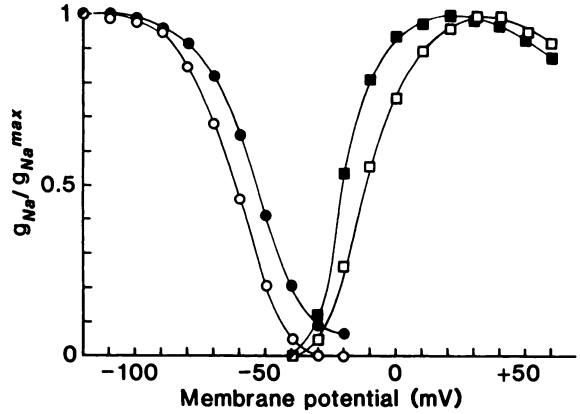

Fig. 2. Effect of GPT on the voltage dependence of activation and inactivation of sodium channels. For activation, N18 cells were maintained at a holding potential of −80 mV, hyperpolarized to −120 mV for 100 msec to remove inactivation of sodium channels, and depolarized to test pulse potentials from −40 to +60 mV as indicated on the *abscissa* for 8.75 msec. The resulting inward sodium currents were recorded and the peak sodium conductance at each potential was calculated as described under Experimental Procedures. The conductance was normalized to the maximum value observed for each cell and plotted versus membrane potential for measurements in the absence of GPT (□) or in the presence of 500 nM GPT (■). For inactivation, N18 cells were maintained at a holding potential of −80 mV, stimulated with a prepulse from −140 to −20 mV for 100 msec as indicated on the *abscissa*, and then depolarized with a test pulse to +10 mV for 8.75 msec to elicit sodium currents. The resulting peak sodium currents were measured as described under Experimental Procedures, and the ratio of the recorded current to that for a prepulse to −140 mV was plotted versus membrane potential for measurements in the absence of GPT (○) or in the presence of 500 nM GPT (●).

nels and bind to sodium channels in a voltage-dependent manner (7, 8, 12–16). The effect of holding potential on the amplitude and the time course of the sodium current in the presence of 60 nM GPT is illustrated in the *inset* to Fig. 3A. At a holding potential of +40 mV, 60 nM GPT slowed inactivation of only a small portion of the sodium current (Fig. 3A, *top trace*). After repolarization, sodium currents increase in magnitude, due to both reversal of slow inactivation processes and onset of the effect of GPT, and reach a new steady state amplitude and time course within 3 min. The effect of GPT at the two different membrane potentials can be assessed from the time course of the sodium current. At −120 mV, a large fraction of the sodium current inactivates slowly in contrast to +40 mV, where most sodium channels inactivate normally. Thus, the fraction of sodium channels with a rate of inactivation that is slowed by GPT is greater at −120 mV than at + 40 mV, consistent with voltage-dependent binding and action of the toxin.

The effect of GPT on the sodium conductance at each holding potential was quantitated as the ratio of the sodium conductance remaining 3 msec after the peak of the sodium conductance (7). The sodium conductance remaining after 3 msec is practically zero in the absence of the toxin (see Fig. 1) and is increased in proportion to the number of channels modified by the toxin. Fig. 3 shows that the effect of 30 or 60 nM GPT becomes progressively smaller when the membrane potential is depolarized between −80 and +40 mV. At potentials more negative than −80 mV, the effect of GPT is approximately constant. Fig. 3B illustrates apparent K_D values of the toxin calculated from the following relation derived by assuming one-to-one binding of the toxin to the sodium channels.

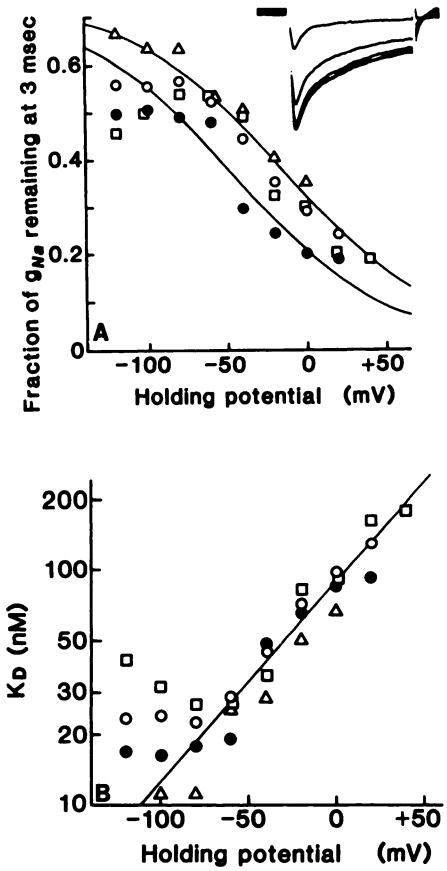

Fig. 3. Voltage dependence of GPT effect on sodium currents of N18 neuroblastoma cells. A. *Inset*, An N18 cell was incubated with 60 nM GPT for 30 min at 37°. After a seal was made, the membrane potential was maintained at +40 mV for 3 min. The cell was then repolarized to −120 mV, and sodium currents elicited by a test pulse to +10 mV for 10 msec were measured 100 msec after repolarization and each 2 sec thereafter. Measured sodium currents increased in each succeeding pulse. *Main panel*, Three N18 cells (□, ○, △) were incubated with 60 nM GPT and a fourth (●) was incubated with 30 nM GPT. The cell was maintained at each holding potential for at least 5 min to allow GPT binding to reach equilibrium at the new membrane potential. Sodium currents were then measured by hyperpolarizing to −120 mV for 100 msec (□, ○, △) or 300 msec (●) to remove inactivation, and then recording the current elicited by depolarization to +10 mV for 10 msec. This procedure was repeated at holding potentials from +40 mV to −120 mV in 20-mV steps for each cell. The fraction of sodium current remaining 3 msec after the peak is plotted as an index of slowing of inactivation by GPT. B. Apparent K_D values were calculated from the results of panel A and the relationship given in the text and plotted as a function of holding potential. The regression line corresponds to an *e*-fold increase in apparent K_D for each 48.3-mV depolarization.

$$K_D = [\text{GPT}]\,(\bar{F}_G/F_G - 1)$$

Here F_G and $\bar{F}_G$ are the fractions of sodium conductance remaining 3 msec after the peak, and the maximum fraction at saturating GPT, respectively. $\bar{F}_G$ is 0.75 ± 0.05 ($n = 3$) following an experiment in the presence of 500 nM GPT, a saturating concentration (see Fig. 1). A linear relationship was obtained between the holding potential and logarithm of the K_D from −80 to +40 mV. Between these potentials the apparent K_D increases *e*-fold for each 48.3 mV of depolarization. The apparent K_D of GPT was also estimated from other cells in the presence of 1 nM ($n = 3$) and 10 nM ($n = 4$) toxin. The holding potential was kept at −80 mV for 10 min before recordings to obtain a steady state at this potential. The K_D calculated as

described above is 11.5 ± 6.9 nM and is similar to the K_D value of 15.9 nM estimated from Fig. 4 at −80 mV.

Dependence of the GPT effect on Na^+ in the external medium. We examined the effect of ionic composition of the bathing medium on GPT action since preliminary biochemical experiments on GPT action did not detect toxin effects in medium which contained 120 mM choline and only 10 mM Na^+. Fig. 4, A and B, shows families of outward sodium currents from two different cells in choline recording medium (see Experimental Procedures) in the absence and presence of 200 nM GPT, respectively. In these experiments, the micropipette contained 140 mM NaF and 20 mM CsCl to allow measurement of outward currents in sodium-free external medium. The holding potential was maintained at −80 mV and, following a prepulse to −120 mV for 100 msec, sodium currents were stimulated by test pulses from −40 mV to +60 mV in intervals of 10 mV. The presence of GPT did not slow the inactivation process of sodium channels activated by each test pulse in sodium-free external medium (Fig. 4, compare panels A and B). In two experiments, the time constants of the decay of the sodium current during a test pulse to +10 mV in choline medium and in normal medium were 0.55 msec and 0.64 msec, respectively. Fig. 4C shows, for comparison, a family of outward sodium currents recorded in choline medium containing 200 nM Lqtx. The inactivation of the sodium channels was markedly slowed. In two experiments, the slowing of the decay of sodium current by 200 nM Lqtx in the choline medium was similar to that in normal medium. The mean decay time constants were 6.94 and 7.02 msec for a test pulse to +10 mV in normal and choline medium, respectively. The modified sodium currents with *Leiurus* toxin were completely blocked after addition of TTX (Fig. 4D). Thus, the effect of GPT on inactivation is lost in Na^+-free medium, whereas the effect of Lqtx is retained. This is due to the reduction of extracellular Na^+ rather than the change in direction of current flow since both inward and outward currents in normal recording medium are prolonged by GPT (Fig. 1).

The dependence of the effect of GPT on external sodium ion concentration was determined in the experiments illustrated in Fig. 5. Prior to recordings, cells were incubated in choline medium containing 200 nM GPT at 37° for 30 min as described under Experimental Procedures. Soon after making a seal between the cell membrane and a micropipette, the amplitude of the outward sodium currents increased as Na^+ from the pipette diffused into the cell. After 20 min, the current amplitude became constant. Fig. 5A shows a family of currents in choline medium recorded 30 min after making a seal. There was little prolongation of the inactivation process of the sodium channels in the absence of external Na^+. The concentration of Na^+ in the bathing medium was increased stepwise every 6 min to a final level of 75 mM by adding normal recording medium containing 200 nM GPT. Fig. 5, B and C, shows example recordings in the presence of 12.4 and 68.5 mM Na^+, respectively. Finally, the Na^+ concentration was raised above 75 mM by addition of 2.5 M NaCl to the bath 2 cm from the cell. Fig. 5, D and E, shows recordings after the extracellular Na^+ was raised to final concentrations of 124.5 and 197.0 mM, respectively. It is evident that the inactivation of the sodium channels was markedly slowed by increasing the concentration of extracellular Na^+. The increase of the inward currents and change of the reversal potential of the currents result from increased inward movement of Na^+ from the external medium.

Fig. 6 illustrates the relationship between Na^+ concentration in the medium and the fraction of sodium conductance remaining 3 msec after the peak of sodium current during a test pulse to +40 mV, as an indicator of the toxin effect on sodium channel inactivation. A linear relationship is observed between 0 and

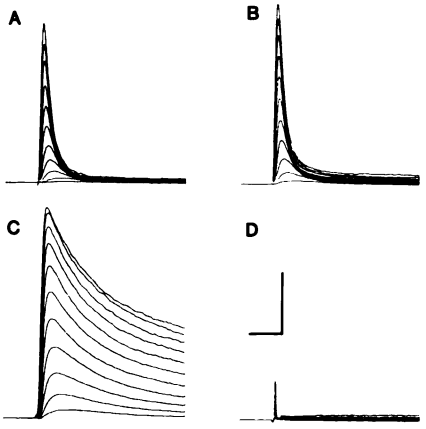

Fig. 4. Substitution of choline for sodium in the extracellular medium prevents GPT action. A. A seal was made on an N18 cell using a pipette filled with 140 mM NaF, 20 mM CsF in choline medium. The cell was maintained at a holding potential of −80 mV, hyperpolarized once per second to −120 mV for 100 msec, and depolarized by test pulses to potentials from −40 mV to +60 mV for 8.75 msec in intervals of 10 mV. Outward sodium currents were recorded as described under Experimental Procedures. B. The cell was incubated with 200 nM GPT for 30 min at 37° and stimulated identically. C. Another N18 cell was incubated for 30 min at 37° with 200 nM *Leiurus* scorpion toxin and outward currents were elicited by an identical stimulus regimen. D. The cell in C was incubated for 5 min with 1 μM TTX and stimulated identically. Calibration: A and B, 2 namp, 2 msec; C and D, 5 nA, 2 msec.

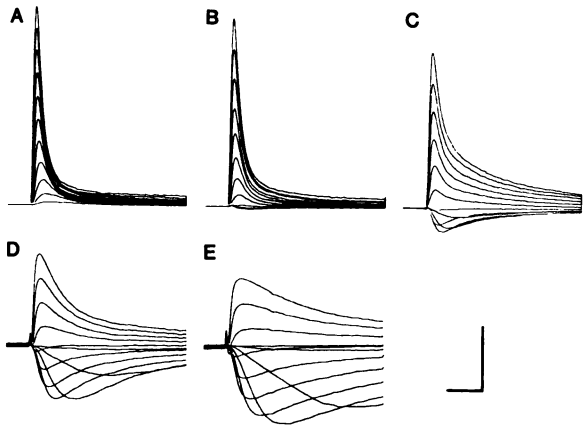

Fig. 5. Determination of the dependence of the GPT effect on sodium ion concentration in the external medium. N18 cells were incubated in choline medium containing 200 nM GPT at 37° for 30 min. After a seal was made between the cell and a micropipette, the holding potential of the cell was maintained at −80 mV. For recordings of the sodium currents, the cell was stimulated once per second by a prepulse to −120 mV for 100 msec followed by the test pulse to potentials from −40 to +60 mV in intervals of 10 mV for 8.75 msec. Thirty min after the seal was made, the sodium ion concentration of the external medium was raised step by step every 6 min up to 197 mM by adding normal recording medium containing 150 mM Na^+ and 200 nM GPT to increase $[Na^+]_{out}$ to 75 mM, or 2.5 M NaCl to increase it beyond 75 mM. A. A family of sodium currents in the absence of sodium ions, recorded 28 min after the seal was made. B. At 34 min after the seal in 12.4 mM Na^+. C. At 46 min after the seal in 68.5 mM Na^+. D. At 71 min after the seal in 124.5 mM Na^+. E. AT 77 min after the seal in 197 mM Na^+. Calibration: 2 namp, 5 msec.

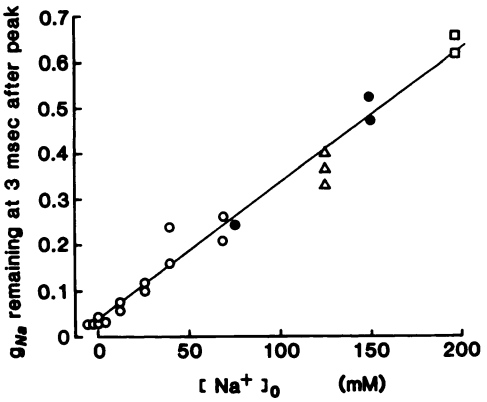

Fig. 6. Concentration dependence of the effect of Na^+. The ratio of sodium conductance remaining at 3 msec after the peak conductance during +40 mV test pulse is plotted versus sodium ion concentration in the external medium from the experiment described in Fig. 5 (*open symbols*). Similar experiments were carried out on three additional cells in Na^+ medium with 150 mM Na^+ or 75 mM Na^+ and 75 mM choline (●). Final Na^+ concentrations of 124.5 mM (△) and 197 mM (□) were achieved by adding 2.5 M NaCl to the recording medium. The resulting increase in Cl^- concentration alters the electrode potential of the $Ag^+/AgCl$ recording electrode resulting in a shift of the g_{Na} versus V curve in control cells of +10.4 mV at 124.5 mM NaCl and +19.2 mV at 197 mM NaCl. These shifts were compensated by plotting currents for test pulses to +50 mV at 124.5 mM NaCl (△) and +60 mV at 197 mM NaCl (□). This correction altered the measured values less than 5%.

197 mM Na^+ for cells examined as illustrated in Fig. 6 (*open symbols*). In addition, values for cells examined in normal recording medium containing 150 mM Na^+ or in choline medium with 75 mM Na^+ also fall near the same line (Fig. 6, *solid circles*). Thus, Na^+ acts at one or more low affinity binding sites which are not saturated within the physiological range of Na^+ concentration for vertebrates but may saturate at the higher concentration in sea water, the normal environment for coral toxin action.

Effect of other cations on GPT action. Li^+, K^+, Rb^+, and Cs^+ were tested to determine whether they would substitute for Na^+ in sustaining the action of GPT on sodium channels. For Na^+ and for Li^+, which are transported well by the sodium channel, the internal pipette medium was 90 mM CsF, 60 mM CsCl, and 10 mM NaCl, and inward currents were measured. For K^+, Rb^+, and Cs^+, which are only slightly permeant, the internal pipette medium was 140 mM NaF, 20 mM CsCl, and outward currents were measured. In the absence of GPT, the kinetics of the outward and inward currents flowing through the sodium channels and blocked by TTX in the presence of these cations were similar to those in normal medium or choline medium. Prior to the recordings in the presence of toxin, the cell was incubated in the normal or cation-substituted recording medium containing 200 nM GPT at 37° for 30 min. In the K^+ medium, GPT slowed the inactivation of the outward sodium currents markedly (Fig. 7D), and TTX blocked those currents completely, leaving only capacitive currents. The effect of GPT was significantly smaller in the Rb^+ medium than in K^+ medium (Fig. 7E), but the inactivation of a small portion of the sodium channels was slowed and small sodium currents remained until the end of the test pulse period. Those outward currents were blocked by TTX. GPT also slowed inactivation noticeably in Cs^+ medium and Li^+ medium (Fig. 7, B and F). Leak currents were always observed during test pulses to positive membrane potentials in Li^+ medium containing GPT.

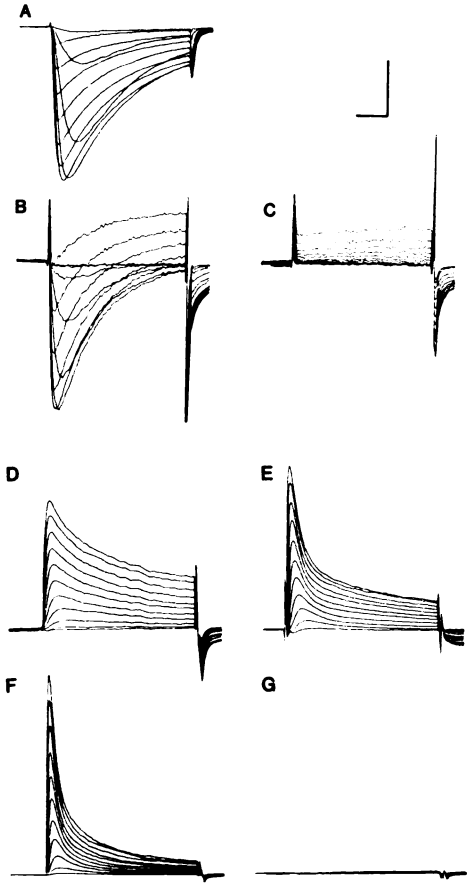

Fig. 7. Effects of other cations on GPT action. A. An N18 cell was incubated for 30 min at 37° in normal recording medium containing 200 nM GPT. A seal was made and inward sodium currents were elicited once per second with a prepulse to −120 mV for 100 msec and test pulses to potentials from −40 mV to +60 mV for 8.75 msec in intervals of 10 mV. Similar experiments were carried out in the presence of other cations: (B) Li^+ medium, (C) Li^+ medium plus 1 μM TTX, (D) outward currents in K^+ medium, (E) outward currents in Rb^+ medium, (F) outward currents in Cs^+ medium, and (G) outward currents in Cs^+ medium plus 1 μM TTX. Calibration: A, 5 namp, 2 msec; B and C, 1 namp, 2 msec; D–G, 5 namp, 2 msec.

These currents were not blocked by 1×10^{-6} M TTX (Fig. 7C) in contrast to the complete block in the presence of Cs^+ (Fig. 7G) or other ions. The nature of the leak current was not studied further.

The effectiveness of these cations on the action of GPT on sodium channels was compared by estimating the sodium channel conductance remaining at 3 msec after the peak conductance during the test pulse to +10 mV. The middle column in Table 1 shows the ratio of the remaining conductance in the presence of 200 nM GPT and the values are normalized to those in normal medium in the right column. Sodium ion has the strongest effect on sustaining the actions of GPT on sodium channels, and the order of the effectiveness of the other five cations tested was $Na^+ > K^+ > Rb^+ > Li^+ > Cs^+ \gg$ choline$^+$.

Elimination of Ca^{2+} ions from normal medium or addition of 5 mM EGTA to Ca^{2+}-depleted medium did not abolish the effect of GPT on sodium channels when tested after 30-min incubation in the presence of 200 nM GPT (data not shown).

Site of action of GPT. The effect of GPT to slow sodium channel inactivation without markedly altering channel activation resembles the actions of α-scorpion toxins and sea

TABLE 1

Comparison of the effects of different ions on GPT action

The ratio of the sodium conductance remaining at 3 msec after the peak to the peak conductance was measured in experiments like the one in Fig. 7 for 2–4 cells for each cation. Mean ratios and standard deviation are given along with values normalized to those in Na^+ medium.

Cation	$\frac{g_{Na}\ (t = 3\ msec)}{g_{Na}\ (peak)}$	Normalized values
Na^+	0.60 ± 0.07	1.0
K^+	0.51 ± 0.17	0.84
Rb^+	0.40 ± 0.02	0.67
Li^+	0.35 ± 0.04	0.58
Cs^+	0.16 ± 0.02	0.26
Choline	0.02 ± 0.02	0.04

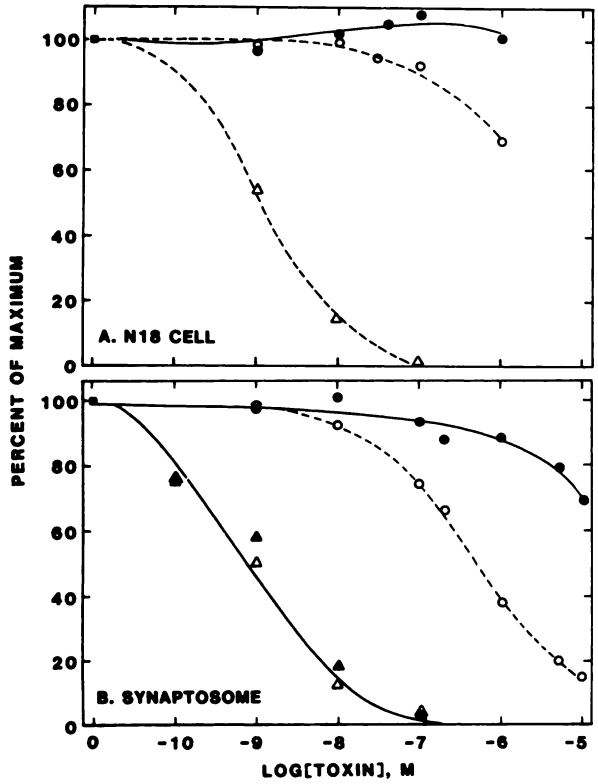

Fig. 8. Inhibition of scorpion toxin binding by GPT. A. N18 cells in 16-mm multiwell dishes were incubated for 60 min at 37° with 1 nM ^{125}I-Lqtx and the indicated concentrations of Lqtx (△) or GPT (○, ●) in Na^+ recording medium (*open symbols*) or choline recording medium (*solid symbols*). The cells were washed three times for 1 min each with either choline or Na^+ recording medium, respectively, and dissolved, and bound ^{125}I-Lqtx was determined by gamma counting. B. Synaptosomes (75 μg of synaptosomal protein) suspended in 20 μl of 0.32 M sucrose, 5 mM Hepes/Tris, pH 7.4, were diluted to 200 μl in Na^+ recording medium (*open symbols*) or choline recording medium (*solid symbols*) containing 1 μM TTX and Lqtx (△, ▲) or GPT (○, ●) to give the final toxin concentrations indicated on the *abscissa*. The samples were incubated for 15 min at 36°, the binding reaction was terminated by dilution into 3 ml of choline wash medium (9), and the synaptosomes were trapped on glass fiber filters (Whatman GFC). Bound ^{125}I-Lqtx was measured by gamma counting.

anemone toxins which act at neurotoxin receptor site 3 on the sodium channel. In order to determine whether GPT acts at this same receptor site, we examined its effect on specific binding of ^{125}I-labeled scorpion toxin from *L. quinquestriatus* to sodium channels in N18 cells. Specific binding of ^{125}I-Lqtx was competitively blocked by unlabeled Lqtx with half-maximal effect at 1–2 nM (Fig. 8A), as expected from previous work (K_D = 1.7 nM; Refs. 8 and 10). In choline medium, no effects of GPT on specific binding of ^{125}I-Lqtx were observed at concentrations ranging from 10 nM to 1 μM (Fig. 8A). In sodium medium, a small inhibition of binding was observed at concentrations above 100 nM. However, this may have resulted from depolarization of the cells since it was reduced 60% by 1 mM TTX (data not shown). At the resting membrane potential of N18 cells (−41 mV), the apparent K_D for GPT action on sodium channels is approximately 36 nM (Fig. 3B). Therefore, the failure of GPT to competitively block binding of ^{125}I-Lqtx at much higher concentrations indicates that its physiological effects on sodium channel inactivation are not mediated by binding at neurotoxin receptor site 3.

We also examined the effect of GPT on ^{125}I-Lqtx binding in synaptosomes (Fig. 8B). In this experimental preparation, GPT caused more marked inhibition of ^{125}I-Lqtx binding. Inhibition in Na^+ medium (Fig. 8B, *open circles*) was greater than in choline medium (Fig. 8B, *solid circles*). As for N18 cells, the inhibition in Na^+ medium was partially reversed by TTX, suggesting that it may result in part from depolarization. Tenfold higher concentrations of GPT were required to block ^{125}I-Lqtx binding than to slow inactivation of sodium channels. Thus, these results with synaptosomes also support the conclusion that GPT action to slow sodium channel inactivation does not require interaction at neurotoxin receptor site 3.

Enhancement of neurotoxin activation of sodium channels. Sea anemone toxins and α-scorpion toxins enhance the persistent activation of sodium channels caused by veratridine, batrachotoxin, and other neurotoxins acting at receptor site 2 on the sodium channel through an allosteric mechanism (9). Since GPT slows sodium channel inactivation in the same manner as α-scorpion and sea anemone toxins, we have examined whether it would also enhance veratridine-stimulated $^{22}Na^+$ influx into N18 cells. Fig. 9 shows that GPT does increase the initial rate of $^{22}Na^+$ influx in the presence of a saturating concentration of veratridine (200 μM). However, it is much less effective than Lqtx, causing only a 1.9-fold enhancement at 1

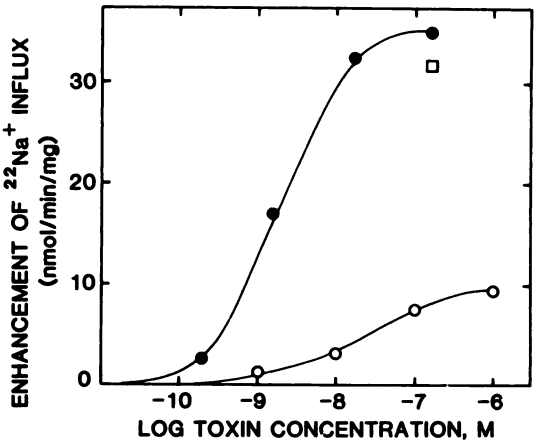

Fig. 9. Enhancement of veratridine activation of sodium channels. N18 cells in 16-mm multiwell dishes were incubated for 30 min at 37° with the indicated concentrations of Lqtx (●), GPT (○), or 1 μM GPT in the presence of Lqtx (□) in Na^+ recording medium. The incubation medium was removed by aspiration and the initial rate of $^{22}Na^+$ influx was measured in the presence of 200 μM veratridine and 5 mM ouabain in assay medium containing 10 mM NaCl and $^{22}Na^+$ (9). The cells were washed with choline wash medium (9) and dissolved, and $^{22}Na^+$ was measured by liquid scintillation counting. The increase in $^{22}Na^+$ influx over that in the presence of veratridine alone is plotted on the *ordinate*.

μM. In agreement with the neurotoxin binding results, addition of 1 μM GPT to samples treated with 200 nM Lqtx (Fig. 9, *open squares*) does not reduce the rate of $^{22}Na^+$ influx appreciably, indicating that it does not compete with Lqtx for binding at neurotoxin receptor site 3.

Discussion

Two classes of polypeptide neurotoxins that slow sodium channel inactivation have been described previously: the α-scorpion toxins and the sea anemone toxins (reviewed in Ref. 17). Although these two families of toxins do not share detectable amino acid sequence homology, they both bind in a voltage-dependent manner to neurotoxin receptor site 3 on the sodium channel and slow channel inactivation. The amino acid sequence of GPT has no homology with the sequence of scorpion or sea anemone toxins.[1] Although GPT also slows sodium channel inactivation, several features of its action distinguish it from the scorpion and sea anemone toxins that have been studied, as outlined in the sections below.

Voltage dependence of activation and inactivation. In N18 neuroblastoma cells, many agents which slow inactivation cause increased activation of sodium currents in response to small depolarizing stimuli and thereby shift conductance-voltage relationships to the left (18). *Leiurus* scorpion toxin and GPT share this action (18). In contrast, the voltage dependence of sodium channel inactivation is altered in different ways by these toxins. Sea anemone toxins and α-scorpion toxins, which act at neurotoxin receptor site 3, reduce the steepness of the steady state inactivation curve without shifting its midpoint appreciably (13, 15, 19, 20). In contrast, we find that GPT shifts the steady state inactivation curve of sodium channels to more positive membrane potentials without altering its shape. All three toxins cause incomplete inactivation, even after long pulses at depolarized membrane potential. Thus, although many aspects of the action of these toxins are similar, their effects on the voltage dependence of inactivation are distinct.

Voltage dependence of toxin action. Depolarization increases the K_D value for binding of α-scorpion toxins (7, 8, 10–13), sea anemone toxins (14–16), and GPT (this report). However, there are quantitative differences in the dependence of K_D on membrane potential. The K_D for α-scorpion toxins is increased *e*-fold for each 15–21-mV depolarization in a variety of experimental preparations between −90 mV and 0 mV (7, 8, 10–13). The dependence of sea anemone toxin action on membrane potential appears to occur at similar membrane potentials (14–16), but a quantitative comparison over a range of voltages has been reported in only one study (15). In this case, the K_D changed *e*-fold per 21 mV at the midpoint of the voltage curve. In contrast, the voltage dependence of GPT action is less steep, with an *e*-fold increase in K_D for each 48.3-mV depolarization, and extends at least over the range of −80 mV to +40 mV. These results suggest that GPT interacts preferentially with different voltage-sensitive states or alters different voltage-sensitive transitions of the channel as compared to scorpion and sea anemone toxins.

Cation dependence of toxin action. The most striking difference between GPT action and that of scorpion and sea anemone toxins is its strong dependence on the nature of the monovalent cation in the extracellular medium. In previous biochemical studies of the action of sea anemone toxins and scorpion toxins, choline-substituted or Tris-substituted incubation media have been used routinely without noticeable effect on toxin binding or action (e.g., Refs. 8, 9, and 14). The results of Fig. 4 confirm that *Leiurus* toxin is active in choline-substituted bathing medium. In sharp contrast, GPT is virtually inactive at 200 nM in choline medium and exhibits increasing activity in Cs^+, Li^+, Rb^+, K^+, and Na^+ recording media, respectively. Even in 150 mM Na^+, the activity of the toxin is not maximal, since the effect of Na^+ increases linearly beyond that concentration. Evidently, the requirement for Na^+ does not involve binding at a high affinity site. The ion specificity for this effect does not follow the ion selectivity sequence of ion permeation through the Na^+ channel for which $Na^+ \sim Li^+ \gg K^+ > Rb^+ > Cs^+$ (21). Thus, we tentatively conclude that occupancy of one or more low affinity cation binding sites on the sodium channel or on GPT itself is required for optimal GPT action. Our results do not distinguish whether the cations are required for toxin binding or for events which follow toxin binding. Further electrophysiological and radioligand binding experiments will be required to address these points.

Site of toxin action. Of the many toxins that act on sodium channels, only the α-scorpion toxins and the sea anemone toxins slow channel inactivation preferentially without marked effects on the time course of channel activation. Both of these toxins act at neurotoxin receptor site 3 (22). Although GPT also slows sodium channel inactivation preferentially, the differences in its mode of action described above raise the possibility that its effect is mediated by interaction with a different receptor site. The failure of GPT to block specific scorpion toxin binding to neurotoxin receptor site 3 in either choline recording medium or normal recording medium at physiologically effective concentrations provides direct support for this conclusion. These results therefore define a new locus on the extracellular aspect of the sodium channel that can selectively alter the process of inactivation. It will be of interest to examine the interactions between GPT and neurotoxins known to act at other receptor sites on the sodium channel and to identify the site of GPT action within the structure of the sodium channel protein.

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