

Sodium Channels in Presynaptic Nerve Terminals

Regulation by Neurotoxins

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ABSTRACT Regulation of Na^+ channels by neurotoxins has been studied in pinched-off nerve endings (synaptosomes) from rat brain. Activation of Na^+ channels by the steroid batrachotoxin and by the alkaloid veratridine resulted in an increase in the rate of influx of ^{22}Na into the synaptosomes. In the presence of 145 mM Na^+ , these agents also depolarized the synaptosomes, as indicated by increased fluorescence in the presence of a voltage-sensitive oxacarboxyanine dye [diO-C₆(3)]. Polypeptide neurotoxins from the scorpion *Leiurus quinquestriatus* and from the sea anemone *Anthopleura xanthogrammica* potentiated the stimulatory effects of batrachotoxin and veratridine on the influx of ^{22}Na into synaptosomes. Saxitoxin and tetrodotoxin blocked the stimulatory effects of batrachotoxin and veratridine, both in the presence and absence of the polypeptide toxins, but did not affect control ^{22}Na influx or resting membrane potential. A three-state model for Na^+ channel operation can account for the effects of these neurotoxins on Na^+ channels as determined both by Na^+ flux measurements in vitro and by electrophysiological experiments in intact nerve and muscle.

INTRODUCTION

A variety of toxins from both animals and plants specifically interact with the voltage-dependent Na^+ channels in excitable membranes. For example, saxitoxin (STX) and tetrodotoxin (TTX) specifically block these channels (Kao, 1966; Narahashi, 1974) and batrachotoxin (BTX), a steroid from the skin secretions of the Colombian poison arrow frog, and veratridine (VER), an alkaloid from the American hellebore (*Veratrum*), open TTX-sensitive Na^+ channels (Albuquerque et al., 1973; Catterall, 1975). Grayanotoxin and aconitine have also been reported to open Na^+ channels (Narahashi and Seyama, 1974; Catterall, 1977 *b*), and yohimbine is a competitive antagonist of these agents (Huang et al., 1978). In electrophysiological experiments, BTX and VER alter the kinetic properties of the rapid, voltage-sensitive activation and inactivation of Na^+ channels (Ulbricht, 1969; Khodorov et al., 1975).

Polypeptide neurotoxins from the venoms of a variety of invertebrate species also affect the properties of excitable Na^+ channels. These polypeptide toxins

block inactivation (Koppenhöfer and Schmidt, 1968; Narahashi et al., 1972; Romey et al., 1976; Okamoto et al., 1977) and in some cases also alter the voltage dependence of Na⁺ channel activation (Cahalan, 1975). Catterall (1976) has shown that a polypeptide toxin (LqTX), purified from the venom of the scorpion *Leiurus quinquestriatus*, potentiates the effects of BTX and VER, but does not, itself, substantially increase Na⁺ permeability of cultured neuroblastoma cells.

Several lines of evidence indicate that synaptosomes (pinched-off nerve endings), prepared by differential centrifugation of mammalian brain homogenates, are a valid model system for studying nerve terminals and neuronal membranes, in vitro (Bradford, 1975; Blaustein et al., 1977). Synaptosomes can maintain a (negative-inside) membrane potential (Blaustein and Goldring, 1975) and have been shown to contain voltage-sensitive Ca²⁺ channels (Blaustein, 1975; Nachshen and Blaustein, 1979) and to exhibit Ca²⁺-dependent release of neurotransmitters (Blaustein, 1975; Cotman et al., 1976). Synaptosomal Na⁺ channels have been indirectly demonstrated by VER-stimulated Ca²⁺ uptake (Blaustein, 1975; Nachshen and Blaustein, 1979), VER-stimulated release of neurotransmitters (Blaustein, 1975; Cotman et al., 1976), VER- (Goldring and Blaustein, 1975) and BTX- (Krueger and Blaustein, 1978) induced synaptosome depolarization, and by the binding of labeled STX (Weigle and Barchi, 1978; Krueger et al., 1979) and LqTX (Ray et al., 1978). Recently, the presence of Na⁺ channels in synaptosomes was demonstrated by Li and White (1977) and by Matthews et al. (1979), who reported a VER-stimulated, TTX-sensitive uptake of Na⁺ into synaptosomes. In the present study, TTX- and STX-sensitive synaptosomal Na⁺ channels are demonstrated by measuring ²²Na influx and by monitoring synaptosome membrane potential changes with a fluorescent probe (Blaustein and Goldring, 1975). We find that LqTX and structurally different polypeptide toxins from the sea anemones *Anthopleura xanthogrammica* (AxTX) and *Anemonia sulcata* (AsTX) potentiate the effects of BTX and VER on Na⁺ channels in rat brain synaptosomes.

A steady-state model for Na⁺ channel regulation by neurotoxins has been proposed as a result of these studies. This model predicts that inhibition of Na⁺ channel inactivation (e.g., by polypeptide toxins) also causes a potentiation of the opening of the channels by the activators BTX and VER at constant membrane potential.

Preliminary reports of some of these results have been published (Krueger and Blaustein, 1978; Blaustein et al., 1979).

MATERIALS AND METHODS

Materials

3,3'-dipentyl-2,2'-oxacarbocyanine [diO-C₅(3)] was a gift from Dr. A. Waggoner, Amherst College; BTX was provided by Dr. John Daly, National Institutes of Health; AsTX I-III were gifts of Dr. Lazlo Beress, University of Kiel, W. Germany; [³H]STX was provided by Dr. Gary Strichartz, State University of New York, Stony Brook. AxTX was purified by the method of Norton, et al. (1976) from anemones obtained

from Bodega Marine Laboratory, Bodega, Calif. Dr. T. Norton kindly provided a sample of AxTX that was used in preliminary studies. Venom from the scorpion *Leiurus quinquestriatus* was from Sigma Chemical Co., St. Louis, Mo.; TTX was from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif., and VER was from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Preparation of Synaptosomes

Synaptosomes were prepared from rat forebrains (150 g, female, albino) by a modification of the method of Hajos (1975) as described by Krueger et al. (1979). The 0.8 M sucrose layer, containing synaptosomes, was diluted slowly with the addition of 1–5-ml aliquots of ice-cold 145 Na + 5 K (145 mM NaCl; 5 mM KCl; 1.4 mM MgCl₂; 1.0 mM CaCl₂; 2.0 mM NaH₂PO₄; 10 mM glucose; 20 mM Tris-HEPES, pH 7.4). The synaptosomes were pelleted by centrifugation at 20,000 *g* for 6 min, and the pellet was suspended in either 145 Na + 5 K or in a solution in which 140 mmol/liter of the NaCl was replaced by choline-Cl (140 Ch + 5 Na + 5 K).

Measurement of Fluorescence

Synaptosomes were suspended (~1 ml/rat; 6–9 mg protein/ml) in 140 Ch + 5 Na + 5 K and warmed to 30°C for 10 min. Aliquots (90- μ l) were added to tubes containing 10 μ l of 140 Ch + 5 Na + 5 K plus various neurotoxins to be tested. Incubations continued for 3–5 min at 30°C. Aliquots (70- μ l) were then added to 1.8 ml of 145 Na + 5 K containing 2.5 μ M of diO-C₅(3) in 10 \times 75-mm glass culture tubes. The tubes were vortexed for 15 s, and fluorescence was read in a Turner Model 110 fluorometer (Turner-Amsco Instrument Co., Carpinteria, Calif.) (λ_{ex} = 448 nm; λ_{em} = 511 nm). In early experiments, the dye-containing solution was maintained at 36°C, the ambient temperature of the fluorometer; in later experiments, the temperature was maintained at 30°C throughout the experiment with the use of a constant temperature sample holder (Turner). Similar results were obtained at both temperatures. The synaptosomes were viable for at least 2 h when maintained in either 145 Na + 5 K or 140 Ch + 5 Na + 5 K at 30°C.

The fluorometer was set to zero (blank), with the cuvette containing 145 Na + 5 K plus diO-C₅(3) before the addition of synaptosomes. The fluorescence (*F*) obtained upon addition of synaptosomes was read in arbitrary units. The change in fluorescence (ΔF) for each condition is expressed as the percent of maximal fluorescence according to the equation:

$$\Delta F = \frac{(F \text{ at indicated condition}) - (F \text{ with no addition in 5 mM K})}{(F \text{ in 150 mM K}) - (F \text{ in 5 mM K})} \times 100\%$$

Measurement of ²²Na Influx into Synaptosomes

Synaptosomes were prepared and equilibrated as described above and were suspended in 140 Ch + 5 Na + 5 K. After preincubation at 30°C for 10 min, aliquots (0.18-ml) were added to tubes containing various neurotoxins to be tested in 20 μ l of 140 Ch + 5 Na + 5 K, and preincubation was continued for various time periods (normally 10 min). 50 μ l of 140 Ch + 5 Na + 5 K containing 140 nCi ²²Na was added, and the incubation was continued (normally for 2 s). The tracer was added while the synaptosomes were vigorously agitated by a vortex mixer; timing was with an electronic metronome sounding at 2/s. The uptake was terminated by the rapid addition of 5 ml of ice-cold wash solution (140 mM choline-Cl; 10 mM NaCl; 1.4 mM MgCl₂; 1.0 mM CaCl₂; 0.5 mM NaH₂PO₄; 20 mM Tris-HEPES, pH 7.4), and

the synaptosomes were filtered on glass fiber filters (GF/C, Whatman, Inc., Clifton, N. J.) under suction (400 mm Hg). Each filter was washed with two additional aliquots of wash solution. The total elapsed time for uptake termination, including washes, was <5 s. The amount of ^{22}Na trapped on the filters was determined by liquid scintillation counting with a Triton X-100-toluene-Omnifluor cocktail (Rohm and Haas, Co., Philadelphia, Pa., and New England Nuclear, Pilot Chemicals Div., Watertown, Mass.) (Blaustein and Russell, 1975).

Determination of [^3H]STX Binding to Synaptosomes

Binding of STX to synaptosomes was determined as described by Krueger et al. (1979). In the experiment shown in Figs. 5 and 6, ^{22}Na uptake and [^3H]STX binding were measured in the same experiment. The two isotopes were counted simultaneously by double-label liquid scintillation spectroscopy.

Purification of Scorpion Polypeptide Neurotoxin

A polypeptide neurotoxin (LqTX) was purified from the venom of the scorpion *Leiurus quinquestriatus* by a modification of the method of Catterall (1976). 40 mg of toxin was dissolved in 40 ml of 10 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, pH 7.0. The insoluble material was removed by centrifugation, and the sample was applied to a cation exchange column (Amberlite CG-50, 200–400 mesh; 1.5×60 cm Rohm and Haas Co.). The column was eluted (0.5 ml/min) first with 40 ml of 10 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, then 60 ml of 100 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, followed by 400 ml of a linear gradient of $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ from 100 to 350 mM. Finally, the column was washed with 50 ml of 500 mM $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$. Polypeptide neurotoxin activity was assayed by monitoring toxin-induced synaptosome depolarization in the presence of 0.3 μM BTX by the fluorescent dye method. The principal peak of activity eluted at ~ 0.2 M $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$. The protein content of the fractions was estimated from the absorbance at 280 nm. The active fractions were analyzed by SDS-polyacrylamide slab gel electrophoresis; the toxin (LqTX) used for subsequent experiments was found to consist of a single polypeptide with a molecular weight of 6,000–7,000.

Other Methods

Protein was determined by the method of Lowry et al. (1951).

RESULTS

The fluorescence of a suspension of synaptosomes containing the voltage-dependent probe 3,3'-dipentyl-2,2'-oxacarbocyanine [diO-C₅(3)] increased as the K^+ concentration in the medium was raised from the normal value of 5 mM to 150 mM. The fluorescence increase was proportional to the logarithm of the K^+ concentration. These results confirm those of Blaustein and Goldring (1975); they calculated that synaptosomes incubated under these conditions (5 mM K^+) have a membrane potential of about -50 mV. We conclude that the changes in synaptosome fluorescence reflect changes in synaptosome membrane potential.¹

¹ The fluorescence of diO-C₅(3) may be directly proportional to the particle membrane potential as indicated by experiments using phospholipid bilayer vesicles (liposomes) made selectively permeable to K^+ with valinomycin (B. Krueger, unpublished). The fluorescence of the liposome suspension was directly proportional to the logarithm of the K^+ gradient ($\text{K}_o^+/\text{K}_i^+$).

In standard physiological solution (e.g., 145 Na + 5 K) increasing membrane Na⁺ permeability would be expected to depolarize the synaptosomes. BTX and VER are steroidal neurotoxins that are known to open neuronal Na⁺ channels (Ulbricht, 1969; Albuquerque et al., 1973; Catterall, 1975). As shown in Figs. 1 and 2, BTX and VER depolarized synaptosomes, as indicated by increased fluorescence. The effects of both toxins were complete within 5 min, whereas the fluorescence of control synaptosomes was unchanged during this time (data not shown). The effects of both VER and BTX were completely blocked by TTX (10 μ M, data not shown).

Catterall (1976) has purified a polypeptide toxin from the venom of the scorpion *Leiurus quinquestriatus* that greatly potentiates the effects of BTX and

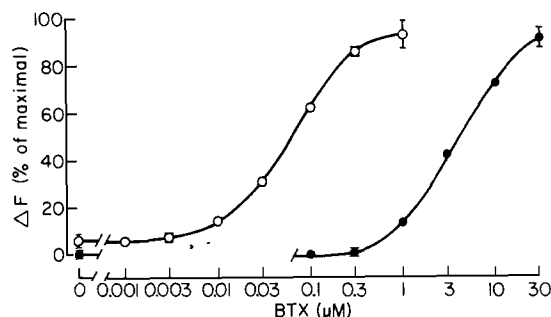


FIGURE 1. Effect of varying the concentration of BTX in the absence and presence of LqTX on synaptosome fluorescence. Synaptosomes were warmed to 30°C for 10 min and were incubated in 140 Ch + 5 Na + 5 K for 5 min at 30°C in the presence of various concentrations of BTX and either in the absence (●) or presence (○) of 0.1 μ M LqTX. Aliquots (65- μ l) of the synaptosomes were then transferred to cuvettes containing 1.8 ml 145 Na + 5 K containing diO-C₅ (3) at 30°C. The tubes were vortexed for 15 s, and the fluorescence was read (in arbitrary units) after it had reached a stable value 15 s later. The fluorescence of the suspensions is expressed as the percent of maximal fluorescence as described under Materials and Methods. The data points shown are the means of triplicate determinations from a single experiment. The bars show \pm SEM when larger than the dimensions of the symbols. This experiment was repeated three times with similar results. The curves were drawn by eye. LqTX was purified as described under Materials and Methods.

VER on Na⁺ channels in neuroblastoma cells in culture. Fig. 1 shows the effect of this purified scorpion toxin (LqTX) on the depolarization of synaptosomes by BTX. In the absence of LqTX, BTX depolarized the synaptosomes with half-maximal depolarization occurring at about 3 μ M. Maximally effective concentrations of BTX depolarized synaptosomes completely, i.e., to the same extent as 150 mM K⁺ (represented by $\Delta F = 100\%$). A maximally effective concentration of LqTX (100 nM) by itself depolarized the synaptosomes by $\sim 15\%$ and greatly potentiated the effects of BTX. In the presence of LqTX, half-maximal depolarization was observed with ~ 0.05 μ M BTX.

The effect of a maximally effective concentration of LqTX on depolariza-

tion of synaptosomes by VER is shown in Fig. 2. In the absence of LqTX, VER increased synaptosome fluorescence with half-maximal increase at about 20 μM . Even at the highest concentration tested (300 μM), VER depolarized only to $\sim 50\text{--}60\%$ of the level reached with maximally effective BTX (Fig. 1) or 150 mM K^+ (represented by $\Delta F = 100\%$). LqTX potentiated the effects of VER; in the presence of LqTX, half-maximal depolarization occurred at about 0.7 μM VER. In addition, in the presence of a maximally effective concentration of LqTX, VER was able to depolarize the synaptosomes to the same level reached with maximally effective BTX or 150 mM K^+ . The increase in the maximal effect of VER induced by LqTX was similar to that observed by Catterall (1976 and 1977 *b*) and by Jacques et al. (1978) on ^{22}Na influx in neuroblastoma cells in culture.

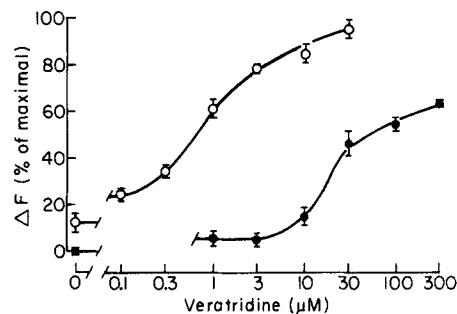


FIGURE 2. Effect of varying the concentration of VER in the absence and presence of LqTX on synaptosome fluorescence. The concentration dependence of VER on synaptosome fluorescence in the absence (●) and presence (○) of LqTX (0.1 μM) was determined as described for BTX in the legend to Fig. 1. The data points shown are the means of triplicate determinations from a single experiment. The bars show $\pm\text{SEM}$ when larger than the dimensions of the symbols. This experiment was repeated twice with similar results. The curves were drawn by eye.

To detect the activation of Na^+ channels by neurotoxins, it is necessary to incubate the synaptosomes with the toxins before the addition of the fluorescent dye diO-C₅(3). Although this dye does not affect depolarization of synaptosomes whose Na^+ channels have been previously opened by neurotoxins, it does inhibit the actions of the toxins themselves. This effect appears to be competitive and does not require irradiation at the absorption maximum of the dye.² Presumably, the dye does not substantially inhibit Na^+ flux through opened channels.

²²Na Influx into Synaptosomes

While membrane potential changes monitored by the fluorescence method reflect relative changes in Na^+ permeability, it is desirable to measure directly the influx of Na^+ ions through Na^+ channels with tracer ^{22}Na . Under

² Krueger, B. K. Unpublished observation.

appropriate conditions, ^{22}Na influx should be proportional to the Na^+ permeability of the synaptosomes and, therefore, to the number of open Na^+ channels.

Because Na^+ ion fluxes through channels should be affected by the membrane potential, it was necessary to ensure that the membrane potential of the synaptosomes remained constant as the Na^+ channels were opened by neurotoxins. This was accomplished by replacing all but 5 mmol/liter of Na^+ in the incubation medium with choline. By fluorescence determinations in the presence of diO-C₅(3), it was found that in this modified medium (140 Ch + 5 Na + 5 K), 30 μM BTX, a concentration that maximally depolarized the synaptosomes in 145 Na + 5 K (Fig. 1), increased the fluorescence by the

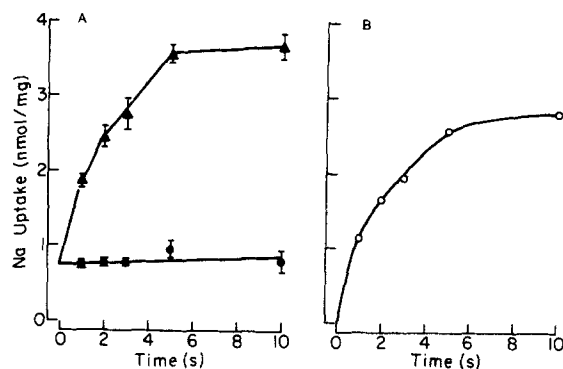


FIGURE 3. Effect of BTX on the rate of ^{22}Na influx into synaptosomes. Uptake of ^{22}Na into synaptosomes was determined as described under Materials and Methods. (A) Aliquots (0.18-ml) of synaptosomes were preincubated at 30°C in 140 Ch + 5 Na + 5 K containing either no additions (●) or 10 μM BTX (▲). After 5 min, 50 μl of 140 Ch + 5 Na + 5 K containing ^{22}Na was added, and the uptake was terminated after the indicated times by filtration on glass-fiber filters. The amount of ^{22}Na trapped on the filters was determined by liquid scintillation counting. The data points shown are means \pm SEM of quadruplicate determinations. This experiment was repeated twice with similar results. The curves were drawn by eye. (B). BTX-stimulated ^{22}Na uptake, i.e., uptake in the presence of BTX minus uptake in the absence of BTX.

same amount as did increasing the external K^+ concentration from 5 to 8 mM (data not shown). Thus, activation of most of the Na^+ channels resulted in only a slight depolarization of the synaptosomes in 5 mM Na^+ . This depolarization may result in a slight underestimation of the ^{22}Na influx at the control membrane potential.

The influx of tracer ^{22}Na will be proportional to Na^+ permeability only when the influx is unidirectional, i.e., when influx is determined before a significant amount of tracer efflux occurs. If this condition is satisfied, Na^+ uptake should be proportional to time. Fig. 3 shows the uptake of ^{22}Na into synaptosomes as a function of incubation time. In the absence of neurotoxins, little time-dependent uptake was observed; in the presence of 10 μM BTX,

Na^+ uptake was about $1 \text{ nmol s}^{-1} \text{ mg}^{-1}$. The BTX-stimulated ^{22}Na uptake (i.e., the uptake in the presence of BTX minus the uptake in the absence of BTX) is shown in Fig. 3 B. The small size of the synaptosomes and a very large surface-to-volume ratio presumably account for the observation that isotopic equilibrium is reached in about 5 s in the presence of BTX. Fig. 3 B shows that BTX-stimulated ^{22}Na influx during the second s of incubation was slightly less than that during the first s. However, because of limitations in the accuracy of the timing of incubation during the shorter interval, 2-s incuba-

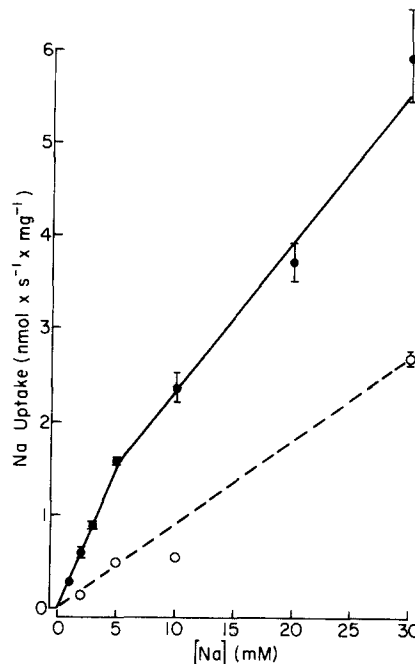


FIGURE 4. ^{22}Na uptake vs. Na^+ concentration. Synaptosomes were preincubated at 30°C for 5 min in the absence (○, dashed line) or presence (●, solid line) of $10 \mu\text{M}$ BTX as described in the legend to Fig. 3. $50 \mu\text{l}$ of a ^{22}Na -containing solution with various Na^+ concentrations was added and uptake was determined after 2 s. The final Na^+ concentration varied from 2.5 to 30 mM and the sum of $\text{Cl}^- + \text{Na}^+ + \text{K}^+$ was 150 mM. The data points shown are the means \pm SEM of triplicate determinations. The curves were drawn by eye.

tions were employed in most experiments. The true, initial rates of ^{22}Na entry may, therefore, be somewhat larger than the reported influxes (Figs. 4–10), which are based on 2-s incubations.

We have measured control and BTX-stimulated ^{22}Na influx into synaptosomes at various concentrations of external Na^+ (Fig. 4). In the absence of neurotoxins, Na^+ influx was proportional to the Na^+ concentration up to 30 mM, the highest concentration tested. This may actually represent binding or influx through another pathway rather than entry through Na^+ channels

since it is not reduced by TTX (data not shown). BTX (10 μM) stimulated Na^+ uptake, and this uptake was proportional to Na^+ only up to 5 mM. From 5 to 30 mM the dependence of Na^+ uptake on external Na^+ was less steep. This may indicate the presence of a saturable binding site for Na^+ in the channel; however, it seems more likely that increased Na^+ permeability at Na^+ concentrations >5 mM results in depolarization and a reduction in the electrical driving force for Na^+ influx. All subsequent measurements of ^{22}Na uptake were carried out in 5 mM Na^+ for 2 s. The Na^+ uptake observed under these conditions should be approximately proportional to the Na^+ permeability and to the number of channels open. The absolute Na^+ permeability was not calculated because of uncertainties in determining the synaptosomal surface area across which the Na^+ influx is measured.

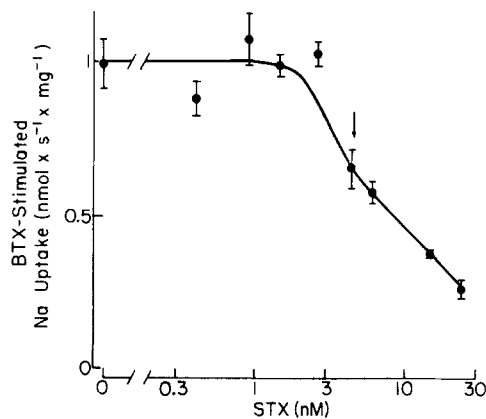


FIGURE 5. Block of BTX-stimulated ^{22}Na uptake by STX. Synaptosomes were preincubated in 140 Ch + 5 Na + 5 K for 5 min at 30°C in the presence of 10 μM BTX and various concentrations of [^3H]STX. ^{22}Na uptake was determined after 2 s as described in the legend to Fig. 3. ^{22}Na uptake in the absence of both BTX and STX was subtracted from each data point. The data points show means \pm SEM of quadruplicate determinations. The curve was drawn by eye. The arrow indicates the dissociation constant for [^3H]STX binding (K_d STX) determined in this experiment (see Fig. 6).

Blockade of Na^+ Channels by STX

Activation of Na^+ channels by either BTX or VER was blocked by TTX. STX, a neurotoxin with similar Na^+ -channel blocking properties also blocked BTX-stimulated Na^+ uptake into synaptosomes. As is shown in Fig. 5, STX produced a concentration-dependent block of Na^+ uptake with half-maximal block observed at 5–10 nM. In the experiment shown, [^3H]STX was used so that STX binding could be measured in the same synaptosomes that were used for measurement of Na^+ uptake. Double-label scintillation counting techniques were used to determine ^3H and ^{22}Na simultaneously. Fig. 6 shows the binding of STX to synaptosomes in this experiment as assayed by counting the amount of [^3H]STX trapped on the filters. The calculated dissociation

constant was ~ 5 nM, in good agreement with the concentration of STX required to produce half-maximal block of Na^+ uptake. When appropriate corrections are made for differences in temperature, this value is similar to the dissociation constants reported for STX binding to frog node of Ranvier (Wagner and Ulbricht, 1975), garfish olfactory nerve and rabbit vagus nerve (Ritchie et al., 1976), synaptosomes (Weigele and Barchi, 1978; Krueger et al., 1979), and neuroblastoma (Catterall and Morrow, 1978).

Effects of Polypeptide Neurotoxins on Na^+ Uptake into Synaptosomes

The effect of varying concentrations of BTX in the absence (●) and presence of LqTX (○) on ^{22}Na uptake into synaptosomes is shown in Fig. 7. BTX

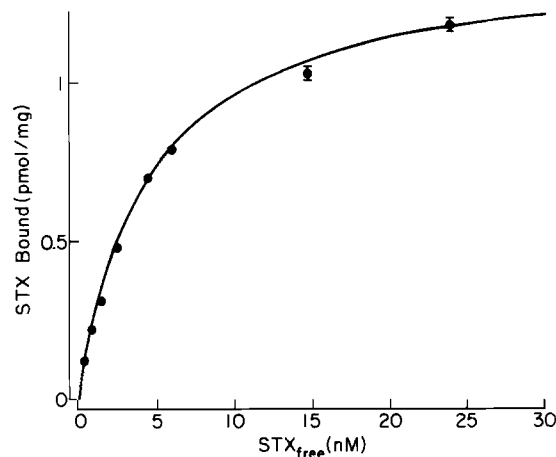


FIGURE 6. Binding of $[^3\text{H}]\text{STX}$ to synaptosomes. ^{22}Na uptake (Fig. 5) and $[^3\text{H}]\text{STX}$ binding were determined in the same experiment. $[^3\text{H}]\text{STX}$ binding was determined as described by Krueger et al. (1979). The data points show the means \pm SEM of quadruplicate determinations. The free STX concentration was calculated as the total $[\text{STX}]$ minus bound $[\text{STX}]$. The curve is a least-squares fit of the data points to the equation:

$$\text{STX Bound} = \frac{B_m}{1 + \frac{K_d}{[\text{STX}]}}$$

The values of B_m and K_d were 1.4 pmol/mg and 4.5 nM, respectively.

caused a concentration-dependent increase in Na^+ uptake, with half-maximal activation observed at $\sim 2 \mu\text{M}$ ($=K_{\text{BTX}}$). Using the fluorescence technique to monitor membrane potential, K_{BTX} was found to be $\sim 3 \mu\text{M}$ (Fig. 1). A maximally effective concentration of LqTX ($0.1 \mu\text{M}$) increased Na^+ uptake by $\sim 0.15 \text{ nmol s}^{-1} \text{ mg}^{-1}$ and greatly potentiated the effect of BTX. In each of three experiments, maximal Na^+ uptake was slightly greater in the absence than in the presence of LqTX. Moreover, in the presence of LqTX, concentrations of BTX $> 0.3 \mu\text{M}$ were slightly inhibitory. The effects of LqTX plus BTX (Δ), LqTX alone, and BTX alone (data not shown) were completely blocked by TTX ($10 \mu\text{M}$).

The effects of various concentrations of VER, in the absence (●) and presence of LqTX (○, 0.1 μM) are shown in Fig. 8. Half-maximal stimulation of Na⁺ uptake was observed with ~20 μM VER (K_{VER}). LqTX greatly potentiated the effects of VER: in the presence of LqTX, half-maximal stimulation was observed at about 1 μM VER; moreover, the maximal effect of VER was increased in the presence of LqTX to the same level observed in the presence of 10 μM BTX (▲). These results agree well with those obtained using the fluorescence method to monitor membrane potential (Fig. 2). The effects of VER in both the absence and presence of LqTX were completely blocked by TTX (data not shown).

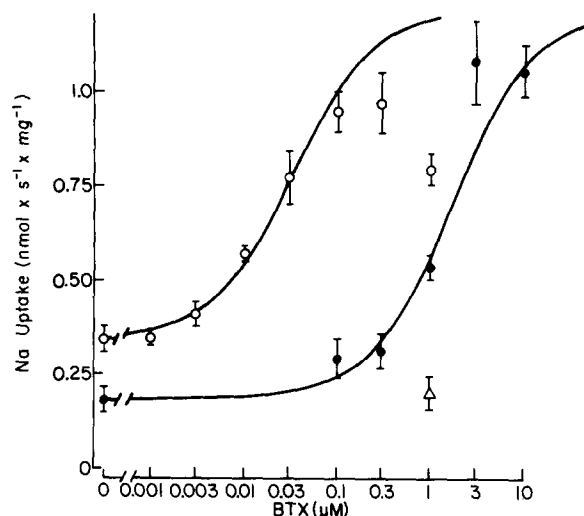


FIGURE 7. Effect of varying the concentration of BTX, in the absence and presence of LqTX, on ²²Na uptake. Synaptosomes (in 140 Ch + 5 Na + 5 K) were preincubated at 30°C for 10 min in the presence of the indicated concentrations of BTX and in the absence (●) or presence (○) of 0.1 μM LqTX. Uptake was determined after 2 s as described under Materials and Methods and in the legend to Fig. 3. (Δ) indicates uptake in the presence of 0.1 μM LqTX, 1 μM BTX, and 10 μM TTX. The data points show the means ± SEM of triplicate determinations. This experiment was repeated twice with similar results. The curves are plots of Eq. 1 with $K_1 = 4.5$, $K_r = 10^4$, $K_2 = 0.003$ (right) or 1.0 (left), and $K_3 = 5$ nM. The maximal increase in f_o was scaled to 1.0 $\text{pmol s}^{-1} \text{mg}^{-1}$.

We have found that a polypeptide toxin purified from the sea anemone *Anthopleura xanthogrammica* (anthopleurin-A; AxTX; see Shibata et al. [1976] and Norton et al. [1976]) affects synaptosomal Na⁺ channels in a way similar to LqTX (Figs. 9 and 10). AxTX (1 μM) potentiated the effects of BTX without itself greatly increasing Na⁺ uptake (Fig. 9). In the presence of AxTX, K_{BTX} was ~0.1 μM, as compared with ~2 μM in the absence of AxTX. This polypeptide toxin has a molecular weight of ~5,000, and its amino acid sequence does not exhibit any structural homologies with that of LqTX.

AxTX also potentiated the effects of VER on synaptosomal Na^+ channels (Fig. 10). K_{VER} was shifted from $\sim 20 \mu\text{M}$ to $\sim 2 \mu\text{M}$ by AxTX ($1 \mu\text{M}$), while Na^+ uptake in the presence of maximally effective VER was increased in the presence of AxTX. AxTX caused a small increase in Na^+ uptake in the absence of VER or BTX.

The effect of various concentrations of LqTX and AxTX on synaptosomal Na^+ channels is shown in Fig. 11. In this experiment, the ability of the polypeptide toxins to depolarize synaptosomes in the presence of a subthreshold concentration of BTX ($0.3 \mu\text{M}$) was measured with the fluorescent probe diO-C₅(3). The data are presented in double reciprocal form. The effects of

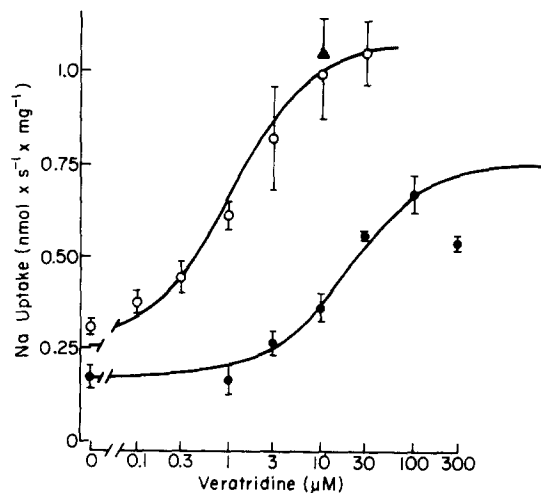


FIGURE 8. Effect of varying the concentration of VER, in the absence and presence of LqTX, on ^{22}Na uptake. Synaptosomes in $140 \text{ Ch} + 5 \text{ Na} + 5 \text{ K}$ were preincubated at 30°C for 10 min in the presence of the indicated concentrations of VER and in the absence (●) or presence (○) of $0.1 \mu\text{M}$ LqTX. Uptake was determined after 2 s as described under Materials and Methods and in the legend to Fig. 3. ▲ indicates uptake in the presence of $10 \mu\text{M}$ BTX and in the absence of LqTX. The data points show the means \pm SEM of triplicate determinations. This experiment was repeated once with similar results. The curves are plots of Eq. 1 with $K_1 = 4.5$, $K_r = 600$, $K_2 = 0.003$ (right) or 0.2 (left), and $K_3 = 50 \text{ nM}$. The maximal increase in f_0 was scaled to $0.91 \text{ pmol s}^{-1} \text{ mg}^{-1}$.

both LqTX and AxTX were saturable, and both toxins were able to completely depolarize the synaptosomes at maximally effective concentrations. Half-maximal effects (K_{LqTX} and K_{AxTX}) were observed at about 4 and 46 nM, respectively. These results suggest that LqTX binds with an affinity about 10 times greater than that of AxTX.

Rathmayer and Beress (1976) and Beress et al. (1975) have reported that the Mediterranean sea anemone *Anemonia sulcata* contains three polypeptide neurotoxins, designated AsTX-I, AsTX-II, and AsTX-III, that have molecular weights of about 4,800, 4,700, and 2,700, respectively. AsTX-II inhibits Na^+

channel inactivation in crustacean nerves (Romey et al., 1976); moreover, Jacques et al. (1978) reported that AsTX-II potentiates the activation of Na^+ channels in neuroblastoma by VER. We have found that all three toxins from *A. sulcata* potentiated the effects of a threshold concentration ($0.2 \mu\text{M}$) BTX on synaptosomal membrane potential (data not shown). None of the three *A. sulcata* toxins were as potent as LqTX. The effects of the *A. sulcata* toxins, both in the absence and the presence of BTX, were blocked by TTX. AsTX-I and AsTX-II have amino acid sequences that are very similar to that of AxTX, but that do not appear to be homologous with either AsTX-III or with LqTX.

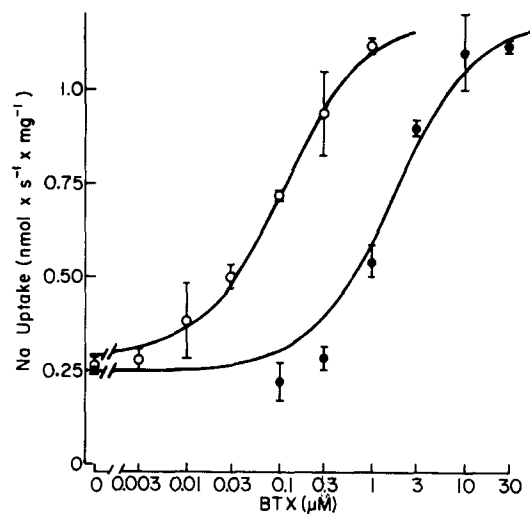


FIGURE 9. Effect of varying the concentration of BTX, in the absence and presence of AxTX, on ^{22}Na uptake. Synaptosomes were preincubated at 30°C for 10 min in the presence of the indicated concentrations of BTX and in the absence (●) or presence (○) of $1 \mu\text{M}$ AxTX. Uptake was determined after 2 s as described under Materials and Methods and in the legend to Fig. 3. The data points show the means \pm SEM of triplicate determinations. This experiment was repeated once with similar results. The curves are plots of Eq. 1 with $K_1 = 4.5$, $K_r = 10^4$, $K_2 = 0.003$ (right) or 0.6 (left), and $K_3 = 5 \text{ nM}$. The maximal increase in f_0 was scaled to $0.94 \text{ pmol s}^{-1} \text{ mg}^{-1}$.

DISCUSSION

Three Types of Neurotoxin Binding Sites on Synaptosome Na^+ Channels

The results presented demonstrate that in synaptosomes, as in neuroblastoma (Catterall, 1976), there are at least three regulatory sites on Na^+ channels. These sites bind, respectively, the activators BTX and VER, the blockers TTX and STX, and the polypeptide toxins from scorpions and sea anemones. The polypeptide toxins potentiate the activation of Na^+ channels by BTX and VER. Our results confirm those obtained on excitable neuroblastoma cells and muscle cells in culture (Catterall, 1975, 1976, and 1977 *b*; Stallcup, 1977; Jacques et al., 1978). Using [^{125}I]LqTX, Catterall (1977 *a*) and Ray et

al. (1978) have demonstrated binding of LqTX to neuroblastoma cells and synaptosomes, respectively, with dissociation constants of $\sim 1\text{--}3$ nM. Recently, Catterall and Beress (1978) reported that AsTX-II competes for the same binding site on neuroblastoma, as does LqTX, but with about 100 times lower affinity. A similar competition between AsTX-II and a polypeptide toxin from the scorpion *Androctonus australis* was observed in neuroblastoma (Couraud et al., 1978).

One interesting finding in the present study is that several polypeptide toxins from very diverse animal species (scorpion and sea anemone), with very

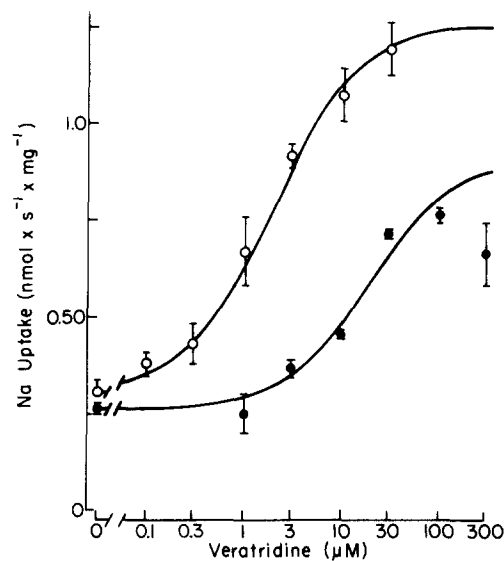


FIGURE 10. Effect of varying the concentration of VER in the absence and presence of AxTX on ^{22}Na uptake. Synaptosomes were preincubated at 30°C for 10 min in the presence of the indicated concentrations of VER and in the absence (●) or presence (○) of $1\ \mu\text{M}$ AxTX. Uptake was determined after 2 s as described under Materials and Methods and in the legend to Fig. 3. The data points show the means \pm SEM of triplicate determinations. The experiment was repeated once with similar results. The curves are plots of Eq. 1 with $K_1 = 4.5$, $K_r = 600$, $K_2 = 0.003$ (right) or 0.06 (left), and $K_3 = 50$ nM. The maximal increase in f_0 was scaled to $1.03\ \text{pmol}\ \text{s}^{-1}\ \text{mg}^{-1}$.

different primary amino acid sequences, have virtually identical effects on synaptosomal Na^+ channels. The primary structures of the five polypeptide toxins tested in this study have been determined (Kopeyan, et al., 1978; Tanaka, et al., 1977; Wunderer and Eulitz, 1978; Wunderer, et al., 1976; Martinez, et al., 1977; Beress, et al., 1977). The amino acid sequences of the sea anemone toxins AxTX, AsTX-I, and AsTX-II are homologous. There are, however, no obvious amino acid sequence homologies between this group of toxins and either LqTX or AsTX-III, nor are LqTX and AsTX-III homologous to one another. Despite the structural differences, all of these toxins

potentiate the actions of BTX and VER and, apparently, bind to the same site on excitable Na^+ channels (Catterall and Beress, 1978). This suggests that, despite differences in amino acid sequence, each toxin may fold up in such a way as to present a common structural determinant to the Na^+ channel binding site that is capable of a functional interaction.

Na^+ Channel Inactivation and the Actions of Anemone and Scorpion Toxins

Another striking effect of the polypeptide toxins (in some cases, the crude scorpion venoms) is observed only in electrophysiological experiments in which measurements are made on a millisecond time scale. This effect is a reduction in the rate of inactivation of Na^+ channels during membrane depolarization (Koppenhöfer and Schmidt, 1968; Narahashi et al., 1972; Bergman et al.,

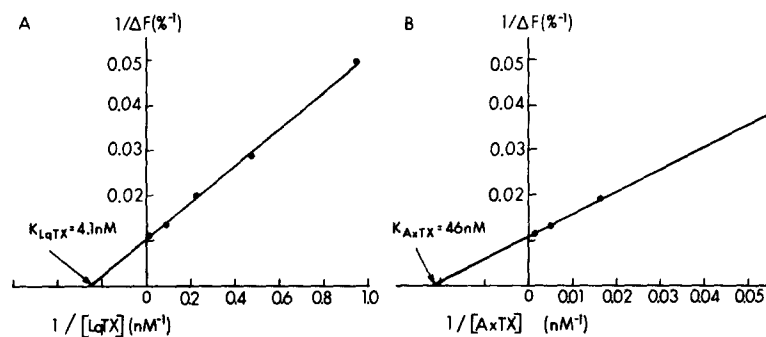


FIGURE 11. Effect of varying the concentration of polypeptide toxins on synaptosome fluorescence. Synaptosomes were preincubated for 10 min in the presence of various concentrations of LqTX (A) and AxTX (B) and in the presence of $0.3 \mu\text{M}$ BTX. Fluorescence was determined as described in the legend to Fig. 1. (Temperature = 36°C). The reciprocal of the fluorescence increase due to polypeptide toxins (expressed as ΔF , see Materials and Methods) is plotted as a function of the reciprocal of the toxin concentration. The lines are least-squares fits to the data points. Each data point is the mean of duplicate determinations. The indicated values of K_{LqTX} and K_{AxTX} are the reciprocals of the respective abscissa intercepts. This experiment was repeated once with similar results.

1976; Romey et al., 1976; Okamoto et al., 1977; Catterall, 1979). Although the Na^+ channels of synaptosomes are, presumably, capable of being inactivated, we are unable to study this phenomenon directly with currently available methods. Nevertheless, as discussed below, it may be possible to obtain some indirect information about Na^+ channel inactivation in this preparation.

A Three-state Model for Na^+ Channel Activation and Inactivation

In the following discussion, we present a model that accounts for both the potentiation of BTX activation of Na^+ channels by polypeptide toxins and the inhibition of Na^+ channel inactivation by these toxins in terms of their interaction with a single, high-affinity site on the Na^+ channel. The Na^+

channel is assumed to exist in three interconvertible states, as shown in Fig. 12. The closed state (*C*) is nonconducting and is favored by hyperpolarization. The open state (*O*) is the only state that conducts Na^+ ions; the transition from *C* to *O* is favored by membrane depolarization. The inactivated state (*I*) is also nonconducting and is favored by sustained depolarization. In the Hodgkin-Huxley description (Hodgkin and Huxley, 1952*a* and *b*) depolarization results in a transient opening of Na^+ channels and a triggering of inactivation.

In accord with electrophysiological evidence, we assume that the transition from the closed to the open states depends on the membrane potential; the transition from the open to the inactivated states is assumed to be independent of membrane potential (although this assumption is not essential). It is of some interest that recent reports, based on detailed voltage-clamp analysis of

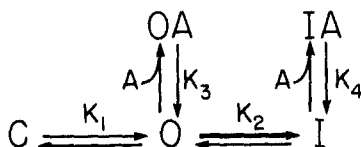


FIGURE 12. Model of Na^+ Channel. Each Na^+ channel is assumed to exist in one of three interconvertible states: State *C* (closed) is nonconducting and is favored by hyperpolarization, state *O* (open) conducts Na^+ ions, and state *I* (inactivated) is nonconducting; states *O* and *I* are favored by depolarization. K_1 is the ratio of closed to open states and is decreased by membrane depolarization; K_2 is the ratio of open to inactivated states. K_3 and K_4 are the dissociation constants for binding of the activators (BTX or VER) to the open and inactivated states, respectively. *OA* is the open state with activator bound and conducts Na^+ ions. *IA* is the inactivated state with activator bound and does not conduct Na^+ ions. The bold arrow indicates the rate constant for the transition from the open to the inactivated state. It is postulated that this rate constant is reduced by polypeptide toxins resulting in an increase in K_2 and a decrease in the rate of Na^+ channel inactivation. See Discussion and Appendix for further details of this model.

the kinetics of Na^+ channels in giant axons, indicate that inactivation may not be an independent, voltage-dependent process (Goldman and Schauf, 1972; Bezanilla and Armstrong, 1977). Rather, inactivation appears to be coupled to voltage-dependent channel activation (cf. Cahalan and Almers, 1979).

In voltage-clamp experiments, Bezanilla and Armstrong ([1977]; and Armstrong and Bezanilla [1977]) obtained evidence for additional closed, open, and inactivated states of the Na^+ channel that have rather complicated and rapid kinetics. However, since our data are based on ion flux experiments made over long periods relative to the time constants of the Na^+ channel state transitions, the analysis of the three-state model will be limited to steady-state considerations.

Activation of Na⁺ Channels by BTX and VER

To account for the activation of Na⁺ channels by BTX and VER, we assume that these activators bind preferentially to the open (*O*) state of the channel (i.e., $K_3 \ll K_4$, K_3 and K_4 being equilibrium dissociation constants for binding of activators to the open and inactivated states of the channel, respectively; see Fig. 12). Moreover, open channels with activator bound (*OA*) are functionally indistinguishable from open channels without activator (*O*). The same assumption was made by Catterall (1977 *b*), who proposed a two-state model to explain the interactions between activators and polypeptide toxins. This assumption is supported by the observation that the effects of the activators in intact nerve preparations are use dependent; that is, shifting the channels to the open state by repetitive depolarization enhances the activation of the channels by BTX and VER (Ulbricht, 1969; Albuquerque et al., 1973; Bartels-Bernal et al., 1977; Revenko, 1977).

Analysis of the three-state model in Fig. 12 (see Appendix) indicates that the fraction of Na⁺ channels open (f_o) is given by:

$$f_o = \frac{\frac{[A]}{K_3} + 1}{K_1 + \frac{1}{K_2} + 1 + \frac{[A]}{K_3} + \frac{[A]}{K_3} \cdot \frac{1}{K_2 K_r}}, \quad (1)$$

where K_1 and K_2 are equilibrium constants for the state transitions defined in Fig. 12, K_3 and K_4 are dissociation constants for the binding of activators to the open and inactivated states, respectively, $[A]$ is the concentration of activator, and $K_r = K_4/K_3$. The value of f_o is proportional to the TTX-sensitive ²²Na influx, which is determined experimentally.

Fig. 13 shows plots of calculations based on Eq. 1: the fraction of channels in the open state (f_o) is graphed as a function of $[A]/K_3$ for various values of K_1 , K_2 , and K_r . It is assumed that K_1 will remain constant, provided the membrane potential is constant. The latter condition is satisfied in the present study (see Results). We postulate that the polypeptide toxins will greatly increase K_2 , resulting in an inhibition of Na⁺ channel inactivation (see above and Fig. 12).

Polypeptide Toxin Effects on Full Activators

In Figures 13 A-C, K_r is set at 10^4 or greater, i.e., the activator binds to the open state of the Na⁺ channel at least ten thousand times more tightly than to the inactivated state. This condition may apply to BTX. Under these conditions, ~100% of the channels are open at maximally effective concentrations of activator. At $K_1 = 300$ (Fig. 13 A), a 1,000-fold increase in K_2 (the postulated effect of the polypeptide toxins) causes only a fourfold decrease in K_A (the concentration of activator required to half maximally activate the channels). The same increase in K_2 causes no significant increase in the fraction of channels open in the absence of activator. As K_1 is decreased to 3 (the postulated effect of depolarization), a 1,000-fold increase in K_2 causes a

250-fold decrease in K_A , and $\sim 20\%$ of the channels are open in the absence of activator.

The values of the parameters that most nearly fit the results obtained with BTX and LqTX (Fig. 7) are: $K_1 = 4.5$, $K_r \geq 10^4$, and $K_2 = 0.003$ and 1.0 , in

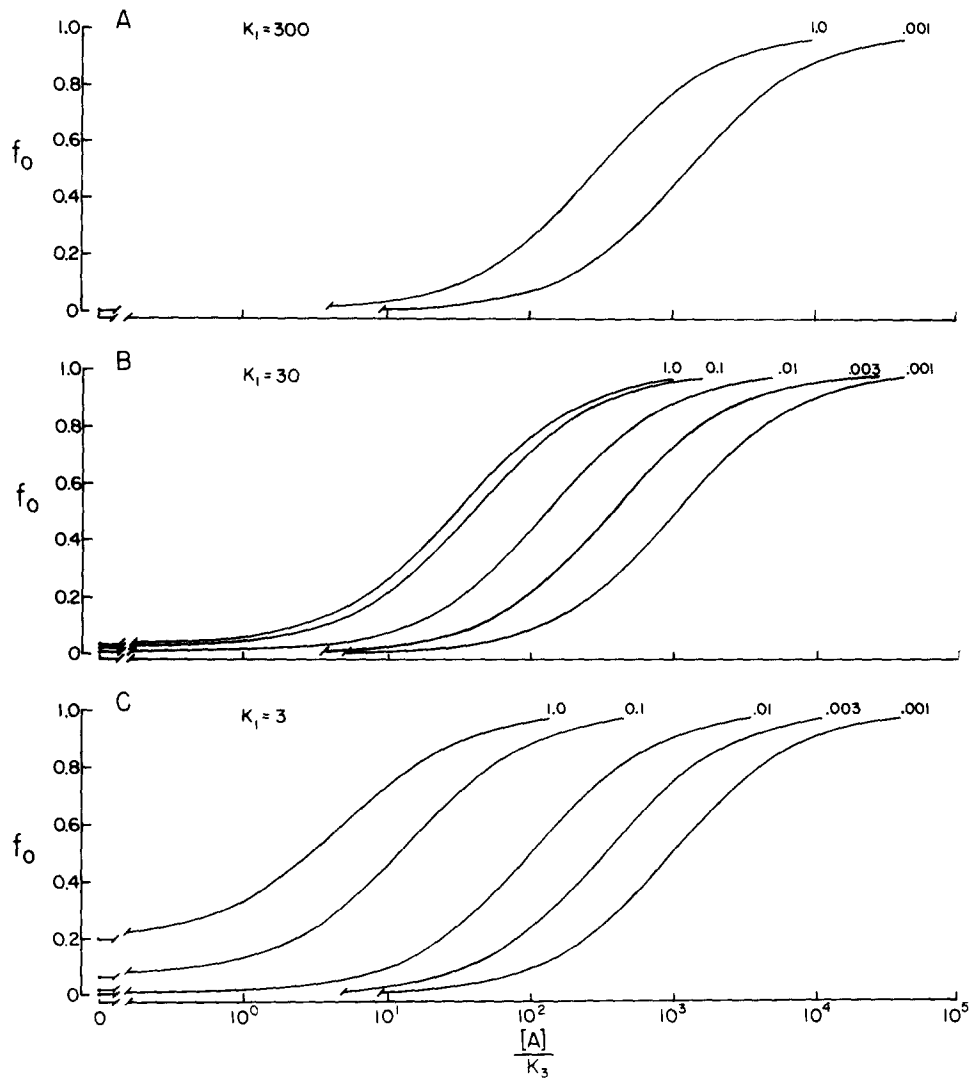


FIGURE 13. Activation of Na^+ channels by full activators (*A-C*) and partial activators (*D-F*) as predicted by the model described in this communication. The fraction of Na^+ channels open (f_0) has been plotted as a function of $[A]/K_3$ according to Eq. 1. For full activators (*A-C*), $K_r = 10^4$ (or greater). This condition may apply to BTX. For partial activators (*D-F*), $K_r = 600$. This condition may apply to VER. The values of K_2 are shown at the *top* or *right* of each panel. The value of K_1 is shown at the *top* of each panel. See Discussion for further details of this model.

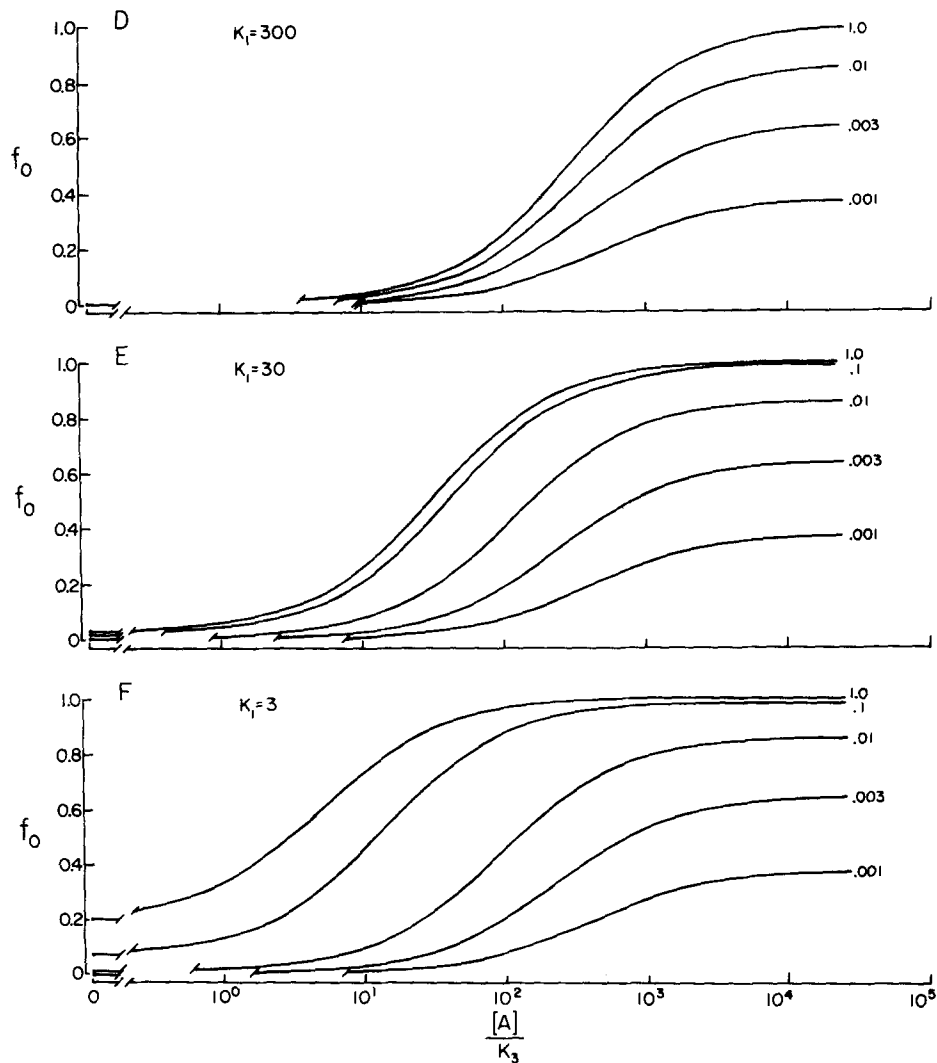


FIGURE 13.

the absence and presence of LqTX, respectively. The results obtained with BTX and AxTX (Fig. 9) are approximated when $K_1 = 4.5$, $K_r \geq 10^4$, and $K_2 = 0.003$ and 0.6 , in the absence and presence of AxTX, respectively. Plots of Eq. 1, using these parameters, have been superimposed on the data of Figs. 7 and 9. The best fit to the data points for both sets of curves is obtained when values on the abscissa are scaled so that $K_3 = 5$ nM, suggesting that this is the dissociation constant for binding of BTX to the open state of the channel.

Polypeptide Toxin Effects on Partial Activators

In Fig. 13 D-F, K_r is 600, i.e., the activator binds to the open state only 600 times more tightly than to the inactivated state. These conditions may apply

to VER. Catterall (1977 *b*) also presented evidence that VER binds to the open state of the channel about 600 times more tightly than to nonopen states. Under these conditions, and when $K_2 = 0.003$, only ~65% of the channels are in the open state in the presence of maximally effective concentrations of activator. At $K_1 = 300$ (hyperpolarized), increasing K_2 from 0.003 to 1.0 (the effect of polypeptide toxins) causes a less than twofold shift in K_A and no increase in the fraction of channels open in the absence of activators. However, under these circumstances, virtually 100% of the channels are open in the presence of a maximally effective concentration of activator. As K_1 is reduced to 3, increasing K_2 not only increases the fraction of channels open at maximally effective activator concentration but also decreases K_A by nearly 30-fold.

The values of the parameters that most nearly fit the results obtained with VER and LqTX (Fig. 8) are: $K_1 = 4.5$, $K_r = 600$, and $K_2 = 0.003$ and 0.2 in the absence and presence of LqTX, respectively. The results obtained with VER and AxTX (Fig. 10) are approximated when $K_1 = 4.5$, $K_r = 600$, and $K_2 = 0.003$ and 0.06 in the absence and presence of AxTX, respectively. Plots of Eq. 1, using the above parameters, have been superimposed on the data in Figs. 8 and 10. The best fit to the data points for both sets of curves is obtained when the values on the abscissa are scaled so that $K_3 = 50$ nM, suggesting that this is the dissociation constant for the binding of VER to the open state of the channel.

Choice of Parameters in the Three-state Model

Since there is not a unique set of parameters (K_1 , K_2 , K_3 , K_r) that fit the experimental data, the choice of these parameters in Figs. 7-10 was arbitrary. The following rationale was used: (a) Activation of 15% of the Na^+ channels by polypeptide toxins alone occurs only when $K_1 \leq 4.5$. This maximum value of K_1 was used. Interestingly, Hodgkin and Huxley (1952 *b*) found that α_m/β_m , which should be equivalent to K_1 , was ~5 at -50 mV. α_h and β_h of Hodgkin and Huxley are apparent voltage-dependent rate constants for inactivation that do not correspond to any parameters in the three-state model presented here. (b) The largest values of K_2 , in the absence and presence of polypeptide toxin, that will fit the data for LqTX are 0.003 and 1.0, respectively. Although the data can be fit with lower values for K_2 , this would require that K_3 , the dissociation constant for binding of activator to the open state of the channel, be very low ($K_3 \leq 10^{-9}$ M). When $K_2 = 0.003$ in the absence of polypeptide toxin, K_3 for BTX was 5 nM and K_3 for VER was 50 nM. These values are in the same range as the dissociation constants for STX, LqTX, and AxTX. (c) For $K_1 = 4.5$, $K_2 = 0.003$ and 1.0 in the absence and presence of LqTX, respectively, the data are uniquely fit for $K_r \geq 10^4$ for BTX and $K_r = 600$ for VER.

Three Effects of Polypeptide Toxins on Na^+ Channels in the Steady State

Inspection of the curves in Fig. 13 reveals that increasing K_2 (the postulated effect of the polypeptide toxins) can cause any one of three effects on the fraction of Na^+ channels open (f_o): (a) In the absence of activators (BTX or

VER), increasing K_2 increases the fraction of channels in the open state (see Eq. 6 in Appendix). The magnitude of this effect depends on the value of K_1 (which is postulated to vary with the membrane potential), becoming significant only at $K_1 < 100$. (b) Increasing K_2 decreases K_A , the concentration of activator required to cause a half-maximal increase in f_o (Fig. 13). The magnitude of this reduction increases as the value of K_1 decreases. (c) For partial activators ($K_r < 10^4$), increasing K_2 from 0.003 to 1.0, increases the fraction of channels in the open state at maximal $[A]$ to nearly 100% at all values of K_1 (Fig. 13 D-F). This can also be seen in Eq. 7 (Appendix) which shows that the fraction of channels open at maximally effective activator concentration is a function of only K_2 and K_r . Each of the three effects of polypeptide toxins predicted by the model is observed in synaptosomes (Figs. 7-10).

The most conspicuous difference between the results obtained in neuroblastoma (Catterall, 1977 *b*) and in synaptosomes (this paper) is that, in the latter system, polypeptide toxins open a substantial fraction of Na^+ channels in the absence of activators. (Jacques et al. [1978] have reported that $\Delta_s\text{TX-II}$ alone opens a significant number of channels in a different neuroblastoma cell line.) A possible explanation may be found in the analysis of the three-state model for Na^+ channels presented above and in the Appendix. In this model, the fraction of channels open in the absence of activators depends both on K_2 (which is increased by polypeptide toxins) and on K_1 (which is decreased by membrane depolarization). Thus, increasing K_2 (the effect of polypeptide toxins) will open a substantial fraction of the channels only when $K_1 < 100$. Thus, the fact that LqTX alone does not open Na^+ channels in neuroblastoma, suggests that K_1 in synaptosomes may be lower than in neuroblastoma. This may reflect a difference in membrane potential or possibly a difference in the voltage dependence of K_1 in the two preparations. As discussed below, the determination of the effects of varying the membrane potential on the opening of Na^+ channels by polypeptide toxins and activators provides a means to test the three-state model.

Some Predictions Based on the Three-state Model for Na^+ Channels

At constant membrane potential, the three-state model presented in this communication is formally identical to the two-state model proposed by Catterall (1977 *b*). Indeed, both models fit the available data from neuroblastoma and synaptosomes equally well. Because it takes into account the voltage dependence of Na^+ channel activation, the three-state model leads to the prediction that changes in the membrane potential should have profound effects on the activation of Na^+ channels by neurotoxins. Specifically: (a) Within a fixed range, the number of channels open in the presence of maximally effective concentrations of polypeptide toxins (maximum K_2), but in the absence of activators, should increase upon membrane depolarization (lower K_1) and should decrease upon hyperpolarization. (b) The extent of shift of K_A for any activator with maximally effective concentrations of polypeptide toxins should increase with membrane depolarization and should decrease with membrane hyperpolarization. (c) For partial activators, raising K_2 (the

effect of the polypeptide toxins) should result in full activation at all membrane potentials.

It may be possible to test these predictions of the model in synaptosomes by examining toxin-stimulated ^{22}Na influx while varying the membrane potential with various concentrations of external K^+ . Alternatively, these predictions could be tested in intact nerve cells (e.g., squid giant axon or snail neuron) by measuring toxin-stimulated, TTX-sensitive, inward current while voltage clamping at various potentials.

Conclusion

The model for neurotoxin regulation of Na^+ channels presented in this communication provides an explanation for two effects of certain polypeptide toxins from scorpions and sea anemones: (a) the inhibition of voltage-sensitive Na^+ channel inactivation observed in electrophysiological experiments, and (b) the potentiation of Na^+ channel activation by BTX and VER at constant membrane potential. Both of these effects can be explained on the basis of a single action of the polypeptide toxins, viz., a reduction in the rate of inactivation (reflected in an increase in K_2). Though direct measurement of inactivation is not presently possible in particle suspensions such as synaptosomes, it may be possible to study this process indirectly. The potentiation of activation by BTX or VER, observed in steady-state ^{22}Na uptake experiments, may be a reflection of the effect of the polypeptide toxins on the inactivation mechanism.

APPENDIX

Eq. 1 in the text is based on the model defined in Fig. 12. Under any constant conditions, the relative distribution of Na^+ channels among the three states will be determined by the values of the equilibrium constants K_1 and K_2 , which are defined as:

$$K_1 = \frac{C}{O} \quad \text{and} \quad K_2 = \frac{O}{I}. \quad (2a \text{ and } b)$$

In this formalism, K_1 and K_2 are equal to the ratios of the backward to the forward rate constants of the respective state transitions. For simplicity, only the ratios will be used in this analysis. Each Na^+ channel activator binds to open Na^+ channels with a dissociation constant:

$$K_3 = \frac{[A] \cdot O}{OA}. \quad (3)$$

To account for partial activation of Na^+ channels by certain activators (e.g., VER), it is assumed that these agents also bind significantly to inactivated channels. The dissociation constant for this binding is:

$$K_4 = \frac{[A] \cdot I}{IA}. \quad (4)$$

In Eqs. 3 and 4, $[A]$ is the concentration of unbound activator (BTX or VER), OA is the number of channels in the open state with activator bound, and IA

is the number of channels in the inactivated state with activator bound. As mentioned in the text, the data obtained with the partial activator VER require binding of activators to both the open and inactivated states with $K_3 \ll K_4$. The data do not exclude binding of activators to the closed state; however, such an interaction would not substantially affect the results of the following analysis.

The fraction of channels that are open (f_o) under any conditions will be given by the equation:

$$f_o = \frac{O + OA}{C + O + OA + I + IA} \quad (5)$$

Substitution of Eqs. 2a, 2b, 3, and 4 into Eq. 5 gives:

$$f_o = \frac{\frac{[A]}{K_3} + 1}{K_1 + \frac{1}{K_2} + 1 + \frac{[A]}{K_3} + \frac{[A]}{K_3} \cdot \frac{1}{K_2 K_r}} \quad (1)$$

where $K_r = \frac{K_4}{K_3}$. In the absence of activator ($[A] = 0$), Eq. 1 reduces to

$$f_o = \frac{1}{\frac{1}{K_2} + K_1 + 1} \quad (6)$$

At very high concentrations of activator,

$$f_o = \frac{1}{1 + \frac{1}{K_2 K_r}} \quad (7)$$

Thus, for K_r very large, f_o will approach 1 at saturating concentrations of activator. As K_r decreases (i.e., binding of activator to the open state decreases relative to binding to the inactivated state), f_o at maximally effective concentrations of activator becomes < 1 .

In this analysis, only the interaction between activators and the open and inactivated states of the channel have been taken into account. If the binding of activators to closed channels is also considered, Eq. 1 must be modified as follows:

$$f_o = \frac{\frac{[A]}{K_3} + 1}{K_1 + \frac{1}{K_2} + 1 + \frac{[A]}{K_3} \left(1 + \frac{1}{K_2 K_r} + \frac{K_1}{K_r'} \right)} \quad (8)$$

where $K_r' = \frac{K_5}{K_3}$ and K_5 is the dissociation constant for binding of activator to the closed state of the Na^+ channels. Since K_1 depends on the membrane

potential, Eq. 8 indicates that depolarization (lower K_1) should increase the maximal effect of an activator if the activator binds to any significant extent to channels in the closed state. This prediction has not been tested.

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