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Na channels from rat brain have been studied at the single channel level in planar bilayer membranes and by using ^{22}Na tracer flux and ^{3}H-STX binding methods. The opening and closing (gating) of BTX-activated Na channels can be modulated by divalent cations. Neither the STX binding site nor the "selectivity filter" is involved in the effects of divalents on gating. External divalents preferentially slowed the rate of channel opening whereas internal divalents preferentially slowed the closing rate. This suggests that divalents are capable of alternately interacting with elements of the voltage-sensing machinery of the channel that are alternately exposed to the outside and inside as the channel closes and opens. In cultured neonatal rat brain glial cells (astrocytes), high affinity STX binding sites and STX-sensitive ^{22}Na influx are virtually absent until day 8 when the density of STX binding sites and STX blockable flux begin to increase. This spontaneous change can be prematurely initiated by placing the cells in a defined culture medium. One or more medium components may regulate the differential expression of STX-sensitive and STX-insensitive Na channels.

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FUNCTIONAL CONSEQUENCES OF CHEMICAL MODIFICATION OF THE SAXITOXIN BINDING SITE ON NEURONAL SODIUM CHANNELS.

Annual Report

Bruce K. Krueger, Ph.D.

November 6, 1988

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SUMMARY

Sodium channels from rat brain have been studied at the single channel level in planar phospholipid bilayer membranes and by using ^{22}Na tracer flux and ^{3}H-saxitoxin binding methods. The opening and closing (gating) of batrachotoxin-activated sodium channels can be modulated by external and internal divalent cations with external divalents causing a depolarizing shift in the open probability vs membrane potential relation and internal divalents causing a hyperpolarizing shift. Although some of these effects are due to interactions of the divalent cations with negatively charged phospholipids, the predominant effect is due to binding to one or more sites on the intrcellular and extracellular sides of the channel protein. The selectivity for various divalent cations on both sides differs from that for open channel block and competition with saxitoxin binding indicating that neither the saxitoxin binding site nor the "selectivity filter" within the channel pore is likely to be involved in the effects of divalents on gating. External divalents preferentially slowed the rate of channel opening whereas internal divalents preferentially slowed the closing rate. This suggests that internal and external divalents are capable of alternately interacting with elements of the voltage-sensing machinery of the channel that are alternately exposed to the extracellular or intracellular spaces as the channel closes and opens. In cultured neonatal rat brain glial cells (astrocytes), high affinity saxitoxin binding sites and saxitoxin sensitive ^{22}Na influx are virtually absent until day 8 when the density of saxitoxin binding sites and saxitoxin blockable flux begin to increase. This spontaneous change can be prematurely initiated and greatly accelerated by placing the cells in a defined culture medium without fetal calf serum but with various growth factors. One or more of these medium components may regulate the differential expression of saxitoxin-sensitive and saxitoxin-insensitive sodium channels in these cells.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. NIH 85-23, Revised 1985).

Animals are maintained in the Central Animal Facility of the University of Maryland School of Medicine. Animals are housed, cared for, and used strictly in accordance with USDA regulations. The University of Maryland School of Medicine Central Animal Facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. The program of animal care is directed by a full-time, specialty trained, laboratory animal veterinarian. This institution has an Animal Welfare Assurance on file with the NIH Office for Protection from Research Risks (OPRR), Assurance Number A3200-01

The person responsible for the Central Animal Facility is:

Director
Central Animal Facility
University of Maryland School of Medicine
Medical School Teaching Facility, Redwood and Pine Streets
Baltimore, Maryland 21201 Phone (301) 528 7612

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EXPERIMENTAL RESULTS

A. Specific Aims of Original Proposal.

The original specific aims (listed below) have not changed. During the first year, substantial progress was made on aims 1, 2, and 4 (see report AN-1-1986, 31 October 1986). During the second year, progress was made on specific aims 4 and 5 and work was expanded on aim 1 in new area related to the development of STX and TTX binding and block in brain glial cells in culture (see report AN-2-1987, 15 December 1987). As described in the present report, during Year 3, effort has been concentrated on the modulation of STX sensitive and insensitive Na channels in cultured neonatal rat brain astrocytes (glia) and on characterizing the effects of internal and/or external divalent cations on Na channel gating.

1. to determine the molecular basis of the voltage dependence of saxitoxin (STX) and tetrodotoxin (TTX) block of neuronal sodium channels.

2. to examine the effects of trimethyloxonium (TMO; a modifier of the negatively-charged toxin binding site) on ion permeation through the channels and on channel block by calcium and strontium.

3. to examine the effect of other carboxyl modifying reagents on ion permeation and block. Special attention will be paid to carbodiimides which render sodium channels insensitive to TTX.

4. to utilize the information in 1 - 3 above to derive a rate theory model for ion permeation through the channel.

5. to determine the rates of opening and closing of single sodium channels at varying membrane potentials and the effects of TMO treatment on these processes.

B. Publications and Scientific Meetings.

Publications:

S. Cukierman, W. C. Zinkand, R. J. French and B. K. Krueger. Effects of membrane surface charge and calcium on the gating of rat brain sodium channels in planar bilayers. Journal of Generaly Physiology 92: 431-447 (1988).

P.J. Yarowsky and B.K. Krueger. Development of saxitoxin-sensitive and insensitive sodium channels in cultured neonatal rat astrocytes. Journal of Neuroscience 9: 1055-1061 (1989).

S. Cukierman and B.K. Krueger. Modulation of sodium channel gating by external divalent cations studied in planar lipid bilayers: Differential effects on opening and closing rates. submitted to Journal of General Physiology.

S. Cukierman, F.C. Albuquerque, and B.K. Krueger. Modulation of sodium channel gating by internal divalent cations provides evidence for strings of negative intrachannel gating charges. submitted to Nature.

A.M. Corbett and B.K. Krueger. Polypeptide neurotoxins modify gating and single channel conductance of veratridine-activated sodium channels in planar lipid bilayers. submitted to Journal of Membrane Biology.

Abstracts:

Corbett, A. M. and B. K. Krueger. 1988. Purification and characterization of two sodium channels from rat brain with different alpha subunits and distinct functional properties. Biophysical Journal 53: 15a.

Cukierman, S., W. C. Zinkand, R. J. French, and B. K. Krueger. 1988. Effects of calcium and lipid surface charge on sodium channel gating. Biophysical Journal 53: 536a.

O'Leary, M.E. and B.K. Krueger. Gating of batrachotoxin-activated Na channels is altered by scorpion venom in planar lipid bilayers. Society for Neuroscience Abstracts, in press (1988).

Yarowsky, P.J., D.S. Brougher and B.K. Krueger. Induction of STX-sensitive sodium channels in cultured astrocytes. Society for Neuroscience Abstracts, in press (1988).

Scientific Meetings.

Drs. Cukierman, French and Krueger and Mr. Zinkand attended the 1988 Biophysical Society meeting in Phoeniz, AZ, and presented a poster on the effects of lipid surface charge on sodium channel gating.

Dr. Krueger and Mr. D. Brougher will present a poster on development and modulation of saxitoxin-sensitive sodium channels in astrocytes

Drs. Krueger and O'Leary will present a poster on voltage-independent sodium channels at the Society for Neuroscience Meeting in November, 1988, in Toronto, Canada.

C. Modulation of Sodium Channel Gating by Divalent Cations.

We have been studying the effects of external and internal divalent cations on the opening and closing (gating) of BTX-activated sodium channels in planar bilayers (1-3). It has been known for about 20 years that increasing external Ca^{2+} depresses excitability primarily by reducing the probability of opening of sodium channels (4). It has been unclear whether this effect is due to interaction of the divalent cations with negatively charged phospholipids or the channel itself, or both. We have addressed this problem by studying the effects of divalent cations on sodium channel gating in uncharged (phosphatidylethanolamine) or negatively charged (phosphatidylserine) bilayers. As shown in Figure 1, external Ca^{2+} causes a depolarizing shift in the probability of being open (P_o) vs V_m in both neutral (left) and negatively charged (right) bilayers. The

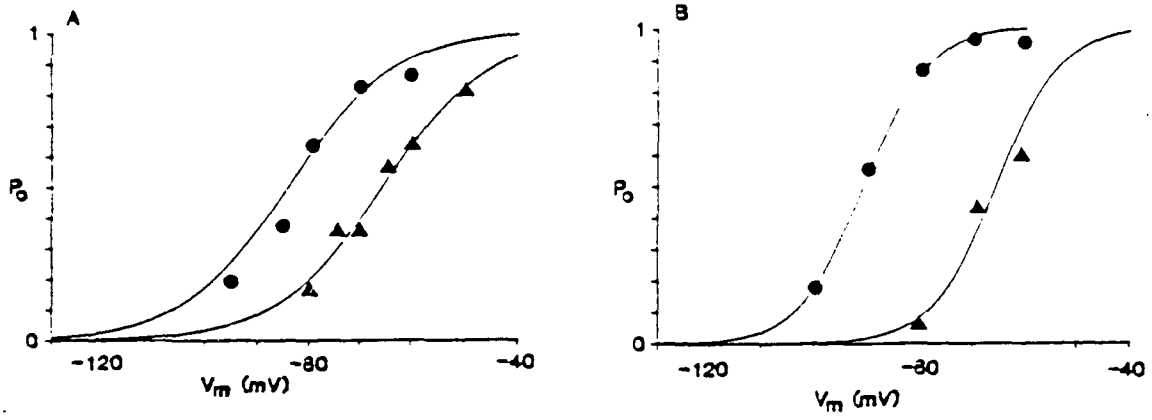

Figure 1. P_o vs V_m for a single sodium channel in a neutral (PE) bilayer (A) and in a negatively charged (PS) bilayer (B). Methods are described in reference 1. (●), control; (▲), + 10 mM Ca^{2+}_o.

shift in negative bilayers was always larger. Thus, some of the effects of divalent cations are due to an interaction with the protein, although addition of negatively charged lipids also causes a small amount of hyperpolarizing shift. Complementary results were obtained with internal Ca^{2+}, viz., a hyperpolarizing shift was observed that was larger in negative bilayers. This is summarized in Figure 2. In neutral bilayers (A), external Ca^{2+} caused a

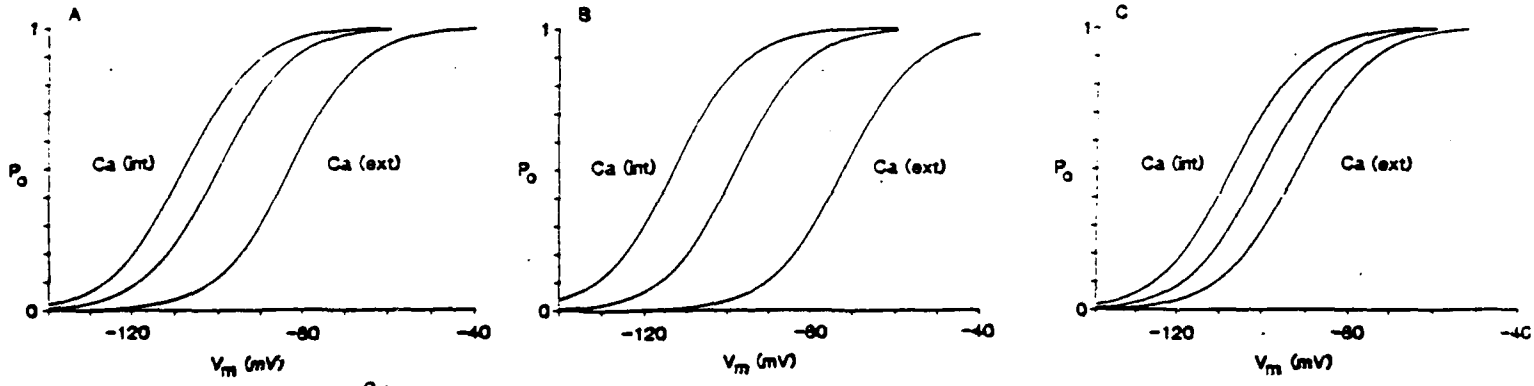

Figure 2. Ca^{2+}-induced shifts in sodium channel gating. Curves show the average shift in P_o vs V_m relations for external or internal Ca^{2+} (10 mM). A. results in neutral bilayers; B, negatively charged bilayers; C. Effect attributable to negatively charged lipids, i.e., shift in negative lipids minus shift in neutral lipids.

depolarizing shift whereas internal Ca^{2+} caused a hyperpolarizing shift. Both effects were larger in negative bilayers (B). In both bilayers, the effect of external Ca^{2+} was larger than that of internal Ca^{2+} possibly due to a higher density of binding sites on the outside of the channel. The net shifts due to the negative lipids (C: B-A) were symmetrical (i.e., the same for internal and external Ca^{2+}) indicating that the influence of the negatively charged lipid headgroups was of equal strenth from both the external and internal sides of the bilayer.

Since the STX binding site at the external mouth of the channel pore is negatively charged (5), it was of interest to determine if Ca^{2+} binding at this site accounted for some of the observed effects on gating. To address this question, we compared the shifts in the midpoint of the Na channel P_o vs V_m relations with the degree of block of the channel, for each of several divalent cations. As shown in Table I, the order of efficacy for shifting was similar but not identical to that for block. Thus, it is unlikely that the STX binding site is involved in the modulation of channel gating by divalent cations.

Table IA. Divalent Cation-Induced Shifts in $V_{0.5}$

	$\Delta V_{0.5}$	
	NEUTRAL	NEGATIVELY CHARGED
0.5 mM Zn^{2+}	10.2 ± 1.3 mV (3)	14.4 ± 1.7 mV (3) *
5.0 mM Ca^{2+}	16.3 ± 1.0 mV (4)	21.6 ± 3.0 mV (4) *
5.0 mM Ba^{2+}	13.6 ± 0.8 mV (5)	18.1 ± 1.1 mV (5) **
5.0 mM Sr^{2+}	8.7 ± 1.2 mV (4)	16.9 ± 2.3 mV (5) #
5.0 mM Mg^{2+}	8.5 ± 2.0 mV (3)	15.0 ± 0.5 mV (3) ##

$\Delta V_{0.5}$ is the depolarizing shift in the midpoint of the P_o versus V_m relation induced by external divalent cations. Neutral bilayers were composed of 80% PE, 20% PC. Negatively-charged bilayers were composed of 56% PE, 44% PS. The data show means ± s.e.m (number of observations). For each divalent cation, $\Delta V_{0.5}$ was significantly larger in negatively charged bilayers (*, $p<.05$; **, $p<.005$; #, $p<.07$; ##, $p<.02$). Methods are described in ref. 1.

Table IB. Block of Na Channels by External Divalent Cations

	NEUTRAL	NEGATIVELY CHARGED
5.0 mM Zn^{2+}	77 ± 1% (3)	77% (2) *
0.5 mM Zn^{2+}	39 ± 1% (4)	36 ± 1% (4) #
5.0 mM Ca^{2+}	46 ± 1% (3)	50 ± 1% (4) #
5.0 mM Mg^{2+}	42 ± 1% (5)	39 ± 2% (5) #
5.0 mM Sr^{2+}	29% (2)	31 ± 3% (4) #
5.0 mM Ba^{2+}	16 ± 2% (5)	13 ± 1% (6) #

The membrane potential was -80 mV. Neutral bilayers were composed of 80% PE, 20% PC; negatively-charged bilayer were composed of 56% PE, 44% PS. Block is expressed as $i_{Me^{2+}}/i_{max}$ x 100%. The data show means ± s.e.m (number of observations). For each divalent cation, block was no greater in negatively charged bilayers than in neutral bilayers (*, $p>0.98$; #, $p>.99$).

We have also studied the effects of external and internal divalent cations

on the rates of channel opening and closing (2,3). For this purpose, we used Ba^{2+} which is a good shifter (Table IA) but a poor blocker (Table IB). This problem was addressed by determining the single channel open and closed dwell time distributions which were well described by single exponentials at the potentials where gating is most voltage-dependent. Under these conditions, the mean closed time is the reciprocal of the opening rate and the mean open time is the reciprocal of the closing rate. As shown in Figure 3A, external Ba^{2+} both increased the closing rate and decreased the opening rate, but had a larger effect of the opening rate. Internal Ba^{2+} decreased the closing rate and increased the opening rate but had a larger effect of the closing rate (Figure 3B). In 14 experiments, external Ba^{2+} doubled the closing rate and tripled the opening rate. In 12 experiments, internal Ba^{2+} tripled the closing rate and only doubled the opening rate.

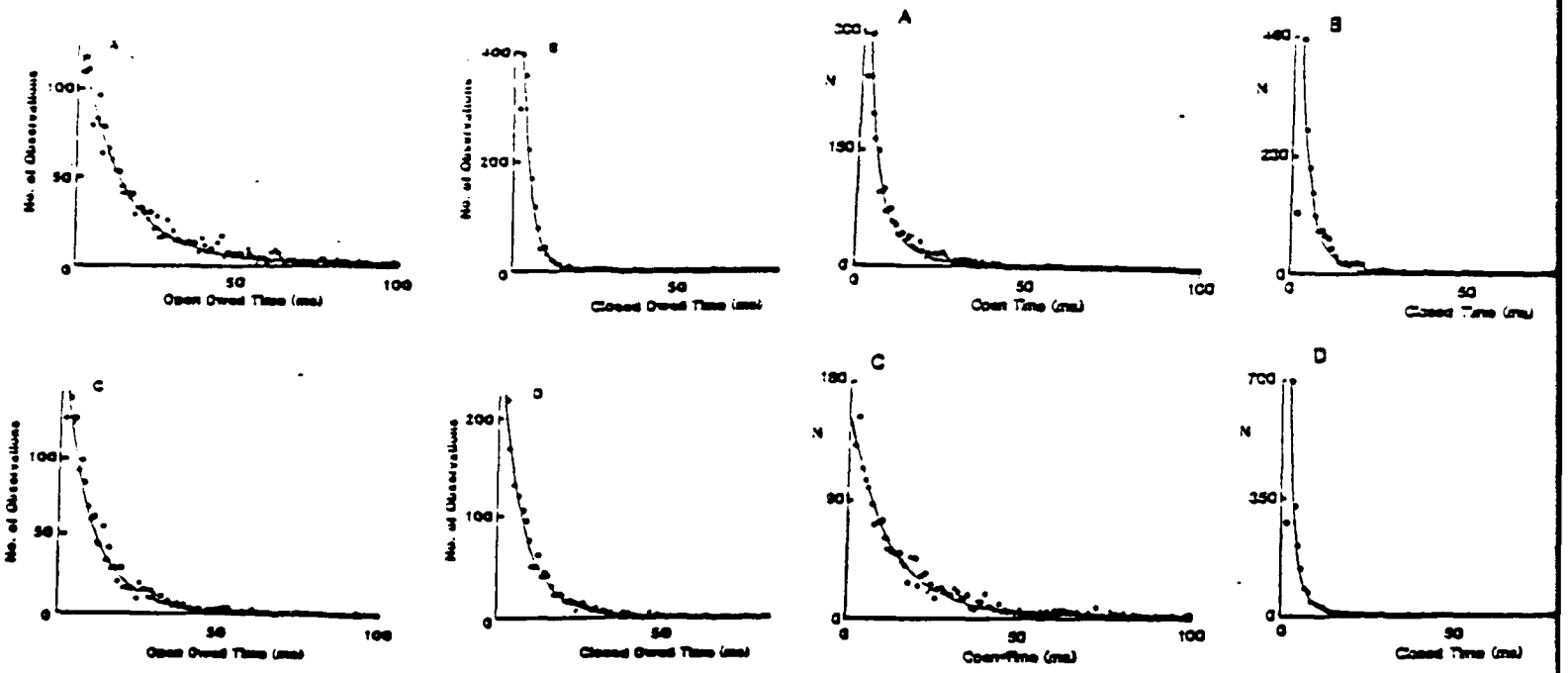

Figure 3. Distributions of open (A and C) and closed (B and D) dwell times of a single Na channel in the absence of divalent cations (A, B) and after addition of 5 mM Ba^{2+} (C, D) to the extracellular side (left) or the intracellular side (right). Membrane potential = -84 mV (l) and -98 mV (r). The graphs are plots of the number of openings (N) of duration t versus duration. Opening and closing rate constants were computed as the reciprocals of τ_c and τ_o, respectively. Methods are described in refs. 2 and 3.

We interpret our data to suggest that in the closed state a negatively charge component of the channel's gating apparatus is exposed to the outside where it can bind a divalent cation. The charged group moves into the channel protein as the channel opens but can only do so when the divalent cation dissociates, thus accounting for the preferential slowing of the opening rate. The effect of internal divalent cation is complementary, causing preferential slowing of the opening rate. As shown in Figure 4, we believe that these charges are the terminal charges on strings of negatively charges within the channel protein that move outward as the channel closes. Recent molecular biological studies have identified segments in the primary amino acid structure of the channel that might be candidates for these gating charges (6).

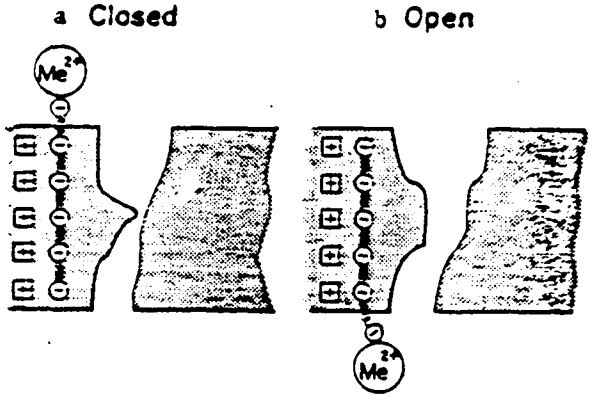

Figure 4. A hypothetical model for certain structural elements of the voltage-gated Na channel that is consistent with our results. Negative charges (⊖) may be aspartate or glutamate residues on a transmembrane helix, of which there are probably four. Countercharges (⊞) may be arginine residues on S4 (7). The terminal negative charge on the extracellular side of the channel is exposed and able to bind divalent cations only when the channel is closed (a). The internal terminal charge is exposed only when the channel is open (b); intracellular divalent cations can bind to this charge, immobilize the gating mechanism, and stabilize the open state.

D. Development and Modulation of STX/TTX-sensitivity of Sodium Channels in Rat Cortical Astrocytes.

In collaboration with Dr. Paul J. Yarowsky, Department of Pharmacology and Experimental Therapeutics, University of Maryland, we have been studying changes in the STX and TTX sensitivity of sodium channels in neonatal rat cortical astrocytes (7). These glial cells, previously thought to have few if any voltage-dependent channels, have recently been shown to have a variety of channel types similar to those in nerve and muscle cells (8). In early cultures (up to about 7 days), there is substantial BTX-activated ^{22}Na-influx but virtually no detectable high affinity ^{3}H-STX binding activity. Block of influx by STX behaved as a single site blocking reaction with a K_i of more than 100 nM. From day 7 to 12, high-affinity (K_d = 1 nM) ^{3}H-STX binding activity increased rapidly (Figure 5) with no overall change in BTX-activated influx. At two weeks or later, there were two components of block, one with a K_i estimated to be somewhat less than 1 nM and the other with a K_i of 100 - 500 nM (Figure 6). We interpret these results to indicate that initially, the astrocytes have only STX/TTX-insensitive sodium channels and that from 7 - 12 days in culture, some of these are replaced with (possibly newly synthesized) STX-sensitive channels that may be similar to

those in neurons with a K_d of about 1 nM.

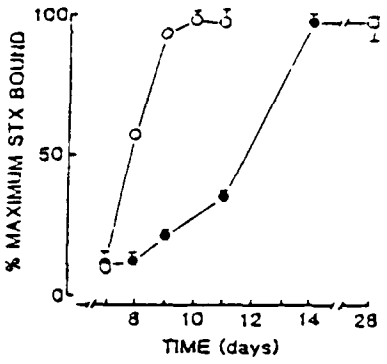

Figure 5. High affinity ^{3}H-STX binding to neonatal rat brain astrocytes. The density of STX binding sites spontaneously increases between day 8 and day 12. (●), control; (○), chemically defined medium added on day 7.

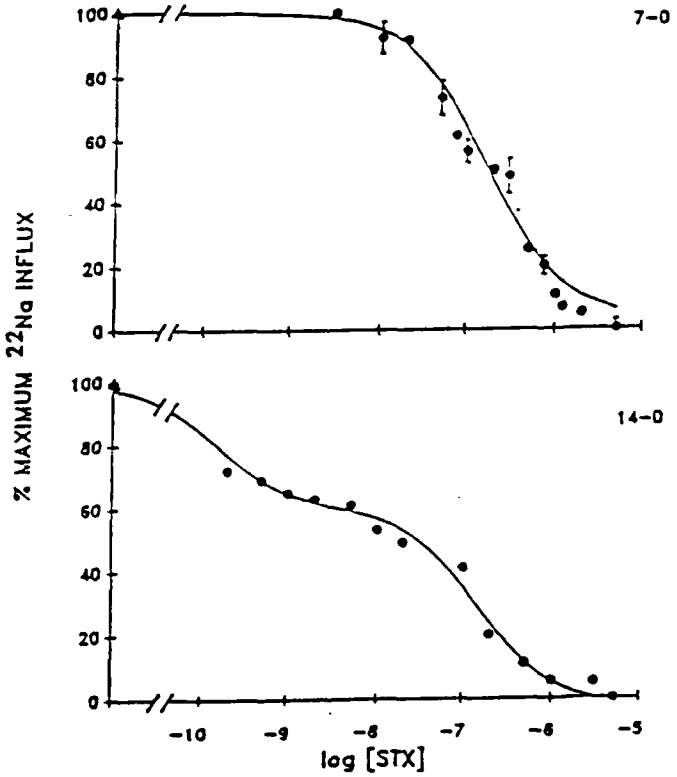

Figure 6. Two components of neurotoxin-stimulated ^{22}Na influx into neonatal rat brain astrocytes. Top (7 day) : All influx is blocked by STX with a single K_i of about 100 nM. Bottom (14 day): About 40% of the influx is blocked by about 0.5 nM STX and the remainder by 100-200 nM STX.

E. Batrachotoxin + scorpion venom activated sodium channels do not close.

In the presence of batrachotoxin (BTX), Na channels display voltage-dependent gating with a midpoint of the open probability (P_o) vs V_m relation being -90 to -100 mV (c.f., Fig. 1 and ref. 1). Scorpion venom (*L. quinquestriatus*) or α scorpion toxin, a polypeptide toxin purified from that venom, affects Na channel gating by slowing inactivation and by potentiating the activating effects of BTX. Scorpion toxin binds to the channels with high affinity (≈ 1-10 nM) at a site distinct from that of BTX or STX (9). We have found that addition of either scorpion venom or α scorpion toxin, in the presence of BTX holds the channels in an open configuration a potentials as negative as -140 mV (Fig. 7)

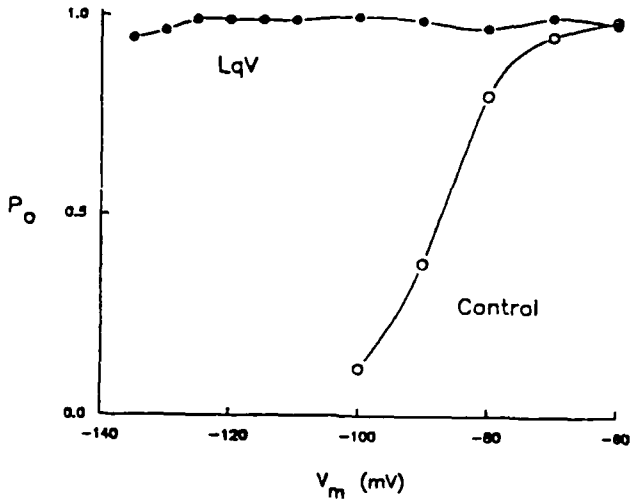

Figure 7. Probability of being open (P_o) vs V_m for single BTX-activated Na channels in the absence or presence of scorpion venom (*L. quinquestriatus quinquestriatus*). The channel is open nearly all of the time at potentials up to -140 mV, the most negative potential normally tolerated by the planar bilayer. (○), control; (●), + 100 nM scorpion venom (LqV).

This capability to monitor single channel current at very large negative potentials provides the opportunity to study previously unaccessible aspects of ion permeation and block and the mechanisms of STX/TTX block. For example, Ca^{2+} is only slightly permeable through Na channels and detection of single channel currents carried by Ca^{2+} is not possible with present methods. At very negative potentials, some Ca^{22+} may be driven past the blocking site resulting in release of block which may be detectable. STX block has been demonstrated to be voltage-dependent with hyperpolarizing potentials favoring block (decreasing K_i; refs. 10 and 11). The voltage-dependence of STX and TTX block is similar suggesting that the voltage dependence arises from a voltage-dependent conformational change in the toxin binding site rather than from entry of the toxin into the channel pore (12). This hypothesis could be directly tested by measuring block at very large negative potentials where a limiting high affinity state would be expected to be reached (13). The extended potential range available with the BTX+scorpion toxin-activated channels should provide this capability.

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