

Effects of Strychnine on the Sodium Conductance of the Frog Node of Ranvier

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ABSTRACT Strychnine blocks sodium conductance in the frog node of Ranvier. This block was studied by reducing and slowing sodium inactivation with scorpion venom. The block is voltage and time dependent. The more positive the axoplasm the greater the block and the faster the approach to equilibrium. Some evidence is presented suggesting that only open channels can be blocked. The block is reduced by raising external sodium or lithium but not impermeant cations. A quaternary derivative of strychnine was synthesized and found to have the same action only when applied intracellularly. We conclude that strychnine blocks sodium channels by a mechanism analogous to that by which it blocks potassium channels. The potassium channel block had previously been found to be identical to that by tetraethylammonium ion derivatives. In addition, strychnine resembles procaine and its derivatives in both its structure and the mechanism of sodium channel block.

INTRODUCTION

Strychnine has been shown to affect sodium currents in the squid giant axon (Shapiro et al., 1974). It has also been shown to produce a block of potassium currents in frog node of Ranvier that resembles in all respects that caused by tetraethylammonium ion and its derivatives (Shapiro, 1977). This paper is designed to elucidate the nature of the action of strychnine on sodium conductance in the node of Ranvier. There was some indication from the experiments on squid axons that strychnine produces a voltage- and time-dependent block of sodium channels. After depolarizing pulses, repolarizations generate sodium tail currents with a pronounced hump or hook on them. This might be an indication of the removal of strychnine block at more negative potentials. Should strychnine produce a voltage- and time-dependent block of sodium channels, the question arises as to what extent this block resembles the block of potassium channels. Further, are the blocks independent or somehow linked? Last, does any block of sodium conductance resemble that produced by other agents and, if so, does strychnine have any chemical features in common with these agents?

MATERIALS AND METHODS

The methods for voltage clamping the frog (*Rana pipiens*) node of Ranvier are identical with those of the previous paper (Shapiro, 1977). The internal composition of the

axoplasm can be changed by cutting the ends of the axon in a desired solution. Small molecules appear to diffuse readily along the core of the axon and to reach a final concentration in the axoplasm (as judged from Nernst potentials) of 40–80% of that in the pools in which the cut ends are immersed. The ends seem to seal and in order to introduce a compound later in an experiment one end was recut in a pool of altered composition. In some experiments the ends were initially cut in isotonic (120 mM) KCl. This is assumed to approximate the normal internal potassium concentration and gives action potentials and voltage clamp ionic currents close to those seen in uncut axons. In other experiments some of the potassium was replaced by sodium to produce an elevated level of internal sodium. In these cases some of the potassium was replaced by cesium to insure the abolition of potassium currents.

N-Methylstrychnine (NMS) was synthesized as in the previous paper. It was added to one end pool after the nerve was recut subsequent to measurement of control currents.

The standard Ringer's solution bathing the node contained 115 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 2 mM Tris (hydroxymethyl) aminomethane buffer, pH 7.4, and 10 mM tetraethylammonium chloride (TEA). The TEA was sufficient to abolish potassium currents, although in the nodes cut in 60 mM CsCl none were detected before TEA application. In low sodium solutions some or all of the sodium was replaced by tetramethylammonium chloride (TMA) (Aldrich Chemical Corp., Milwaukee, Wis.).

In all experiments the nodal membrane was held at -80 mV between stimuli. Each depolarizing pulse was immediately preceded by a conditioning pulse to -144 mV for 60 ms. This was found to be more than sufficient to remove all sodium inactivation. All measurements were carried out at 3°C.

RESULTS

Strychnine Blocks Sodium Channels

Strychnine reduces the peak sodium currents at low concentrations. The sodium currents before and after addition of 50 μ M strychnine are compared in Fig. 1. After strychnine treatment the currents during depolarization show a smaller and slightly earlier peak and a faster decay. After repolarization the tail currents increase to a peak and then decline much more slowly than in the control.

The peak sodium currents before and after strychnine for two different sodium concentrations are plotted against membrane potential in Fig. 2. As would be expected, the outward current in sodium-free Ringer's always exceeds that in standard Ringer's at corresponding potentials. In contrast, at membrane potentials above 45 mV the outward current in strychnine-treated nodes is higher in standard Ringer's. This implies that strychnine block is voltage and sodium dependent. The peak currents may actually not be a good measure of strychnine block since strychnine alters the kinetics of the decay of sodium currents. Strychnine either alters sodium inactivation or blocks sodium channels in a time-dependent way. Thus, the size of the peak currents in strychnine probably depends on the kinetics of strychnine block and of sodium activation and inactivation. However, if the kinetics of the block are relatively ion independent, then one important observation still holds. At large voltages strychnine block can produce an anomalous ion dependence so that an increase in external sodium results in a decreased outward sodium current.

A time-dependent strychnine block is better studied with nodes whose sodium inactivation is reduced, slowed, or abolished. Then sodium currents have a time

course similar to that of potassium currents and the magnitude and time course of strychnine block is directly observable.

Koppenhöfer and Schmidt (1968) described the reduction and slowing of sodium inactivation in an amphibian node of Ranvier treated with scorpion venom from *Leiurus quinquestriatus*. Using venom from this scorpion from two sources—Sigma Chemical Co., St. Louis, Mo., and Prof. E. Zlotkin, Hebrew University, Jerusalem—I found the reported selective effect on sodium inactivation after application of these venoms. A concentration of 1 $\mu\text{g}/\text{ml}$ was found to be ideal. It greatly slowed sodium inactivation and the magnitude of the effects was stable for periods up to 30 min. Higher concentrations produced a larger but less stable effect and often hastened the deterioration of the node. In all experiments an identical concentration of venom was added to all control and

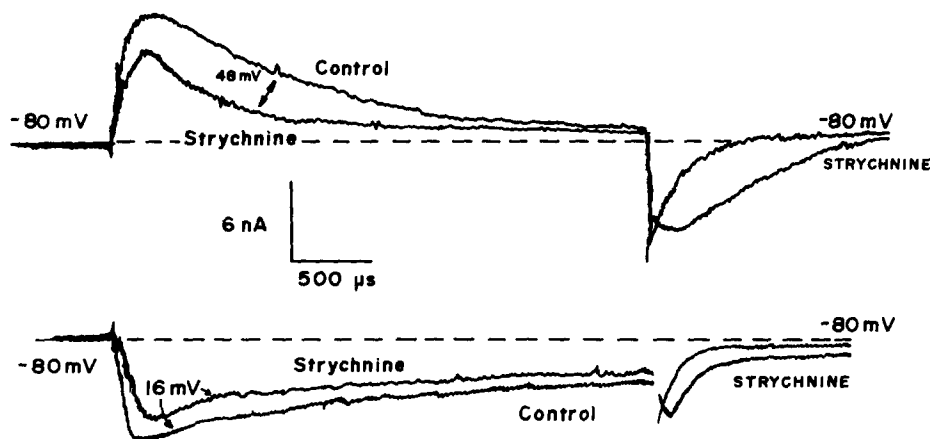


FIGURE 1. Sodium currents in a node cut in 60 mM NaCl, 60 mM CsCl. Control and 15 min after addition of 50 μM strychnine to bath. Top traces pulse to 48 mV; bottom to -16 mV.

test solutions. Only experiments which showed little change in sodium current shape and magnitude after washing were considered.

The effect of *Leiurus* venom treatment is shown in the sodium current trace labeled "control" in Fig. 3. Sodium inactivation is incomplete and slowed. The effects are more pronounced at more positive potentials. Upon repolarization a small residual inward current persists. The current tails decline monotonically to this low but measurable value. The residual inward current resembles the effects of venom from the scorpion *Centruroides* which has been found to alter sodium activation kinetics (Cahalan, 1975). The slowing of sodium inactivation by scorpion venom permits us to see the time dependence of the strychnine block. The decay of sodium current is much faster after strychnine application and there is a hump in the tail current after repolarization and an exceptionally slow decay of current. The time constant of this slow tail is also longer after a pulse which produces greater strychnine block.

In the venom-treated controls the sodium currents do decay during a depolarization, even if more slowly than before venom exposure. After strychnine

exposure the sodium currents decay in two major phases, one more rapid than in the control and other other slower and noticeable even 6 ms after the start of depolarization. The slow phase was found to be close to that seen in the control after 4 ms. Fig. 4 shows the ratio of sodium currents before and after strychnine

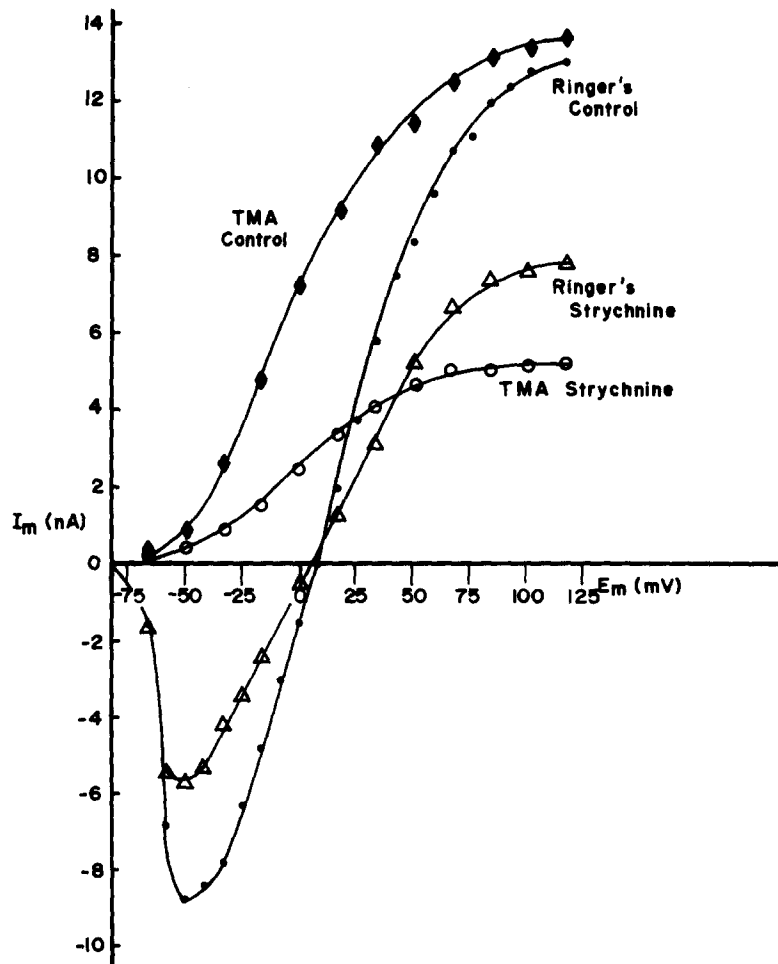


FIGURE 2. Peak sodium current as a function of voltage before and after addition of 50 μ M strychnine. Node cut in 60 mM NaCl, 60 mM CsCl. Filled circles: Ringer's, control; filled diamonds: Na-free, control; open triangles: Ringer's + strychnine; open circles: Na-free + strychnine.

treatment. The ratio approaches an asymptote, declining to within 5% of its final value after 5 ms. If there are two independent parallel inactivation or blocking processes, one introduced by strychnine and the other present both before and after strychnine treatment, then Fig. 4 would be a plot of the kinetics of the strychnine-dependent block of sodium channels. In no case was this function

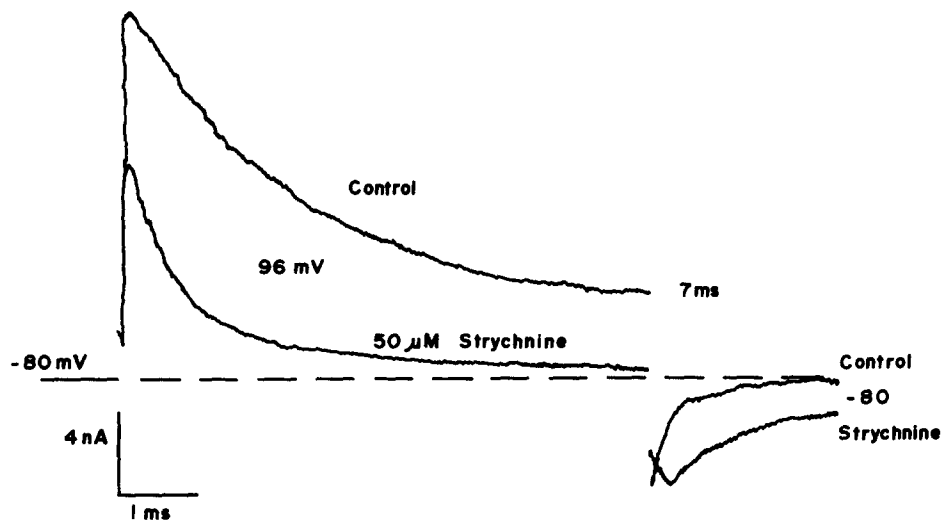


FIGURE 3. Sodium currents after addition of 1 mg/ml scorpion venom. Node cut in 60 mM NaCl, 60 mM CsCl. Pulse to 96 mV.

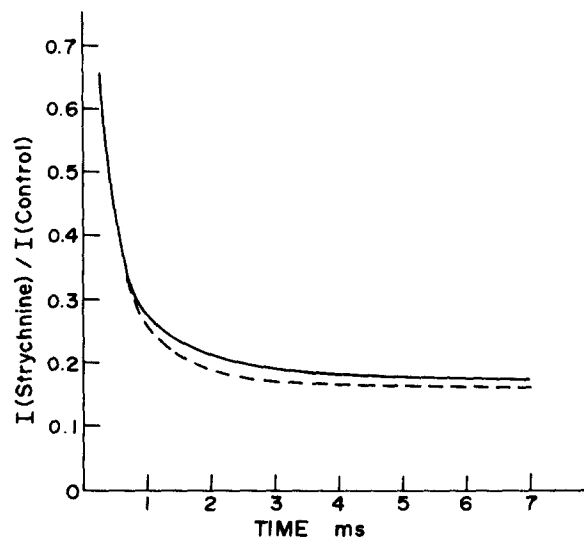


FIGURE 4. Ratio of sodium currents before and after strychnine during a pulse from -80 to $+96$ mV. Data from same experiment as Fig. 3. Node bathed in 1 mg/ml scorpion venom. Cut in 60 mM CsCl. Solid line is experimental measurements. Dashed line is an exponential decay function fit to the initial decay phase of the data. It has a time constant of 0.45 ms.

found to be fit by a simple exponential. If the early decay is fit by first-order blocking kinetics, then a small, slower residual block is always found. The strychnine block always was very close to its equilibrium level by 7 ms and the currents at that time are compared in the following section.

The Block is Voltage Dependent

The time course and magnitude of the current for several potentials is shown in Fig. 5. The outward equilibrium currents have a negative conductance region. That is to say, more depolarization produces less outward current. In addition,

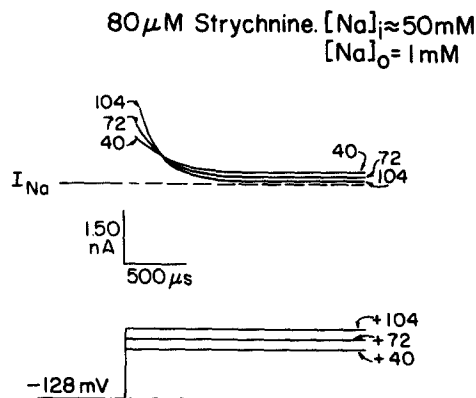


FIGURE 5. *Top traces*, Na currents in a node cut in 60 mM sodium and bathed in 1 mM NaCl, 80 μ M strychnine, and 1 μ g/ml venom. $[Na]_i$ calculated from Goldman equation is close to 50 mM. *Bottom traces*, Voltage records.

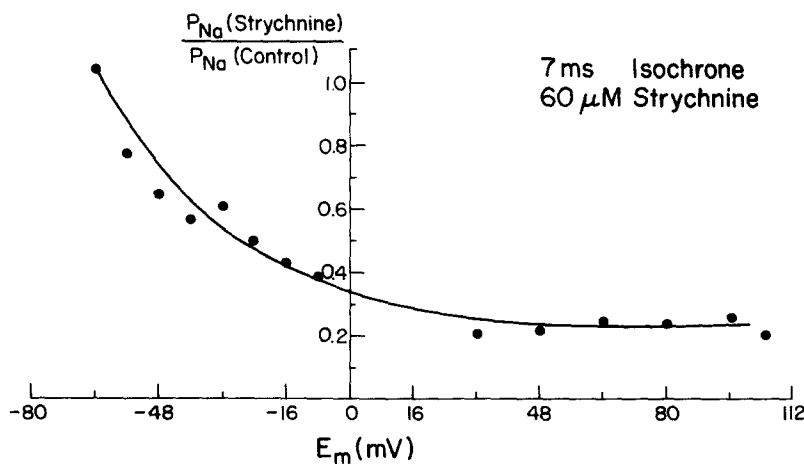


FIGURE 6. Strychnine block as a function of E_m for a node bathed in 60 μ M strychnine and 1 μ g/ml venom and cut in 60 mM NaCl, 60 mM CsCl. Block is determined by the ratio of P_{Na} s after depolarization of 7 ms (7 ms isochrone).

the time constants of this block are voltage dependent. The greater the depolarization, the faster the approach to equilibrium. Finally, the block is fast. In this case the time constant at 104 mV is about 100 μ s. Higher strychnine concentrations result in a greater, faster block. At concentrations of 1 mM, the block at large positive potentials is too fast to resolve with this voltage clamp.

The voltage dependence of the block for one node is shown in Fig. 6. The gap

in the data is around E_{Na} . The value for the small currents in the poisoned node is susceptible to considerable misestimation due to small errors in the leakage compensation. Errors introduced by the incomplete elimination of sodium inactivation by venom also make the curve more qualitative than quantitative.

The Block Depends on Sodium and Lithium Concentration

Reduction of the external sodium during strychnine exposure increased the block in venom-treated nodes (Fig. 7) as it did in nodes without venom treatment (Fig. 2). It is apparent from Fig. 5 that strychnine blocks inward as well as

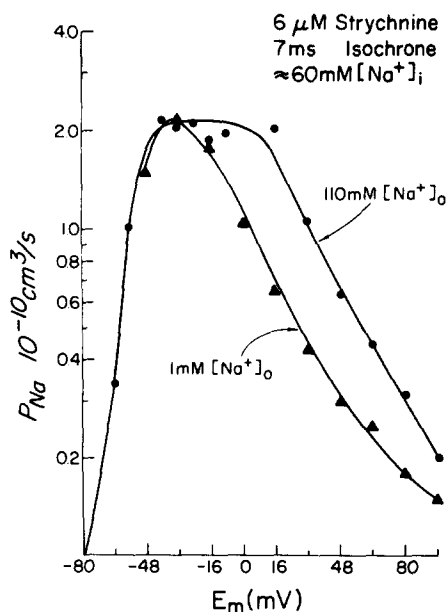


FIGURE 7. Sodium permeability (7 ms isochrone), calculated from the Goldman equation, as a function of E_m for high and low $[Na]_o$. Node bathed in $6 \mu M$ strychnine and $1 \mu/ml$ venom. Node cut in $60 mM NaCl$, $60 mM CsCl$. Filled circles, $110 mM [Na]_o$; filled triangles, $1 mM [Na]_o$.

outward current. There is the possibility that some of the block is current or sodium flow-dependent. If that were true the block should increase as E_{Na} is shifted to more negative potentials. That does indeed happen but the shift in the P_{Na}/E_m curve is less ($\approx 30 mV$) than the shift in E_{Na} ($>80 mV$).

Replacement of sodium by potassium, cesium, or tetramethylammonium ions gave in each case an approximately equivalent increase in the strychnine block. The lower the external sodium, the greater the block. In all cases there is a potential region over which reduction of $[Na]_o$ gives the anomalous decrease of outward I_{Na} . By contrast, replacement of Na by Li did not enhance strychnine block. Hille (1972) has observed in normal fibers that the ionic conductance is reduced by about 20% in lithium Ringer's and that the permeability-voltage curve is shifted about 3 mV in the depolarizing direction. I confirm the small

reduction in both inward and outward currents after replacement of external sodium by lithium. Fig. 8 shows the ratio of ionic permeabilities before and after strychnine in 110 mM Na_o and 110 mM Li_o. In this experiment and in four others in which P_{Li} and P_{Na} were compared after strychnine, the permeabilities in lithium were never reduced by more than 25%. This replacement by lithium was quite different from replacement by cesium, potassium, or TMA which enhanced the block of sodium channels.

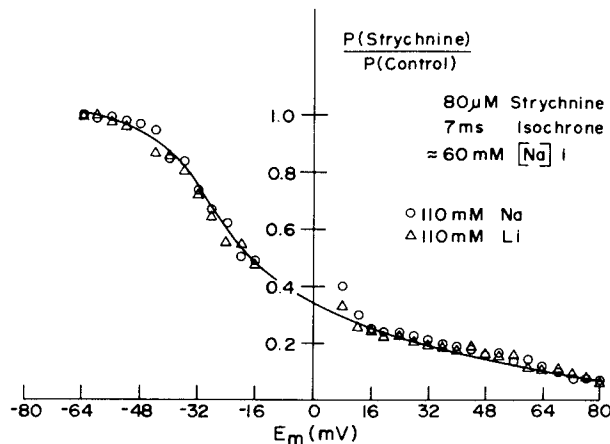


FIGURE 8. Blocking ratio (7 ms isochrone) as a function of membrane potential for a node bathed in either 110 mM sodium (circles) or 110 mM lithium (crosses). Strychnine 80 μ M. All solutions contain 1 μ g/ml venom. Node cut in 60 mM NaCl, 60 mM CsCl.

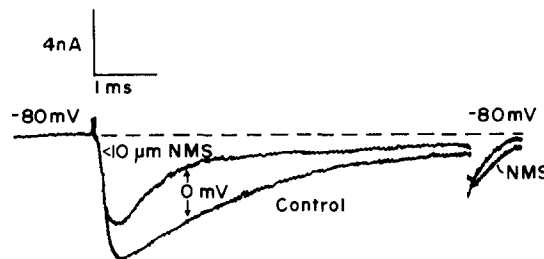


FIGURE 9. Sodium currents in a node before and after cutting in 10 μ M NMS. Node bathed in Ringer's, cut in 120 mM KCL. Pulse to 0 mV.

NMS Blocks Sodium Channels from the Inside

The sodium currents of nodes were compared before and after recutting one end of the fibers in NMS. A characteristic strychnine-like voltage and time-dependent block was always observed after recutting in NMS (Fig. 9). In contrast, addition of 500 μ M NMS to the external bath had no observable effect. The progressively developing block seen in potassium channels with external NMS (Shapiro, 1977) is not seen in sodium channels. Since this block is only very slowly and partially reversible, it may mean that the potassium channels are

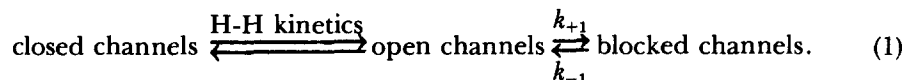
more susceptible to a generally toxic, perhaps detergent, effect of 0.5–1 mM NMS. The study of the effects of internal NMS is much less complete than that of those on the tertiary base. However, as for potassium channels, the effects of the tertiary base and its derivative are similar or identical in all particulars studied.

DISCUSSION

By reducing and slowing sodium inactivation we have been able to observe strychnine block of sodium channels. Strychnine produces a time- and voltage-dependent block. The rate and extent of the block increases with depolarization. The block is inversely related to the external sodium or lithium concentration. Both net inward and outward sodium currents are blocked but outward currents are always blocked more.

NMS produces a strychnine-like block when applied internally but not when applied externally. If we assume that the mechanisms of action of NMS and strychnine are the same, then strychnine must act from the axoplasmic side after crossing the membrane. NMS, which is a quaternary derivative of strychnine, must cross the membrane very slowly if at all, and thus require direct introduction into the axoplasm in order to block sodium channels.

How does strychnine block of sodium channels compare with its block of potassium channels? Evidence is presented in the previous paper (Shapiro, 1977) that strychnine blocks potassium channels in the same manner as do certain quaternary amines (QA) when applied intracellularly. These have been well studied by Armstrong (1969, 1971). The basic mechanism postulated by Armstrong (1969) is that amines block only open channels in the following fashion.



Armstrong deduced his mechanism from several kinds of two-pulse experiments. The equations, both in the original form and with some modification, have been solved on a computer and found to reproduce closely the experimental behavior of the currents in almost all regards.

In this paper two-pulse experiments were not attempted. The persistence of some inactivation even after venom treatment makes it far more difficult to measure and interpret such experiments. However, one feature of strychnine block of potassium channels is also seen in strychnine-treated sodium channels. The tail currents after repolarization are altered by strychnine. In the untreated nodes the tails decline monotonically to zero. After strychnine exposure the tails first peak and then decline more slowly than the controls. Conditions which increase the amount of block during the depolarization—more positive pulse, longer pulse duration, higher strychnine concentration—produce a more conspicuous hump in and a slower decay of the tail currents (Fig. 1). The same characteristic humps in the potassium tail currents, modified by the same conditions, are seen after strychnine exposure (Shapiro, 1977). The explanation proposed by Armstrong for potassium tail currents would also fit the sodium tail currents. If a large percentage of the open channels have been blocked by

strychnine during the depolarization, then upon repolarization some channels become unblocked while open channels are closing. If enough channels are initially blocked then we may observe a transient increase in the number of open channels. Subsequently, the rate of decline of the number of open channels depends on both the H-H rate constant for closing and the rate of removal of strychnine block. Such behavior is well modeled by sequential reactions of the form of Eq. (1).

The simplest blocking model postulates that strychnine block obeys first-order kinetics. The block should approach its equilibrium value exponentially as is observed in strychnine block of potassium channels. The data in this paper are not sufficiently precise to ascertain the order of reaction for sodium channels.

Sodium channel block is faster than that of potassium channels at comparable levels of block. The difference is more than an order of magnitude. Strychnine blocks sodium channels at lower concentrations; NMS is also effective at lower concentrations. If the sodium channel block is by the same mechanism, then both k_{+1} and k_{-1} are much larger for sodium channels, and the dissociation constant of the site is lower.

Much of the analysis of potassium channel block relies on the shape of the block-voltage function and the fact that it can be derived directly from the Boltzmann equation. The sodium channel data are simply not reproducible enough to allow any conclusions based on the form of the relationship. However, the results are qualitatively consistent with the model proposed for potassium channel block. As the axoplasm is made more positive, strychnine cation or NMS is driven into the binding site by raising the free energy of the charged base in the axoplasm relative to base at the binding site. The binding site is part-way down the membrane electric field. This drives the equilibrium in Eq. (1) toward the right and speeds up the approach to equilibrium.

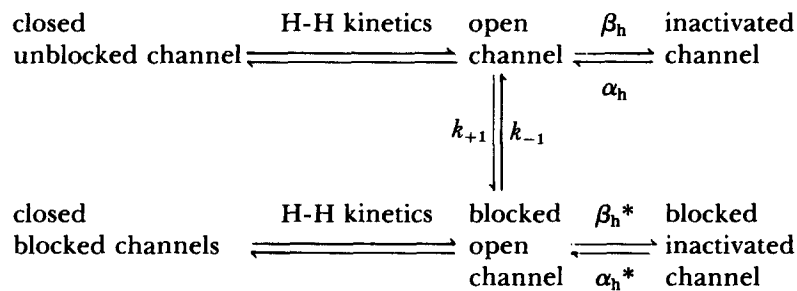
If we increase the inward flow of cations through sodium channels we decrease the amount of block. The direction of the voltage dependence is consistent with this. Increasing the external concentration of sodium or lithium, two ions which most readily transit sodium channels, should increase inward flux. Strychnine block is also decreased by this change. Changes in impermeant monovalent cations have no effect on strychnine block. These results are analogous to the findings on strychnine-blocked potassium channels. There, only external potassium ions interfere with potassium channel block.

Better kinetic data are required before we can distinguish among various possible mechanisms of interference of strychnine block by inward sodium current. One possibility is that the positive charge on the permeant cations could repel cationic strychnine or NMS. Inward-flowing ions must first cross the selectivity filter before approaching the blocking site closely enough to remove the strychnine.

Hille (1975) has discussed a four-barrier model for the sodium channel. In this model external H^+ crosses one barrier to bind at a site part-way down the membrane field. This blocks the passage of sodium ions which must also bind at this site as they pass through the pore (see Woodhull, 1973). Strychnine could block in a similar way from the other side of the membrane, crossing one or two energy barriers to bind at a different site from that of H^+ but a site which is also

occupied by sodium ions as they transit the pore. However, Hille's "one-ion" model allows only one ion in any of the three energy wells. An H^+ or other impermeant ion should still interfere with strychnine binding since there is a binding site external to the selectivity filter. One alternative model permits each energy valley to be occupied by an ion but prohibits the ions from passing one another. This is a "single-file" pore. In such a model strychnine block would only be reduced by permeant ions. Armstrong (1971) has shown that a single-file model of potassium pores explains many of the details of amine block of such pores. The strychnine experiments provide some of the first evidence for similar kinetic behavior of ions through sodium pores.

The most notable agents which block sodium channels in a voltage-dependent manner are certain lidocaine derivatives (Strichartz, 1973; Courtney, 1975) and perhaps lidocaine itself (Tritthart et al., 1971). The evidence strongly supports the hypothesis that these and other local anesthetics block sodium channels only from the axoplasmic side and act in their cationic forms. Courtney has proposed a model for the mechanism of block of one lidocaine derivative, GEA 968:



If we eliminate the two inactivation reactions then GEA 968 block would greatly resemble strychnine block of venom-treated channels (Eq. [1]). However, if the inactivation reactions are not altered by venom or other agents, then the course of block during a depolarizing pulse would only be apparent if $k_{+1} + k_{-1}$ were sufficiently large so that the channel was blocked at least as fast as it was inactivated. If the blocking reaction were slow then block would not be apparent during a single depolarizing pulse. Courtney proposes that blocked channels have an altered forward inactivating rate constant, β_h^* . The change is such that at a given potential the fraction (blocked-inactivated channels)/(total blocked channels) is greater than the fraction (unblocked-inactivated channels)/(total unblocked channels). This is such that at resting potential almost all of the blocked channels are inactivated while most unblocked are not. In such a model, although amine block is not apparent during an individual depolarization, the number of blocked channels is dependent on the frequency of stimulation. During a train of depolarizations the peak sodium current declines as more and more channels are tied up in the blocked-inactivated pool.

M. Cahalan and I (Cahalan and Shapiro, 1976) have seen a similar frequency-dependent block of squid giant axon sodium channels by strychnine. If sodium inactivation is destroyed by internal perfusion with pronase then we observe a strychnine-induced block of sodium currents to some plateau level. Most notably, pronase perfusion abolishes the frequency dependence. This would be

predicted by Courtney's model. The difference between strychnine and lidocaine derivatives could be explained by differences in k_{+1} and k_{-1} alone.

Last, the structural resemblance between strychnine and lidocaine and their derivatives is noteworthy. Lidocaine contains a tertiary nitrogen atom and an amide nitrogen adjacent to a benzene ring. The amide and tertiary nitrogens are separated by two carbons. The derivatives studied by Strichartz differ from this pattern in that one nitrogen is quaternary rather than tertiary. GEA 968 differs in the distance between amide and tertiary nitrogens. There are one nitrogen and four carbon atoms between them. Strychnine also contains a tertiary nitrogen and an amide nitrogen adjacent to a benzene ring. They are separated by three carbons. In structure as well as action strychnine resembles both local anesthetics and Armstrong's quaternary amine blockers.

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