

Trimethyloxonium Modification of Single Batrachotoxin-activated Sodium Channels in Planar Bilayers

Changes in Unit Conductance and in Block by Saxitoxin and Calcium

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ABSTRACT Single batrachotoxin-activated sodium channels from rat brain were modified by trimethyloxonium (TMO) after incorporation in planar lipid bilayers. TMO modification eliminated saxitoxin (STX) sensitivity, reduced the single channel conductance by 37%, and reduced calcium block of inward sodium currents. These effects always occurred concomitantly, in an all-or-none fashion. Calcium and STX protected sodium channels from TMO modification with potencies similar to their affinities for block. Calcium inhibited STX binding to rat brain membrane vesicles and relieved toxin block of channels in bilayers, apparently by competing with STX for the toxin binding site. These results suggest that toxins, permeant cations, and blocking cations can interact with a common site on the sodium channel near the extracellular surface. It is likely that permeant cations transiently bind to this superficial site, as the first of several steps in passing inward through the channel.

INTRODUCTION

Saxitoxin (STX) and tetrodotoxin (TTX) are highly specific and potent blockers of the voltage-dependent sodium channels responsible for the inward sodium current during the action potential in nerve and muscle. The specificity of the interaction between STX and TTX and their binding site offers the opportunity to use these toxins as probes of the molecular structure of the channel (cf. Ritchie and Rogart, 1977). Several lines of evidence suggest that an ionized carboxyl group, located near the extracellular surface of the voltage-activated sodium channel, is associated with the binding site for the blocking neurotoxins STX and TTX. Radiolabeled toxin binding experiments using membrane vesicles

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(Reed and Raftery, 1976; Weigele and Barchi, 1978*a, b*) and intact excitable membranes (Henderson et al., 1973), as well as electrophysiological recordings (Hille, 1968), have demonstrated the involvement of an acidic site (pK_a , ~ 5) in toxin binding and block. These results suggest that a carboxyl or phosphate group might be associated with the toxin receptor. Henderson et al. (1973, 1974) studied the effects of monovalent and divalent metal cations and pH on TTX and STX binding to intact nerve fibers and a nerve membrane preparation. Their results led to the suggestion that the toxin binding site may be a coordination site for the cations as they pass through the sodium channel.

Evidence for an electronegative binding site also comes from studies of cation permeation through sodium channels. This site is apparently located deep within the channel, such that ions sense a significant fraction of the transmembrane voltage as they move toward the site. It is thought that this site forms part of the "selectivity filter" (Hille, 1975) that serves to limit the transit rates of permeant ions. Hydrogen ions cause a voltage-dependent reduction in the sodium current, with an apparent pK_a close to 5.0 (Hille, 1968; Woodhull, 1973; Begenisich and Danko, 1983; but see Campbell, 1982). Extracellular cations have also been shown to reduce sodium currents in a voltage-dependent fashion (Woodhull, 1973; Yamamoto et al. 1984; French et al., 1986*b*). In addition, the metal cation selectivity sequence for the sodium channel, in a wide variety of tissues, closely follows that expected for a channel with a carboxyl group in the permeation pathway (Eisenman, 1962; Hille, 1972). These studies suggest that there is an electronegative group, within the sodium channel pore, which appears to be important in lowering the free energy required to replace the water of hydration from a metal cation as it passes through the sodium channel. Because of the similarities between the cation binding site revealed by toxin interactions and that revealed from ion permeation rates, Hille (1975) and Reed and Raftery (1976) have suggested that the toxin binding site and the selectivity filter are identical.

Evidence that the selectivity filter is separate from the TTX/STX binding site has come from studies using the carboxyl side-chain modifiers carbodiimide or trimethyloxonium tetrafluoroborate (TMO), which render sodium channels insensitive to TTX without greatly altering ion permeation through the channels (see discussion and references in Brodwick and Eaton, 1982). Sigworth and Spalding (1980) and Spalding (1980) demonstrated that sodium currents in frog node of Ranvier and skeletal muscle are insensitive to TTX after TMO modification. They also found that, after TMO modification, sodium currents were reduced, selectivity among organic cations was unaffected, and the sensitivity of sodium currents to low pH was reduced. These results suggested that, in contrast to the conclusions of Hille (1975) and Reed and Raftery (1976), the toxin receptor site is distinct from the domains of the sodium channel responsible for ion selectivity, which are apparently inaccessible to larger, complex cations, such as TTX and STX.

STX and TTX block of batrachotoxin (BTX)-activated sodium channels in planar lipid bilayers is voltage dependent, with hyperpolarizing potentials favoring block (Krueger et al., 1983; French et al., 1984; Moczydlowski et al., 1984*a, b*). The voltage dependence of toxin block is apparently due to a voltage-

dependent conformational change in the structure of the toxin binding site rather than to entry of the toxin into a site deep within the membrane electric field (Moczydlowski et al., 1984*a, b*; Green et al., 1984). It has been suggested that voltage-dependent block by STX and TTX is unique to BTX-activated channels (Rando and Strichartz, 1985).

We report here the effects of modification by TMO of the STX binding site on single BTX-activated sodium channels in planar lipid bilayers. TMO, via what appears to be methylation of a single site, affected the interaction between the channel and permeant ions (sodium), nearly impermeant cations (calcium), and blocking neurotoxins (STX, TTX). We suggest that this site is the binding site for STX and TTX and that it may also act as a prefiltering site for permeant cations before their translocation through the sodium channel pore in the absence of blockers. We suggest further that some of the inhibitory effects of calcium and other divalent cations on ion movement through the channel might be due to competitive binding of calcium and permeant cations at this site.

MATERIALS AND METHODS

Materials

STX was provided by J. E. Gilchrist, FDA, Cincinnati, OH. [³H]STX was obtained from Dr. G. R. Strichartz, Harvard Medical School, Boston, MA, and from Dr. S. Davio, U.S. Army Medical Institute for Infectious Diseases, Fort Detrick, MD. BTX was a generous gift of Dr. John Daly, National Institutes of Health, Bethesda, MD. Phospholipids were obtained from Avanti Polar Lipids, Inc., Birmingham, AL. Decane was repurified on layers of neutral, acidic, and basic alumina before use. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Planar Bilayers

Planar lipid bilayers were painted from a solution of bovine brain phosphatidylethanolamine (PE) in decane across a 250- μ m hole in a polystyrene or Lexan partition separating two buffered salt solutions. These two solutions contained identical concentrations of NaCl (125 mM), HEPES (10 mM), MgCl₂ (100 μ M), and EGTA (50 μ M), pH 7.0. Calcium was also present where indicated. All experiments were done at ambient temperature (22–24°C). Command potentials were applied and transmembrane current was measured via Ag/AgCl electrodes connected to a homemade voltage-clamp circuit (French et al., 1986*a*). Current records were stored on FM tape and replayed on a stripchart recorder or a digital oscilloscope (2090, Nicolet Instrument Corp., Madison, WI) for measurement of single channel currents. In experiments where 10 mM calcium was added asymmetrically, a correction was made for a 3-mV electrode offset.

To incorporate sodium channels, P₃ (2–10 μ g protein/ml, see below) was added to the *cis* side of a preformed bilayer. BTX (60 nM) was normally present on the *cis* side, although identical results have been obtained with BTX on only the *trans* side. Transmembrane current was monitored at a holding potential of –60 mV and channel incorporation was detected by a stepwise jump in current of 1.5 to 5 pA, which reflects the incorporation of one to three channels. Approximately equal rates of channel incorporation were obtained in the nominal absence of Ca²⁺ (50 μ M EGTA, 100 μ M Mg²⁺) or with 100 μ M free Ca²⁺ on both sides of the bilayer. In nearly all cases, a single incorporation event occurred and experiments were completed without perfusion of the *cis* chamber and without further channel incorporation. Subsequent changes in solution composition were made by additions from concentrated stock solutions with gentle stirring.

The voltage convention used was the normal cell physiological convention of $E_{\text{mem}} = E_{\text{inside}} - E_{\text{outside}}$, with the outside of the bilayer defined as the side from which STX or TTX blocked before TMO modification. With this convention, channels tended to close with increasingly negative potentials.

[³H]STX Binding Assay

The binding of [³H]STX to membrane vesicles was determined by filtration on glass fiber filters (Weigele and Barchi, 1978a; Krueger et al., 1979). Membrane fractions (~1 mg/ml protein) were incubated for 20 min at 0°C in the presence of various concentrations of [³H]STX and 125 mM NaCl, 10 mM HEPES, pH 7.0. Excess unlabeled STX (5 μM) was also present in some samples for determination of nonspecific binding. Samples were rapidly filtered over glass fiber filters (type 25, Schleicher & Schuell, Inc., Keene, NH, or GF/C, Whatman, Inc., Clifton, NJ) under vacuum and washed with 10 ml of ice cold 125 mM NaCl, 10 mM HEPES, pH 7.0. The filters were counted by liquid scintillation spectroscopy and the amount of specifically bound STX was calculated from the radioactivity retained on the filters after subtraction of nonspecific binding.

Membrane Preparation

Rat brain membrane vesicles (P₃) were prepared by a modification of the method of Krueger et al. (1979). Four forebrains were placed in 60 ml chilled sucrose solution containing 0.32 M sucrose and 10 mM HEPES, adjusted to pH 7.4 (0°C) with 5 M NaOH and homogenized for 30 s using an Ultra-Turrax (Tekmar Co., Cincinnati, OH) homogenizer. The suspension was diluted with 3 vol of sucrose solution and centrifuged at 1,000 g for 15 min. The supernatant (S₁) was gently aspirated and saved. The pellet was resuspended in 40 ml sucrose solution, homogenized in a glass-Teflon homogenizer, and centrifuged again at 1,000 g for 15 min. The pellets (P₁) were discarded and the supernatants (S₁) were aspirated and pooled with the first S₁ fraction. The pooled supernatants were then centrifuged at 10,000 g for 20 min. The supernatant (S₂) was gently aspirated and saved on ice as before. The pellets (P₂) were homogenized and resuspended again as described above and centrifuged at 10,000 g for 20 min. The pellets were discarded and the supernatant was collected and pooled with the first S₂ fraction. The pooled supernatants (S₂) were then centrifuged at 100,000 g for 45 min. The supernatants (S₃) were discarded (and contained no measurable [³H]STX binding) and the pellets (P₃) were homogenized, resuspended, and centrifuged again at the same speed. The pellet (P₃) was resuspended in 0.4 M sucrose in 7–10 ml, giving a final protein concentration of ~10 mg/ml. The P₃ suspension was then divided into 0.1-ml aliquots and stored at -77°C. For bilayer experiments, P₃ was diluted 1:10 with 0.4 M sucrose.

The binding of [³H]STX to the P₃ fraction was specific and saturable, with an apparent dissociation constant between 0.8 and 1.2 nM at 0°C (Krueger et al., 1979). For each new P₃ preparation, the K_d was determined from a complete binding curve from 0.25 to 25 nM STX. The maximal binding ranged from 5 to 10 pmol/mg protein. Although fraction P₃ contained at least 70% of the [³H]STX binding sites of the whole brain homogenate and was used in all experiments reported here, the other subfractions (P₁, P₂) were routinely prepared as described above and tested for [³H]STX binding activity. P₃ gave a more consistent incorporation of sodium channels in bilayers than did P₁ and P₂. The highest specific activity was found in the P₃ fraction and represented a 5–10-fold enrichment in [³H]STX binding activity over the crude homogenate.

Virtually all of the toxin binding sites are located on the outside surface of the vesicles (Krueger et al., 1979). This implies that the channels in these vesicles are in an outside-out orientation, as both block and binding of STX and TTX occur only on the extracel-

lular side of intact nerve fibers (Narahashi et al., 1966). Incorporation of sodium channels into planar bilayers usually results in the STX blocking site facing the *cis* side.

Modification of Sodium Channels by TMO

For modification of the toxin binding site in rat brain membrane vesicles, solid TMO was added to ~1 mg/ml of membrane vesicles (P_3), which had been resuspended in 125 mM NaCl, 50 mM HEPES buffer, pH 7.0 (sodium solution), to give a final concentration of 50 mM. The pH of the membrane vesicle suspension was maintained at constant pH by adding microliter quantities of 1 M NaOH as necessary. The reaction mixture was incubated on ice for 8–10 min after the addition of TMO. All reaction byproducts were removed by centrifugation for 10 min (15,000 g). The pellet containing the modified sodium channels was resuspended in fresh sodium solution. In protection experiments, the membrane vesicle suspension was preincubated on ice with STX or calcium for 1 h before the addition of TMO. The binding of [3 H]STX to brain membrane vesicles before and after modification by TMO was determined by filtration on glass fiber filters as described above.

Single sodium channels were also modified by TMO after their incorporation into planar bilayers. One or two channels were incorporated as described above, except that the HEPES concentration was 50 mM. Control records were then taken to verify normal sodium channel behavior and to measure the single channel conductance. An appropriate amount of solid TMO was then added to the extracellular side of the channel to give a final concentration of 50 mM. Then, within 5 s, 50 μ l of 1 M NaOH was added to the same side to maintain the pH at 7.0. After 1 min of stirring, 50 nM STX or TTX was added to the same side (extracellular side) to determine whether TMO had rendered the channels insensitive to toxins. Current records were then recorded at different voltages. At the end of each experiment, the pH of the solution was measured to confirm that the final pH was between 6.8 and 7.2.

In ~20% of the attempts to modify sodium channels, we failed to observe any change in toxin sensitivity or single channel conductance after the addition of the carboxyl-modifying reagent. We presume that this was due to the labile nature of the reagent in aqueous solution, particularly at room temperature (~24°C). The half-life of alkyloxonium derivatives in an aqueous solution is between 1 and 8 min at 5°C and is very temperature dependent (see Baker and Rubinson, 1977; Spalding, 1980). In order to minimize reagent breakdown, solid TMO, which had been preweighed and stored at 4°C no longer than a few hours in a sealed test tube, was added directly to the extracellular side of the channel with brisk stirring.

RESULTS

Effect of TMO Modification on the Properties of Single Sodium Channels

Fig. 1 illustrates the modification of single BTX-activated sodium channels by TMO. Sodium channels from rat brain were incorporated into membranes composed of PE, with the identical solution composition on both sides to minimize asymmetries in membrane surface potential. Fig. 1A shows a normal, BTX-activated sodium channel that displayed occasional transitions between the open and closed states, as shown by the brief closing events lasting up to a few tens of milliseconds. Many of these closing events were too brief to be resolved at the recording bandwidth (150 Hz). These events reflected the voltage-dependent closing and opening of BTX-activated channels and became more frequent at

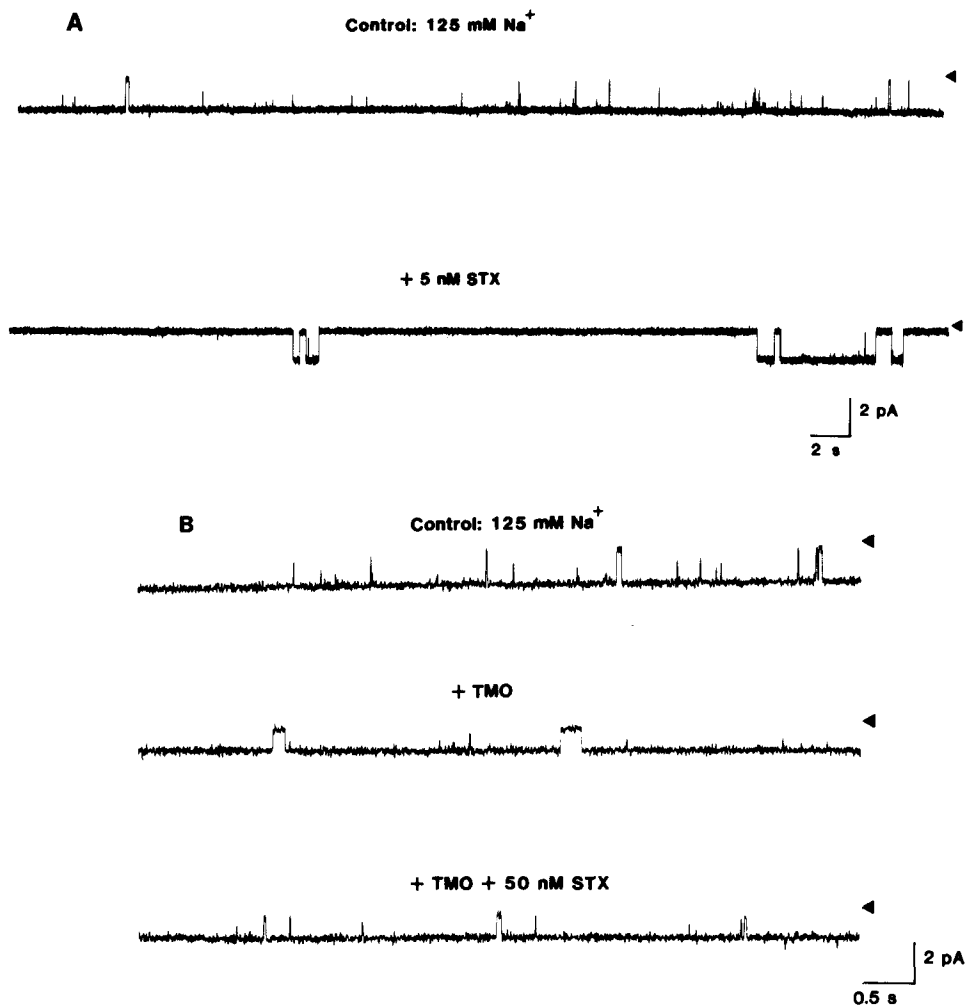


FIGURE 1. Consequences of TMO modification. (A) A single sodium channel was incorporated into a planar bilayer with 125 mM sodium on both sides. The current fluctuations were recorded at -60 mV. The zero-current levels are indicated by arrowheads, so that upward current deflections represent channel closings. In the absence of STX, the channel spent $>98\%$ of the time in the open, conducting state (top). When 5 nM STX was added to the extracellular side (bottom), the channel was in the closed or blocked state most of the time, with only infrequent openings as the STX molecule dissociated from the blocking site. (B) Current recordings from a different channel under the same conditions as A. When TMO (50 mM) was added to the extracellular side, the single channel current was reduced by 37% (middle record). In this experiment, modification had already occurred by the end of the stirring period, which lasted 30 s. When 50 nM STX was added to the extracellular side of the TMO-modified channel, there were no STX-induced, long-lived closing or blocking events as in A. The STX concentration was 10 times higher in B than in A. Note the difference in time scale for A and B. Records were filtered at 150 Hz.

hyperpolarized potentials (Krueger et al., 1983; French et al., 1984). At -60 mV, the channel remained open most of the time ($>98\%$). Previous studies have shown that these channels are selective for sodium over potassium and are sensitive to STX and TTX (Krueger et al., 1983). Upon addition of STX (5 nM) to the extracellular side, long-lived "closing" events caused by toxin block were observed. The single channel conductance before and after toxin addition was unchanged. Toxin block is clearly resolved as an all-or-none event, in contrast to the effect of less potent blockers like calcium (see below), which reduce the apparent single channel conductance (cf. Yellen, 1984). These channels also displayed voltage-dependent STX and TTX block in which depolarizing potentials favor an unblocked state (French et al., 1984; Moczydlowski et al., 1984a).

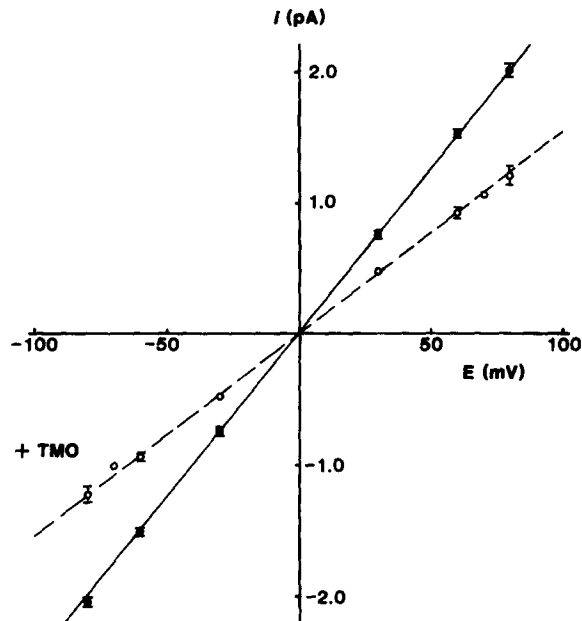


FIGURE 2. Single channel current-voltage relationships for normal and TMO-modified sodium channels. The conductance before the addition of TMO was 25 ± 0.6 (SEM) pS (solid circles). After TMO modification, the single channel conductance was reduced to 15.8 ± 0.3 (SEM) pS (open circles, nine experiments).

Fig. 1B illustrates the consequences of modification by TMO. After the addition of 50 mM TMO, the single channel current at -60 mV was reduced by 37% . Moreover, STX failed to block the channels at a concentration (50 nM) that would normally block unmodified sodium channels $>96\%$ of the time at this potential. TMO-modified sodium channels were also insensitive to TTX (100 nM). It has not been determined whether toxin binding is completely lost or the affinity is greatly reduced; however, the affinity for toxin would have to be decreased by at least two orders of magnitude to account for this result. Fig. 2 shows the single channel current-voltage relationship before and after TMO modification. TMO lowered the single channel conductance from 25 ± 0.6 to

15.8 ± 0.3 pS; the relationships were linear over the voltage range studied both before and after TMO modification.

Reduction of Single Channel Conductance and Toxin Sensitivity

When STX (50 nM) was added before TMO, alterations in channel function were not observed (four experiments). With two or more channels in the bilayer, it was occasionally observed that one was modified, whereas the other was not

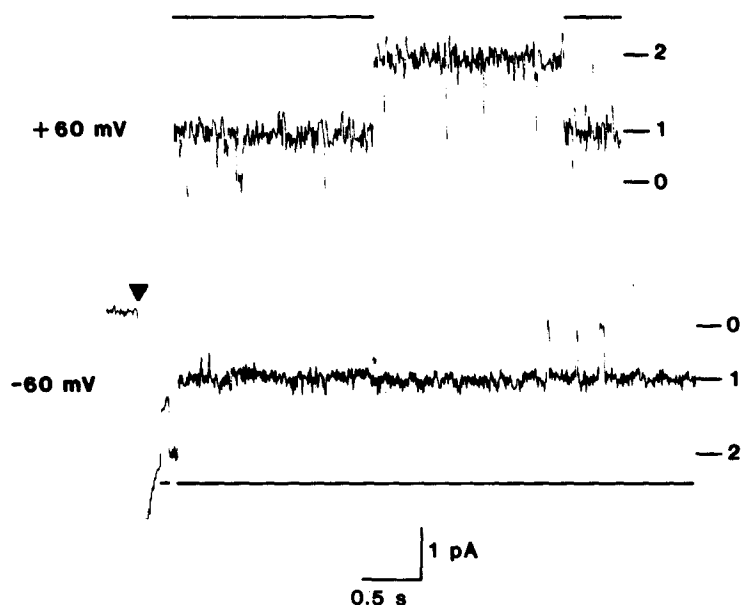


FIGURE 3. Single channel current fluctuations from a planar bilayer containing both an unmodified sodium channel and a TMO-modified sodium channel. These records were taken at +60 and -60 mV in the presence of 50 nM STX. For the current record at the bottom, the arrowhead indicates when the potential was changed from 0 to -60 mV. The open states of the two channel types (unmodified channel at level 2; TMO-modified channel at level 1) are indicated at the right. The horizontal lines indicate periods during which the unmodified channel was blocked by STX. The channel with the larger conductance displayed voltage-dependent block by STX, whereas the STX-insensitive channel was open most of the time. Note the STX blocking and unblocking events on the capacitive transient when the voltage was stepped to -60 mV.

(Fig. 3). At +60 mV, an unblocked state is favored for normal, unmodified STX-sensitive sodium channels with a K_d for block of ~ 80 nM. At -60 mV, block is much more potent and the K_d is ~ 2 -4 nM (French et al., 1984). Fig. 3 shows recordings from a bilayer containing two sodium channels, only one of which was modified by addition of TMO. The modified, lower conductance sodium channel was never blocked and the single channel current was ~ 0.9 pA. However, the unmodified sodium channel, which could be blocked by STX, had a single

channel current of ~ 1.4 pA. Even after extended periods of recording, TMO-modified channels with reduced conductance never displayed the characteristic long-lived blocked states of unmodified channels. At -60 mV, the unmodified sodium channel was blocked by STX very soon after the voltage step and did not become unblocked again in this trace. On the other hand, the TMO-modified channel was never blocked by STX. We observed that reduced single channel conductance and toxin insensitivity always occurred concomitantly upon TMO addition and we conclude that both of these consequences of TMO result from modification of a common site (see Discussion and Appendix).

A concern in these studies was that the byproducts of the TMO reaction with water (protons, methanol, and ether) were producing changes in sodium channel properties rather than the reagent itself. To test this, TMO was allowed to react completely with solutions bathing a preformed bilayer before the addition of biological material. The pH was maintained at 7.0 by the addition of concentrated NaOH. Membrane vesicles (P_3) were then added, which resulted in incorporation of normal, unmodified sodium channels that were STX sensitive and showed no differences in single channel conductance or any other functional property.

Effect of Extracellular Calcium on Single Channel Currents

Divalent cations have been shown to influence sodium ion movement through sodium channels in excitable tissues (Woodhull, 1973; Yamamoto et al., 1984; French et al., 1986*b*). Fig. 4*A* shows the effect of extracellular calcium addition on single sodium channel currents before TMO addition. At -60 mV, with <100 nM divalent cations, the sodium channels were mostly open, with occasional fluctuations to the closed state. When 10 mM calcium was added to the extracellular side, the single channel current was reduced by $\sim 60\%$. The long "closing" event at the end of the record shown in Fig. 4 is due to the presence of a low concentration of STX (5 nM), which was added to provide an unambiguous determination of the closed state current level. This calcium-induced current reduction is presumably due to a rapid movement of calcium ions onto and off of a blocking site, located in the sodium channel, producing a time-averaged reduction in the single channel current (cf. Yellen, 1984).

In addition to reducing the single channel current, extracellular calcium can affect the channel's activation kinetics. The sodium channel appears to spend a greater fraction of time in the closed, nonconducting state in the presence of extracellular calcium. This effect of extracellular calcium on the kinetic properties of sodium channels has been described previously (Frankenhaeuser and Hodgkin, 1957; Hille et al., 1975*b*; Hahn and Campbell, 1983; French et al., 1986*b*) and may be due to a steepening of the intramembrane electric field as a consequence of screening of fixed negative charges on the outer surface of the membrane lipid or on the channel protein itself. We attribute the decreased single channel current to calcium block of the open channel rather than to a failure to resolve rapid gating fluctuations induced by extracellular calcium.

The single channel current-voltage relationships before and after calcium addition are shown in Fig. 4*B*. The inward single channel current is reduced by

extracellular calcium. There is greater block at -90 mV ($\sim 74\%$) than at -30 mV ($\sim 53\%$), which indicates that block by calcium is voltage dependent, such that more hyperpolarized potentials favor greater block. One interpretation of this result (Woodhull, 1973; Yamamoto et al., 1984) is that calcium blocks by binding to a site within the channel that is located a significant distance into the membrane electric field from the extracellular surface.

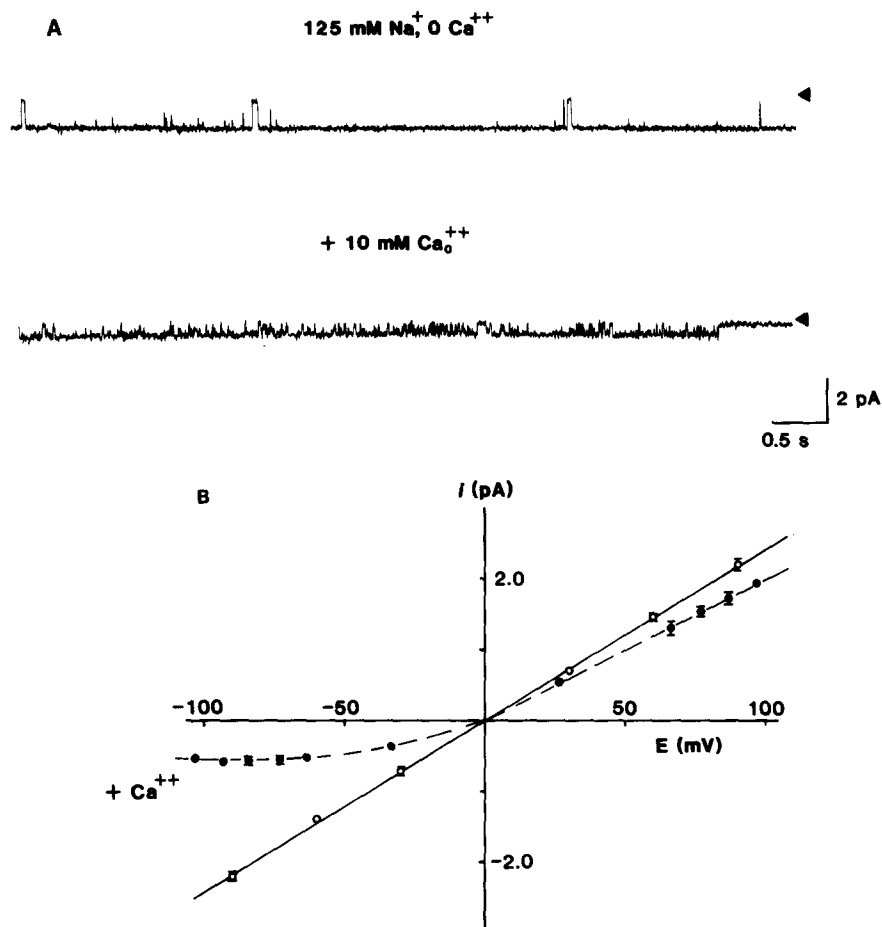


FIGURE 4. Effects of extracellular calcium on single sodium channels. (A) A single sodium channel was incorporated into a bilayer separating identical 125 mM sodium solutions. The zero-current levels are indicated by the arrowheads. Single channel current fluctuations were recorded at -60 mV in the absence (top trace) and presence (bottom) of 10 mM extracellular calcium. STX (1 nM) was added to the extracellular solution upon addition of calcium and is responsible for the long closing observed at the end of the current record at the bottom. (B) Current-voltage relationships for sodium channels in the absence (open circles) and presence (solid circles) of 10 mM extracellular calcium. Each point represents data from six experiments; the smooth curves were drawn by eye.

Table I shows the effect of changing membrane potential on the block of inward currents by extracellular calcium. The apparent K_i for calcium evaluated at each potential was calculated from the fraction of maximal conductance blocked (F_b) according to the following relationship:

$$K_i = (F_b^{-1} - 1)[Ca^{2+}]. \tag{1}$$

The more negative the membrane potential, the higher the apparent affinity for calcium. By adopting the formalism used by Woodhull (1973), one can determine the apparent voltage dependence of block, or the fraction of the transmembrane voltage sensed by calcium, from the following relationship:

$$K_i(E) = K_0 \exp(DzFE/RT), \tag{2}$$

where $K_i(E)$ and K_0 are the calculated inhibition constants for calcium at the indicated potential, E , and 0 mV, respectively, D is the fraction of the applied electric field sensed by the divalent blocker, z is the valence of the ion, and R , T ,

TABLE I
Effect of Voltage on Block by Extracellular Calcium

<i>E</i>	Fraction of current blocked	K_i
<i>mV</i>		<i>mM</i>
-34	0.51±0.04	9.6±1.6
-64	0.64±0.03	5.6±0.9
-74	0.68±0.03	4.7±0.7
-84	0.71±0.02	4.1±0.5
-94	0.74±0.02	3.5±0.3
-104	0.79	2.6

Data were obtained from planar bilayers each containing a single sodium channel. K_i was calculated from the fraction of current blocked, using Eq. 1. Data were collected from four to seven membranes, except for the value at -104 mV, which was obtained from a single membrane. Errors shown are SEMs.

and F have their usual meanings. From the fraction of current blocked at each potential, the K_i can be determined from Eq. 1 (see Table I). These results are consistent with the hypothesis that calcium binds to a site located ~23% of the electrical distance from the extracellular surface and are in close agreement with the results of macroscopic voltage-clamp and patch-clamp studies (Woodhull, 1973; Yamamoto et al., 1984).

Calcium Competitively Inhibits STX Block and Binding

Fig. 5 illustrates the effect of calcium on block of a single sodium channel by STX. STX (10 nM) was present on the extracellular side of the channel. At -60 mV, the channel spent >95% of its time blocked by STX. When 10 mM calcium was added to both sides of the bilayer, the channel, on the average, spent a longer time in the open, unblocked state, as indicated by the increased open (unblocked) dwell times. This suggests that the on (or blocking) rate for STX was reduced in the presence of calcium. Furthermore, the overall fraction of

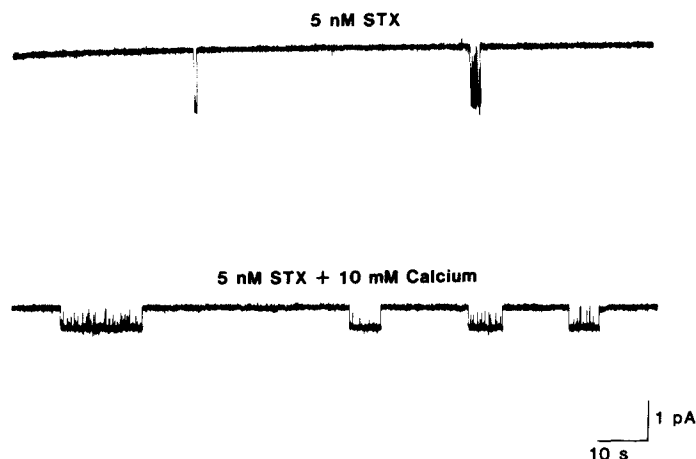


FIGURE 5. Effect of calcium on STX block. A single sodium channel was incorporated into a planar bilayer with 125 mM sodium on both sides and 5 nM STX only on the extracellular side. The current fluctuations were recorded at -60 mV and the zero-current level is upward, so that downward fluctuations in the current record represent channel openings. The current record at the top shows that the channel was blocked by STX $>90\%$ of the time, and became unblocked only briefly. When 10 mM calcium was added to both sides of the membrane, the single channel current was reduced, the mean unblocked dwell time increased, and the channel spent a larger fraction of the time in the open, unblocked state.

time the channel spent unblocked increased, which indicates that the overall sensitivity to toxin was decreased. Also, the single channel current was reduced $\sim 60\%$ in the presence of 10 mM calcium (see Fig. 4 for comparison). Calcium was added symmetrically to avoid a change in the voltage dependence of channel gating, as was observed in Fig. 4A. Current fluctuations between the open and closed states caused by STX blocking and unblocking events are clearly distinguishable from channel gating behavior. Similar effects on STX block were obtained with calcium addition to the extracellular side alone.

Table II shows the computed rate constants and fractional block by STX from

TABLE II
Effect of Calcium on STX Block

[Ca ²⁺] _o	k_{on}	k_{off}	K_d from	
			Rate constants	Mean current (f_b)
	$10^8 \text{ M}^{-1} \text{ s}^{-1}$	s^{-1}	nM	nM
0 mM	0.310 ± 0.010	0.034 ± 0.020	1.3 ± 0.3	1.5 ± 0.4
10 mM	0.065 ± 0.010	0.058 ± 0.020	9.6 ± 0.4	9.8 ± 0.6

These determinations were obtained at -60 mV from three separate single channel membranes. K_d was calculated from the ratio of the rate constants, $k_{\text{off}}/k_{\text{on}}$, for each experiment (mean \pm SEM shown) and from the mean reduction in current, f_b (mean \pm SEM shown).

analysis of records such as those of Fig. 5 (cf. French et al., 1984). There was about a fivefold reduction in the on (blocking) rate for STX and a small (less than twofold) effect on the STX off (unblocking) rate. Overall, calcium caused a six- to sevenfold increase in the apparent dissociation constant for STX block, as determined from either the blocking and unblocking rate constants or a reduction in the mean current (fractional block). The off rate for STX was not significantly affected by calcium. These results suggest that calcium reduces the apparent STX affinity by competing with STX for the blocking site.

[³H]STX binding to membrane vesicles (P₃) was also reduced in the presence of calcium, as shown in Fig. 6. The double-reciprocal plots illustrate a shift in the K_d , without altering B_{max} . Calcium (10 mM) caused a five- to sixfold increase in the dissociation constant for [³H]STX binding, with the characteristics of

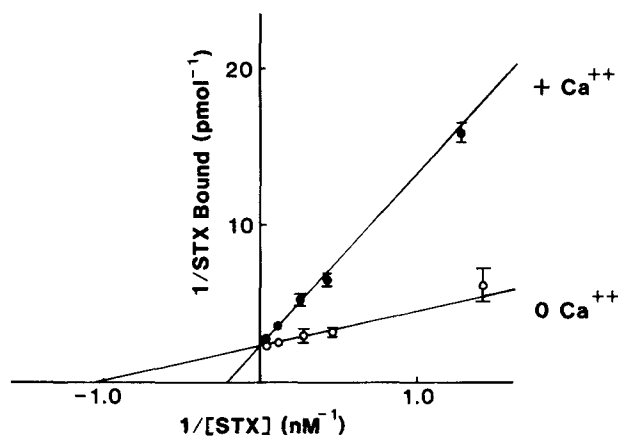


FIGURE 6. Double-reciprocal plot of [³H]STX binding to membrane vesicles (P₃). Binding was measured under equilibrium conditions at 4°C in the presence of 125 mM sodium at pH 7.0 with (solid circles) and without (open circles) 10 mM calcium. Each point represents four determinations of radiolabeled STX bound to membrane vesicles (0.2 mg/ml) at the indicated concentration. In the presence of calcium, the STX affinity was reduced. As determined from double-reciprocal plots such as this one, 10 mM Ca²⁺ increased the apparent dissociation constant from 0.87 ± 0.06 to 5.12 ± 0.04 nM [³H]STX ($n = 3$).

competitive inhibition. We assume that there was little or no vesicle membrane potential under these conditions. These values are similar to those obtained from [³H]STX binding experiments on *Torpedo* electroplax and rat brain synaptosomes under similar conditions (Henderson et al., 1974; Weigele and Barchi, 1978b).

Calcium and STX Protect Against TMO Modification

Competition between calcium and STX could reflect interactions between the blockers and the sodium channel at separate sites. However, Fig. 7 illustrates that both STX and calcium were able to protect against TMO modification. The reduction in the number of chemically modified STX binding sites occurred in

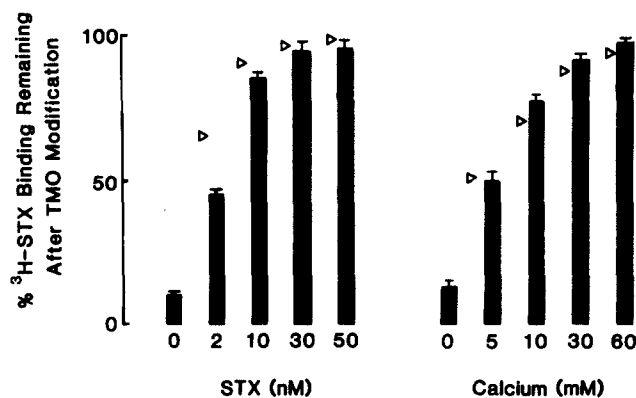


FIGURE 7. STX and calcium protect against TMO modification. [^3H]STX binding was measured after the addition of TMO (50 mM) in the presence of STX or calcium at the indicated concentration. The data are plotted as the means \pm SEM ($n = 4$). In the absence of STX or calcium, TMO eliminated 80–90% of the [^3H]STX binding sites. The arrowheads indicate the expected protection from TMO modification as described in the text.

a dose-dependent manner that closely followed the potency for block by STX and calcium obtained from single channel measurements in planar bilayers. As the STX or calcium concentration was increased, the number of [^3H]STX binding sites recovered after TMO treatment increased. The arrowheads indicate

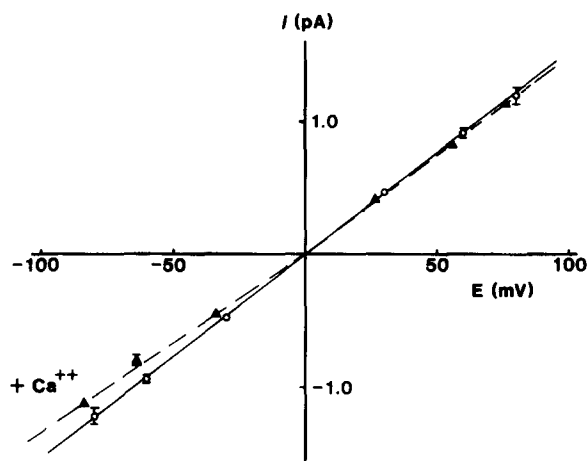


FIGURE 8. The effect of calcium on TMO-modified channels. Current-voltage relationships were obtained from sodium channels incorporated into bilayers in symmetrical 125 mM sodium. Single channel currents were recorded after modification by TMO in the absence of calcium (open circles, $n = 9$; see Fig. 2) and then after addition of 10 mM Ca^{2+} to the extracellular side (solid triangles). Data points show the means \pm SEM. STX (5 nM) added after TMO modification did not block the channels. Each point in the presence of calcium (dashed line) represents the single channel current at the indicated potential in three to five membranes, except that the data point at -80 mV was obtained from a single membrane.

the predicted occupancy of the site by STX or calcium, as determined from their effects on the single sodium channels in the bilayer. The parallel potencies of channel blockade by calcium and STX and protection from modification provide further evidence that calcium and STX can bind to a common site on the sodium channel.

TMO Modification Reduces Block by Extracellular Calcium

Fig. 8 illustrates the current-voltage relationships of a TMO-modified sodium channel before and after the addition of 10 mM calcium to the extracellular side. This toxin-insensitive sodium channel was nearly insensitive to block by calcium, when compared with unmodified, STX-sensitive sodium channels (Fig. 5). In Fig. 9, the inhibition of single channel current (fraction blocked) by calcium is plotted as a function of membrane potential for a normal and a TMO-modified

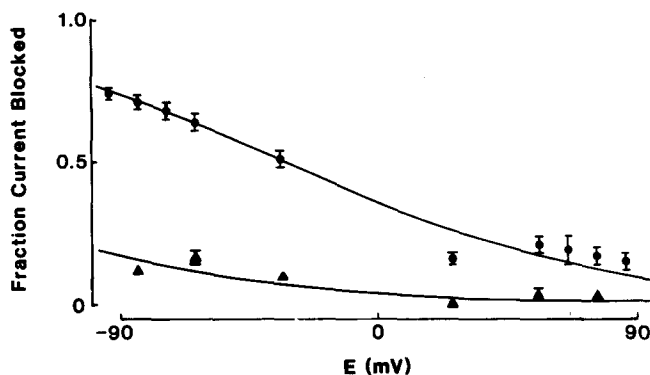


FIGURE 9. Voltage dependence of block by calcium. The fraction of current blocked by 10 mM extracellular calcium vs. membrane potential is plotted for normal, STX-sensitive sodium channels (circles) and for TMO-modified sodium channels (triangles). The fraction of current blocked by calcium was determined from the relationship $F_b = (1 - I/I_0)$, where I and I_0 are the magnitudes of the single channel currents in the presence and absence of 10 mM calcium, respectively, and were obtained from the data in Figs. 4 and 8. The smooth curves were calculated from Eq. 2 with $D = 0.23$ and $K_0 = 17.7$ (top) or 240 mM (bottom) calcium.

channel. At all potentials, the TMO-modified channel was less sensitive to extracellular calcium than the unmodified sodium channel. The smooth lines were determined from Eqs. 1 and 2, in which the apparent fraction of the field sensed by the blocking ion was constant ($D = 0.23$), while the affinity for calcium at 0 mV (K_0) was varied. The lines were calculated using K_0 's of 17.7 and 240 mM calcium. We cannot determine with certainty whether the small amount of block of TMO-modified sodium channels by calcium was almost independent of voltage or was simply much less potent with the same voltage dependence.

DISCUSSION

TMO, when added to the extracellular side of a single sodium channel in a planar bilayer, had three conspicuous effects on channel properties. First, TMO application eliminated STX and TTX sensitivity (Fig. 1). In addition, [^3H]STX

binding to the rat brain membrane vesicle preparation (P_3) was also eliminated (Fig. 7). Second, the single channel current was reduced by 37% after TMO modification (Figs. 1 and 2). A third, and unprecedented, effect of TMO was to render the toxin-insensitive channel nearly insensitive to extracellular calcium (Figs. 8 and 9), which normally blocks inward sodium currents through the channel (Figs. 4 and 9). These three effects are consistent with TMO modifying a single site, with which sodium, calcium, and toxin interact, that is in or near the permeation pathway.

Chemistry of Modification Reagents Suggests Methylation of a Carboxyl Group

TMO is a highly reactive agent that esterifies carboxyl groups, producing a methyl ester and dimethylether as a byproduct. This reaction converts a normally negatively charged, hydrophilic group to a neutral, more hydrophobic residue. Although we cannot be certain that it is a carboxyl residue on the sodium channel that is being modified in these experiments, the observation that water-soluble carbodiimide, in the presence of glycine methyl ester, had a similar effect on action potential block by TTX (Baker and Rubinson, 1975) points strongly toward a carboxyl group as the modified residue, because both TMO and carbodiimide act primarily on carboxyl groups and have no other overlapping specificities.

A Single Hit by TMO Can Account for All Three Changes in Channel Properties

We believe that the three consequences of TMO addition, viz., elimination of block by TTX and STX, reduced single channel conductance, and reduced calcium block, are due to the methylation of a single, specific carboxyl group. In 36 attempts, we successfully modified 30 channels, but in no case did a channel exhibit insensitivity to STX without having a reduced single channel conductance or vice versa. We calculate that if the reduction of single channel conductance and the elimination of toxin block were independent events, the probability of observing only doubly modified channels would be $<10^{-6}$ (see Appendix). This suggests that the toxin binding and the single channel conductance cannot be modified independently. We did not test the six channels that we failed to modify for sensitivity to calcium, but all channels with reduced calcium sensitivity were toxin insensitive and had reduced single channel conductance under control conditions.

Reduction in Single Channel Conductance Produced by TMO Modification

In a previous study, a TMO-induced reduction in the single channel conductance was estimated from nonstationary fluctuation analysis of sodium currents in frog node of Ranvier (Sigworth and Spalding, 1980). We have now directly demonstrated this effect of TMO with single channel recordings (Fig. 2). There are some quantitative differences between the two studies. Sigworth and Spalding reported that TMO treatment reduced the single channel conductance from 7 to 2.4 pS in frog node of Ranvier bathed in Ringer solution. In planar bilayers,

we found that the single channel conductance was reduced from ~25 to ~16 pS in symmetrical 125 mM NaCl. The difference in conductance before TMO treatment was not surprising and may have been due to differences in ionic conditions (in particular, different extracellular calcium concentrations) or to errors in computing unit conductance from noise measurements with a limited bandwidth (see Levis et al., 1984). In light of our observation that TMO-modified channels are nearly insensitive to calcium, the larger relative reduction in single channel conductance reported by Sigworth and Spalding (1980) (70 vs. 37% in the present study) cannot be attributed to differences in the degree of block by external calcium. This discrepancy is puzzling, but could be due to differences between cell types or differences between normal and BTX-activated channels.

Gulden and Vogel (1985) reported a TMO-induced reduction in the number of channels that was independent of the effects of TMO on TTX sensitivity. We have not observed any loss of sodium channels from planar bilayers after TMO addition.

TMO-modified Channels Are Less Sensitive to Block by Extracellular Calcium

A novel and unexpected effect of TMO modification was the marked reduction in the degree of calcium block of sodium ion permeation through open channels (see Fig. 9). At 10 mM, the highest concentration tested, calcium blocked unmodified channels in a voltage-dependent manner, but had very little effect on TMO-modified channels. From the small amount of block at negative potentials, we calculate that there was a dramatic reduction in the affinity of the channel for calcium (the apparent dissociation constant, at 0 mV, changed from 18 to >200 mM).

STX, Sodium, and Calcium Compete with One Another

STX binding is inhibited by monovalent and divalent cations (Weigele and Barchi, 1978b; Reed and Raftery, 1976; Henderson et al., 1973, 1974). We found that calcium was able to compete with STX for binding to sodium channels in rat brain membranes (Fig. 6). Calcium also relieved block of these sodium channels in planar bilayers (Fig. 5; Table II), as if calcium were competing with STX for binding to the channel (indicated by increased unblocked dwell times) as well as blocking the channel (indicated by reduced single channel conductance; see above). The apparent K_i for STX block was increased about six- to sevenfold in the presence of calcium, as determined from both the reduction in mean current and the kinetic analysis of unitary blocking and unblocking dwell times. A five- to sixfold increase in the K_d for [^3H]STX binding was found. We have also quantitatively evaluated the effect of calcium on the kinetics of block (Table II). The on (blocking) rate for STX was decreased in the presence of calcium, with little or no change in the off (unblocking) rate, as predicted for simple competition. Some component of the inhibition of STX action could be due to alternative mechanisms such as a calcium-induced, allosteric modification of the toxin binding site or a reduction in the surface potential near the toxin binding

site¹ (Henderson et al., 1974; Hille et al., 1975a; Strichartz and Hansen Bay, 1981), but simple competition between calcium and STX is sufficient to account for both our kinetic and equilibrium data. In addition, sodium has been shown to competitively inhibit STX binding (Weigele and Barchi, 1978b) and TTX block (Moczydlowski et al., 1984a), and sodium also competes with calcium to relieve block by calcium of single channel current (Worley et al., 1985; Worley, J. F., R. J. French, and B. K. Krueger, manuscript in preparation). These results suggest that sodium, calcium, and STX compete with one another to bind at a common site. This is probably a site with which sodium ions normally interact before reaching the "selectivity filter" as they pass inward through the channel.

Calcium and STX Can Protect the TMO-vulnerable Site

The addition of 50 nM STX to the outside of sodium channels in planar bilayers before TMO addition prevented modification. This protection can be studied quantitatively with binding of radiolabeled STX to membrane vesicles. In fact, the ability of STX to protect against TMO modification can be directly related to its intrinsic affinity for that site, as shown in Fig. 7. The concentration of STX required to prevent half of the STX binding sites from being modified was ~3 nM, which is higher than the K_d for [³H]STX binding (~1 nM). Presumably, this reflects the amount of time the modification reagent is active: in the presence of 1 nM STX, on the average, half of the sites would be occupied by STX, the remainder being available for irreversible modification by TMO. To the extent that TMO is active for longer than the mean bound time (1/dissociation rate), STX will dissociate from some of the blocked sites, rendering more of them accessible to modification.

Concentration-dependent protection of the STX binding site was also observed when calcium, instead of STX, was present (see Fig. 7). The K_i for calcium blockade of single channel currents can be estimated from Table I to be between 2 and 9 mM; 5 mM calcium was able to prevent half the sites from being modified by TMO. Protection against TMO modification by STX and calcium with potencies similar to those for binding and block provides further evidence that the toxin binding site was being modified and that calcium and toxin can both interact with this site.

The Locus at Which Toxins Bind Is Complex

Toxin binding is highly sensitive to even minor alterations in STX and TTX structure, which suggests that there is a complex interaction between the toxin

¹ The most direct evidence that calcium inhibits toxin binding by affecting surface potential comes from a report by Hille et al. (1975a), in which calcium increased the K_d for STX to a larger extent than that for TTX in frog node of Ranvier. In that study, the apparent K_i for calcium inhibition of STX block was about five times greater than we observed. In addition, calcium block of sodium channel currents ($K_{Ca} = 90$ mM) was approximately ninefold less potent than in our observations ($K_{Ca} = 10$ mM at positive voltages; Worley, J. F., R. J. French, and B. K. Krueger, manuscript in preparation). Thus, in the frog node experiments of Hille et al., the influence of calcium binding, both competing directly with STX and blocking the channel, was weaker. Effects on toxin block caused by modification of the surface potential near the binding site would be expected to be more prominent in such a case, where direct calcium binding is relatively weak.

molecule and its receptor that involves multiple site liganding (Strichartz, 1984). The modification of one or more of these groups (sites) on the channel protein, such as the conversion of a carboxyl to a carboxymethyl ester by TMO, would be expected to substantially decrease the affinity for toxins.

While a carboxyl group within this locus is apparently crucial to toxin binding, that group has a less dramatic influence on the access of small ions, such as sodium and calcium, to the channel pore. We envision it simply as a way station, occupied transiently and with low affinity, en route to deeper sites in the pore. This might explain why toxin block is virtually eliminated, whereas the rate of sodium permeation is reduced by only about one-third, and the apparent affinity for calcium is decreased ~10-fold. These effects of TMO on single sodium channel conductance and calcium block could be due to a combination of factors, both steric (addition of a methyl residue) and electrostatic (removal of negative charge on a carboxyl group). Such changes in a carboxyl group at the binding site could both impede sodium permeation and reduce binding of cations to the channel. One or more deeper sites, inaccessible to TMO, would determine the similar selectivity sequences for permeant organic cations observed with unmodified and TMO-modified channels (Spalding, 1980).

Voltage Dependence of STX and Calcium Block Probably Arise from Different Mechanisms

Calcium and STX can interact with a common site on the extracellular side of the sodium channel, and block of sodium permeation by both substances is voltage dependent, with hyperpolarizing potentials favoring block. If these blockers entered the membrane electric field to block the channels, the fraction of the membrane potential that would have to be sensed by the blocking cation to account for this result is similar for calcium and STX (in our experiments, ~30% for STX and 23% for calcium). Hydrogen ions have also been reported to have a similar voltage-dependent blocking behavior, sensing ~25–30% of the membrane potential in intact nerve fibers (Woodhull, 1973; Begenisich and Danko, 1983) but not in amphibian skeletal muscle (Campbell, 1982). Moczydlowski et al. (1984b) showed that the voltage dependence of block by TTX, STX, and several STX derivatives with net charges of 0, +1, and +2 was the same (~40 mV/e-fold), which suggests that the voltage dependence did not arise from the charged toxin penetrating a significant distance into the transmembrane electric field. They proposed that the voltage dependence of block by STX and TTX was a consequence of a voltage-dependent conformational change in the channel protein that resulted in an alteration of the molecular structure of the toxin binding site. In contrast, voltage-dependent block by calcium could be better explained if calcium (and possibly protons) competed with permeant ions at a site deep within the membrane electric field, at which selectivity among permeant ions is determined (the "selectivity filter"). The observation that calcium ions can carry inward current through sodium channels (Baker et al., 1971; Meves and Vogel, 1973) indicates that calcium has access to all sites on the path through the channel. The less superficial sites are apparently affected little, if at all, by TMO. We believe that the similar voltage dependence of block by STX and calcium is coincidental and reflects actions of toxin and calcium at

different sites, with different mechanisms responsible for the voltage dependence. This hypothesis predicts that TMO-modified sodium channels should exhibit very low-affinity ($K_i > 100$ mM) block by calcium having the same voltage dependence as in unmodified channels. We are undertaking experiments to test this possibility.

Possible Energetic Bases of the Changes Produced by TMO Modification

We have shown that it is possible to chemically modify the structure of a single channel molecule and study the functional properties of that particular molecule both before and after modification.² Rate theory modeling of the current-voltage relations offers the opportunity to associate specific changes in the energy barrier profile that the channel presents to entering ions with a specific change in its molecular structure induced by the modifying reagent. For example, the reduction in conductance caused by TMO could be due to a change in the affinity for sodium or to a change in the maximum rate of sodium ion passage through the channel. If it is assumed that the maximum transport rate of ions through the channel was unaltered, we calculate that the affinity for sodium must be reduced ~3.5-fold by TMO. On the other hand, if the affinity for sodium was not altered by TMO, our data suggest that the modified residue would increase the activation energy for sodium ion passage through the channel by ~0.5 kT, thus lowering the overall ion permeation rate. In a subsequent paper, we will present detailed calculations based on the data in this paper and on additional experiments, which suggest that the TMO-induced modifications result in localized changes at the extracellular end of the channel's energy barrier profile.

APPENDIX

We can test the hypothesis that the decrease in conductance and the removal of STX binding occur independently as follows. This calculation was suggested to us by Dr. Richard Horn. The calculations are based on observations of 36 channels, in 24 one-channel experiments and 6 two-channel experiments. The statistical test is described by Lehmann (1959). Using our data, we set up the following contingency table:

Toxin binding	Conductance	
	Modified	Not modified
Modified	30	0
Not modified	0	6

If the conductance and toxin could be modified independently, the probability of seeing

² Analysis of modified sodium channels at the single channel level is not unprecedented, however. Using the patch-clamp technique, Horn and colleagues (Patlak and Horn, 1982; Horn et al., 1984; Vandenberg and Horn, 1984) studied single sodium channels in rat myotubes and cultured pituitary tumor cells after removal of inactivation by *N*-bromoacetamide and trypsin.

such an extreme deviation from a random pairing of modifications of each characteristic would be:

$$P_y = \frac{\binom{n_{tm}}{n_{gmtm}} \binom{n_{tu}}{n_{gmtu}}}{\binom{N}{n_{gmtm} + n_{gmtu}}} = \frac{\binom{30}{0} \binom{6}{0}}{\binom{36}{30}} = 1/1,947,792 < 10^{-6},$$

where n_{tm} is the number with modified toxin binding; n_{tu} is the number with unmodified toxin binding; n_{gmtu} is the number with modified conductance and unmodified toxin binding; n_{gmtm} is the number with modified conductance and modified toxin binding; and N is the total number of observations.

Thus, we can discard the hypothesis that conductance and toxin binding are modified independently.

We are grateful to Dr. John Daly (National Institute of Arthritis, Metabolic, and Digestive Diseases, National Institutes of Health) for providing BTX, to Drs. Gary Strichartz and Steven Davio for supplying [^3H]STX, and to Dr. Richard Horn for suggesting the calculation presented in the Appendix. We thank Drs. M. Cannell, W. J. Lederer, and M. T. Nelson for helpful discussions during the course of this work. We also thank Marc Blaustein, Felipe Albuquerque, and William Zinkand for excellent technical assistance, Gus Ay for fabricating the bilayer chambers, and Jeff Michael for fabricating the voltage-clamp circuits used in this study.

This work was supported by National Institutes of Health grants NS16285 (to B.K.K.), NS20106 (to R.J.F.), contract DAMD17-82-C-2188 from the U.S. Army Medical Research and Development Command (to B.K.K. and R.J.F.), and by a University of Maryland Graduate Fellowship to J.F.W. This report is derived from parts of a thesis submitted by J. F. Worley III, in partial fulfillment of the requirements for the Ph.D. in physiology from the University of Maryland. A preliminary report of some of these results has appeared (Worley et al., 1985).

Original version received and accepted 23 September 1985.

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