Charge Immobilization Caused by Modification of Internal Cysteines in Squid Na Channels

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ABSTRACT We studied the effects of modification of native cysteines present in squid giant axon Na channels with methanethiosulfonates. We find that intracellular, but not extracellular, perfusion of axons with positively charged [(2-trimethylammonium)-ethyl]methanethiosulfonate (MTSET), or 3(triethylammonium)propyl]methanethiosulfonate (MTS-PTrEA) irreversibly reduces sodium ionic (I_{Na}) and gating (I_g) currents. The rate of modification of Na channels was dependent on the concentration of the modifying agent and the transmembrane voltage. Hyperpolarized membrane potentials (e.g., −110 mV) protected the channels from modification by MTS-PTrEA. In addition to reducing the amplitudes of I_{Na} and I_g, MTS-PTrEA also altered their kinetics such that the remaining I_{Na} did not appear to inactivate, whereas I_g was made sharper and declined to baseline more quickly. The shape and amplitude of I_g after modification of channels with MTS-PTrEA appeared to be "charge-immobilized," as if the modified channels were inactivated. MTS-PTrEA did not affect I_{Na} or I_g when inactivation was removed by internal perfusion of the axon with pronase. In addition, we find that the steady-state inactivation curve of modified Na channels is made much shallower and is markedly shifted to hyperpolarized potentials. The rates of activation, deactivation, or open-state inactivation were not altered in MTS-PTrEA-modified channels. The uncharged sulfhydryl reagent methymethanethiosulfonate (MMTS) did not affect either I_{Na} or I_g, but prevented the irreversible effects of MTS-PTrEA or MTSET on Na channels. It is proposed that the positively charged methanethiosulfonates MTS-PTrEA and MTSET modify a native internal cysteine(s) in squid Na channels, and by doing so promote inactivation from closed states, resulting in charge immobilization and reduction of I_{Na}.

INTRODUCTION

One strategy used to probe the structure and function of ion channels has been that of chemical modification of specific groups such as sulfhydryls, amines, and histidines. As early as 1958 it was noted that chemical modification of sulfhydryls in frog sciatic nerve or giant axons of lobster abolishes excitability (Smith, 1958), and it was shown consequently in crayfish giant axons that this is brought about by changes in the steady-state inactivation of Na channels (Shrager, 1976, 1977). Two recent advances, however, have made the use of chemical modification of specific groups a more powerful tool for studying channel topology and structure function. The first is single amino acid mutagenesis—the ability to introduce and functionally express proteins with known alterations in the amino acid sequence. Thus a single reporter cysteine, for example, can be introduced into the channel structure at a known position and the effect of chemical modification of the cysteinyl sulfhydryl on channel function studied. The second advance is the synthesis, by Karlin and co-workers (Akabas et al., 1992; Stauffer and Karlin, 1994), of three charged reagents that react rapidly and specifically with thiols under physiological conditions (see Table 1). These compounds are based on a methanethiosulfonate developed by Kenyon and colleagues (Smith et al., 1975) and often alter channel function (e.g., activation, deactivation, or inactivation kinetics or single-channel conductance) when they react with the reporter cysteine.

Clearly, functional effects of modification of sulfhydryls with thiosulfonates can be attributed to modification of the engineered reporter cysteine only if the wild-type channel is devoid of native cysteines, or alternatively, if the native cysteines are located within the protein structure such that they are not accessible to water-soluble thiol-reactive compounds. Several recent studies have reported that despite the presence of >30 native cysteines in the α-subunit of the adult human skeletal muscle sodium channel (hSKM1) or the rat skeletal muscle sodium channel (μ1), these channels, in the wild type, are (functionally) resistant to modification by methanethiosulfonates (Yang and Horn, 1995; Yang et al., 1996; Lerche et al., 1997; Vedantham and Cannon, 1998). However, the results of these studies differ from the observations reported earlier showing that modification of sulfhydryls abolishes excitability in neurons (Smith, 1958) by altering the inactivation properties of Na channels (Shrager, 1976, 1977). One possible explanation for this difference may be that the reagents used in the earlier reports (such as *N*-ethylmaleimide) are not as specific as methanethiosulfonates used later. Alternatively, however, it may be that the topology of neuronal Na channels is different from those of the muscle such that at least one native

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TABLE 1 Structures of commonly used methanethiosulfonates and MTS-PTrEA

Full name	Abbreviation	Chemical formula
Methyl methanethiosulfonate	MMTS	CH_3-SO_2-S-CH_3
[2-(Trimethylammonium)ethyl]methanethiosulfonate bromide	$MTSET^+$	CH_3-SO_2-S-CH_2-CH_2-$N^+(CH_3)_3Br^-$
2-(Aminoethyl)methanethiosulfonate hydrobromide	$MTSEA^+$	CH_3-SO_2-S-CH_2-CH_2-NH_2 · HBr
Sodium (2-sulfonoethyl) methanethiosulfonate	$MTSES^-$	CH_3-SO_2-S-CH_2-CH_2-$SO_3^-Na^+$
[3-(Triethylammonium)propyl]methanethiosulfonate bromide	$MTS\text{-}PTrEA^+$	CH_3-SO_2-S-CH_2-CH_2-$N^+(CH_2$-$CH_3)_3Br^-$

cysteine is accessible to thiol-reactive compounds and alters channel function when modified.

We examined the effects of the more selective methanethiosulfonates on native neuronal Na channels of squid giant axons. Squid giant axons are ideally suited for such a study as the intracellular and extracellular solutions can be changed with relative ease and speed. We find that internal, but not external, application of a custom-made, positively charged methanethiosulfonate, methanethiosulfonate propyl-triethylammonium (MTS-PTrEA), irreversibly reduces Na ionic (I_{Na}) and channel gating currents (I_g). This is caused, in part, by a large hyperpolarizing shift in the steady-state inactivation properties of the modified Na channels. We provide evidence that MTS-PTrEA affects I_{Na} and I_g by modification of a native cysteine and show that another positively charged methanethiosulfonate, MTSET, has similar effects on Na channels. Interestingly, neither MTS-PTrEA nor MTSET affects activation, deactivation, or open-state inactivation of modified channels; they shift the steady-state inactivation curve and cause charge immobilization by increasing the rate of inactivation from closed states. Preliminary reports of this study has been presented (Khodakhah et al., 1997).

METHODS

Experiments were performed at the Marine Biological Laboratory (Woods Hole, MA) on voltage-clamped, internally perfused giant axons of the squid *Loligo pealei* at 8°C. The main extracellular solution contained (mM) 200 NaCl, 100 $CaCl_2$, 10 TRIZMA 7.0, and enough tetramethylammonium chloride (TMACl) to obtain an osmolality of 1000 mOsmol/kg. When Na channel gating currents were recorded in isolation from ionic currents, NaCl was omitted from the extracellular solution and the concentration of TMACl was increased to maintain osmolality. The intracellular solution contained (mM) 550 *N*-methyl-D-glucamine, 50 HF, 50 HCl, 395 glutamic acid, and 55 HEPES adjusted to pH 7.0 with glutamic acid, and sucrose to increase the osmolality to 1000 mOsmol/kg.

Traces are shown after subtraction of linear leak and capacity transients. To obtain these, in the presence of permeating ions (e.g., Na or K), the axon was hyperpolarized to −130 mV and a series of −50-mV voltage pulses was applied. The appropriately scaled average of these pulses was subtracted from the recorded ionic currents. Although this procedure was adequate for correction of I_{Na}, we find that it distorted the shape of Na channel gating currents. To correct gating current records we obtained the linear leak and capacity transients by depolarizing the axon to +50 mV and, after allowing inactivation of the Na channels, applying a series of +50-mV voltage steps. The capacity transients thus obtained allow for a more accurate estimation of the shape and kinetics of Na channel gating currents.

Synthesis of MTS-PTrEA

[3(Triethylammonium)propyl]methanethiosulfonate bromide (MTS-PTrEA) was synthesized from sodium methanethiosulfonate (NaMTS) and (3-bromopropyl)-triethylammonium bromide (PTrEA) according to the procedure described by Stauffer and Karlin (1994) for the synthesis of MTSET. NaMTS was prepared as described by Kenyon and Brucie (1977). (3-Bromopropyl)-triethylammonium bromide was obtained as a product of the reaction of 1,3-dibromopropane with triethylamine in acetone in the molar ratio of 5:1. Equal volumes of 1,3-dibromopropane and acetone (100 ml) were placed in a 1-liter flask (round bottom), heated to 50°C, and stirred vigorously. Acetone solution of triethylamine (5:1, v/v) was added to the mixture drop by drop over the course of 4 h. The reaction was allowed to continue overnight under reflux at the same temperature. The reaction mixture was concentrated, chilled, and stored at 4°C for 48 h. The precipitate was separated from the liquid phase on Whatman filter paper and recrystallized twice in an acetone:methanol mixture (2:1, v/v), yielding small, yellowish crystals. The structure of MTS-PTrEA was confirmed with NMR. ^{1}H NMR spectra were recorded on a Brucker AMX-300 instrument. ^{1}H NMR (CD_3OD, δ_H ppm, 300 MHZ): 1.26 (9H, t ($J_{H\text{-}H}$ = 7.23 Hz), CH_3CH_2), 2.17 (2H, m, CH_2), 3.29 (10H, m, CH_2), 3.42 (3H, s, CH_3SO_2).

The purity of MTS-PTrEA and its rate of hydrolysis were measured with 5-thio-2-nitrobenzoate (TNB) assay as described by Stauffer and Karlin (1994). The compound was 95% pure. The half-time for hydrolysis of MTS-PTrEA in 150 mM NaCl solution at room temperature was ~2 h.

RESULTS

MTS-PTrEA irreversibly reduces I_{Na} and I_g

The effect of intracellular perfusion of an axon with MTS-PTrEA was studied in voltage-clamped axons. Fig. 1 *A* shows membrane currents resulting from a 2.5-ms voltage step to 0 mV before and after perfusion of MTS-PTrEA (in the absence of intra- or extracellular potassium ions). The voltage step resulted in an initial outward Na channel gating current (I_g), which was followed by the inward ionic sodium current (I_{Na}). Before application of MTS-PTrEA, I_{Na} increased in amplitude during the first 500 μs as more channels opened, and then declined because of inactivation of Na channels. Perfusion of the axon with MTS-PTrEA irreversibly reduced I_{Na} and I_g. The reduction in the amplitudes of I_{Na} and I_g after modification was accompanied by changes in their kinetics such that the remaining I_{Na} did not inactivate, and I_g was smaller and sharper (the changes in the shape and kinetics of I_g will be described in more detail later). Similar results were obtained in all 14 axons studied.

At high concentrations (e.g., 10 mM) MTS-PTrEA immediately reduced I_{Na} without affecting I_g (see Fig. 1 *B* and its *inset*). This initial reduction of I_{Na} by MTS-PTrEA was reversible and reflects its ability as a low-affinity pore

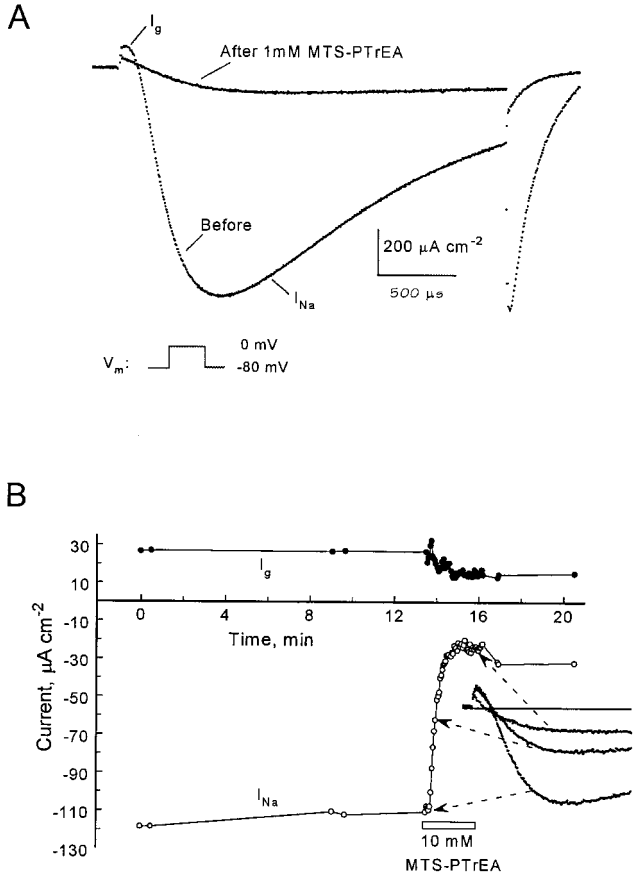


FIGURE 1 Internal perfusion of an axon with MTS-PTrEA irreversibly reduces I_{Na} and I_g. (*A*) The rate of modification of I_{Na} as a function of transmembrane voltage. Shown are Na channel gating (I_g) and ionic currents (I_{Na}) in response to a 2.5-ms voltage step to 0 mV from a holding potential of −80 mV before and after perfusion of an axon for 24 min with 1 mM MTS-PTrEA. After application of MTS-PTrEA both I_g and I_{Na} are reduced in amplitude. Experiment SE076C. (*B*) Time dependent changes in the peak amplitudes of I_{Na} and I_g determined from 2.5-ms voltage steps from −80 mV to 0 mV (as in *A*) in an axon perfused with 10 mM MTS-PTrEA (indicated by the *open bar*). Amplitudes of I_g and I_{Na} were stable before perfusion of axon with MTS-PTrEA, but declined irreversibly after exposure to MTS-PTrEA. MTS-PTrEA initially decreased I_{Na} to approximately half its amplitude without affecting I_g (see data traces in the *inset*), and then reduced both I_{Na} and I_g concurrently. Experiment SE046A.

blocker ($K_i \approx 15$ mM, discussed in more detail later). With continued perfusion of MTS-PTrEA, I_g and I_{Na} reduced concurrently, and their kinetics changed, as described earlier.

Extracellular application of 10 mM MTS-PTrEA for 5 min did not affect I_{Na} or I_g, indicating that MTS-PTrEA modifies an intracellular moiety of squid Na channels. This finding also demonstrates that MTS-PTrEA does not readily permeate the membrane.

Modification of Na channels with MTS-PTrEA is voltage and state dependent

We examined whether the rate of modification of Na channels by MTS-PTrEA was dependent on the holding potential or the gating state of the channel. Intracellular perfusion of an axon with 0.5 mM MTS-PTrEA affected neither I_{Na} nor I_g when the axon was held at −110 mV (Fig. 2 A), indicating that at this potential MTS-PTrEA could not modify the channels. Changing the holding potential to −70 mV allowed modification of Na channels, as manifested by the decline in the amplitude of I_{Na} and I_g. Returning the holding potential to −110 mV recovered some of the Na channels from steady-state inactivation and prevented further modification by MTS-PTrEA. A subsequent change in the mem-

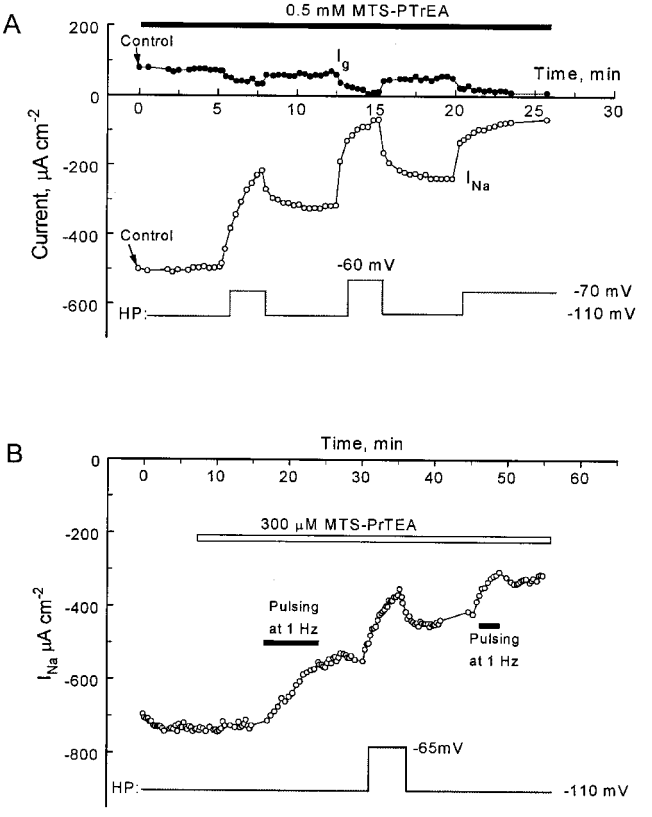


FIGURE 2 The rate of modification of Na channels with MTS-PTrEA is voltage and state dependent. (*A*) Time course of changes in the peak amplitudes of I_{Na} and I_g determined from 2.5-ms voltage jumps to 0 mV in an axon perfused with 0.5 mM MTS-PTrEA. When the axon was clamped at a holding potential of −110 mV (note HP in the graph), MTS-PTrEA did not affect I_{Na} or I_g, even though the channels were exposed to the compound for 5 min. Changing the membrane potential to −70 mV resulted in the concurrent decrease of I_{Na} and I_g, mainly because of modification of channels with MTS-PTrEA. Although return of the holding potential to −110 mV partially increased I_{Na} and I_g by removing steady-state inactivation (see also Fig. 5 for additional information on the effect of MTS-PTrEA on steady-state inactivation), at equilibrium I_{Na} and I_g remained decreased in amplitude, indicating that a number of Na channels had been irreversibly modified by MTS-PTrEA. Two subsequent changes in the holding potential to −60 and −70 mV resulted in further modification of the channels. Experiment SE086B. (*B*) The state dependence of modification of Na channels with MTS-PTrEA was tested by perfusing an axon voltage-clamped at −110 mV with 300 μM MTS-PTrEA (duration indicated by the *open bar*). Peak I_{Na} and I_g were measured from infrequent (approximately every 25 s) 2.5-ms voltage jumps to 0 mV. Repeated 2.5-ms voltage jumps to 0 mV at 1 Hz, applied when indicated by the solid bars, increased the rate of modification of Na channels by MTS-PTrEA, as did changing the holding potential to −65 mV. Experiment SE086A.

brane potential to −60 mV caused an additional decline in I_{Na} and I_g, again suggesting that at these less hyperpolarized potentials MTS-PTrEA could access a site to modify Na channels. This voltage dependance in the rate of modification of Na channels with MTS-PTrEA was seen in all three axons tested. We find that Na channels are modified by MTS-PTrEA not only when the membrane is continuously kept depolarized, but also when the channels are repeatedly opened and closed with short depolarizing pulses. Fig. 2 *B* shows that perfusion of an axon held at −110 mV with 0.3 mM MTS-PTrEA did not affect I_{Na}. I_{Na} declined, however, when the axon was repeatedly depolarized at 1 Hz to 0 mV for 2.5 ms to open the channels, or continuously depolarized to −65 mV. Qualitatively similar results were seen in all three axons studied.

The irreversible effect of MTS-PTrEA on squid Na channels is due to modification of cysteines

What is the nature of the interaction of MTS-PTrEA with Na channels? The most likely possibility is that it modifies a cysteine (or cysteines) in the intracellular part of the channel. If its observed effects are due to modification of a cysteine, then other cysteine-reactive agents should be capable of reacting with this site as well. Indeed, we find that intracellular perfusion of MTSET (Table 1) has effects comparable to those produced by MTS-PTrEA (eight of eight axons studied; Fig. 3 *A*). As with MTS-PTrEA, the rate of modification of Na channels is also voltage dependent.

The hydrophobic thiol-reactive compound MMTS (see Table 1) modifies cysteines by attaching its relatively small, uncharged methyl side chain to them. Interestingly, perfusion of axons with low concentrations of MMTS (e.g., 2 mM) affected neither I_{Na} nor I_g, although MMTS prevented the irreversible actions of MTS-PTrEA in all four axons studied (Fig. 3 *B*). Apparently MMTS modifies the internal cysteine(s) without affecting channel function, and by doing so makes them unavailable for further modification by MTS-PTrEA, confirming that the irreversible actions of MTS-PTrEA are due to the modification of cysteines. Preexposure of axons to MMTS also prevented the irreversible effects of MTSET in the one axon studied.

Although MMTS prevented the irreversible action of MTS-PTrEA, the latter could reversibly reduce I_{Na} while present in the axon (Fig. 3 *B*). This indicates that MTS-PTrEA blocks Na channels with an affinity of 15 mM, which is similar to the affinity of tetraethylammonium (TEA) for Na channels.

Methanethiosulfonates hydrolyze in aqueous solutions (the half-time of hydrolysis of MTS-PTrEA is ~2 h) and thereby lose their ability to modify sulfhydryls. Perfusion of an axon with hydrolyzed MTS-PTrEA solution (10 mM, kept for 3 days at room temperature) caused reversible block but had no irreversible effects. The inability of hydrolyzed MTS-PTrEA to irreversibly affect I_{Na} or I_g is in agreement with the proposed hypothesis that MTS-PTrEA modifies Na channels by reacting with cysteines.

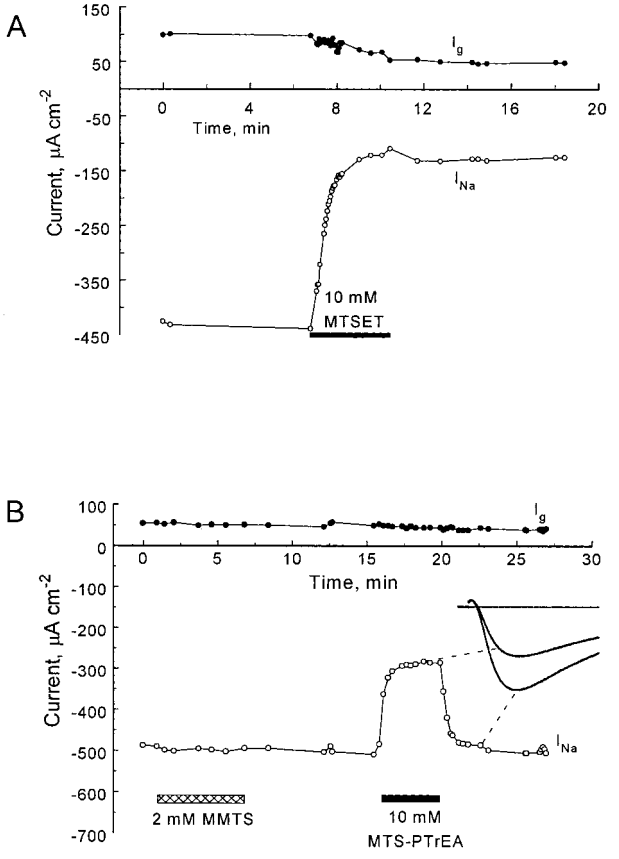


FIGURE 3 MTS-PTrEA reacts with cysteines. (*A*) Peak amplitudes of I_{Na} and I_g determined from voltage steps to 0 mV in an axon voltage-clamped at a holding potential of −80 mV. The positively charged methanethiosulfonate MTSET was perfused into the axon during the time indicated by the solid bar. Like MTS-PTrEA, MTSET irreversibly reduced I_{Na} and I_g. Experiment SE046B. (*B*) Perfusion of the hydrophobic sulfhydryl modifying reagent MMTS did not reduce either I_{Na} or I_g but did prevent the irreversible effects of subsequently perfused MTS-PTrEA on the channels. (*Inset*) Two representative traces of I_g and I_{Na} are shown in the presence and absence of MTS-PTrEA. Note that I_g (the initial outward current) is not affected by MTS-PTrEA, but I_{Na} is reduced reversibly. Experiment SE056B.

The modification rate of target cysteines by MTS-PTrEA at −70 mV is ~5 M^{-1} s^{-1} (see Discussion).

Modification of cysteines with MTS-PTrEA results in charge immobilization

As shown earlier (see Fig. 1), MTS-PTrEA not only reduced I_{Na}, but also I_g. The effect on I_g was studied in detail in axons bathed and perfused with solutions devoid of permeant ions. Fig. 4 *A* demonstrates On-gating currents in an axon before and after exposure to MTS-PTrEA. The compound reduced I_g and quickened the time course. These changes resemble the effects of inactivation on I_g (Armstrong and Bezanilla, 1977). The figure compares I_g of normal channels inactivated with a 10-ms prepulse to +40 mV to I_g after MTS-PTrEA. In the latter case there was no

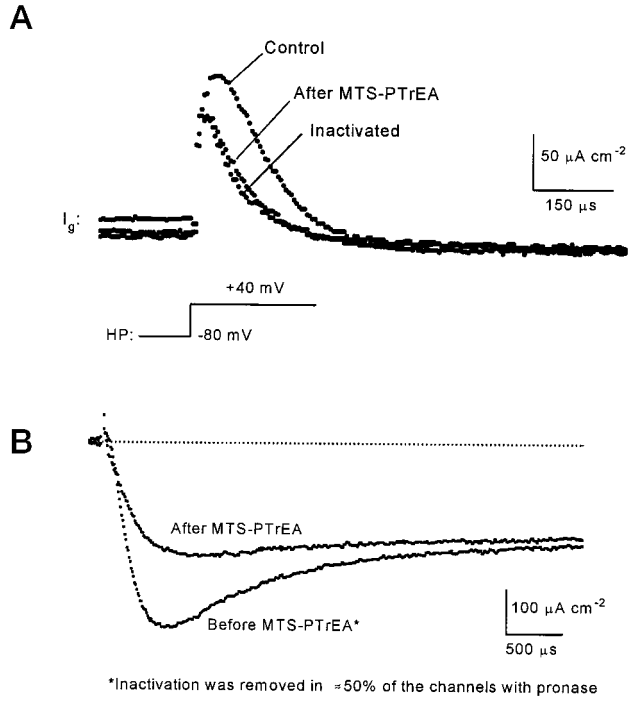


FIGURE 4 MTS-PTrEA alters inactivation and results in charge immobilization. (*A*) Na channel gating currents were measured in an axon where no permeating ions were present in the intra- or extracellular solutions. On-gating currents are shown after a voltage step from −80 mV to +40 mV, before (labeled "Control") and after modification of channels with MTS-PTrEA (labeled "After MTS-PTrEA"). After modification with MTS-PTrEA the amplitude of On-gating currents was reduced, and the current became sharper and declined more quickly to baseline. The charge-immobilized appearance of the On-gating currents after modification by MTS-PTrEA is similar to On-gating currents recorded from inactivated unmodified Na channels (labeled "Inactivated," Na channels were prompted to inactivate with a 10-ms prepulse to +40 mV). Experiment AU316B. (*B*) Inactivation was removed in approximately half of the Na channels by internal perfusion of the axon with pronase (labeled "Before MTS-PTrEA"). Note that I_{Na} inactivates promptly to a steady-state level equal to half its peak amplitude. After perfusion of the axon with 10 mM MTS-PTrEA I_{Na} does not inactivate, and its amplitude is reduced to that of the steady-state level before modification. Experiment SE066E.

prepulse. The similarities suggest that modifying Na channels with MTS-PTrEA promotes inactivation.

We explored the connection between inactivation and the irreversible action of MTS-PTrEA on Na channels in more detail by removing inactivation with pronase (Armstrong et al., 1973). The result of one such experiment is shown in Fig. 4 *B*. The axon was perfused with pronase long enough to remove inactivation in approximately half the channels. This is evident in the trace labeled "Before MTS-PTrEA" in Fig. 4 *B* because the steady-state amplitude of I_{Na} after full inactivation is approximately half of the peak amplitude. When the axon was subsequently perfused with MTS-PTrEA, the peak amplitude of I_{Na} was reduced without altering the steady-state current much, and the remaining current did not appear to inactivate at all. This suggests that only Na channels with intact inactivation (i.e., those not affected by pronase) could be irreversibly modified by MTS-PTrEA. Alternatively, it may be that the target cysteine could be modified even in channels that had their inactivation removed by pronase, but that its modification was ineffective in altering channel function. Interestingly, even channels that did not inactivate after treatment with pronase were subject to reversible block by the compound (not shown).

MTS-PTrEA alters steady-state inactivation of Na channels

The correlation between inactivation and reduction of I_{Na} and I_g by MTS-PTrEA suggests that perhaps MTS-PTrEA reduces I_{Na} and immobilizes charge movement by promoting inactivation of Na channels. Given such a premise, it may be expected that a hyperpolarizing prepulse applied to remove inactivation would partially restore I_{Na} in MTS-PTrEA-modified channels. The result of one such experiment is shown in Fig. 5 *A*, where the axon was subjected to 50-ms prepulses to different potentials (see also *traces* in Fig. 7 *A*). Hyperpolarization maximally recovered 32 ± 9% (mean ± SD, $n = 5$) of I_{Na}. After modification with MTS-PTrEA the h_∞ curve is very shallow and does not saturate at −130 mV (Fig. 5 *B*).

Interestingly, the rate at which channels recovered from inactivation at −130 mV was not different in MTS-PTrEA-modified channels compared with control channels (Fig. 5 *C*). The inactivation rate at −80 mV, however, was significantly increased in modified channels with a time constant for inactivation of ~3 ms as compared with ~12 ms under control conditions (Fig. 5 *D*). In contrast, the rate of inactivation from the open state was essentially unaltered after modification by MTS-PTrEA (Fig. 6).

As shown earlier, perfusion of axons with MMTS did not affect I_{Na} but prevented the irreversible effects of MTS-PTrEA on I_{Na} and I_g. Similarly, MMTS did not directly alter the h_∞ curve, but prevented MTS-PTrEA from altering it in the two axons studied. This provides further evidence that MTS-PTrEA affects Na channels by modifying a cysteine or cysteines located in the intracellular part of the channel.

MTS-PTrEA does not affect activation and deactivation kinetics of Na channels

The Na channel kinetics before and after modification by MTS-PTrEA are compared in Fig. 7. Inactivation was removed with a hyperpolarizing prepulse, and I_{Na} in response to a 1-ms voltage jump to 0 mV was recorded. It can be noted that neither the activation (Fig. 7 *A*) nor the deactivation (Fig. 7 *B*) kinetics are significantly altered.

DISCUSSION

Our results show that an internal cysteine residue in squid Na channels can be labeled with MTS compounds. That the residue is closely tied to the inactivation gate is shown by

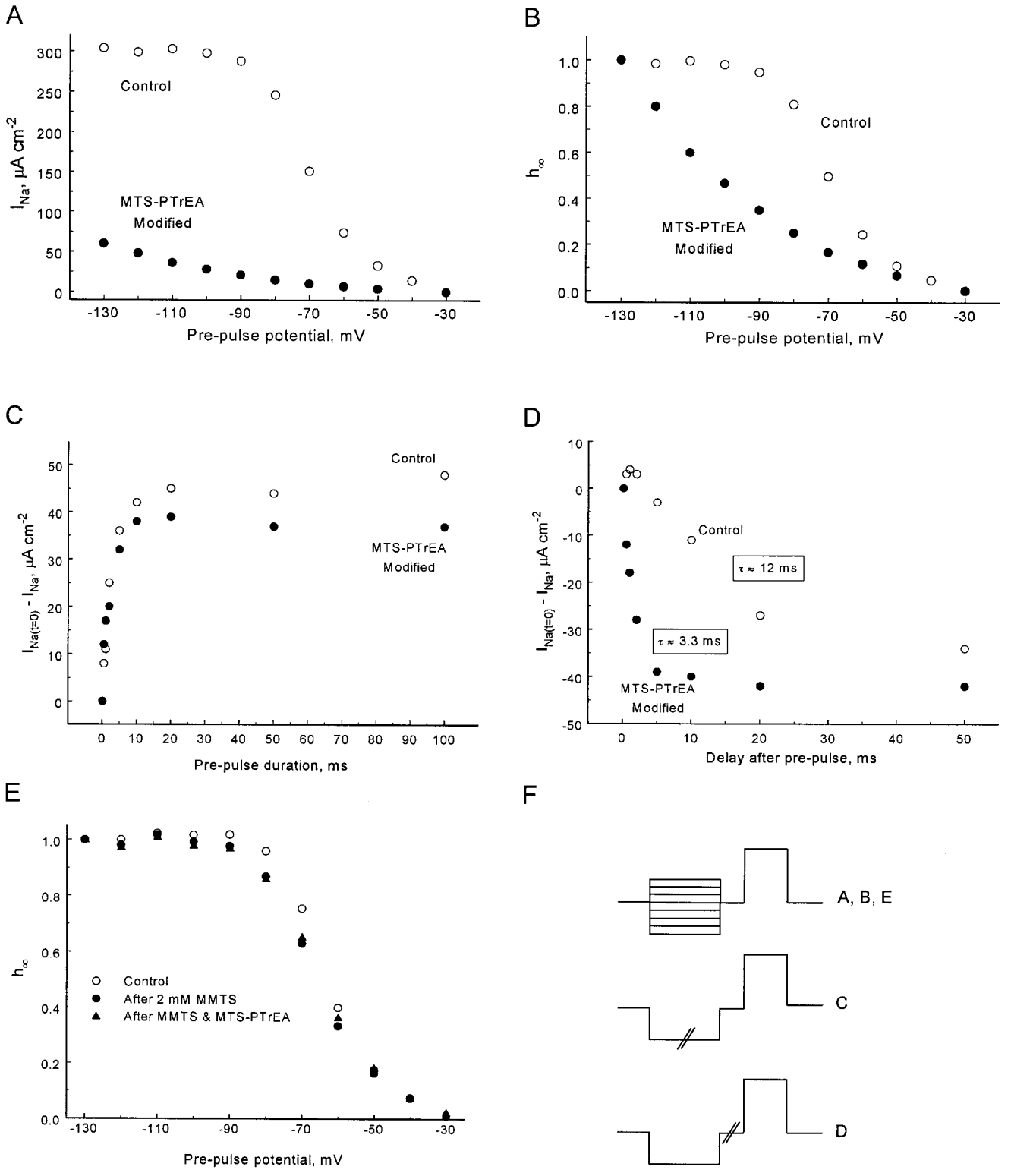


FIGURE 5 MTS-PTrEA alters steady-state inactivation of Na channels. (*A*) Axons were voltage-clamped at a holding potential of −80 mV, and the membrane potential was stepped to the potentials shown in the abscissa for 50 ms. Test pulses of 2-ms duration to 0 mV were applied 0.2 ms after each prepulse, and the peak amplitude of I_{Na} was recorded (consult *F* for the protocol). Shown is the amplitude of I_{Na} before (○) and after (●) modification of channels with MTS-PTrEA. Hyperpolarizing prepulses recover proportionally larger currents in modified channels than in control channels. (*B*) The data from part A were normalized to construct steady-state inactivation curves (h_∞ curves). After modification by MTS-PTrEA the h_∞ curve is made shallower and shifts toward hyperpolarized potentials. Thus whereas normally less than 20% of channels are inactivated at a holding potential of −80 mV (○), the fraction of inactivated channels after modification by MTS-PTrEA was increased to 75% (●). (*C*) The time course of recovery from steady-state inactivation at −130 mV was compared in control and MTS-PTrEA-modified channels. The axon was clamped at a holding potential of −80 mV and hyperpolarized to −130 mV for varying durations shown in the abscissa (refer to the middle protocol in *F*). The peak amplitude of I_{Na} after a 0.2-ms delay was measured

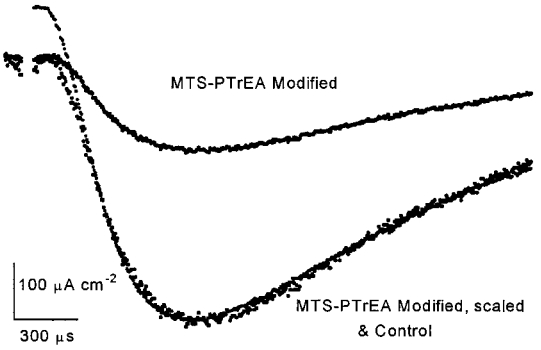


FIGURE 6 MTS-PTrEA does not alter inactivation from the open state. Activation and inactivation of I_{Na} before and after modification with MTS-PTrEA are shown resulting from a voltage jump to 0 mV (holding potential = −80 mV). In both cases the axon was hyperpolarized to −130 mV for 50 ms to remove inactivation. The noninactivating component of MTS-PTrEA-modified I_{Na} was removed by subtracting a trace for which the axon was depolarized to 0 mV for 50 ms before the test pulse. When arbitrarily scaled, the inactivation kinetics of MTS-PTrEA-modified I_{Na} closely follows that of the control. Experiment SE076C.

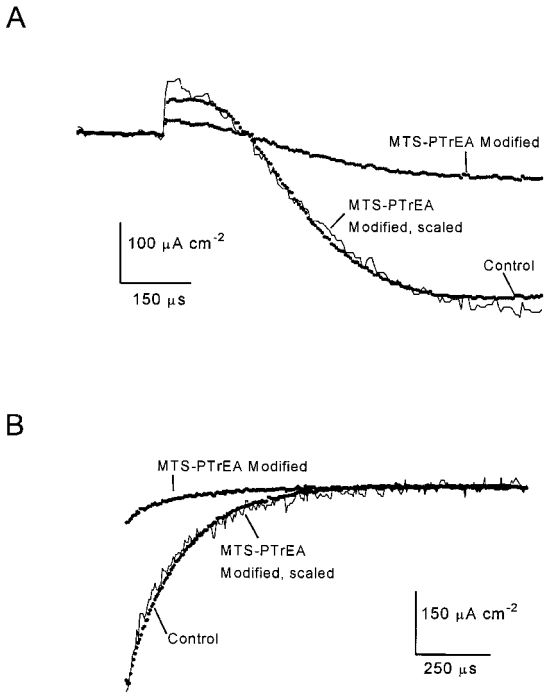


FIGURE 7 MTS-PTrEA does not alter activation and deactivation kinetics of Na channels. (*A*) I_{Na} before and after exposure of the axon to MTS-PTrEA was recorded in response to a 1-ms test pulse to 0 mV from a holding potential of −80 mV. The test pulse was preceded by a 50-ms prepulse of −130 to recover channels (particularly the MTS-PTrEA-modified ones) from steady-state inactivation. Scaling the current obtained after modification of channels with MTS-PTrEA allows for reasonable superimposition with control records. (*B*) Tail currents in control and MTS-PTrEA-modified channels when the membrane potential was repolarized to −80 mV at the end of a 1-ms depolarizing test pulse to 0 mV. When scaled, the deactivation kinetics of MTS-PTrEA-modified current closely followed that of control channels. Experiment SE046C.

several facts. First, the evidence suggests that channels are preferentially labeled when they are inactivated. Second, the labeling compound has no effect on the channels unless the inactivation machinery is intact. Third, labeling the residue with MTS-PTrEA affects the gating current of the channels in the same way as inactivation. Finally, the steady-state inactivation curve is strongly affected in labeled channels. We discuss these points in the following and present a tentative model.

MTS-PTrEA probably modifies inactivated Na channels

The alkanethiosulfonate family, of which MTS-PTrEA is a member, reacts rapidly with cysteinyl sulfhydryls of proteins with high selectivity (Kenyon and Brucie, 1977). The most likely explanation for the irreversible actions of MTS-PTrEA on I_{Na} and I_g is reaction with a cysteine (or cysteines) present in the Na channel. That certain states of the channel are selectively susceptible to labeling is strongly suggested by the fact that the rate of cysteine modification, monitored from reduction of I_{Na}, depended on V_m. Hyperpolarization of axons to −110 mV completely prevented modification, whereas at −70 mV the rate of modification was ~5 M^{-1} s^{-1}. A possible interpretation of the results, therefore, may be that only inactivated Na channels are modified by MTS-PTrEA. Hyperpolarization to −110 mV removes all steady-state inactivation, making channels unavailable for modification at this potential, whereas a fraction of them would be inactivated at −70 mV and capable of reaction with MTS-PTrEA. Two further observations are in agreement with this interpretation. The first is that repeatedly opening and inactivating the Na channels with short depolarizing pulses from a holding potential of −110 mV speeded modification. And the second is that removing inactivation by pronase prevented MTS-PTrEA from irreversibly affecting the channels.

The rate of modification of Na channels with MTS-PTrEA at −70 mV is much lower than the speed by which methanethiosulfonates react with sulfhydryls in solution (in

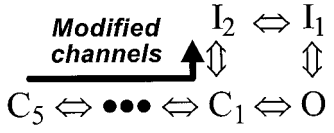
with 2-ms-long voltage steps to 0 mV. Control (○) and MTS-PTrEA-modified (●) channels recovered from steady-state inactivation with a similar time course at −130 mV. (*D*) The time course of inactivation of control and MTS-PTrEA-modified channels at −80 mV was studied in the same axon. The axon was hyperpolarized to −130 mV for 50 ms, and the peak I_{Na} was measured from 2-ms test pulses to 0 mV after varying delays shown in the abscissa (see bottom protocol in *F*). MTS-PTrEA-modified channels (●) inactivated four times more quickly at −80 mV than did unmodified channels (○). *A–D* are from experiment SE076D. (*E*) Comparison of the h_∞ curve of MMTS-modified channels (●) with control (○) shows that MMTS does not affect the h_∞ curve. Preexposure of axons to MMTS, however, does prevent subsequently perfused MTS-PTrEA from changing the h_∞ curve (▲). Experiment SE056B. (*F*) Voltage protocols used for experiments shown in earlier panels.

the range 10^4-10^5 M^{-1} s^{-1}; see Stauffer and Karlin, 1994) and is within the lower range of rates reported for modification of cysteines in protein structures (see, for example, Holmgren et al., 1996a). This suggests that the target cysteine(s) is not readily accessible to MTS-PTrEA, even in inactivated channels.

MTS-PTrEA-modified channels inactivate avidly from closed states

Labeling with MTS-PTrEA has clear effects on the inactivation mechanism. After modification the steady-state inactivation curve is much shallower than normal and does not saturate at −130 mV. Even at this very negative voltage it is possible to recover ionic current from only about one-third of the labeled channels. Gating current of modified channels has the rapid, monotonic decay characteristic of inactivated channels for steps from a holding potential of −80 mV. Gating current recovers its amplitude and rounded time course after 50 ms at −130 mV, analogous to the recovery of ionic current at this voltage.

For a population of normal channels, stepping to −130 from −80 mV for 50 ms increases the current in a subsequent depolarization by ~20–30%. When V_m is returned to −80 mV, this recovered current inactivates once again with a time constant of ~3.3 ms. The recovered current from MTS-labeled channels subjected to the same protocol inactivates about four times faster. Furthermore, the gating current of the labeled channels returns to the "inactivated" shape with the same time course. Interestingly, when measured at 0 mV, the inactivation rate of modified channels is the same as for normal channels. This strongly suggests that inactivation from closed states is facilitated in the MTS-labeled channels, whereas inactivation from the open state occurs normally. A possible model for this effect can be seen by using the state diagram that has been proposed for Na channels (Armstrong and Gilly, 1979; see Scheme 1). Normally the activation path goes from C_5 to O through the intervening closed states. The channels then inactivate, moving primarily from the open state to the inactivated state I_1. Recovery from inactivation bypasses the open state, as shown: the preferred path is from I_1 (the open inactivated state) to I_2 (the closed inactivated state), and thence to the closed states C_1–C_5. Thus channels do not leak Na^+ during recovery from inactivation (Armstrong and Croop, 1982; Bean, 1981). A consequence of this recovery path combined with the theoretical necessity that all steps be reversible is that channels must be able to inactivate from state C_1. Inactivation from one or more closed states was in fact measured by Bean (1981), and it occurs primarily at slightly depolarized voltages where, in terms of the state diagram, occupancy of state C_1 is at maximum. The results presented in this paper are explainable by postulating that MTS labeling facilitates inactivation from state C_1 or opens pathways to I_2 from the other closed states. The results thus open an experimental window into this little-studied but important aspect of Na channel physiology. The importance of these details of Na channel inactivation is due to the numerous diseases that result from perturbations of the mechanism.



Scheme 1.

Comparison with actions of local anesthetics

Although the interactions of local anesthetics with Na channels are quite complicated (for a review see Hille, 1992), it is of interest to note that the effect of MTS-PTrEA on Na channels is similar to actions of some local anesthetics. For example, much like MTS-PTrEA, ionizable amine anesthetics lidocaine and tetracaine, or the neutral anesthetic benzocaine, alter the inactivation curve by reducing its slope and producing a large hyperpolarizing shift in it (Hille, 1977; unpublished I_g affected similarly). Despite the similarities there are two distinguishing differences between the mechanism of action of local anesthetics and that of MTS-PTrEA. The first is that local anesthetics alter the inactivation curve by reducing the rate of recovery from inactivation (Khodorov et al., 1976; Hille, 1977), but MTS-PTrEA does not alter the recovery rate. And the second is that some local anesthetics alter "slow" inactivation in addition to fast inactivation (Khodorov et al., 1976; Hille, 1977), whereas actions of MTS-PTrEA are restricted solely to affecting fast inactivation.

Comparison with literature

Early reports on the modification of sulfhydryls in crayfish giant axons with extracellularly applied *N*-ethylmaleimide (NEM) reported a change in steady-state inactivation properties of Na channels and attributed this to a change in slow inactivation (Shrager, 1976, 1977; Starkus and Shrager, 1978). Using selective thiol-reactive compounds, we also find that modification of native cysteine(s) in squid giant axons affects steady-state inactivation. Our results differ, however, from the mentioned reports in two aspects. The first is that we are unable to affect Na channels with methanethiosulfonates applied externally. Since it is well documented that NEM readily and rapidly crosses lipid bilayers (Holmgren et al., 1996b), its effect in crayfish giant axons may have been a consequence of its diffusion across the membrane. Second, we find that MTS-PTrEA affects steady-state inactivation apparently by increasing the closed state(s) inactivation rate rather than by altering "slow" inactivation. This is supported by the fact that in squid giant axons pronase removes fast inactivation without affecting slow inactivation (Rudy, 1978), and as shown, pronasing

precludes the irreversible effects of MTS-PTrEA or MTSET on Na channels.

The effects of methanethiosulfonates on squid Na channels described here are in contrast to their inertness in adult human (hSKM1) and rat (μ1) skeletal muscle Na channels (Yang and Horn, 1995; Yang et al., 1996; Lerche et al., 1997; Vedantham and Cannon, 1998). The reason for this is not clear but most likely arises from differences in the amino acid sequence of squid Na channels. In the absence of the sequence for squid *Loligo pealei* Na channels, a detailed comparison cannot be made at this time.

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REFERENCES

Akabas, M. H., D. A. Stauffer, M. Xu, and A. Karlin. 1992. Acetylcholine receptor channel structure probed in cysteine-substitution mutants. *Science.* 258:307–310.

Armstrong, C. M., and F. Bezanilla. 1977. Inactivation of sodium channel. II. Gating current experiments. *J. Gen. Physiol.* 70:567–590.

Armstrong, C. M., F. Bezanilla, and E. Rojas. 1973. Destruction of Na channel conductance inactivation in squid axons perfused with pronase. *J. Gen. Physiol.* 62:375–391.

Armstrong, C. M., and R. S. Croop. 1982. Simulation of Na channel inactivation by thiazin dyes. *J. Gen. Physiol.* 80:641–662.

Armstrong, C. M., and W. F. Gilly. 1979. Fast and slow steps in the activation of sodium channels. *J. Gen Physiol.* 74:691–711.

Bean, B. P. 1981. Sodium channel inactivation in the crayfish giant axon. Must channels open before inactivating? *Biophys. J.* 35:595–614.

Hille, B. 1977. Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.* 69:497–515.

Hille, B. 1992. Ionic Channels of Excitable Membranes, 2nd Ed. Sinauer and Associates, Sunderland, MA.

Holmgren, M., M. E. Jurman, and G. Yellen. 1996a. N-type inactivation and the S4–S5 region of the Shaker K^+ channel. *J. Gen Physiol.* 108:195–206.

Holmgren, M., Y. Liu, and G. Yellen. 1996b. On the use of thiol-modifying agents to determine channel topology. *Neuropharmacology.* 35: 797–804.

Kenyon, G. L., and T. W. Brucie. 1977. Novel sulfhydryl reagents. *Methods Enzymol.* 47:407–430.

Khodakhah, K., A. I. Melishchuk, and C. M. Armstrong. 1997. Cysteine labeling and charge immobilization in squid Na channels. *Biophys. J.* 72:A262.

Khodorov, B., L. Shishkova, E. Peganov, and S. Revenko. 1976. Inhibition of Na currents in frog Ranvier node treated with local anesthetics. Role of slow inactivation. *Biochim. Biophys. Acta.* 433:409–435.

Lerche, H., W. Peter, R. Fleischhauer, U. Pika-Hartlaub, T. Malina, N. Mitrovic, and F. Lehmann-Horn. 1997. Role in fast inactivation of the IV/S4–S5 loop of human muscle Na^+ channel probed by cysteine mutagenesis. *J. Physiol. (Lond.).* 505:345–352.

Rudy, B. 1978. Slow inactivation of the sodium conductance in squid giant axons. Pronase resistance. *J. Physiol. (Lond.).* 283:1–21.

Shrager, P. 1976. Specific chemical groups involved in the control of ionic conductance in nerve. *Ann. N.Y. Acad. Sci.* 264:293–303.

Shrager, P. 1977. Slow sodium inactivation in nerve after exposure to sulfhydryl blocking agents. *J. Gen Physiol.* 69:183–202.

Smith, D. J., E. T. Maggio, and G. L. Kenyon. 1975. Simple alkanethiol groups for the temporary blocking of sulfhydryl groups of enzymes. *Biochemistry.* 14:766–771.

Smith, H. M. 1958. Effects of sulfhydryl blockade on axonal function. *J. Cell. Comp. Physiol.* 51:161–171.

Starkus, J. G., and P. Shrager. 1978. Modification of slow sodium inactivation in nerve after internal perfusion with trypsin. *Am. J. Physiol.* 235:C238–C244.

Stauffer, D. A., and A. Karlin. 1994. Electrostatic potential of the acetylcholine binding sites in the nicotinic receptor probed by reactions of binding-site cysteines with charged methanthiosulfonates. *Biochemistry.* 33:6840–6849.

Vedantham, V., and S. C. Cannon. 1998. Slow inactivation does not affect movement of the fast inactivation gate in voltage-gated Na^+ channels. *J. Gen. Physiol.* 111:83–95.

Yang, N., A. L. George, and R. Horn. 1996. Molecular basis of charge movement in voltage-gated sodium channels. *Neuron.* 16:113–122.

Yang, N., and R. Horn. 1995. Evidence for the voltage-dependent S4 movement in sodium channels. *Neuron.* 15:213–218.