MODIFICATION OF SODIUM CHANNEL INACTIVATION IN SINGLE MYELINATED NERVE FIBERS BY METHIONINE-REACTIVE CHEMICALS

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ABSTRACT Several methionine-reactive reagents, including *N*-bromoacetamide, *N*-bromosuccinimide, chloramine-T, and *N*-chlorosuccinimide, irreversibly slowed and prevented Na channel inactivation in single myelinated nerve fibers, whereas sulfhydryl- or tyrosine-modifying reagents had little effect. The activation process was not modified by the reagents that altered inactivation and could be modulated normally by Ca^{++} ions and *Centruroides* scorpion toxin IIα. These results suggest that externally applied *N*-bromoacetamide and its related compounds specifically affect Na channel inactivation by modifying methionine residues on the channel.

The Na permeability of excitable membranes increases quickly (activation) and subsequently decreases (inactivation) during a maintained depolarization. The structure that controls the Na channel inactivation process is susceptible to a wide variety of agents. For example, pronase (proteolytic enzymes) and *N*-bromoacetamide (NBA) when applied internally in squid axons completely prevent Na channel inactivation (1, 2). Because NBA can both modify the side-chain residues of proteins and, like pronase, cleave peptide bonds, the identification of the critical residues involved in the inactivation process remains ambiguous.

I have sought to remove Na channel inactivation in single myelinated nerve fibers by applying chemicals to the external surface. To date, no such chemical method has been available for the node of Ranvier except by internally applied iodate (3). When applied externally to single myelinated nerve fibers, NBA and *N*-bromosuccinimide (NBS) slowed and prevented Na channel inactivation dramatically (Fig. 1, *A* and *B*). Often the peak Na currents were also somewhat reduced, but the activation time course and the peak current-voltage relationship were little changed. Prolonged depolarization of the membrane revealed that the half-time for decay of the inactivatable Na current in the treated fibers was at least threefold longer than it was in the control fibers, depending upon the duration of exposure to the reagents. Eventually most of Na channel inactivation could be inhibited (Fig. 1, *C* and *D*). This effect was not reversed upon washing, implying that a covalent reaction had led to an irreversible change.

NBA and NBS cleave peptide bonds of soluble proteins at various amino acid residues including tryptophan, tyrosine, and histidine. They also modify the side chain of cysteine and methionine residues (see Table I). Oxford et al. (2) have suggested that the action of NBA on Na channel inactivation is to cleave the carboxyl end of tyrosine residues on the axoplasmic side of membrane, since they observed that another reagent which reacts with tyrosyl residues, *N*-acetylimidazole (NAI), also partially removed inactivation when applied internally in squid axons. However, I found that NAI had little effect on Na currents in single myelinated nerve fibers when applied externally (at 1.8 mM for 30 min), and that it did not antagonize the effect of NBA. Assuming that external NAI has similar accessibility to the protein residues as does external NBA (since both reagents are small, <140 dalton, and are predominantly in neutral form at physiological pH), this result then suggests that tyrosine residues are not critical for the function of the inactivation process, at least not in myelinated nerves. In the following sections, I will present the results of selective chemical modifications to demonstrate a possible critical amino acid residue for Na channel inactivation.

Two other reagents, *N*-chlorosuccinimide (NCS) and chloramine-T (CT, *N*-chloro-*p*-toluenesulfonamide), also selectively modify methionine and cysteine residues of proteins at neutral pH (4). This mode of action resembles the side-chain modification of proteins by NBA and NBS (see Table I). Similarly, NCS and CT slowed and prevented Na channel inactivation when applied externally to the nerve fibers. Detailed studies of CT effects on Na channels in myelinated fibers have revealed that, like NBA and NBS, this compound has no effect on channel activation (5). Taken altogether, these results strongly implicate

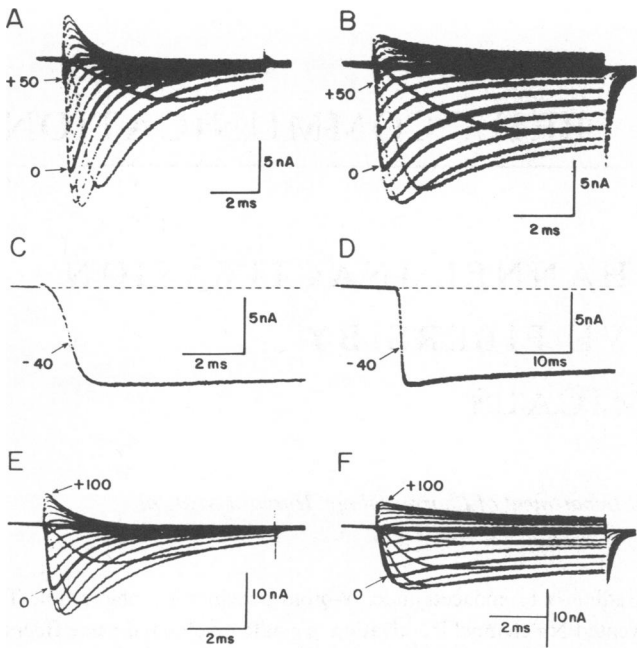

FIGURE 1 Effects of various chemicals on Na currents. Single myelinated fibers were isolated from sciatic nerves of the toad *Bufo marinus* and voltage-clamped as described (17). Na currents were measured before (*A*) and after (*B*) the node was treated with 0.36 mM NBA for 13 min. Most of Na channel inactivation was prevented as shown in *C* and in *D* (note different time scales). (*E*) Na currents in a nerve fiber first treated with 2 mM NEM for 20 min and (*F*) further treated with 0.37 mM NCS for 10 min. The control Na current family before NEM treatment is not shown here; it appeared similar to (*E*) and only a small reduction (<10%) in peak Na currents was found after NEM treatment. All potassium currents were blocked by internal 0.12 M CsCl and external 12 mM TEA-Ringer's solution. Leak and capacitive currents were subtracted by analog circuitry. Na currents were calibrated by assuming an internodal resistance of 20 MΩ. Temperature, 8°C. Holding potential, −100 mV. Prepulse potential, −130 mV for 50 ms. Numbers indicated in the current traces are the test potentials. Frog TEA-Ringer's solution contained 12 mM tetraethylammonium chloride, 110 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.2.

that reactive cysteine or methionine residues, or both, are critical for the normal inactivation processes of Na channels.

In principle, demonstration of a cysteine residue that is essential for the function of proteins can be accomplished by using several sulfhydryl group-specific reagents (6, 7); two of them, *N*-ethylmaleimide (NEM) and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), were tested on the node. I found that neither sulfhydryl reagent had much effect on the fast inactivation of Na channels in this preparation (Fig. 1 *E*), in agreement with the findings of others in squid and crayfish axons (2, 8). Furthermore, pre-treatment with NEM or DTNB did not prevent methionine-reactive reagents from removing inactivation, as shown in Fig. 1 *F*. NEM has a relatively slow reaction rate at pH 7.2, since it must react with the unprotonated side chain of cysteine by nucleophilic addition across the NEM double bond. But even when the solution pH was raised to 8.4 (~pK_a of cysteine side chain) NEM still did not change the Na current kinetics as compared with the control currents at that pH. Thus, unless NEM, a small (125 daltons) and neutral molecule, has different accessibility to the protein residues than NBA or NCS, the results indicate that cysteine does not appear to be a critical residue for Na channel inactivation. Besides, NBA and NBS modify the amino acid cysteine to cysteic acid (4, 9) while NCS and CT modify it to cystine (4). Two very different products of cysteine are presumably produced by these reagents, yet they have a common effect on Na channel inactivation. These observations, along with others (2, 8), are inconsistent with the assignment of cysteine residues as protein side chains essential for Na channel inactivation. On the other hand, methionine residues are modified by all of these reagents to either methionine sulfoxide (-SO-CH_3) alone or both methionine sulfoxide and sulfone (-SO_2-CH_3) (4). The selectivity of these reagents for methionine residues strongly supports the notion that the modification of

TABLE I
SPECIFICITIES OF VARIOUS CHEMICALS AT NEUTRAL pH AND THEIR EFFECTS ON Na CURRENTS

Chemicals	Effective concentration	Amino acid modified and products				Sites of peptide cleaved			Effects on Na channel inactivation	
		Methionine	Cysteine	Cystine	Tyrosine	Tryptophan	Tyrosine	Histidine	Slowed	Prevented
	mM									
NBA (9)	0.36	sulfoxide sulfone	cysteic acid	cysteic acid	—*	+*	+	+	+	+
NBS (4, 9)	0.28	sulfoxide sulfone	cysteic acid	cysteic acid	—	+	+	+	+	+
NAI (18)	1.82	—	(+)*	—	+	—	—	—	—	—
NEM (6)	2.00	—	+	—	—	—	—	—	—	—
DTNB (7)	0.63	—	+	—	—	—	—	—	—	—
NCS (4)	0.37	sulfoxide	cystine	—	—	—	—	—	+	+
CT (4)	1.07	sulfoxide	cystine	—	—	—	—	—	+	+

*—, no effect; +, positive; (+), positive but less reactive than tyrosine. All chemicals were obtained from commercial sources and dissolved freshly in frog TEA-Ringer's solution.

methionine residues destroys normal Na channel inactivation.

A summary of the effects of various reagents on Na channel inactivation is shown in Table I. The ionic currents of this nerve preparation, unfortunately, will not survive at certain pH values, high reagent concentrations, and long incubation times, so it is expected that modification of inactivation by some reagents cannot be demonstrated physiologically. For example, I tested several reagents such as benzyl bromide (16.8 mM), iodoacetamide (1.35 mM), iodoacetic acid (2.69 mM), and H_2O_2 (8.5 mM), which presumably also react with cysteine and methionine residues under certain conditions (for review, see reference 10); no effects of these drugs were found on Na channel inactivation, except that there was a scaled reduction of the Na current at all times and voltages, consistent with a decrease in the number of channels. Furthermore, these reagents did not protect Na channel inactivation from being slowed by NCS or CT indicating that the reaction of these reagents with methionine residues does not occur or occurs very slowly under neutral pH conditions.

It may be pertinent at this point to discuss the difficulties of interpreting the negative results in chemical modifications of Na channels. Negative results do not demonstrate unequivocally that the specific residues are not required for normal inactivation. Only those residues that are accessible to the chemical and are in the appropriate environment in terms of hydrophilicity and local pH will be expected to be modified (4, 9, 18). A case in question, then, is the negative external NAI result in nodes, which clearly contradicts the positive result obtained in squid axons when NAI was perfused internally. It is almost certain that tyrosine residues cannot be the only important functional groups for Na channel inactivation in the node, since both CT and NCS remove inactivation without modifying tyrosine residues. One possible explanation to reconcile these different results of NAI in nodes and in squid axons is that perhaps several different functional groups in the Na channel are required for the normal inactivation mechanism. The tyrosine residues might be critical for inactivation in nodes, but that cannot be proved directly by using external NAI. Obviously, a detailed study of the involvement of tyrosine residues in Na channel inactivation is needed to address this question.

Although NBA strongly affects Na channel inactivation, its actions on myelinated fibers differed from those that have been reported for squid axons (2). NBA at 1 mM removed Na channel inactivation only when perfused internally in squid axons, whereas all the effective chemicals tested in our preparation were applied externally. No effects on Na channel inactivation were found when NBA was applied from the outside of squid axons. Also significant was the slowing of Na channel inactivation at the node of Ranvier after NBA or similar treatments. In squid axons NBA removed Na channel inactivation in an all-or-none manner; no slowing of the residual inactivation was detected. Interestingly, external NBA at 0.72 mM also slowed Na channel inactivation in frog skeletal muscle (11). These differences cannot be easily explained at this time. However, since most of the test chemicals are electrically neutral and can permeate the nerve membrane, the location of the modified residues cannot be determined by noting whether application of reactive chemicals was internal or external.

The activation process of the Na channel appears to be insensitive to the chemical modifications (1–3, 11, and Fig. 1). Activation not only showed normal voltage-dependence after inactivation was removed by methionine-reactive reagents but also could be further modulated by *Centruroides* toxin IIα. Previous results have shown that several purified *Centruroides* scorpion toxins shift the activation process by 40–50 mV in the hyperpolarized direction after a brief membrane depolarization (12, 13). These toxins presumably affect the activation process by a voltage-dependent binding reaction as proposed by Cahalan (14). Fig. 2 shows the effects of *Centruroides* toxin IIα on Na

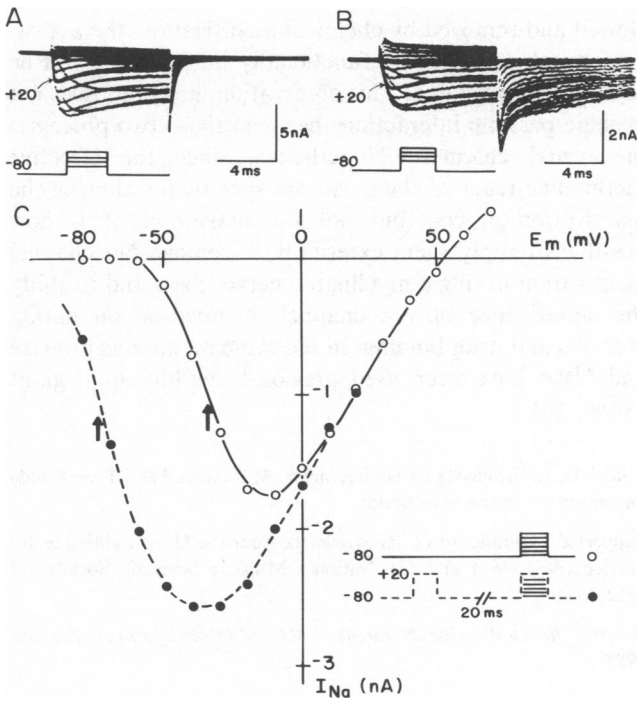

FIGURE 2 Modulation of Na channel activation after chloramine-T treatment. (*A*) Na currents were recorded from a nerve fiber treated with 1.07 mM chloramine-T for 28 min and washed with TEA-Ringer's solution. Note that Na channel inactivation was slowed and prevented. (*B*) Na currents were measured after the same nerve had been further treated with 250 nM *Centruroides* toxin IIα for 11 min. Note that the induced currents appear after the membrane repolarization. (*C*) The shift of voltage dependence of peak Na currents during test pulse was measured in the absence (○) and in the presence (●) of a +20 mV conditioning pulse. The shift was ~40mV in the hyperpolarized direction as shown in arrows. Pulse schemes (*A–C*) are shown at the bottom of each panel with a constant pulse duration of 8 ms. Holding potential, −80 mV. *Centruroides* toxin IIα was purified from *Centruroides sculpturatus* scorpion venom as described (13).

currents in a chloramine-T treated fibers. The peak currents were reduced to ~40% of the original value, and the activation process was shifted during a depolarization so that following repolarization toxin-induced currents appeared (Fig. 2, *A* and *B*). The extent of the apparent shift in activation was ~40 mV in the hyperpolarized direction as shown in Fig. 2 *C*. The kinetics of toxin-induced currents were identical to those of myelinated fibers in the presence of both *Leiurus* (which also slows and prevents Na channel inactivation) and *Centruroides* toxins. Both activation and inactivation processes can be modified separately and simultaneously by the two classes of scorpion toxins (12, 13), or by chemicals and *Centruroides* toxin IIα (Fig. 2).

Similarly, after the Na channel inactivation was removed by CT treatment, elevating the external calcium concentration still caused the same shift in the voltage-dependence of peak Na permeability as in a control fiber, 9.0 mV/*e*-fold Ca^{++} change, a shift in the depolarized direction that is similar to one in normal frog myelinated nerve fibers reported previously (8.7 mV/*e*-fold Ca^{++} increase, reference 15). Thus, even when inactivation was slowed and removed by chemical modification, the activation structure remained functionally intact and could be separately modulated. This observation, however, does not exclude possible interactions between those two processes in normal channels. Nonetheless, since the effective methionine-reactive chemicals are specific for altering the inactivation process but not the activation, it is now possible to apply them externally to remove Na channel inactivation in single myelinated nerve fibers and to study the significance of Na channel inactivation on gating currents and drug binding, in the same manner as pronase and NBA have been used previously within squid giant axons (16).

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