EFFECTS OF SOME CHEMICAL REAGENTS ON SODIUM CURRENT INACTIVATION IN MYELINATED NERVE FIBERS OF THE FROG

MICHAEL RACK,* NORBERT RUBLY,‡ AND CHRISTEL WASCHOW*
*Institut für Physiologische Chemie; and ‡I. Physiologisches Institut, Universität des Saarlandes, D-6650 Homburg/Saar, Federal Republic of Germany

ABSTRACT The effect of several chemical reagents on the sodium current was studied in voltage-clamped single nerve fibers of the frog. The oxidants halazone and hypochlorous acid drastically inhibited inactivation. Their effect was similar to that of chloramine T (Wang, 1984*a*). The curve relating the steady-state inactivation parameter h_∞ to the conditioning potential E became nonmonotonic after treatment with the oxidants, i.e., $dh_\infty/dE > 0$ for $E > -20$ mV. By contrast, the oxidants periodate, iodate, and hydrogen peroxide (applied for the same time, but at higher concentrations) merely produced a parallel shift of the $h_\infty(E)$ curve to more negative values of membrane potential. Diethylpyrocarbonate, a reagent that preferentially modifies histidine groups, had one marked effect: a strong shift of the $h_\infty(E)$ curve to more negative values of membrane potential. Almost no effect was observed after application of the tyrosine-reactive reagent *N*-acetylimidazole. Similarly, the arginine-reactive reagent glyoxal had only minor effects on the Na permeability. The results suggest that methionine is not critically involved in the kinetics of Na current inactivation. Similarly, an essential tyrosine or arginine residue seems to be unavailable to chemical reagents from outside on the frog node of Ranvier. Deduced from the reactivities of (some of) the reagents used, modification of membrane lipids is a tentative explanation for the effects observed on inactivation kinetics.

INTRODUCTION

When given from outside on voltage-clamped myelinated nerve fibers, the chemical reagents glutaraldehyde (Schmidtmayer, 1985), *N*-bromoacetamide (NBA) (Wang, 1984*b*) and chloramine T (CT) (Wang and Strichartz, 1983; Wang, 1984*a*, *b*) partially remove inactivation of the sodium permeability irreversibly. Among these agents CT is of major interest because its application does not cause a deterioration of the fibers and, under certain conditions, CT modifies only sulfhydryl- and methionine residues among the amino acids common in proteins (Shechter et al., 1975). Modification of sulfhydryl groups does not result in a maintained Na current (Shrager, 1977; Rack et al., 1984; Wang, 1984*b*). Consequently, modification of a methionine residue critically involved in the process of inactivation was proposed (Wang, 1984*a*, *b*; Wang et al., 1985). However, external application of other reagents known to modify methionine (and other) amino acid residues does not alter the kinetics of inactivation in myelinated nerve fibers (see Wang, 1984*b*). Moreover, CT removes a large part of inactivation when applied internally (or externally) on squid axons (Wang et al., 1985), while cyanogen bromide (BrCN), another reagent with a high reactivity toward methionine residues, is without effect on inactivation in this preparation (Oxford et al., 1978).

In view of these conflicting results it seemed of interest to study the effects of several chemical reagents closely related to CT, and of others able to modify the amino acid residue(s) proposed to be involved in sodium channel inactivation. The results do not favor the idea of a critical involvement of a methionine residue in sodium channel inactivation.

METHODS

Single nerve fibers were dissected from the sciatic nerve of the frog *Rana esculenta* (Stämpfli, 1969). A node of Ranvier was voltage clamped at 12°C by the method of Nonner (1969). The fiber was cut on both sides of the node at a distance of ~0.75 mm. The ends of the fiber were in 113 mM CsCl, 7 mM NaCl, 2 mM MOPS (morpholinopropanesulfonic acid), pH 7.3. This solution was used for blocking K currents. The potential at which 30% of the Na channels are inactivated was taken as the normal resting potential and defined as $E = -70$ mV. The fibers were held at $E = -70$ mV. The command voltage pulses were generated by a 12-bit D/A converter under computer control. Membrane currents were filtered by a 10 kHz lowpass filter and sampled in 10 or 100-μs intervals by means of a 12-bit A/D converter, also operating under computer control. Absolute membrane currents were calculated by assuming a longitudinal axoplasmic resistance of 10 MΩ, corresponding to a value of 140 MΩ/cm for the resistance per unit length of a 14 μm frog nerve fiber (Stämpfli and Hille, 1976). All potentials are given as absolute potentials. To correct for capacitative and leakage currents, the current produced by a -30-mV pulse was scaled appropriately and subtracted from the currents produced by the depolarizing pulses.

Address all offprint requests to M. Rack.

To measure the $h_\infty(E)$ curve, 40-ms conditioning pulses to varying potentials followed by a constant test pulse to +10 mV were used. Normalized test pulse current was plotted against membrane potential during the conditioning pulse. The equation

$$h_\infty = [(1 - C)/(1 + \exp((E - E_h)/k))] + C \qquad (1)$$

was fitted to the experimental points. In this equation, k is the slope parameter and E_h is the potential at which $h_\infty = 0.5 \times (1 + C)$. C is used for the description of a noninactivating component of the sodium permeability.

SOLUTIONS

The node was superfused continuously with Ringer's solution with or without a chemical reagent. The control Ringer's solution contained 110 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 12 mM TEA, and 4 mM MOPS. The pH was adjusted to 7.2 with 1 N NaOH. In several experiments (with halazone, CT, and hypochlorous acid) the NaCl was replaced by RbCl and the pH adjusted to 7.2 with tetramethylammonium hydroxide instead of NaOH, with no change in the concentration of the other salts. This solution was used during treatment with the chemical reagents to prevent shifts of the reversal potential that occur by Na-ion influx (Rack and Woll, 1984). The sodium hypochlorite stock solution contained ~5% available chlorine and was diluted 1:100 with RbCl-Ringer. The sodium ion content before dilution is >2 M, thus sodium ions were present during application of this reagent. Halazone was dissolved in a small amount of 1 N tetramethylammonium hydroxide, added to the Ringer's solution and the pH was readjusted to 7.2 with 1 N HCl.

Three of the reagents tested (iodate, periodate, diethylpyrocarbonate) were applied in a Ringer's solution with a pH of 5.5. This solution contained 4 mM MES and the pH was adjusted with 1 N NaOH.

Glyoxal was applied in a Ringer's solution with a pH of 9.0. The concentrations of the salts were the same as in standard Ringer's, but 4 mM CHES instead of 4 mM MOPS was used as buffer. The pH was adjusted with 1 N NaOH.

Because most of the reagents are hydrolyzed by water, the individual Ringer's solutions were freshly prepared for each experiment and applied to the nerve fiber immediately after preparation. After application the fiber was washed with reagent-free Ringer's solution for at least 6 min before measurements were continued.

MATERIALS

Morpholinopropanesulfonic acid (MOPS), morpholinoethanesulfonic acid (MES), 2-(cyclohexylamino)-ethanesulfonic acid (CHES), *N*-acetylimidazole (NAI), *N*-bromosuccinimide (NBS) and glyoxal were from Serva, Heidelberg, Federal Republic of Germany. CT, rubidium-chloride, tetraethylammonium chloride (TEA), and tetramethylammonium hydroxide (TMA) were from Fluka, Neu-Ulm. Diethylpyrocarbonate (DEP) and halazone were from Sigma, München. Sodium hypochlorite was from Ega, Steinheim. All other chemicals were analytical grade or in the purest form available and were purchased from Merck, Darmstadt, Federal Republic of Germany.

RESULTS

Hypochlorous Acid

HOCl has a long history of use as disinfectant and for other purposes like bleaching of textiles. This strongly oxidizing compound is developed when *N*-chloroamides are dissolved in aqueous solution (O'Connor and Kapoor, 1970; Alexander, 1973). Thus, HOCl will be present after dissolving CT or halazone (see below) in Ringer's solution. To study the effects of hypochlorous acid directly, an aqueous solution of sodium hypochlorite was diluted in Ringer's solution (see Methods) and applied to single nodes of Ranvier at a pH value of 7.2 for 10 min. After washout of the reagent the Na currents were reduced to ~40% of their original size and most of the inactivation of the remaining currents was removed (see Fig. 1). Plotting I_{Na} vs. pulse potential revealed that HOCl treatment shifted the descending branch of the $I_{Na}(E)$ curve by 10 mV in the depolarizing direction, while the reversal potential was shifted in the hyperpolarizing direction (Fig. 2). In three experiments, the shift of the descending branch of the $I_{Na}(E)$ curve was 7.9 (±2.7) mV, (N = 3). The shift of the descending branch of the $I_{Na}(E)$ curve is partly due to the voltage drop across the series resistance R_s. Assuming R_s = 220 kΩ (Drouin and Neumcke, 1974), the shift resulting from the reduction of I_{Na} (after HOCl-treatment) accounts for <2 mV.

The shift of the reversal potential can be explained by a gradual rise of the internal sodium concentration during application of the modifying Ringer's solution (containing >20 mM NaCl, see Methods). This assumption was confirmed by the use of calcium hypochlorite. The use of this sodium free Ringer's solution prevented most of the shift of the reversal potential, while sodium current inacti-

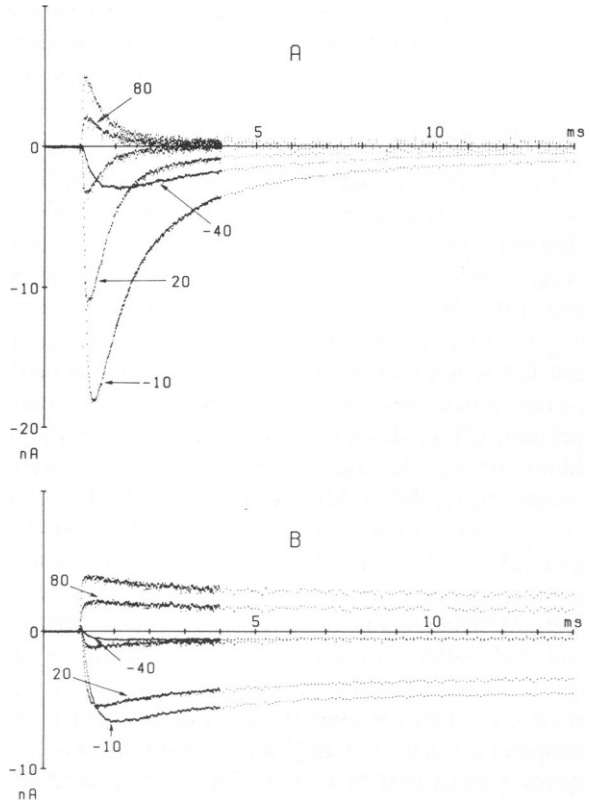

FIGURE 1 Na currents associated with 14-ms depolarizing pulses to potentials between −40 and 110 mV (30 mV steps) before (*A*) and after (*B*) treatment with hypochlorous acid for 10 min at pH 7.2. K currents blocked by internal Cs and external TEA. Holding potential was −70 mV.

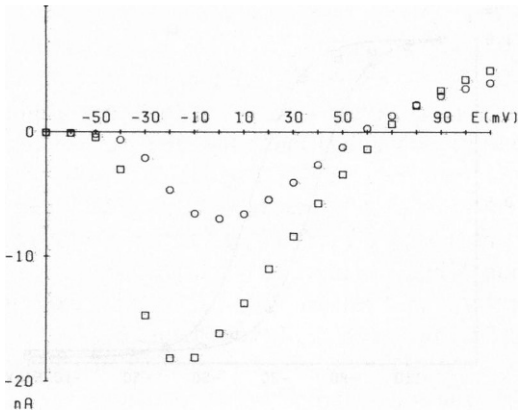

FIGURE 2 Peak sodium current-voltage curve, $I_{Na}(E)$, before (□) and after (○) treatment with hypochlorous acid for 10 min at pH 7.2.

vation was inhibited as after the application of sodium hypochlorite.

Fig. 3 shows the steady-state inactivation curve, $h_\infty(E)$, after application of hypochlorous acid. Inactivation is not only incomplete, moreover the curve becomes nonmonotonic, i.e., h_∞ increases again on depolarizations to $E > -20$ mV. In three experiments with HOCl the minimum of h_∞ was 0.267 (±0.043), $N = 3$. The half potential E_h (at which $h_\infty = 0.5 \times (1 + C)$, see Methods) is shifted to more negative values of membrane potential by −14 mV and the slope of the curve is drastically decreased. At −120 mV, the current after HOCl-treatment was 42,1(±5,3)% of the current measured before in the same Ringer's solution (see also Table I). When HOCl was applied at pH 9, the reagent had almost no effect on the decaying phase of the Na currents.

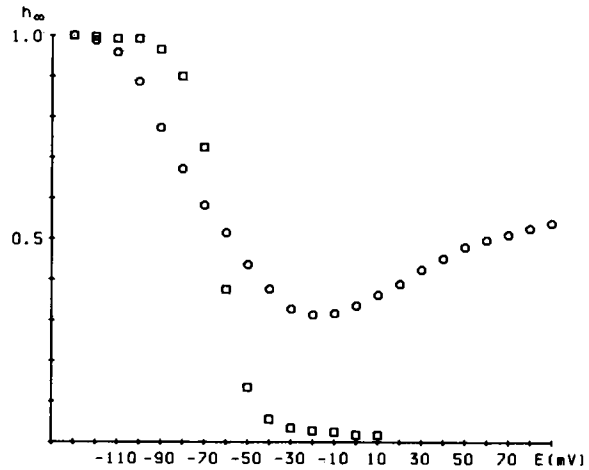

FIGURE 3 Steady-state inactivation curve, $h_\infty(E)$, of the sodium system before (□) and after (○) treatment with hypochlorous acid for 10 min. Holding potential was −70 mV. h_∞ reaches a minimum at −20 mV and rises again at more positive potentials. E_h (as defined in Methods) is shifted from −64 to −80 mV.

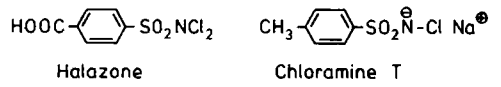

FIGURE 4 The structures of halazone and chloramine T.

Halazone

p-(*N*,*N*-Dichlorosulfamyl)-benzoic acid (halazone) tablets are used for disinfection of water (see O'Connor and Kapoor, 1970). This compound is chemically closely related to CT, the nitrogen atom is linked with two instead of one chlorine atom and, certainly more important here, a methyl group is replaced by a carboxyl group (see Fig. 4). Therefore, at neutral pH, halazone has an additional negative charge when compared with CT.

5 mM halazone acted like HOCl in every respect, except that the shift of the descending branch of the $I_{Na}(E)$ curve was three times smaller (see Table I). When applied at pH 9 the effect of halazone on the decaying phase of the Na current was much smaller than at pH 7.2; the noninactivating component C of the $h_\infty(E)$ relation remained <0.1.

Chloramine T

Schmidtmayer (1985) showed that the $h_\infty(E)$ curve becomes nonmonotonic after application of CT (similar to our results with HOCl and halazone, see above), while the $h_\infty(E)$ curve shown by Wang (1984*b*) is almost monotonic.

TABLE I
CHANGES IN SODIUM CURRENTS PRODUCED BY THE CHEMICAL AGENTS TESTED. DATA ARE GIVEN AS MEANS ± SD, $N = 3$

	Minimum of h_∞	$I_{Na}(E)$ curve* ΔE	Na current‡ remaining
		mV	%
0.05% HOCl§	0.267 (± 0.043)	7.9 (± 2.7)	42 (± 5)
5 mM Halazone	0.233 (± 0.075)	2.4 (± 3.0)	43 (± 9)
1 mM CT	0.395 (± 0.056)	8.0 (± 0.5)	41 (± 7)
	h_∞ curve‖ ΔE_h	$I_{Na}(E)$ curve ΔE	Na current remaining
	mV	*mV*	%
20 mM Periodate	−17.0 (± 3.2)	0.9 (± 1.8)	79 (± 15)
20 mM Iodate	− 4.9 (± 1.3)	−4.7 (± 1.8)	103 (± 8)
20 mM H_2O_2	− 5.4 (± 1.2)	−0.3 (± 2.6)	75 (± 10)
10 mM DEP	−15.1 (± 2.2)	0.6 (± 1.4)	77 (± 22)
10 mM NAI	< − 1	0.7 (± 0.6)	102 (± 10)
5 mM Glyoxal	− 2.0 (± 2.1)	−2.5 (± 1.5)	85 (± 16)

*Shifts of the descending branch of the $I_{Na}(E)$ curve.
‡Na currents remaining were measured after application of the chemical reagents and compared with the currents measured before in the same Ringer solution. Test pulse to 10 mV was preceded by a pulse to −120 mV of 50 ms duration. Holding potential was −70 mV.
§NaOCl-solution with 5% available chlorine, diluted 1:100 and buffered at pH 7.2 (see Methods).
‖Shifts of the half potential E_h of the $h_\infty(E)$ curve.

To compare the effects of the three reagents (CT, halazone, HOCl) on the same preparation and under identical conditions, we have carried out some experiments with CT.

As shown in Table I, application of 1 mM CT for 10 min at pH 7.2 had the same effect as HOCl. Wang (1984*a*) reported that CT has almost no effect on activation. By contrast, we have found that this reagent shifts the descending branch of the $I_{Na}(E)$ curve by 8 mV. When series resistance artifacts are minimized by performing all Na current measurements in Ringer's solution plus 6 nM tetrodotoxin, a small shift of the m_∞ curve to more positive values of membrane potential is also observed (Meves and Rubly, 1986). When 1 mM CT was applied at pH 9, the reagent had almost no effect on the decaying phase of the Na currents.

N-Bromosuccinimide (NBS)

NBS and NBA inhibit inactivation in squid axons (Oxford et al., 1978) and in myelinated nerve fibers of the toad (Wang, 1984*b*). Similar to halazone and CT, the *N*-bromo compounds NBS and NBA are a source of positively polarized halogen atoms.

In four experiments with 0.5 mM NBS in Ringer's solution pH 7.2, a drastic increase of the leakage current and "break-down" of the fibers occurred within 5–8 min after application of the reagent. This result is in accordance with previous findings of Keana and Stämpfli (1974) on the same preparation. In two of the four experiments a partial inhibition of Na current inactivation was observed, before the increase in leakage current made further measurements impossible.

Periodate

Periodate is known to modify methionine, sulfhydryl, and a number of other amino acid residues (see below). After addition of sodium periodate to a standard Ringer's solution, a white precipitate appears. A clear solution is obtained in Ca-free Ringer's or at low pH. In consequence, periodate was applied at pH 5.5 in a concentration of 20 mM for 10 min. The decaying phase of the sodium current was not affected significantly. The descending branch of the $I_{Na}(E)$ curve and the reversal potential remained unchanged, too. Periodate treatment shifted the steady-state inactivation curve, $h_\infty(E)$, to more negative values of membrane potential (Fig. 5 *a*). In a total of three experiments the average shift of E_h was -17.0 (± 3.2) mV (see Table I), while k increased slightly by 0.4 (± 0.2) mV.

Iodate

There is not much known about the groups modified by iodate. However, as internally applied iodate has frequently been used to inhibit sodium channel inactivation in myelinated nerve fibers (e.g., Stämpfli, 1974; Schmidt-

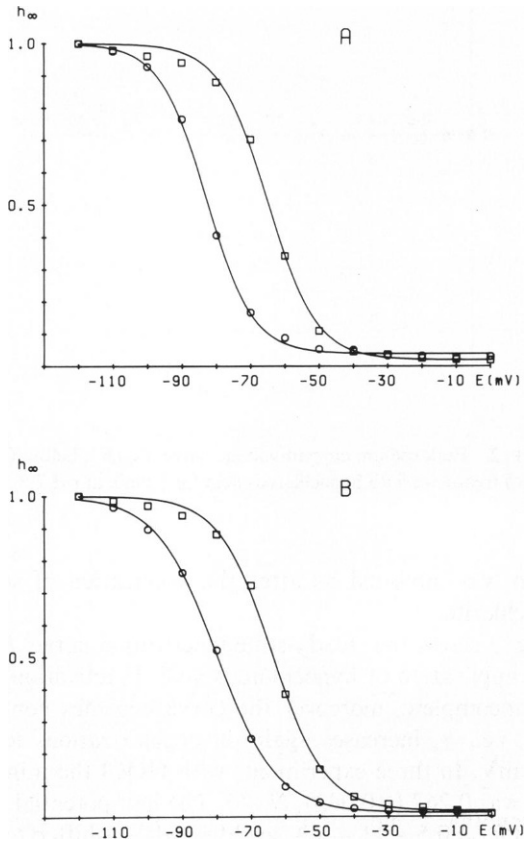

FIGURE 5 Parallel shift of the steady-state inactivation curve $h_\infty(E)$ by periodate *A* or diethylpyrocarbonate *B*. □, control measurements: ○, after treatment with 20 mM periodate *A* or 10 mM diethylpyrocarbonate *B* for 10 min. Points were fitted by Eq. 1 with $E_h = -64.9$ mV, $k = 7.1$ mV, $C = 0.015$ for □ in *A*, $E_h = -83.4$ mV, $k = 7.3$ mV, $C = 0.033$ for ○ in *A*, $E_h = -64.0$ mV, $k = 8.0$ mV, $C = 0.016$ for □ in *B*, $E_h = -80.6$ mV, $k = 9.4$ mV, $C = 0.009$ for ○ in *B*. At -120 mV, the current after treatment was 84% (*A*) or 83% (*B*) of the current measured before in the same Ringer's solution.

mayer et al., 1983) it was of interest to study in detail possible effects of iodate from outside.

As shown in Table I, a 10-min treatment with 20 mM iodate at pH 5.5 shifted the steady-state inactivation curve and the descending branch of the $I_{Na}(E)$ curve by -4.9 and -4.7 mV, respectively. Thus, the effect of iodate on the $h_\infty(E)$ curve is much smaller than that of periodate. The reagent had no significant effect on the decaying phase of the sodium current.

Hydrogen Peroxide

H_2O_2 oxidizes the sulfur containing amino acid residues cysteine and methionine. Treatment of the fibers with 20 mM H_2O_2 for 10 min at pH 7.2 did not significantly alter the decaying phase of the Na currents; the descending branch of the $I_{Na}(E)$ curve and the reversal potential remained unaffected. The $h_\infty(E)$ curve was slightly shifted to more negative values of membrane potential (see Table I).

Diethylpyrocarbonate (DEP)

DEP has a high degree of specificity for histidine modification, amino groups may also be modified more slowly. The specificity of DEP is more pronounced at low pH (Osterman-Golkar et al., 1974); therefore the fibers were treated with 10 mM DEP for 10 min at pH 5.5. DEP acted similarly to periodate; it produced a large negative shift of the $h_\infty(E)$ curve (Fig. 5 *b*) without significantly affecting the descending branch of the $I_{Na}(E)$ curve (see Table I), or the decaying phase of the sodium current.

N-Acetylimidazole (NAI)

NAI modifies tyrosine residues and a side reaction with amino groups may also occur. In three experiments with 10 mM NAI applied for 10 min at pH 7.2, no effect on the size of I_{Na} or its decay, nor on the $I_{Na}(E)$ or on the $h_\infty(E)$ curve was observed.

Glyoxal

Glyoxal reacts with arginine residues and a side reaction with amino groups will also occur (see Means and Feeney, 1971). In three experiments with 5 mM glyoxal applied for 10 min at pH 9.0 almost no alteration of the decaying phase of the sodium currents and only a small effect on the descending branch of the $I_{Na}(E)$ curve and the $h_\infty(E)$ curve were observed (see Table I). In a single experiment, the glyoxal treated fiber was exposed further to 50 mM of the reagent for 10 min at pH 9.0, which produced a further -2-mV shift of E_h.

DISCUSSION

The experiments show that part of the chemical reagents able to modify methionine (and other amino acid) residues remove a large part of inactivation of the Na current, while others do not (see Table II). Similar results with methionine reactive reagents have been obtained by Oxford et al. (1978) and Wang (1984*b*). There are at least five reagents known to be highly reactive with methionine residues (for review see Means and Feeney, 1971) and none of them has an effect on the time course of the Na current (Oxford et al., 1978; Wang, 1984*b*; this study). It is important to note that these reagents (BrCN, benzyl bromide, iodoacetamide, iodoacetic acid, hydrogen peroxide, periodate) largely differ in their structure, two of them are negatively charged in solution (like CT) and their lipid solubility is very different.

Therefore, a critical role of a methionine residue for the time course of Na current inactivation can hardly be regarded as well-established; rather, the negative results with very different methionine reactive reagents suggest that the effect of CT is probably not mediated by methionine modification. Thus, the question appears which part of the membrane is modified by CT. Among the other amino acids present in proteins only sulfhydryl groups are

TABLE II
REACTIVITY OF CHEMICAL REAGENTS TOWARD METHIONINE OR DOUBLE BONDS COMPARED WITH THEIR ABILITY TO INHIBIT INACTIVATION OF Na CURRENTS

	Modifies methionine	Modifies double bonds	Inhibits inactivation
HOCl	+	+	+
Halazone	+	+	+
CT	+	+	+
NBS	+	+	+
Periodate	+	–	–
Iodate	?	–	–
H_2O_2	+	–	–
Cyanogen bromide*	+	–	–
Benzyl bromide‡	+	–	–
Iodoacetic acid‡	+	–	–

*See Oxford et al. (1978).
‡See Wang (1984*b*).

modified by CT (when applied at neutral or slightly alkaline pH) and under acidic conditions tryptophan is oxidized, too (Shechter et al., 1975). As outlined by Wang (1984*a*), there is no indication that SH-group modification removes inactivation (see Shrager, 1977; Rack et al., 1984; Wang, 1984*b*) and also in this study neither periodate nor iodate or hydrogen peroxide, reagents easily oxidizing SH-groups (see Means and Feeney, 1971; Gorin and Godwin, 1966), had an effect on the time course of Na current inactivation.

Tryptophan modification is very unlikely under previous conditions (Wang, 1984*a*, 1985) or in our experiments, although an exposed tryptophan in a special environment may become extremely reactive. Periodate, however, is also a tryptophan-reactive reagent (Clamp and Hough, 1965) and does not remove Na current inactivation. Taking together the points discussed above, it appears, that for modification with CT, there is no likely candidate among the amino acids common in proteins.

When CT is dissolved in water, hypochlorous acid is developed (Alexander, 1973; O'Connor and Kapoor, 1970). Hypochlorous acid reacts with aliphatic double bonds (Detoeuf, 1922; Stroh, 1962). Therefore, another constituent of a biological membrane (beside methionine or sulfhydryl groups) that might be modified by CT are the double bonds of membrane lipids. Halazone, a reagent chemically closely related to CT and used here, has been proposed for use in the determination of iodine numbers of fats (Windholz et al., 1976). As was to be expected from the known chemistry of CT and halazone in aqueous solution, application of hypochlorous acid on the nerve fiber also led to the development of a maintained current. Thus, these three reagents may act in the same way. A further indication for a common reaction mechanism of these reagents is that their ability to inhibit Na-current inactivation is reduced considerably (or almost completely)

when applied at pH 9. A fivefold higher concentration of halazone is needed to obtain approximately the same effects as with CT. This might be due to the different charge of these reagents in Ringer's solution (see, e.g., Fig. 4).

The nondecomposed R-NHCl compounds of the equilibrium R-NHCl + $H_2O \rightleftharpoons RNH_2$ + HOCl may have different access to functionally important parts of the membrane. HOCl from CT, therefore, could be developed in a compartment not easily reached by halazone. Alternatively, the positively polarized chlorine atom at the nitrogen of R-NHCl may act directly (see Balasubramanian and Thiagarajan, 1975). The amount of HOCl present is strongly pH-dependent and may be described as

$$NaOCl \xrightarrow{+HCl} HOCl + NaCl \xrightarrow{+HCl} Cl_2 + H_2O + NaCl.$$

Thus, at high pH values the equilibrium between HOCl and OCl^- is shifted to the latter; at low pH values the reagent is destroyed while chlorine is developed. The situation is even more complex, as the rate of reaction is also dependent on the concentration of the Cl^- added (e.g., from the Ringer's solution) (see Balasubramanian and Thiagarajan, 1975).

Removal of inactivation by *N*-chlorosuccinimide (NCS), NBS, or NBA (see Wang, 1984*b*) can be explained by the same mechanism; release of halogen atom from *N* atom and reaction of the halogen with the double bonds of membrane lipids. Unfortunately, in our experiments with NBS, there was a drastic effect of this reagent on the leakage current, making detailed studies impossible. Our observations after NBS application confirm earlier results of Keana and Stämpfli (1974) on the same preparation. The reason for membrane "break-down" in *Rana esculenta* after NBS application is unknown. However, inhibition of inactivation by NBA in squid axons (e.g., Oxford et al., 1978) and by NBA and NBS in myelinated nerve fibers of the toad *Bufo marinus* (Wang, 1984*b*) fits the model of modification of double bonds by positively polarized halogen atoms.

With regard to the possible importance of membrane lipids in the process of inactivation, it is pertinent to note that application of the detergent deoxycholate removes inactivation in squid axons when applied from inside or outside (Wu et al., 1980). This supports the idea that the effect of CT on sodium current inactivation is due to modification of double bonds of membrane lipids. However, other (unknown) constituents of the excitable membrane may be attacked by hypochlorous acid and, consequently, by CT or halazone as well.

No Effect of Tyrosine and Arginine-Reactive Reagents

Treatment with NAI and glyoxal had no effect on the decaying phase of the Na currents. The lack of effect is surprising because glyoxal (Eaton et al., 1978) and NAI (Oxford et al., 1978; Brodwick and Eaton, 1978) are effective when applied intracellularly in squid axons and both reagents are considered to be membrane permeant. Almost no effect of NAI after external application on myelinated nerve fibers was also observed by Wang (1984*b*).

On the Alterations of the Steady State Inactivation Curve, $h_\infty(E)$

DEP shifts the $h_\infty(E)$ curve in the hyperpolarizing direction. This reagent has a high specificity for modification of histidine side chains in proteins; however, amino group modification may also occur (Melchior and Fahrney, 1970). Modification of amino groups by highly specific reagents (Cahalan and Pappone, 1981; Rack et al., 1984; Rack, 1985) induced a shift of E_h of the $h_\infty(E)$ curve very similar to that caused by DEP, but in contrast to the amino group modifying reagents, DEP does not significantly change the slope of the curve. In model systems, primary amines react ~50 times more slowly than imidazole at neutral pH values and especially at low pH values the specificity of DEP for histidine is increased (Osterman-Golkar et al., 1974). It is very unlikely that DEP (applied at pH 5.5) shifts E_h via a side reaction to an extent similar to that observed with the highly reactive amino reagent ethyl acetimidate (see Rack, 1985). Consequently, modification of a histidine residue is likely to occur.

DEP and periodate produce a large negative shift of the $h_\infty(E)$ curve; however, the descending branch of the $I_{Na}(E)$ curve remains almost unaffected. This selective effect is clearly different from the effect of divalent cations or hydrogen ions, affecting activation and inactivation simultaneously, albeit to a different degree (for review see: Stämpfli and Hille, 1976). Even shifts in the voltage dependence of h_∞ and m_∞ in opposite directions have been observed earlier (see, e.g., Elliott et al., 1984; Rack, 1985).

Iodate and H_2O_2 cause a small shift of the $h_\infty(E)$ curve in the hyperpolarizing direction. There is not much known about the effects (and side effects) of iodate on amino acid side chains except that this reagent oxidizes SH-groups and disulfide bonds (Gorin and Godwin, 1966). H_2O_2 readily oxidizes methionine and sulfhydryl groups (see Means and Feeney, 1971). The effects of H_2O_2 were very similar to those of iodate and the shifts of E_h in the hyperpolarizing direction by both reagents closely resemble that caused by the relatively high SH-specific reagent *N*-ethylmaleimide (Rack et al., 1984). The effects of H_2O_2 and iodate differ from that of CT in at least one important point: from outside, both reagents do not inhibit sodium current inactivation.

Periodate is known to react with methionine, SH-groups, disulfide bonds, tyrosine, histidine, tryptophan in a peptide chain; furthermore serine and threonine would be attacked as NH_2-terminal residues (Clamp and Hough,

1965). A shift of -17 mV of the $h_\infty(E)$ curve is not seen after application of iodate, H_2O_2 or NAI, suggesting that SH-groups, disulfide bond, methionine, or tyrosine modification do not account for the major part of the shift observed after periodate application. As discussed for DEP (see above), modification of a histidine residue is most likely to occur. Additionally, periodate may have shifted the $h_\infty(E)$ curve by a chemical attack on a tryptophan residue. A direct investigation of this point seems difficult, as, unfortunately, the reaction of 2-hydroxy-5-nitrobenzyl bromide (a highly specific tryptophan reagent) with water is extremely rapid and complete within <30 s (Horton and Koshland, 1965).

Little is known about the chemistry of iodate (see above), and the group(s) modified by this agent from inside the myelinated fiber (Stämpfli, 1974) is still unknown. The internal effects of endopeptidases show the importance of proteinous structures for the process of inactivation (see Armstrong et al., 1973; Sevcik and Narahashi, 1975); however, these data do not allow identification of the functional groups associated with the process of inactivation (see also Rojas and Rudy, 1976). The nature of these groups remains still to be elucidated.

CONCLUSIONS

In summary, our data on ten reagents of very different specificity for reactions with different membrane constituents suggest that on myelinated nerve fibers: (*a*) a critical role of a methionine residue in the process of inactivation is unlikely; (*b*) unsaturated lipids (or unknown membrane constituents others than amino acid side chains) might be critically involved in the process of Na-current inactivation; (*c*) steady-state inactivation is affected by histidine modification; and (*d*) there is no indication for the presence of exposed, essential tyrosine or arginine groups.

We thank Professor H. Meves for his support and advice.

This investigation was supported by the Deutsche Forschungsgemeinschaft SFB 38 "Membranforschung".

Received for publication 15 January 1986 and in final form 28 May 1986.

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