Removal of Sodium Channel Inactivation in Squid Axon by the Oxidant Chloramine-T

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ABSTRACT We have investigated the effects of a mild oxidant, chloramine-T (CT), on the sodium and potassium currents of squid axons under voltage-clamp conditions. Sodium channel inactivation of squid giant axons can be completely removed by CT at neutral pH. Internal and external CT treatment are both effective. CT apparently removes inactivation in an irreversible, all-or-none manner. The activation process of sodium channels is little affected, as judged from the voltage dependence of peak sodium currents, the rising phase of sodium currents, and the time course of tail currents following the repolarization. The removal of inactivation by CT is pH-dependent; higher pH decreases the removal rate, whereas lower pH increases it. Internal metabisulfite, a strong reductant, does not protect inactivation from the action of external CT, nor does external metabisulfite protect from internal CT application. CT slightly depresses the peak potassium currents at comparable concentrations but has no apparent effects on their kinetics. Our results suggest that the neutral form of CT modifies an embedded methionine residue that is involved in sodium channel inactivation.

INTRODUCTION

The function of the sodium channel is to allow the transient passage of sodium ions through excitable membranes during the nerve impulse. Under voltage-clamp conditions, depolarization induces the sodium conductance to rise rapidly (activation) and then to decline slowly (inactivation) back to a steady state level (Hodgkin and Huxley, 1952).

Sodium channels in excitable membranes are membrane-bound glycoproteins. Biochemical characterization of these proteins has recently begun (Agnew et al., 1980; Barchi et al., 1980; Costa et al., 1982). Nonetheless, the “functional” amino acid residues of the sodium channel that mediate the permeability changes during depolarization remain obscure. Chemical modification of a protein can yield important information about its amino acid structure and the functional
relationships of the amino acid residues. In fact, the proteinaceous nature of the sodium channel was demonstrated by modification procedures long before the actual purification of these proteins. Internal perfusion with pronase (Armstrong et al., 1973), chymotrypsin (Sevcik and Narahashi, 1975), N-bromoacetamide (NBA) (Oxford et al., 1978; Patlak and Horn, 1982), glycoxal, 2,3-butanedione (Eaton et al., 1978), tetranitromethane, or iodination (Brodwick and Eaton, 1978) irreversibly removes sodium channel inactivation. On the basis of the specificities of these reagents and the difference in their effects when applied internally or externally, it has been proposed that tyrosine and arginine residues at the internal membrane surface are essential for the inactivation process.

We report here that chloramine-T (CT), a specific sulfur-containing amino acid-modifying reagent, can also remove the sodium channel inactivation in squid axons after either internal or external application. This reagent does not cleave peptide bonds. Our results suggest that the amino acid that was modified may be a methionine residue located within the membrane and therefore not exposed to the aqueous environment on the cytoplasmic surface of the membrane.

MATERIALS AND METHODS
Squid giant axons (400–650 μm in diameter) were isolated from Loligo pealei, supplied by the Marine Biological Laboratory, Woods Hole, MA. The axons were cannulated, briefly exposed to 0.1 mg/ml of pronase in the internal perfusate for <3 min, and then perfused with internal perfusate alone and voltage-clamped as described by Wu and Narahashi (1973). Leakage currents and capacitive transients were removed by a P/3 sequence similar to that originally described by Armstrong and Bezanilla (1975). The records were digitized at various rates (usually 2 or 4 μs/point). The analog bandwidth of the system was 100 kHz. The records were plotted for presentation on an analog X-Y plotter. Under such conditions, pronase removed some of the axoplasmic matrix and improved the perfusion efficiency, while the sodium current kinetics remained unchanged. The reference electrode was made of an agar pipette containing 0.5 M KCl since a silver pellet electrode was not desirable when an oxidant reagent was present. The interaction of CT with the internal platinum wire floating in the internal voltage-sensing electrode produced, at most, only small voltage offsets that were never more than 5 mV. All experiments were performed at 10 ± 0.2°C.

Solutions
The external artificial seawater (ASW) contained 440 mM NaCl, 10 mM KCl, 50 mM CaCl₂, and 10 mM HEPES, pH 7.2. The standard internal perfusate (SIP) contained 325 mM potassium glutamate, 50 mM NaF, 15 mM K₂HPO₄, and 333 mM sucrose, pH 7.27. Unless indicated otherwise, SIP also contained 25 mM tetroethylammonium bromide (SIP/TEA), which blocks the voltage-dependent outward potassium currents. Alternatively, a Cs/SIP was used that contained 350 mM CsF, 50 mM NaF, 300 mM sucrose, and 20 mM HEPES, pH 7.27. The other buffers used in this report were 10 mM MES [2(N-morpholino)ethanesulfonic acid] for external buffer, at pH 6.2, and 10 mM Tris [tris(hydroxymethyl) aminoethane] at pH 8.0; the internal perfusate in potassium phosphate buffer was adjusted from pH 7.27 to 8.27 in some experiments. CT was purchased from Fisher Scientific Co., Pittsburgh, PA, and dissolved in solution just before application. Pronase (isolated from Streptococcus griseus) was from Calbiochem-Behring Corp., San Diego, CA. Tetrodotoxin (TTX) was from Sigma Chemical Co., St. Louis, MO; TEA was
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FIGURE 1. The structure of CT and the end products of methionine oxidation by CT. Under the conditions present during our experiments, the oxidation of methionine was unlikely to progress beyond the sulfoxide at step 2.

from Eastman Kodak Co., Rochester, NY. Sodium metabisulfite was purchased from Fisher Scientific Co. All other chemicals were reagent grade from commercial sources.

The structure of CT and the formal reaction of the oxidation of methionine by CT are shown in Fig. 1.

RESULTS

CT, at a concentration of 3.55 mM (1 mg/ml), could remove sodium inactivation when applied externally in ASW at pH 7.2. Fig. 2A shows the sodium currents at different times after the application of CT. Within 5–10 min of CT treatment, nearly all inactivation was removed. The removal of sodium channel inactivation by CT was not reversed by washing with ASW, which indicates that chemical modification had occurred. If we lowered the CT concentration, the removal of

FIGURE 2. Removal of sodium channel inactivation by CT. (A) Sodium currents at a test potential of 0 mV were recorded at various times during external CT (3.55 mM) treatment. Each current trace is displaced by ~0.1 ms to reveal the activation and inactivation time course. (B) Sodium currents at a test potential of 0 mV as in A except that CT (3.55 mM) was applied internally. The numbers indicated correspond to incubation times (in minutes) of CT with the axons. The holding potential was ~70 mV in each panel.
the inactivation usually proceeded more slowly. The results of external CT treatment, therefore, are very similar to those of internal pronase or NBA perfusion, each of which destroys sodium channel inactivation.

When applied internally at the same concentration (3.55 mM), CT also removed sodium channel inactivation, as shown in Fig. 2B. As was the case for the external application, the reaction was time- and concentration-dependent and was irreversible. Since the actions of CT after either external or internal application are quite similar, we will present results from one of the treatments and mention only briefly results obtained from CT application to the opposite side of the membrane.

**CT and Peak Sodium Current**

Peak sodium current (\(I_{Na}\)) at a given voltage reflects the maximal permeability of sodium ions at that membrane voltage. The Hodgkin-Huxley model (Hodgkin and Huxley, 1952) predicts that after removal of sodium channel inactivation, the peak sodium current should increase significantly. On the other hand, chemical modification or cleavage of sodium channel proteins could functionally damage some of the sodium channels. The results of CT treatment show that the peak sodium current is only slightly inhibited by the application of external CT. The ratio of \(I_{Na}\) in treated axons to that of control axons was 0.95 ± 0.16 (mean ± SD, \(n = 6\)); for internal application, the ratio was 0.88 ± 0.09 (\(n = 7\)), measured after nearly all inactivation was removed under the conditions described in Fig. 2. This slight inhibition of peak sodium current after CT treatment is similar to the inhibition produced by NBA or pronase treatment (Oxford et al., 1978; Armstrong et al., 1973).

Sodium currents normally inactivated with an exponential time course (Fig. 3, open circles). During progressive removal of inactivation by CT, the time course of the inactivation that remained was similar to that of the control currents (half-time, 0.9–1.0 ms). No significant differences in the half-time for inactivation were detected after partial removal of sodium channel inactivation by CT. These results indicate that CT, like NBA and pronase, can destroy sodium channel inactivation in an all-or-none manner.

**Effects of CT on Sodium Channel Activation**

CT has little effect on the voltage dependence of sodium activation. The voltage dependence of sodium currents before and after CT treatment is shown in Fig. 4, A and B, respectively. The current-voltage relationship for peak sodium currents before and after CT treatment remained similar except for the negative conductance limb at negative potentials (Fig. 5). The removal of sodium inactivation at those negative potentials may have had a bigger effect on the peak sodium currents, or there may have been a minor interaction of the CT with the internal platinum electrode. However, the shift in this region was always <5 mV.

The time course of sodium tail currents is another measure of the activation kinetics of sodium channels, since it is an indication of the closing rate of the channel. The tail currents recorded before and after CT treatment were only slightly altered. The axon was held at −70 mV, depolarized to the test potential for a short time, and then returned to the holding potential. The time constants
of the tails were obtained from semilog plots fitted by eye. Again, no significant differences were observed in a control axon (τ = 180 μs) and a CT-treated axon (τ = 210 μs). The ratio of the time constants was 1.18 ± 0.22 (CT treated/control) in four separate experiments. However, prolonged CT incubation, which produces substantial reductions in peak sodium current, also tends to make the time constants for tail currents longer than those observed in control axons or ones treated with CT for short durations. This difference is probably due to nonspecific modifications of the channel.

Pronase has previously been shown to have no apparent effects on the activation of sodium channels (Armstrong et al., 1973); therefore, we treated axons with internally applied pronase first and then perfused with CT. The result of this study is shown in Fig. 6. No apparent changes were found either in the activation time course or in the tail current kinetics. After CT or pronase treatments, all the sodium current could be blocked by external 100 nM TTX application.

**Effects of CT on Slow Inactivation**

The slow inactivation process, like the fast inactivation process, also attenuates the sodium conductance but with a much longer time constant (Adelman and Palti, 1969; Chandler and Meves, 1970; Schauf et al., 1976). Pronase and NBA do not remarkably alter slow inactivation after removing fast sodium inactivation.
These results indicate that slow and fast inactivation can be dissociated and probably represent separate processes.

CT, when applied externally to remove fast inactivation, did not alter slow inactivation \( (S_s) \), as shown in Fig. 7. Slow inactivation developed when the holding potential was held constant for 1 min. The holding potential that corresponded to a 50% attenuation of the maximal sodium conductance (at \(-80\) mV holding potential) was about \(-35\) mV. This value is almost identical to that of the axons treated with pronase or NBA. After CT treatment, neither the time course for development of slow inactivation nor the 50% attenuation level was significantly different from that observed in control axons (our observations and those of Oxford et al., 1978). Similar results were found in axons when CT was applied internally (50% attenuation was in the range of \(-45\) to \(-35\) mV).

**pH Dependence of CT Reaction**

In solution at pH 7.0, most CT molecules are in an ionic form (anions) (Bishop and Jennings, 1958). The pK\(_a\) of CT at 50 mM measured in distilled water is
~5. Below pH 5, a white precipitate appears that is, presumably, the neutral form of CT.

Since CT gave almost identical results whether it was applied to the inside or outside of axons, we suspected that a common amino acid residue was modified by both treatments. In order to reach a common site from both membrane surfaces, CT would have to be permeable or be sufficiently hydrophobic to penetrate the membrane. It seems likely, then, that CT in its neutral form reacts with a common residue(s), whether applied internally or externally, particularly since the ionic form of CT is not permeable (Montelaro and Rueckert, 1977).

An increase of the pH, therefore, should reduce the rate of the removal of sodium inactivation, as fewer molecules would be in the neutral form; conversely, a decrease of the pH would increase the reaction rate. Fig. 8 shows that the rate of the removal of sodium inactivation is dependent upon the pH of the solution in which the CT is applied. By raising the external pH by ~0.8 unit (from pH 7.2 to 8.0), the reaction rate for externally applied CT was significantly reduced. Lowering the pH had the opposite effect; however, at low pH (such as pH 6.2), CT also dramatically inhibited the peak sodium conductance, probably because of the loss of CT specificity. Below pH 6.5, CT also reacts with tryptophan (Alexander, 1974; Shechter et al., 1975). These results, therefore, are consistent
FIGURE 6. Effects of CT pronase-treated axons. Sodium current after internal pronase (0.1 mg/ml) treatment for 20 min is shown in the solid trace. Little inactivation is left under these conditions. The axon was further treated with CT (3.55 mM) internally for 5 min (dashed trace). No apparent effects on the activation and the tail currents were found except that there was a 17% reduction of peak sodium current.

with the notion that the neutral form of CT is required in solution for the removal of sodium inactivation.

**Effects of Metabisulfite and CT on the Removal of Sodium Channel Inactivation**

Previous reports have indicated that the inactivation gate is located near the $S_{0.5}$.

FIGURE 7. Slow inactivation ($S_w$) of the sodium channel is not removed by CT treatment. Peak sodium currents at a test pulse potential of 0 mV from different holding potentials were measured and normalized to the peak $I_{Na}$ at a holding potential of -80 mV. Holding potentials were changed for a duration of 1 min. The sequence of holding potentials used to obtain the figure was -90, -80, -60, -50, -40, -30, -20, -10, and then repeating -20, -30, -40, and -70 mV. CT (3.55 mM) was applied internally for 80 min in SIP/TEA. Control data in the absence of CT are not shown; however, in other untreated axons, the slow inactivation was virtually the same as in this figure.
internal membrane surface or is even loosely connected to the inner end of the sodium channel (for a review, see Armstrong, 1981). To test whether a functional residue modified by CT is exposed to internal solution, we used metabisulfite, a reducing reagent, which reduces CT and destroys its oxidizing ability. When CT and metabisulfite were mixed together, CT immediately lost its potency to remove sodium inactivation. Metabisulfite alone did not have effects on sodium current kinetics but slightly inhibited the peak sodium current.

After internal perfusion of 5.3 mM metabisulfite, we found that external CT at 3.55 mM still removed sodium inactivation. Similarly, the application of external metabisulfite did not prevent the action of internal CT. These results imply that CT does not pass through the membrane to act later near or at the internal or external surfaces in an aqueous environment.

**FIGURE 8.** CT reaction rate is pH-dependent. Times required for external CT to remove the sodium channel inactivation were determined at pH 7.2 (two axons, circles), pH 8.0 (two axons, squares), and pH 6.1 (one axon, triangles). The ratio of the current amplitude at the end of a 7.5-ms pulse over the peak current amplitude is plotted on the ordinate. All axons were treated internally with CT at a concentration of 3.55 mM.

**Effects of CT on Potassium Current**

No apparent effects of CT on the kinetics of potassium current were found in two separate experiments. All the sodium currents were blocked by external 100 nM TTX, while the SIP solution contained only 325 mM K⁺ without TEA. Internal perfusion of CT (pH 7.27) at 3.55 mM reduced the peak potassium current by ~30% and at 14.2 mM by ~50%. Similar results were found when CT was applied externally. This reduction in peak current was not reversible.

**DISCUSSION**

In this report, we have demonstrated that the inactivation process of sodium channels in squid axons can be removed by internal as well as external application of CT. No significant modifications were found in the activation process. The efficacy of the removal of sodium channel inactivation by CT is very similar to
that of internal NBA or pronase treatments. In contrast, the action of CT is strongly dependent on the pH of the solutions, and the modified residues are perhaps not exposed to the aqueous environment at the internal membrane surface. In the following sections, we will compare our results with those from single myelinated fibers treated with CT and squid axons treated with other chemical reagents. We will also discuss the possible CT-modified residue and its relative location.

Comparison with Single Myelinated Fibers

Because of the small diameter of single myelinated fibers, the control of the internal aqueous environment is not possible. Nevertheless, CT, when applied externally, can remove >80% of the inactivation process (Wang, 1984a). Two major differences were observed. First, the peak sodium current in squid axons was not much inhibited by CT at the concentration of 3.55 mM, while the same concentration inhibited most of the sodium current in single myelinated fibers. An apparently high CT concentration (~2 mM) can damage a significant number of sodium channels in myelinated fibers. Second, the time course of the remaining sodium current inactivation of CT-treated squid axons was not much changed compared with that of untreated axons, but in myelinated fibers the residual inactivation was considerably slowed by CT. The steady state inactivation \( h_m \) of the remaining sodium current after partial removal of sodium channel inactivation shifted by ~20 mV to the depolarizing direction in single myelinated fibers, whereas in squid axons, \( h_m \) was not appreciably shifted (Wang, G., preliminary results). We concluded that at least two separate amino acid residues of the sodium channel protein were modified in myelinated fibers, one of which was responsible for the \( h_m \) shift, while the other was responsible for the removal of sodium channel inactivation.

These different results in invertebrate and vertebrate preparations might reflect subtle structural differences in their sodium channels. Chiu (1977) and Nonner (1980) both have shown that, in single myelinated fibers, the sodium channel inactivation has at least two exponential components. They proposed that multiple inactivation processes (states) exist in single myelinated fibers. In squid axons, only one exponential is needed to fit the inactivation time course (Hodgkin and Huxley, 1952). It would be interesting to investigate whether the multiple inactivation processes are due to separate inactivation gating moieties in the sodium channel.

Comparison of the Action of CT with the Action of Other Agents That Remove Inactivation

Several enzymes and a variety of group-specific reagents can remove sodium channel inactivation when applied to the internal surface of axonal membranes. Like CT, they all remove inactivation in an all-or-none manner without significantly affecting the activation or slow inactivation processes or greatly changing the magnitude of the peak inward current.

Although the various agents all produce a common end effect, their modes of action are likely to be significantly different. The specificities of the various
agents strongly suggest that they are reacting with several different amino acid residues, all of which are necessary for proper functioning of the peptide responsible for sodium channel inactivation. Of the agents that have been previously examined, alkaline proteinase b, the active principle of pronase (Armstrong et al., 1973; Rojas and Rudy, 1976), cleaves peptide bonds at lysine or arginine residues. Cleavage alone, however, only indicates the presence, and not the functional importance, of these residues. Subsequent experiments with lysine- and arginine-modifying reagents and with agents that mimic peptides containing arginine (Eaton et al., 1978; Kirsch et al., 1980; Lo and Shrager, 1982; Nonner et al., 1980) have demonstrated that an arginine residue was probably present and was an integral part of the inactivation mechanism.

Similarly, chymotrypsin (Sevcik and Narahashi, 1975) and NBA have a common mode of action in cleaving peptides at tyrosine or phenylalanine residues. Tyrosine residues were subsequently implicated as a functional component of the inactivation peptide after tyrosine-modifying procedures were shown to remove inactivation (Oxford et al., 1978; Brodwick and Eaton, 1978).

Less specific peptide-reactive reagents such as tannic acid (Shrager et al., 1969a; Horn et al., 1980a), glutaraldehyde (Shrager et al., 1969b; Horn et al., 1980b), and iodate (Stämpfli, 1974) all react with tyrosine or arginine residues (among others) and remove inactivation in the manner of pronase, NBA, or the other, more specific agents.

In contrast to these reagents, CT does not react with arginine or tyrosine, but rather shows a marked specificity for the sulfur-containing amino acids methionine and cysteine (Schechter et al., 1975). Only NBA, of the reagents mentioned above, can react with cysteine or methionine residues (Means and Feeny, 1971). However, Shrager (1977) and Oxford and co-workers (1978) have used various sulfhydryl-modifying reagents, such as N-ethylmaleimide, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and p-chloromercuriphenylsulfonic acid, to react with sodium channels and have found no significant effects on fast sodium channel inactivation. We also applied DTNB and found no effect. These results imply that neither NBA or CT is likely to be reacting with the sulfhydryl group of an essential cysteine residue.

A Possible CT-modified Residue and Its Location

The specificity of the reaction of CT with protein is well defined. CT can react only with cysteine and methionine residues (Schechter et al., 1975). Since sulfhydryl-reactive reagents have little effect on fast sodium channel inactivation, a methionine residue becomes a likely candidate to explain the action of CT in removing inactivation (Wang, 1984b). Unfortunately, cyanogen bromide (CNBr), another reagent specific for methionine residues, had no effect on fast sodium channel inactivation (Oxford et al., 1978). We can only surmise that the extremely rapid hydrolysis rate of CNBr did not allow the presentation of sufficient reagent to the modifiable residue, or that the reaction conditions at pH 6.3 were unfavorable for the CNBr reaction (reaction in vitro is usually accomplished at pH values of 5 or lower [Means and Feeny, 1971], which are not suitable for use in perfused axons).
Whichever residue is being modified by CT, there is indirect evidence that it is not located near the internal membrane surface accessible to the aqueous perfusate. The reasons for suggesting a location other than the internal surface are threefold. First, both internal and external CT treatments yield almost identical results in removing sodium inactivation. Second, CT action is highly pH-dependent: at higher pH, the reaction rate is much slower, which suggests that CT is active in the neutral form, which presumably can penetrate the membrane. Another possible theory to explain the pH dependence of CT is that the modified residue (probably methionine) has an altered reactivity at different pH values. This seems unlikely since the in vitro reaction between methionine and CT is not pH-dependent (Schechter et al., 1975). Third, internal metabisulfite does not protect inactivation from external CT application (although an extremely slow reaction of CT and metabisulfite at the membrane surface might also explain this result). These three results suggest that the modified residue is not exposed to the internal aqueous environment. It is more likely that the residue is located within the cell membrane.

Of course, chemical modification can give only indirect evidence about the structure and function of the peptides that constitute the sodium channel. This approach, however, will provide us with some information until more definitive information can be obtained from other approaches such as the determination of the primary amino acid sequence of the sodium channel peptides.

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