REMOVAL OF SODIUM INACTIVATION AND BLOCK OF SODIUM CHANNELS BY CHLORAMINE-T IN CRAYFISH AND SQUID GIANT AXONS

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ABSTRACT Modification of sodium channels by chloramine-T was examined in voltage clamped internally perfused crayfish and squid giant axons using the double sucrose gap and axial wire technique, respectively. Freshly prepared chloramine-T solution exerted two major actions on sodium channels: (*a*) an irreversible removal of the fast Na inactivation, and (*b*) a reversible block of the Na current. Both effects were observed when chloramine-T was applied internally or externally (5–10 mM) to axons. The first effect was studied in crayfish axons. We found that the removal of the fast Na inactivation did not depend on the states of the channel since the channel could be modified by chloramine-T at holding potential (from −80 to −100 mV) or at depolarized potential of −30 mV. After removal of fast Na inactivation, the slow inactivation mechanism was still present, and more channels could undergo slow inactivation. This result indicates that in crayfish axons the transition through the fast inactivated state is not a prerequisite for the slow inactivation to occur. During chloramine-T treatment, a distinct blocking phase occurred, which recovered upon washing out the drug. This second effect of chloramine-T was studied in detail in squid axons. After 24 h, chloramine-T solution lost its ability to remove fast inactivation but retained its blocking action. After removal of the fast Na inactivation, both fresh and aged chloramine-T solutions blocked the Na currents with a similar potency and in a voltage-dependent manner, being more pronounced at lower depolarizing potentials. A similar voltage-dependent block was observed with aged chloramine-T solution in an axon with intact inactivation. In contrast to the action of the fresh solution, the aged chloramine-T solution was found to accelerate the decay of Na currents. These results suggest that chloramine-T solution contains at least two active molecular forms that act at different sites in the Na channel.

INTRODUCTION

The structure–function relation of the Na channel has been approached from many fronts. An approach that has used both electrophysiological technique and protein modification has generated much information giving rise to functionally defined channel structures. The in situ modification of amino acids will become more powerful in elucidating the molecular structure of the functional subunits of the Na channel, since the amino acid sequence of the Na channel has been recently determined (Noda et al., 1984). The channels can be modified in situ with various amino acid–specific reagents or chemical bond specific cleaving agents, and the degree of modifications is monitored by observing changes in the kinetics of Na currents under the voltage clamped condition.

When the membrane is depolarized, the transient opening of the Na channels is reflected at the macroscopic level as a transient current that increases rapidly and then decreases during a maintained depolarization. The rising phase of the macroscopic current is referred to as the activation phase and the declining phase as the fast inactivation phase. The voltage and time dependence of the two phases of Na currents are the basis of the kinetic analysis pioneered by Hodgkin and Huxley (1952*a*,*b*) and subsequently by many others (see review of Armstrong, 1981). The change in the kinetic and steady-state properties of Na currents has been taken to be indicative of a change in the conformational states of the channel protein.

Numerous reagents have been used to slow or remove the fast Na inactivation. Pronase (Armstrong et al., 1973; Rojas and Rudy, 1976) and *N*-bromoacetamide (Oxford et al., 1978; Oxford, 1981) are widely used to remove the inactivation process to study both the activation process itself and the drug–channel interaction. Recently, chloramine-T has been investigated in myelinated nerves (Ulbricht and Stole-Herzog, 1984; Wang, 1984*a*,*b*; Schmidtmayer, 1985) and squid axons (Wang et al., 1985) for its action on the fast inactivation process observed when

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chloramine-T is applied either internally or externally. We report here our studies of chloramine-T on crayfish and squid giant axons. In addition to its irreversible effect on the Na inactivation process as previously reported, we found that chloramine-T can block the Na currents reversibly in both crayfish and squid axons.

METHODS

Single crayfish giant axons of 100–200-μm diam were isolated from the circumesophageal segment of nerve trunk of crayfish, *Procambarus clarkii*, and carefully cleaned. The isolated axons were mounted in a sucrose gap chamber, canulated, and internally perfused with K- or Cs-internal solution (Lund and Narahashi, 1981). The kinetics of Na currents were studied using the double sucrose gap voltage clamp technique (Julian et al., 1962*a,b*). In some experiments, a long axon was isolated to test the potential-dependent effect of chloramine-T on the removal of the fast inactivation, with the same technique as previously described (Salgado et al., 1985). The nodal membrane was usually held at −100 mV, and the temperature was maintained at 9°–10°C.

Giant axons from squid, *Loligo pealei*, were obtained at the Marine Biological Laboratory, Woods Hole, MA. Internal perfusion was initiated after squeezing the axoplasm by the roller method (Baker et al., 1961). The axons were voltage clamped with the axial wire technique as described before (Yeh and Tanguy, 1985). The holding potential was −80 mV, and the temperature was maintained at 8°–10°C for all experiments.

In both preparations, the sodium current was isolated by blocking the potassium current with external K-free solution and internal K-free Cs-containing solution. The voltage clamp pulse was generated from a digital-analog converter and its sequence was controlled through a preprogrammed protocol with the aid of a DEC PDP11/73 computer. The membrane current was normally sampled at 20 μs per point through a 14-bit A/D converter on line with the computer. The linear leakage current and capacitative current were digitally subtracted using the standard P-P/4 method (Armstrong and Bezanilla, 1974).

The solutions used in the experiments with crayfish and squid axons are listed in Table I. In crayfish experiments, 5 μM $LaCl_3$ was added to the sucrose gap solution (adjusted to 500 mosmol) to stabilize the membrane, which was immersed in sucrose streams (Pooler and Valenzeno, 1983). The sodium salt of chloramine-T was purchased from Sigma Chemical Co., St. Louis, MO. In crayfish axons experiments, the increase in internal sodium concentration as a result of the addition of sodium salt of chloramine-T to internal solution was not corrected for. In squid axon experiments, the internal Na concentration was kept constant at 50 mM as indicated in Table I (internal Ch-T solution). For both preparations, the freshly prepared chloramine-T solution was made immediately before application. The aged chloramine-T solution was prepared 24 h earlier and kept at room temperature (20°C).

TABLE I
IONIC COMPOSITIONS OF EXTERNAL AND INTERNAL SOLUTIONS

	Crayfish				Squid		
	External		Internal		External	Internal	
	V-H	K	Cs	K	TMA	50 Na	Ch-T
Na	205	15	15	15	—	50	50
K	—	265	—	220	—	—	—
Cs	—	—	220	—	—	275	275
TMA	5.4	—	—	—	485	—	—
Ca	10	—	—	—	50	—	—
Mg	2.6	—	—	—	—	—	—
Cl	235.6	280	15	15	575	—	—
Glutamate	—	—	170	170	—	275	275
F	—	—	50	50	—	35	35-[Ch-TT]*
HEPES	5	5	5		10	—	—
Phosphate	—	—	—	—	—	15	15
Sucrose	—	—	—	—	—	400	400
Ch-T							[Ch-T]*
pH	7.5	7.5	7.35	7.35	7.3	7.3	7.3
mOSmol	470	470	410	410	980	1,040	1,040

*[Ch-T] indicates the concentration of sodium salt of chloramine-T.

RESULTS

I. Effects of Chloramine-T on Na Currents in Crayfish Axons

A freshly prepared chloramine-T solution exerted two major effects on the Na currents when it was applied either internally or externally to crayfish axons. It removed fast inactivation of the Na currents and reduced the amplitude of the Na currents. The former effect was irreversible and the latter effect reversible upon washing out chloramine-T solution.

Time Course of Chloramine-T Action. The modification of the Na current during internal application of 10 mM chloramine-T is illustrated with four selected traces of Na currents in Fig. 1 *A*. As illustrated by trace *1*, an initial transient increase in the peak of inward Na current, albeit small, was always seen during the first few minutes of drug exposure despite the decrease in the driving force for the inward Na current due to the use of the sodium salt of chloramine-T (see Methods). The increase in the peak Na current was not accompanied by any change in the decay phase of the Na current, as previously observed by Rojas and Rudy (1976) during pronase treatment.

Trace *2* in Fig. 1 *A* shows a decrease in the peak and an increase in the steady-state current, which reflects the removal of the fast Na inactivation. Trace *3* represents the Na current at its final stage of treatment: the peak Na

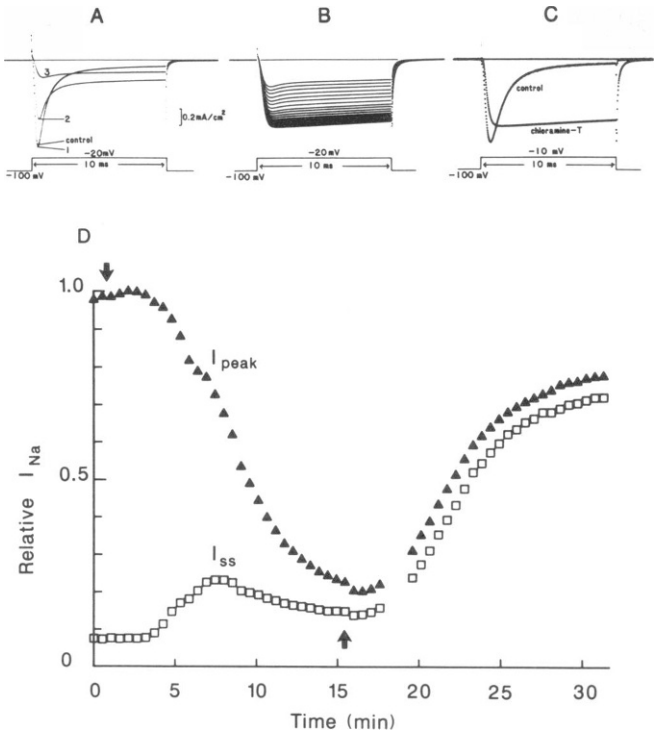

FIGURE 1 The time course of modification of the Na current by internally applied fresh chloramine-T solution in crayfish axon. The development of modification during internal application of 10 mM chloramine-T solution is illustrated in *A*. Traces *1*, *2*, and *3* were selected from Na currents during the wash-in period to represent an increasing degree of modification. *B* shows Na currents that were elicited at an interval of 30-s (from the top to the bottom traces) between the test pulses during washing out of chloramine-T from the internal solution. In *C*, the Na currents before and after washing out of the chloramine-T show that the fast Na inactivation had been completely removed by chloramine-T treatment. In *D*, the peak (*solid triangles*) and the steady-state (*open squares*) values of Na currents were plotted as a function of time for wash-in and wash-out. The downward arrow represents the time of switching to chloramine-T solution and the upward arrow the time of switching to drug-free solution.

current was reduced to ~20% of the control one and the decaying phase was almost completely suppressed. This decrease of the Na current suggests a blocking action of chloramine-T solution. Upon washing out chloramine-T, this blocking action was reversible, whereas the removal of Na inactivation was not, as illustrated in Fig. 1 *B* (depolarizing pulses were applied every 30 s to monitor the time course of recovery from the chloramine-T block). However, when an aged chloramine-T solution (see Methods) was used, the removal of Na inactivation was not observed, whereas the decrease of the peak Na current still occurred. This blocking action of the aged chloramine-T was studied in detail in squid axons (see Section II).

Fig. 1 *C* shows the Na currents before and after chloramine-T treatment. The fast Na inactivation had been completely removed and the Na current recovered to 80% of its peak control value. On average of four experiments, the amplitude of Na current after treatment was 79 ± 3% of the control peak Na currents. This decrease probably reflects the loss of channels during chloramine-T treatment. The time course of modification of Na currents during internal application of a fresh chloramine-T solution and the time course of recovery from the blocking action are illustrated in Fig. 1 *D*. The onset of modification and the recovery were very slow, reflecting mainly the slowness in exchanging internal solution in the crayfish axon. This notion was verified in squid axon preparations, in which solution exchange was very rapid (see Fig. 4).

Voltage Dependence of Na Activation after Chloramine-T Treatment. Families of Na currents before and after chloramine-T treatment are illustrated in Fig. 2, *A* and *B*, respectively. The decay phase of the control Na currents was completely abolished at all test potentials in Fig. 2 *B*, indicating a total removal of the fast Na inactivation. The current–voltage relations before and after chloramine-T treatment shown in Fig. 2 *C* were obtained from the peak and the steady state values of Na currents shown in Fig. 2, *A* and *B*, respectively. After removal of the fast Na inactivation by chloramine-T, the steady-state currents increased at potentials more negative than −40 mV and decreased at potentials more positive than −40 mV, without any change in the reversal potential. Fig. 2 *D* illustrates changes in the conductance–voltage (g-V) relation after chloramine-T treatment. In the control, as the membrane was depolarized the conductance increased, reached a maximum at ~+10 mV, and declined with increasing depolarization above +10 mV. The decline in the control maximum conductance at high depolarizations can be interpreted as a consequence of the fast Na inactivation, which can truncate full development of the maximum conductance (Stimers et al., 1985), or as a result of rectification of the open channel conductance. The chloramine-T treatment had three major effects on the g-V relation. First, it reduced the maximum conductance to ~70% of the control. Second, it increased the conductance at lower voltages (membrane potential less negative than −40 mV) even though that the maximal conductance was reduced. This effect may simply reflect removal of Na inactivation since the time course of activation and inactivation overlap, especially at lower depolarizing potentials (Patlak and Horn, 1982). Third, the Na conductance did not reach its maximum until the membrane was depolarized to +30 mV. The latter effect was also observed by Stimers et al. (1985) in squid axon after removal of the fast Na inactivation by pronase treatment. Since the fast inactivation occurs 4 to 5 times faster in crayfish axons than in squid axons (Swenson, 1980) and is more voltage-dependent (Bean, 1981), the effect of Na inactivation on the g-V relation would be more pronounced in crayfish than in squid axons. However, no significant change in the steepness of voltage-dependence was observed after removal of the fast inactivation by chloramine-T: the slope

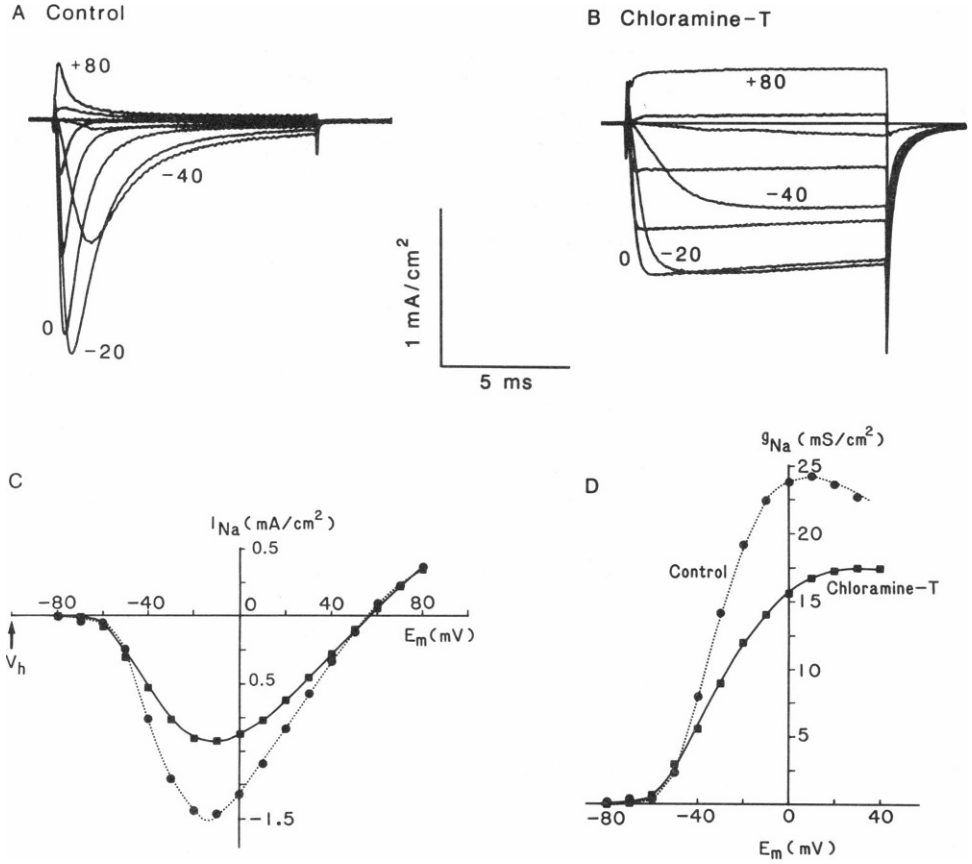

FIGURE 2 Voltage-dependence of the Na activation after chloramine-T treatment in crayfish axon. Families of Na currents before (*A*) and after complete treatment by internally applied 10 mM fresh chloramine-T solution (*B*) were obtained by applying 10-ms test pulses ranging from −80 to +80 mV in 20-mV increments, from a holding potential of −100 mV. After treatment, there is no sign of fast decay in Na currents during maintained depolarization at all tested potentials. *C* depicts the current–voltage relationship for the peak current in control (*solid circles*) and for the steady-state values of the chloramine-T modified Na currents (*solid squares*) presented in *A* and *B*, respectively. Both I-V curves have an identical reversal potential of 56.6 mV. The chord conductances calculated from the I-V relations shown in *C* were plotted as a function of membrane potentials in *D*. The slope factor for conductance increase is 6mV/e-fold in control and 8 mV/e-fold after chloramine-T treatment. The midpoint ($V_{1/2}$) for activation is −39 and −35 mV before and after chloramine-T treatment, respectively.

factor was 6.3 ± 0.3 (n = 3) and 7.0 ± 1.0 (n = 3) mV per e-fold conductance increase in control and after chloramine-T treatment, respectively.

Slow Inactivation Is Not Removed by Chloramine-T Treatment. Besides the fast Na inactivation, a slow inactivation process with time constants varying from several hundreds of milliseconds to several tens of seconds has been previously described in various preparations (see Rudy, 1981). In crayfish axons, inactivation with a time constant of several hundred milliseconds has been reported before (Shrager, 1977; Starkus and Shrager, 1978) and was not investigated here. We report the effect of chloramine-T on the slow inactivation with a time constant on the order of 10 s, which was not removed by the chloramine-T treatment.

Fig. 3 shows the recovery from slow inactivation produced by a 30-s depolarizing pulse, which produced steady-state slow inactivation. 1 s after the conditioning pulse, 70% of the channels recovered from slow inactivation in control (Fig. 3 *A*), whereas only ~15% of the channels recovered in the chloramine-T–treated axon (Fig. 3 *B*). This result shows that Na channels recovered initially faster in control than after chloramine-T treatment. Fig. 3 *C* plots the time course of recovery from slow inactivated states. The data after 10 s of recovery were fitted to a sum of two exponential functions. The time constants of recovery were 17 and 184 s in control and 21 and 164 s in the chloramine-T–treated axon. These values are rather similar. Thus, the principal effect of chloramine-T on slow inactivation is that more channels can undergo slow inactivation when the fast inactivation is removed. This result does not support the notion that the slow inactivation occurs only through the fast inactivated state. On the contrary, it appears that the fast inactivation can compete with the slow inactivation when the Na channel is subjected to a prolonged depolarizing pulse, as proposed by Rudy (1981).

Chloramine-T Action Is Not Modulated by Holding Potential. Salgado et al. (1985) reported that membrane depolarization in crayfish axon slows the action of *N*-bromoacetamide and pronase in removing the fast Na inactivation. To test whether membrane depolarization had any effect on chloramine-T action on the Na inactivation, we took advantage of the double sucrose-gap voltage clamp technique. Namely, the same axon could have two membrane segments, each having different membrane potentials: the nodal region was maintained at negative holding potentials (−80 to −100 mV) through voltage clamp feedback, while the axon in the left-hand pool was depolarized to ~−30 mV by K-free external and internal solutions (see Table I). After the removal of the fast inactivation of the channels in the nodal region, the chloramine-T solution was thoroughly washed out, and then a new node was created by moving the axon segment

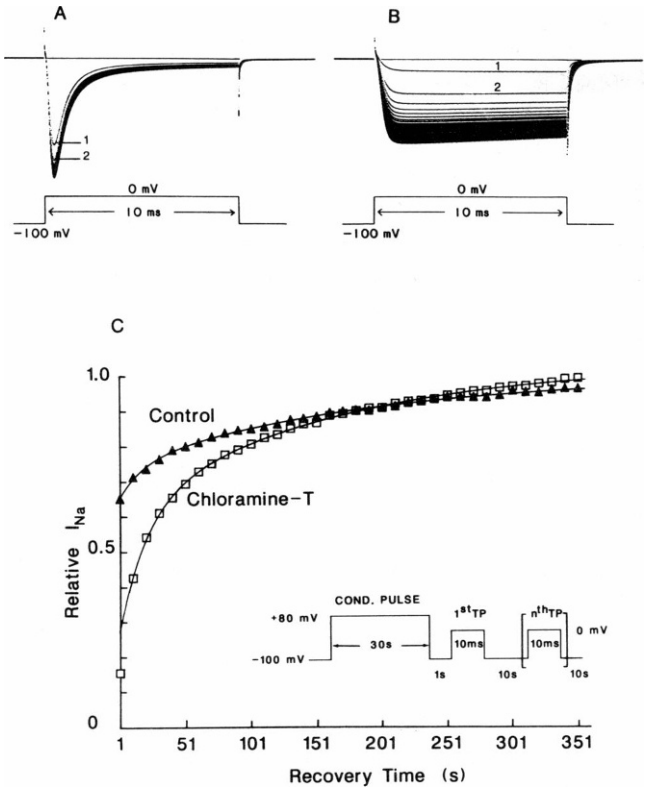

FIGURE 3 Recovery of Na currents from slow inactivation before and after chloramine-T treatment in crayfish axon. The slow inactivation was produced by a 30-s conditioning pulse to +80 mV and recovery from inactivation was monitored with test pulses to 0 mV: the first test pulse (1st TP) was applied 1 s after the conditioning pulse and then subsequent test pulses (nth) were applied every 10 s (see inset of *C*). The traces labeled *1* and *2* in *A* and *B* were recorded 1 and 11 s after the conditioning pulse respectively, and the following traces with 10-s increment. In *C*, the peak values in *A* and steady-state values in *B* were normalized to the maximum recovery amplitude and plotted as a function of recovery time. The control (*solid triangles*) was fitted to a sum of two exponentials of the form of $1 - 0.1\exp(-t/17.3) - 0.26\exp(-t/184.0)$. A similar two exponential function, which fit the data points after chloramine-T treatment for recovery times from 11 s on to complete recovery, had the form of $1 - 0.35\exp(-t/20.7) - 0.42\exp(-t/163.5)$.

that had been depolarized in the left-hand pool and had been exposed to the same concentration of drug for the same duration as the old nodal membrane. We found that in the new node the fast inactivation was gone. Thus, membrane depolarization does not protect the channel from chloramine-T action. These results suggest that chloramine-T can modify the channel in either the resting or inactivated states.

II. Blocking Effects of Chloramine-T on Na Currents in Squid Axons

The Na channel blocking action of chloramine-T seen in crayfish axons was studied in detail in squid axon because solution changes could be completed within 2–3 min. In squid axons, the freshly prepared chloramine-T solution exerted two effects on Na currents as observed in crayfish axons: first, an irreversible removal of the fast inactivation; second, a reversible block of Na currents. The first effect had been reported by Wang et al. (1985), therefore, it will not be repeated here. The second effect was the main focus of this study. The blocking action was compared between a freshly prepared solution and an aged solution of chloramine-T. In addition, the blocking action in axons with intact Na inactivation was compared with that observed in axons after removal of the Na inactivation by freshly prepared chloramine-T solution.

Effect of Freshly Prepared Chloramine-T Solution. During chloramine-T treatment, the peak Na current was decreased, while the steady-state Na current was increased (compare Fig. 4, *A* and *B*). The time-dependent decay was extant in the presence of chloramine-T even after prolonged chloramine-T treatment. When chloramine-T was washed out with drug-free solution, both the peak and steady-state Na currents were increased, more markedly for the steady-state current. This increase could be explained by recovery from the chloramine-T block

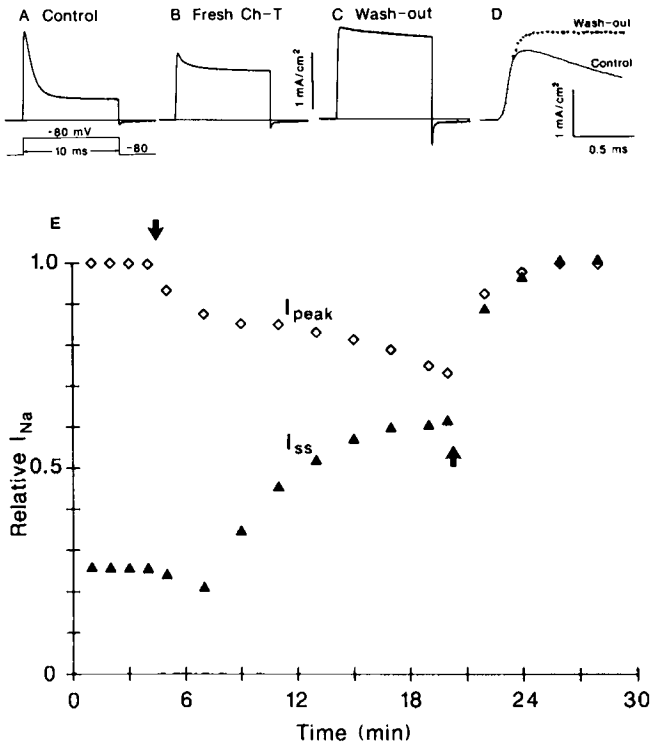

FIGURE 4 Effects of freshly prepared chloramine-T solution on Na currents in squid axon. The steady-state effects of 10 mM chloramine-T on Na currents are illustrated in *B*: the peak Na current (I_{peak}) was reduced, whereas the steady-state current (I_{ss}) was increased by washing out chloramine-T solution (*C*). The currents shown in *A–C* have same scale as indicated between *B* and *C*. The Na current, after removal of the inactivation process, had same rising phase as the control, as shown in *D* (where the wash-out trace has been scaled up by a factor 1.15). The time course of effects during wash-in (*downward arrow*) and wash-out (*upward arrow*) of the chloramine-T solution is illustrated in *E*. The peak Na current was normalized to the initial control peak current, whereas the steady-sate current was normalized to the final I_{ss} after washing out of chloramine-T solution.

(compare Fig. 4 *B* with Fig. 4 *C*). The Na current did not decay appreciably during maintained depolarization, indicating that chloramine-T had effectively removed most of Na inactivation (Fig. 4 *C*). After modification, the Na current rose with a similar time course as the control one, as illustrated in Fig. 4 *D*. This result suggests that the activation process was not affected by chloramine-T treatment. In contrast to the experiments performed in crayfish axons, the time course of chloramine-T action was very rapid. As shown in Fig. 4 *E*, the steady-state Na current increased rapidly during wash-in period, reflecting the removal of Na inactivation, while the peak Na current decreased with a similar time course, mainly reflecting the blocking action of chloramine-T. Upon washing out chloramine-T solution, both peak and steady-state Na currents increased up to their initial respective value with a similar time course, indicating that the blocking effect was completely reversible.

Blocking Action of an Aged Chloramine-T Solution. Fig. 5 shows that in an axon with intact inactivation, 10 mM aged chloramine-T solution did not remove the fast Na inactivation. In contrast, it accelerated the decay phase of Na current with respect to the control one (compare Fig. 5 *A* with 5 *B*). The decaying phase of the Na current could be fit to a single exponential time course, which has been interpreted as to reflect the Na inactivation process. The Na current at +80 mV decayed with a time constant of 0.83 ms in control and 0.60 ms in the presence of an aged chloramine-T solution. This acceleration of the Na decay kinetics was observed at all potentials between 0 and +80 mV; the time constant was on the average decreased by 30%. This effect was completely reversible, as illustrated in Fig. 5 *C*. After wash-out, the Na current decayed with a time constant of 0.82 ms, a value similar to the control one.

The onset of action and wash-out experiments was detailed in Fig. 5 *D*. After switching to an aged 10 mM chloramine-T solution, both the peak and steady-state Na currents decreased immediately, reaching their final level within 10 min. The peak Na current was reduced to 61% of the control peak and the steady state was reduced to 15% of their respective values in the control. Both the blocking action and the effect on the decay kinetics of Na currents were reversible upon washing out the aged chloramine-T solution.

Blocking Action of Aged and Fresh Chloramine-T Solutions after Removal of Inactivation. Fig. 6 *A* shows another example of the blocking action of an aged solution containing 5 mM chloramine-T in an axon with

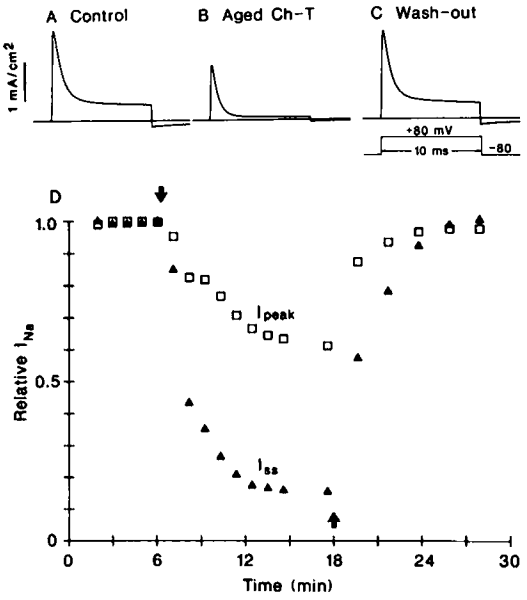

FIGURE 5 An aged chloramine-T solution reversibly blocks Na currents in squid axon. The Na currents were recorded in response to depolarizing pulse to +80 mV applied from a holding potential of −80 mV before (*A*), during (*B*), and after (*C*) internal application of an aged 10 mM chloramine-T solution. The Na current in *B* represents the full effects of chloramine-T (10-min exposure): the peak was reduced by 35% and the steady-state by 84%. The decay kinetics was accelerated: the time constant decreased from 0.83 ms in the control (*A*) to 0.60 ms. Both effects were reversible upon washing with the drug-free solution, as illustrated in the *C*. The time course of wash-in and wash-out was shown in *D*. The downward arrow indicates the time of switching to the drug solution, and the upward arrow the time of switching to the drug-free solution.

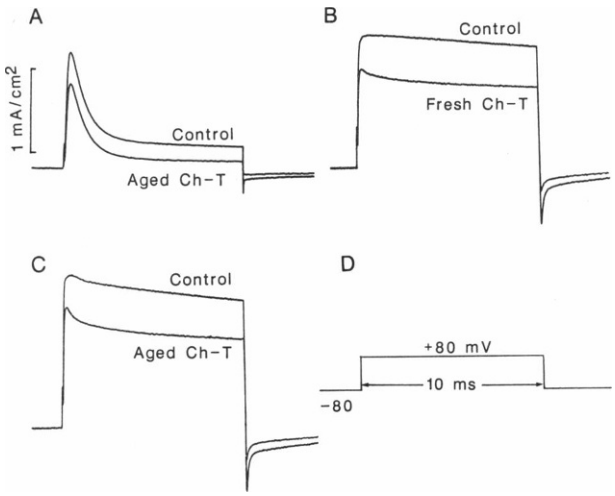

FIGURE 6 Blocking action of an aged and a fresh chloramine-T solution after removal of Na inactivation in squid axons. *A* shows that the aged solution containing 5 mM chloramine-T blocked the peak Na current 27% and the steady-state sodium current by 64% in an axon with intact inactivation. Upon washing out the aged chloramine-T solution, the Na current returned to the control level (not shown here). In the same axon, the subsequent application of a freshly prepared chloramine-T solution could remove the Na inactivation process, as shown in *B* (trace labeled as control). The re-admission of 5 mM fresh chloramine-T solution caused a time-dependent block of the Na current: the peak current was reduced by 22% and the steady-state by 35%. *C* depicts the blocking action of an aged chloramine-T solution in another axon in which the Na inactivation had been removed by using freshly prepared chloramine-T. The peak current was reduced by 20% and the steady-state current by 31%. The pulse protocol is shown in *D*.

TABLE II
COMPARATIVE Na CHANNEL BLOCKING ACTION BETWEEN FRESH AND AGED CHLORAMINE-T SOLUTIONS*

	(−) Inactivation‡			(+) Inactivation		
	I_p	I_{ss}		I_p	I_{ss}	
mM	%	%	*N*	%	%	*N*
Fresh 5	20 ± 5.0	35 ± 2.0	3			
10	34 ± 2.0	50 ± 8.0	4			
Old 5	22 ± 2.0	32 ± 4.0	4	22	62	2
10	32	40	1	38	84	1

*The Na channel blocking action of chloramine-T was measured as the percentage of decrease in I_p and I_{ss}, measured, respectively, at 0.5 and 10 ms after the beginning of depolarizating pulse to +80 mV.
‡The inactivation had previously been removed by chloramine-T treatment.

intact inactivation. After washing out the aged solution, the Na current returned to the original control level (not shown here). In the same axon, the blocking action of a freshly prepared chloramine-T solution was examined after removal of Na inactivation by internally applied chloramine-T fresh solution. The decay phase of Na current was eliminated, suggesting that inactivation of Na current was removed (labeled as the control in Fig. 6 *B*). Upon introducing another freshly prepared solution containing 5 mM chloramine-T, the Na current was decreased to 78 and 65% of its control value at 0.5- and 10-ms time points after the beginning of the depolarizing pulse, respectively. A similar blocking action was obtained with an aged solution of chloramine-T as illustrated in Fig. 6 *C*.

Table II summarizes the blocking action of both fresh and aged chloramine-T on the Na currents before and after removal of Na inactivation by chloramine-T. Both solutions blocked the Na current equally well. When the blocking potency was determined at 0.5 ms after the beginning of the depolarizing pulse, the suppression of the Na current was 20 ± 5% (n = 3) in the presence of a fresh solution of 5 mM chloramine-T and was 22 ± 2% (n = 4) in the presence of an aged solution originally containing 5 mM chloramine-T. When the blocking action was measured at 10 ms after the beginning of the depolarizing pulse, both solutions exerted a stronger blocking action: 35 ± 2% (n = 3) and 32 ±4% (n = 4) for the fresh and aged chloramine-T solutions, respectively. However, for the aged solution, this time-dependent block was more pronounced in axons with intact inactivation than after removal of inactivation (compare Fig. 6 *A* and 6 *C*). At 10 ms after the beginning of the depolarizing pulse, the Na currents were decreased by 62% in axons with intact inactivation instead of 32% after removal of inactivation by chloramine-T. This result suggests that the aged chloramine-T solution can actually enhance the Na inactivation process or that the inactivation process enhances the time dependency of the blocking action.

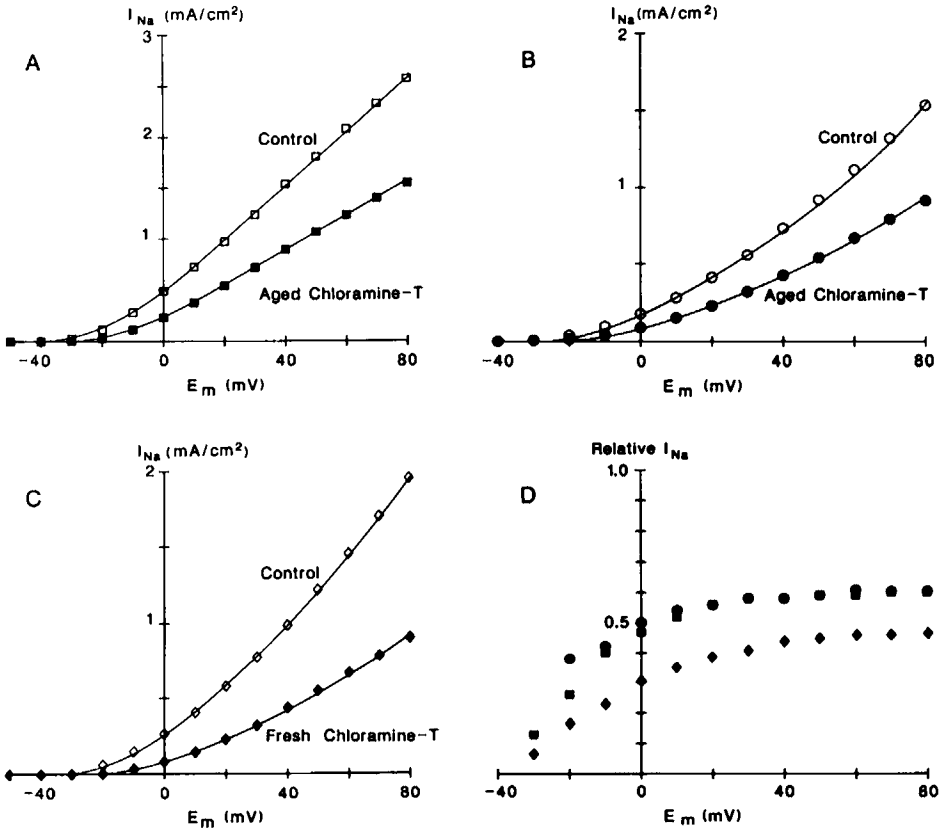

FIGURE 7 Voltage-dependent block of Na currents by aged and fresh chloramine-T solutions in squid axons. The I-V curves shown in *A* were obtained from the peak Na currents in an axon with intact inactivation before (*open squares*) and during (*solid squares*) internal application of 10 mM aged chloramine-T solution. The ratio of the peak Na current measured in the presence of aged chloramine-T over its control value at each potential was plotted as a function of membrane potential in *D* (*solid squares*). In *B* and *C*, the Na inactivation had been removed by chloramine-T pretreatment, which is referred to as control. The I-V curves were obtained before (*open symbols*) and during (*solid symbols*) application of 10 mM aged (*B*) or fresh (*C*) chloramine-T solution. The ratios of the Na current in the presence of the aged (*solid circles*) and fresh (*solid diamonds*) chloramine-T solution over their respective control values at each potential were plotted as a function of membrane potential in *D*.

Voltage Dependence of Chloramine-T Blocking Action. The Na channel blocking action of chloramine-T, freshly prepared or aged solution, was determined from the I-V relations shown in Fig. 7. In axons with intact inactivation (Fig. 7 *A*), a 10 mM aged chloramine-T solution reduced the Na current in a voltage-dependent manner. At membrane potentials less positive than +20 mV, the reduction became more pronounced as the membrane potential became less depolarized. In contrast, at membrane potentials more positive than +20 mV, there was a constant degree of reduction in Na currents in the presence of chloramine-T (Fig. 7 *D*). After removal of Na inactivation, a similar voltage dependence of block was obtained with either aged (Fig. 7 *B*) or fresh (Fig. 7 *C*) solutions. The removal of Na inactivation did not affect the voltage-dependence of the block induced by aged chloramine-T solution (Fig. 7 *D*).

DISCUSSION

We have demonstrated here that chloramine-T has two major effects on the Na channels in crayfish and squid axons: (*a*) it irreversibly removes the fast inactivation as previously reported with myelinated nerve fibers and squid axons (Wang, 1984*a,b*; Wang et al., 1985; Schmidtmayer, 1985), and (*b*) it reversibly blocks the Na currents. The first effect was observed only with freshly prepared chloramine-T solution, whereas the second effect was observed with both fresh and aged solutions. After removal of the fast inactivation, the chloramine-T modified channels can still undergo slow inactivation (Ulbricht and Stole-Herzog, 1984; Wang et al., 1985). We found a similar result in crayfish axon, suggesting that the fast inactivated state is not a prerequisite for slow inactivation to occur. In addition, we found that more channels can undergo slow inactivation after removal of the fast inactivation with chloramine-T. This effect contrasts with the effect of pronase treatment, which did not affect the proportion of the Na channels undergoing the transition into slowly inactivated state in squid axons (Rudy, 1978). Finally, the lack of voltage-dependent removal of the fast inactivation by chloramine-T in crayfish axon also contrasts with the removal of Na inactivation by pronase and N-bromoacetamide (Salgado et al., 1985). This result suggests that chloramine-T act at a different site from that of pronase and *N*-bromoacetamide in crayfish axon.

In squid axons, we found that the fresh chloramine-T solution lost its ability to remove the fast inactivation with time, whereas its Na channel blocking action remained unchanged. This result suggests that there are at least two different molecular forms of chloramine-T present in solution: an active and labile one, which removes the fast Na inactivation, and an inactive and stable one, which blocks the channel. Since the fresh and aged solutions are equally potent in blocking the Na channel, it is possible that the active form could also block the Na channel if the active and inactive forms have a similar affinity for the blocking site. Alternatively, the active form could be only responsible for the removal of fast inactivation, if the inactive is the predominant form in the fresh solution. However, it is difficult to determine the exact molecular species involved since chloramine-T (Na salt of *N*-chloro-*p*-toluenesulfonamide) is thought to exist in a complex series of equilibria in aqueous solution: sodium hypochlorite (hypochlorous acid), protonated chloramine-T, dichloramine-T, and *p*-toluenesulfonamide (Alexander, 1973; Balasubramanian and Thiagarajan, 1975; Campbell and Johnson, 1978). Since all the products, with the exception of hypochlorous acid, possess the aromatic ring and since the blocking action of aged chloramine-T solution is similar to that seen with phenol and benzocaine (unpublished data), it is tempting to conclude that aromatic moiety is involved in the blocking action.

The active species involved in the removal of Na inactivation must be labile inasmuch as removal of Na inactivation is observed only in freshly prepared solution. Several molecular species could be involved, including chloramine-T, hypochlorous acid, and dichloramine-T. Two different mechanisms for the removal of the fast Na inactivation have been recently proposed: one involves the methionine residues of the Na channel protein (Wang et al., 1985) and the other, the unsaturated lipids in the membrane (Rack et al., 1986). In the first hypothesis, the methionine residues are thought to be oxidized by chloramine-T since chloramine-T has been shown to oxidize the methionine residues selectively under controlled conditions (Shechter et al., 1975). This hypothesis merits testing in view of the recent Na channel model (Noda et al., 1984) in which 13 methionine residues out of 1,820 amino acid residues are found in various regions of the Na channel (i.e., at the positions of 331, 358, 369, 590, 754, 765, 1,179, 1,182, 1,201, 1,219, and 1,495).

In the second hypothesis, the modification of double bonds of membrane lipids by chloramine-T may be responsible for removal of Na inactivation (Rack et al., 1986). Both hypochlorous acid (Swain and Crist, 1972; Rack et al., 1986) and dichloramine-T have been postulated to mediate the chlorination of double bonds (Higuchi and Hussain, 1967). A variant of this hypothesis is that the specific unsaturated lipids that are covalently bound to the Na channel structure (Levinson et al., 1987) may be the site of action of the active species. This notion would imply that these specific lipids play a determinant role in the fast Na inactivation process.

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