J. Membrane Biol. 169, 83–90 (1999)

The Journal of
Membrane
Biology

Involvement of Methionine Residues in the Fast Inactivation Mechanism of the Sodium Current from Toad Skeletal Muscle Fibers

M. Quiñonez, M. DiFranco, F. González

Laboratorio de Fisiología y Biofísica del Músculo, Instituto de Biología Experimental, Facultad de Ciencias, Universidad Central de Venezuela, Apartado 47114, Caracas, Venezuela

Received: 10 November 1998/Revised: 4 January 1999

Abstract. The role of methionine residues on the fast inactivation of the sodium channel from toad skeletal muscle fibers was studied with the mild oxidant chloramine-T (CT). Isolated segments of fibers were voltage clamped in a triple Vaseline® gap chamber. Sodium current was isolated by replacing potassium ions by tetramethylammonium ions in the external and internal solutions. Externally applied chloramine-CT was found to render noninactivating a large fraction of sodium channels and to slow down the fast inactivation mechanism of the remainder fraction of inactivatable channels. The action of CT appeared to proceed first by slowing and then removing the fast inactivation mechanism. The voltage dependence of the steady-state inactivation of the inactivatable CT-treated currents was shifted +10 mV. CT also had a blocking effect on the sodium current, but was without effect on the activation mechanism. The effects of CT were time and concentration dependent and irreversible. The use of high CT concentrations and/or long exposure times was found to be deleterious to the fiber. This side effect precluded the complete removal of fast inactivation. The effects of CT on the fast inactivation of the sodium current can be explained assuming that at least two methionine residues are critically involved in the mechanism underlying this process.

Key words: Chloramine-T — Methionine residues — Sodium current — Fast inactivation — Toad muscle fibers — Triple Vaseline® gap chamber

Introduction

The study of the structure-function relation of the ion channels has been long approached by combining electrophysiological techniques with biochemical and molecular biology methods (Catterall, 1988). One successful approach in providing valuable information about the structure-function relation of the ion channels is based on the specific *in situ* chemical modification of the ion channels (Brodwick & Eaton, 1982). With this approach either specific groups present in the amino acid residues of the native channels can be covalently modified, or covalent bonds broken, thus allowing the determination of the role of specific chemical structures in the function of these membrane proteins. The chemical modification of the ion channels is even more useful for those channels of known primary structure.

Chloramine-T (CT), is a noncleaving, mild oxidant reagent known to react, at slightly alkaline pH (7.0–8.5), with exposed methionine residues of noncysteine proteins and with free sulfhydryl groups of cysteine residues (Shechter, Burstein & Patchornik, 1975). At low millimolar concentration, CT has been shown to specifically remove sodium current fast inactivation of both vertebrate and invertebrate nerve fibers, without affecting the activation process (Wang, 1984*a*,*b*; Wang, Brodwick & Eaton, 1985; Huang, Tanguy & Yeh, 1987). By contrast, sulfhydryl-reactive reagents are without effect on the inactivation mechanism (Shrager, 1977; Rack et al., 1984; Wang, 1984*a*). As a result, it was proposed that methionine residues are involved in the channel inactivation mechanism of nerve fibers.

The study of the effect of CT on sodium currents has become more relevant as several methionine residues have been shown to be present in all the sodium channels sequenced (Noda et al., 1984, 1986; Catterall, 1986, 1988). As the effects of CT on sodium current have only been studied in nerve fibers, we decided to study the effects of this reagent on the sodium current from skeletal muscle fibers, using the tropical toad *Bufo marinus* as a model.

Correspondence to: M. Quiñonez

We found that CT affects the sodium channel in a manner similar to that initially reported for myelinated nerves from *B. marinus* (Wang, 1984*b*). At low CT concentrations a large fraction of the channels became noninactivating. The remaining inactivatable channels showed a slower inactivation process and modified voltage dependence for inactivation, whereas the activation process was not significantly affected. The amplitude of the sodium current showed a biphasic change in the presence of CT; the peak current initially increased above and then decreased below control. The irreversibility of the changes in the inactivation mechanism indicates that they resulted from a covalent modification of the channels. Additionally, it was found that large CT concentrations and/or prolonged exposures to the reagent had deleterious effects on the fiber. An optimal exposure time/concentration combination was determined. Our results suggest that the sodium channel from toad skeletal muscle has at least two accessible methionine residues, which are involved in the mechanism of fast inactivation.

Materials and Methods

Fiber Dissection and Mounting Procedure

Segments of single muscle fiber, 80–100 μm in diameter, were isolated from the sartorius muscle of the tropical toad *B. marinus.* Fiber segments were dissected in toad Ringer solution (*see* Solutions), and mounted in a triple Vaseline® gap chamber for voltage clamping (Hille & Campbell, 1976; Vergara et al., 1991). Briefly, isolated fiber segments were contractured at fixed slack length in isotonic potassium solution (*see* Solutions) and once relaxed were transferred to the experimental chamber, flooded with the same solution. Exposure to the air-liquid interface was avoided. The fibers were placed across Vaseline® pre-seals, previously applied to the chamber's partitions, and the Vaseline® seals were completed. Excess solution was removed to electrically isolate the chamber's compartments. The lateral pools' solutions were perfused with internal solution (*see* Solutions) and the ends of the fiber were cut again close to the Vaseline® seals. Finally, the content of the experimental compartment was exchanged for Ringer and a continuous flux of solution maintained. A 30–40 min period was allowed for equilibration of intracellular content and the end pools' solution before experiments started. Sodium currents were measured in K-free Ringer solution (*see* Solutions). The perfusion system had a dead time of about 30 sec. All experiments were carried out at 22°C.

Electrophysiological Methods

The voltage clamp circuit was similar to that described by Hille and Campbell (1976). Holding potential was set at −100 mV, unless otherwise stated. Sodium current was isolated by replacing potassium ions by tetramethylammonium ions (TMA) in the external and internal solutions. Leak and capacitative currents were subtracted online from the records. Electrical signals were filtered at 10 kHz and acquired with a TL-125 data acquisition system (Axon Instruments, Foster City, CA).

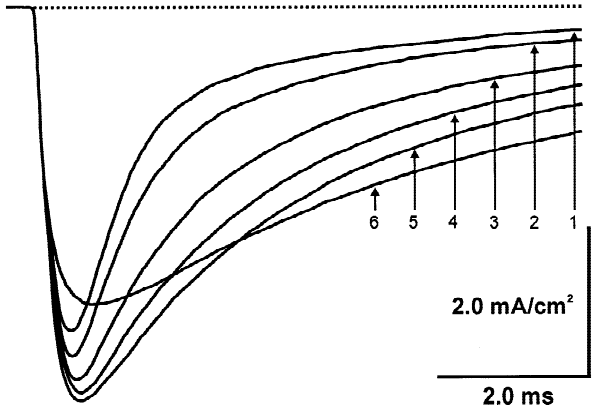

Fig. 1. Effect of CT on the sodium current amplitude and fast inactivation. Currents were elicited by continuously stimulating the fiber at 0.1 Hz with 7 msec, 70 mV depolarizing pulses. The fiber was treated with 1 mM CT during 1 min. The traces are sample records of currents obtained at 0.0, 1.5, 2.0, 2.2, 2.5 and 5.0 min after CT treatment started, respectively. The dotted line represents the zero current level.

Solutions

Normal toad Ringer solution contained (in mM): 125 NaCl, 6 KCl, 1.8 $CaCl_2$, and 5 HEPES (N-2-hydroxyethyl piperazine-N′-2-ethane sulphonic acid). K-free Ringer solution was identical to normal Ringer except that potassium was exchanged for TMA-Cl. The internal solution contained (in mM): 120 TMA-Aspartate, 2 $MgCl_2$, 5 ATP-Na, 5 phosphocreatine-Na, 10 MOPS-Na (3-[N-morpholino]propane sulfonic acid), and 0.1 g/ml creatine phosphokinase. The isotonic K-solution contained 85 mM K_2SO_4 and 10 mM HEPES. The pH and osmolality of the solutions were adjusted to 7.4 and 250 mOsmol, respectively. Chloramine-T solutions were prepared fresh for each experiment by adding the required amount to K-free Ringer solution.

Results

Effect of CT on the Fast Inactivation Mechanism and the Amplitude of the Sodium Current

When externally applied, chloramine-CT was found to differentially modify the fast inactivation and the amplitude of the sodium current. While fast inactivation changed monotonically with time, the effect on current amplitude was biphasic. These effects depended on the exposure time and the reagent concentration (Figs. 1–3). To follow the progression of the effects of CT, short (7 msec) depolarizing pulses of fixed amplitude were applied at 0.1 Hz, and the resulting sodium currents recorded as a function of time. The results of an experiment using 1 mM CT and 70 mV pulses are shown in Fig. 1. The record obtained in control conditions (trace 1) shows that, due to the fast inactivation mechanism, the sodium current rapidly decays to a value close to zero by the end of the pulse. In this fiber, changes in fast inactivation were first detectable about 1 min after the per-

fusion with CT-containing Ringer started. In the presence of CT the fast inactivation was progressively slowed (traces 2–6). Consequently, an increasing fraction of sodium channels were not completely inactivated by the end of the pulse as the reagent effect progressed. After about 5 min of treatment (trace 6), the effect of CT on fast inactivation seemed to reach an apparent steady-state condition, in which only ~60% of the sodium current inactivated at the pulse end.

The changes in fast inactivation were accompanied by an increase in the time of peak. This parameter increased from 0.51 ± 0.08 msec in control conditions to 0.78 ± 0.21 msec after 5 min exposure to CT ($n = 6$). As can be seen in Fig. 1, the exposure to CT also modified the peak sodium current. The current amplitude initially increased to a maximum of about 122% the control amplitude (trace 5), and finally decreased to about 10% below the control value (trace 6). In six experiments a mean peak current of 4.43 ± 0.21 mA/cm^2 and 3.65 ± 1.15 mA/cm^2 was found in control and experimental conditions, respectively (*see also* Figs. 2–3). The changes in current amplitude resulted from the combination of two mechanisms. The initial increase in current amplitude is predicted by the Hodgkin-Huxley model as a consequence of the partial removal of fast inactivation described above. Accordingly, an increase in the time to peak of the sodium current is also expected. With the caveat that the single channel conductance is not modified (Cukierman, 1991), the subsequent decrease in sodium current amplitude suggests that a fraction of sodium channels became in some way nonconductive or blocked in the presence of CT-containing solutions.

The changes in fast inactivation could not be reversed by extensive fiber washout (~1 hr). This result is consistent with a covalent modification of sodium channels. It was also found that the effects of CT on fast inactivation were not use- or voltage-dependent as indicated by the fact that results similar to those above were obtained using other pulse amplitudes, holding potentials and without repeatedly pulsing the fiber.

The results described are concurrent with those obtained in squid and crayfish nonmyelinated fibers and toad myelinated fibers (Wang, 1984*a*,*b*; Wang et al., 1985; Huang et al., 1987), and in frog muscle fiber externally treated with CT (Hahin, 1988).

When long voltage pulses were applied it became evident that CT produced a partial removal of fast inactivation, rather than just slowing the inactivation mechanism. As shown in Fig. 2, the control current (Fig. 2*A*) quickly reverted to zero, whereas about 25% of the sodium channels remained open after CT treatment (Fig. 2*B*), as indicated by the steady level of inward current observed by the end of the voltage pulse. In control conditions, the fraction of sodium current measured at the end of 80 mV depolarizing pulses was indistinguishable from the baseline, whereas after CT treatment it increased to 28.1 ± 5.2% of the peak current ($n = 6$).

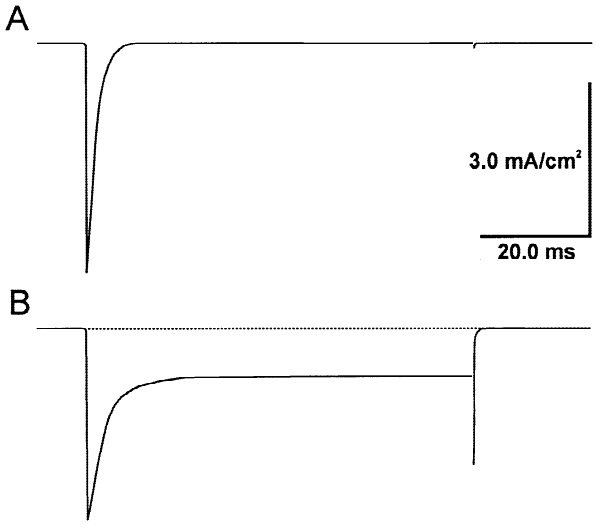

Fig. 2. Partial removal of fast inactivation by CT treatment. (*A*) Control current. (*B*) Sodium current obtained 7 min after the start of CT treatment. Sodium currents were elicited by 75 msec, 80 mV depolarizing pulses. The fiber was treated with 1 mM CT during 5 min. The dotted line represents the zero current level.

The presence of a fraction of noninactivating sodium channels is also clearly indicated by the apperance of a large tail current at the pulse end (Fig. 2*B*). It can also be appreciated in this figure that the fast inactivation of the inactivatable fraction of the sodium current is slowed by CT treatment (*see below*).

The identity of the channels involved in the responses reported above was probed with TTX. Both the control ($n = 2$) and the CT-treated ($n = 5$) currents were completely blocked by TTX (100 nM). This result confirms that the recorded currents corresponded to currents passing through the voltage-sensitive sodium channel.

As in our experimental conditions CT is expected to react with both methionine and cysteine residues, controls experiments were carried out with the sulfhydryl group-specific reagent 5,5′-dithiobis-2-nitrobenzoic acid (DNTB). At the concentrations used (1.0 mM, $n = 2$; 5.0 mM, $n = 3$), DNTB was found without effect on the fast inactivation of the sodium current. Similar results were found previously in nerve fibers (Shrager, 1977; Rack et al., 1984; Wang, 1984*b*; Wang, 1985; Rack, Rubly & Waschow, 1986).

Dependence on Concentration and Time of the Chloramine-T Effects

The time and concentration dependence of the effect of CT is illustrated in Fig. 3. Results from two long lasting sample experiments in which two intermediate concen-

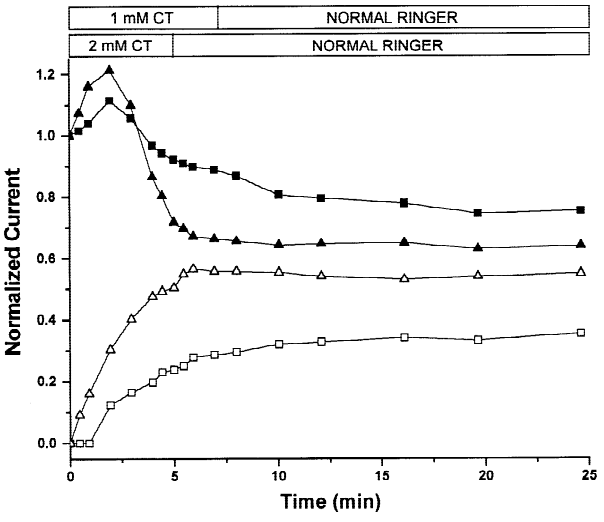

Fig. 3. Time course and concentration dependence of the effects of CT. Currents were activated by depolarizing the fiber at –20 mV during 25 msec. Data were normalized with respect to the control values. The filled and open symbols indicate the peak current amplitude, and the current measured at the end of the pulse, respectively. The results obtained in two different fibers using 1 mM (triangles) and 2 mM (squares) CT are shown. The exposure times to CT are indicated at the top of the figure.

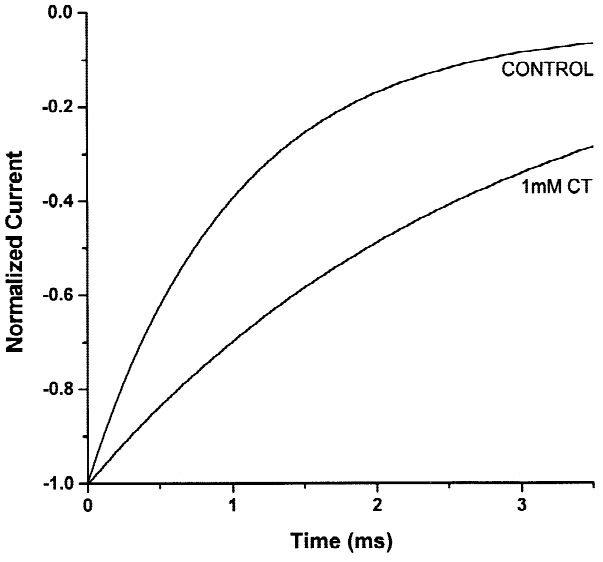

Fig. 4. Time course of control and CT-treated fast inactivation. The normalized decaying phase of sodium currents obtained in response to pulses of 5 msec in length and 80 mV in amplitude are shown. The non-inactivating current fraction of the CT-treated current was obtained by subtracting the steady level of current at the end of pulse to the total CT-treated current. The fiber was treated with 1 mM CT for 5 min.

trations were used (1.0 mM, triangles; and 2.0 mM, squares) are shown. The amplitude of the changes was concentration dependent and the effects of CT on both current amplitude and fast inactivation were faster the higher the concentration used. The noninactivating fraction of sodium current increased by 33 and 56% for 1 and 2 mM CT, respectively. The dual effect of CT on the current amplitude, described above, led to an early peak change occurring before maximal fast inactivation removal was reached. The peak sodium current increased by 10 and 18% with 1 and 2 mM CT, respectively, and the maximum blocking effect was 25 and 37% for 1 and 2 mM CT, respectively. Final steady values of current amplitude and fast inactivation seem to be obtained at similar times for a given concentration.

Is it worth noting that, contrary to that found in invertebrates' nerve fibers (Wang et al., 1985; Huang et al., 1987; Wang et al., 1987) and concurrent with the results from vertebrates' nerve fibers (Wang, 1984*a*,*b*), a complete removal of fast inactivation could not be obtained in toad muscle fibers. It was due to the fact that prolonged exposure times or high CT concentrations produced a progressive increase in leakage current, leading to fiber death. From experiments similar to that described above, using a CT concentration between 0.1–10.0 mM and exposure times up to 10 min, it was found that applying 2 mM CT for 5 min produces a maximal removal of inactivation with the less effect in leakage current.

Fast Inactivation Kinetics of the Inactivatable Sodium Current Fraction

On the assumption that single sodium channel conductance is insensitive to CT (Cukierman, 1991), the results can be interpreted as resulting from the appearance of three different channel populations. A fraction of channels would represent blocked or nonfunctional channels. The functional channel fraction would comprise two subpopulations, differing in their ability to inactivate. As the current records shown in Figs. 1–2 suggest that CT does not modify the fast inactivation in an all-or-none fashion, we compared the decaying phase of control and CT-treated sodium currents in order to determine if the residual inactivation was kinetically modified.

When the records of the decaying phase of the control currents and the inactivating fraction of the current after exposure to 1 mM CT were normalized (Fig. 4) it became evident that the inactivatable fraction of the CT-treated currents decays more slowly than control currents. The time constants of these records were 1.10 msec and 2.58 msec for control and CT-treated currents, respectively. An average ratio of treated to control time constants of 2.35 ± 0.3 msec was found from 6 other similar experiments using 1 mM CT. These results suggest that CT modified the fast inactivation mechanism of a sodium channel in at least two steps. The first step probably involved the slowing of the fast inactivation

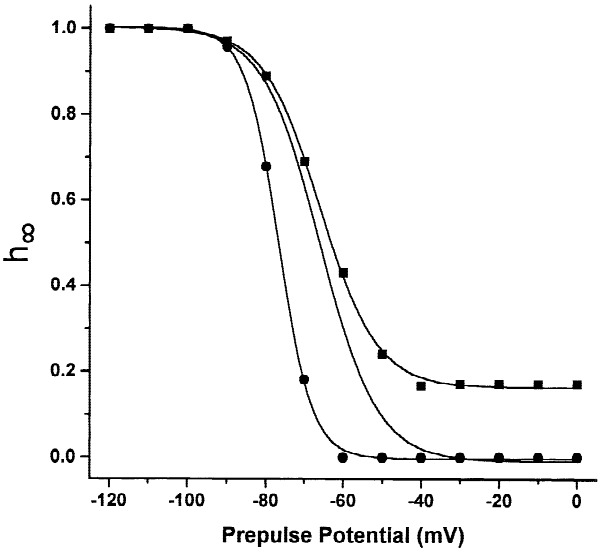

Fig. 5. Shift of the voltage dependence of the steady-state inactivation of the sodium current from fibers treated with CT. Steady-state inactivation of the control and the total CT-treated current are represented by the filled circles and squares, respectively. The steady-state inactivation of the inactivating fraction of the CT-treated sodium current is represented by the solid line curve without symbols. The fiber was treated with 1 mM CT during 5 min.

mechanism and the second the complete removal of this mechanism.

Steady-State Inactivation of the CT-Treated Sodium Current

CT has been reported to induce a shift in the steady-state inactivation (h_∞) curve of the sodium current from nerve fibers (Wang, 1984*b*), but this effect has not been studied before in skeletal muscle fibers. To this purpose a double pulse protocol was used. Long (60 msec) conditioning pulses of different amplitude were given before a test pulse to −20 mV was applied. The ratio of nonconditioned to conditioned peak current was plotted as a function of the conditioning potential for both the control and the CT-treated current. Figure 5 shows that control sodium currents were completely inactivated by conditioning pulses more positive than about −60 mV. On the contrary, after CT treatment a large fraction of channels remained open at 0 mV.

To determine the voltage dependence of the isolated inactivating fraction of current, the noninactivating current fraction was subtracted from the total CT-treated current. The resulting curve (solid line without symbols) shows that the voltage dependence of the inactivating fraction of channels was shifted about 10 mV to the right. The half-inactivation voltage of the control and CT-treated curves were −76 and −66 mV, respectively. An average shift of 10.5 ± 1.2 mV was determined from 4 fibers treated with 1.0 mM CT.

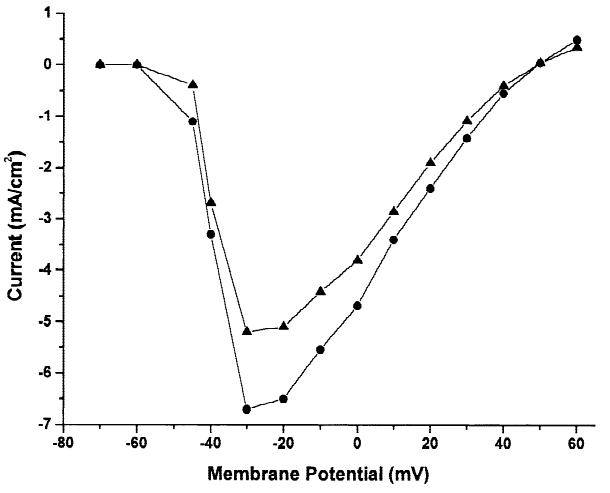

Fig. 6. Current-to-voltage relation of the sodium current. The circles represent the current obtained in control conditions, and the triangles the current measured after the fiber was exposed 4 min to 1.5 mM CT.

Voltage Dependence of CT-Treated Currents

The relation between the applied potential and the current was determined before and after exposing the fibers to CT in order to assess the possible effect of this reagent on the voltage dependence of the sodium current. The sodium current density was measured before and after CT treatment and plotted as a function of voltage (Fig. 6). Both current-to-voltage curves showed similar voltage dependence. The activation and reversal potentials of the control and CT-treated sodium currents were not significantly different. Reversal potentials of 45 ± 1.2 mV and 47 ± 2.5 mV were found before and after 1 min exposure to 1.5 mM CT, respectively ($n = 6$). Similar results were obtained with 1.0 and 2.0 mM CT. The curves for both conditions differed only in the maximal current amplitude, as a result of the blocking effect of CT. The peak current was 6.7 ± 0.3 mA/cm^2 and 4.6 ± 1.1 mA/cm^2, for the currents measured before and after treatment with 1.5 mM CT, respectively. The difference between the control and CT-current depended on CT concentration. The constancy of the activation and reversal potential indicates that activation and ion selectivity of the sodium channel were not affected by CT treatment.

Kinetic of Tail Sodium Current in Control and CT-Treated Fibers

The previous result showed that CT has no effect on the voltage dependence of activation. Additionally, it can be

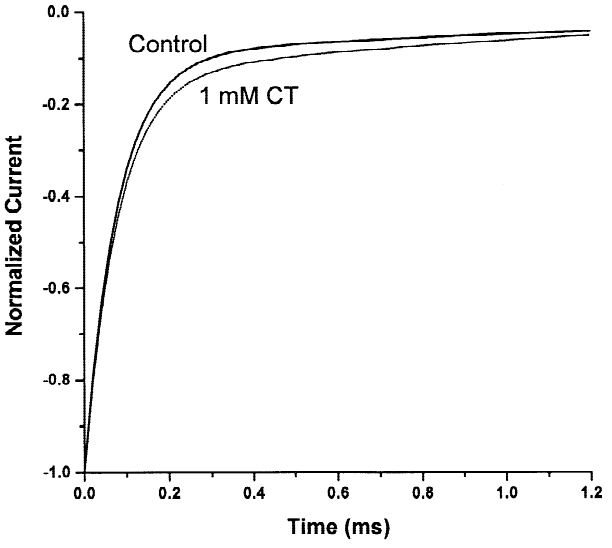

Fig. 7. Comparison of tail sodium currents from control and CT-treated fiber. Records were normalized and superimposed. Sodium currents were elicited by depolarizing pulses of 1 msec in duration and 80 mV in amplitude.

seen in Fig. 1 that the initial part of the sodium currents recorded at different times of CT exposure seem to superimpose to the control record. This result suggests that CT has little, if any, effect on the sodium current activation kinetics. To further study this point, the kinetics of the deactivation of control and CT-treated sodium currents was studied in the absence of significant inactivation contamination. To this end, sodium currents were elicited by applying short voltage pulses (1 msec) and the decay time constants of the tail currents determined. This approach was based on the fact that the activation and deactivation processes are governed by the same parameters. Normalized tail currents recorded before and after CT treatment (1.0 mM) are shown in Fig. 7. It can be seen that both records nearly completely superimposed, indicating that the activation parameter of control and total CT-treated currents were similar. The decay time constants for control and treated tail currents, were 0.10 and 0.11 msec, respectively. A ratio of control/CT-treated time constants close to unity was obtained (1.04 ± 0.03 msec; $n = 6$). Using other CT concentrations (1.5 and 2.0 mM) it was determined that the decay time was not concentration dependent. Using longer pulses (10 msec), the deactivation time constant of the noninactivatable fraction of channels could be separately measured. The results obtained with this procedure were similar to those presented above (*not shown*). Taken together these results indicate that the activation process is not affected by CT treatment.

Discussion

The results presented here demonstrate that CT produces a selective effect on the fast inactivation of the sodium channel from toad skeletal muscle. After CT treatment a large fraction of the sodium channels became noninactivatable, whereas the remainder of the channels showed an inactivation mechanism with abnormal kinetics and voltage dependence. On the contrary, the activation process does not seem to be altered at all, as suggested by the fact that the activation and deactivation kinetics and the voltage dependence of control and CT-treated currents were almost identical. The ionic selectivity of the sodium channel was also found to be insensitive to CT. In addition to the effects on the sodium current, CT also was found to produce an increase in the duration of the action potential (under current clamp conditions), and to elicit a transient contracture of the fiber. The first effect can be explained as the result of the partial removal of the fast inactivation mechanism. The second effect could result from an alteration of one or more steps of the excitation-contraction coupling.

The effects of CT on the fast inactivation would result from an irreversible covalent modification of the sodium channel, as they could not be reversed by extensive washing. On the basis that the fast inactivation mechanism was found insensitive to DNTB it can be suggested that the oxidation of the thioether group of the lateral chain of the methionine residues are in all likelihood the mechanism underlying the CT effects (Wang, 1984*b*). As this reaction does not involve net charge modification (Shechter et al., 1975), neither the removal nor the voltage-dependence modification of the fast inactivation rely on a nonspecific charge screening mechanism. An alternative explanation for the CT effects is based on the action of hypochlorous acid, which is known to be formed after dissolving CT in water (Rack et al., 1984). Nevertheless, when used alone, the effects of hypochlorous acid on the voltage dependence and reversal potential of the sodium current, and the steady-state inactivation differ from those of CT-containing solutions (Rack et al., 1984).

The effects of CT on fast inactivation of sodium channel from *B. marinus* muscle fibers were similar to those previously found in myelinated nerves and skeletal muscle from amphibians (Wang et al., 1987; Hahin, 1988). CT produced a pronounced deleterious effect in toad fibers, manifested as a large increase in leakage current, which prevented a complete removal of inactivation. This result was opposite to that found in invertebrate nerve fibers (Wang et al., 1985; Huang et al., 1987), and concurrent with results from myelinated fibers (Wang 1984*a*,*b*). Differences in membrane proteins or lipid composition may probably underlie the differential effect to CT on leakage current. In our hands, typi-

cally, under optimal conditions, a maximal removal of 50–60% of the inactivation process could be obtained.

The effect of CT on the steady-state inactivation was not studied before in skeletal muscle. We found a +10 mV shift of the steady-state inactivation curve for the inactivatable fraction of the CT-treated sodium current. This value was smaller, but of the same sign as that previously reported for nerve fibers (Wang, 1984*b*; Wang et al., 1985; Huang et al., 1987). As previously pointed out, this effect is unique of CT, and not found with other procedures known to remove fast inactivation (Wang, 1984*b*).

The kinetics of the inactivation process of the inactivatable fraction of CT-treated sodium current suggests that the removal of the fast inactivation by CT take place in at least two steps. The first step would lead to the slowing down of the inactivation process and the second its removal. One way to explain this is by assuming that at least two accessible methionine residues are involved in the mechanism of fast inactivation. This result, also observed in vertebrate nerves (Wang, 1984*b*), is in contrast to that reported for invertebrate nerve fibers, in which an all-or-none removal of fast inactivation was found (Wang et al., 1985; Huang et al., 1987). Differences in the number and/or accessibility of methionine residues of the sodium channels from those preparations might explain this differential effect of CT. This result indicates that the sodium channel from vertebrate skeletal muscle is more related to the sodium channel from myelinated nerves than to that from nonmyelinated nerves. The shift of the voltage dependence of the steady-state inactivation of CT-treated currents is further evidence suggesting that there is more than one methionine residue involved in the fast inactivation mechanism.

Opposite to that reported for other treatments known to remove fast inactivation from nerve fibers (Salgado, Yeh & Narahashi, 1985), we found that the removal of fast inactivation by CT was not voltage- or use-dependent. These results indicate that CT is acting at a different site from that of pronase and N-bromoacetamide (Huang et al., 1987).

In addition to its effect on fast inactivation, CT-containing solutions also showed a blocking effect on the sodium channels, which could overcome the increase in peak sodium current resulting from the partial removal of the fast inactivation, and reduce the current amplitude below the control value. Nevertheless, the final current reduction never exceeded ~20% of the control amplitude. This effect has been previously explained as the result of the interaction of the hydrophobic moiety of CT with lipids associated with the sodium channel or due to the blocking effect of side-products of CT (Rack, 1984; Huang, 1985). Nevertheless, none of this hypothesis can satisfactorily explain the irreversibility of the blocking effect.

Although our results suggest that the target of CT action are located at the extracellular side of the sodium channels, the alternative possibility cannot be ruled out because the diffusion of protonated CT molecules could occur even at the pH used (Bishop & Jennings, 1958). The slowness of diffusion from the lateral pools to the experimental pool prevented this issue to be clarified in our preparation. The determination of location of the CT target methionine residues is pivotal to challenge tridimensional models of the sodium channels. To conform to our results, an accurate model should have methionine residues exposed to the aqueous phase. Nevertheless, whether or not the location of CT target remains undefined, the fact that this reagent acts from the extracellular side makes it a valuable tool to study the behavior of the sodium channels in the absence of inactivation (Wang et al., 1987; Hahin, 1988; Cukierman, 1991). This is particularly relevant for preparations unsuited for intracellular perfusion techniques, such as small diameter nerve fibers and skeletal muscle fibers. Unfortunately, the usefulness of CT in skeletal muscle fibers is impaired by the deleterious side effects shown in this preparation.

In conclusion, our results suggest that at least two accessible methionine residues are involved in the fast inactivation mechanism of the sodium channel from toad skeletal muscle fibers.

This work was partially supported by grants CDCH-03-34-2614-97 (M.Q.), CDCH-03-10-3914-97 (M.DiF.) and CONICIT-S1-2289 (M.DiF.).

References

Bishop, E., Jennings, V.J. 1958. Titrimetric analysis with chloramine-T. I. The status of chloramine-T as a titrimetric reagent. *Talania.* **1:**197–212

Brodwick, M.S., Eaton, D.C. 1982. Chemical Modification of Excitable Membranes. Proteins in the Nervous System: Structure and Function. pp. 51–72. Alan R. Liss, New York

Catterall, W.A. 1986. Voltage dependent gating of sodium channels. Correlating structure and function. *Trend. Neurosci.* **9:**7–10

Catterall, W.A. 1988. Structure and function of voltage-sensitive ion channels. *Science* **242:**50–61

Cukierman, S. 1991. Inactivation modifiers of Na^+ currents and the gating of rat brain Na^+ channels in planar lipid membranes. *Pfluegers Arch.* **419:**514–521

Hahin, R. 1988. Removal of inactivation causes time-invariant sodium current decays. *J. Gen. Physiol.* **92:**331–350

Hille, B., Campbell, D.T. 1976. An improved Vaseline gap voltage clamp for skeletal muscle fibers. *J. Gen. Physiol.* **67:**265–293

Huang, J.M.C., Tanguy, J., Yeh, J.Z. 1987. Removal of sodium inactivation and block of sodium channels by chloramine-T in crayfish and squid giant axons. *Biophys. J.* **52:**155–163

Noda, M., Ikeda, T., Kayano, T., Susuki, H., Takeshima, H., Kurasaki,

M., Takahashi, H., Numa, S. 1986. Existence of distinct sodium channel messenger RNAs in the rat brain. *Nature* **320:**188–192

Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, K., Kangawa, K., Matsuo, H., Raftery, M.A., Hirose, T., Inayama, S., Hayashida, H., Miyata, T., Numa, S. 1984. Primary structure of the *Electrophorus electricus* sodium channel deduced from cDNA sequence. *Nature* **312:**121–127

Rack, M., Hu, S.L., Rubly, N., Waschow, C. 1984. Effects of chemical modification of amino and sulfhydryl groups on the voltage-clamped frog node of Ranvier. *Pfluegers Arch.* **400:**403–408

Rack, M., Rubly, N., Waschow, C. 1986. Effects of some chemical reagents on sodium current inactivation in myelinated nerve fibers of frog. *Biophys. J.* **50:**557–564

Salgado, B.L., Yeh, J.Z., Narahashi, T. 1985. Voltage-dependent removal of sodium inactivation by N-bromoacetamide and pronase. *Biophys. J.* **47:**567–571

Shechter, Y., Burstein, Y., Patchornik, A. 1975. Selective oxidation of methionine residues in proteins. *Biochemistry* **14:**4497–4503

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

Vergara, J., DiFranco, M., Compagnon, D., Suarez-Isla, B.A. 1991. Imaging of calcium transients in skeletal muscle fibers. *Biophys. J.* **59:**12–24

Wang, G.K. 1984*a*. Modification of sodium channel inactivation in single myelinated nerve fibers by methionine-reactive chemicals. *Biophys. J.* **46:**121–124

Wang, G.K. 1984*b*. Irreversible modification of sodium channel inactivation in toad myelinated nerve fibres by the oxidant chloramine-T. *J. Physiol.* **346:**127–141

Wang, G.K., Brodwick, M.S., Eaton, D.C. 1985. Removal of sodium channel inactivation in squid axon by the oxidant chloramine-T. *J. Gen. Physiol.* **86:**289–302

Wang, G.K., Brodwick, M.S., Eaton, D.C., Strichartz, G.R. 1987. Inhibition of sodium currents by local anesthetics in chloramine-T-treated squid axons: The role of channel activation. *J. Gen. Physiol.* **89:**645–667