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Na Activation Delays and Their Relation to Inactivation in Frog Skeletal Muscle

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Summary. Delays in the development of activation of Na currents were studied using voltage-clamped frog skeletal muscle fibers. Na currents elicited by a depolarizing voltage step from a hyperpolarized membrane potential were delayed in their activation when compared to Na currents elicited from the resting potential. The magnitude of the delay increased with larger hyperpolarizing potentials and decreased with larger depolarizing test potentials. Delays in activation observed following chloramine-T treatment that partially removes inactivation did not differ from delays observed before treatment. Longer exposures of the muscle fiber to chloramine-T led to a complete loss of inactivation, coincident with an elimination of the hyperpolarization-induced delays in activation. Steady-state slow inactivation was virtually unaffected by prolonged exposures of the fibers to chloramine-T that eliminated fast inactivation. The results show that chloramine-T acts at a number of sites to alter both activation and inactivation. Markov model simulations of the results show that chloramine-T alters fundamental time constants of the system by altering both activation and inactivation rate constants.

Key Words Na channels · skeletal muscle · kinetics · chloramine-T · electrophysiology · current inactivation

Introduction

Voltage-clamped Na currents elicited by a voltage step from a hyperpolarized membrane potential have previously been shown to be delayed in their activation in squid and *Myxicola* axons (Armstrong & Bezanilla, 1974; Hahin & Goldman, 1978) and frog node (Neumcke, Nonner & Stampfli, 1976) when compared to Na currents elicited from the resting potential. In all the studies, the magnitude of the delay increased when the membrane was held at larger hyperpolarized potentials before eliciting Na currents with a depolarization. The magnitude of the delays was also shown to increase when longer duration hyperpolarizing pulses preceded the activating pulse (Hahin & Goldman, 1978; Taylor & Bezanilla, 1983; Rayner et al., 1989). Taylor and Bezanilla (1983) also showed that Na gating currents undergo a shift or delay in their appearance which correlates with the shift in activation of Na current.

The delays are hypothesized to arise from the distribution of initial openings of Na channels. If channel openings and closings are interpreted as time- and voltage-dependent events of a Markov process, the delays can be shown to depend on the fundamental time constants (reciprocal Eigenvalues) and the initial conditions of the process. If the fundamental time constants are altered, the development of the delays would be expected to be changed.

To test this hypothesis, I have observed the delays in the activation of Na currents in normal fibers and those that have modified kinetics (altered fundamental time constants) to assay for an effect on the delays. The simplest method to modify Na channel inactivation kinetics was to eliminate inactivation. Activation kinetics were changed by using different tests pulses.

In this work I report that partial elimination of fast inactivation using chloramine-T treatment does not significantly alter the development of steady-state delays in activation. However, full removal of inactivation produced by long duration chloramine-T treatments eliminates the hyperpolarization-induced delays in activation. Also, delays in activation increase in magnitude as activation is slowed. The work demonstrates that delays in activation depend upon activation and inactivation. The results demonstrate that chloramine-T acts in a two-step irreversible manner, causing changes in both inactivation and activation of Na channels. Finally, the results show that the delays in activation and their alteration by agents provide kinetic information about both activation and inactivation and can be used in model discrimination.

Materials and Methods

Single fibers were dissected from the semitendinosis muscles of either bullfrogs (*Rana catesbeiana*) or grass frogs (*Rana pipiens*) and studied under voltage-clamp conditions using the Vaseline-

gap voltage-clamp technique (Hille & Campbell, 1976). Several changes in the original method have been employed in these experiments and have been described previously in Campbell (1983), Campbell & Hahin (1983*a*), and Hahin & Campbell (1983). These changes have reduced the series resistance to 0.5–1.5 Ωcm^2 and have functionally uncoupled the surface membrane from the transverse tubular system (Campbell & Hahin, 1983*b*; Campbell, 1986).

Pulse Generation and Data Acquisition

The methods for the generation of command pulses and the acquisition and storage of data were identical to methods previously described (Hahin, 1988). Data were sampled typically at 10 μsec per point; however, in a few experiments data were sampled at 5 μsec per point.

In most of the experiments, series resistance compensation was employed to reduce experimental artifacts induced by the flow of large currents. Over one-half (50–60%) of the series resistance was compensated for when employed. The maximum error in voltage in all the experiments was always less than 2.3 mV, and in most of the experiments the error was less than 1.5 mV. In all experiments, linear capacitive and leakage currents were subtracted using a three time constant electronic leakage subtractor.

Measurement of Delays in Activation

To measure delays, an arbitrary reference standard holding potential was chosen. In these experiments, Na currents elicited from a holding potential of −70 mV were considered to be standard Na currents and exhibited no delays in their activation. Sodium currents recorded using holding potentials that were hyperpolarized compared to −70 mV were considered to be delayed in their activation if they did not superimpose onto the standard Na current record and were delayed in their rise to a peak value. After compensating for the delays, the activation of untreated control Na currents were typically identical and independent of the holding voltage and would superimpose well when overlaid on each other (Hahin, 1989). In a few instances, (e.g., Fig. 1) the early portion of the record (activation) was delayed less than later portions (inactivation) of the record. In those cases, the superimposition method was not used. Instead a Hodgkin-Huxley fitting method was used as follows: Delays were measured using a second technique; modified Hodgkin-Huxley model fits to the data were made using an equation (*see* Fig. 1) that included a delay parameter and two time constants of inactivation (Hahin, 1989). Application of least-squares fitting procedures to the data produced estimates of the delays. Both methods produced almost identical results. For those cases where the methods differed, or when then the first method could not be used, the Hodgkin-Huxley fitting method was used instead of the superimposition method. In 23 cases where both methods could be used, the correlation between the two estimates was 0.98.

Solutions

Na currents were measured using frog Ringer solution containing (in mM): 115 NaCl, 2 KCl, 2 $CaCl_2$, and 4 HEPES. The pH was adjusted to 7.4 using NaOH. Fiber ends were cut in pools containing 115 mM CsF, 5 mM NaF, and 4 mM HEPES titrated to pH 7.4. Fibers were transiently exposed (10–15 min) to the fluoride end pool solution before immersing the A pool in Ringer solution to maximize the uncoupling of the surface membrane from the T-tubular membrane. Fibers were cut into the end pool solutions and allowed to equilibrate with the solution for at least 40 min before recording to ensure that T-system Na currents were eliminated from the records.

Test solutions contained control Ringer; a sufficient amount of freshly prepared chloramine-T was added to produce a 1–2 mM solution. After chloramine-T application, Na currents were repetitively elicited to observe the continual loss of inactivation. At various stages of inactivation loss, the chloramine-T test solution was washed out with Ringer, and then Na currents were measured to study the effect of inactivation removal on the delays in activation. In one experiment, Na current delays were recorded while the fiber was exposed to the chloramine-T test solution. All control and test solutions were precooled to 12°C before applying the solution to the experimental chamber. All experiments were conducted at 12°C.

Results

Hyperpolarizing Prepulses Delay the Activation of I_{Na}

Na currents elicited from holding potentials more negative than −70 mV are delayed in their activation. Figure 1 illustrates the appearance of a delay in the activation of Na currents by comparing I_{Na} elicited from a −90 mV (leftmost record) and a −150 mV holding voltage via a step to 10 mV. The Na currents elicited by a step from −150 mV are delayed in this experiment by about 100 μsec. In this particular experiment, the current kinetics are not simply delayed along the time axis. In order to fit the current kinetics obtained from a −150 mV holding potential using a modified Hodgkin-Huxley (1952) model prediction (*see* Fig. 1 legend) a delay of 100 μsec must be used (Hahin, 1989).

Delays in activation have been previously observed in other prepartions; this is the first report of this effect in muscle. Hahin and Goldman (1978) showed that the delay developed with a time course that depended upon the duration of a hyperpolarizing prepulse applied from the resting potential, and this effect was confirmed by Taylor and Bezanilla (1983) and Rayner, Starkus and Ruben (1989). Thus, a long duration prehyperpolarization could produce a delay that equals the shift seen by holding the membrane at this same hyperpolarized value. This effect is similarly seen in muscle (*not shown*). Prepulse hyperpolarizations greater than 50 msec always produced a steady-state delay. To maximize the effect, the membrane was held at −150 mV for more than 3 sec in between Na current-eliciting depolarizing pulses.

Figure 2 shows how the magnitude of the delay

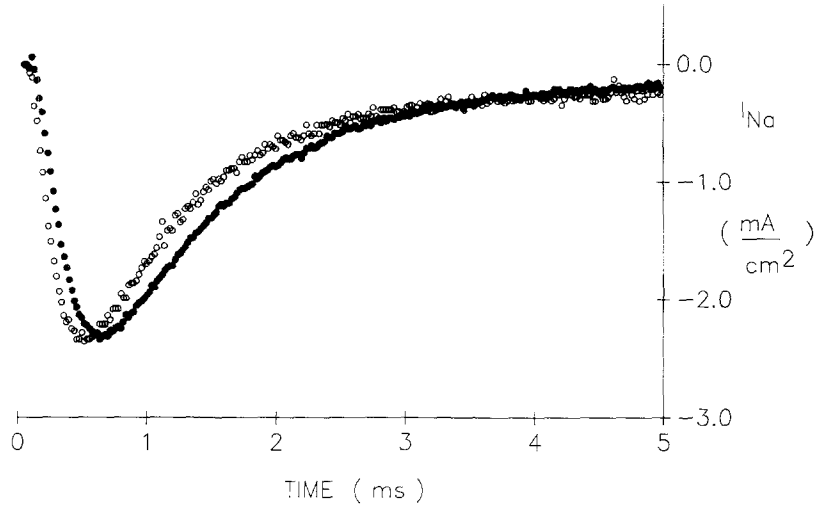

Fig. 1. Normal Na channel activation delay in muscle. Na currents were elicited from a holding potential of either −90 (open circles) or −150 mV (filled circles) via a 5-msec pulse to +10 mV. The Na current record elicited from a −90-mV holding potential was scaled by 1.4. In this instance the Na current elicited from −150 mV is delayed, but not simply shifted along the time axis. To obtain the delay, the record obtained using −150 mV was fit with the following equation:

$$I_{Na} = I_{Namax}[1 - \exp(-(t - d)/\tau_m]^3\{(F)\exp(-(t - d)/\tau_{hf}) + (1 - F)\exp(-(t - d)/\tau_{hs})\}$$

where $I_{Namax} = 4.1$ mA/cm^2, $\tau_m = 0.18$ msec, $\tau_{hf} = 1.05$ msec, $\tau_{hs} = 10$ msec, $F = 0.95$ (fraction of rapidly inactivating channels), and $d = 100$ μsec. If a delay of either 90 or 110-μsec were used, the above equation would not fit I_{Na}

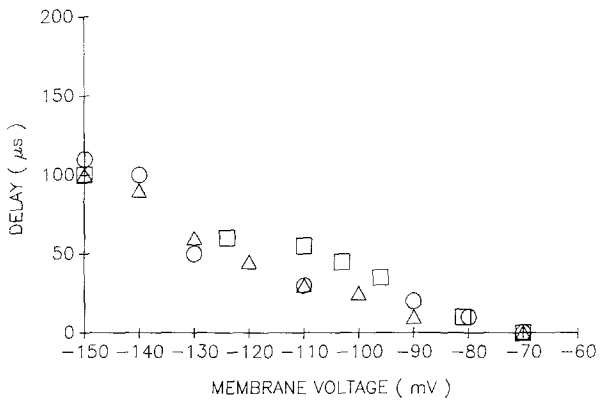

Fig. 2. Delays in the development of activation in muscle. Delays in activation were determined using two different methods that produced identical results. The symbols (triangles, squares, and circles) represent delays obtained from muscle fibers obtained from different frogs. In the first method (overlay method), delays were obtained using a comparison procedure similar to that shown in Fig. 1, except that all currents were compared to a standard current elicited by a 10-mV step from a holding potential of −70 mV. Hyperpolarizing the holding potential relative to −70 mV caused a delay to appear. The second method utilized Hodgkin-Huxley (Hodgkin & Huxley, 1952) fits to the data. The best fits to the kinetics required a particular delay to be incorporated into the equations (Hahin, 1988)

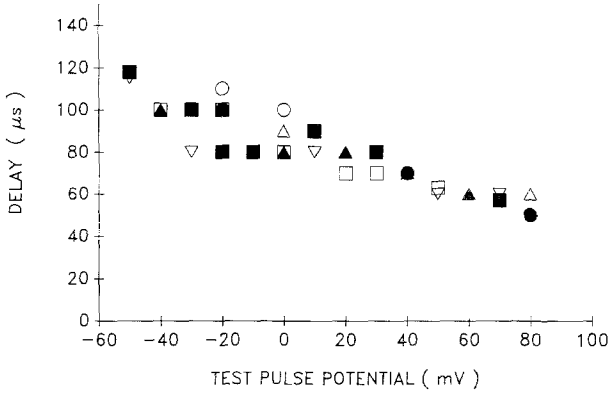

Fig. 3. Activation delays depend upon the test potential. Delays in activation were determined using the overlay method and confirmed using Hodgkin-Huxley (Hodgkin & Huxley, 1952) fits. The symbols (filled and open squares, triangles, inverted triangles, and circles) represent delays obtained from mucle fibers obtained from different frogs. Smaller depolarizing test voltages produced larger hyperpolarization-induced delays than large depolarizing test voltages at the same holding voltage

is affected by the magnitude of the holding potential used. In the figure, all delays have been arbitrarily measured relative to a −70 mV holding potential; hyperpolarizing the membrane from −70 mV produces a delay in the activation of I_{Na}. This holding potential (−70 mV) was chosen to ensure that comparisons could be made with previously reported delays in axonal preparations. The size of the delays recorded in muscle are similar in magnitude to corresponding values reported in neuronal preparations.

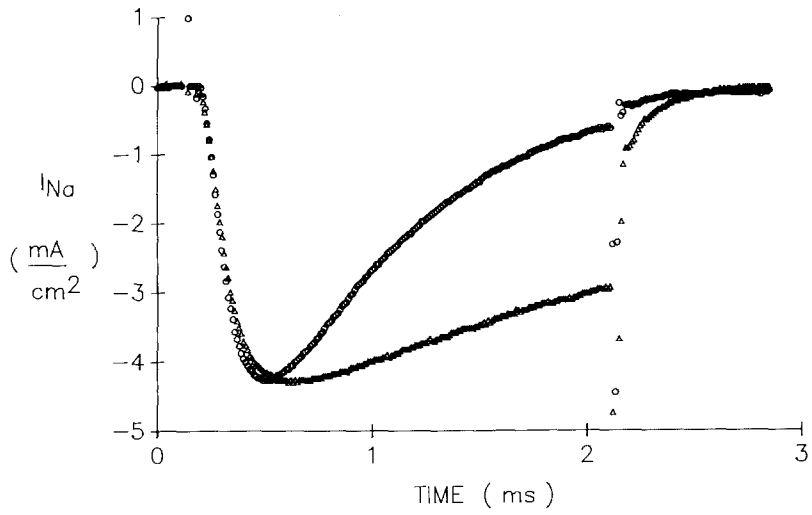

Fig. 4. Partial removal of inactivation does not affect delays. Na currents were elicited by a test pulse to −20 mV from a holding voltage of −140 mV before (circles) and after (triangles) application of 1.5 mM chloramine-T for 1 min. The post treatment current (triangles) was recorded in Ringer following a 5 chamber volume washout. Since oxidant treatment reduced the amplitude of the Na current, the treated current (triangles) was scaled by a factor of 1.14 and superimposed upon the untreated record for comparison purposes. The treated current, displaying partial inactivation removal, shows an initial activation that is coincident with normal activation. Similar results were seen at other voltages

Test Pulse Dependency of the Delays

Hahin and Goldman (1978) obtained delays using test pulses in the range of −10 to 30 mV. They did not systematically study the effect of the size of the test pulse upon the delay produced. Since the measured delay may depend upon the test pulse used to elicit the Na current, a set of experiments was undertaken to determine if the delay depended upon both the test pulse and the holding voltage used to elicit Na current. Figure 3 illustrates the results of six experiments designed to determine the delay magnitude produced by a number of test pulses from a holding potential of −140 mV. Figure 3 shows that the observed delay depends upon the test pulse used to elicit the sodium current. Small depolarizing test pulses (e.g., −50 mV) produce the largest delays. The relationship of delay *versus* voltage is relatively linear over the whole voltage range.

Partial Removal of Fast Inactivation Does Not Affect Delays

Since the delays in activation may be affected by altering both activation and inactivation, a series of experiments was conducted to determine if partial or full removal of fast inactivation would alter the measured delays.

The oxidant, chloramine-T, has been shown in frog node (Wang, 1984) and squid axon (Wang, Brodwick & Eaton, 1985) and in muscle (Hahin, 1988) to eliminate virtually all fast inactivation of Na channels at all voltages. Since chloramine-T selectively modifies methionine residues to form methionine sulfones and can be applied in a controlled way externally, it was used to modify the inactivation of muscle Na channels.

After many applications, I determined that 1–2 mM chloramine-T was an optimal dose. Doses larger than this rapidly eliminated inactivation, but usually led to large leak currents and the ultimate death of the fiber. Smaller doses produced little loss of inactivation over an interval of 10–15 min. The optimal dose produced a loss of inactivation that was large and variable over a 1–5 min interval. Most of the loss of inactivation occurred early on in the exposure; thus, solutions containing chloramine-T were washed out of the chamber once it was evident that continued exposure of the fiber to the oxidant would lead to no further loss of inactivation. Those applications of chloramine-T that produced only a partial loss of inactivation were analyzed and compared with experiments that produced virtually a full loss of inactivation.

In 6 out of 10 experiments, chloramine-T application to the fibers caused a substantial prolongation of the inactivation process, but did not completely eliminate it. One of these six experiments is illustrated in Fig. 4. In Fig. 4, the original control, sampled record exhibiting rapid inactivation is shown superimposed upon a more slowly inactivating record obtained after exposure of the fiber to 1.5 mM chloramine-T for 1 min. Both records superimpose well during the early rise to the peak but differ in the final climb to the peak; this effect was also seen at all other voltages. The delays in activation were identical. At the termination of the pulse, a comparison of the tails shows that the chloramine-T treated currents decay more slowly than the untreated currents, suggesting that the distribution of initial channel openings is little affected by the oxidant, but that channel closure is substantially altered (Hahin, 1988).

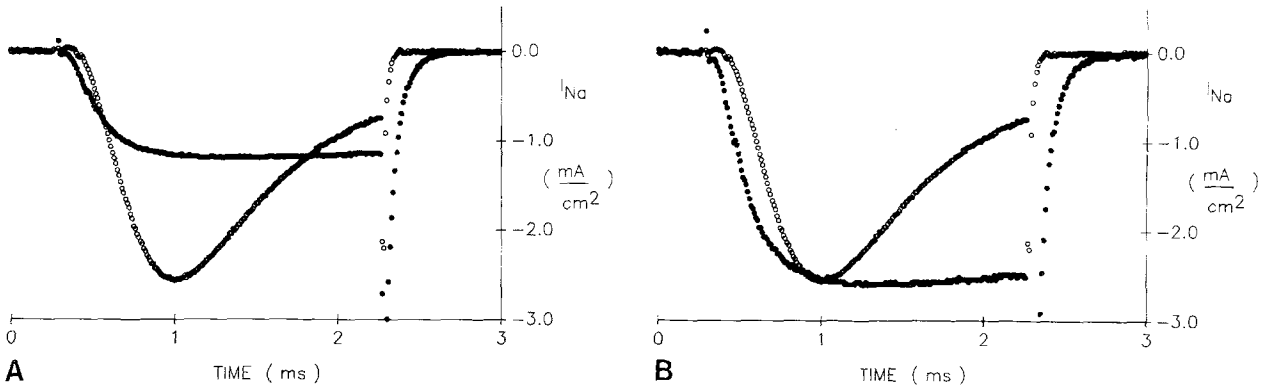

Fig. 5. Full removal of inactivation eliminates hyperpolarization-induced delays. Na currents were elicited by a test pulse to 0 mV from a holding voltage of −140 mV before (open circles) and after (filled circles) application of 1.5 mM chloramine-T for 1 min (different fiber than shown in Fig. 4). In this treatment, inactivation was completely removed from the current records, coincident with a decrease in amplitude of the current. (*A*) The untreated control current was superimposed upon the chloramine-T treated current for comparison purposes. (*B*) The treated record showing complete loss of inactivation observed in *A* was scaled by a factor of 2.2 and superimposed upon the control current for comparison purposes. In both comparisons, the chloramine-T treated records activate prior to untreated control currents. The kinetics of activation in the treated and untreated cases are similar but not identical

Full Removal of Inactivation Eliminates Activation Delays

Figure 5 shows the effect on the hyperpolarization-induced delay of a complete loss of fast inactivation. In this particular experiment, the fiber was exposed to 1.5 mM chloramine-T in Ringer for 1 min and was then washed with normal Ringer solution before current records were obtained. Inactivation was removed quickly in this experiment and produced current records that activated to a sustained current exhibiting very little decline in amplitude over the duration of the activating pulse; this effect was seen at all test voltages. Figure 5*A* shows the original control Na current superimposed upon the chloramine-T treated current; this comparison shows that there is a diminution of the amplitude of the current along with a loss of fast inactivation.

If the initial time course of the activation of the treated and untreated currents are compared in Fig. 5*A*, the hyperpolarization-induced delay seen in the control records is absent in the chloramine-T treated current. To better observe this effect, the oxidant-treated current was scaled so that its maximum coincided with the peak current of the control record and then superimposed onto the control current (Fig. 5*B*). Figure 5*B* shows that the activation of the current is quite similar to the untreated current after full removal of inactivation; however, the treated current is shifted leftward on the page compared to the untreated current. Similar results were seen in three other experiments. Thus, in all four experiments, in which chloramine-T fully removed inactivation, the hyperpolarization-induced delays were eliminated. In the five experiments in which inactivation was only partially removed, the delays were left unchanged by chloramine-T treatment. In one experiment, inactivation appeared to be only partially removed, yet the hyperpolarization-induced delays in activation were eliminated (Fig. 6).

To further test whether the delays were actually eliminated by inactivation removal, the holding voltage was changed to a number of different values, and a fixed test pulse was used to elicit currents in a fiber in which inactivation was removed. Figure 6 shows the results of one such experiment. In this particular experiment, inactivation was removed, and the currents elicited from pulses from −150 mV after treatment were not delayed in their activation when compared to similar currents elicited before oxidant treatment. Similarly, the chloramine-T treated currents evoked by a test pulse were not advanced or delayed compared to each other when the holding voltage was altered. Currents elicited from a −150-mV holding potential were found not to be delayed in their activation compared to currents elicited from a −90-mV holding potential.

Specific Modification of Methionine Residues

Since chloramine-T can potentially modify both methionine residues and sulfhydryl groups, it is possible that the oxidant can exert more than one effect on inactivation by acting at either of these sites, there are many potential sulfhydryl groups and methionine residues available for attack. To test whether sulfhydryl group modification could remove inactivation and eliminate the delays in activation, Na currents were treated with sulfhydryl-reactive reagents. In three experiments, the application of

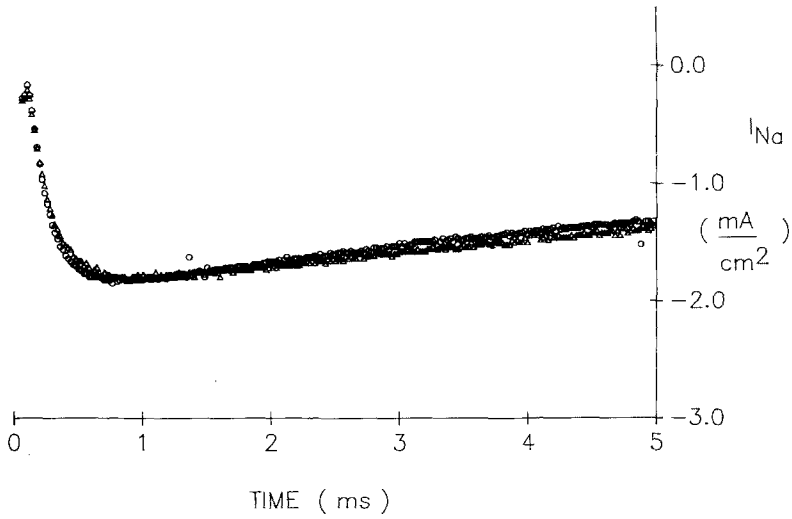

Fig. 6. Loss of hyperpolarization-induced delays after inactivation removal. Chloramine-T treated Na current records were elicited by voltage steps to 10 mV from a holding of −90 mV (circles) and −150 mV (triangles) and were superimposed. The hyperpolarization-induced delays seen (*cf.* Fig. 1) prior to treatment were eliminated by the alteration of the inactivation process. In this experiment, inactivation was not completely removed; however, the hyperpolarization-induced delays were eliminated

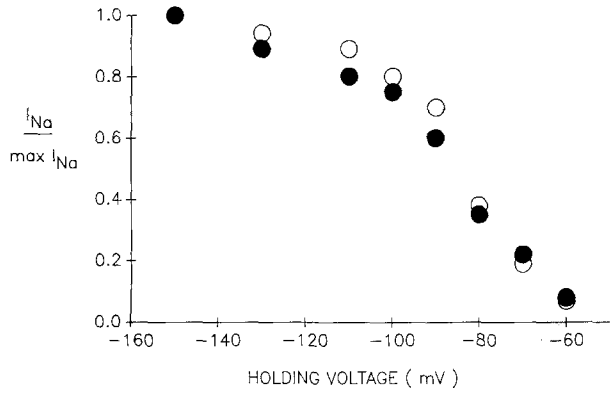

Fig. 7. Slow inactivation remains after fast inactivation loss. Open circles represent the normalized peak Na current obtained from a −20-mV test pulse from the holding voltages shown on the abscissa. All current amplitudes were normalized by dividing the current by the peak Na current obtained from a holding potential of −150 mV. Filled circles represent a similar experiment on the same fiber after removal of inactivation using 1 mM chloramine-T for 1 min

of dansyl aziridine (2–3 mM), a sulfhydryl-selective reagent, to muscle fibers produced only minor alterations of the Na channel kinetics (*not shown*) and did not alter inactivation or the delays in activation; similar results were obtained using 5-[2-(iodoacetyl)aminoethyl]aminonaphthalene-1-sulfonic acid (IAEDANS) at 1 mM. These observations suggest that sulfhydryl groups are not likely involved in the loss of either inactivation or the hyperpolarization-induced delays.

Slow Inactivation Remains Intact After Fast Inactivation Removal

In all experiments, partial or full removal of inactivation did not alter the slow inactivation process. Changing the holding potential to more depolarized values significantly reduced the current amplitude in all chloramine-T treated fibers. Figure 7 illustrates the effect of chloramine-T treatment on slow inactivation. The open circles represent normalized Na currents elicited via a 10-mV test pulse from various holding voltages plotted on the abscissa using a normal fiber. After exposing this fiber to 1 mM chloramine-T for 1 min, inactivation was almost completely removed, and slow inactivation was not much altered by the treatment (filled circles), even though activation delays were eliminated. Similar results were seen in two other experiments.

Discussion

Comparison of Results

Hyperpolarization Delays in Activation

Hyperpolarization-induced delays in activation have been previously observed in squid and *Myxicola* axons (Armstrong & Bezanilla, 1974; Hahin & Goldman, 1978), frog node (Neumcke et al., 1976), and crayfish axons (Rayner et al., 1989). The delays reported in muscle increase their magnitude as the holding potential is hyperpolarized in a manner quite similar to delays reported in all other preparations. The maximum delay observed (110 μsec) is slightly larger than similarly reported values because it is easier to hold muscle fibers at large negative holding potentials than squid axons or node of Ranvier. In contrast, shifts close to 100 μsec have been observed in *Myxicola* axons (Hahin & Goldman, 1978).

Hahin and Goldman (1978) did not test whether

the delay depended upon the test voltage used to elicit the Na current in the measurement protocol. This paper shows that the delay is nearly linearly dependent upon the test potential used.

Effects of Chloramine-T or Other Modifiers of Inactivation

All reports of the action of chloramine-T (Wang, 1984; Wang et al., 1985; Rack, Rubly & Waschow, 1986; Hahin, 1988) on the kinetics of Na channels suggest that the oxidant partially removes inactivation without affecting the activation process. Similarly reports of the action of many other modifiers of inactivation, such as N-bromoacetamide (Oxford, 1981) trypsin, papain, or ficin (Gonoi & Hille, 1987) suggest that activation was little affected or shifted in its voltage dependency. Consistent with previous results, chloramine-T acts to cause no change in the activation of muscle Na channels when inactivation is partially removed; however, prolonged exposure of Na channels typically leads to a full removal of inactivation with a consequent loss of activation delays.

Mode of Action of Chloramine-T

Previous work by Wang (1984) has suggested that chloramine-T acts to selectively oxidize methionine residues to produce the loss of inactivation. Rack et al. (1986) alternatively argues that modification of methionine residues is not responsible for the loss of inactivation, and from a further investigation of other modifiers, that modification of membrane lipids or Na channel histidine residues is the basis for the loss of inactivation. Gonoi and Hille (1987) showed that Na channels are particularly vulnerable to attack by many types of proteolytic or nonproteolytic agents which either slow or remove inactivation by acting at many sites including sulfhydryl groups at lysyl and argenyl bonds. The results of this paper suggest that chloramine-T treatment acts to remove inactivation by oxidizing methionine residues.

My working hypothesis is that a short duration exposure of the membrane to chloramine-T alters an exposed, reactive methionine to produce a partial loss of inactivation that causes no change in the distribution of initial channel openings (activation) of Na channels, but does increase the probability for channel reopening at all voltages. A prolonged exposure in some cases produces further modification of a less reactive group(s) in the channel and produces a further and more complete loss of inactivation coincident with a change in the distribution of initial opening latencies of Na channels. The actual groups oxidized can not be identified with any of the available data. Noda et al. (1984) have reported that Na channels possess 13 methionine residues; therefore; the potential for multistep modifications of these residues is great.

The hypothesis explains why chloramine-T treatment does not always lead to a complete elimination of inactivation and also a loss of the hyperpolarization-induced delays in activation. In some experiments, a brief exposure to chloramine-T produces a rapid loss of inactivation that incompletely removes inactivation; further prolongation of the treatment may prove ineffective in altering inactivation further before cell death occurs. This observation is consistent with at least two hypothesized sites of actions for chloramine-T.

Loss of Hyperpolarization-Induced Delays at Reduced Current Densities

A number of tests and precautions were undertaken to ensure that the loss of hyperpolarization-induced delays produced by chloramine-T treatment were not artifactually produced by abnormally large currents. In one experiment, chloramine-T treatment produced a full removal of inactivation and a loss of the hyperpolarization-induced delay. Application of tetrodotoxin (TTX) at 20 nM reduced the current density but did not alter the kinetics of the current record. Similarly in another experiment, reducing the current density by reducing the external Na concentration by replacement with NH_4 (75% replacement) did not alter the current kinetics. Also, a set of experiments was performed to ensure that the loss of hyperpolarization-induced delays was not dependent upon the current density: Using three chloramine-T treated fibers that exhibited virtually no inactivation, hyperpolarization-induced delays were not seen no matter which test potential (range: -40 to $+70$ mV, in steps of 10 mV) was used to elicit a Na current; each test potential produced different-sized Na currents, and each lacked a delay.

Patch-Clamp Experiments and Single-Channel Interpretations

In patch-clamp studies (McCarthy & Yeh, 1987), chloramine-T's effects on the mean open time varied widely, even within a given patch, from showing virtually no change to increasing the open time to as long as 70 msec. At the single-channel level, chloramine-T treated channels continually opened and closed during 1-sec steps to -30 mV from a hyperpolarized holding voltage and appeared to eliminate

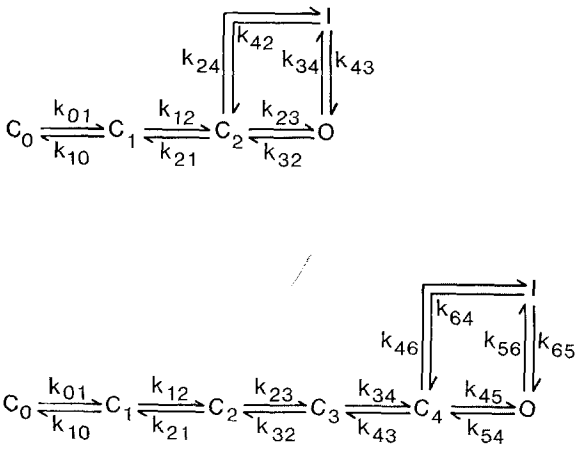

Fig. 8.

the inactivation of closed Na channels (McCarthy & Yeh, 1987).

The above results are consistent with the idea that chloramine-T acts in a multistep manner in altering gating. Chloramine-T can oxidize many methionine residues and alter both the distribution of initial opening and the probability of reopening; this could produce the variability of action of chloramine-T in the present study and in single-channel observations.

Relationship Between Activation and Delay

Delays in activation can be interpreted to represent the mean time taken for the population of Na channels to first open and contribute to the current. If the system of channels is assumed to be homogeneous and Markovian, the probability density function for the channels to remain closed until time t, when they open, given that they were originally closed, can be used to obtain a simple expression for the mean delay for the channels (Colquhoun & Hawkes, 1981). The delay, δ, is simply a linear combination of the fundamental time constants (reciprocal eigenvalues) of the system

$$\delta = a_1T_1 + a_2T_2 + \ldots + a_nT_n$$

where T_n are the time constants of the system.

The fundamental time constants of the system of channels can be modified by agents that modify inactivation or by changing the test pulse potential used to activate the channels. Changes in test pulse potential and chloramine-T induced loss of inactivation cause changes in the delays (Figs. 3 and 5). If the Na current kinetics are modeled by a Markov model in which activation is coupled to inactivation, the delays and their alteration by chloramine-T treatment can be simulated.

Markov Model Simulations

Many different Markov models could be used to simulate the results since the simulations are not unique to any particular model. Markov models containing 5 or 6 states could not be used since they did not predict the Na current kinetics elicited by depolarizing steps from holding potentials of -140 or -150 mV. In all cases the rapid rise in Na current could be reproduced, but the simulations did not possess enough delay to accurately predict the experimental records.

Alternatively, a 7-state model was used. The 7-state model produced predictions which matched the data best, and the 7-state model shown in Fig. 8 was used to simulate the results.

7-State Model Simulations

The 7-state model chosen for study was simply a modification of one of the 5-state models (Fig. 8) used by Horn and Vandenberg (1984). Figure 9 shows a simulation of a Na current record elicited by a step to -30 mV from a -150-mV holding potential. The rate constants used to simulate the data are included in the figure legend. The model transient is shown as a trace superimposed upon a voltage-clamp record. The model prediction fits the early rise of the current to the peak well, but does not fit the inactivation decay well at all. This disparity occurs because there are two types of Na channels, a majority current carrier and a minority current carrier (Hahin, 1989) and evidence for two open states (Patlak, 1989) in frog skeletal muscle. Hodgkin-Huxley model predictions produce greater discrepancies than those shown in Fig. 9, unless inactivation is assumed to have a fast and slow phase (Hahin, 1989). To better fit the records an 8-state model that includes two open states could have been used; however, the conclusions of this study do not depend upon the accuracy of the fit of the inactivation decay, therefore the 7-state model was used to reduce computation time.

Chloramine-T acts to slow inactivation and decrease the current density. A 30-fold reduction in k_{56} and a 60% decrease in the total number of channels available to open simulated the effects of chloramine-T, for the case of incomplete inactivation removal. Figure 10*A* shows the effect of the above changes on the inactivation process by comparing

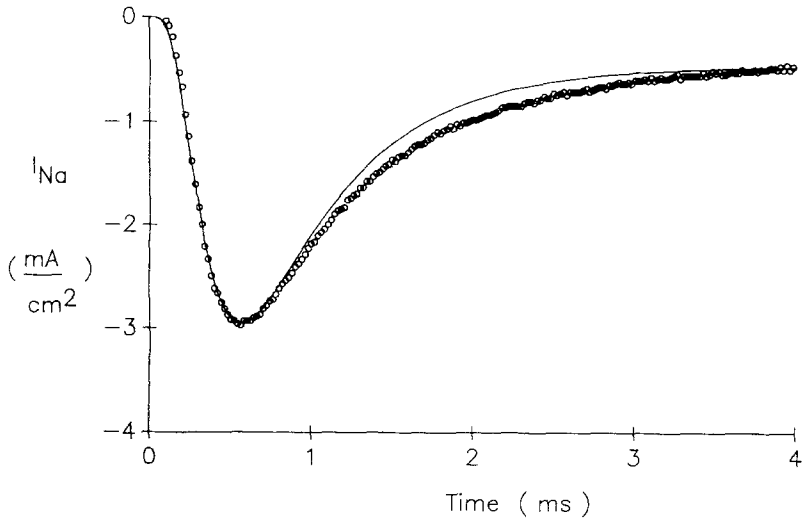

Fig. 9. Seven-state model Na current kinetic simulation. Open circles represent a Na current elicited by a −30-mV pulse from −150 mV. Superimposed upon the current record is 7-state model simulation of the current record. Inactivation displays a fast and slow component that is not predicted by the 7-state model; however, the initial delay and rise to the peak of the current are easily simulated. The rate constants used to simulate the data are (in sec^{-1}): k_{01} = 20,000, k_{10} = 2000, k_{12} = 19,000, k_{21} = 3000, k_{23} = 18,000, k_{32} = 3000, k_{43} = 2500, k_{34} = 12,000, k_{45} = 12,000, k_{54} = 70, k_{64} = 0.03, k_{46} = 50, k_{56} = 1500, while k_{65} was determined by the principle of detailed balance

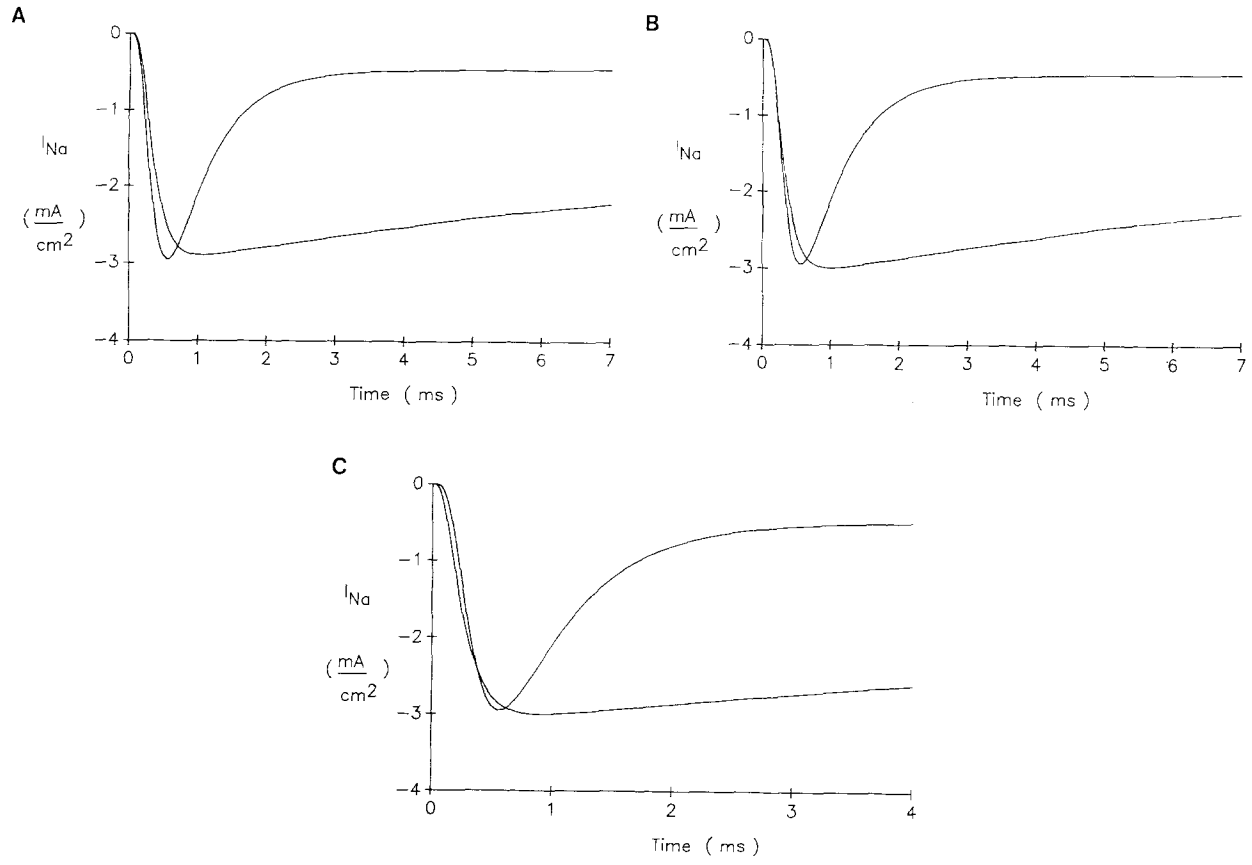

Fig. 10. Seven-state model simulations of the effects of chloramine-T treatment. (*A*) Simulates the effect of a 60% reduction in the total number of channels and a reduction of k_{56} to 50 sec^{-1} on the kinetics of Na currents. The altered current is scaled up so that its peak current is identical to a control current and superimposed upon the control current. All other rate constants except k_{65}, which changes to maintain detailed balance, have values identical to those represented in Fig. 9. (*B*) Simulates the effect of an increase of k_{01} to 50,000 sec^{-1} in addition to the changes made in *A*. Superimposition of the simulated control current onto the altered current shows that the currents are identically delayed in time, despite the fact that one current is slowed in its activation and inactivation. (*C*) Shows the effect of an increase in both k_{01} and k_{12} to 100,000 sec^{-1} in addition to a reduction of k_{56} to 20 sec^{-1}. The resulting current is shown superimposed upon the control current in *C* and simulates the effect of a prolonged chloramine-T treatment that fully eliminates inactivation

the kinetics of Na currents before and after changes were made in k_{56}. The comparison shows that a reduction in k_{56} and the number of channels slightly slows the activation kinetics and also adds a delay to it. Shifting the slowed simulated current to the left slightly would reproduce the behavior seen in experimental chloramine-T treated and control records (Fig. 4). To simulate this effect k_{01} was increased 2.5-fold (Fig. 10*B*). Systematic adjustments of individual rate constants showed that no single rate constant could be altered to simulate the effects of chloramine-T treatment. Chloramine-T slows the rate of inactivation, which introduced a delay in activation when simulated by changing k_{56} and k_{65} (*see* Fig. 10*A*). However the delay is not seen experimentally, suggesting that chloramine-T also affects the rate of activation to cause the loss of the hyperpolarization-induced delays.

In those cases where inactivation is fully removed, the reduction in the delay in activation is even greater. Figure 10*C* shows a simulation of the experimental observations. To simulate the effects, the inactivation rate constant, k_{56}, was slowed and the activation rate constants, k_{01} and k_{12}, were increased by a factor of 10. This is a conservative change in these rate constants, since a small delay remains, while in the experiments, the delay is completely eliminated.

The simulations show that chloramine-T treatment acts to affect both activation and inactivation simultaneously. Partial removal or full removal of inactivation produces effects on activation delays that can not be modeled by simply slowing inactivation (decreasing k_{56}). In all experimental cases, activation (k_{01} or k_{12}) and inactivation (k_{56} or k_{46}) rate constants must be changed to simulate the experimental results. The simulations and experiments suggest that at least two different reactive groups on the channel protein are modified to produce the loss of hyperpolarization-induced delays.

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