Removal of Inactivation Causes Time-invariant Sodium Current Decays

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ABSTRACT The kinetic properties of the closing of Na channels were studied in frog skeletal muscle to obtain information about the dependence of channel closing on the past history of the channel. Channel closing was studied in normal and modified channels. Chloramine-T was used to modify the channels so that inactivation was virtually removed. A series of depolarizing prepulse potentials was used to activate Na channels, and a −140-mV postpulse was used to monitor the closing of the channels. Unmodified channels decay via a biexponential process with time constants of 72 and 534 μs at 12°C. The observed time constants do not depend upon the potential used to activate the channels. The contribution of the slow component to the total decay increases as the activating prepulse is lengthened. After inactivation is removed, the biexponential character of the decay is retained, with no change in the magnitude of the time constants. However, increases in the duration of the activating prepulse over the range where the current is maximal 1–75 ms) produce identical biexponential decays. The presence of biexponential decays suggests that either two subtypes of Na channels are found in muscle, or Na channels can exist in one of two equally conductive states. The time-invariant decays observed suggest that channel closure does not depend upon their past history.

INTRODUCTION

If channel gating is Markovian and the channel can exist in only one open state, the dwell time of the channel in the open state should be exponentially distributed. Also, the time constant describing the rate of closing of open channels exhibiting a single open state should not depend upon the duration or amplitude of the voltage used to initially activate the channels. In essence, open channels should close identically in time and should not depend upon their past history. Early observations of the closing of Na channels were made using the method of tail currents, and produced results inconsistent with the tenets of a Markov process; Frankenhaeuser and Hodgkin (1957) recorded squid axon Na current tails in a high-Ca solution. They found a change in the apparent time constant of the current decay as a function of the duration of the depolarizing pulse used to stimulate the Na current.

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Goldman and Hahin (1978) repeated the experiments in *Myxicola* axons, and suggested that the apparent time constant previously observed arose from a biexponential decay process, and the resulting decays could be modeled by a single-open-state Markov model. Sigworth (1980) showed that Na tail currents in frog node also exhibit this effect, and suggested that a two-open-state channel may provide a better explanation. Sigworth also pointed up a problem in interpreting tail currents; with inactivation intact, Na current tails arise from the closing of previously open channels and the opening and subsequent closing of previously inactivated or closed channels. If inactivation is removed, and an activating pulse designed to open channels is followed by a return to a very hyperpolarized potential, a tail of Na current will be seen and will represent only the closing of open Na channels, assuming that inactivated channels will not reopen at −140 mV.

Chloramine-T has been shown (Wang, 1984) to eliminate inactivation in the node of Ranvier. It has been employed similarly to eliminate inactivation in frog skeletal muscle fibers to study the closing of open Na channels. Two specific questions have been addressed. First, do channels close identically, no matter how many times they have closed and reopened? Second, can the closing of Na channels be interpreted as a single-open-state Markov model? The evidence presented below suggests that there are either two open states to the Na channel or two subtypes of Na channels in muscle, and also suggests that Na channel closing is independent of the past history of the channel.

METHODS

Single fibers were dissected from the semitendinous 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 and Campbell, 1976). Several changes in the original method have been employed in these experiments and have been described previously in Campbell (1983), Campbell and Hahin (1983), and Hahin and Campbell (1983). These changes have reduced the series resistance to 0.5–1.5 $\Omega \cdot cm^2$ and have functionally uncoupled the surface membrane from the transverse-tubular system to increase the fidelity of the recording of Na currents from the surface membrane of the muscle fiber.

Pulse Generation and Data Acquisition

Voltage-clamped command pulses were generated by a fabricated digital stimulator whose timing was controlled by a Digitmer (D4030; Medical Systems Corp., Great Neck, NY). Subtraction of linear leakage and capacity currents was performed using an analog electronic transient generator. The subtracted current records were filtered using a 100-kHz filter. Current records were sampled at 10-μs intervals using a digital oscilloscope (2090, Nicolet Instrument Corp., Madison, WI) and stored on 5-1/4-in mini-diskettes for later analysis.

To eliminate the effects of long-term inactivation, fibers were held at −140 mV between pulses. In a few experiments, the holding potential was varied over the range −140 to −110 mV to test for the potential dependence of the Na current decay. In all other experiments, the membrane potential of the fiber was held at −140 mV between command pulses.

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. Since typical series resistance values, estimated from the initial "hop" in voltage resulting from a large step of current applied under

current-clamp conditions, were between 0.5 and 1.5 $\Omega \cdot cm^2$, a 3-mA/cm^2 current typically produced a 1.5-mV voltage shift with 50% compensation. With this current density and 50% compensation, the maximum error in voltage was always <2.3 mV. Current densities were always kept to <3 mA/cm^2 and were typically 1–2 mA/cm^2.

A number of control experiments were undertaken to ensure that linear capacitive current subtractions were adequate. In some experiments, tail currents were obtained by recording Na currents without electronic subtraction, followed by a point-to-point experimental subtraction of capacitive currents; capacitive currents were derived by repeating the protocols in the presence of 100 nM tetrodotoxin (TTX). Since tail current experiments are subject to errors in capacitive current subtraction, control experiments were conducted to ensure that the subtraction methods were reliable and did not contribute artifacts to the records. In addition to the above control experiments, another control experiment was used: in a few experiments designed to test whether Na current tails change as a function of the duration of the current-activating pulse after inactivation removal, no subtraction or correction for capacitive currents was made at all. Since the linear capacitive transient does not change with the duration of the pulse, it would not contribute any detectable differences in the decay. The only differences in capacitive currents between records are attributable to changes in Na or K gating currents, which are quite small compared with the linear capacitive currents. The results of these control experiments suggested that the current transients could be reliably recorded after a 50–60-μs wait. Small differences between current transients elicited by pulses of two different durations at early times (50–75 μs) during the decay were presumed to arise because of changes in gating currents. All other experiments were conducted using linear capacitive current subtraction techniques.

In many of the experiments, decays with time constants between 70 and 100 μs are reported. Currents were initially observed only after a 60-μs wait, and the time constants were determined from the decay of the currents from times after this 50–60-μs wait.

Solutions

Na currents were measured using frog Ringer solution containing: 115 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, and 4 mM HEPES. The pH was adjusted to 7.4 at 20°C using NaOH. Fibers were cut into 115 mM CsF, 5 mM NaF, and 4 mM HEPES titrated to pH 7.4. Fibers were cut into these solutions and allowed to equilibrate with the solution for at least 40 min before recording. Test solutions contained control Ringer; a sufficient amount of chloramine-T was added to produce a 1.5-mM 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.

Statistical Tests

The means reported in this article are given plus or minus the standard error, with the number of observations given in parentheses. Statistical significance was determined by applications of the double-tailed Student's t test, and the significance of differences between two means was tested using a 5% confidence interval.

RESULTS

Muscle Na Current Tails Vary with Prepulse Duration

Na currents were elicited in frog skeletal muscle fibers by a variable-duration depolarizing pulse and their decays were observed upon returning the voltage to the

holding potential. Typically, the time course of the decay of the tail of current changed as a function of the duration of the depolarizing pulse. The holding potential was kept at large negative values to ensure that the channels closed quickly. In these experiments, the holding potential was maintained at −140 mV.

In order to compare the time course of the tails of currents at two different durations, the experiment illustrated in Fig. 1 was performed. In this experiment, a +10-mV depolarizing prepulse was used to activate Na current. Two different pulse durations were used in Fig. 1 to compare the time courses of Na current decays. In the experiment, five different durations were used, but only the durations 0.7 and 1.5 ms were used to illustrate the effect in Fig. 1. Inactivation proceeded quickly

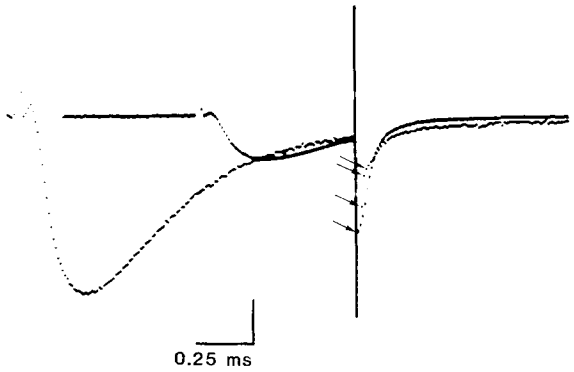

FIGURE 1. Muscle Na current tails vary with the duration of the prepulse. Shown above are two frog skeletal muscle Na currents elicited by pulses to +10 mV from a holding potential of −140 mV. Two different durations (0.7 and 1.5 ms) of pulses were used, and the records are superimposed so that the ends of the pulses coincide. The tails of Na current produced at the ends of the pulses are compared. To facilitate comparison, the Na current produced by the 1.5-ms pulse was scaled by a factor of 8 to ensure that the instantaneous Na currents following the pulse will be nearly identical. To clarify the comparison, the initial decay after the 1.5-ms pulse is initially denoted by four arrows. The arrows point to the samples of current observed at 50, 60, 70, and 80 μs after the return to −140 mV. These sampled values of current can be compared to the first four samples of current (unmarked) obtained after the 0.7-ms pulse. The values are nearly identical initially and then diverge from one another. After diverging from one another, the two traces intersect, diverge again, and eventually approach each other and merge (not shown). The vertical scale bar represents 2 mA/cm^2 for the 0.7-ms duration record and 0.25 mA/cm^2 for the 1.5-ms duration record. The Na current decay following a 1.5-ms pulse is slowed when compared with the decay following a 0.7-ms pulse. The data were obtained at 12°C.

enough that the Na current was substantially reduced after a 1.5-ms pulse. To ensure an appropriate comparison, the Na current elicited by the 1.5-ms pulse was scaled up by a factor of 8 to compensate for the diminution of the current amplitude. This scaling factor caused the Na currents to approximate one another at the termination of the two pulses. Fig. 1 shows the two Na current records superimposed so that both tail currents begin at the same time; this time is marked on the figure by a vertical cursor. The cursor also marks the end of both voltage-clamp pulses. The scaling used also equalized the magnitudes of the extrapolated instantaneous currents observed in the two tail current records.

The above scaling procedure was used to show that the two current decays are quite dissimilar. The 1.5-ms pulse produced a current decay that was definitely biexponential; there is a rapid early decay followed by a slower decay. Four arrows point to the 50-, 60-, 70-, and 80-μs samples obtained after the 1.5-ms pulse. Although the two currents have nearly identical initial amplitudes, the current produced by the 1.5-ms pulse decays quickly and then eventually intersects with the other record, slowly approaches the other current in time, and eventually merges with it. The effect was also seen by using other pairs of pulses with different durations. Observations similar to these have been reported previously by Goldman and Hahin (1978) using *Myxicola* axons, and Sigworth (1980) using frog node of Ranvier.

Using data from another experiment, illustrated in Fig. 2, decays of Na current were plotted semilogarithmically for 1- and 2-ms duration pulses. A +44-mV depolarization was used to activate Na channel opening, followed by a return to −140 mV to elicit a tail of Na current. To make a comparison of the decays, the currents were normalized at 60 μs. Normalizations initiated at somewhat later times also produced similar results, so this time was arbitrarily chosen as a starting point for the observations.

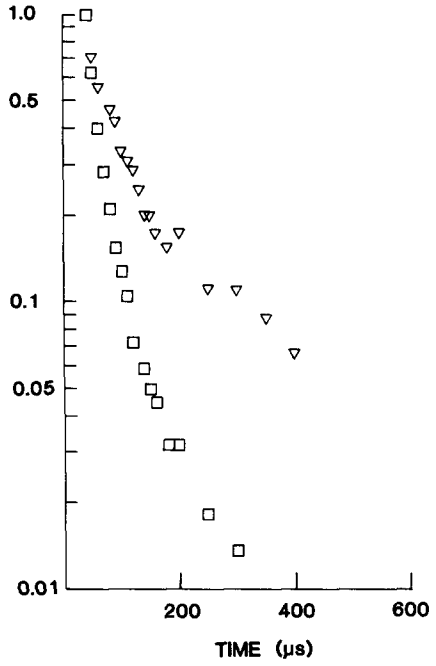

FIGURE 2. Muscle Na current tails vary with the duration of the prepulse. Data from an experiment similar to the one illustrated in Fig. 1 were used to construct a semilogarithmic plot of the decays of Na currents elicited by 1- and 2-ms pulses to 44 mV from −140 mV. The currents were normalized at 40 μs to compare the decays. If the decays are observed for only 400 μs, and the small changes in current decay are ignored thereafter, the semilogarithmic plot shows two "apparent" single-exponential decays. The 2-ms pulse produces a slower "apparent" decay. Neither decay is actually a single exponential (see Fig. 3). Data were obtained at 12°C.

Fig. 2 shows that the two pulses of different durations produce two disparate decays. If the decays are observed only for a limited period of time, they could be approximated by single-exponential decay processes and fit with time constants. If this approach were taken, the 2-ms pulse would produce a decay with a larger time constant for decay. This approach was used originally by Frankenhaeuser and Hodgkin (1957) to fit tail currents in squid giant axons. A close observation of the

records reveals that the decays are not simply exponential, but contain a second, slowly decaying component that can only be observed if the tail of current is recorded over a longer interval of time. Gilly and Armstrong (1984) have reported the existence of a similarly observable second component to the tail of current in squid axons.

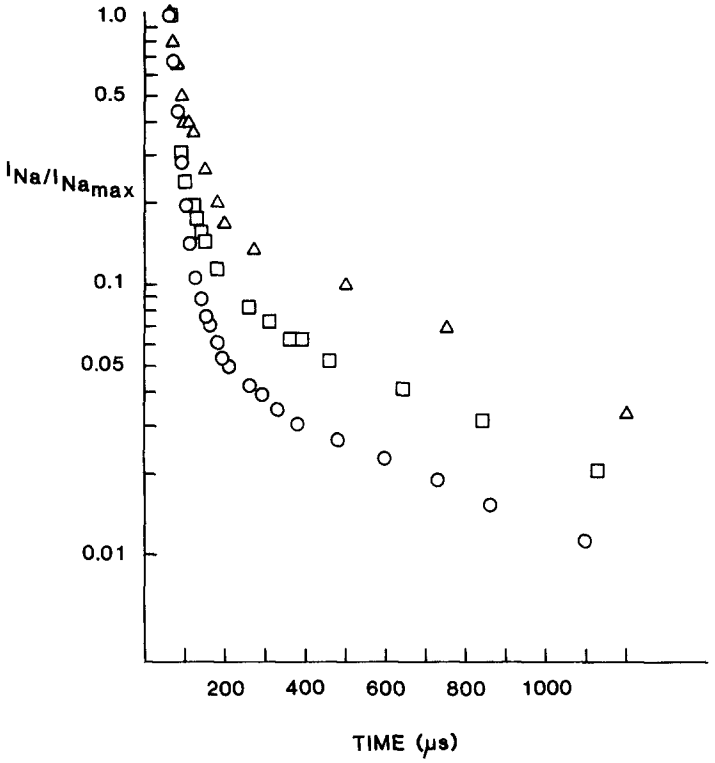

FIGURE 3. Muscle Na current tails vary with the duration of the prepulse: semilogarithmic plots reveal biexponential decays. Data from a third experiment were used to construct a semilogarithmic plot of the decays of Na currents elicited by pulses of 0.5, 1.0, and 2.0 ms to −22 mV from −140 mV. The currents were normalized at 60 μs to compare the decays. If the decays are observed for >1 ms, a small but detectable slow component of the tail of current is evident in each plot. Fitting time constants to the two exponentials for each plot shows that the fast and slow exponential time constants do not change as the duration changes (0.5-ms pulse: $\tau_f = 65$ μs, $\tau_s = 700$ μs; 1.0-ms pulse: $\tau_f = 70$ μs, $\tau_s = 690$ μs; 2.0-ms pulse: $\tau_f = 70$ μs, $\tau_s = 670$ μs). Only the initial relative amplitudes of the exponentials change, producing the slowing of the tail of current. Temperature, 12°C.

Muscle Na Current Tails Show Biexponential Decays

Fig. 3 illustrates an example of an experiment designed to record the tail of current for a longer period of time. A −22-mV test pulse was used to elicit Na currents using pulses of seven different durations. Three of the seven pulse durations used (0.5, 1.0, and 2.0 ms) are shown. The observed decays of Na currents for each pulse

duration are plotted semilogarithmically. The current decays were observed for >1 ms. In each case, a biexponential decay was seen. The same behavior was observed for all the other durations used. Tetrodotoxin eliminated both the slow and fast components of the decay.

All records decay as a biexponential decay process. The decay of currents can be represented by the following equation:

$$I_{Na} = I_{Na1} \exp(-t/\tau_1) + I_{Na2} \exp(-t/\tau_2),$$

where I_{Na1} and I_{Na2} represent the amplitudes of the two exponential components, and can be determined graphically as the zero time intercept values of the two respective exponential components. The 0.5-ms record (denoted by the circles) decays quickly because the slow exponential decay has a small amplitude, and the second rapid exponential decay has a much larger amplitude. The 2-ms record (denoted by the triangles) decays more slowly since the slow exponential component has a greater amplitude than that observed in the 0.5- and 1-ms records. Longer prepulse durations increased the amplitude of the second exponential to an even greater degree than that shown for the 2-ms pulse (not shown). Similar results were seen in eight other experiments using six other fibers.

The time constants of the two exponentials were determined by first fitting a straight line to the slower exponential, subtracting this line from the data, and then fitting the remaining data. The time constants obtained for 0.5- and 1-ms pulse durations are tabulated for nine determinations using six fibers. The mean slow time constant obtained for the 0.5-ms pulses is 529 ± 33 μs (9); this did not differ statistically from the value (518 ± 35 μs [9]) obtained for the 1-ms-duration pulses. In a number of other experiments, pulse durations of 0.7 and 1.5 ms were also used to obtain estimates of the slow time constants, and the pooled values obtained for those experiments were similar to the time constants obtained for the 0.5- and 1.0-ms pulses. All values are presented in Table I. The grand mean of all observations of the slow time constant was 534 ± 21 μs (26) at 12°C.

Similarly, the fast time constant did not vary with the duration of the pulse over the range 0.5–1.5 ms. The fast time constant obtained for the 0.5-ms pulse (69 ± 16 μs [9]) did not differ statistically from that obtained for a 1.0-ms pulse (74 ± 6 μs [9]). Other pulse durations produced fast exponential components with similar time constants. Table I shows the results for nine experiments using seven fibers. The mean of all the observations was 72 ± 3 μs (26).

These results are qualitatively similar to those first reported in *Myxicola* giant axons (Goldman and Hahin, 1978). In both cases, lengthening the duration of the activating pulse increases the amplitude of the slow exponential component relative to the fast component. The time constants (reciprocal eigenvalues) of the system do not change.

Table II provides the relative amplitudes of the slow components observed in seven control fibers. Determinations of the relative amplitude of the slow components observed for prepulse durations of 0.5, 1, and 2 ms were made. It was difficult to make accurate determinations for 2-ms pulses because the currents inactivated to such an extent that both the rapid and slow components were reduced to small values; therefore, most determinations were made using 0.5 and 1 ms. A num-

TABLE I

Fast- and Slow-Component Time Constants in Normal Frog Muscle Fibers

Duration	VT	τ_f	τ_s	Fiber
ms	*mV*	*μs*	*μs*	
0.5	−42	60	520	15A86/4
1.0	−42	60	400	15A86/4
0.5	−40	75	630	14A86/3
0.7	−40	60	530	14A86/3
1.0	−40	65	660	14A86/3
1.0	−28	70	460	25J86
0.5	−22	65	700	15A86/6
0.7	−22	70	660	15A86/6
1.0	−22	70	690	15A86/6
1.5	−22	70	685	15A86/6
2.0	−22	70	670	15A86/6
0.5	−12	50	490	14A86/3
1.0	−12	60	580	14A86/3
1.0	−12	80	460	25J86
2.0	−12	70	450	25J86
0.5	−10	40	470	14A86/1
0.5	−10	60	420	14A86/2
1.0	−10	45	520	14A86/1
1.5	−10	90	590	14A86/1
0.3	+8	75	300	14A86/2
0.5	+8	80	510	14A86/2
0.5	+8	125	410	14A86/1
0.7	+8	70	570	14A86/2
1.0	+8	100	410	14A86/1
0.5	+22	70	610	15A86/4
1.0	+22	90	480	15A86/4
Mean ± SEM		72 ± 3	534 ± 21	

TABLE II

Slow-Component Amplitudes in Tail Currents in Frog Muscle

Fiber	$A_{0.5}$	A_1	A_2	Prepulse *V*	A_2/A_1	$A_1/A_{0.5}$
15A86/3	0.002	0.009	—	−42	—	4.5
14A86/3	0.007	0.017	—	−40	–	2.4
25J86	—	0.006	0.039	−28	6.5	—
5A86/6	0.009	0.018	0.042	−22	2.3	2.0
25J86	—	0.006	0.025	−12	4.2	—
14A86/3	0.010	0.021	—	−12	—	2.1
14A86	0.020	0.036	—	−10	—	1.8
14A86	0.023	0.041	—	−10	—	1.8
14A86	0.030	0.0566	—	+8	—	1.9
14A86/2	0.033	0.065	—	+8	—	2.0
15A86/4	0.004	0.011	—	+22	—	2.8
Mean ± SEM	0.013 ± 0.003	0.026 ± 0.006	0.035 ± 0.005	4.3 ± 1.2	2.4 ± 0.3	

ber of prepulse voltages were used. The table shows that the slow component comprises 1.3% of the total current when elicited with a 0.5-ms prepulse, up to 2.6% for a 1-ms pulse, up to 3.5% for a 2-ms prepulse. The last column in the table, denoted by $A_1/A_{0.5}$ compares the magnitudes of the two slow components for 1- and 0.5-ms prepulse durations. The mean ratio is 2.4 ± 0.3, which suggests that the slow component more than doubles its amplitude as the prepulse duration increases. Only three determinations of the relative magnitude of the slow component were made at 2 ms. The voltages used were by necessity kept below 0 mV so that the inactivation rate was slow enough to ensure that a sizable tail current remained at the termination of the pulse. However, from the results, it is clear that there is still an increase in the relative amplitude of the slow component over this time range.

The two-component decays can be interpreted in a number of ways. The two most likely interpretations of the decays are: (*a*) two-component decays arise from the presence of two subtypes of Na channels, or (*b*) two-component decays arise from the presence of two open states of the Na channel. No matter which one is correct, it was important to eliminate inactivation to study the closing of Na channels.

The tail of current reflects the closing of open Na channels, the reopening of inactivated Na channels, and the opening of previously closed channels. If a very hyperpolarized pulse (−140 mV) is used to elicit the decay, the contribution to the decay from the opening of previously closed channels will be assumed to be negligible, and if inactivation is removed, the decay will reflect only the closing of open Na channels. Thus, the elimination of inactivation provides a simple method for observing channel closing. The dependence of channel closing upon the past history of the channels can then be easily studied.

Chloramine-T Removes Inactivation

The oxidant chloramine-T was used to remove inactivation in skeletal muscle fibers. The oxidant acts to modify methionine residues to form methionine sulfoxides, which are subsequently oxidized to form methionine sulfones (Lundblad and Noyes, 1984). Chloramine-T was first used externally by Wang (1984) to remove inactivation in frog node of Ranvier, and was subsequently shown to remove inactivation in squid axons (Wang et al., 1985) when applied internally to perfused fibers. The reagent acts externally in frog muscle to virtually eliminate inactivation. Fig. 4 illustrates the effect of a 10-min exposure of a single muscle fiber to 1.5 mM chloramine-T in normal frog Ringer. The top panel shows the Na current elicited by a 1.5 ms pulse to −22 mV from a holding potential of −140 mV. The lower panel shows a Na current record obtained after exposing the fiber to 1.5 mM chloramine-T, followed by a washout and a return to control Ringer. The lower record does not inactivate and has a maximum amplitude of only 40% of the original peak current. Similar results were observed in all the experiments.

Continued long-term exposure of the fiber to chloramine-T led to increases in the leak current, eventually producing cell death. The use of larger concentrations led to a more rapid loss of inactivation; however, the likelihood of obtaining a stable and viable preparation was decreased. Therefore, fibers were routinely exposed to 1–1.5 mM chloramine-T for 1—30 min, until inactivation was maximally removed,

and washed with Ringer solution; test Na current experiments were then conducted. Continued washing in Ringer did not alter the kinetics of the currents in time.

Chloramine-T Treatment Produces Time-invariant Tail Currents

After removing inactivation by chloramine-T treatment, depolarizing pulses produced Na currents that activated to a sustained plateau. Channels opened to produce the activation portion of the curve and thereafter produced a steady plateau of Na current. Single-channel studies (McCarthy and Yeh, 1987) confirmed this but showed much variability; however, in many instances, chloramine-T minimally affected the single Na channel lifetime. Thus, after a depolarizing pulse was applied, some fraction of the channels will have opened, closed, and reopened; longer pulse durations increase the likelihood that all of the channels will have opened, closed, and then reopened. The kinetics of Na currents at the termination of pulses of various durations was studied to obtain information about the dependence of channel closing on the past history of the channels.

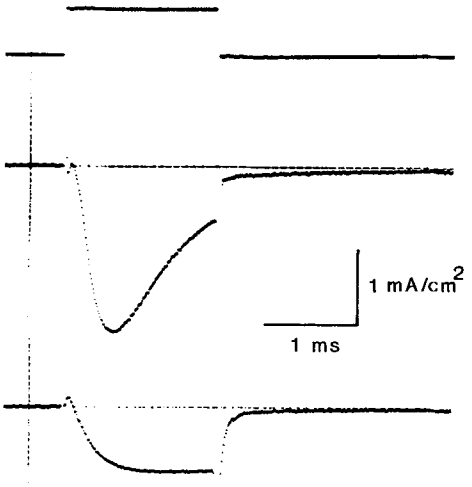

FIGURE 4. Chloramine-T eliminates inactivation. Shown above are frog skeletal muscle Na currents recorded before and after a 10-min exposure of the fiber to 1.5 mM chloramine-T in normal frog Ringer. The currents were elicited by two voltage pulses shown in the panel above the current records. A voltage pulse to −20 mV from a holding potential of −120 mV was used. The current was recorded at 12°C.

Fig. 5 shows one such experiment. In this experiment, a single fiber was pretreated with 1.5 mM chloramine-T for 10 min to eliminate inactivation. Na currents were produced by applying 3- and 4-ms pulses to −20 mV from a holding voltage of −120 mV to the fiber. The top panel of the figure shows the 3- and 4-ms pulses used to elicit the corresponding currents shown in the lower panel. The voltage and current traces are superimposed so that they terminate at the same time. The superimposition shows that both Na currents rise to an identical steady plateau value. At the termination of the pulse, the currents are virtually identical, as shown by the good superposition of the current decays. The same results were observed using other test pulse voltages and using holding voltages of −130 and −140 mV. Similar results were obtained using other pulse durations (range, 0.5–75 ms). Results similar to this were observed in 17 fibers at 12°C. The results suggest that the changes in

the time course of the tail currents observed in normally inactivating fibers appear only when inactivation is intact.

To test whether changes in the Na tail current time course are correlated with inactivation, a number of experiments were performed on fibers that were exposed to chloramine-T for shorter periods of time, so that inactivation was only partially removed. In these experiments, partial removal of inactivation produced tail current decays that still depended on the duration of the test pulse used to elicit the Na currents. However, the differences in the kinetics of the decays were reduced, and the degree of reduction depended upon the degree to which inactivation was removed.

These experiments show that the loss of inactivation nearly eliminates the changes in the kinetics of decay of Na currents observed upon increasing the duration of the depolarizing pulse used to open the Na channels. However, these experiments yield little information about the kinetics of the decay process. To obtain information

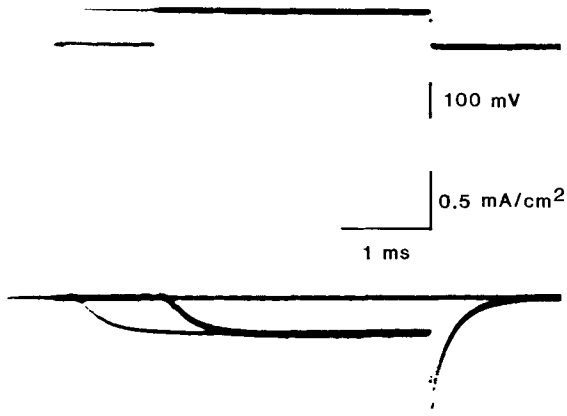

FIGURE 5. Without inactivation, Na current tails do not vary with the duration of the prepulse. Shown above are chloramine-T–treated Na currents elicited by two depolarizing pulses to −20 mV from −120 mV of duration 3 and 4 ms. The two current records are superimposed. The two pulses employed to elicit the currents are shown above the current records. At the termination of the pulses, a tail of Na current is observed. The current records are superimposed so that the two tails of currents can be compared. The two tails of current superpose well and are indistinguishable in their amplitude and time course.

about whether the basic decay processes are altered by the presence of the drugs, semilogarithmic plots of the decays were obtained.

Decay Modes for the Channels Are Unaffected by Chloramine-T

Fig. 6 show decays of Na currents plotted semilogarithmically. The data were obtained from experiments similar to those described in Fig. 2. Data were obtained from two different fibers to obtain these plots. In both experiments, the fiber was exposed to 1.5 mM chloramine-T for >15 min before washing out the oxidant and recording the currents. A 26-mV test pulse was used to elicit the current in *A*, while a 16-mV pulse was used in *B*. The decays observed after 1-ms (circles) and 2-ms (triangles) pulses are plotted in *A*; decays recorded after 1-ms (circles), 3-ms (hexagons), or 6-ms (squares) pulses are plotted in *B*.

In both fibers, the decays retain the biexponential appearance observed before

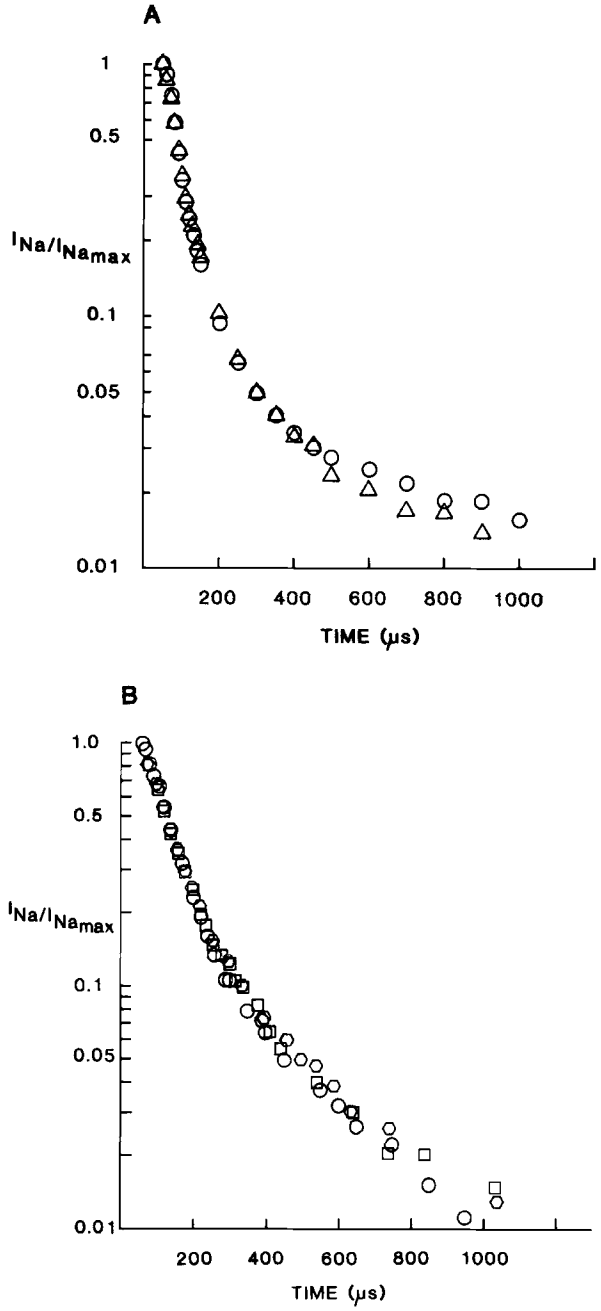

FIGURE 6. Without inactivation, Na current tails are typically biexponential. Shown above are semilogarithmic plots of Na current tails from two different experiments. 1.5 mM chloramine-T was applied in both cases to virtually eliminate inactivation. Panel *A* shows the current decays produced by 1- and 2-ms 26-mV pulses, after returning to −140 mV. Two prominent exponentials are seen (910 and 70 μs). Panel *B* shows the current decays produced by pulses of 1, 3, and 6 ms to +16 mV upon returning to −140 mV. Two exponentials are again observed (537 and 88 μs; mean of three fits). In both cases, the biexponential decay characteristic seen in Na current tails in normal fibers is retained; however, the relative amplitudes of the slow and fast exponentials are unchanged after chloramine-T treatment.

inactivation was removed. Also, as was qualitatively shown in Fig. 5, the kinetics of the decays are very similar. In other experiments, superposition of biexponential decays was seen over a wider range of prepulse durations (up to 75 ms). The method of sampling of the data precluded extending the duration to >75 ms.

Fig. 6 also shows that the slow time constants vary considerably from fiber to fiber and the relative amplitude of the slow component also varies considerably. To quantitate this effect, estimates of the time constants and relative magnitudes of the two components were obtained. Using five fibers, 48 determinations of the two time constants were made. In each case, the currents were observed at −140 mV. However, the activating pulse used to elicit the current was varied to determine whether there was a test voltage dependence to the time constants. The prepulses ranged between −42 and +48 mV. A number of durations were used to elicit the tails of current. The majority of them were either 1 or 2 ms; however, durations from 3 to 8 ms in 1-ms increments were also used. The data were analyzed using the procedure described below.

Data similar to those shown in Fig. 6 were used to extract the two time constants of decay. Over a range where the slow exponential component predominated, a single-exponential decay was drawn to the records, and the rapidly decaying component was obtained after subtracting this exponential component from the original data. Another exponential was drawn to the rapidly decaying component. Similar results were obtained using nonlinear least-squares fitting procedures. Only the current decays that had a reasonable signal-to-noise ratio during the period of the slow component were used to obtain estimates of the slow and fast components. All of the data showed two-component decays; however, some of the data displayed too much noise to be used to obtain a reasonable estimate of the slow component and were not used in the analysis.

The composite mean values for the slow and fast components were 554 ± 37 and 77 ± 3 μs for 48 determinations on five fibers. Determinations made for 1-ms pulses did not differ statistically from those made at 2 or 3 ms or greater. The relative amplitudes of the slow components compared with the total current were also tabulated for determinations made using 1- and 2-ms prepulses. Table III shows the results. To obtain the relative amplitudes, the amplitude of the zero-time intercept of the slow component was obtained and divided by the extrapolated value of the total instantaneous zero-current amplitude. This relative amplitude provides a measure of the total fraction of current carried by the slow component. Table III shows the relative amplitudes of the slow components obtained using a number of different prepulses to activate the current and the ratio of the relative slow component amplitudes for 1-ms (column A_1) and 2-ms (column A_2) durations.

The mean relative amplitude of the slow component for a 1-ms pulse (0.037 ± 0.008) does not differ from the corresponding value for a 2-ms pulse (0.037 ± 0.009). This implies that, on average, the slow component comprises only ~4% of the total current. Table III shows that changes in the relative amplitude of the slow component do occur. However, the mean value of ratio of the amplitudes, A_2/A_1, is 1.1 ± 0.09 and this demonstrates that, on average, the current decays observed are nearly identical and do not depend upon the duration of the prepulse used to elicit the current. Similar results were seen at other durations. The differences observed between records obtained at 2 ms, compared with those at durations of ≥3 ms, were always much smaller than those seen between the 1- and 2-ms records. When plotted semilogarithmically, the 2-ms records routinely superposed well on records obtained using durations ≥3 ms. These data quantitate the visual similar-

ity of the tails seen in Fig. 5. Loss of inactivation removes the changes in the relative amplitude of the slow component compared with the fast component for current tails recorded using test pulse durations of ≥1 ms.

Finally, a comparison of the fast and slow component time constants for normal fibers with chloramine-T–treated fibers reveals that there are no significant changes in the values after removal of inactivation. Untreated control fibers exhibit fast and slow time constants of 72 ± 3 (26) and 534 ± 21 μs (26) (see Table I), while fibers with inactivation removed show corresponding time constants of 77 ± 3 (48) and 554 ± 37 μs (48). Only the changes in the relative amplitude of the slow component observed as a function of the prepulse duration are lost with inactivation removal.

TABLE III

Slow-Component Amplitudes in Chloramine-T–treated Fibers

Fiber	A_1 1-ms prepulse	A_2 2-ms prepulse	Prepulse V	A_2/A_1
			mV	
19J86/2	0.034	0.029	−52	0.9
19J86/3	0.025	0.048	−52	1.9
19J86/2	0.044	0.026	−42	0.6
19J86/3	0.031	0.037	−22	1.2
19J86/2	0.030	0.036	−28	1.2
19J86	0.06	0.019	−22	1.2
19J86	0.017	0.018	−22	1.1
15A86/6	0.023	0.026	−22	1.1
19J86	0.013	0.012	−8	0.9
19J86/2	0.038	0.028	−8	0.7
19J86/2	0.15	0.15	−8	1.0
19J86	0.011	0.020	−2	1.8
19J86	0.017	0.015	−2	0.9
19J86/3	0.040	0.035	−2	1.5
19J86/2	0.076	0.104	−2	1.4
19J86	0.015	0.011	+42	1.4
19J86/2	0.022	0.011	+48	0.5
Mean ± SEM	0.037 ± 0.008	0.037 ± 0.009		1.1 ± 0.09

DISCUSSION

Biexponential Decays of Na Tails of Current

Changes in tail currents have been observed previously using squid axons by Frankenhaeuser and Hodgkin (1957). In all of their experiments, they interpreted the decay as a single exponential, and thus reported changes in an apparent time constant. Therefore, they reported an initial increase in the time constant with increases in the duration of the prepulse, followed by subsequent decreases in the apparent time constant.

Alterations in Na tail currents as a function of the duration of the prepulse were also reported (Goldman and Hahin, 1978) in *Myxicola* giant axons. Na current tails exhibited a fast and slow component; the slow component became more prominent

as the duration of the prepulse was increased. Goldman and Hahin (1978) suggested that the biexponential decays arose from the properties of the state transitions the channel undergoes upon closing, but also presented evidence for the existence of two populations of Na channels.

Mozhaeva et al. (1980) made observations of Na tail currents in frog myelinated nerve. They reported the existence of two components to the tail and showed that increases in the duration of the activating prepulse increased the relative fraction of current carried by the slow component. Upon adding venom from the scorpion *Leiurus quinquestriatus*, which slows inactivation, prepulse duration–induced changes in the amplitude of the slow component were reduced. They also reported similar results, which were not shown, with the use of sea anemone venom.

Mozhaeva et al. (1980) thus report results quite consistent with the observations reported in this article. If chloramine-T treatment is applied so that inactivation is slowed, but not eliminated, the tail currents show changes as the duration of the activating pulse is increased. If inactivation is removed, the changes in the relative amplitude of the slow and fast component are eliminated. The results of Mozhaeva et al. suggest that other agents besides chloramine-T can alter inactivation and are likely to cause the same effect. Unfortunately, most other agents slow inactivation, but do not eliminate it.

Gilly and Armstrong (1984) also reported two components to Na current tails in squid axons and suggested that two populations of Na channels (normal and threshold) produce the effects. Using a depolarizing pulse to activate Na current and a postpulse potential of −80 mV to observe the current decays, they observed the presence of a slow component to the tail of Na current. The magnitude of the slow component increased as the potential of the activating pulse increased until the voltage reached approximately −40 mV, where its magnitude was maximized. The putative threshold channels activate and inactivate; thus, a +40-mV pulse for 3 ms inactivates them sufficiently so that at the termination of the pulse, the tail of Na current is devoid of a slow component. In their experiments, the second component attributable to the threshold channels contributes ~3% of the total Na permeability. They report a ratio of slow to fast time constants of ~10 at 8°C, compared with a corresponding ratio of 7 found for muscle at 12°C.

In muscle, the second component of the tail current similarly averages ~3–4% of the total Na permeability. However, large depolarizing pulses (40–50 mV) of 2 or 3 ms in duration do not virtually eliminate the slow component. If the slow tail of current in muscle arises from the activation of a subtype of Na channel, its kinetics of inactivation differ from the threshold channels reported in squid axons. The striking dependence of the magnitude of the slow component on the prepulse potential observed in squid axons (Gilly and Armstrong, 1984) saturating with voltage at around −40 mV was not studied systematically in muscle.

Dependence of Channel Closing on Past History

In a number of kinetic or mathematical models for predicting Na channel currents or channel opening, the assumption that channels close independently of their past history is tacitly assumed. Most of the kinetic models (reviewed by French and Horn,

1983) for Na channels assume the existence of a set of discrete kinetic states and transitions between any of the states that are not time dependent. These assumptions are the tenets of a Markovian model. If channel gating is Markovian, the dwell time of the channel in a given kinetic state is exponentially distributed (French and Horn, 1983). If only one open state exists, the Markov assumption predicts that tail currents elicited with a −140-mV voltage at the termination of activating depolarizations should be single exponential and their kinetics should not depend upon the duration of the activating pulse used to elicit the currents.

Van der Kloot et al. (1979) and Cohen et al. (1981) used a linear convolution integral approach to predict the channel opening rate as a function of time during an endplate potential. A similar approach was applied to Na channels (Aldrich et al., 1983) to predict the probability that a Na channel will open at any given time during a voltage pulse. In both of these approaches, a key assumption is made: channel closing is independent of the channels' past history. These mathematical methods of predicting channel opening, as well as many of the multistate models of channel gating reported in the literature, are predicated upon this Markovian assumption. The primary purpose of the experiments reported in this article was to test this critical assumption. As will be described below, this important assumption is supported by the experimental evidence.

Na Current Tail Kinetics Change with the Prepulse Duration

In normal Ringer, Na channels activate and inactivate in response to depolarizing pulses applied to the fiber membrane. Upon termination of the depolarizing pulse at various times, the observed tail of Na current has both a fast and slow component. The amplitude of the slow component increases in response to the duration of the depolarizing pulse, while its time constant remains unchanged. These results show that the tail time course changes as the duration of the pulse used to elicit the current increases. Two alternative explanations of these effects are consistent with a Markov process.

If, as described by Gilly and Armstrong (1984), two populations of Na channels are present, then the fast and slow tails represent two independent processes exhibiting exponentially distributed channel closing. The increased amplitude of the slow component of the composite tail of Na current as the prepulse duration is increased could be explained by a differential activation of the two subtypes of channels during the prepulse. Presumably, the slow component increases slowly as a function of the duration of the prepulse, because the slow subtype of channel (threshold channel) activates slowly with membrane depolarizations.

If the Na channel has two open, conductive states, the closing of open channels observed at −140 mV would be biexponential. During the application of a depolarizing pulse, channels would open and would become distributed between the two open states. As the depolarizing pulse is lengthened, the distribution of channels between the two open states would be altered. With inactivation intact, open channels could also enter into an inactivated state. If some fraction of inactivated channels can reopen before closing, the tail of Na current would remain biexponential and the relative amplitude of the slow and fast components would be determined by the distribution of channels among the two open states and the inactivated state.

When long-duration prepulses are used, a greater fraction of channels is inactivated, and only a small fraction of these need reopen to increase the relative amplitude of the second component. These observations can be reproduced by two open-state model simulations. Single-channel observations provide further support for this hypothesis.

Patlak and Ortiz (1986) provided single-channel evidence that Na channels can inactivate and reopen; only a fraction of Na channels exhibit this behavior, observed as bursts of sequential openings, which contributes to the late Na current. Patlak et al. (1986) also observed open-time distribution heterogeneities when they studied different bursts of Na channel opening. The observations of bursting could be explained by a two-open-state kinetic model. However, Patlak and Ortiz (1986) preferred the hypothesis that Na channels undergo a change in kinetic "mode" to produce bursting.

Either a two-open-state model or two kinetically different subtypes of Na channel could describe the tail of Na current seen in normal frog muscle fibers. Both are consistent with predictions of a Markov process. In squid axons, Gilly and Armstrong (1984) show that a 3-ms pulse to 40 mV eliminates the slow component found in the tail of Na current upon returning the voltage to −80 mV. For this reason, they preferred the first description. In muscle, a long-duration strong depolarizing pulse does not eliminate the slow component; therefore, either explanation could be invoked to describe the results.

Chloramine-T Eliminates the Kinetic Changes in the Na Tails

The two-open-state prediction that the changes in the relative amplitude of the slow and fast components of the decay arise because a fraction of inactivated channels reopen can be tested. The assumption that a fraction of channels reopens and causes the above changes is reasonable, but can be obviated experimentally by using chloramine-T treatment to virtually eliminate inactivation. The kinetics of channel closing at −140 mV can be observed at various times after activating Na channels with depolarizing pulses.

The results of a series of these experiments reveals the presence of two components to the decay of the Na currents. The time constants of the fast and slow components are not significantly different from those observed with inactivation intact. If depolarizing pulses of sufficient duration and magnitude are applied so that a plateau of Na current is reached, all durations produce identical current decays. For most all the depolarizing potentials used, 1-ms pulses produced decays that were virtually indistinguishable from those produced by pulses of 2, 3, 5, 10, 20, or even 50 or 75 ms. There were no statistically significant changes in the fast or slow components. Thus, it appears that the changes in the relative amplitudes of the slow and fast components seen upon changing the prepulse duration are driven by the presence of inactivation. Once inactivation is removed, the changes are eliminated.

Evaluation of analytical solutions and simulations from equations derived from coupled activation-inactivation Markov models (Hahin, 1988) describing the behavior of single open-state channel ensembles exhibiting three, four, five, or more states show that models with a single open state fail to reproduce the kinetic behavior observed.

The experimental results confirm that the biexponential character of the decay of Na currents is not altered after removing inactivation. The characteristic time constants of the system are similarly unaffected. Regardless of whether the results are interpreted in terms of two populations of single open-state channels, or a single population of two-state channels, the changes in the tail currents seen as a function of the duration of the prepulse are produced by the presence of inactivation. The biexponential decay that is observed can be predicted to arise as a consequence of channels closing in a Markovian way.

Single-Channel Experiments and Implications

McCarthy and Yeh (1987) applied chloramine-T to excised inside-out patches from mouse neuroblastoma cells to remove inactivation. Chloramine-T acted to cause a continuous opening and closing of Na channels during 1-s-long depolarizations to −30 mV. They found a great variability on the effects on mean open time within particular patches. In a number of cases, no changes in mean open time were seen; however, some patches showed increases to a maximum change of a 70-ms mean open time at −30 mV. These single-channel results suggest that the plateau of Na current seen in frog muscle arises from a steady state equality of the opening and closing rate of Na channels.

In the chloramine-T–treated fibers, depolarizing potentials activate Na channels to a maximum value. Subsequent experiments with longer durations cause a greater fraction of the channels to open and reopen during the pulse. Long durations of ~50–70 ms to +40–50 mV will cause almost all of the channels to open and reopen at least once. Some of the channels will have opened and reopened a number of times. The decays observed for these pulses are not significantly different than those seen for 1-ms-duration pulses. These experimental results are consistent with the predictions of a Markov process. Channel closure is not dependent upon the past history of the channel.

Concluding Remarks: Two Open States or Two Different Channels

In the vast majority of the experiments, prepulse potentials and durations were chosen to ensure that the tails of current were observed after the peak of Na current had occurred. This was done to focus on the relationship between inactivation and the kinetics of the tails of current. The results can be interpreted to arise either from a two-open-state channel or two subtypes of channels.

If a two-open-state channel model is used, the results suggest that activating pulses that are long enough to maximize the Na current distribute channels between the two possible open states in a constant ratio. Further increases in the duration of the activating pulse will not change the relative distributional ratio of open channels.

If a model with two single-open-state channels is used to describe the data, then some restraints are placed upon the density of the two channels and their closing rates to produce the biexponential decays. Chloramine-T treatment also must eliminate inactivation in both types of channels; prepulses greater in duration than that used to maximize the Na current activate the two different channel types, so that

there is a constant fraction of slow closing channels compared with fast closing channels.

The present experiments do not provide a definitive clue that would allow us to discard one of the models. However, no matter which possibility proves to best describe the results, channels appear to close independently of their past history.

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