

# Slow Currents Through Single Sodium Channels of the Adult Rat Heart

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**ABSTRACT** The currents through single Na<sup>+</sup> channels from the sarcolemma of ventricular cells dissociated from adult rat hearts were studied using the patch-clamp technique. All patches had several Na<sup>+</sup> channels; most had 5–10, while some had up to 50 channels. At 10°C, the conductance of the channel was 9.8 pS. The mean current for sets of many identical pulses inactivated exponentially with a time constant of  $1.7 \pm 0.6$  ms at –40 mV. Careful examination of the mean currents revealed a small, slow component of inactivation at pulse potentials ranging from –60 to –30 mV. The time constant of the slow component was between 8 and 14 ms. The channels that caused the slow component had the same conductance and reversal potential as the fast Na<sup>+</sup> currents and were blocked by tetrodotoxin. The slow currents appear to have been caused by repeated openings of one or more channels. The holding potential influenced the frequency with which such channel reopening occurred. The slow component was prominent during pulses from a holding potential of –100 mV, while it was very small during pulses from –140 mV. Ultraslow currents through the Na<sup>+</sup> channel were observed occasionally in patches that had large numbers of channels. They consisted of bursts of 10 or more sequential openings of a single channel and lasted for up to 150 ms. We conclude that the single channel data cannot be explained by standard models, even those that have two inactivated states or two open states of the channel. Our results suggest that Na<sup>+</sup> channels can function in several different “modes,” each with a different inactivation rate.

## INTRODUCTION

Na<sup>+</sup> currents give rise to the fast initial depolarization of the cardiac action potential. They are very similar to the Na<sup>+</sup> currents in nerve and skeletal muscle: their voltage and time dependence are essentially identical (Ebihara and Johnson, 1980), and they are blocked by tetrodotoxin (Baer et al., 1976). Neither cardiac nor nerve Na<sup>+</sup> currents are exactly described by the classic Hodgkin-Huxley (1952) model. Chandler and Meves (1970) and Bezanilla and Armstrong (1977) have shown that steady Na<sup>+</sup> currents flow at large depolarizations in the squid axon, and models must include a second open state of the Na<sup>+</sup> channel to

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describe the data. Inactivation kinetics of  $\text{Na}^+$  currents have multiple time constants in the node of Ranvier (Chiu, 1977; Sigworth, 1981), and the onset of inactivation is preceded by a short delay (Bezanilla and Armstrong, 1977; Goldman and Hahn, 1978); both characteristics contradict the Hodgkin-Huxley theory, which predicts monoexponential inactivation without a delay in its onset.

Several of these phenomena have also been shown in cardiac muscle. Reuter (1968) and Dudel and Rudel (1970) have shown that  $\text{Na}^+$  current inactivation in Purkinje fibers has more than one time constant. Brown et al. (1981) have confirmed this result using single dissociated cardiac cells. Very slow and steady  $\text{Na}^+$  currents appear to flow as well, as indicated by tetrodotoxin (TTX) blockage of one component of the plateau current (Attwell et al., 1979; Carmeliet, 1984). While such steady currents could be explained by an overlap between the steady state activation and inactivation curves (referred to as a "window" current), the existence of an overlap large enough to explain the observed currents remains controversial (Colatsky and Tsien, 1979).

The significance of observed differences between cardiac  $\text{Na}^+$  currents and the predictions of the Hodgkin-Huxley theory has been questioned, however, because of the limitations of cardiac voltage-clamp methods. Complex tissue geometry prevents fast voltage-clamp recordings, and artifacts may arise from complex membrane capacitance, series resistance, and ion accumulation. Since not all of the slow currents in cardiac muscle have been clearly identified,  $\text{Na}^+$  currents are usually distinguished from other currents by their sensitivity to TTX. The measurement of small, slow components thus depends on the assumption that TTX has no secondary effects.

We report here the results of an investigation of cardiac  $\text{Na}^+$  current using the patch-clamp technique (Sakmann and Neher, 1983) on dissociated adult rat ventricular cells. This method permitted recording from small numbers of individual  $\text{Na}^+$  channels within a membrane patch with an area of several square microns. This recording method is particularly useful for the measurement of slow  $\text{Na}^+$  currents because it allows direct observation of the underlying single channel currents, thus removing doubts as to their origin. We observed both slowly inactivating and noninactivating  $\text{Na}^+$  currents in patches that had between 4 and 50  $\text{Na}^+$  channels. Our results are compared with the predictions of several theories that have been used to account for slow and steady  $\text{Na}^+$  channel currents.

#### METHODS

All experiments were performed on 200–300-g, adult, female Sprague-Dawley rats. After the animals were anesthetized with ether or pentobarbital, the hearts were removed and rapidly mounted on a Langendorf-type perfusion apparatus. Cell dissociations were performed with a protocol very similar to that used by Isenberg and Kloeckner (1982), using 0.1% collagenase (type I, Worthington Biochemical Corp., Freehold, NJ) and 0.1% hyaluronidase (Boehringer Mannheim Biochemicals, Indianapolis, IN). After dissociation and centrifugation, the cells were stored in (mM): 85 KCl, 30  $\text{K}_2\text{PO}_4$ , 5  $\text{MgSO}_4$ , 5 K-pyruvate, 5 K-succinate, 20 taurine, 5  $\text{Na}_2\text{ATP}$ , 5 creatine, 20 glucose, 5% dextran T-500, 40  $\mu\text{M}$  EGTA, and 10 HEPES, pH 7.4 (KB medium).

The dissociated cells were transferred to a 1-ml, glass-bottomed chamber that was mounted on the stage of a Zeiss inverted microscope with Nomarski optics. After the cells

had settled, they were perfused with fresh KB medium. Patch recordings were made in KB medium, which had two important advantages: (a) the cells were quiescent, without spontaneous beats or propagated waves of contraction; this allowed long, stable recordings from individual patches; (b) the resting potential of the cell was near 0 mV. This small, unchanging resting potential permitted accurate control of the transmembrane potential of the membrane patch (resting potential minus patch electrode potential). The chemical components of the KB medium did not interfere with the formation of gigaohm seals when the electrode was filled with normal saline solutions.

Patch electrodes were made from Corning (Corning, NY) type 7052 glass, which had excellent electrical and sealing properties (Rae and Levis, 1983). The electrodes were filled with a HEPES-buffered saline solution that contained (mM): 140 NaCl, 5 KCl, 1.8 CaCl<sub>2</sub>, 5 MgCl<sub>2</sub>, 10 glucose, and 10 HEPES, pH 7.4. Perfusion of the cells was stopped during recordings. The bath was cooled with a Peltier element mounted near the chamber. The temperature was measured with a small thermocouple element near the recording electrode at the bottom of the chamber. The temperature was controlled with a micro-computer, which sampled the temperature periodically and determined output levels for the Peltier cooler. Measurement and control of the temperature were accurate to 0.5°C. Recordings were made at 10°C.

The recording electronics were standard (Hamill et al., 1981). The patch signal was filtered with an analog Bessel filter set to 2 kHz and sampled by computer at 5 kHz. In some experiments, the patch currents were sampled at 1 kHz and filtered at 500 Hz to permit recording of slow currents. Records consisting of 256 time points were stored for subsequent analysis. For each test pulse potential, the averaged response to 48 pulses of equal and opposite amplitude was also stored and used for transient cancellation. During data analysis, the data were further filtered to 1 kHz with a digital filter that had Gaussian characteristics (Colquhoun and Sigworth, 1983).

Mean currents were determined by averaging the current signals at each time point over the "ensemble" (a set of identical pulses given sequentially) of 100–300 pulses. Ensembles were checked for a slow decline in the magnitude of the current ("rundown") by plotting the current after 1 ms, which was near the peak response, against the pulse number. Ensembles that had measurable rundown were not used for analysis.

For analyses that fitted exponential curves to the mean current, the accurate determination of the zero-current baseline was very important. The baseline was determined by inspecting the individual current traces within the ensemble and averaging only those that had no channel openings during the last 6 ms of the test pulse. The baseline was taken to be the average of all the points within this interval, and it was accurate to 0.001 pA.

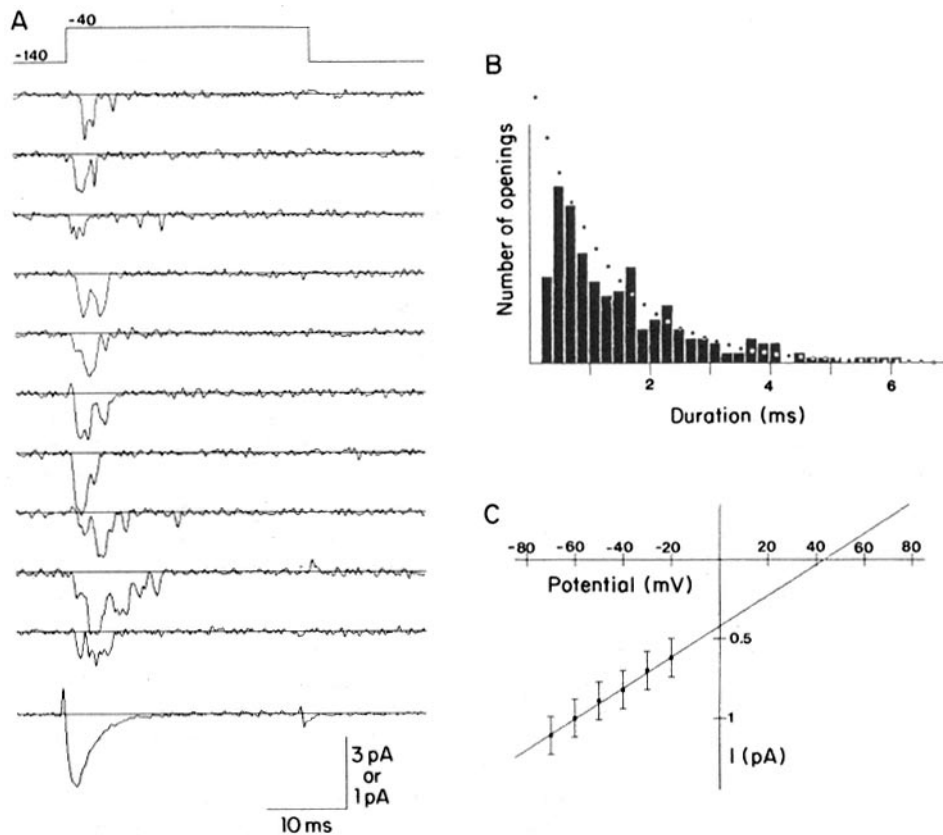
Time constants for the inactivation process were determined by fitting one exponential or the sum of two exponentials to the falling component of the mean currents. A general-purpose, nonlinear, least-squares fitting routine was used to determine the best fit and its standard error in each case. The squared deviations between observed and calculated points were weighted by the inverse square of the observed values (Bevington, 1969). The level of significance for fitting the same data with two rather than one exponential was determined using the  $F_2$  test (Bevington, 1969).

## RESULTS

### *Single Na<sup>+</sup> Channel Properties*

Na<sup>+</sup> channels were present in almost every patch studied. In 29 of these patches, we obtained stable recordings consisting of at least 300 pulses. The results presented here are from those recordings. Sequential traces from one patch are

shown in Fig. 1A. The single channel amplitude was 0.8 pA during the test pulse to  $-40$  mV. The  $\text{Na}^+$  channel's time-dependent behavior after the start of the test pulse can best be summarized by averaging the response to many identical



**FIGURE 1.**  $\text{Na}^+$  channels from rat heart. (A) 10 sequential traces from a patch with relatively few (4–5)  $\text{Na}^+$  channels. The patch, in the “cell-attached” configuration, was held at  $-140$  mV for 1 s between pulses to  $-40$  mV. The temperature was  $10^\circ\text{C}$ . The straight lines are the zero-current levels as determined from the entire ensemble. Below is the average of all 161 pulses in this ensemble. Note the different amplitude scales for the individual pulses (3 pA) and the mean current (1 pA). (B) Open time histogram from the same pulse ensemble. Only open times of single channel currents for which both the beginning and the end could be determined unambiguously within  $200 \mu\text{s}$  (the bin width) were included. The histogram was fitted with a single-exponential curve using a nonlinear least-squares algorithm to determine the best value for the time constant. The curve shown is the best-fitted single exponential, with a time constant of 1.2 ms. (C) Single channel current amplitudes at several pulse potentials from another patch. Amplitudes were determined by averaging up to 10 time points (where possible) before and after a channel opening or closing. Each amplitude is the average of 40–60 such measurements within an ensemble. Standard deviations for the individual measurements are indicated for each point. The slope of the best-fitted straight line (linear regression) is 9.8 pS. The zero-current intercept is  $+43$  mV.

test pulses. The curve at the bottom of Fig. 1A is the mean of the 161 pulses in this ensemble. After reaching the peak, the current's decay caused by inactivation is dominated by a single-exponential component. The time constant for the inactivation of the mean currents in this patch was 2.4 ms. We observed considerable patch-to-patch variation in the inactivation time constant. The average time constant was  $1.72 \pm 0.6$  ms for 16 separate estimates of the predominant component of inactivation at a pulse potential of  $-40$  mV.

Most patches had between 4 and 10  $\text{Na}^+$  channels, and some patches had up to 50. We did not observe any patches that had only one channel. The number of channels in the patches was estimated in two ways: (a) maximum likelihood analyses of the frequency of occurrence of 0, 1, 2 . . .  $N$  simultaneously open channels (Patlak and Horn, 1982); (b) ensemble fluctuation analysis (Sigworth, 1980). Neither method was particularly sensitive, and neither held for heterogeneous populations of channels. However, these methods were generally self-consistent for any one patch and thus allowed rough estimates ( $\pm 50\%$ ) of the number of channels.

The patch illustrated in Fig. 1 had four or five channels. Since most of the channel openings occurred simultaneously, the duration of the channel's open time could not be determined rigorously. However, approximately one-third of the opening events were observed without overlapping channel currents. While the open times thus sampled are biased because long openings have a higher probability of overlapping, the procedure gives a general indication of the channel kinetics. The histogram of channel open times thus measured is shown in Fig. 1B. The open time histograms was fitted with a single exponential that had a time constant of 1.2 ms. (The actual mean open time would have been somewhat longer because of the bias discussed above.)

Single channel amplitudes decreased linearly as the test pulse potentials were more depolarized. Fig. 1C shows the mean values of the single channel currents (and standard deviation bars) during pulses to potentials ranging from  $-60$  to  $-20$  mV. The slope of the line fitted to these points with linear regression is 9.8 pS. Its intercept is  $+43$  mV.

Mean currents from one patch with a large number of  $\text{Na}^+$  channels are shown in Fig. 2A. The average currents from ensembles of 138 pulses were quite smooth because they represent the activity of thousands of channel openings. Fig. 2A shows the family of curves that was obtained by test pulses of various amplitudes from a holding potential of  $-120$  mV. This family of currents shows the basic features of the kinetic behavior of  $\text{Na}^+$  currents in cardiac muscle—a gradual increase in current amplitude at potentials more positive than  $-70$  mV, and an increasing rate of activation and inactivation with greater depolarization.

The holding potentials that were necessary in this preparation to remove steady state inactivation and the potentials that caused channel activation were shifted in the hyperpolarized direction from their physiological levels. In most preparations, steady state inactivation was nearly complete at  $-80$  mV; it was completely removed by holding potentials more negative than  $-120$  mV. Hyperpolarized shifts of the steady state inactivation curve have also been seen in cardiac Purkinje fibers that had been cooled (Dudel and Rudel, 1970). Further characterization of this phenomenon is beyond the scope of this paper.

*Two Components of Inactivation*

All of the patches that we studied had late channel openings that could not be explained by a single-exponential decay of the inactivation process. These late openings appeared to be a reflection of a slowly inactivating component of

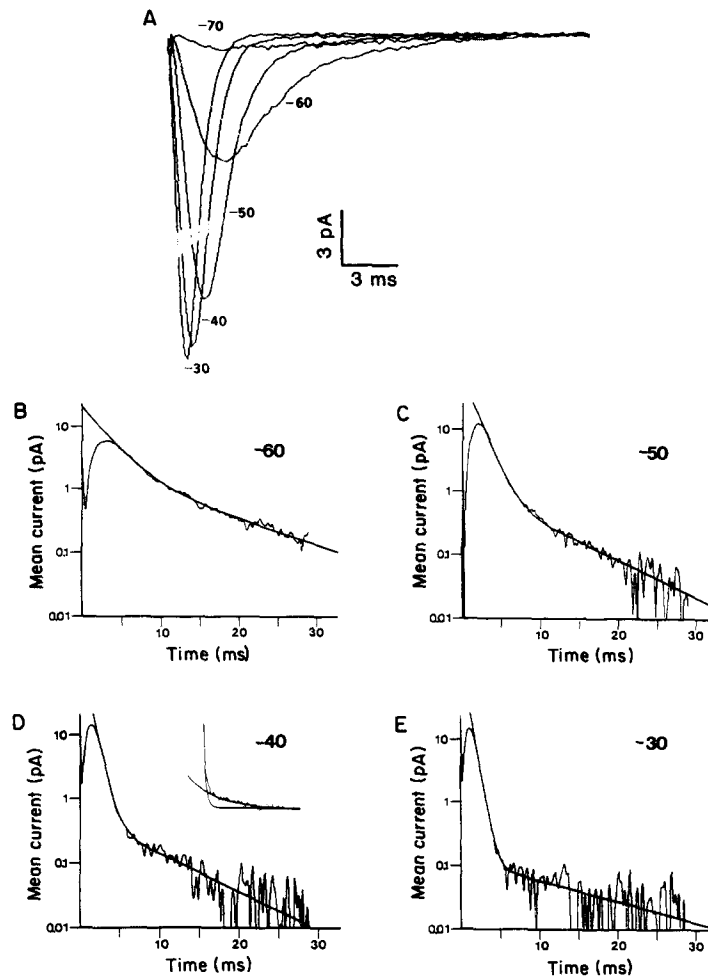


FIGURE 2. Mean currents through the  $\text{Na}^+$  channel. (A) A family of average currents from a patch. Each trace is the average of 138 individual pulses that were given once per second from a holding potential of  $-120$  mV. The membrane potential during the pulse is given next to each trace. This patch had an estimated 50 channels. (B–E) Four of the mean currents shown in A are plotted on semilog axes. The pulse potential is given with each plot. The zero-current level was determined independently by averaging segments of the pulse currents where no channels were open. Values of the mean current that were less than zero are plotted just beneath the abscissa. The solid curves are the best-fitted sum of two exponentials (fitted to the actual data, not to the semilog plots). Values for the curves are given in Table I. The inset in D is the linear plot of the data and fitted curves at  $-40$  mV. The calibration bars in A have the values of 1 pA and 15 ms for this inset.

current. The exponential form of the slow inactivation could be resolved in patches, like that shown in Fig. 2A, with large numbers of channels. The inactivating phase of these currents for test pulses between  $-60$  and  $-30$  mV could not be fitted with a single exponential. Semilog plots of the mean current vs. time have two clear linear segments (Fig. 2, B-E).

Table I shows the results of curve fits to the mean currents that are shown in Fig. 2. The fits were made to the original data (not to the semilog transforms illustrated in Fig. 2), as indicated by the inset of Fig. 2D, which shows the original data at high gain and the two best-fitting exponential curves. The zero-current baseline was determined independently by measuring the current level found during individual pulses when no channels were open (see Methods). The use of

TABLE I  
*Best-fitted Time Constants and Zero-Time Amplitudes of the Mean Currents in Fig. 2*

$V_m$	$\tau_f$	$A_f$	$\tau_s$	$A_s$
mV	ms	pA	ms	pA
-70	$14.8 \pm 0.3$	1.5	—	—
-60	$2.7 \pm 0.2$	22.8	$10.6 \pm 0.5$	2.4
-50	$1.3 \pm 0.1$	91	$7.4 \pm 0.3$	1.3
-40	$0.75 \pm 0.05$	231	$7.1 \pm 0.5$	0.62
-30	$0.60 \pm 0.03$	251	$13.9 \pm 1.9$	0.13
-20	$0.52 \pm 0.03$	218	—	—

$V_m$  = pulse potential.

$\tau_f$  = fast time constant of inactivation ( $\pm$  SE).

$\tau_s$  = slow time constant of inactivation ( $\pm$  SE).

$A_f$  = amplitude of fast component at zero time.

$A_s$  = amplitude of slow component at zero time.

two exponentials improved the fits significantly ( $P \ll 0.1\%$ ) at pulse potentials between  $-60$  and  $-30$  mV. No significant improvement was obtained using two exponentials at  $-70$  mV, and at  $-20$  mV the slow component was too small to be resolved. Fast inactivation became progressively faster as the pulse potential became more positive, as expected for  $\text{Na}^+$  channels. Our measurements were not sufficiently precise to resolve potential-dependent changes in the time constant of the slow component, although the amplitude of this component clearly declined at more depolarized potentials.

#### *Slow Component Caused by Reopening of $\text{Na}^+$ Channels*

The single channel currents that underlie the slow component can be seen in individual traces, as shown in Fig. 3A. The amplitude of these late openings was also 0.8 pA at  $-40$  mV, and the slope conductance was  $\sim 10$  pS. Both the slow and the fast currents were blocked by  $10^{-5}$  g/ml TTX (three experiments), and they were eliminated by depolarizing the holding potential to  $-80$  mV, which caused steady state inactivation of virtually all current in our recordings.

The late openings are due either to the reopening of channels that were active earlier in the pulse, or to channels that waited  $>10$  ms to open for the first time. Although we cannot yet distinguish quantitatively between these two possibilities,

many of the late currents are caused by a sequential series of channel openings, with fewer overlapping channel currents than would be expected if the openings were from different, independent channels. We conclude that the slow currents are caused by channels that reopen late in the pulse.

#### *Influence of Holding Potential on Pulse Kinetics*

Holding potentials sufficiently negative to remove resting inactivation of the Na<sup>+</sup> channel (−100 mV in our preparation) affected the magnitude of the fast and

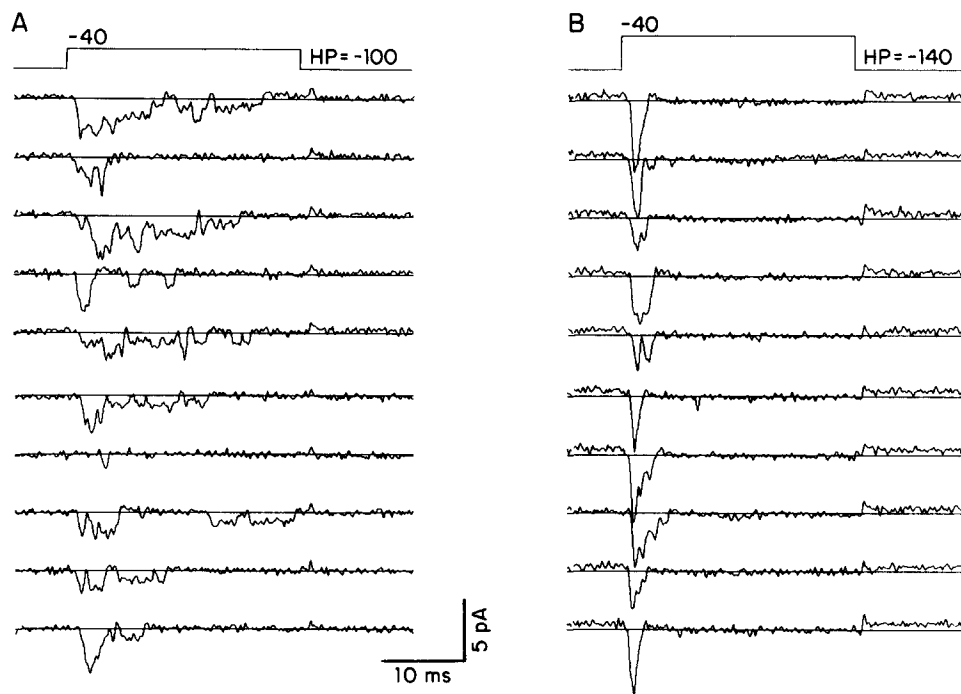


FIGURE 3. Holding potential modifies channel currents during pulse. (A) 10 sequential pulses from a holding potential −100 mV to a −40-mV pulse potential. (B) 10 sequential pulses from a holding potential −140 mV to a pulse potential −40 mV.

the slow component of inactivation. As the holding potential was made more negative, the amplitude of the fast component increased, as would be expected if resting inactivation were being removed. However, the amplitude of the slow component initially increased, but then decreased again when the membrane potential was made more negative than −100 mV. These changes are evident in the currents elicited by individual pulses, as shown in parts A and B of Fig. 3, which are from the same patch. Fig. 4A shows the time course of mean currents in another patch that was held at either −100 or −140 mV for 1 s between test pulses to −40 mV. Although the peak current at a holding potential of −140 mV is considerably greater, it decays faster than at −100 mV. At late times, the current for −100 mV is larger than that for −140 mV. These changes are caused



by alterations in the amplitudes of the two components. Fig. 4, *B* and *C*, shows the semilogarithmic plots of the currents from Fig. 4*A*. The solid lines through the data are the results of least-squares fits of the sum of two exponentials to the data. Although the amplitudes of the fast and slow components are clearly different, the fits were not sufficiently precise to determine whether the time course of the currents had changed.

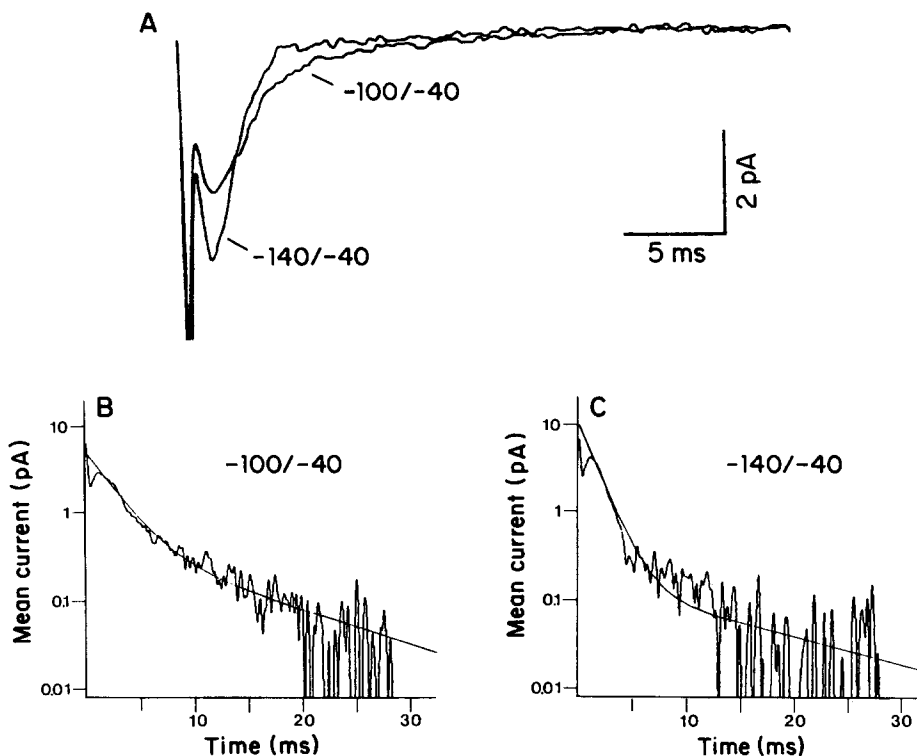


FIGURE 4. Mean currents at two holding potentials. (*A*) The mean currents from pulse ensembles to  $-40$  mV from holding potentials of  $-100$  or  $-140$  mV are superimposed for comparison. Each trace is the average of 138 pulses, which were given once per second. The temperature was  $12^{\circ}\text{C}$ . The initial downward deflection was caused by an incompletely compensated capacity transient at the start of the pulse. (*B* and *C*) Semilog plots of the currents shown in *A*. The solid lines are the best-fitted sums of two exponentials. Details of fitting and plotting are the same as in Fig. 2.

#### *Ultraslow $\text{Na}^+$ Currents*

Some pulses elicited currents that had  $>10$  sequential reopenings and continued for up to 150 ms (the maximum pulse length used to date), as shown in Fig. 5. These openings must have been due to the activity of only one channel since no overlapping currents were seen, despite the high proportion of open time during the burst. In these cases, the channel must have inactivated very slowly or failed

to inactivate. We have seen eight instances with such extended responses in four patches in which we used a slow time base. These currents were seen at pulse potentials of  $-50$ ,  $-40$ , and  $-30$  mV. The amplitude of the individual openings is very close to the amplitude measured for the fast openings at all three pulse potentials. We also observed numerous instances of channels that were still

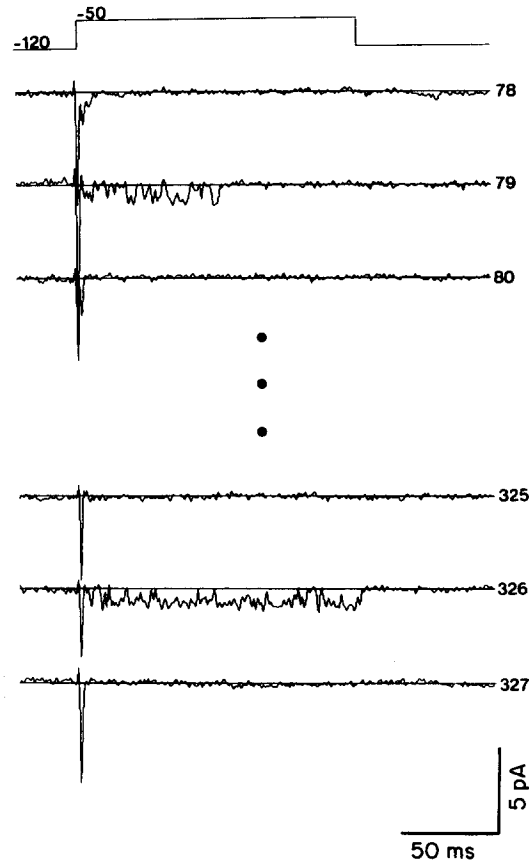


FIGURE 5. Ultralong  $\text{Na}^+$  channel activity. Two exceptional  $\text{Na}^+$  channel currents are bracketed by the preceding and following pulse currents. Note the slow time base. These two bursts of openings occurred during a 332-pulse ensemble. The pulse number is given with each trace. This patch was at room temperature, but similar currents were observed at  $10^\circ\text{C}$ . Currents were sampled at 1-ms intervals and filtered at 500 Hz.

functioning at the end of the 30-ms pulses that we used to record most of our data. Although these data are not yet conclusive, they indicate that the ultraslow responses are also due to  $\text{Na}^+$  channel activity.

The 332 pulses that were given during the ensemble from which Fig. 5 was taken elicited the two bursts that are illustrated. No other current during this ensemble lasted for  $>20$  ms, and, in most pulses, very few reopenings were seen after  $\sim 5$  ms. 11 sequential openings were detected during the first burst, with a

mean open time of 3.3 ms and a mean closed time of 3.8 ms. The second burst had 13 openings, with mean open time of 9.5 ms and a mean closed time of 1.9 ms. However, because of the limitations of our on-line data-sampling algorithm, these slow currents were sampled once each millisecond. Short closing events that might have interrupted the channel openings could not be resolved. For this reason, we cannot yet show that the channel's open time during these ultraslow currents was different than during the fast currents.

The patch shown in Fig. 5 had between 10 and 20 channels. If we assume that all channels had an equal chance of such extended responses, then the frequency of occurrence for each channel can be estimated roughly as the observed frequency divided by the number of channels. Ultraslow currents would be expected once in 3,000 pulses for each channel in this patch. Although the total number of observations is still too low for accurate statistical analysis, the responses in other patches were consistent with an estimate of one burst in 2,000–5,000 pulses for each channel.

#### DISCUSSION

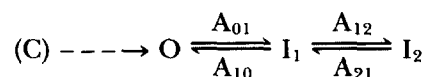
The two major findings of this paper are: (a) a slow component of Na<sup>+</sup> channel inactivation ( $\tau \cong 10$  ms) is caused by channels that undergo multiple reopenings after a depolarizing pulse and (b) in a very small fraction of pulses, a channel can continue to function for up to 150 ms, undergoing repeated openings and closings. The currents that we observed appear to underlie both the slow and the ultraslow (or steady) TTX-sensitive currents that have been seen in cardiac tissue (Carmeliet, 1984). Assuming a channel density of  $10/\mu\text{m}^2$  and a surface area of  $35,000 \mu\text{m}^2/\text{cell}$ ,  $\sim 100$  nA peak Na<sup>+</sup> current would flow in a cell clamped to  $-40$  mV (corresponding to a rate of rise of 300 V/s in an unclamped preparation). Approximately 100 pA of steady current would flow in these same conditions.

#### *Possible Causes for Slow and Steady Na<sup>+</sup> Currents*

The observation that currents inactivate with at least two time constants implies that inactivation is a complex process. Since the two types of slow Na<sup>+</sup> currents described here appear to be related, it is instructive to see whether they can both be explained by a common mechanism. At least four hypotheses could be advanced to explain these data: (a) the currents are not due to Na<sup>+</sup> channel activity; (b) channels have two inactivated states; (c) channels have two open states; (d) channels are heterogeneous or function in two or more "modes." Our data can be used to help distinguish between these hypotheses, as discussed below.

(a) The slow component could be a fundamentally different current, through channels other than the Na<sup>+</sup> channel, or it is a poorly compensated leak or capacity current. This possibility can be eliminated because the single channel signals that underlie the mean currents can be observed directly. The slow currents are due to channels with the same slope conductance, extrapolated reversal potential, and TTX sensitivity as the Na<sup>+</sup> channels that make up the fast component. The ultraslow currents appear to share these properties as well. We conclude that the slow and ultraslow currents are due to Na<sup>+</sup> channel activity.

(b) Each  $\text{Na}^+$  channel may have two inactivated states, with sequential or parallel reaction steps leading from the open state. Usually, all channels are assumed to be independent and identical, and their kinetics are modeled as a Markov chain reaction (French and Horn, 1983). The observation of multiple rates of inactivation can be easily explained by models that include more than one inactivated state. Chiu (1977) successfully described the double-exponential inactivation at the node of Ranvier using two sequential inactivated states. His model leads to several predictions that can be tested with single channel data. Chiu used the following model of inactivation (ignoring activation steps):



His data allowed him to specify the four rate constants at all potentials. At a moderate depolarization to  $-30$  mV, both components were prominent. The rate constants had the values  $A_{01} = 0.61$ ,  $A_{10} = 0.08$ ,  $A_{12} = 0.17$ , and  $A_{21} = 1.4 \times 10^{-4}$  (all values are in reciprocal milliseconds for an experiment at  $4^\circ\text{C}$ ). The mean open time for a single channel would be  $1/A_{01}$ , or 1.6 ms (ignoring reclosing to state C). The chance that the channel reopens after entering  $I_1$  is 33% [ $A_{10}/(A_{10} + A_{12})$ ], and the mean dwell time in state  $I_1$  is  $1/(A_{10} + A_{12}) = 4.0$  ms, and in  $I_2$  it is  $1/A_{21} = 7.1$  s.

Our observations of  $\text{Na}^+$  channels in cardiac muscle fit these predictions during most pulses. The channel's open time was between 1 and 2 ms, as shown in Fig. 1, and the channels usually had very few reopenings. The probability that the channel returns from inactivation was very low after the initial period of activity.

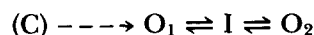
The slow component of Chiu's model is caused primarily by channels that reopen several times before entering state  $I_2$ . For example, the chance of a channel having four sequential openings is  $\sim 2\%$ . We saw multiple channel openings in the cardiac  $\text{Na}^+$  channel, as shown in Fig. 3A. These signals appear to underlie the second component of the mean current. Although our data are quantitatively different from the predictions of Chiu's model, the slow currents that we saw could be described by his model using appropriate rate constants.

However, two of our observations are contrary to the predictions of Chiu's model. Although a second inactivated state could explain the slow exponential component of inactivation that we observed, it cannot simultaneously explain both the 10-ms decay and the ultraslow currents, as shown in Fig. 5. The chance that a current which was part of an exponential distribution with a time constant of 10 ms would continue for 150 ms is  $\exp(-15)$ , or  $3 \times 10^{-7}$ . In contrast, we estimate that  $\text{Na}^+$  channels in rat heart continue to function this long once in  $\sim 2,000$ – $5,000$  pulses. Therefore, the ultraslow currents must be generated by a mechanism that is not accounted for by Chiu's model.

Our observation of an influence of holding potential on the frequency of occurrence of late channel openings, and thus on the amplitude of the slow component, is also contrary to the predictions of models that have two inactivated states. The rate constants in such Markovian models are determined by the pulse potential alone. The holding potential can influence the initial conditions (i.e., the fraction of channels that are already inactivated at the start of the pulse), but

not the reaction rates during the pulse. Chiu's model predicts that at a depolarized holding potential, some of the channels are already in the inactivated state and do not participate in the current response. Both the fast and slow components would have a low amplitude. As the holding potential becomes more negative, both components would increase. Although our observations of the fast component fit this prediction, the decrease in the slow component with hyperpolarization does not. The increased tendency for channels to reopen late in the pulse must be due to channels that have been altered in some way by the holding potential (see below).

(c)  $\text{Na}^+$  channels might have two open states. Chandler and Meves (1970) first proposed a second open state for  $\text{Na}^+$  channels to account for their observations of steady  $\text{Na}^+$  currents in squid axons. A second open state was also included for the same reason in the model of Armstrong and Bezanilla (1977). Such models can be schematically represented as:



Sigworth (1981) analyzed ensemble fluctuations from the node of Ranvier using a two-dimensional autocovariance analysis, and concluded that a second open state of the channel was needed to account for the apparently longer channel open times late in the test pulse.

This model might be used to explain the ultraslow  $\text{Na}^+$  currents that we observed. However, the infrequent occurrence of these currents indicates that the rate constant from I to  $O_2$  must be very small. The repeated openings of the channel could not be explained unless additional closed states were added to the right of  $O_2$ .

Furthermore, this model does not predict a slow component of inactivation with a time constant of 10 ms, nor does it explain the effect of holding potential on the amplitude of the slow component. Although further modification of the model might improve its ability to predict our results, the number of extra states that must be added would make it unattractive.

(d)  $\text{Na}^+$  channels might be heterogeneous or might function in two or more different "modes." Suppose there were several fixed subpopulations of  $\text{Na}^+$  channel, each with a unique rate of inactivation. Recordings from a large number of such channels would be the sum of all activity and would thus have multiple rates of inactivation. Alternatively, suppose that each  $\text{Na}^+$  channel interconverted slowly between several modes of activity. The currents would appear to be caused by discrete subpopulations during each test pulse, but the fraction of channels in each would be in dynamic equilibrium from pulse to pulse, not fixed as in the first example.

We believe that the easiest explanation for our observations is the latter, that the  $\text{Na}^+$  channel can function in two or more modes. For example, the model of Chiu (1977) could be modified so that the rate constant  $A_{12}$  could have one of several values at the start of the pulse, and the channel would function in this mode for the duration of the pulse. Most of the time,  $A_{12}$  would be fast. During a small fraction of pulses, it would be somewhat slower, and occasionally it would be very slow. If the value of this rate constant were influenced by the holding potential, then all of our data could be explained.

The use of models that permit changes in the reaction rates controlling the channel's gates is a major deviation from the standard time-homogeneous Markovian models that are currently popular. However, such models have both experimental and theoretical precedents. The rate of binding or unbinding of substrate to an enzyme molecule is influenced by numerous environmental factors (Stryer, 1981). Single glutamate-activated channels in locust muscle undergo sudden, major shifts in the distributions of their open and closed times (Patlak et al., 1979). Similarly, Na<sup>+</sup> channels that have been inserted into bilayers and treated with batrachotoxin suddenly shift their kinetics (Weiss et al., 1984). One possible physical basis for such changes in the reaction rate is that the channel protein can have various conformations that influence the ability of the channel's gates to respond to membrane voltage. Hoyt (1984) proposed a model for Na<sup>+</sup> channel activity based on the assumption that the reaction rates controlling the channel's gates are controlled by a set of secondary reactions and are thus variable with time during the pulse. Her model is an excellent predictor of mean current, gating current, steady currents, and conditional probability measurements of single channels. Her model might also be accommodated to include our observations.

Models of the Na<sup>+</sup> channel that use Markov chains must include extra states to account for observations like ours. To model all the channel's behavior, these models must be extremely complex. Models with variable reaction rates are attractive because they are simpler than the Markov chain models. We do not yet have enough data to formulate or critically test such models. Clearly, the best experiments would record the activity of an individual channel for thousands of pulses. In the absence of this ideal experiment, various statistical measures may be used to test aspects of the model. We are currently undertaking such studies.

The kinetic properties of Na<sup>+</sup> channels that we report here might be unique to cardiac muscle. The slow and ultraslow currents that we describe have not been reported in other patch-clamp investigations of Na<sup>+</sup> channels (Sigworth and Neher, 1980; Patlak and Horn, 1982; Aldrich et al., 1983). On the other hand, the slow activity that we report is rare for any individual channel, and the above studies concentrated on patches that had as few channels as possible. Our use of patches that had large numbers of Na<sup>+</sup> channels reveals such exceptional behavior much more frequently. Our preliminary investigations show that bursts similar to those in Fig. 5 can also be observed in tissue-cultured skeletal muscle, and in adult frog skeletal muscle when large patches are used. Since the existence of slow currents through the Na<sup>+</sup> channel constrains the models of the Na<sup>+</sup> channel's activity, it is important to re-examine other preparations for the presence of such slow currents.

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