Mechanisms of Closure of Cardiac Sodium Channels in Rabbit Ventricular Myocytes: Single-Channel Analysis

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We have examined the kinetics of closure of sodium channels using single-channel recordings in cell-attached and excised membrane patches of rabbit ventricular myocytes. Sodium-channel closure was dependent on membrane potential. The closing rate initially decreased with depolarization. The rate then passed through a minimum and increased at strongly depolarized potentials. We attempted to determine the separate voltage dependence of the deactivation and inactivation rate constants using the method of Aldrich, Corey, and Stevens. In a majority of experiments, the method did not give internally consistent results. As an alternative approach, batrachotoxin was used to remove inactivation and determine the voltage dependence of deactivation rate. The deactivation rate decreased with depolarization. To account for the increase in the closing rate at strongly depolarized test potentials, one must postulate voltage dependence of inactivation. The ensemble average current relaxed with a time course that was usually best described by the sum of two exponentials. The larger of the two rate constants that described the relaxation was strongly voltage-dependent, increasing with depolarization. The larger rate constant may reflect voltage-dependent inactivation. We found evidence of two possible mechanisms for the slow component of relaxation: 1) cardiac sodium channels may open repetitively during a given depolarizing epoch, and 2) channels may return from the inactivated state with low probability and burst for as much as 200 msec with open times that are longer than those during usual gating. The slow component appears to be more prominent in cardiac muscle than in nerve and may play an important role in the control of the action potential duration and the inotropic state of the heart. (Circulation Research 1987;60:897–913)
Materials and Methods

Cell Preparation

Experiments were performed on enzyme-dissociated cardiac myocytes of adult rabbits. Rabbits (1.5–3.5 kg) were anesthetized with ketamine 5–10 mg/kg i.m. and pentobarbital 40–70 mg/kg i.p. Additional supplements of pentobarbital were given intravenously to produce adequate anesthesia. A tracheostomy was performed, and each rabbit was ventilated using a pediatric ventilator (Loosro Instrument Company). Following thoracotomy, the ascending aorta was dissected free. The heart was rapidly excised and perfused in a retrograde fashion using the Langendorff technique. The heart was perfused with calcium-free Krebs-Henseleit solution for 5 minutes. The heart was then perfused with enzyme medium for 45 minutes, followed by perfusion with Kraft-Bruehe (KB) medium for 5 minutes. Each ventricle was cut into small segments and gently agitated in KB medium. The isolated myocytes were separated from the tissue chunks by filtration through a 200-μm nylon mesh. The isolated myocytes were washed twice with KB medium using centrifugation at low speed. The cells were suspended in KB medium for 1 hour at room temperature. They were then transferred to DME/F12 and kept at 37°C prior to use. Cells stored at 37°C in DME/F12 had more negative resting potentials than cells kept in KB medium only. They also maintained their rod-like structure with cross striations for greater than 24 hours.

Solution and Electrodes

The Krebs-Henseleit solution had the following composition (in mM): NaCl 118.2, CaCl₂ 2.7, KCl 4.7, MgSO₄·7H₂O 1.2, HCO₃ 25, NaH₂PO₄ 1.2, glucose 11. Calcium-free Krebs-Henseleit solution had no added calcium. The Krebs-Henseleit solution was gassed with 95% O₂ and 5% CO₂. Enzyme medium was made by addition of 69.4 U/ml collagenase and 50 g/ml hyaluronidase to calcium-free Krebs-Henseleit solution. KB medium contained (in mM) KC1 85, K₂HPO₄ 30, MgSO₄·7H₂O 5, Na₂ ATP 5, pyruvic acid 5, β- OH butyric acid 5, creatine 5, taurine 20, glucose 20, EGTA 0.04, polyvinyl povidone-40 40 g/l. The pH was adjusted to 7.2 with KOH. DME/F12 was made by mixing equal parts of DME and Hams F-12 (Gibco Scientific Company, Grand Isle, N.Y.). They were then sampled at 10 kHz before further analysis. Voltage command pulses were provided by Digital Equipment Corporation LSI-11/23 microcomputer (Merrimack, N.H.). The voltage command pulses were filtered at 4 kHz before they were applied to the noninverting input of the amplifier.

Recording Techniques

We used an EP-C 5 or 7 patch clamp amplifier (List Electronics Company, West Germany) to measure the single-channel currents. Each microelectrode was coupled to the headstage of the amplifier with an Ag/AgCl wire coated with Teflon up to its tip (In Vivo Metric Systems). The current output of the amplifier was recorded on an analog tape recorder (Racal Recorders Incorporated 4DS) at 7½ in./sec. During off-line analysis, records were filtered at 1.5 kHz with a Frequency Devices Incorporated 8 pole Bessel filter (Haverhill, Mass.). They were then sampled at 10 kHz before further analysis. Voltage command pulses were provided by Digital Equipment Corporation LSI-11/23 microcomputer (Merrimack, N.H.). The voltage command pulses were filtered at 4 kHz before they were applied to the noninverting input of the amplifier.

Experimental Protocol

A 22 × 22 mm glass coverslip formed the base of the recording chamber. The base of the recording chamber was coated with laminin. The recording chamber was mounted on an American Optical Company Biostar inverted microscope (New York). A drop of cell suspension was introduced onto the base of the chamber, and the cells were perfused at a rate of 1 ml/min after they had been allowed to settle for 15 minutes. Currents were recorded from cell-attached, inside-out, and outside-out membrane patches according to the method of Hammill et al. In the cell-attached configuration, the holding potential was set at 10–40 mV negative to the cell resting potential (holding potential designated Rp). Any electrode offset potential (usually less than 5 mV) is nullified prior to gigaseal formation. It is assumed that with no voltage applied to the pipette, the potential across the patch is equal to the transmembrane poten-
tial of the cell. Positive-applied voltages hyperpolarize the patch. In the inside-out and outside-out configurations, the holding potential was set at −110 to −140 mV. We elicited single sodium-channel currents with 40-msec voltage clamp pulses applied to 0.66 Hz. In most experiments, 60–80 voltage pulses were applied at each potential. We also applied 10 voltage pulses of opposite polarity at each potential. The current records so elicited were used for leakage and capacitive transient subtraction. To determine steady-state sodium-channel inactivation, the membrane was held continuously at the conditioning potential (−140 to −90 mV in outside-out patches, Rp = 20 to −80 mV in cell-attached patches), except during the test pulse. Because of the marked negative shift in the sodium inactivation curve, hyperpolarizing pulses to potentials more negative than −180 mV, which were required for capacitive transient subtraction, often led to membrane breakdown and so were avoided. Unless otherwise stated, experiments were performed at room temperature (22° C).

Data Analysis

Each group of 60–80 records at a given potential was reviewed. Depolarizing epochs with no events (nulls) were averaged and subtracted from epochs with events to minimize leakage and capacitive transients. In patches that contained several channels, no nulls were observed at some test potentials. In these experiments, leakage and capacitive transients were minimized by adding the average current responses to hyperpolarizing voltage pulses. Further analysis was performed on the corrected records. Each depolarizing epoch was reviewed for well-defined openings of single-channel events. The amplitudes of the well-defined openings were averaged, and the average amplitude was used to set thresholds for channel opening and closing.23 We estimated the number of channels in a patch from the maximum number of overlaps of single-channel events at a depolarized test potential. A hyperpolarized holding potential was used to make the availability (h) close to 1. The test voltage was such that channels opened with high probability after short latency. This approach has been reported to provide good lower-limit estimates of the number of channels in the patch, comparable to those obtained with complex statistical procedures, provided the number was less than 5.21

Idealized records of channel events were obtained from the digitized representation of the channel currents (10 kHz sampling rate) by setting a threshold between the open event and the closed event. From the idealized record, an event list was constructed of each unitary event that contained the time of channel opening and the time of channel closing. When several events overlapped, the closing events were randomly assigned with equal probability to the opening events. From the event list, closed-time intervals and open-time intervals were computed. From these parameters, mean intervals and histograms were computed. For the histograms, the bin width was set to an integer-multi-ple of the basic data sampling interval, Δt, (bin width = αΔt, where α is usually 4). Histograms, starting with the bin with the maximum number of events, were fit to a Poisson density function, λ exp (−λt), using a minimum χ² procedure:

\[ \chi^2 = \sum_{i=0}^{\text{max}} \frac{(n_i - [N\pi_i + 0.5])^2}{[N\pi_i + 0.5]} \]

where \( n_i \) is the observed frequency in the ith histogram bin, \( N \) is the total number of events, and \( \pi_i \) is the probability of events occurring in the ith bin (further defined below). The instrumentation and sampling rate limit the accuracy of event frequencies in the first few bins, so the observed histogram appears to have an initial upward trend that resolves into a decreasing exponential trend. The decreasing exponential phase of the histogram was fit, and \( N \) was defined as the number of events in the exponential region. To do this, the probability mass for the ith bin was set as that conditional on \( i > i_0 \), where \( i_0 \) is the index of the bin with the maximum frequency, as defined by

\[ \pi_i = \int_{a\Delta t_i}^{(i+1)a\Delta t_i} \lambda e^{-\lambda t} dt = \frac{\lambda e^{-\lambda i_0 a\Delta t}}{\lambda a\Delta t} \]

We constrained the expected bin frequencies, \( N\pi_i \), to integer values by rounding to maximize the goodness of fit.24 This is consistent with considering theoretical frequencies to be integers, and it results in a better fit than that obtained using noninteger expected values. For the analysis of macroscopic currents derived by summing across all epochs at a particular potential, a non–least-squares procedure25 was used to estimate the amplitude and rate of either single or double exponentials for the relaxation of the current. Plots of the relaxation of the current on a logarithmic scale clearly showed deviations from a single exponential. Therefore, we initially attempted to fit a double exponential to all experiments. Initial parameter estimates were provided. When the two exponential rates are nearly equal, the nonlinear estimation procedure becomes unstable because of a singular design matrix. When this occurred, we then refit the data to a single exponential.

Results

The results are based on the analysis of data from 25 cell-attached, 4 inside-out, and 10 outside-out membrane patches. Each recording mode has specific advantages. In the cell-attached configuration, the milieu of the membrane patch is least disturbed. This configuration has the disadvantage that the resting potential during a given record is not known. In 19 cells, the membrane was ruptured following gigaseal formation, and average resting potentials of −62 ± 17 mV (mean ± SD) were measured. Four of the cells had resting potentials of −35 mV or less. The maximum hyperpolarization of 40 mV used in this study probably would not have removed the resting inactivation from these cells. When these 4 cells were excluded, the
mean resting potential was $-67 \pm 6 \text{ mV}$. The resting potential in 4 cells at $37^\circ \text{C}$ was measured and a mean of $-84 \pm 2 \text{ mV}$ was obtained, which suggests that the low resting potential of the cells at room temperature results, in part, from the low temperature. In the inside-out and outside-out configurations, the transmembrane potential is always known. However, as we shall describe, the kinetic parameters of channel gating are shifted in the hyperpolarizing direction on the voltage axis.\textsuperscript{14,26} We shall present a brief description of the characteristics of single sodium channels in rabbit ventricular myocytes before presenting the detailed analysis of the kinetics of channel closure.

Figure 1 shows the membrane currents for 8 depolarizing epochs in an inside-out membrane patch. The holding potential was fixed at $-110 \text{ mV}$, and 60-mV voltage clamp steps were applied. This level of depolarization was just positive to the threshold level for this membrane patch. During two of the voltage steps, no events were evident. After a variable latency, single-channel events of 0.7-8.1-msec duration could be clearly resolved. Depolarization positive to this level did not show any overlapping events, suggesting that there was a single channel in the patch. The single-channel events are similar to those reported in neuronal tissue and cardiac and skeletal muscle.\textsuperscript{26-28} Patches

![Figure 1](image-url)

**Figure 1.** Single-channel recording of sodium current. Single sodium channel currents were recorded in inside-out patch. Holding potential was $-110 \text{ mV}$, and 60 mV (test potential, $E_m$ = $-50 \text{ mV}$) voltage clamp pulse was applied. Leakage and capacitive currents were partially subtracted by analog circuitry, and residual was subtracted digitally during analysis. Vertical line, onset of depolarizing step; horizontal line, 0 current level. In this and all subsequent figures, inward current is shown downwards. Eight steps shown were obtained consecutively.
with 4 or fewer channels, such as those in Figure 1, are suitable for detailed analysis of single-channel currents.

In other patches, a large number of channels were present (up to 75 channels). This was especially true for outside-out membrane patches that were slowly excised. For voltage steps close to thresholds, it was sometimes difficult to identify the baseline. For large depolarizations, there was considerable overlap of channel openings, such that single events were difficult or impossible to identify (e.g., Figure 2). Data from such patches were not suitable for detailed single-channel analysis. On the other hand, smooth ensemble average currents could readily be constructed from 20-40 depolarizing epochs. Therefore, they were used to investigate the time course of the average current and the voltage dependence of steady-state inactivation.

### Kinetic Analysis

Most kinetic schemes of the sodium channel postulate closure of the sodium channel from a single open state:

\[
C_N \rightarrow C_{\text{O}} \rightarrow I_a \rightarrow I_b
\]

where \( C_N \) is the state or group of states preceding channel opening, \( O \) is a single open state, \( I \) is inactivated state, and \( a \) and \( b \) are deactivation and inactivation rate constants, respectively. Such a scheme predicts that the open time should have an exponential distribu-

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**Figure 2.** Sodium-channel currents from outside-out patch with large number of channels. Holding potential was \(-140\) mV. Three consecutive voltage steps of 60, 70, and 80 mV (test potential, \( E_m = -80, -70, \) and \(-60\) mV) are shown in Panels A, B, and C, respectively. Capacitive transients were partially subtracted by analog circuitry only. Because of large number of channels in patch, it was not possible to discern single-channel events as in Figure 1. However, fluctuations in current waveform are clearly evident.
tion with closing rate, $\lambda$, equal to the sum of the rate constants for exit from the open state.\textsuperscript{30} Figure 3A shows the distribution of open times of a cell-attached membrane patch. A single exponential provided a good fit as assessed by $\chi^2$ distribution. The closing rate, $\lambda$, was 1.0 msec$^{-1}$. In all histograms with a total of more than 100 events (some constructed with as many as 1,134 events), we found a single exponential to be an adequate fit. At threshold potentials, opening probability was low, so the total number of events with 60–80 steps was less than 100. Histograms constructed from such a low total number of events may not provide accurate estimates of the true closing rate at threshold test potentials. The single exponential fit to the open times suggests that cardiac sodium channels have a single open state. In a later section, we present evidence that a second open state may occur, albeit with low probability.

For an exponentially distributed random variable, the reciprocal for the mean of the open time also provides an estimate of the closing rate, $\lambda$.\textsuperscript{31} Provided the total number of events used in constructing the histogram was large, there was very good agreement between $\lambda$ and the reciprocal of the mean open time. The closing rate was voltage-dependent (Figure 3B), with $\lambda$ decreasing with increasing depolarization. We had estimates of $\lambda$ at 2–5 voltage levels, each separated by at least 5 mV in 11 cell-attached patches. For a range of voltage steps of 30–70 mV, $\lambda$ decreased 0.6 ± 0.3 msec$^{-1}$ (mean ± SD) per 10-mV decrease in the level of the test potential. For very large depolarizations, first-event latency was short, and events tended to be over the initial capacitive transient. This could lead to an underestimate in the closing rate at these voltages. In two experiments where $\lambda$ was measured over a large voltage range, it appeared to pass through a critical voltage range. 16 We felt it was crucial to resolve this point because it would provide specific information as to the nature of the voltage dependence of the inactivation rate constant.

We performed experiments in 3 cell-attached patches in which the temperature was lowered to 15°C and cell membrane depolarized to 0 mV in isotonic KCl. The electrode contained the standard pipette solution used in the cell-attached patches. The low temperature slowed the activation kinetics so that good separation of events from the initial capacitive transient was obtained. The KCl depolarized the membrane close to 0 mV. Therefore, the potentials may be compared with other studies performed in depolarized cells (e.g., Patlak and Ortiz).\textsuperscript{13} In all three experiments shown in Figure 3C, the closing rate declines as the size of the depolarizing step is made more positive. The closing rate passes through a minimum and then may double at the most depolarized test potentials. The results clearly show that in cell-attached patches, the closing mechanism of the channels is voltage-dependent. The combined voltage dependence of inactivation and deactivation lead to a U-shaped voltage dependence of the mean closing rate.

The mean closing rate in excised membrane patches was also measured. These offer the advantage that the membrane potential is known and can be fixed during the collection of a data set. They suffer from the disadvantage that the channel kinetics may be altered by the internal perfusion solution. In all inside-out patches, a closing rate of 0.5 msec$^{-1}$ or less was observed at one or more test potentials. The smallest closing rate in a cell-attached patch at room temperature was 0.77 msec$^{-1}$. It would appear that some component of the internal solution, possibly $F^-$, slows the channel kinet-
ics. We did not compute closing rates for outside-out patches because of considerable overlap of events and because of the large number of channels in these patches.

The voltage dependence of the overall closing rate does not exclude the possibility suggested by Aldrich et al\textsuperscript{11} that the inactivation rate is voltage-independent. The closing rate could have the dependence on membrane potential we describe if the deactivation rate constant had a complex dependence on membrane potential while the inactivation rate constant was voltage-independent. To decide between these possibilities, it was important to determine the individual deactivation and inactivation rate constant of each test voltage. Aldrich et al\textsuperscript{11} and Aldrich and Stevens\textsuperscript{32} described a method to determine the individual rate constants. The prior detailed study in neonatal cardiac myocyte by Kunze et al\textsuperscript{14} indicated that the method frequently failed. Our data obtained from a different cardiac preparation of myocytes of adult rabbit provided another opportunity to examine the applicability of the method to cardiac preparations.

In our initial series of experiments, we had eight experiments that were suitable for analysis. The method provided realizable answers in only two experiments. The low frequency with which the method could be applied indicated to us that little reliability could be placed on the individual rate constants provided by the method. The analysis by Kunze et al\textsuperscript{14} suggests there is a problem with the actual model on which the analysis is based. An alternative possibility is that the data set used to test the model was incomplete. Multichannel patches were the rule rather than the exception. Particularly at markedly depolarized potentials, very few or no nulls in the 60–80 depolarization at a given potential were observed. This put a low degree of reliability on one of the parameters used in the estimate of the rate constants. Drop-out of channels during the data set could have vitiated the analysis.

We attempted to address some of these problems in a single experiment by obtaining a large number of voltage steps at each of two test potentials, which were separated by 20 mV. We were able to verify that the estimate of the number of channels in the patch was unchanged at the end of collection of the data. The membrane potential was held at 40 mV negative to the resting potential (i.e., Rp – 40 mV) and stepped 40 and 60 mV. The ensemble average currents are shown in Figure 4. A single exponential was adequate to describe the current relaxation at both potentials. The relaxation rate constant was obtained from the least-squares fit of the declining phase of the ensemble average current. For the 40-mV step, the relaxation rate constant, $\beta_a$, was 0.095 msec$^{-1}$. This is considerably slower than the overall closure rate constant of 1.65 msec$^{-1}$ measured from the histogram of single-channel open times. Similarly, $\beta_a = 0.38$ msec$^{-1}$, while the overall closure rate constant was 0.94 msec$^{-1}$ for the 60-mV step. It is apparent that the relaxation rate was slower for the 40-mV step, while the microscopic closure rate was faster for this step. For the 40-mV step, despite 66 nulls in a total of 240 steps, the probability of channel closure by inactivation (B in the nomenclature of Aldrich et al\textsuperscript{11}) was 0. However, the ensemble average current clearly shows relaxation towards 0, suggesting entry of channels into an absorbing state. We, therefore, conclude that even when the data set appears optimal, the method may provide unreliable results.

An alternative approach was tried to estimate the individual rate constants or, at least, to get some qualitative measure of their voltage dependence by studying channels in which inactivation has been removed. A large number of compounds have been shown to remove inactivation of the cardiac sodium channel (reviewed by Honerjager\textsuperscript{33} and Katzung\textsuperscript{34}). In preliminary experiments, we tried a number of these agents, such as chloramine T, batrachotoxin (applied externally to the intact cells), or N-bromoacetamide applied to the inner-cell membrane in inside-out patches.\textsuperscript{35–37} Chloramine T proved to be extremely toxic, and cells did not survive exposure for longer than 5 minutes. Following exposure to N-bromoacetamide, the probability of channel opening decreased rapidly and was 0 within 5...
minutes. Stable records were obtained with batrachotoxin only. Therefore, batrachotoxin was selected for our studies. Batrachotoxin interacts with the neurotoxin site number two of the sodium channel. Inactivation is completely removed, and therefore channel closure is by deactivation only. Batrachotoxin also has other effects on the sodium channel. The activation rate is slowed. All gating parameters are shifted in hyperpolarizing direction on the voltage axis, and channel selectivity is reduced leading to a decrease in conductance. From the multiplicity of effects, it is clear that channel kinetics observed after batrachotoxin exposure cannot be compared quantitatively with those of the unmodified channel. For our experiments, 1 μM batrachotoxin was included in the microelectrode during the study of 2 cell-attached membrane patches. A series of depolarizing voltage steps to potentials that produce sodium-channel opening was applied. After several depolarizations, two populations of channel events emerged: normal sodium channels and channels with long open times and reduced conductance. The latter were assumed to be batrachotoxin activated. These channels did not require voltage steps to initiate channel openings, and they continued to open and shut repetitively, even after the patch potential was held at a fixed level for several minutes. As shown in Figure 5, the channel open times increased progressively with increasing depolarization. As the channels are presumed to close by deactivation only in the presence of batrachotoxin, these experiments suggest that the deactivation rate constant declines with increasing depolarization.

How then may we account for the U-shaped distribution of the mean closing rate? We propose a simple scheme in which deactivation and inactivation have opposite voltage dependence. For small depolarizations, channel closure is primarily by deactivation. With increasing depolarization, the closing rate decreases because of a decrease in the deactivation rate constant. With strong depolarizations, a greater proportion of the channel closed by inactivation. As the inactivation rate constant increases with further depolarizations, the closing rate would increase at the most depolarized potentials. Such a scheme would predict multiple openings of the sodium channel around the threshold potential, and this was observed. Nonabsorbing inactivation would produce a similar result. However, nonabsorbing inactivation and deactivation have the same expression at the single channel level.

In 5 cell-attached patches, a different pattern of re-

**Figure 5.** Batrachotoxin-activated single sodium channels. Single sodium channel currents were recorded in cell-attached membrane patch with microelectrode containing 1 μM batrachotoxin. In the presence of batrachotoxin, voltage steps were not required for channel activation. Level of steady holding potential is shown on right. Records were filtered at 0.5 KHz. Mean closing rate decreased from 0.04 to 0.007 msec⁻¹ as the holding potential was decreased from Rp — 60 to Rp — 10 mV. [K⁺]o was 5.4 mM.
petitive channel opening was observed during single voltage steps. Figure 6 shows 10 consecutive voltage clamp steps from one of the experiments. The holding potential was $R_p - 40$, and the voltage clamp step was 60 mV. The mean open-channel closing rate was 1 msec$^{-1}$ (i.e., mean open time of 1 msec). However, it appears that 1 channel opened repetitively through the sixth 40-msec voltage step. During the burst, there were 2 long events of 10 and 8 msec. Based on the distribution of open times, events of this duration should occur with a probability of $4.5 \times 10^{-5}$ and $3.3 \times 10^{-4}$. Despite the low probability of occurrence of events of long duration, they tended to cluster in the same depolarizing epoch. This suggests that an alternative mode of gating is entered with low probability. The low probability did not permit detailed analysis in the cell-attached experiment patches. This property of the channel was recently reported by Patlak and Ortiz.13

If the bursting mode is a true property of the channel that is entered with low probability, then records from patches with a large number of channels should show the phenomenon more frequently. This was the case in outside-out patches, which often contained 50 or more channels. In two preliminary experiments, the duration of the voltage step was increased to 200 msec. We saw late opening channels that had a tendency to burst throughout the test pulse. An example is shown in Figure 7A. To see single-event current levels throughout the tracing, the amplifier was used in configuration with the 50 GΩ resistor in the feedback loop. At this gain, the initial surge of sodium current saturated the amplifier. The holding potential was $-120$ mV, and the voltage step was to $70$ mV. Throughout most of the 200 msec of this step, a channel opened and closed repetitively. In 300 steps at this potential, we saw four examples in which a channel burst for 100 msec or longer. We do not think the bursting represents win-
**Figure 7.** Single sodium channel with slow kinetics. Record in Panel A was obtained in outside-out membrane patch. Holding potential was $-120$ mV, and voltage step was 70 mV ($E_m$, $-50$ mV). Gain of patch clamp amplifier was adjusted such that feedback resistor was 50 GΩ. Initial surge of inward current saturated patch clamp amplifier. For most of remainder of 200-msec pulse, prolonged burst of single channel is evident. Record was not leakage subtracted. Zero current level is shown on far right. Record in Panel B was obtained in cell-attached membrane patch. Cell was depolarized in high K solution ($[K^+]_o$ 125 mM). Holding potential was $V_p$ $-120$ mV, and voltage steps of 60 mV ($E_m$, $V_p$ $-60$) were applied. Six consecutive voltage steps are illustrated. Sodium-channel bursts for most of 40-msec epoch in last 3 voltage steps.

down current with overlap of the $m_\infty h_\infty$ curves. $h_\infty$ was 0 at $-80$ mV in all outside-out patches. The small component of sodium current could be easily lost in the leakage current in macroscopic current measurements. We have a single experiment that suggests that bursting does not occur as a uniform phenomenon. A sample of 6 consecutive voltage steps is shown in Figure 7B. The records were obtained on a KCl depolarized cell with a holding potential of $V_p = 110$ and an applied voltage step of 60 mV. The patch contained 4 channels. The first 3 epochs show typical sodium-channel currents. The last 3 steps show bursting for the entire 40-msec duration of the epoch. Bursting for the full 40 msec was observed in 33 of 80 depolarizing epochs in this patch. These 33 epochs did not occur in a random manner throughout the 80 steps. Rather, they were grouped together in five runs with 1–9 bursting epochs (mean number of bursting epochs, 6.6 per group).

**Macroscopic Kinetics**

The time course of relaxation of the sodium current in outside-out and cell-attached patches was examined. The outside-out patches had the advantage that the membrane potentials were accurately known. The observed time course could be directly compared with that measured in other preparations. Outside-out patches had five to ten times as many channels as cell-attached patches obtained with microelectrodes of similar resistance. Seal resistances were often greater than 50 GΩ, so leakage currents were small. Very smooth ensemble average currents could be constructed with few depolarizations. Also, channel-gating behavior that occurs with low probability is more likely
to be represented by averages of a large number of channels. The time course of the averaged current in 5 outside-out patches was determined. The holding potential was set at −140 mV, and 40-msec voltage clamps were applied in increments of 10 mV. Except at the threshold potential level, double exponentials were required to fit the relaxation of the current. The ratios of the rate constants varied from 44:1−3:1, so they could be reliably separated at most potentials. In Figure 8, the rate constants as a function of membrane potential were plotted in our most complete experiment. Both rate constants increase with increasing depolarization. However, the larger rate constant shows steeper voltage dependence. The rate constants are similar to those reported by Brown et al in internally perfused rat ventricular myocytes. In outside-out patches, the voltage dependence of the gating variables was shifted to negative potentials. Data collection was usually started 5 minutes after isolating the patch. The shift was already established at that time, so we do not have an estimate of the size of the shift. In six preparations, the potential for 50% steady-state inactivation ($V_{1/2}$) was −113 ± 4 mV. Similar shifts have been observed by Brown et al and Cachelin et al.

The time course of relaxation of the sodium current was more variable in cell-attached patches. In 5 patches, two exponentials were required to describe the relaxation of the current at 1 or more test voltages (e.g., Figure 9A). In five other experiments, one exponential was sufficient at all test voltages (e.g., Figure 5). Some of the variability of the results with cell-attached patches may be because too few points were available at some times to adequately characterize the time course of the current. Also, gating behavior that occurs with low probability may not be well represented in averages constructed from few events. In two experiments, single-channel current in a cell-attached patch was obtained using a 1-MΩ patch electrode. The results of one of these experiments is shown in Figure 9. The holding potential was 20 mV negative to the resting potential (i.e., $R_p$ = 20), and voltage steps of 90, 80, 70, 60, and 50 and a repeat of 80 mV were applied. The patch contained at least 21 channels. Because of the large number of channels, smooth ensemble average currents were readily obtained. For the voltage steps of 90, 80, and 70 mV, two exponentials were required to describe the current relaxation. The larger rate constants showed definite voltage dependence (Figure 9A). The smaller rate constant was not voltage-dependent. The record of a single step of 80 mV is shown in Figure 9B. We estimate that an 80-mV voltage step would place the test potential close to 0 mV where the single-channel level signal-to-noise ratio is
Figure 9. Panel A: Kinetics of relaxation of sodium-channel current in cell-attached patch. Single sodium channel currents were recorded in cell-attached membrane patch. Holding potential was $V_p = -20$. Current during 30–40 voltage clamp steps at each test potential was averaged. Relaxation of current was fitted with 2 exponentials for the 90, 80, and 70 mV steps ($E_m, V_p + 70, V_p + 60$, and $V_p + 50$). For 60 and 50 mV steps ($E_m, V_p + 40$ and $V_p + 30$), single exponential provided adequate fit. Large ($\Delta$) and small (•) rate constants are plotted as functions of voltage step. Large rate constant was strongly voltage-dependent, while small rate constant showed no voltage dependence.

Panel B: Single-channel current during one of the 80-mV voltage steps ($E_m, V_p + 60$) shows initial surge of inward sodium current. This is followed by prolonged opening of at least 1 channel. Such prolonged opening could account for slow component of relaxation of average current. $[K^+]_o$ was 5.4 mM.

Reduced. Despite this, the figure shows persistent current through at least 1 channel long after the major portion of the current relaxation is complete. Such activity could clearly contribute to the slowly relaxing component of the current. For the voltage steps of 50 and 60 mV, a single exponential was sufficient to describe the current relaxation.

The rate constants at specific test voltages could not be directly compared in cell-attached and outside-out patches because of uncertainties about the test voltage in cell-attached patches. Double exponentials were more clearly defined at intermediate levels of depolarization. In general, at the extremes of test voltage, a single exponential was adequate to describe the current relaxation in cell-attached patches.

Most kinetic schemes of the sodium channel are time-homogenous, i.e., the rate constants are assumed to be functions of voltage alone. For a kinetic scheme with $k$ states, the overall time course of the current is the sum of $(k-1)$ exponentials. The exponential time constants are eigenvalues of the overall transition matrix. The transition matrix is composed, in turn, of the individual time-independent rate constants in the kinetic scheme. The implication is that while the time course of the current at a given test potential may vary with the initial conditions (e.g., the holding potential), because of differences in the weighting of each exponential, the exponential time constants of relaxation should be independent of the initial conditions. The study of Patlak and Ortiz showed a difference in the time course of the current as the holding potential was varied. However, it was not clear that the actual rate constants changed as holding potential was varied. This question was examined by analyzing the time course of the relaxation of the sodium current as a function of holding potential in 8 outside-out and 4 cell-attached patches. Again, the outside-out patches had a large number of channels, and smooth curves of average currents could be obtained after few (40) depolarizing voltage steps. For the outside-out patches, the holding potential was varied between $-140$ and $-90$ mV, and the test potential was fixed at $-50$ mV. Superimposed current records from one experiment are shown in Figure 10A. Two exponentials were required to fit the relaxation of the current. The rate constants for the exponentials describing the relaxation are shown in Figure 10C. For holding potentials of $-140$ to $-120$ mV, the rate constants were similar. At potentials just positive to threshold (e.g., $-110$ mV), there was a clear deviation of the time course from that at other test potentials. A single exponential was required, and the time constant was between the
values of the fast and slow time constants observed at other holding potentials. When the current just positive to threshold was scaled to equal the magnitude of the current at the most positive potentials, the slower time course of currents elicited from the more positive holding potential became clearly evident (Figure 10B). In cell-attached patches with few (<4) channels where single-channel events were well resolved, the result was the same. The relaxation rates were similar except for currents elicited at potentials just positive to threshold. Time-homogenous schemes are therefore adequate for most holding voltages.

Discussion

Microscopic Kinetics

Our results show that the closing rate of cardiac sodium channels is voltage-dependent. It decreases with increasing depolarization, passes through a minimum, and increases at strongly depolarized test potentials. Studies in neonatal rat ventricular myocytes,26 tunicate egg,28 skeletal muscle,27 neuroblastoma cells,40,41 and adrenal chromaffin cells42 showed voltage dependence of the closing rate. Only Aldrich and Stevens32 have reported little or no voltage dependence of the closing rate on transmembrane potentials. At least four studies in noncardiac tissue suggest that the closing rate increases at strongly depolarized test potentials. However, previous studies analyzing the kinetics of cardiac sodium channels have not reported much data on the kinetics of closure. Cachelin et al28 reported that the single-channel mean open time increased with depolarization. In the study of Kunze et al,14 sodium open times did not show much voltage dependence over a range of test potential Rp - 30 to Rp + 10. Our study shows that the closing rate is not a simple monotonic function of voltage. Sodium-chan-
nel closure occurs by deactivation and inactivation, and the experimentally derived closure rate is the sum of the deactivation and inactivation rate constants. These rate constants must have an appropriate functional dependence on transmembrane potential to account for the U-shaped distribution of the overall closure rate. It is therefore desirable to decompose the closing rate constant into separate deactivation and inactivation rate constants.

The deactivation and inactivation rate constants were unable to be determined separately for the unmodified sodium channel. Like a number of other investigators, e.g., Vandenberg and Horn\(^1\) and Kunze et al,\(^14\) we failed to obtain consistent results using the method of Aldrich, Corey, and Stevens.\(^11\) We presume that one or more of the explicit or implied assumptions in the method is incorrect. At least around threshold potential, we have observed that failure of channels to open during a 40-msec epoch does not necessarily reflect passage into the inactivated state. When the pulse duration was increased to 200 msec, first opening could occur with a latency of over 100 msec (A.O. Grant, unpublished observations). Of course, the possibility that such openings arise from channels that passed directly into the inactivated state without opening and are now returning from inactivation cannot be excluded. We used the alternative approach of removing inactivation and determining channel-closure rate of the modified channel. Channel-closure rate decreased progressively with depolarization in the presence of batrachotoxin. Huang et al\(^3\) showed a monotonically declining function of the inactivation rate of batrachotoxin-treated sodium channels in mouse neuroblastoma cells. Horn et al\(^4\) showed in GH\(_3\) cells that when inactivation was removed with N-bromacetamide, the inactivation rate declines with increasing depolarization. The evidence in cardiac, neuroblastoma, and GH\(_3\) cells is that the deactivation rate constant declines with increasing depolarization. This would account for the initial decline in closing rate of unmodified sodium channels with depolarization. To account for the increase in the closing rate with strong depolarization, we must postulate that the inactivation rate constant increases with increasing depolarization in cardiac muscle. Such a scheme would be similar to that proposed for the transition of excitability-inducing material in lipid bilayers.\(^45\)

This interpretation of the mechanism of closure of cardiac sodium channels is in qualitative agreement with earlier studies in noncardiac preparations. Sigworth and Neher\(^27\) observed an initial increase of the mean open time of skeletal muscle sodium channels, followed by a decrease with further depolarization. They interpreted their results according to the Hodgkin-Huxley scheme, with closure being dominated by deactivation and inactivation (\(\beta_d\) and \(\beta_i\)) for small and large depolarizations, respectively. Similar results were obtained by Fukushima\(^28\) using an \(m'h\) model for the tunicate sodium channel. Vandenberg and Horn\(^12\) used a maximum likelihood procedure to fit various kinetic schemes and derive parameters for the sodium channel of clonal rat pituitary cells. Although the voltage dependence of the mean open time was not as prominent as in the present study, their results were compatible with a scheme of closure by voltage-dependent deactivation and inactivation. Even in simple model systems like excitability-inducing material in lipid bilayers, closure by voltage-dependent process(es) was observed.

In relating the microscopic kinetics of closure to the relaxation rate of the sodium current, Aldrich et al\(^11\) proposed that the increase in the relaxation rate of the sodium current with depolarization results from decreases in latency to first opening. Channels opened earlier with strong depolarization but closed at a relative fixed rate by voltage-independent inactivation. In our own and other studies of the sodium channel (e.g., Kunze et al\(^14\)) event first latency is shown to decline with increasing depolarization. However, to this must be added the factor that the inactivation rate constant is also increasing. We speculate that at the peak of the cardiac action potential at room temperature, closure would be dominated by inactivation. We shall return to the question of the relation between microscopic kinetics when the nature of the relaxation of the macroscopic current is discussed.

Histograms of the distribution of open times were well fitted by single exponentials. This is consistent with a single open state of the sodium channel. Other evidence in this study, though, forces us to question this conclusion. Figures 6 and 7 show prolonged bursting of sodium channels throughout the duration of 40- and 200-msec pulses, respectively. The open times during the prolonged burst are longer than those during normal opening (Figure 6). Such events occurred with low frequency. However, one has to postulate a scheme such as the following to account for this type of gating:

\[
\begin{align*}
C_{n} & \xrightarrow{k_1} O_1 \xrightarrow{k_2} O_2 \\
& \leftarrow I
\end{align*}
\]

where \(k_1\) and \(k_2\) are very small. Further, to account for the bursting behavior, we must postulate an additional closed state to the right of \(O_2\). The grouping of epochs with bursting (e.g., Figure 7) suggests that the rate constant \(k_2\) is the least of the order of the reciprocal of the interstimulus interval, i.e., \(6.6 \times 10^{-4}\) msec. A similar scheme had been proposed by Bromm and Schwarz\(^46\) to explain the inactivation of the macroscopic sodium current in myelinated nerves. The scheme is formally similar to the multiple modes of gating recently proposed for the calcium channel by Hess, Lansmann, and Tsien.\(^47\) Patlak and Ortiz\(^48\) have recently reported similar slow sodium-channel kinetics in adult rat ventricular myocytes. More recently, Patlak and Ortiz\(^44\) demonstrated dual mode gating in frog sartorius muscle. On the other hand, such bursting was not reported by Kunze et al\(^\text{al}\)\(^14\) in rat neonatal cells at room temperature. Our study in rabbit ventricular myocytes confirms that this is a genuine property of the sodium channel in adult cells. In our laboratory,
bursting has also been observed in the rat neonatal cells (J. Hurwitz, unpublished observation). Patlak and Ortiz proposed a scheme of multiple modes of gating to explain their results. A relatively simple explanation would be that bursting results from intermittent failure of a channel to inactivate. Gating would then be reduced to the scheme:

\[ \cdots C_s \rightarrow O \]

From this scheme, specific predictions about the relation of the shut times to the rising phase of the current can be made. Because the events that show slow kinetics occur with low probability, it will require patches with very large numbers of channels or prolonged recordings of patches with a few channels to provide detailed kinetic analysis. Channels with slow kinetics have been recorded in patches in which sodium and calcium are the only cations at the external membrane surface. Single calcium channels should have amplitudes that cannot be resolved above the background noise when measured with 2.7 mM [Ca\(^{2+}\)]. Therefore, we are confident that the slow channels we have recorded are sodium channels. They have amplitudes similar to those of other sodium-channel events recorded in the same patch. Patches that showed prolonged bursting as the exclusive mode of sodium-channel gating were never observed. However, it remains to be demonstrated whether channels with slow kinetics are an entirely different class of sodium channels or if they represent the usual sodium channel but exhibit slow gating kinetics. Detailed studies of voltage dependence of gating, conductance, and tetrodotoxin sensitivity are needed to address some of these issues.

Differences in single-channel current amplitude may also suggest multiple open states or multiple populations of sodium channels. Experiments describing such results have recently been reported by Nagy et al.,\(^{40}\) Cachelin et al.,\(^{26}\) and Kunze et al.\(^{14}\) We have results from 2 patches that show two conductance levels of single sodium channels that cannot be explained by band-width limitation of the recordings. We have not reported them in detail in this paper since they were obtained in adult myocytes that had been maintained in culture for over 48 hours. All of the results reported in this paper were obtained in freshly dissociated cells.

**Macroscopic Kinetics**

In outside-out and cell-attached patches containing a large number of channels (>10), two exponentials were required to fit the relaxation of the current. The larger of the two relaxation rates was strongly dependent on transmembrane voltage. Schemes for double exponential relaxation of the sodium current in neuronal membranes have been analyzed in detail.\(^{46,49,50}\) These schemes propose a greater number of inactivated states than the two originally proposed by Hodgkin and Huxley.\(^{3}\) Our results suggest two possible bases for the slow component of the relaxation. If a fraction of open sodium channels initially close by deactivation during a depolarizing epoch, they may reopen and contribute to the slow component of current relaxation. Our results support reopening of cardiac sodium channels. They are consistent with those recently reported by Kunze et al.\(^{14}\) and Scnaley et al.\(^{15}\) and show that reopening of cardiac sodium channels occurs at most test voltages. These results are in contrast to those reported in neuroblastoma cells,\(^{11}\) where sodium channels close largely by inactivation and rarely reopen during a given depolarizing epoch. We have also shown that cardiac sodium channels may enter a long-lived bursting state for up to 200 msec (the longest pulse examined to date). This bursting occurred with low probability (less than 1% of steps). However, when summed over the thousands of sodium channels in a cell, the slow kinetic states may provide a slow component of current that is different from that provided during the transient inward current surge.

There is now a larger body of evidence suggesting that sodium ions carry a significant current component during the action potential plateau. Dudel, Peper, Rudel, and Trautwein\(^{39}\) reported that 10\(^{-5}\) M tetrodotoxin (TTX) shortened the action potential plateau of Purkinje fibers of sheep. Using TTX-sensitive current as a measure of sodium current, they showed that 10 msec after a suprathreshold voltage step, there was residual sodium current of about 1% of the amplitude of the initial current. This declined to 0.1% in 140 msec. Coraboeuf, Deroubaix, and Coulombe\(^{50}\) showed that concentrations of TTX that had little or no effect on the action potential upstroke shortened the cardiac action potential duration in canine cardiac Purkinje and ventricular muscle fibers. Purkinje fibers were more sensitive to this action of TTX. A similar TTX sensitivity of the action potential duration has been reported by Bhattacharya and Vassalle.\(^{52}\) An analysis of steady-state sodium current in sheep Purkinje fibers showed a TTX-sensitive component between -65 and -15 mV.\(^{55}\) They suggested that this current resulted from an overlap of the m\(_{\infty}\) and h\(_{\infty}\) curves. The voltage range where the current is observed would require considerable overlap of the m\(_{\infty}\) and h\(_{\infty}\) curves. Carmeliet\(^{55}\) has examined the slow sodium-current components in detail in rabbit Purkinje fibers under voltage clamp. He showed slow TTX-sensitive components of membrane current over a wider potential range than that reported by Attwell et al.\(^{56}\) Voltage clamp experiments by Colatsky\(^{7}\) in the same preparation showed very little overlap between the m\(_{\infty}\) and h\(_{\infty}\) curves. Gintant et al.\(^{58}\) also reported TTX-sensitive components of sodium current even at positive potentials when h\(_{\infty}\) is likely to be 0. In our experiments, we could identify single sodium channels with slow kinetics at potentials where h\(_{\infty}\) was 0. It is likely that the slow kinetics displayed by the sodium channels can account for the TTX-sensitive component of slow sodium current reported in previous studies. We doubt that overlap at the m\(_{\infty}\) and h\(_{\infty}\) curves is the principal basis for this current (e.g., see Figure 13 in Kunze et al\(^{14}\)). The observation of very slow sodium-channel inactivation kinetics by Dubois and Bergman\(^{57}\) and Fox\(^{58}\) indicates that these slow kinetics are not unique to cardiac muscle.
Influence of Holding Potential on Inactivation Kinetics

Frankenhaeuser\textsuperscript{40} showed that very brief (<0.5 msec) activating prepulses accelerated the relaxation of the sodium current during a subsequent test pulse. The interpretation was that schemes with time-independent rate constants provided an erroneous description of sodium-channel kinetics. Dudel and Rudel\textsuperscript{4} examined the effects of 2–4,000-msec conditioning pulses on the course of the sodium current during a subsequent step to a fixed test potential in sheep Purkinje fibers. The conditioning potentials of −180 and −140 mV were at the extremes of the steady-state inactivation (h\textsubscript{i}) curve. The time course of the sodium-channel relaxation was slowed by depolarizing conditioning prepulses. Patlak and Ortiz\textsuperscript{13} reported a similar slowing of sodium-channel relaxation with crossover of the declining phases in rat ventricular myocytes when conditioning potentials of −140 and −100 mV were employed. On the other hand, Kunze et al\textsuperscript{10} found no crossover for holding potentials between −90 and −130 mV. The effects of conditioning prepotentials on the relaxation of the sodium current was studied over a wide range of conditioning potentials (h\textsubscript{i} = 1 to h\textsubscript{i} close to 0). Over most of the potential range studied, a significant difference in the relaxation rate constants was not found. At holding potentials where h\textsubscript{i} was markedly reduced, the relaxation rate could be fitted by a single exponential, and the time course of the current was slowed when compared with more hyperpolarized potentials (Figure 10). We are not sure whether the change in time course is related to a reduction in the amplitude of the current per se or to the more depolarized holding potentials. Recently, Kiss and Nagy\textsuperscript{60} reported single sodium channel data in neuroblastsoma cells, which suggests that channel kinetics may be influenced by the state (open or closed) of neighboring channels. This could provide a mechanism for overall kinetics that depends on the magnitude of the current. At the present time, it would appear that schemes with time-independent rate constants can account for the kinetics of the sodium channel over most test potentials.

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**Grant and Starmer  Closure of Cardiac Sodium Channels**

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