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Sodium channels in cardiac Purkinje cells

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Summary. Sodium (Na^+) currents are responsible for excitation and conduction in most cardiac cells, but their study has been hampered by the lack of a satisfactory method for voltage clamp. We report a new method for low resistance access to single freshly isolated canine cardiac Purkinje cells that permits good control of voltage and intracellular ionic solutions. The series resistance was usually less than 3Ω cm^2, similar to that of the squid giant axon. Cardiac Na^+ currents resemble those of nerve. However, Na^+ current decay is multiexponential. The basis for this was further studied with cell-attached patch clamp recording of single Na^+ channel properties. A prominent characteristic of the single channels was their ability to reopen after closure. There was also a long opening state that may be the basis for a small very slowly decaying Na^+ current. This rare long opening state may contribute to the Na^+ current during the action potential plateau.
Key words. Cardiac Purkinje cells; Na^+ channels; voltage clamp; single channel recording.

Introduction

Silvio Weidmann and his colleagues demonstrated in a series of papers the importance of sodium (Na^+) ions for excitation and conduction in cardiac Purkinje fibers[14, 16, 38]. Detailed studies of Na^+ current (I_{Na}) have been made in the giant axon of the squid, with good spacial voltage control[23] intracellular perfusion[3] and identification of channel gating currents[2]. However, equivalent studies were not possible in cardiac cells because of their small size and complex geometry. Such detailed study in cardiac cells is important and necessary, since it is clear that the cardiac Na^+ channel is a separate molecule, different from that of nerve and skeletal muscle[26, 29].

The first step toward systematic study of cardiac I_{Na} was the development of the two-micropipette voltage clamp in a shortened Purkinje strand by Deck et al.[15] and Hecht et al.[22]. This method was used by Dudel and Rudel[17] in their systematic study of I_{Na} in cooled Purkinje fibers. An alternative sucrose-gap voltage clamp method was used for voltage clamp studies in ventricular muscle by Beeler and Reuter[5]. While in retrospect we know that these investigators had relatively poor voltage control, they nevertheless were able to make valuable measurements. The data they obtained were then used as the justification for modeling I_{Na} in heart muscle in a modified Hodgkin-Huxley form[6, 28, 37].

In a continuing effort to improve the quality of the voltage clamp, Colatsky and Tsien[13] were able to control I_{Na} in small, shortened rabbit Purkinje strands in which the current was reduced by low temperatures and low Na^+ solutions. Ebihara and Johnson[18] used very small cultured aggregates of cardiac cells in a similar way. Several valuable studies on the mechanism of action of antiarrhythmic drugs on the Na^+ channel were forthcoming[4], but these studies on multicellular preparations were inevitably less reliable than desired.

Cell culture methods have provided us the means to isolate single cardiac cells. Normally cardiac cells are wrapped in connective tissue and are electrically coupled by large-conductance gap junctions. Isolation of single cells for physiological studies has been achieved by use of gentle enzyme treatment to digest away the connective tissue and supportive ionic environments to promote cell survival while the disrupted gap junctions close. Brown et al.[8] first took advantage of these single cells and used large-bore sealed pipettes to perform voltage clamp studies of cardiac I_{Na}.

Recently, a new and dramatic way of studying Na^+ current has become available with the development of the patch clamp method for recording of single channel openings[21]. For most of us who are accustomed to thinking of currents in

terms of the Hodgkin-Huxley formulation, the stochastic properties of these single channels has been difficult to translate into whole cell behavior. However, some good progress already has been made in determining the characteristics of single cardiac Na^+ channels[10, 20, 25, 32].

A particularly desirable approach to the study of Na^+ currents and their role in excitation and conduction is to perform single channel measurements in the same preparation with uniform membrane voltage clamping of the quality found in squid axon. Both methods of study bring their special opportunities and advantages, and they both have their problems and artifacts. They represent complementary approaches to understanding the function of the cardiac Na^+ channel. We have recently been able to adapt the method used by Kostyuk and colleagues[24] to control the internal solution of canine cardiac Purkinje cells and to achieve excellent voltage control for detailed study of I_{Na}[27]. Other applications of this method have been used by Bodewei et al.[7] and Bustamante and McDonald[9].

We will outline our methods and the salient characteristics of cardiac Na^+ currents. We will combine the membrane clamp with patch clamping in the cardiac Purkinje cells in an attempt to demonstrate the special values of each technique, and to illustrate these with combined studies of the complex nature of Na^+ current decay, which is of such importance in determining the shape of the cardiac action potential.

Methods

Cell preparation

The method for preparing single canine cardiac Purkinje cells has been described in detail elsewhere[35]. Briefly, canine Purkinje fibers from the hearts of adult mongrel dogs were cut into short segments (2–3 mm) and placed in Eagle's minimal essential medium modified to contain 0.1 mM free Ca^{2+} (by the addition of EGTA), 5.6 mM Mg^{2+}, 5.0 mM Hepes, 1 mg/ml albumin, and 5 mg/ml Worthington type I collagenase buffered to pH 6.2. The fibers were incubated in this digestion solution at 37 °C, gassed with 100% O_2, and gently agitated in a water bath shaker for 3–4 h. After 15 min in 130 mM K-glutamate, 5.7 mM Mg, 0.1 mM EGTA, 5.0 mM glucose and 5.0 mM Hepes (pH 6.2) the cells were mechanically dispersed and maintained in Eagle's minimal essential medium with 1.0 mg/ml albumin and 5.0 mM Hepes (pH 7.2) at room temperature. They were studied within 12 h of isolation.

Perfused cell experiments

All experiments were performed with single canine cardiac Purkinje cells prepared as above. A single glass suction pipette with a large pore of about 25 μm in diameter was used for both voltage clamp and internal perfusion as described in Makielski et al.[27]. In brief, a single cell with normal striation pattern and no membrane blebs was selected that had the same diameter as the pipette aperture. The cell was drawn into the aperture until about one-third remained outside of the pipette. The cell was allowed to seal to the aperture walls and then the segment of the cell within the pipette was ruptured by a manipulator-controlled wire, to allow free access of the pipette solution to the cell interior. The response time of the voltage clamp circuitry was within 1% of the final potential by 10 μs, while the membrane capacity time constant, τ_{cap}, with compensation was usually < 5 μs and series resistance (R_s) was usually < 3 ohm-cm^2 [27]. Currents recorded from such segments of membrane were derived from about one-third of the cell. This will be identified as 'membrane current', to distinguish it from single channel current.

Command voltage steps were generated by a 16-bit D/A converter and currents were recorded by a 12-bit A/D converter (Masscomp 5500 System, Wesford, MA). For peak I-V relationships, current responses to step depolarizations were digitized at 10 μs per point, digitally filtered (Gaussian) at 5 KHz and peak I_{Na} calculated by subtracting the leak current at the end of the 25-ms step depolarization from the peak transient current. Capacity subtraction was not performed because the short τ_{cap} allowed for clear separation of peak I_{Na} from I_{cap} and thus the differences between measurements with and without subtraction were less than 1 nA. All voltage protocols had a cycle frequency of 1 Hz.

External solution changes were made within 30 s by moving the cell from the inlet of one bath chamber to the inlet of another bath chamber separated by a 10-mm length of plexiglass. Complete exchange of bath solution was confirmed when the experimental reversal potential no longer changed with time and the current response to a step depolarization stabilized. For internal solution changes, a four-way mechanical valve controlled the internal solution just proximal to the inlet of the suction pipette. Internal solution changes were usually accomplished in less than 2 min.

The table shows the solutions (in mM) for the experiments in which sodium concentration was varied. Concentrations and not activities were used for the calculations since the activity coefficients of Na^+ and Cs^+ are nearly identical[33] and the ionic strength remained constant between the solution changes. All experiments were carried out at 10–16 °C. The pH was adjusted to 7.15 with CsOH.

Junction potentials between the internal and external solutions were corrected by the method of Oxford[30]. Briefly, the junction potential required to null the current across the circuit of Ag-AgCl pellet/3.0 M KCl agar bridge/internal solution/3.0 M KCl agar bridge/external solution/3.0 M KCl agar bridge/Ag-AgCl pellet was subtracted from the command potential. For solutions when the major cations were either Cs^+ or Na^+, the junction potential was less than 0.6 mV and was therefore neglected.

Solutions for studies in perfused cell experiments

Extracellular solutions (mM)						
Na	Cs	Ca	Mg	Cl	glucose	Hepes
15	134	3.0	1.0	157	5.5	10
45	104	3.0	1.0	157	5.5	10
120	29	3.0	1.0	157	5.5	10

Intracellular solutions (mM)								
Na	Cs	EGTA	F	H_2PO_4	glucose	Hepes	Mg-ATP	8, Br-cAMP
15	134	5.0	134	15	5.5	10	0.1	0.1
45	104	5.0	134	15	5.5	10	0.1	0.1
120	29	5.0	134	15	5.5	10	0.1	0.1

Patch clamp studies

Patch pipettes were made according to the method of Hamill et al.[21]. They were drawn in two stages using 1.5 mm outer diameter, fiber filled borosilicate glass capillary tubes (1B150F, WPI). The tip was fire polished to an inside diameter of about 1 μm and its resistance when filled with isotonic salt solution was about 5 Mohm. The tapered end of the pipette was coated with Sylgard 184 resin (Dow Corning) to reduce capacitance and electrical noise. The pipettes were usually filled with a solution of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES buffer at pH 7.4.

Freshly prepared single canine Purkinje cells were placed in a bath solution containing either 150 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4), and 10 mM glucose or 140 mM NaCl, 5.4 mM HCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, (pH 7.4) and 10 mM glucose at a temperature of 10–12 °C. The pipette was advanced until the cell membrane

barely dimpled, where it sealed after gentle suction. All studies were on cell-attached patches containing four or fewer channels and in which seal resistances were stable.

Single channel recordings were made with a Dagan 8900 patch clamp/whole cell clamp. Initial experiments were done with the Dagan headstage and were filtered at 1 kHz. Later experiments used a head stage constructed according to a custom design (J. Rae and R. Levis, Rush Medical College, Chicago, Ill). The signal was filtered at 2 kHz with an 8-pole Bessel filter (Frequency Devices, Inc.). A capacity compensation circuit was used to minimize the capacity transient, and further correction was performed during analysis by the subtraction of averaged sweeps without openings. Experimental protocols were controlled by an IBM-PC with a TM-100 digital-to-analog converter (Tecmar, Inc.). Data acquisition was at 10 kHz. The membrane patch was depolarized repetitively at 1 Hz to test potentials for 45 ms, from a holding potential that was usually −120 to −140 mV (assuming a resting membrane potential of 0 mV). Data were stored directly on floppy disk. Average single channel currents were derived from histograms of the set of all sampled current values during the test step. The open durations were measured as the time above half-amplitude currents[34]. Mean channel open times were obtained from histograms of open durations fitted by a single exponential using a least squares method. The first 0.5 ms was discarded for these fits because the frequency response of the recording system prevented accurate measurement of these very short openings. Latencies (waiting times) were measured from the onset of the clamp step to the first opening or to each opening.

Results

Adequacy of voltage control and internal perfusion

The parameters of importance for judging the quality of the voltage clamp are the speed of voltage control and the series resistance (R_s). Without R_s compensation, the decay of the capacity transient could be fitted with an exponential with a time constant of about 15 µs over a period of 6–7 time constants, and this was reduced to about 5 µs after R_s compensation. In general, the capacity current approached baseline noise level by 30–40 µs. If the cell was cooled to 10–15 °C to slow the onset of I_{Na} somewhat, it was not necessary for us to subtract the capacity current during onset of Na^+ current flow. The measured R_s after compensation was $< 3\ \Omega\ cm^2$, which compares favorably with the squid voltage clamp. To reassure ourselves that there were no significant voltage gradients in the segment of the cell studied, we also made separate impalements at the cell end with a fine-tipped KCl-filled micropipette. The transmembrane potential recorded was the same as the holding potential, ruling out any significant junction potentials, and the voltage deviation at the peak I_{Na} was 3–4 mV, as predicted by the R_s measurements[27]. Since both internal and external solutions were controlled and the P_{Cs}:P_{Na} ratio is very low (0.02), the current reversal potential could be predicted by the Nernst equation. In a set of three cells, the solutions were systematically changed from 120/15, through 45/15, 45/45, 15/45, and 15/120 (where, for example, 120/15 means Na_o = 120 mM and Na_i = 15 mM), the reversal potentials agreed within 1 mV of the predicted values (fig. 1). This result could have occurred only if the internal concentration of Na^+ was well controlled.

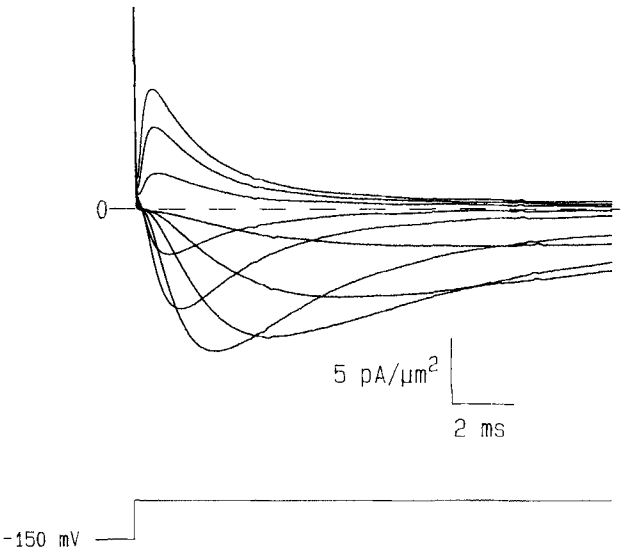

Figure 2. Family of I_{Na} measurements during steps to the indicated voltage from a holding potential of −150 mV. The currents were filtered at 5 KHz. No leak or capacity subtraction was made. 45 mM Na_o/15 mM Na_i. (Cell 45.03; temp. 10 °C).

Development of Na^+ currents

Depolarizing steps from a negative holding potential reached a threshold beyond which I_{Na} developed and decayed (fig. 2). Larger depolarizations resulted in larger and faster currents.

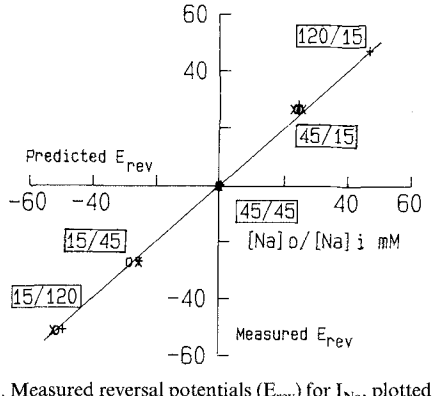

Figure 1. Measured reversal potentials (E_{rev}) for I_{Na}, plotted for comparison to E_{rev} predicted by the Nernst equation for the Na^+ gradients (Na_o/Na_i) in mM, indicated in the boxes. Five different gradients were used in three cells (x, o, +). The solid line represents the theoretical line calculated from the Nernst equation. (Cells 19-02, 19-03, and 21-04; temp. 14 °C).

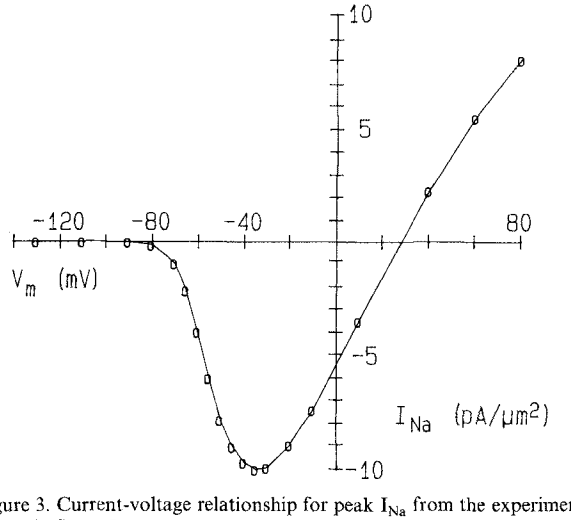

Figure 3. Current-voltage relationship for peak I_{Na} from the experiment shown in figure 2.

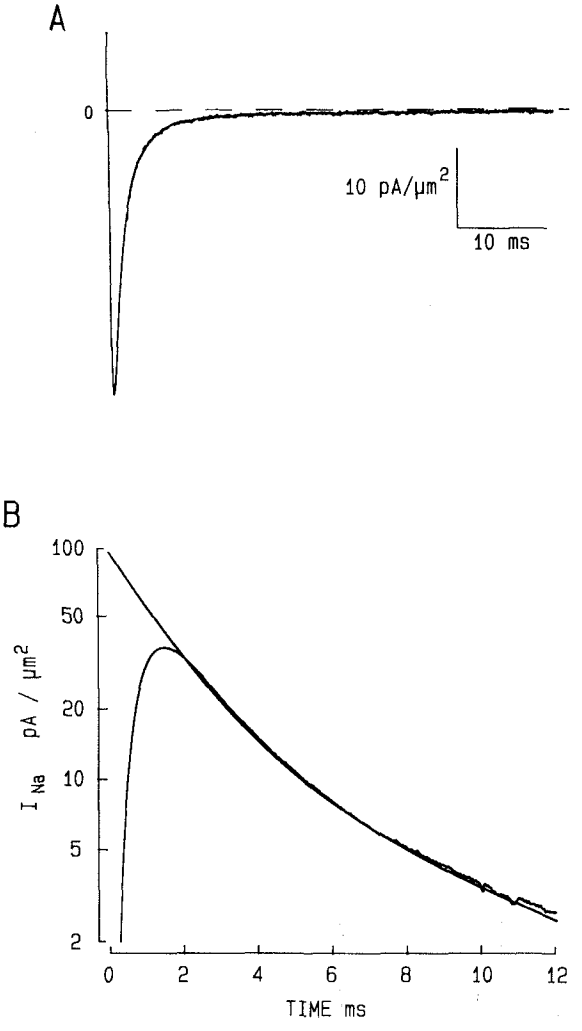

Figure 4. Decay of I_{Na}. Panel A illustrates a recording of I_{Na} (downward deflection) during a 100 ms depolarizing step to −50 mV from a holding potential of −150 mV for a cell in 120 mM Na_o/15 mM Na_i. The current was filtered at 5 KHz. A slowly decaying component of I_{Na} can be seen. A three exponential fit of the current time course yields a third exponential time constant of about 30 ms. This slow component represents only about 1–2% of the peak current. (Cell 37.02; temp 12.6 °C).
Panel B shows the early I_{Na} plotted upward on a logarithmic scale. The current decay shows clearly more than one exponential. The fitted line is a best 3-exponential fit. The first two exponentials have time constants of about 2 and 6 ms, and comprise 68% and 30% respectively of the peak I_{Na}.

This transient current has the general shape that has been well described for nerve Na^+ current, in that both its activation rate and its decay rate are speeded by depolarization. The interpretation of this behavior in terms of the Hodgkin-Huxley model is that there are two independent voltage-dependent processes, one for activation and one for inactivation. The current-voltage relationship shows that the peak I_{Na} was small in the threshold region and increases with depolarization. Further depolarization reduced the Na^+ gradient, and the peak current fell to zero at the Na^+ electrochemical reversal potential (fig. 3). The increase in the current in the threshold voltage region requires that more Na^+ channels be open and that they open more synchronously.

Sodium current decay

The Hodgkin-Huxley model assumes that activation and inactivation are independent. Their model predicts that I_{Na} decay will be by a first order process (single exponential decay). However, in the intermediate voltage range (from about −50 mV to 0 mV) cardiac I_{Na} decayed with a more complex time course. We can fit the I_{Na} decay over 100 ms with the sum of three exponentials, as shown in figure 4. There is a rapidly decaying component with a time constant of 1–2 ms at these temperatures that comprises most of the current decay. A second component has a time constant of 5–6 ms that completes most of the remaining current decay. Finally, there is also a very slowly decaying component that represents only 1–2% of the total current. Single channel recordings help in identifying the probable basis for this complex and slow current decay.

Single Na^+ channel properties

Single channel recordings were made with cell-attached patches containing NaCl as the charge carrier. After sealing, the pipette is set at a positive potential, so that the patch has a normal transmembrane electrical field. Most of the studies were done from a holding potential such that the field was equivalent to a hyperpolarized interior potential of −120 mV, in order to prevent resting inactivation of Na^+ channels. The patch recordings showed that depolarization resulted in the opening of one or more channels in the patch, with most openings occurring within a few ms of the onset of the step. The number of openings then rapidly fell, as expected for inactivation. Sometimes the channel did not open at all during the depolarizing step, indicating that the channel may inactivate without passing through the open state.

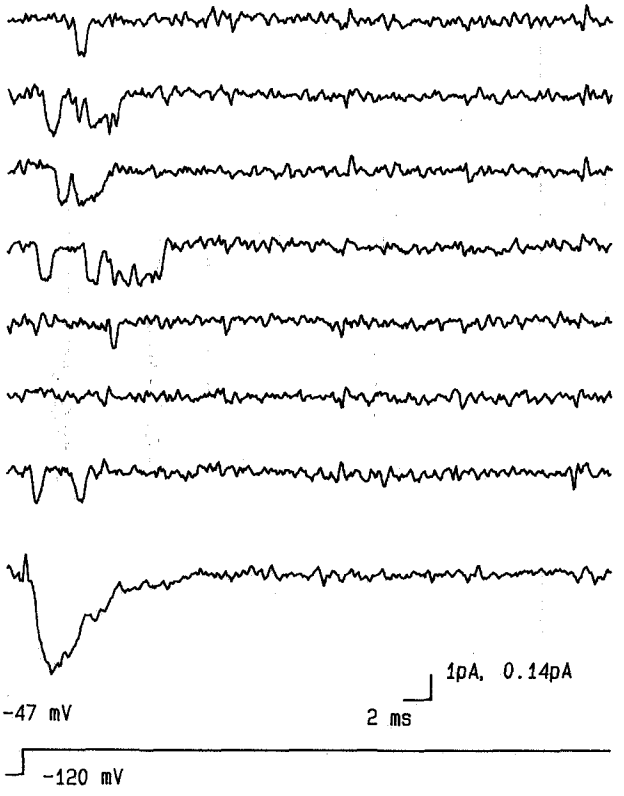

Figure 5. Recordings of single channel currents during sequential steps in a patch containing only one Na^+ channel. Membrane potentials in this experiment are relative to the cell resting potential, and are estimated to be from −120 mV to −47 mV. Na^+ current is recorded as a downward deflection of the current trace, and it is filtered at 1 KHz. The timing of onset of the step is illustrated below. The lowest current trace is an ensemble, representing an average of the currents of 109 traces. The 0.14 pA calibration refers to the ensemble current. (Patch 1/29).

Figure 5 illustrates representative recordings from a patch having only one channel. These recordings were from a series of depolarizing steps to an estimated potential of −47 mV for 45 ms. Evidence that these single channel events are indeed the elementary units of the total membrane I_{Na} is shown in the lower panel of figure 5. This is an ensemble current, obtained by averaging the single channel current recordings from a large number of depolarizing steps. The resulting current resembles the membrane I_{Na} in its activation and inactivation properties, emphasizing that the full kinetic properties of I_{Na} are found in each single channel.

The single channel characteristics of importance are the single channel current, the time of opening after onset of depolarization, and the duration of its opening. In general, the amplitude of the current is the same for all openings, yielding a constant single channel conductance. At 10–12 °C the conductance was 12.8 pS, measured between −50 and −15 mV. The duration of opening was variable. A histogram of open durations shows an exponentially distributed open duration, with a mean open time (time constant of the exponential function) of 1–2 ms (fig. 6). The mean open time was voltage-dependent, with largest values near −40 mV, the voltage at which the peak I_{Na} is largest. The mean open times were independent of the time at which the openings occurred in the step. The two values of single channel current and mean open time allow us to calculate that a typical channel opening permits entry into the cell of less than 10^4 Na^+ ions.

The time of opening after depolarization is called the waiting time or the latency. The time at which the first opening occurs after depolarization is called the 'first latency', while the times to all openings are identified here as 'all latencies'. A histogram of all latencies is shown in the lower panel of figure 7. The top of figure 7 shows records from a patch during steps to −50 mV; below this is an ensemble from this patch. The latency plot below is from the sweeps that make up the ensemble. It is clear from the comparison that it is the time course of the frequencies of channel opening that largely determines the time course of I_{Na}.

A comparison of the single channel records with the ensembles in figures 5 and 7 shows that sometimes the first channel opening during a step depolarization occurs after the peak of the ensemble current. This supports the proposal that the rate of channel activation, determined by the time to the first opening, is close to the rate of inactivation determined from the ensemble.

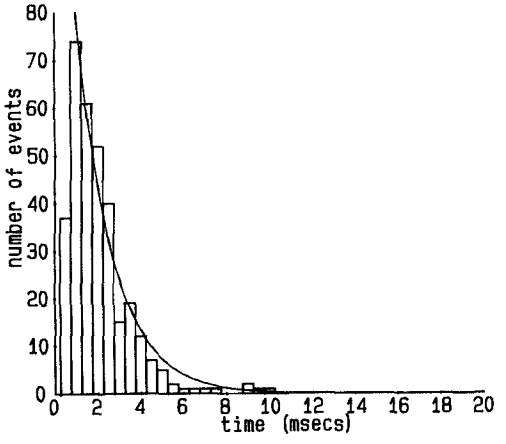

Figure 6. Open durations of single channel openings from the experiment shown in figure 5. The events are grouped in bins of 0.5 ms width. The first bin is not accurate because short openings are missed as a consequence of the necessary filtering. The solid line is an exponential fit to the histogram with a time constant of 1.7 ms.

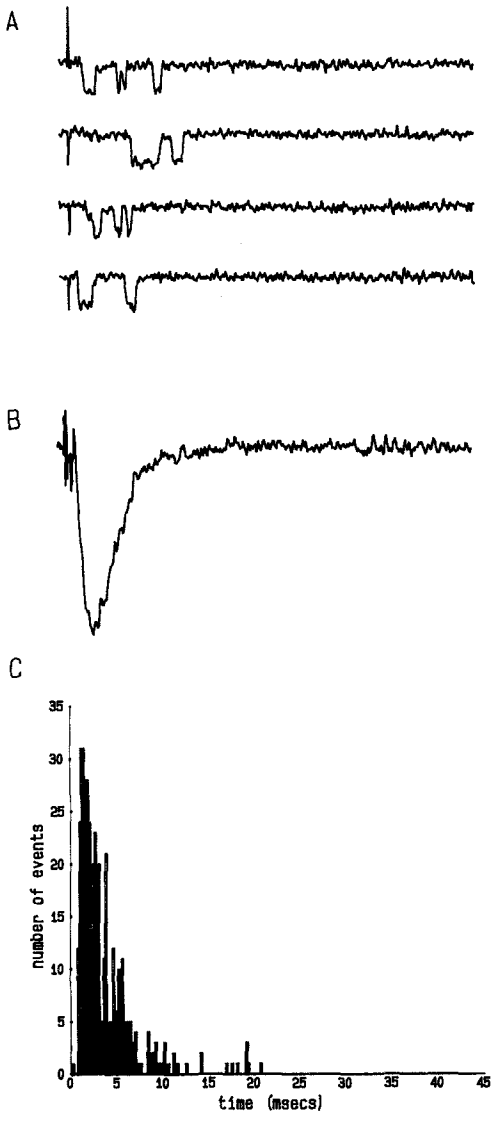

Figure 7. Relationship between single channel recordings (panel A), ensemble current (panel B), and the histogram of all latencies (panel C). See text for explanation.

The recordings in figure 5 were obtained from a patch with only one Na^+ channel. Verification that the patch contained only one channel was obtained by use of the Patlak-Horn method[31]. As is evident at this step potential, many depolarizations are associated with multiple successive openings, indicating that the channel has the ability to open and close several times before inactivating. This can also be seen in figure 8, which compares a histogram of first latencies (top panel) to a histogram of all latencies (bottom panel) for the single channel patch. This reopening of the channel contributes to a second slower component of I_{Na} decay, corresponding to the second exponential component.

The small very slowly decaying I_{Na}, however, is not explained by channel reopening. Patlak and Ortiz[32] reported the occasional occurrence of very long openings of Na^+ channels in rat ventricular myocytes, and we searched for such events in the Purkinje cells. Figure 9 shows examples of these very long openings, which lasted 30–40 ms. In one instance, an opening persisted for more than one second. These long openings represented less than 1% of the channel openings seen, but they might be sufficient to account for the very long, small membrane I_{Na} seen in figure 4.

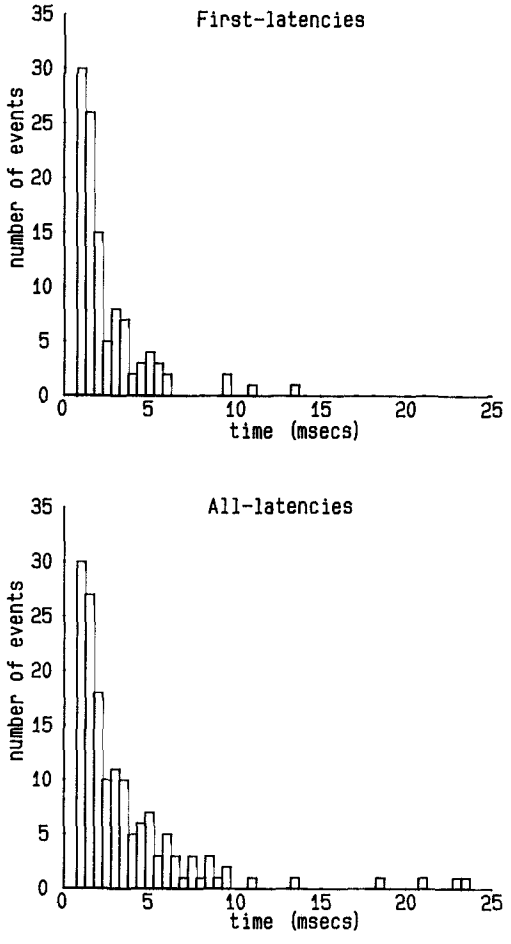

Figure 8. Histograms of first latencies and all latencies for the patch with one channel. The step potential was to −47 mV (estimated), as for the ensemble in figure 5.

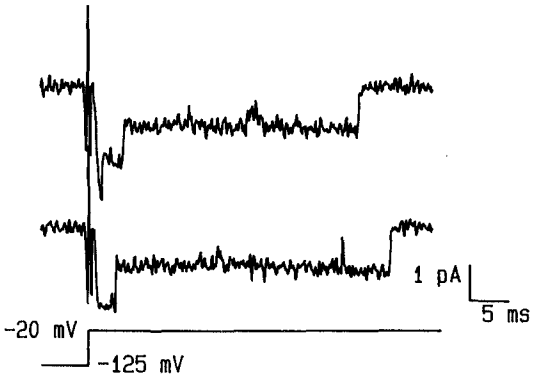

Figure 9. Recording of single Na channel currents with a patch clamp. The patch had at least 3 channels. During a 45-ms step from a holding potential of −125 mV to −20 mV a channel opened and remained open for many milliseconds. Filtered at 2 kHz. (Patch 10/10B).

Discussion

The availability of single cardiac cells has been a key element in achieving dramatic progress in voltage clamp techniques for the study of Na^+ channels in cardiac muscle. The characteristics of the voltage clamp used in our lab demonstrate voltage control equivalent to that achieved for the squid axon. Furthermore, this technique permits control and systematic alteration of the intracellular ionic contents of the cell. These advances now make possible quantitative study of cardiac membrane currents similar to those in nerve. The methods also allow investigation of the role of cytoplasmic processes in the regulation of membrane channels.

Membrane currents are the result of the opening and closing of many thousands of channels of diverse types. While inferences can be made regarding single channel behavior, these depend on several assumptions that cannot be directly tested by membrane current study. The second important new technique is single channel recording with the patch clamp. This method permits detailed study of one or a few channels of a single type. Full understanding of membrane electrical events requires that the two methods of study yield the same description of channel behavior.

We have combined the membrane voltage clamp in a perfused Purkinje cell with single channel recording of Na^+ channels with cell-attached patches in the same cell type. I_{Na} kinetics determined from the time course of membrane current demonstrate impressive similarity of behavior of Na^+ channels between cardiac cells and nerve. One area of some difference is in current decay, the process usually called inactivation. Squid axon I_{Na} decays with a single exponential, but some nerve cells[12,19] show I_{Na} decays by two exponentials. Multiexponential decay has also been described for cardiac muscle[8,25,32]. Most cardiac I_{Na} decays rapidly, similar to nerve, but there are some very slowly decaying components that could be important to the maintenance of the cardiac action potential plateau[11] and to Na^+ entry into cardiac cells. Addition of information from single Na^+ channel recording to our understanding of Na^+ channel behavior has been of great value. Na^+ channels appear to have only two conductance states – open or shut. When open, they all have nearly the same conductance, although there may be a small population of Na^+ channels with a low conductance level[34a]. The kinetic properties of I_{Na} are contained in each channel, as indicated by the similarity between the ensemble current and the membrane current. The channel exists in at least three states: closed, open, and inactivated (fig. 10).

There are probably several closed states through which the channel must pass before opening. The closed states are favored at more hyperpolarized potentials. Depolarization increases the probability that the channel will pass to the open state. From the open state the channel may switch to the inactivated state or it may return to the closed state and reopen. The channel may inactivate from the closed state, as well as the open one. The most useful model at this time is a series of states related by stationary voltage-dependent transition rates. Calculation of the rates for simple models is possible by use of maximum likelihood or minimal entropy methods (unpublished observations), indicating that the principal voltage-dependence of the transition rates is between the closed states and from the closed state to the open one[36]. The transition rate from open to inactivated state is slightly voltage-dependent.

This model accounts for the membrane current in a way not anticipated by the Hodgkin-Huxley model[1]. One example is the decay of I_{Na}. The principal decay rate is determined by the rate of activation of channel openings after the peak of

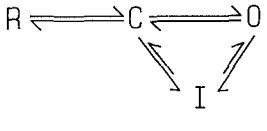

Figure 10. A state model of the Na^+ channel. The channel is predominantly in the R state at rest. Upon depolarization it passes through a C (closed) state to the O (open) state. Inactivation (I state) may occur from either the O state or the C state. See text for further explanation.

the membrane current. It is also influenced by the tendency of channels to open repeatedly at some voltages. Finally, an unusual state exists for Na^+ channels that can occasionally be seen. The channel in this state behaves as if the inactivation process were missing, so it either does not close or it repeatedly opens and closes without inactivating for many milliseconds. This state may be very important in cardiac muscle by determining I_{Na} during the action potential plateau. An intriguing further question is whether this state is regulated by the cell.

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