A Comparison of Calcium Currents in Rat and Guinea Pig Single Ventricular Cells

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SUMMARY. The slow inward calcium currents were compared in rat and guinea pig heart using enzymatically dissociated, single ventricular cells. A single electrode voltage clamp was used, in which current and voltage were sampled separately using a time-sharing method. Spatial homogeneity of membrane potential during peak slow inward calcium current was assessed by measuring the potential with two microelectrodes 50 μm apart; the potentials were within 3 mV of each other. Peak current-voltage relations for slow inward calcium currents were similar for the two species, but the individual currents showed a faster time course of inactivation and a slower time course of recovery from inactivation for rat, compared with guinea pig. The potassium current blockers 4-aminopyridine and tetraethylammonium chloride did not produce significant effects on the net membrane currents recorded at the holding potentials (−50 to −40 mV) used in this study. The underlying mechanism for the inactivation of the slow inward calcium currents was explored using a double pulse procedure. In both rat and guinea pig heart cells prepulses to very positive potentials were associated with a partial restoration of the slow inward calcium current in the following test pulse. In addition, internal ethylene glycol-bis N,N,N′,N′-tetracetic acid or substitution of barium for calcium slowed the rate of inactivation of the slow inward calcium current in rat heart cells. Calcium activation of nonspecific currents was thought less likely to have produced these results due to the lack of effect of depolarizing prepulses on hyperpolarizing test pulses. A calcium-dependent component of inactivation may be responsible for the differences observed in both the inactivation and the recovery time courses of the slow inward calcium current in these species. (Circ Res 54:144–156, 1984)

THE plateau of the vertebrate cardiac action potential is associated with an influx of Ca ions, which serves to initiate muscle contraction (see reviews by Reuter, 1973, 1979). However, marked differences can be observed between certain mammalian species in respect to the shape and duration of the cardiac plateau. A striking example of such variation can be found in a comparison of the ventricular action potentials recorded from rat and guinea pig hearts (Coraboeuf and Vassort, 1968). In the rat, the plateau is brief and occurs at more negative potentials, as compared to the guinea pig action potential which displays a long-lasting plateau at positive potentials. This observation leads one to question: Do the dissimilar plateaus of these action potentials result solely from differences in the voltage-dependence or kinetics of the slow inward Ca++ currents, or are additional currents involved?

In this paper, and in the following paper (Josephson et al., 1984b), the membrane currents which contribute to the plateau of the ventricular action potential in rat and guinea pig single ventricular cells will be compared. The present paper will focus on the properties of the slow inward Ca++ currents (iso) in these two species. The following paper will present a description of an early outward current (iso), found only in rat heart cells, which appears to play an important role in determining the unusual shape of the rat action potential.

A preliminary account of these experiments has been presented (Josephson et al., 1982).

Methods

Cell Dispersion

The method used for enzymatic dissociation of single heart cells was similar to that reported previously (Powell and Twist, 1976; Powell et al., 1980). In brief, adult rats or guinea pigs were killed by cervical dislocation. The chest was opened, the heart was rapidly removed and was washed twice in Krebs solution (composition in mM: NaCl, 140; KCl, 5.4; MgCl₂, 1.2; CaCl₂, 3.6; glucose, 10; HEPES, 5; adjusted to pH 7.4 with NaOH). Retrograde perfusion through the aorta was performed at 37°C using a Langendorff apparatus. The elapsed time from excision of the heart to cannulation and perfusion was less than 1 minute. The heart was perfused with nominally Ca++-free Krebs solution ([Ca++]; 10–20 μM) at a rate of 12 ml/min for 5 minutes. After this period, collagenase (Sigma type II), at a concentration of 0.04%, was added to the Krebs solution and was recirculated through the heart for 10–20 minutes. The heart then was removed from the cannula, the atria were cut off and discarded, and the ventricles were finely minced into 1- to 2-mm pieces by a mechanical tissue chopper. Incubation in the collagenase-containing solution proceeded for an additional 5–15 minutes, during which time gentle agitation was provided using a 10-ml pipette. The resulting cell suspension was filtered through a 250-μm nylon mesh and centrifuged (22 g). The cell pellet was resuspended in 20 ml of Krebs solution (O-
Ca\textsuperscript{2+}} and recentrifuged. This washing procedure was repeated a second time to remove any residual collagenase. The final cell pellet was suspended in Krebs-Ringer containing 0.5 to 3.6 mm CaCl\textsubscript{2} and was kept at 4°C.

Alternatively, the dispersed cells were washed and stored in "Power Soup" or "Kraftfrühne" (Iseben and Klockner, 1980). The composition of this medium was (mm) KCl, 80; KH\textsubscript{2}PO\textsubscript{4}, 30; Na\textsubscript{2}ATP, 5; MgSO\textsubscript{4}, 5; pyruvic acid, 5; succinic acid, 5; creatine, 5; Na\textsubscript{2}EGTA, 0.1; adjusted with KOH to pH 7.2. The cells were generally used after incubation for 2–4 hours; however, they could be stored in Power Soup at 4°C for periods up to 5 days.

**Electrophysiology**

**Glass Microelectrodes**

Conventional glass micropipettes were made on a D. Kopf vertical puller. They were filled with filtered solutions of 3 mM CsCl; (3 mM KCl or 3 mM K\textsuperscript{+}-citrate was used for studies of the outward currents; see following paper), and had tip resistances of 10–50 MΩ. Lower resistance pipettes (3–10 MΩ) were used to improve the speed of the voltage clamp for tail current measurements and for pressure injection experiments. These pipettes were filled with 150 mM KCl.

**EGTA Injection**

In experiments designed to test the effect of the [Ca\textsuperscript{2+}] on the inactivation of I\textsubscript{Ca}, a Ca-chelating agent was pressure-injected into a heart cell through the same electrode used for voltage clamp. For this purpose, pipettes were filled with filtered (Millipore Corp) solutions containing 5 mM ethylene glycol-bis N,N,N',N'-tetraacetic acid (EGTA) in 150 mM KCl, buffered to pH 7.2 (resistances of 3–10 MΩ). Hydraulic pressure was applied to the solution in the pipette (held by a WPI holder, designed for pressure injection) by advancing a microliter syringe.

**Voltage Clamp**

Two approaches were employed initially for voltage clamping single heart cells: a standard two-microelectrode voltage clamp and a single-microelectrode (switching) voltage clamp. Both were used to study the flow of current through cell contraction and made it difficult to maintain the double impalement. This led to more frequent use of a single-microelectrode clamp method for studying I\textsubscript{Ca} (see Results). A Dagan 8100 amplifier (Minneapolis, Minn) was employed for the single-electrode voltage clamp experiments. This instrument switches rapidly (from 0.5 to 25 kHz) from voltage recording to current passing through a single microelectrode. The voltage is measured only when the discontinuous or chopped current is zero, yielding an accurate measurement of membrane potential. To determine the maximal response time of the Dagan, a model heart cell membrane (50 MΩ resistance in parallel with 200 pF) in series with a model electrode (10 MΩ in parallel with 5 pF) was voltage clamped. Under these conditions, the fastest risetime of a 40-mV voltage step was 0.5 msec and the capacitive current decayed (90%) in 0.7 msec. In practice, the speed of the voltage clamp was limited by the RC characteristics of the microelectrode tip, which determined the maximal switching frequency. Switching frequencies ranging between 10 and 20 kHz (depending on the individual electrode) were used without attenuation of either the voltage or current signals. These high switching frequencies were made possible by careful adjustment of the capacity compensation (0–10 pF) and phase controls. The overall gain of the voltage clamp ranged from 5 to 50000X, and was adjusted to provide the fastest risetime during the voltage step and maximal control of the cell voltage (within 2% of the command voltage) without producing oscillations.

For the two-microelectrode voltage clamp (used in preliminary studies for the following paper), the cell potential was monitored with a microelectrode which was connected to one channel of a WPI KS-700 electrometer, and compared with the command potential using a high-grain differential amplifier (Tektronix 502). The output of this amplifier was fed through the bridge circuit of the electrometer to the current-passing microelectrode. The bridge circuit also allowed potential measurement through the current microelectrode. Membrane currents were measured with a virtual ground circuit connected to the bath via an agar bridge.

The command signals for both the Dagan and the two-microelectrode voltage clamp were obtained from a WPI interval generator and pulse modules which were connected to buffered attenuators. Voltage steps were applied at a rate of 12/μm unless otherwise noted.

**Experimental Setup**

A small (0.5-ml) plexiglass cell chamber was mounted on the stage of a Leitz inverted microscope. Solutions were superfused through the chamber at a rate of 5 ml/min. Microelectrodes were held by WPI Ag:AgCl reversible half-cells which were connected to the input probes of the Dagan or WPI KS-700 amplifier. The probes were mounted directly to two Narashige pneumatic micromanipulators. The entire apparatus rested on a vibration-free table. All experiments described were performed at room temperature (18–22°C) in order to slow the activation kinetics of I\textsubscript{Ca} and, thereby, improve the voltage control.

**Agents**

The following agents were added to the solution reservoirs from concentrated stock solutions during some experiments to give the desired final concentrations: tetrodotoxin (TTX) (10–6 m) to block the fast Na\textsuperscript{+} current; CoCl\textsubscript{2} (3 X 10–3 M) or CdCl\textsubscript{2} (10–6 m) to block L\textsubscript{Ca} 4aminopyridine (4-AP) (2 X 10–3 M) and tetraethylammonium chloride (TEA) (2 X 10–3 m) were used to test for the presence of transient, Ca-activated, or delayed outward currents (Kenyon and Gibbons, 1979; Meech, 1978).

**Data Recording and Analysis**

Current and voltage signals were displayed on a Tektronix D11 storage oscilloscope and were photographed with a Polaroid camera. In addition, current and voltage signals were digitized (at 50 or 100 μsec per point) on a DEC LSI 11 microcomputer for storage, and transferred to a PDP 11/70 for plotting and analysis. The time constants of inactivation of I\textsubscript{Ca} were determined with a multi-exponential curve-fitting program which minimizes the sum of the squared error using a Marquardt algorithm.

**Results**

**Membrane Potentials**

With our procedure, the yield of rod shape cells with uniform striations was approximately 30–50% per preparation in normal Krebs solution containing 3.6 mM Ca\textsuperscript{2+}. Rat ventricular cells measured approximately 100 μm in length and 20 μm in diameter,
FIGURE 1. Action potentials recorded from typical guinea pig (part A) and rat (part B) single isolated ventricular cells in response to brief depolarizing current pulses. Horizontal traces indicate the zero potential. Note the difference in the time calibration for parts A and B. Part C. Electroneutral potentials recorded at two sites in a single rat ventricular cell (recorded immediately after impalement with the second electrode). Intracellular hyperpolarizing current (I) was applied through the microelectrode connected to the bridge circuit (V1); voltage was also recorded by an independent microelectrode (V2). Interelectrode distance was approximately 50 μm; cell length was approximately 100 μm.

The cells of both species were mechanically quiescent, both before and after impalement with microelectrodes, and they displayed stable resting potentials of −80 to −90 mV (−82.7 ± 3.6; n = 12) for rat. −85.4 ± 4.3 for guinea pig (n = 8). Action potentials were elicited with brief (0.5- to 5-msec) depolarizing current pulses. Typical examples from a guinea pig and a rat heart cell can be seen in Figure 1, A and B. The action potentials obtained from isolated single rat and guinea pig heart cells were similar to those recorded in the intact heart; that is, they had large resulting potentials, fast rates of rise, and the characteristic shapes and durations for their species (see Coraboeuf and Vassort, 1968).

Following maintained depolarization (by intracellular current injection) to a region between −50 and −40 mV, to inactivate the fast Na+ conductance, current steps generated slowly rising (<10 V/sec) action potentials which were insensitive to tetrodotoxin (10−5 M) (TTX) (for an example of a slow action potential, see Fig. 1, following paper). Both the normal and the slow action potentials were accompanied by twitch contractions. The preceding observations provided evidence that the slow inward Ca++ current system appeared to be functional after cell isolation.

Resting Space Constant

In preparation for voltage clamp experiments, a determination of the space constant for the resting membrane was conducted. For these experiments, two microelectrodes were employed: the first simultaneously injected current and recorded potential (V1) via an active bridge circuit, and the second microelectrode independently recorded the potential V2 at a known distance from the first. The results of such an experiment are shown in Figure 1C in which 0.5 nA was injected through V1 (located near the middle of a rat heart cell of approximately 100 μm length) and V2 was located 50 μm from V1. If we assume that the heart cell membrane can be modeled as a short cable (of length L), terminated by open circuits at both ends, then the steady state response is given by:

\[ V_2 = V_1 \frac{\cosh[(L - X)/\lambda]}{\cosh[L/\lambda]} \]  

(1)

where X is the interelectrode distance and λ is the cell length constant (Weidmann, 1952; see also Brown et al., 1981a; Powell et al., 1980). Using this equation, the length constant calculated from the experiment shown in Figure 1 was 375 μm. Values (ranging from 320 to 360 μm) were obtained in three other cells; however they are, most probably, underestimates of the true space constant, since the introduction of a second microelectrode had a deleterious effect on the membrane which was detected as a reduction of the input impedance and irreversible cell contracture (Rm). Before the second microelectrode impalement, Rm (measured through the bridge electrodes) was typically 40–80 MΩ (measured near the resting potential of approximately −80 mV), and after the second impalement, Rm progressively fell to values of 10–20 MΩ, reflecting the additional leakage pathway.

Voltage Clamp

Due to the difficulties in doubly impaling single mammalian heart cells in Ca++-containing solutions, voltage clamp experiments were conducted using a single-electrode (switching) voltage clamp. The single-microelectrode impalement introduces relatively little damage, as judged by a high input impedance, large resting potential, and long plateau duration. These characteristics are indicative of a relatively high shunt resistance between the microelectrode tip and the membrane which minimizes the amount of leakage current flowing across this site under voltage clamp conditions. Another advantage of the one-electrode method for the study of I Na is that the heart cell is less damaged by contraction than with the two fixed microelectrodes of the conventional voltage clamp. In addition, since a voltage clamp with discontinuous feedback measures the cell potential only in the absence of current flow, potential measurement is effectively independent of changes that may occur in any resistance in series with the membrane (Brenecke and Lindemann, 1974). A critical assessment of the advantages and limitations of the single-electrode voltage clamp methods, using Aplysia neurons, has been presented by Wilson and Goldner (1975).
The Slow Inward Current

After the successful microelectrode impalement of a single heart cell (maintenance of a steady resting potential of —80 to —90 mV) and the generation of typical action potentials, the voltage clamp was switched on and the holding potential was set to —50 or —40 mV. The holding potential was adjusted to inactivate the fast Na⁺ conductance completely; in addition, in several experiments, TTX (10⁻⁵ M) was used to block the fast Na⁺ channels. Since it was found that there were no differences discernible in the slow inward currents elicited from this holding potential after the addition of TTX, subsequent experiments were done in its absence.

As can be seen in Figure 2, the application of depolarizing voltage clamp steps (in 10-mV increments from the holding potential) led to the appearance of voltage-dependent, slow inward currents (Iᵢ) in both rat (part A) and guinea pig (part B) ventricular cells. The time course for the activation of Iᵢ was rapid; the half-time for the activation at 0 mV was 2.9 msec in the rat, as compared to 4.6 msec in the guinea pig. Slow inward currents were routinely recorded from over 100 single cells, and the time-to-peak Iᵢ was significantly faster than that reported previously for the activation of Iᵢ in most multicellular preparations of cardiac muscle (see review by Carmeliet and Vereecke, 1979, but see also Noma et al., 1980). However, the true activation time for Iᵢ may be even more rapid (Lee and Tsien, 1982) since the switching voltage clamp circuit, when used with high resistance microelectrodes provides limited resolution of kinetics of activation.

Spatial Control

In several experiments, the degree of spatial control present during the peak Iᵢ was directly evaluated using a second, independent voltage-sensing micro-

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**Figure 2.** The slow inward currents obtained from single rat (part A) and guinea pig (part B) ventricular cells. Holding potential in part A was —50 mV (holding current 0.5 nA); in part B, it was —40 mV (holding current 0.6 nA). Voltage steps were applied in 10-mV increments. Current traces were digitized and subsequently replayed on an oscilloscope for photography.

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**Figure 3.** A test of the voltage control during the flow of Iᵢ in a rat ventricular cell. The command voltage was stepped from —50 to —10 mV. Trace labeled A is the transmembrane potential recorded by the microelectrode connected to the switching voltage clamp circuit. This microelectrode was positioned near the center of the cell. Trace B is the transmembrane potential recorded by an independent microelectrode, positioned near one end of the cell, approximately 50 μm from the voltage clamping microelectrode. Trace C is a differential record (trace A—trace B) which demonstrates the spatial and temporal aspects of the voltage control. Trace D is a record of the membrane currents during the voltage step. Vertical calibration is 20 mV in traces A and B, 10 mV in C, and 5 nA in D. Horizontal calibration is 10 msec in traces A–D.
voltage traces from an experiment in which the command voltage was stepped from —50 to —10 mV. Trace A is the voltage recorded by the voltage clamping (switching) microelectrode, trace B is the voltage recorded near the end of the cell by the independent microelectrode, and trace C is the difference trace (trace A minus trace B). Trace D shows the membrane current. In this case, the deviation in the potential near the end of the cell was 3.0 mV. Thus, voltage control during the flow of peak $I_{\text{si}}$ was adequate in these experiments. The relatively long-setting time of the voltage step, as measured near the end of the cell (trace B), precluded a quantitative assessment of the kinetics of activation of $I_{\text{si}}$ in these experiments.

**Current-Voltage Relation of $I_{\text{si}}$**

The current-voltage relations for $I_{\text{si}}$ obtained from a typical single rat (part A) and guinea pig (part B) ventricular cell, are shown in Figure 4. A holding potential of —50 or —40 mV was used in this study since, at these potentials, there was no inactivation of $I_{\text{si}}$, and it was below the activation threshold for $I_{\text{si}}$. In both species, the threshold for the peak $I_{\text{si}}$ (circles) is near —35 mV and the maximum peak $I_{\text{si}}$ occurs at approximately +10 mV. The magnitude of the maximum $I_{\text{si}}$ ranged from 1 to 5 nA and might be accounted for by the variations in the dimensions of single cells or, more likely, the use of two cell aggregates. The net inward current crossed the voltage axis at +50 mV (in part A) and +60 mV (in part B). However, this need not imply that $I_{\text{si}}$ is an outward current beyond these potentials. The apparent reversal of $I_{\text{si}}$ may be due to the contribution of a time-independent, outwardly rectifying leakage current, whose magnitude becomes relatively large at high voltages with large depolarizing voltage steps. When the currents obtained after the administration of CdCl$_2$ (10$^{-4}$ M) were digitally subtracted from the net $I_{\text{si}}$, they yielded similarly shaped I-V curves up to ~+20 mV (e.g., with no shift in the voltage for peak $I_{\text{si}}$). However, the uncertainty concerning the effects of Cd$^{++}$ on the outward leakage current at high potentials (i.e., above +20 mV) led to the use of the net I-V curves to describe the behavior of $I_{\text{si}}$ (see Brown et al., 1981).

An example of the blocking effect of Cd$^{++}$ (10$^{-4}$ M) on $I_{\text{si}}$ in a rat heart cell is shown in Figure 5A. The control trace is a digitized record of the current elicited by a voltage step from —50 to —10 mV. Exposure to Cd$^{++}$ for 2 minutes completely abolished the inward current, leaving a small, time-varying outward current. Similar results were obtained with Cd$^{++}$ in guinea pig cells.

**Figure 4.** The current-voltage relationships for the rat (part A) and guinea pig (part B) slow inward currents. Circles represent the peak values of $I_{\text{si}}$ (measured as the difference from the holding current level); squares represent the values of the maintained component of $I_{\text{si}}$ measured at 70 msec.

**Figure 5.** Part A: effects of Cd$^{++}$ (10$^{-4}$ M) on $I_{\text{si}}$ in a rat heart cell. Lower trace: $I_{\text{si}}$ elicited by a voltage step from —50 to —10 mV. Upper trace: residual current recorded 3 minutes after the addition of CdCl$_2$ (10$^{-4}$ M). Part B: Steady state current-voltage relation after blockade of $I_{\text{si}}$ with 10$^{-4}$ M Cd$^{++}$. Holding potential was —50 mV.
The fact that the current traces appeared flat after Cd++, even during voltage steps of several hundred milliseconds, as well as the absence of any significant effect of K+ channel blockers (such as 4-AP and TEA) on the net I\textsubscript{Na} suggests the possibility that a delayed K+ current may be absent or minimal in these two species. Additional evidence suggesting that a delayed K+ current does not add a major contribution to the net current is the absence of outward tail currents upon the repolarization of long duration (350 msec) depolarizing steps to the holding potential (−50 mV). However, anomalous or inward rectification of the background current in the voltage region between −60 and zero mV was evident in both rat and guinea pig heart cells even after blockade of I\textsubscript{Na} with Cd++, as can be seen in the steady state I-V curve for the net currents after removal of the Cd++-sensitive current (Fig. 5B).

Another possible source of contamination of the net currents was a transient outward current, which was found to be present in the rat ventricular cells, but not in the guinea pig ventricular cells (see accompanying paper). This current, however, was almost completely inactivated at the holding potentials (−50 to −40 mV) used in this study. In addition, these experiments were conducted in the presence of 4-AP, which would block any residual transient outward current. Thus, it is unlikely that the transient outward current can account for the differences in the I\textsubscript{Na} seen in the rat and guinea pig ventricular cells, under these conditions.

**Maintained Component of I\textsubscript{Na}**

A most conspicuous difference between the rat and the guinea pig I\textsubscript{Na} was found in the magnitude of the maintained component of I\textsubscript{Na}, which may be either slowly inactivating, or non-inactivating. The latter has also been termed the "steady state" I\textsubscript{Na} and is, presumably, a reflection of the degree of overlap of the steady state inactivation curve with the activation curve (Reuter and Scholz, 1977; Kass et al., 1976). As can be seen in Figure 2, the decay or inactivation of I\textsubscript{Na} in the guinea pig is not complete at certain potentials (i.e., −30 to 0 mV). Indeed, in some cases, this steady state I\textsubscript{Na} persisted without inactivation for voltage steps lasting several seconds. The magnitude of the maintained component of I\textsubscript{Na} (measured at 70 msec, i.e., not steady state for all potentials) in a guinea pig heart cell is plotted in Figure 4B. In comparison, the rat (Fig. 3A) displayed only a small maintained I\textsubscript{Na}. It seems reasonable to assume that this primary difference in I\textsubscript{Na} is reflected in the different action potential configurations displayed in these two species.

Therefore, the following experiments were conducted in order to ascertain the amount of steady state I\textsubscript{Na} predicted, as well as to gain information concerning the voltage dependence of activation and inactivation (Hodgkin and Huxley, 1952). The voltage dependence of activation for I\textsubscript{Na} (d\textsubscript{a}) was estimated in rat heart cells by a tail current method, as shown in Figure 6. Since the capacitive currents (r for decay of 250 msec) contaminate the early values for the I\textsubscript{Na} tails, isochronal measurements (at 1 msec after repolarization of the voltage step) of the tail currents were made. In doing this, we have assumed that the values at 1 msec are proportional to the instantaneous values of the current at a given potential. The decay of the tail currents at −50 mV was fit by a single exponential with a r of 1.7 to 1.9 msec following steps from −30 to −50 mV. The magnitude of the inward tail currents flowing at −50 mV (following depolarizing voltage steps) were normalized against the maximum tail currents (elicited at +50 mV) and plotted as a function of potential. It is interesting to note that in these experiments activation of I\textsubscript{Na} did not become maximal at 0 mV, as has been reported for multicellular preparations of cardiac muscle (see Carmeliet and Vereecke, 1979), but it continued to increase up to +50 mV (see Brown et al., 1981c). The steady state inactivation variable, f\textsubscript{a}, was studied by applying 2-second conditioning prepulses to potentials between −50 and +20 mV. The potential was then stepped to +20 mV; a voltage which almost maximally activates I\textsubscript{Na}. After normalization, the data were plotted in Figure 7 on the same axes as the d\textsubscript{a} curve. The maximal degree of overlap between d and f is about 0.3, and occurs at a potential of approximately −18 mV. If it is assumed that both activation and inactivation of I\textsubscript{Na} represent first-order processes, then the largest value predicted for the steady state I\textsubscript{Na} would be only 9% of the maximal peak I\textsubscript{Na}. This predicted value agrees well with the experimentally obtained value of 10% at −20 mV (see Fig. 4A), since, at 70 msec, the
CONTROL
EGTA
0.6nA
10 msec

FIGURE 7. Part A: the effects of intracellular EGTA on the decay of $I_{\text{Na}}$ in a single rat heart cell. EGTA was pressure injected into the cell during voltage clamp. Top trace shows the control current obtained during a voltage step from $-50$ mV to $0$ mV (holding current $0.9$ nA). Lower trace shows the current $3$ minutes after injection of EGTA.

Part B: the effect of Ba ion on the inactivation of $I_{\text{Na}}$ during a voltage step from $-50$ to $-10$ mV in a rat myocyte. Trace 1 shows $I_{\text{Na}}$ recorded in Tyrode's solution containing $3.6$ mM Ca$^{++}$. Traces 2-8 show the currents (superimposed) at $5$-second intervals after switching the superfusion to Ba (3.6 mM) Tyrode. Holding current, $0.3$ nA.

Current (at this potential) is approaching its steady state value.

Time Course for Inactivation of $I_{\text{Na}}$

As mentioned previously, the $I_{\text{Na}}$ in the rat and guinea pig cells appeared to differ in time course for decay or inactivation. The computer curve-fitting algorithm described in Methods was employed for extraction of the time constants for the inactivation of $I_{\text{Na}}$. Using this method, it was determined that a two time constant model provided the best fit to the current traces. As can be seen in Table 1, several trends are evident in the values for the time constants as a function of the clamp potential ($V_m$). The fast time constant, $\tau_1$, in both the rat and guinea pig cells has values that were large near threshold, became smaller near the voltages for maximal $I_{\text{Na}}$ (0 to $+10$ mV), and then become large again at more positive potentials. The values for the second time constant of inactivation, $\tau_2$, were always larger in the guinea pig than the rat.

Mechanism for $I_{\text{Na}}$ Inactivation

There is a substantial body of evidence suggesting that the Ca$^{++}$ currents flowing across various excitable membranes may display Ca$^{++}$ current-dependent inactivation (Tillotson, 1979; Eckert and Tillotson, 1981; Brehm et al., 1980; Brown et al., 1981c; Hagiwara and Byerly, 1981). Several types of experiments that were conducted were designed to test whether inactivation of $I_{\text{Na}}$ in ventricular myocytes is modulated by calcium ion. If the inactivation of $I_{\text{Na}}$ in rat and guinea pig heart cells is due to the binding of Ca$^{++}$ at the inner surface of the membrane, then a reduction of the [Ca] (by chelation of the free Ca$^{++}$) might reduce the amount, and slow the rate of inactivation.

Internal EGTA

The result of a test of this hypothesis is shown in Figure 7A. In this experiment, a pipette used in voltage clamping a rat heart cell was filled with a solution of $150$ mM KCl containing $5$ mM EGTA (buffered to pH 7.2).

The cell was clamped and depolarized (at a rate of 12/min) with pulses, $70$ msec in duration, and from $-50$ mV to $-10$ mV, at which time the current labeled "control" was recorded. Hydraulic pressure then was applied to the solution in the pipette, and, after a short delay, the magnitude of the peak $I_{\text{Na}}$ was found to be slightly increased, the rate of decay of the current slowed to half its original value, and the maintained component of $I_{\text{Na}}$ was increased (EGTA trace). After several minutes, the peak inward current declined, but the inactivation remained slower than the control. There was no effect on $I_{\text{Na}}$ during control experiments with equivalent pressure injections of $150$ mM KCl without EGTA. It seems possible that the Ca-chelating agent, EGTA, acted to reduce the amount of free calcium near the inner surface of the membrane and, in doing so, reduced the amount of inactivation which developed during the current. Consistent with this interpretation is the observation that the peak $I_{\text{Na}}$ was slightly delayed and increased in magnitude after EGTA, possibly reflecting a slowing of the development of inactivation. A similar effect of EGTA on $I_{\text{Na}}$ of guinea pig ventricular myocytes has been reported by Kurachi (1982).

Ba$^{++}$ Currents

Barium ion has been shown to carry inward current during the flow of $I_{\text{Na}}$ in ventricular muscle (see Reuter, 1973; Kohlhardt et al., 1973). Therefore, a
set of experiments was conducted in which the Ca ions in the Tyrode’s solution were replaced with an equimolar concentration of Ba ions in order to test the effects of this divalent cation on the inactivation of I\. Figure 7B shows the current elicited during a voltage step from \(-50\) mV to \(+10\) mV in Ca\(^{2+}\) (3.6 mM) Tyrode (trace labeled 1), and at 5-second intervals (traces 2–8) after switching to Ba\(^{2+}\) (3.6 mM) Tyrode. The resulting Ba\(^{2+}\) currents were larger in peak amplitude and inactivated much more slowly than did the Ca\(^{2+}\) currents. The inactivation phase of the Ba\(^{2+}\) current was best fit by a two exponential model, yielding time constants of 31.7 and 155 msec at \(-10\) mV. These values are several times greater than the time constants for inactivation found in Ca\(^{2+}\) solutions (see Table 1). Thus, the slow decay of the Ba\(^{2+}\) currents suggests that this ion may not be able to substitute for Ca ion at a membrane component site that modulates inactivation.

**Double Pulse Experiments**

As a further test to determine whether the influx of Ca\(^{2+}\) is involved in the inactivation of I\(_{\text{I}}\), a double-pulse protocol was used (Tillotson and Horn, 1978; Marban and Tsien, 1980). In these experiments, the prepulse voltage was varied and the effects on the peak I\(_{\text{I}}\) elicited by a second test pulse was observed. As was shown in Figure 6A, the peak I\(_{\text{I}}\) in a rate ventricular cell was greatly reduced by 50-msec prepulses to \(-10\) and \(+40\) mV. However, when the prepulse amplitude was increased to \(+80\) mV, there was a substantial return (58%) of the test I\(_{\text{I}}\) towards the control value. A relatively long (100 msec) interpulse interval was used in this experiment to diminish any effects of Ca\(^{2+}\)-activated K\(^{+}\) currents.

In order to test the possibility that the Ca\(^{2+}\) entry during the prepulse activated a non-voltage-dependent outward current which might have affected the apparent magnitude of the test pulse I\(_{\text{I}}\) the following experiments were conducted. As can be seen in the inset of Figure 8A, prepulses to varying potentials were applied which were followed by a hyperpolarizing voltage step. In all cases, the currents flowing during the test hyperpolarization remained unchanged, regardless of the prepulse voltage and its associated Ca\(^{2+}\) influx. In addition, exposure of the heart cells to solutions containing 4-AP and TEA (agents which block transient and Ca\(^{2+}\)-activated outward currents) gave essentially identical results to those depicted in Figure 6.

The depression and return of I\(_{\text{I}}\) was a graded function of the prepulse amplitude, as shown in Figure 8B. This plot describes the effects of varying the prepulse (PI) amplitude using different prepulse durations (5 and 50 msec). It can be seen that the 5-msec PI produced only approximately half of the maximum depression of the test (PII) I\(_{\text{I}}\), compared with the 50-msec PI. It is also interesting to note that this effect occurred at a PI potential of 0 mV for the 5-msec pulse, as compared with \(+20\) mV in the case of the 50-msec pulse. The return of the test I\(_{\text{I}}\) to control levels was almost complete at very positive potentials with the 5-msec prepulse, but only partial restoration was seen with the 50-msec prepulse.

The results described above suggest that the inactivation of I\(_{\text{I}}\) may be related, at least in part, to the prior entry of Ca\(^{2+}\). Therefore, a quantitative estimate was obtained for the amount of charge (assumed to be proportional to the amount of Ca\(^{2+}\)) which entered the cell during each prepulse in the experiments plotted in Figure 8B. To accomplish this, the areas bounded by the prepulse current-time records (corrected for the small leakage currents) were cut out and weighted (Brown et al., 1981c). The resulting current-time integrals were converted to coulombs by calibration against a known current-time integral.

The relationship between Ca\(^{2+}\) entry and peak I\(_{\text{I}}\) during the test pulse is shown in Figure 8C. It is apparent that neither prepulse duration displays a purely linear correlation of Ca\(^{2+}\) entry and the test I\(_{\text{I}}\), which would be expected if the inactivation of I\(_{\text{I}}\)

---

**Table 1**

<table>
<thead>
<tr>
<th>Model: (Ae^{-t/\tau_1} + Be^{-t/\tau_2} + C)</th>
<th>(V_m) (mV)</th>
<th>(\tau_1) (msec)</th>
<th>(A) (nA)</th>
<th>(\tau_2) (msec)</th>
<th>(B) (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>(-20)</td>
<td>16.40</td>
<td>(-0.314)</td>
<td>26.4</td>
<td>(-0.378)</td>
</tr>
<tr>
<td></td>
<td>(-10)</td>
<td>6.29</td>
<td>(-9.72)</td>
<td>52.7</td>
<td>(-0.778)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>8.50</td>
<td>(-11.9)</td>
<td>52.8</td>
<td>(-1.22)</td>
</tr>
<tr>
<td></td>
<td>(+10)</td>
<td>13.4</td>
<td>(-5.98)</td>
<td>33.1</td>
<td>(-3.12)</td>
</tr>
<tr>
<td></td>
<td>(+20)</td>
<td>19.0</td>
<td>(-1.93)</td>
<td>33.2</td>
<td>(-4.35)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>(-10)</td>
<td>11.9</td>
<td>(-0.326)</td>
<td>84.8</td>
<td>(-0.564)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10.4</td>
<td>(-4.93)</td>
<td>107</td>
<td>(-0.973)</td>
</tr>
<tr>
<td></td>
<td>(+10)</td>
<td>6.74</td>
<td>(-3.48)</td>
<td>116</td>
<td>(-2.72)</td>
</tr>
<tr>
<td></td>
<td>(+20)</td>
<td>18.0</td>
<td>(-1.68)</td>
<td>146</td>
<td>(-2.22)</td>
</tr>
</tbody>
</table>

A computer curve-fitting algorithm was used to determine the fast \((\tau_1)\) and slow \((\tau_2)\) time constants for inactivation of I\(_{\text{I}}\) in rat and guinea pig ventricular cells. The model used was the sum of two exponentials (where A and B are the magnitudes of the fast and slow components) plus a constant C.
were determined solely by the Ca\textsuperscript{2+} influx. However, in the case of the 50-msec prepulse, an additional mechanism prevented the return of I\textsubscript{Ca} at high prepulse potentials, in spite of the fact that the Ca\textsuperscript{2+} entry was relatively small. In contrast, the curve for the 5-msec prepulse never shows this “crossing over” at high potentials. One possible explanation for the differences obtained with the long and short prepulse durations is that during the 5-msec pulse, less voltage-dependent inactivation may have occurred than during the 50-msec pulse. Alternately, it is possible that, after the longer prepulse and the greater Ca\textsuperscript{2+} entry, the mechanism involved could not completely sequester or extrude the Ca\textsuperscript{2+} which has accumulated and, therefore, I\textsubscript{Ca} was in a partially inactivated state for the subsequent test pulse.

A series of experiments, similar to those described above, also were conducted with guinea pig ventricular cells, for comparison with the results obtained for rat cells. Figure 8A shows the depression of the test pulse I\textsubscript{Ca} in a guinea pig ventricular cell following a prepulse to +10 mV, and its subsequent return following a prepulse to +70 mV. The results from experiments using interpulse intervals of 100 msec and 10 msec are presented graphically in Figure 9B. In contrast to the rat heart cells (not shown), the magnitude of the guinea pig I\textsubscript{Ca} during the test pulse was not greatly altered by the pronounced reduction in the interpulse interval. In addition, when compared with rat cells, the guinea pig cells displayed a smaller reduction of the test pulse I\textsubscript{Ca} following prepauses to potentials which elicited maximal I\textsubscript{Ca}. Finally, the tendency of I\textsubscript{Ca} to return to the control values following high prepulse potentials was greater in the guinea pig.

The prepulse currents from the same experiment shown in Figure 8B were converted to current-time integrals, as described previously for the rat I\textsubscript{Ca}, to estimate the Ca\textsuperscript{2+} influx at each prepulse potential. These values are plotted as a function of the normalized I\textsubscript{Ca} elicited at the corresponding potential in Figure 9C. As can be seen, even with a short inter-
pulse interval of 10 msec, there was still a greater tendency for a linear correlation of Ca++ influx and $I_{Na}$ in the guinea pig, than in the rat.

The double pulse experiments suggest that there may be an effect of prior Ca++ entry on the subsequent activity of $I_{Na}$. However, do alterations in the Ca++ entry change the inactivation rate of $I_{Na}$ during a single voltage step? To answer this question, we examined the rates of inactivation of $I_{Na}$ as the currents were diminished by depolarizing prepulses (Figs. 8 and 9). It was found that there was no systematic increase in either the fast or slow time constants for inactivation ($\tau_1$, $\tau_2$) of the test pulse current under prepulse conditions which maximally reduced the test current.

The time course for recovery of $I_{Na}$ from inactivation was studied in rat and guinea pig ventricular cells using two fixed pulses which were separated by a variable interval (Fig. 10A). In this figure, only the current traces elicited by the second pulse are shown. As the interpulse interval ($\Delta t$) was shortened from 2 seconds to 0.05 second, the magnitude of the peak $I_{Na}$ was reduced. The results from experiments in rat cells, using 70- and 300-msec prepulses, are presented in graphical form in Figure 7B. The recovery of $I_{Na}$ in the rat appears to have a fast phase (time constant of 316 msec) and a slower phase (time constant of 1594 msec). The fact that the recovery time constants are markedly longer than those for the development of inactivation is consistent with a hypothesis that channel regulation involves Ca binding and its subsequent removal, and that these processes occur over different time courses (see Brehm et al., 1980).

The double-pulse experiments suggested that there was a significant difference from rats in the time course for the recovery from inactivation of $I_{Na}$ in the guinea pig heart cells. Further evidence is shown in Figure 10C, which graphs the time course for the recovery of the guinea pig $I_{Na}$ using prepulse durations of 70 and 150 msec. As was seen for the rat (Fig. 9B), the recovery follows a fast and a slow phase (time constants of 122 and 433 msec). However, in marked contrast with that of the rat, the
time course of recovery is much more rapid in the
guinea pig. For example, with a 70-msec prepulse,
80% recovery of \( I_{sw} \) occurred in only 160 msec in the
guinea pig, but required 840 msec in the rat.

**Discussion**

Recently, enzymatically dissociated, single cardiac
cells have been successfully employed in the study
of passive membrane properties (Lee et al., 1979;
Powell et al., 1980; Brown et al., 1981; Hume and
Giles, 1981), the fast Na\(^+\) current (Lee et al., 1979;
Brown et al., 1981a, 1981b), and the slow inward
current (Isenberg and Klockner, 1980, 1982; Powell
et al., 1981; Lee and Tsien, 1982; Kurachi, 1982).
The findings in the present paper confirm the use-
fulness of the single-cell preparation in the study of
cardiac membrane currents. Previous studies, using
intact multicellular preparations of cardiac tissues
for voltage clamp, may have been hindered by the
three-dimensional, syncytial nature of the muscle.
This geometry produces difficulties in achieving
temporal and spatial uniformity of potential during
voltage clamp (Johnson and Lieberman, 1970; Carmeliet
and Vereecke, 1971; Reuter, 1979). In addi-
tion, the presence of small clefts between the cells
introduce a resistance, in series with the membrane
resistance, which can result in a further loss of
voltage control. The intercellular clefts may also
present a significant restriction to the diffusion of
ions in the extracellular space, thereby causing ion
accumulation or depletion during activity (Attwell et al., 1979).

The present experiments demonstrate that the
slow inward calcium currents in single rat and
guinea pig ventricular cells are similar in peak mag-
nitude, but inactivate over differing time courses to
dissimilar steady state values. The relatively rapid
decay of \( I_{sw} \) in the rat heart cells, and the relatively
small component of steady state \( I_{sw} \) would seem to
be an economical adaption for an action potential of
such short duration. In contrast, a slower rate of
inactivation and a large maintained component of
\( I_{sw} \) would serve well in prolonging the plateau in the

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**FIGURE 10.** The time course for the recovery of the slow inward current. Part A: recovery of rat \( I_{sw} \). The digitized current records show \( I_{sw} \) during the (second) test pulse as the interpulse interval (\( \Delta t \)) was varied between 0.05 and 2 seconds. Voltage step —50 to —10 mV; holding current, 0.7 nA. Part B: rat \( I_{sw} \) recovery. The percent recovery of the second, test pulse (70-msec duration) compared to its control value (in the absence of a prepulse) is plotted as a function of the interpulse interval. Data from two prepulse durations (70 and 300 msec) are shown. Both the prepulse and the test pulses were stepped from —50 to —10 mV. Part C: guinea pig \( I_{sw} \) recovery. The percent recovery of \( I_{sw} \) during the second, test pulse (duration: 70 msec) (as compared to its control value in the absence of a prepulse) is plotted as a function of the interpulse interval. Data from two prepulse durations (70 and 150 msec) are shown. Both the prepulses and the test pulses were stepped from —40 to —10 mV.
guinea pig ventricular cells; (Reuter and Scholz, 1977; Matsuda et al., 1982) and in bovine ventricular cells (Ishenberg, 1982). Of course, outward current systems may contribute in determining the plateau duration, one of which will be examined in the following paper (Josephson et al., 1984).

Although it may not be a universal mechanism for all Ca++ currents, (see Hagiwara et al., 1975), Ca-mediated inactivation has been demonstrated in such diverse cells as Aplysia neuron (Eckert and Tillotson, 1978, 1981; Brehm et al., 1980), Helix aspersa neurones (Brown et al., 1981c), Paramecium (Brehm et al., 1980), insect skeletal muscle fibers (Ashcroft et al., 1980), and in bundles of calf Purkinje fibers (Marban and Tsien, 1980). In the present study, the inactivation of I.alpha. in both rat and guinea pig heart cells also appeared to be related, at least in part, to Ca++ entry and accumulation. Several lines of evidence lend support to this conclusion. First, a reduction in the internal free [Ca] by injection of EGTA was shown to reduce the rate and magnitude of the inactivation of I_alpha. This result cannot be explained by a change in the driving force for the Ca ion, since the [Ca], is normally maintained at very low levels (10^-7 to 10^-8 M), and its further reduction would not produce a significant change in the current. Although it is possible that part of the slowing of the inactivation of the net inward current after EGTA reflects the elimination of a Ca-activated K+ current, this explanation seems unlikely, since 4-AP, which blocks the early outward current, and TEA did not significantly affect the control Ca++ currents.

Second, it was found that substitution of Ba++ for Ca++ also slowed the inactivation of I_alpha. This result, which has been previously demonstrated in Helix neurons (Brown et al., 1981c), suggests that it is not the flow of current which causes inactivation, but a specific action of Ca++ ion which is responsible. The fact that inactivation persisted in Ba++ solutions, even though I_alpha. > I_car, excludes ion depletion at the extracellular face or ion accumulation at the intracellular surface as major factors in the process.

Third, the double-pulse experiments demonstrate that the inactivation of I_alpha. is related to the magnitude of the prior Ca++ influx. Prepulses to positive potentials, where the Ca++ influx was decreased, allowed a recovery of the subsequent I_alpha. Also consistent with the hypothesis relating the inactivation of I_car with Ca++ influx is the observation that the fast time constant of inactivation (r_i) tended to have the small values at those potentials which elicited larger increases in Ca++ current. Conversely, r_i was larger (i.e., inactivation was slower) at potentials yielding smaller peak Ca++ currents. This correlation need not imply a causal relationship between Ca++ influx during a voltage step, and the rate of inactivation of the Ca++ current. In fact, the rates of inactivation of the prepulse-reduced currents were found to be very similar to those of the larger currents elicited without prepulses. This suggests that the probability of opening of the single Ca++ channel currents may not be determined by the Ca++ influx during the flow of the current.

However, another explanation for the present results might be that the Ca++ influx during a prepulse activated the leakage current described by Colquhoun et al. (1981). This outward leakage current could have resulted in an apparent reduction of the test pulse Ca++ current. Although it is a possibility, this idea seems improbable for the following reasons. We measured the I-V relationship at hyperpolarized potentials using the same double-pulse sequence used to measure I_alpha. inactivation. Since we found no change in this curve using various prepulse potentials, we consider it unlikely that a Ca++-activated leakage current of the type referred to was involved. In addition, the test pulse potential used in the inactivation study was -10 mV, which is near the reversal potential for the Ca++-activated leakage current, thereby minimizing the possibility of interference from this current.

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