Dynamics of the Background Outward Current of Single Guinea Pig Ventricular Myocytes

Ionic Mechanisms of Hysteresis in Cardiac Cells

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Subthreshold potentials are thought to be mediated by time-independent, “passive” background currents. In this study, we show that the background current–voltage (I-V) relation of guinea pig ventricular myocytes is changed significantly by repetitive stimulation, in such a way that cell excitability becomes enhanced. Myocytes were used for whole-cell voltage-clamp experiments. A voltage-clamp ramp (100 mV/sec) to -50 mV was applied from a holding potential of -100 mV. Subsequently, a train of square voltage-clamp pulses to +10 mV (duration, 300 msec; interpulse interval, 300 msec) was delivered from a holding potential of -85 mV. A new ramp was applied again immediately after the train, and the resulting I-V curve was compared with that obtained before the train. Pulsing displaced the I-V relation to the right, the zero-current point becoming 1-2 mV less negative, and increased the degree of inward-going rectification. These changes were insensitive to tetrodotoxin (30 μM); disappeared during superfusion with cobalt (2 mM), verapamil (22 μM), or ryanodine (5 μM); and could not be mimicked by agonists of the protein kinase C system. In the presence of cesium (8 mM), pulsing still displaced the I-V curve to the right. However, the linear portion of the curve became steeper after the train. Subtraction of the cesium-sensitive current from control revealed that, although the zero-current point remained constant, the I-V relation showed a stronger inward-going rectification after pulsing. In accordance with these results, we have demonstrated hysteresis of excitability in ventricular myocytes. We conclude that the observed changes are mediated by an increase in intracellular calcium, which leads to an increase in rectification of I_{K1}, as well as to activation of another membrane-conductance system, perhaps the Na-Ca exchange or the Ca^{2+}-activated, nonselective current. (Circulation Research 1991;69:1316–1326)

In quiescent mammalian ventricular myocytes, most of the background conductance is provided by the potassium-selective, inward rectifier channels that carry the outward current known as I_{K1}.1 These channels are responsible for maintaining a well-polarized level of membrane resting potential, as well as for carrying most of the membrane outward current during the final phase of action potential repolarization.1,2 Another important function of the I_{K1} channels is that they provide the cell with the input resistance necessary for membrane depolarization in response to a current input.3,4 The latter has important implications in excitability because, even in the presence of normal sodium currents, a cell will not generate an active response if it fails to depolarize from its resting potential to the threshold for inward current activation.

Subthreshold depolarization in excitable cells initially was compared with the voltage response to current injection in a parallel resistive-capacitive circuit, where the resistor was a passive, ohmic element.5 More recent evidence, however, shows that the inwardly directed rectification of the I_{K1} channel causes the subthreshold depolarizations to depart drastically from what is expected from a purely resistive-capacitive circuit. In fact, as previously demonstrated by Tourneur6 and later confirmed in our laboratory,3,4 the amplitude and shape of the subthreshold response in a ventricular myocyte can be

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dynamically controlled by the voltage dependence of the $I_{K1}$ channel.

In addition to its strong voltage dependence, $I_{K1}$ conductance is also a function of time.\textsuperscript{5,7} Nevertheless, it has been generally accepted that during the cardiac cycle, $I_{K1}$ acts as a purely voltage-dependent channel, because its activation and inactivation times are too fast to alter the time course of the action potential. However, Mazzanti and DeFelice\textsuperscript{8} have shown recently that the outward conductance of $I_{K1}$ changes as a function of time during the action potential and that such time-dependent changes are a consequence of the exquisite sensitivity of $I_{K1}$ to the intracellular concentration of divalent cations.\textsuperscript{9-10}

In this paper, we demonstrate a much slower time dependence of the background outward conductance, one that becomes apparent only after repetitive stimulation of the single myocyte. Our data demonstrate that repetitive activation of the membrane currents causes a progressive decrease of the outward charge flowing through the membrane, particularly in the voltage range of the subthreshold response. This effect partly is due to a time-dependent increase in the inward-going rectification of the cesium-sensitive $I_{K1}$ current. The process seems to require an elevation in the intracellular concentration of free Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]) but is not replicated by superfusion with agonists of the protein kinase C system. This phenomenon may have important implications in the modulation of cell excitability during repetitive stimulation and explains the presence of active facilitation,\textsuperscript{11} memory, and hysteresis of excitability in isolated ventricular muscle tissue\textsuperscript{12} and in the single ventricular myocyte.\textsuperscript{13}

Materials and Methods

Cell Isolation and Recording Techniques

Adult guinea pigs (300–350 g) were heparinized (500 IU i.p.) and then anesthetized with sodium pentobarbital (75–150 mg/kg). The hearts were removed rapidly from the chest and placed in a Langendorff apparatus for dissociation of the ventricular cells. The procedure for cell isolation has been described elsewhere.\textsuperscript{4}

Single ventricular myocytes were placed on the stage of an inverted microscope and continuously superfused with normal Tyrode’s solution of the following composition (mM): NaCl 150, KCl 5.4, MgCl\textsubscript{2} 1.0, CaCl\textsubscript{2} 1.8, NaHCO\textsubscript{3} 5.8, NaHPO\textsubscript{4} 0.4, glucose 5.5, and HEPES 5.0, pH 7.4. Unless otherwise stated, temperature was maintained at 30±1°C.

Experimental recordings were obtained using patch pipettes (made out of borosilicate glass) in the whole-cell voltage-clamp configuration.\textsuperscript{14} Composition of the internal pipette solution was (mM) KCl 150, MgCl\textsubscript{2} 1.0, EGTA 5.0, HEPES 5.0, β-OH-butyric acid 2.0, ATP (disodium salt) 5.0, and phosphocreatine 5.0, pH 7.2. Electrodes were coupled to an Axopatch 1A amplifier (Axon Instruments, Inc., Foster City, Calif.); signals were filtered at a cutoff frequency of 1 kHz and displayed on a Hitachi VC-6020 oscilloscope. All reported values of membrane potential have been corrected by \(-8\) mV to account for tip potentials.\textsuperscript{4}

Stimulation and Data Acquisition

Voltage-clamp stimulation and data acquisition protocols were computer programmed using the CLAMPPEX routine of the pCLAMP 5.03 software (Axon Instruments) operating on an IBM/AT system. Unless otherwise stated, the stimulation protocol was as follows (see Figure 1A): A control voltage-clamp ramp (labeled C in Figure 1A) was applied from a holding potential of \(-100\) to \(-50\) mV at a rate of 100 mV/sec. Subsequently, a train of 50 pulses to 10 mV (duration, 300 msec; interpulse interval, 300 msec) was delivered from a holding potential of \(-85\) mV. A test ramp (labeled T in Figure 1A) was applied again 10 msec after the train. Current–voltage (I-V) relations were obtained during both control and test ramps. Current obtained within the first 5 mV after the onset of the ramp (i.e., from \(-100\) to \(-95\) mV) was disregarded to eliminate the artifact imposed by the capacitive transient of the membrane.

To test for the presence of calcium currents, membrane potential was held constant at \(-50\) mV, and 300-msec pulses were applied in 5-mV steps to progressively more depolarized levels between \(-45\) and \(+50\) mV.

Data Analysis

Measurements of zero-current potential ($E_m'$; i.e., the expected membrane resting potential, $E_m$, in the absence of clamp), total outward charge ($Q_o$), and maximal outward current ($I_{max}$) were done directly from the CLAMPFIT routine of the pCLAMP system. $Q_o$ was calculated as the integral under the curve for positive values of membrane current (see Figure 1B). The time course of $Q_o$ decay and relaxation was determined using commercially available software (GRAPHPAD INPLOT; Inset Systems, San Diego, Calif.). Differences in outward current as a function of voltage ($\Delta I[V]$; see Figure 1C) were calculated from the averaged values of current sampled within a 1-mV range. Because the computer acquired 500 data points in each 50-mV ramp, each averaged point included 10 values of current. Averaging was done to dampen noise artifacts and minimize the possibility that current differences were simply due to the coincidence of two opposite peaks of noise.

Statistics

Statistical analysis was carried out using the INSTAT software package (Inset systems). The significance of changes in one or more variables resulting from either repetitive stimulation (Table 1) or pharmacological intervention (Tables 2 and 3) was tested using one-way analysis of variance. A one-sample $t$ test was used to evaluate the statistical significance of the shift in $E_m'$ observed during Ca\textsuperscript{2+} channel blockade or during ryanodine superfusion. Differences with two-tailed values of $p<0.05$ were regarded as significant. Averaged data are expressed as mean±SEM.
Chemicals

Cesium chloride (Sigma Chemical Co., St. Louis, Mo.) was dissolved directly in the Tyrode's solution. A stock solution of verapamil HCl (Knoll Pharmaceuticals) was prepared by dissolving the drug in 8% ethanol. Cobalt chloride (Sigma), phorbol 12,13-dibutyrate (Sigma), and tetrodotoxin (Calbiochem Corp., La Jolla, Calif.) were dissolved in distilled water and stored for a maximum of 1 week. Proper amounts of one or another stock solutions were added directly to the Tyrode's solution at the time of the experiment. Ryandine (Calbiochem; lot No. 801920) was prepared at the moment of the experiment by first dissolving it in 1% ethanol and then adding it to the Tyrode's solution. Final content of ethanol in the Tyrode's was 0.01%. This is less than the concentration needed to reduce Ca2+ currents in cardiac cells15 or increase [Ca2+]i in other systems.16,17

Results

Changes in the Background Current–Voltage Relation With Activity

Our results show that repetitive depolarizing pulses significantly alter the background I-V relation of the ventricular myocyte. This is illustrated in Figure 1B. The control I-V curve (C) was obtained after a long (i.e., >30 seconds) period of quiescence, whereas the test curve (T) was recorded after the train (see panel A). Cell activity caused a displace-
Table 2. Effects of Ca\(^{2+}\)-Channel Blockade, Na\(^{+}\)-Channel Blockade, and Ryanodine on Membrane Parameters Associated With Dynamics of the Background Outward Current

<table>
<thead>
<tr>
<th></th>
<th>(E_m) (mV)</th>
<th>(\Delta I_{\text{max}}) (pA)</th>
<th>(\Delta Q_0) (pC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+1.96±0.25</td>
<td>-124.0±13.8</td>
<td>-23.85±6.1</td>
</tr>
<tr>
<td>Calcium channel blockade</td>
<td>+0.54±0.12</td>
<td>-17.9±8.8</td>
<td>-4.78±1.99</td>
</tr>
<tr>
<td>Tetrodotoxin</td>
<td>+2.25±0.77</td>
<td>-92.25±35.8</td>
<td>-24.51±10.11</td>
</tr>
<tr>
<td>RYR</td>
<td>+1.46±0.22</td>
<td>-87.03±15.9</td>
<td>-20.9±2.15</td>
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</table>

\(\Delta E_m\)', mean change in zero-current potential; \(\Delta I_{\text{max}}\), mean maximal outward current change; \(\Delta Q_0\), mean total outward charge variation.

Table 3. Effect of Phorbol 12,13-Dibutyrate on Membrane Parameters Associated With Dynamics of Background Outward Current

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Phorbol 12,13-dibutyrate</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E_m)' (mV)</td>
<td>-84.2±1.4</td>
<td>-83.5±1.2</td>
<td>NS</td>
</tr>
<tr>
<td>(I_{\text{max}}) (pA)</td>
<td>538.7±56.0</td>
<td>545.8±59.6</td>
<td>NS</td>
</tr>
<tr>
<td>(Q_0) (nC)</td>
<td>1.402±0.204</td>
<td>1.378±0.222</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(n=10\). \(E_m\)', expected membrane resting potential, \(E_m\), in the absence of clamp; \(I_{\text{max}}\), maximal outward current; \(Q_0\), total outward charge.

Figure 2. Plots showing the total outward charge (\(Q_0\)) observed during (panel A) and immediately after (panel B) repetitive stimulation. In both cases, data were best-fit by monoeponential functions (continuous lines) with time constants of 10.56 and 6.95 seconds and correlation coefficients of 0.971 and 0.996 for decay (panel A) and relaxation (panel B), respectively. Time constants were calculated using a nonlinear curve-fitting program, based on the least-squares criterion to determine convergence.

maximal \(\Delta I\) was reached at \(-67±1.5\) mV, and it was 21.40±3.56% larger than the value of \(\Delta I\) measured at \(E_m\)' of the T trace.

Time course. To follow the time course of the activity-induced decrease in \(Q_0\), seven experiments were done in which a voltage-clamp ramp was applied after every fifth or 10th pulse in the train. Figure 2A shows the results from one experiment in which ramps were applied after every 10th pulse. The abscissa indicates the time elapsed from the onset of the train and the ordinate, the value of \(Q_0\) (black dots) measured at each point in time. The monotonic decay in \(Q_0\) was best-fit by a monoeponential function (black line) with a time constant (\(\tau_{\text{act}}\)) of 10.56 seconds. Combined results from all seven experiments gave an average \(\tau_{\text{act}}\) of \(Q_0\) decay of 8.95±1.86 seconds.

The time course of relaxation was determined by applying individual 500-msec ramps after progressively longer periods of quiescence. Figure 2B shows the time course of \(Q_0\) relaxation for the same experiment shown in panel A. After the last pulse in a series of six trains, membrane potential was held constant at \(-85\) mV, and voltage-clamp ramps were applied every 3 seconds. With quiescence, \(Q_0\) re-
turned progressively to a value very similar to that obtained in the control. The relaxation process followed a monoexponential time course, with a time constant ($\tau_{\text{dec}}$) of 6.95 seconds. In two other experiments, $\tau_{\text{dec}}$ was 3.85 and 4.98 seconds.

$\Delta Q_o$ is a function of the amplitude of the conditioning pulse. Changes in $Q_o$ were dependent on the amplitude of the voltage-clamp pulses used in the train. This is illustrated by the composite presented in Figure 3. Each open symbol represents the mean value of $\Delta Q_o$ at a particular voltage-clamp level from the holding potential of $-85$ mV ($V_c$). The bars correspond to the SEM, and the small number next to each data point indicates the number of experiments. These results show that $\Delta Q_o$ increases progressively as $V_c$ reaches values higher than $-40$ mV. Repetitive voltage pulses to less than $-40$ mV produced no significant change in $\Delta Q_o$.

Role of temperature. The amplitude of the changes in the I-V relations observed as a result of repetitive pulsing is relatively small. Thus, it was necessary to achieve a great deal of stability in our recordings. In our experience, ventricular myocyte recordings are more stable when the cells are maintained at lower temperatures. Nevertheless, to demonstrate that the same shift in the I-V relation still was apparent at more physiological temperatures, a group of 14 experiments was done in which myocytes were maintained at 36±1°C. The values of $\Delta E_m$, $\Delta I_{\text{max}}$, and $\Delta Q_o$ obtained under these conditions (+1.54±0.18 mV, $-82.82±10.52$ pA, and $-24.36±3.79$ pC, respectively) were not significantly different from those obtained at lower temperatures. Seven of these myocytes also were used for the ryanodine experiments described below.

Effects of Repetitive Pacing on Cesium-Sensitive and Cesium-Insensitive Currents

During quiescence, most (if not all) of the background I-V relation of the ventricular myocyte is provided by $I_{K1}$. However, during activity, other components (e.g., activation of electrogenic transport systems) might come into play. Thus, the next step in our analysis was to identify which of the activity-induced changes in the background current were mediated by $I_{K1}$ and to separate them from those mediated by activation of other current systems. To dissect out the $I_{K1}$ component, the usual voltage-clamp protocol (see Figure 1) was applied before and after

Figure 3. Plot showing the voltage dependence of the difference in total outward charge ($\Delta Q_o$). Each point represents mean values; bars correspond to SEM. Small numbers next to each data point indicate number of experiments included.

Figure 4. Increase in inward-going rectification of $I_{K1}$ after repetitive activity. Panel A: Current–voltage (I-V) relations obtained in normal Tyrode’s solution. Stimulation protocol is the same as described in Figure 1. Panel B: Effect of cesium (8 mM). Repetitive activity still resulted in a downward shift of the I-V relation, but with a steeper slope. Panel C: Cesium-sensitive ($I_{K1}$) currents, obtained by subtracting the currents in the presence of cesium from control. C, control ramp; T, test ramp.
after addition of 8 mM cesium to the superfusate. Figure 4 shows these results. Panel A shows the I-V relations obtained during superfusion with normal Tyrode’s solution; panel B shows results obtained 5 minutes after the onset of cesium exposure. In the presence of cesium, the amplitude of the control background current was significantly reduced. Moreover, repetitive activity still displaced the I-V relation toward the right. However, in contrast to the cesium-free condition (panel A), the T curve was steeper than C, and ΔI became progressively smaller until the two curves intersected. Panel C shows the cesium-sensitive current (I\textsubscript{K1}) obtained by subtraction of currents in the presence and in the absence of cesium. For the most part, the inward portions of the I-V relations are superimposable; however, there was a significant increase in inward-going rectification after activity (T curve). Analysis of the I\textsubscript{K1} I-V relations obtained with this procedure showed that repetitive activity causes a reduction in both Q\textsubscript{0} and I\textsubscript{max} (ΔQ\textsubscript{0} = -11.9±4.9 pC; ΔI\textsubscript{max} = -56.95±17.4 pA; n=20), without major changes in E\textsubscript{m'} (ΔE\textsubscript{m'} = -0.135±0.55 mV). Averaged values of E\textsubscript{m'} before and after activity were -83.27±0.74 and -83.41±0.99 mV, respectively. Two additional parameters were measured: chord resistance between E\textsubscript{m'}, and -95 mV (inward resistance), and chord resistance between E\textsubscript{m'c'} and -65 mV (outward resistance). In the 20 experiments analyzed, we did not observe a statistically significant difference in the inward resistance; values were 29.0±2.6 and 31.6±3.3 Ωm when measured, respectively, before and after the train. However, there was a significant increase in the chord outward resistance, which changed from 56±3.5 Ωm in quiescence to 67.9±5.6 Ωm after activity (n=20; p<0.01; analysis of variance). Yet, we should point out that in six of the experiments, although the control I-V relation changed in the expected direction after activity, no appreciable changes in I\textsubscript{K1} were detected during cesium superfusion. These data demonstrate that the changes in background I-V relation with activity (Figure 4A) are the result of at least two mechanisms: an I\textsubscript{K1}-mediated increase in inward-going rectification (see Figure 4C) and a cesium-insensitive, voltage-independent increase in another background conductance, one that allows the passage of inward current in the voltage range of the subthreshold response (see Figure 4B).

**Possible Modulation by Intracellular Free Ca\textsuperscript{2+} Concentration**

*Effect of Ca\textsuperscript{2+} current blockade.* The results shown in Figure 4C are qualitatively similar to those previously obtained by Mazzanti and DiFrancesco\textsuperscript{9} when testing the effect of various [Ca\textsuperscript{2+}] on the I-V relation of I\textsubscript{K1} cardiac channels. This similarity led us to hypothesize that the shift in the background I-V relation after pacing was a consequence of an increased [Ca\textsuperscript{2+}] after activity. As a first approach to testing such a hypothesis, we repeated our experimental protocol in the presence and in the absence of calcium channel blockers, because the increase in [Ca\textsuperscript{2+}] during cell activation is known to be significantly less after Ca\textsuperscript{2+} channel blockade.\textsuperscript{18} An example of these experimental results is presented in Figure 5. In all panels, the top traces correspond to the background I-V relations before and after activity, and the bottom traces are membrane currents elicited by depolarizing voltage-clamp pulses applied from a holding potential of -50 mV (see “Materials and Methods”). The small numbers next to the current traces in panel A (bottom) indicate the values of membrane potential during the voltage-clamp pulse. Panel A shows the control. In panel B, we present data obtained after 5 minutes superfusion with 2 mM cobalt chloride. Calcium inward currents were blocked (panel B; bottom), and repetitive activity caused only a minor displacement of the I-V relation. As shown in panel C, calcium currents again were apparent (although smaller in amplitude) on washout, and this coincided with the reappearance of the shift in the background I-V relation after pacing.
(panel C). Similar experiments were performed in a total of eight cells. In five of these cells, Ca\(^{2+}\) currents were blocked with 2 mM CoCl\(_2\) whereas in the remaining three cells, verapamil (2.2 \(\mu M\)) was used. Table 2 summarizes global results. The data indicate that the three parameters, \(\Delta Q_0\), \(\Delta E_m'\), and \(\Delta I_{\text{max}}\) were sensitive to calcium channel blockade. However, the remaining shift in \(E_m'\) observed in the presence of the drug still was significantly different from zero (p<0.005; one-sample t test).

**Effect of sodium current blockade.** Although the sodium inward current does not seem to play a major role in intracellular Ca\(^{2+}\) homeostasis,\(^{19}\) it was important to determine whether it had any effect on the changes in background I-V relation with activity. A possible role of the sodium current was analyzed in four experiments. Neither \(\Delta Q_0\), \(\Delta I_{\text{max}}\), nor \(\Delta E_m'\) was modified by the addition of 30 \(\mu M\) tetrodotoxin to the superfusate (Table 2).

**Effect of ryanodine.** Our results with Ca\(^{2+}\) channel blockers suggested that the observed changes in background current after pacing were mediated by an activity-induced increase in [Ca\(^{2+}\)]. To test this hypothesis further, we repeated our experimental protocol in the presence and in the absence of 5 \(\mu M\) ryanodine, a potent antagonist of intracellular calcium release.\(^{18}\) These experiments were carried out at 36°C. Results obtained during normal Tyrode’s superfusion were not different from those recorded at a lower temperature. Figure 6 shows data from one experiment. Panel A depicts the currents obtained under control conditions and panel B, those obtained during ryanodine superfusion. The shift in the I-V relation was strongly reduced after blockade of intracellular calcium release. Cumulative results are shown in Table 2. In some experiments, a small shift in \(E_m'\) still was apparent in the presence of ryanodine. In fact, the value of \(\Delta E_m'\) during ryanodine was still different from zero (p<0.01), as evaluated by a one-sample t test.

**Possible modulation by protein kinase C.** The results obtained with ryanodine supported the hypothesis that activity-induced reduction in \(Q_0\) was mediated by an increase in [Ca\(^{2+}\)]. The effects of [Ca\(^{2+}\)] may be elicited either directly on the membrane channels or indirectly, perhaps through the activation of a protein kinase that could, in turn, phosphorylate the channels and change their conductance states.\(^{20}\) Because the enzyme most likely activated by calcium would be protein kinase C, we attempted to determine the effects, if any, of 10 \(nM\) phorbol 12,13-dibutyrate (a potent agonist of protein kinase C)\(^{21}\) on the background I-V relation of the ventricular myocyte. For these experiments, we were interested in the effect of protein kinase C stimulation per se. Thus, cells were not paced and only a voltage-clamp ramp (10 mV/sec) was applied from -100 to 0 mV before and after the drug. All these experiments were carried out at 36°C because stimulation of ionic currents by protein kinase C is known to be temperature dependent.\(^{21}\) Results from one experiment are shown in Figure 7. The trace labeled C is the I-V relation obtained in normal Tyrode’s solution, and the trace labeled PDB is the one obtained 5 minutes after protein kinase C stimulation began. In this experiment, we noticed a slight displacement of \(E_m'\) toward the left (from -84.0 to -85.2 mV), as well as an increase in \(I_{\text{max}}\) from 820 to 869 pA. However, in other experiments, the curve shifted slightly in the opposite direction. On average, there was no significant change in either \(E_m'\), \(I_{\text{max}}\), or \(Q_0\) (see Table 3).
These results argue against the possibility that protein kinase C stimulation is an intermediate step in the changes in background I-V relation with activity.

**Difference in Membrane Potential for Each Value of Outward Current**

As a result of the nonlinear voltage dependence of the background currents, small changes in the shape of the I-V relation can lead to large differences in the amplitude of a voltage response elicited by a current pulse under current-clamp conditions. This is illustrated in Figure 8. Panel A shows the I-V relations obtained in control (C) and after the train (T) in one of our experiments. To add clarity, only the outward part of the curves is depicted. We define \( \Delta V(I) \) as the difference in membrane potential measured between the two curves for a given value of current. Panel B shows a plot of \( \Delta V(I) \) for 50 regularly spaced current values ranging from \( I=0 \) to \( I=I_{\text{max}} \) of T. Clearly, the separation between the two curves increases progressively as I becomes larger. In fact, \( \Delta V \) changes from 1.4 mV at \( I=0 \) to 9.5 mV at \( I_{\text{max}} \) of T. These large differences in \( \Delta V \) with activity provide a mechanistic explanation for the fact that, under current-clamp conditions, a current pulse of constant amplitude may cause either a subthreshold or a suprathreshold response, depending on previous history (see "Discussion").

**Discussion**

This paper demonstrates the effect of repetitive activity on the background I-V relation of single ventricular myocytes. The data show that pacing leads to a progressive reduction of the outward background current in the voltage range between \( E_m \) and \(-50 \text{ mV} \).

Results obtained with 8 mM cesium superfusion demonstrate that the activity-induced changes in background current are the result of at least two mechanisms: a cesium-sensitive decrease in \( I_{K1} \) outward conductance, and a cesium-insensitive increase in a background inward current. The former causes an increase in inward-going rectification of the background I-V relation, whereas the latter is responsible for a shift in \( E_m \) toward less negative values. Both mechanisms combined lead to a decrease in \( I_{\text{max}} \) and a reduction in \( Q_m \). Furthermore, both mechanisms seem to be sensitive to \([\text{Ca}^{2+}]_o\). Indeed, activity-induced reduction of outward background current is unaltered by exposure to tetrodotoxin but is mostly prevented by blockade of the \( \text{Ca}^{2+} \) currents or by blockade of calcium release from the sarcoplasmic reticulum. Finally, direct stimulation of protein kinase C failed to mimic the effect of repetitive activity, thus rendering unlikely a possible intermediate role for this enzyme.

**Changes in \( I_{K1} \) With Activity**

Our results suggest that repetitive activity causes an increase in \([\text{Ca}^{2+}]_i\) that directly decreases the outward conductance of the \( I_{K1} \) channels. Such a hypothesis is supported by data in the literature showing that a small increase in \([\text{Ca}^{2+}]_i\) would lead to a major reduction in \( I_{K1} \) outward conductance.9,10 More recent work by Mazzanti and DeFelice8 shows that the calcium flowing through the membrane during the action potential still is capable of increasing the rectification of the \( I_{K1} \) channel.

In 1977, Isenberg22 studied the effect of intracellular \( \text{Ca}^{2+} \) injections on the steady-state I-V relation of isolated Purkinje fibers. His results suggested that high \([\text{Ca}^{2+}]_i\) would selectively increase membrane potassium conductance. More recently, Matsuda23 performed a similar experiment in isolated guinea pig ventricular myocytes and failed to detect any increase in potassium conductance. Our results are consistent with those of Matsuda23 and suggest that there is a difference in the behavior of the steady-state conductance of multicellular Purkinje fibers as compared with that of the ventricular cells. It is also possible that Isenberg’s results22 were hampered by technical limitations related to injection of \( \text{Ca}^{2+} \) intracellularly in Purkinje fibers. For example, it is possible that at least part of the injected \( \text{Ca}^{2+} \) may have leaked into the extracellular space, thus increasing \([\text{Ca}^{2+}]_o\) and affecting background conductance.24
Our results show that the decrease in $Q_0$ with activity is a slow process that takes several seconds to reach a steady state. The data also show that $Q_0$ slowly returns to control values after quiescence. These results could be explained by a progressive buildup in intracellular free Ca$^{2+}$ during diastole as a result of repetitive stimulation at short cycle lengths. To our knowledge, there are no data in the literature as to the relation between diastolic [Ca$^{2+}$], and stimulation cycle length in freshly dissociated adult cardiac myocytes. However, experiments in cultured chick embryo myocardial cell aggregates have shown that there is a significant increase in diastolic [Ca$^{2+}$], during repetitive stimulation and a subsequent slow decay during quiescence. It is quite possible that a similar process is present in adult mammalian cells such as those used in our study.

**Mechanism of the Increased Background Inward Current**

One possible mechanism for the activity-induced increase of inward current demonstrated in the presence of cesium (Figure 4B) is the activation of the electrogenic Na-Ca exchange. Indeed, this system is functional at physiological concentrations of [Ca$^{2+}$], and it may provide the extra inward current necessary for displacement of $E_m$ toward the right (see also Figure 4B). In fact, the Na-Ca exchange system is capable of generating current in the presence of verapamil and ryanodine, which would explain the remaining shift in $E_m$ seen in our experiments during superfusion with those drugs. An additional component could be provided by opening of the Ca$^{2+}$-activated, nonselective channel. Yet, it is still controversial whether this channel is active at physiological [Ca$^{2+}$]. In summary, the Na-Ca exchange mechanism may act together with the Ca$^{2+}$-activated nonselective channel to cause a change in background inward current. However, thus far it is not possible to determine the specific contribution of each system to the total displacement of the I-V relation seen in the presence of cesium.

Matsuda has studied the effect of intracellular Ca$^{2+}$ injection on the background I-V relation of guinea pig ventricular myocytes. In contrast with our results, obtained by directly pacing the cardiac cell, Matsuda’s data show that Ca$^{2+}$ injection caused a very large shift in $E_m$, as well as the virtual disappearance of inward-going rectification. This discrepancy may be explained by the fact that pressure injection of a 1 mM Ca$^{2+}$ solution (as in Matsuda’s experiments) probably raises [Ca$^{2+}$]$_i$ to much higher levels than those attained after repetitive stimulation. Thus, other current systems, such as the Na-Ca exchanger or the Ca$^{2+}$-activated, nonselective channel, would be strongly activated and would provide most of the background conductance of the cell.

**Involvement of Other Membrane Conductances**

Our results provide indirect as well as direct information regarding the participation of sodium channels in the activity-dependent increase of $Q_0$. Indeed, trains of pulses at voltages below -50 mV failed to induce a change in $Q_0$ even though large inward currents were elicited during the train. This is consistent with the fact that, in the presence of tetrodotoxin, the effect of pacing remained unchanged. Thus, we have concluded that sodium currents are not involved in this process. The latter results also suggest that the effect of calcium channel antagonists in preventing the activity-induced changes in background currents (see Figure 5) is not associated with the blocking effect that these agents may exert on sodium currents.

The delayed rectifier, $I_{K}$, activates during the action potential and deactivates mostly during diastole. Accordingly, we would expect to find a progressively decaying $I_{K}$ component embedded in the total background current recorded after the train. Yet, given the reversal potential of $I_{K}$ (-77 mV at $[K]_o=5.4$ mM) and its time course of deactivation (time constant=220 msec at -85 mV; see Reference 3), most of the $I_{K}$ conductance should decay during the first half of the ramp and in the voltage range at which the current is inward. Moreover, as a result of the voltage-dependence of $I_{K}$ deactivation, channel closure would be further accelerated by the hyperpolarized levels of membrane potential used at the beginning of the ramp. Accordingly, $I_{K}$ deactivation may affect the amplitude of the background inward current, but it is unlikely to contribute in a significant way to the total background outward current recorded after the train.

Two other mechanisms to be considered are the potassium accumulation/depletion process and the electrogenic current driven by activation of the Na-K pump. It is very unlikely that either of these two systems is involved in the observed shift of the I-V relation, because, to our knowledge, neither of them is sensitive to ryanodine or calcium channel blockers. Furthermore, activation of the Na-K pump would cause an outward current, thus shifting the I-V relation in the opposite direction to what we have observed. Further evidence for the absence of an accumulation/depletion effect is the fact that the reversal potential of the cesium-sensitive current does not significantly shift with activity (see Figure 4C).

**Implications on Cell Excitability**

Previous work from our laboratory has demonstrated the presence of active facilitation, memory, and hysteresis of excitability both in single ventricular myocytes and in whole ventricular muscle preparations. Common to these phenomena is the fact that cell excitability improves during activity and is impaired during quiescence. Furthermore, the results of Lorente et al suggest that the facilitatory effect of suprathreshold stimulation is due to a change in the background I-V relation of the cell. Indeed, that study demonstrated the existence of hysteresis in the excitability of single guinea pig ventricular myocytes when stimulated repetitively
Panel A: Armchair model of changes in cell excitability resulting from the activity-induced reduction in background outward current in the ventricular myocyte. Panel B: The large differences in membrane potential for a given current value explain why, under current-clamp conditions (panel A), a current pulse of constant amplitude may cause either a subthreshold or suprathreshold response, depending on previous history. For further explanation, see text. C, control ramp; T, test ramp.

under current-clamp conditions. In addition, the study showed that after a failed beat, the amplitude of the subthreshold response progressively decreased even though the magnitude of the depolarizing current pulse was held constant.

The voltage-clamp data presented in this paper provide conclusive evidence as to the mechanisms responsible for active facilitation and hysteresis of excitability in the ventricle. Consider a hypothetical cardiac cell whose I-V relations before (C) and after (T) activity are of the form illustrated in Figure 9B (same traces as in Figure 8A). If under current-clamp conditions we were to drive the cell with depolarizing current pulses (panel A), its behavior would vary markedly as a result of the I-V relation shift (panel B). The dotted vertical lines indicate the threshold voltage for activation. After a period of quiescence (curve C) a current pulse of magnitude X should bring the membrane from resting potential a to threshold b. However, after a period of activity, the I-V relation should move downward and to the right (curve T). Under these conditions, a current pulse of magnitude X now would result in a larger voltage response (point c), thus enhancing excitability and reducing the stimulus-response latency (point c in panel A). Consequently, a pulse, Y, of lesser amplitude would be sufficient for excitation (point d). If stimulation is stopped for some time, however, the membrane would return to its original I-V relation, and a pulse of magnitude Y will induce only a subthreshold response (point e).

The above model assumes that threshold does not change with activity. Yet, it is possible that because of the activity-induced reduction in outward current, fewer I_{Na} channels would need to open to generate a net inward current. This means that threshold potential may shift to more negative values, thus increasing hysteresis of excitability.13

It is important to point out that, in spite of the slow time course of the decrease in Q_{o} (Figure 3), changes in the I-V relation already are apparent after trains of only five voltage-clamp pulses. This explains why, under current-clamp conditions, a short train of action potentials may be sufficient to alter the excitability as the cell is shifted from its quiescent to its active state.11

The above model also shows that the cell acts as a positive feedback system in regards to its excitability. In such a system, repetitive activation improves excitability and thus tends to perpetuate activity, whereas quiescence enhances the likelihood of failure. In other words, the system would tend to lock the stimulus-response patterns of the ventricle into one of two major stable states, 1:1 or 1:0. Recent mathematical simulations have provided confirmation for the feasibility of this hypothesis13 and have shown that intermediate Wenckebach patterns are mostly transitional states, although stable Wenckebach periodicities can be observed at specific stimulation cycle lengths.13

Conclusions

Our results show that repetitive activation of the ventricular myocyte causes a shift in the membrane I-V relation in such a way that cell excitability becomes enhanced. The observed changes are mediated by an increase in [Ca^{2+}], which leads to an increase in the inward-going rectification of the I_{K1} current, as well as to activation of other membrane conductance systems, perhaps the Na-Ca exchange or Ca^{2+}-activated, nonselective currents. Our results provide a cellular mechanism for active facilitation and hysteresis of excitability and stress the importance of dynamic changes in the I_{K1} conductance on the modulation of cell excitability in the heart.

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