UltraRapid Communication

Ca$^{2+}$ Influx Through Ca$^{2+}$ Channels in Rabbit Ventricular Myocytes During Action Potential Clamp

Influence of Temperature

José L. Puglisi, Weilong Yuan, José W.M. Bassani, Donald M. Bers

Abstract—Ca$^{2+}$ influx via Ca$^{2+}$ current ($I_{Ca}$) during the action potential (AP) was determined at 25°C and 35°C in isolated rabbit ventricular myocytes using AP clamp. Contaminating currents through Na$^+$ and K$^+$ channels were eliminated by using Na$^+$- and K$^+$-free solutions, respectively. DIDS (0.2 mmol/L) was used to block Ca$^{2+}$-activated chloride current ($I_{Cl(Ca)}$). When the sarcoplasmic reticulum (SR) was depleted of Ca$^{2+}$ by preexposure to 10 mmol/L caffeine, total Ca$^{2+}$ entry via $I_{Ca}$ during the AP was $\approx$12 μmol/L cytosol (at both 25°C and 35°C). Similar Ca$^{2+}$ influx at 35°C and 25°C resulted from a combination of higher and faster peak $I_{Ca}$, offset by more rapid $I_{Ca}$ inactivation at 35°C. During repeated AP clamps, the SR gradually fills with Ca$^{2+}$, and consequent SR Ca$^{2+}$ release accelerates $I_{Ca}$ inactivation during the AP. During APs and contractions in steady state, total Ca$^{2+}$ influx via $I_{Ca}$ was reduced by $\approx$50% but was again unaltered by temperature (5.6±0.2 μmol/L cytosol at 25°C, 6.0±0.2 μmol/L cytosol at 35°C). Thus, SR Ca$^{2+}$ release is responsible for sufficient $I_{Ca}$ inactivation to cut total Ca$^{2+}$ influx in half. However, because of the kinetic differences in $I_{Ca}$, the amount of Ca$^{2+}$ influx during the first 10 ms, which presumably triggers SR Ca$^{2+}$ release, is much greater at 35°C. $I_{Ca}$ during a first pulse, given just after the SR was emptied with caffeine, was subtracted from $I_{Ca}$ during each of 9 subsequent pulses, which loaded the SR. These difference currents reflect $I_{Ca}$ inactivation due to SR Ca$^{2+}$ release and thus indicate the time course of local [Ca$^{2+}$] in the subsarcolemmal space near Ca$^{2+}$ channels produced by SR Ca$^{2+}$ release (eg, maximal at 20 ms after the AP activation at 35°C). Furthermore, the rate of change of this difference current may reflect the rate of SR Ca$^{2+}$ release as sensed by L-type Ca$^{2+}$ channels. These results suggest that peak SR Ca$^{2+}$ release occurs within 2.5 or 5 ms of AP upstroke at 35°C and 25°C, respectively. $I_{Cl(Ca)}$ might also indicate local [Ca$^{2+}$], and at 35°C in the absence of DIDS (when $I_{Cl(Ca)}$ is prominent), peak $I_{Cl(Ca)}$ also occurred at a time comparable to the peak $I_{Ca}$, difference current. We conclude that SR Ca$^{2+}$ release decreases the Ca$^{2+}$ influx during the AP by $\approx$50% (at both 25°C and 35°C) and that changes in $I_{Ca}$ (and $I_{Cl(Ca)}$), which depend on SR Ca$^{2+}$ release, provide information about local subsarcolemmal [Ca$^{2+}$]. The full text of this article is available at http://www.circresaha.org. (Circ Res. 1999;85:e7-e16.)

Key Words: Ca$^{2+}$ current ■ cardiac muscle ■ excitation-contraction coupling ■ sarcoplasmic reticulum Ca$^{2+}$ release

In mammalian cardiac muscle, Ca$^{2+}$ influx during the action potential (AP) triggers the release of additional Ca$^{2+}$ from the sarcoplasmic reticulum (SR) via Ca$^{2+}$-induced Ca$^{2+}$ release and results in muscle contraction. To achieve [Ca$^{2+}$], balance and a steady-state level of contraction, Ca$^{2+}$ influx must equal Ca$^{2+}$ efflux during successive cardiac cycles. An accurate measurement of those fluxes is needed to understand excitation-contraction coupling. We previously studied the Ca$^{2+}$ removal fluxes and their relative contributions to relaxation in different species (rabbit, rat, and ferret) at different temperatures. We also measured Ca$^{2+}$ influx via Ca$^{2+}$ current ($I_{Ca}$) during square voltage-clamp pulses and contractions in rat and rabbit at room temperature. However, the Ca$^{2+}$ entry during an AP will be different than during a square voltage pulse. In the present study, we focus on quantitative measurements of Ca$^{2+}$ influx via Ca$^{2+}$ channels during the rabbit ventricular AP. These values should be useful in the overall quantitative understanding of Ca$^{2+}$ fluxes in rabbit myocytes.

During an AP, Ca$^{2+}$ can enter the cell through sarcolemmal Ca$^{2+}$ channels ($I_{Ca}$) and via Na$^+$/Ca$^{2+}$ exchange ($I_{Na/Ca}$), but under normal conditions, it is generally accepted that $I_{Ca}$ is the major source of Ca$^{2+}$ influx. $I_{Ca}$ has been intensively studied using voltage clamp, mainly with square pulses that allow characterization of channel kinetics and other intrinsic properties. Using such kinetic parameters, the behavior of $I_{Ca}$ during an AP has been modeled. However, this modeling has been limited because the Ca$^{2+}$ influx during an AP is a complicated function of not only time and voltage but also...
local Ca$^{2+}$ at the inner mouth of the channel. The use of the AP clamp technique has provided some unique insight into the time course of $I_{\text{Ca}}$ under more physiological conditions.\textsuperscript{11-13} Nevertheless, it is difficult to assess accurately the integrated Ca$^{2+}$ entry via $I_{\text{Ca}}$ during the normal AP because of contaminating ionic currents (Na$^+$, K$^+$, Cl$^-$, and Na$^+$/Ca$^{2+}$ exchange) and unknown SR Ca$^{2+}$ loading and release. Yuan et al\textsuperscript{14} used AP clamp to study isolated $I_{\text{Ca}}$ during the AP waveform in rabbit and rat ventricular myocytes at room temperature. However, in that study [Ca$^{2+}$], was heavily buffered, specifically to allow comparative study of $I_{\text{Ca}}$ properties free of the normal influence of local [Ca$^{2+}$]. It is clear that Ca$^{2+}$-dependent inactivation of $I_{\text{Ca}}$ is important in affecting the amount of Ca$^{2+}$ entry.\textsuperscript{15-18} Indeed, during Ca$^{2+}$ release from the SR, [Ca$^{2+}$] near the Ca$^{2+}$ channel mouth may be much higher than the bulk [Ca$^{2+}$], measured with fluorescence indicators, consistent with theoretical calculations.\textsuperscript{19,20}

This high local [Ca$^{2+}$], may contribute greatly to $I_{\text{Ca}}$ inactivation during the AP. In the present study, we allowed normal SR Ca$^{2+}$ release to occur and evaluated its effect on $I_{\text{Ca}}$ during the AP. Moreover, we used the effect of SR Ca$^{2+}$ release on $I_{\text{Ca}}$ inactivation to provide indirect information about local [Ca$^{2+}$], near the Ca$^{2+}$ channel.

Having normal Ca$^{2+}$ transients occur during AP clamp creates additional complications in isolating $I_{\text{Ca}}$ from potentially contaminating currents. While K$^+$ free, Cs solutions can block most K$^+$ currents; it is more difficult to perform experiments in Na$^+$-free solutions to prevent Na$^+$ currents and Na$^+$/Ca$^{2+}$ exchange. This is because Na$^+$/Ca$^{2+}$ exchange is so important in the steady state in extruding Ca$^{2+}$ from the cell (which entered via $I_{\text{Ca}}$). We have overcome this problem and measured $I_{\text{Ca}}$ during AP clamp in Na$^+$- and K$^+$-free solution with 0.2 mmol/L DIDS to block Ca$^{2+}$-activated Cl$^-$ current ($I_{\text{Cl(Ca)}}$).\textsuperscript{21,22} AP waveforms were first recorded under more physiological ionic conditions in perforated current clamp mode. These AP waveforms were then applied to cells as the command voltage templates, under conditions where currents other than $I_{\text{Ca}}$ were blocked, but Ca$^{2+}$ transients and contractions were relatively normal. Experiments were done at both 25°C and 35°C using appropriate AP waveforms.

We found that in rabbit myocytes the total amount of Ca$^{2+}$ entry did not change significantly between 25°C and 35°C during AP clamp. However, as the SR Ca$^{2+}$ load and SR Ca$^{2+}$ release increased, the amount of Ca$^{2+}$ entry was reduced to ≈50% of that seen without SR Ca$^{2+}$ release. The quantity of Ca$^{2+}$ that entered in the first 10 ms (which may trigger SR Ca$^{2+}$ release) was larger at 35°C. Kinetic differences in $I_{\text{Ca}}$ inactivation as SR Ca$^{2+}$ release increases were used to infer changes in local [Ca$^{2+}$] near Ca$^{2+}$ channels and indicate peak SR Ca$^{2+}$ release early in the AP.

Materials and Methods

New Zealand White rabbits were obtained from Myrtle’s Rabbittry (Thompson Station, Tenn) and cared for according to AAALAC guidelines. Ventricular myocytes were isolated as described,\textsuperscript{2} and cell shortening, APs, and ionic currents were measured at 25°C and 35°C (using 200 μg mL$^{-1}$ amphotericin B in perforated patch). Steady-state APs were recorded in current-clamp mode in physiological ionic conditions, triggered by depolarizations (0.5 Hz) at both 25°C and 35°C. The bathing normal Tyrode’s (NT) contained the following (mmol/L):

- NaCl 140, KCl 6, MgCl$_2$ 1, glucose 10, and HEPES 5, pH adjusted to 7.4 with NaOH. The pipette solution contained the following (mmol/L): KCl 30, K$^+$-aspartate 110, NaCl 8, and HEPES 5, pH adjusted to 7.2 with KOH (pipette liquid junction potential = −13 mV was corrected during recording).
- $I_{\text{Ca}}$ was measured in cells depolarized by 30 minutes in 0 Na$^+$/Ca$^{2+}$ NT) and in Na$^+$-free NT (Na$^+$ replaced by TEA and K$^+$ by Cs, pH 7.4, with TEA-OH). Perforating patch electrodes contained the following (mmol/L): CsSO$_4$ 80, CsCl 55, MgCl$_2$ 10, HEPES 10, and EGTA 0.1. The liquid junction potential (−3 mV) was not corrected. DIDS (0.2 mmol/L) was usually added to block $I_{\text{Cl(Ca)}}$ without reducing $I_{\text{Ca}}$.\textsuperscript{21,23} Initially, standard I-V curves were assessed with square voltage-clamp pulses (holding potential, $V_m$ = −70 mV, 200-ms steps to −20 to +40 mV). Then, AP waveforms recorded in current clamp were used for trains of AP clamps. These were preceded by application of 10 mmol/L caffeine in NT for 10 seconds to empty the SR. This brief exposure to Na$^+$-containing solution allowed extrusion of the SR Ca$^{2+}$ load via Na$^+$/Ca$^{2+}$ exchange but without appreciable Na$^+$ entry (<0.5 mmol/L). AP clamps were given every 30 seconds to avoid Ca$^{2+}$ overload (with the Na$^+/Ca^{2+}$ exchanger blocked). After 10 AP clamps, twitch contractions achieved steady state and was similar to the value at 0.5 Hz in field-stimulated intact cells. Protocols were repeated at 25°C and 35°C, and the I/V method was used for leak subtraction.

Integrated $I_{\text{Ca}}$ (in pC/pF) was converted to total cellular Ca$^{2+}$ entry by multiplying by the cell surface to volume ratio (4.58 pF/pL cell), resulting in Ca$^{2+}$ flux expressed in mmol/L cytosol.

Currents were normalized to cell capacitance ($C_m$). $C_m$ was measured (with ion currents blocked) by a −5-mV voltage step ($\Delta E_{m}$) from −80 mV, with currents analyzed\textsuperscript{14} using $C_m = \tau / \Delta E_{m}/(1−(I/L))$, where $I$ and $\tau$ are the peak and time constant of capacitance current relaxation, fit by a single exponential (extrapolated to the time of $\Delta E_{m}$), and $L$ is the steady-state current after $\Delta E_{m}$. Series resistance compensation was not used. Cells used had on-cell access resistance <5 MΩ (3.3±0.4 MΩ), $C_m$ = 229±18 pF, and membrane capacitance, $R_m$ = 463±51 MΩ, giving a membrane charging time constant of 0.71±0.05 ms.

Data are presented as mean±SEM. Student’s unpaired or paired $t$ test was used to determine statistical significance. Values of $P$$\leq$0.05 were considered as significant.

Results

AP Measurements

Figure 1 shows examples of APs recorded in perforated whole-cell current-clamp mode in rabbit ventricular myo-
cytotes at 25°C and 35°C. As temperature increased, the resting membrane potential (E_m) was slightly hyperpolarized and AP duration and overshoot were reduced (see Table). In 8 experiments, the action potential duration (APD) measured at the level of 50% repolarization (APD_{50}) decreased from 114±13 ms (mean±SEM) to 74±14 ms (P<0.05). These APD_{50} values indicate a Q_{10} of 1.52±0.11. This is similar to the Q_{10} of 1.46 that can be extrapolated from AP duration recordings in isolated rabbit ventricular muscle, whereas Kiyosue et al. reported an AP duration Q_{10} of 2.5 at 25°C and 35°C. As temperature increased, the resting E_m decreased by 72±1.6 to -81±1.7 mV (P<0.05). Despite changes in resting E_m, the AP amplitude was not significantly different (131±1.2 mV at 25°C, 127±2.5 mV at 35°C). Such changes in APD and resting E_m were reversed after returning to the initial temperature. The AP waveforms in Figure 1 were used as the command E_m templates for subsequent AP clamp experiments.

### I_{ca} Measurements Using Square Pulses

With all K⁺ free conditions (to block K⁺ currents) and Na⁺ free conditions (to block I_{Na}, Na⁺/K⁺-ATPase, and Na⁺/Ca²⁺ exchange), all ionic current is blocked by 1 μmol/L nifedipine or 1 mmol/L Cd. Figure 2 shows that the currents recorded under our experimental conditions were almost completely blocked by 300 μmol/L Cd at all voltages (for both AP clamp and traditional square pulses), and similar results were found for 2 μmol/L nifedipine. The 95.1±0.5% block of I_{ca} by 300 μmol/L Cd is consistent with a K_i of 15 μmol/L, similar to the values (6 to 10 μmol/L) we have measured in concentration-response experiments (not shown). These results indicate that all of the ionic current in Figures 4 through 9 is carried by Ca²⁺ channels. The lack of other ionic currents under our recording conditions ensures that linear leak subtractions (P/4) used in subsequent experiments should be adequate.

Some initial experiments with conventional square voltage-clamp pulses were carried out in the absence of DIDS but with Na⁺- and K⁺-free solutions to prevent Na⁺, K⁺, and Na⁺/Ca²⁺ exchange currents. The I_{ca} records often showed a shoulder or hump, especially at 35°C. To test whether this might be due to I_{Cl(Ca)}, we used 0.2 mmol/L DIDS, which blocks I_{Cl(Ca)} without altering I_{ca} (at [DIDS] up to 0.5 mmol/L). At 25°C, DIDS had little effect on I_{ca} recorded during steps from -70 to 0 mV (Figure 3A), consistent with our previous observations at room temperature. However, at 35°C, the control I_{ca} for the same depolarization had a prominent notch, which was abolished by DIDS (Figure 3B). This DIDS-sensitive current resembles the I_{Cl(Ca)} previously described in rabbit ventricular myocytes. The DIDS-sensitive outward current was largest at potentials where I_{ca} and contraction were also large and that are different from the expected Cl⁻ equilibrium potential of

### Table: Different Parameters Measured at 25°C and 35°C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>25°C (mV)</th>
<th>35°C (mV)</th>
<th>Q_{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td>E_m resting</td>
<td>-72±1.6</td>
<td>-81±1.7*</td>
<td>...</td>
</tr>
<tr>
<td>AP amplitude</td>
<td>131±1.2</td>
<td>127±2.5</td>
<td>...</td>
</tr>
<tr>
<td>APD_{50}, ms</td>
<td>114±13</td>
<td>74±14*</td>
<td>1.52</td>
</tr>
<tr>
<td>Time to peak I_{k}, ms, square pulse</td>
<td>5.90±0.45</td>
<td>2.90±0.37*</td>
<td>...</td>
</tr>
<tr>
<td>I_{k} inactivation, τ fast, ms</td>
<td>12.6±1.9</td>
<td>4.2±0.8*</td>
<td>3.0</td>
</tr>
<tr>
<td>I_{k} inactivation, τ slow, ms</td>
<td>51±6</td>
<td>20±3*</td>
<td>2.6</td>
</tr>
<tr>
<td>Relative amplitude of fast component</td>
<td>0.80±0.03</td>
<td>0.67±0.03</td>
<td>...</td>
</tr>
<tr>
<td>Time to peak I_{k}, ms, AP at SS</td>
<td>6.1±0.38</td>
<td>2.8±0.40*</td>
<td>...</td>
</tr>
<tr>
<td>I_{Ca} (μmol/L cytosol), total AP at SS</td>
<td>5.64±0.23</td>
<td>6.01±0.22</td>
<td>1.06</td>
</tr>
<tr>
<td>I_{Ca} (μmol/L cytosol), during 10 ms</td>
<td>0.79±0.13</td>
<td>1.89±0.20*</td>
<td>2.4</td>
</tr>
</tbody>
</table>

APD_{50} refers to the AP duration measured at 50% amplitude. SS indicates steady state.

*P<0.05, n=8.
On the other hand, there were large changes in the I Ca with ideal voltage clamp. Thus, the activation kinetics of I Ca cannot readily be analyzed, and even the times to peak should be taken only as relative values or approximations. Warming from 25°C to 35°C should affect capacitance currents much less than ion channel gating. Thus, the relative changes in peak I Ca and time to peak I Ca at 25°C versus 35°C are likely to be meaningful (if imprecise in quantitative terms). These voltage-clamp limitations will have much less impact on I Ca decline or integrals. For example, even without any correction for capacitance, the integrated I Ca would then be underestimated by <30% (due mainly to outward capacitative current during depolarization). However, using P/4 to compensate for linear leak and capacitance (as we have) reduces the error by much more than a factor of 10, resulting in an error of much less than 3% in I Ca integrals.

The time course of I Ca decline was fit with a double-exponential decay curve, and both fast and slow time constants were 2.5 to 2.9 times longer at 25°C ($\tau_{\text{fast}} = 12.2 \pm 1.9$ s at 25°C, $4.20 \pm 0.80$ ms at 35°C; $\tau_{\text{slow}} = 51.0 \pm 5.9$ ms at 25°C, 20.1 ± 2.8 s at 35°C). The fraction of I Ca decline in the fast component was 0.80 ± 0.03 at 25°C, 0.67 ± 0.03 at 35°C (Figure 4D). The slower inactivation at 25°C resulted in an absolute value of I Ca that was larger than at 35°C at all times longer than 6 ms in the pulse shown in Figure 4A.

\[ I_{Ca} \text{ Measurements Using AP Clamp} \]

Figure 5 shows I Ca during steady-state AP clamps at 25°C and 35°C. During the AP clamp, I Ca activated rapidly and then inactivated, but the time course differs from that observed for square pulses. The peak value of I Ca and the inactivation kinetics are affected by temperature in a manner qualitatively similar to results obtained with square pulses. At 25°C, peak I Ca is smaller, but there is a striking sustained component of I Ca during the AP plateau phase. This sustained component is less prominent at 35°C and may reflect a balance between the gradually increasing driving force for I Ca as repolarization proceeds and the progression of channel inactivation. The larger peak and more pronounced inactivation at 35°C make the Ca$^{2+}$ influx through the Ca$^{2+}$ channels more concentrated in the early phase of the AP at this temperature.

The I Ca records were integrated to quantify the amount of Ca$^{2+}$ entering through Ca$^{2+}$ channels. Figure 6A shows the amount that enters during a steady-state twitch over the whole AP and also during the first 10 ms. Temperature did not significantly change the total Ca$^{2+}$ influx via I Ca during the steady state, temperature-appropriate AP (5.64 ± 0.23 mmol/L cytosol at 25°C, 6.01 ± 0.22 mmol/L cytosol at 35°C). This fact rules out increased total Ca$^{2+}$ influx as an explanation for hypothermic inotropy, in which contractile force increases several fold upon cooling from 35°C to 25°C. Cooling did affect channel kinetics, and the smaller peak current at 25°C was balanced by longer duration such that the integrated flux is smaller, but there is a striking sustained component of I Ca during the AP plateau phase. This sustained component is less prominent at 35°C and may reflect a balance between the gradually increasing driving force for I Ca as repolarization proceeds and the progression of channel inactivation. The larger peak and more pronounced inactivation at 35°C make the Ca$^{2+}$ influx through the Ca$^{2+}$ channels more concentrated in the early phase of the AP at this temperature.

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-37 mV (Figure 3C, top curve). We refer to this current as $I_{Ca_{cyt}}$. Peak outward $I_{Ca_{cyt}}$ at 35°C in Figure 3A occurred 17 ms after the start of depolarization.

Figure 3C shows the I-V relationship for $I_{Ca}$ measured with square voltage-clamp pulses at 25°C and 35°C in the presence of DIDS. Maximum $I_{Ca}$ was at $\approx 10$ mV at both temperatures. On the other hand, there were large changes in the $I_{Ca}$ amplitude and time course. The maximal peak $I_{Ca}$ at 35°C was 77 ± 19% larger than at 25°C ($Q_{10} = 1.8 \pm 0.2$, $n = 8$). Increasing temperature can also speed activation and inactivation.28 Figures 4A and 4B show raw and normalized traces of $I_{Ca}$ at 25°C and 35°C. The time to peak $I_{Ca}$ decreased significantly from 5.9 ± 0.5 ms at 25°C to 2.9 ± 0.4 ms at 35°C.

Voltage-clamp limitations at very short times hamper precise measurement of the rising phase of $I_{Ca}$. For example, the membrane-charging time constant (0.7 ms) indicates that 3 ms will elapse before achieving 90% voltage control. This will distort the initial $I_{Ca}$ time course measured, compared with ideal voltage clamp. Thus, the activation kinetics of $I_{Ca}$ cannot readily be analyzed, and even the times to peak should.
SR Ca\(^{2+}\) Release Induces \(I_{\text{Ca}}\) Inactivation

Successive AP clamp \(I_{\text{Ca}}\) records, initiated immediately after depletion of SR Ca\(^{2+}\) by a caffeine pulse, allowed us to monitor the effect of SR reloading on \(I_{\text{Ca}}\). Figures 7A and 7B show consecutive contractions during this SR Ca\(^{2+}\) refilling process at 25°C and 35°C. As the SR is refilled, the amplitude (and rate of rise) of contractions increased progressively to reach \(\approx 10\%\) of resting cell length at 25°C. This is consistent with an increasing amount of SR Ca\(^{2+}\) release as the SR refilled toward a level that is normal for steady-state twitch conditions. The natural AP waveform may be expected to change as the SR refills. However, we chose to use the same AP clamp waveform at each sequential pulse because this allows us to infer changes in \(I_{\text{Ca}}\) from pulse to pulse, which are Ca\(^{2+}\) dependent rather than \(E_m\) dependent, as steady state is attained.

Figures 7C and 7D illustrate the corresponding simultaneously recorded AP clamp \(I_{\text{Ca}}\) records. The peak \(I_{\text{Ca}}\) did not change appreciably during successive AP clamps, but \(I_{\text{Ca}}\) traces showed progressively more inactivation. Because the voltage waveform was the same for each pulse and the peak \(I_{\text{Ca}}\) was not different, the greater \(I_{\text{Ca}}\) decline is unlikely to be due to inactivation that is either voltage dependent or dependent on Ca\(^{2+}\) entering via \(I_{\text{Ca}}\). On the other hand, the
progressive increase in SR \(Ca^{2+}\) release may be expected to contribute to progressively stronger \(Ca^{2+}\)-dependent inactivation of \(I_{Ca}\) (see also References 16 through 18).

Subtraction of the first \(I_{Ca}\) trace (where there is no SR \(Ca^{2+}\) release) from the consecutive traces provides a putative SR \(Ca^{2+}\) release–sensitive \(I_{Ca}\) (Figure 7E and 7F). The time course of this difference current may also indicate the time course of local \([Ca^{2+}]_i\), change due to SR \(Ca^{2+}\) release into the region near the L-type \(Ca^{2+}\) channels. If we assume that the peak of those traces corresponds to the maximum inactivation induced by the SR \(Ca^{2+}\) release, those peaks should reflect the time of maximum local \([Ca^{2+}]_i\), due to \(Ca^{2+}\) released from the SR. At 35°C, we infer that maximal local \([Ca^{2+}]_i\), occurred within the first 20 ms, whereas at 25°C, the peak does not
occur until 75 ms. This 20-ms value inferred for peak local [Ca\textsuperscript{2+}], near the Ca\textsuperscript{2+} channel at 35°C is also consistent with the time to peak of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current in Figure 3B.

We can take this analysis one step further. To the extent that the difference currents in Figure 7E and 7F reflect the SR-dependent change in local [Ca\textsuperscript{2+}], near the L-type Ca\textsuperscript{2+} channel mouths, their rates of change may reflect the rate of SR Ca\textsuperscript{2+} release as sensed locally by the Ca\textsuperscript{2+} channel. Figure 8 shows the derivatives of the traces from Figure 7E and 7F. We focus on early times where the change is likely to be dominated by SR Ca\textsuperscript{2+} release rather than reuptake or other processes. Peak Ca\textsuperscript{2+} release appears to occur at 5.4 ms at 25°C and 2.5 ms at 35°C. This coincides with the peak of I\textsubscript{Ca} during the AP at the two temperatures (6.1±0.38 ms at 25°C, 2.8±0.4 ms at 35°C; P<0.05, n=8). This may indicate that there is little delay between I\textsubscript{Ca} activation and SR Ca\textsuperscript{2+} release. It is also notable that the peak of the putative release flux occurred at the same time for pulses with different SR Ca\textsuperscript{2+} loads and released quantities.

Obviously, features of I\textsubscript{Ca} inactivation relate inferentially to the rate of SR Ca\textsuperscript{2+} release, unlike quantifiable measurements with optical indicators. The latter phases of the difference current (or its derivative) may be harder to interpret because of possible cumulative effects of inactivation on subsequent I\textsubscript{Ca}, during a given AP. On the other hand, I\textsubscript{Ca} may respond much more rapidly to highly localized changes in [Ca\textsuperscript{2+}], in a manner that fluorescent indicators cannot. Even with confocal microscopy, Ca\textsuperscript{2+}-dependent signals represent averages over diameters on the order of 500 nm, whereas the space between the release channel and L-type Ca\textsuperscript{2+} channel may be 20 times smaller. Thus, these AP clamp experiments may provide unique insight into the process of excitation-contraction coupling.

Figure 8. Putative SR Ca\textsuperscript{2+} release flux. Assuming that the traces from Figure 7E and 7F reflect the sub sarcolemmal [Ca\textsuperscript{2+}] as sensed locally by the Ca\textsuperscript{2+} channels, the time derivatives of those traces would reflect the rate of Ca\textsuperscript{2+} release from the SR. Peak Ca\textsuperscript{2+} release occurred at 5.4 ms at 25°C and 2.5 ms at 35°C. The units are not explicitly meaningful with respect to Ca\textsuperscript{2+} flux.

SR Ca\textsuperscript{2+} Release Reduces Ca\textsuperscript{2+} Influx During AP by 50%

Figure 9 shows the beat-to-beat change in integrated Ca\textsuperscript{2+} influx during the AP as the SR refills after the caffeine-induced depletion. Regardless of the temperature, the SR Ca\textsuperscript{2+} release progressively inhibited Ca\textsuperscript{2+} influx by ~50%. At 25°C, the integrated Ca\textsuperscript{2+} influx at the first pulse (without SR Ca\textsuperscript{2+} loading) was 12.5±0.7 μmol/L cytosol. The value at 35°C for pulse one was 12.3±0.7 μmol/L cytosol. These values are not very different from those reported by Yuan et al\textsuperscript{14} for rabbit ventricular myocytes under AP clamp at room temperature with EGTA in the pipette. Their reported value was 13.4 μmol/L cell, which corresponds to 20 μmol/L cytosol after accounting for mitochondrial volume, as we have done in the present study.

Discussion

Isolation of I\textsubscript{Ca} in the Physiological Context

A novel aspect of our study is that we have isolated I\textsubscript{Ca}, (by using Na\textsuperscript{+}- and K\textsuperscript{+}-free solutions and DIDS) while simultaneously having the physiological AP waveform and normal Ca\textsuperscript{2+} transients at both 25°C and 35°C. We used very brief exposures to Na\textsuperscript{+}-containing solution with caffeine for periodic unloading of the SR. This prevents progressive Ca\textsuperscript{2+} loading and Ca\textsuperscript{2+} overload in Na\textsuperscript{+}-free experiments in contracting cardiac myocytes. Of course, blocking Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange and Na\textsuperscript{+} current could alter the local [Ca\textsuperscript{2+}], at the inner mouth of the Ca\textsuperscript{2+} channel (due to either altered induction of SR Ca\textsuperscript{2+} release or local action of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange).\textsuperscript{29,30} This is an intrinsic limitation of our approach. However, it is not possible to simultaneously isolate I\textsubscript{Ca} while having normal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange or Na\textsuperscript{+} current. Because the contractions were comparable to normal twitches without voltage clamp, blocking Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange may make only a very minor alteration in the local Ca\textsuperscript{2+} transient and I\textsubscript{Ca} time course. Indeed, Bassani et al\textsuperscript{2} found that Na\textsuperscript{+}-free conditions did not inhibit Ca\textsuperscript{2+} transient amplitude (if SR Ca\textsuperscript{2+} load was constant) and only modestly slowed [Ca\textsuperscript{2+}], decline in rabbit and rat ventricular myocytes. Thus, although we have isolated I\textsubscript{Ca} effectively and used physiological AP waveforms, there might be minor differences in local [Ca\textsuperscript{2+}] that we cause by preventing Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, and those might alter I\textsubscript{Ca}.
slightly. Caffeine can have complicating effects such as phosphodiesterase inhibition and increased myofilament Ca$^{2+}$ sensitivity. The myofilament effects are rapidly reversed on caffeine washout, and our brief (<10 seconds) exposures to caffeine does not result in altered $I_{C_{a}}$ amplitude (which would be expected if phosphodiesterase inhibition had a basal or lasting effect).

$I_{C_{a}}$, has been extensively studied,\textsuperscript{21-23,31,32} $I_{C_{a}}$ was much more prominent at 35°C, although it can be increased at room temperature by isoproterenol.\textsuperscript{21} It is not clear why $I_{C_{a}}$ was so much more pronounced at 35°C, but it could be due to locally higher [Ca$^{2+}$] (due to increased peak $I_{C_{a}}$), higher Ca$^{2+}$ sensitivity at 35°C, or higher intrinsic activity at 35°C versus 25°C. Although our main concern was to block $I_{C_{a}}$, this current may normally facilitate rapid repolarization when SR Ca$^{2+}$ load and release are high. This could shorten AP duration, limit Ca$^{2+}$ entry via $I_{C_{a}}$, and thus provide negative feedback on cell Ca$^{2+}$ load.

Total Ca$^{2+}$ Influx During AP

Some investigators have tried to assess Ca$^{2+}$ influx via Ca$^{2+}$ channels during the AP by measuring the nifedipine-sensitive current.\textsuperscript{7} Although nifedipine can block $I_{C_{a}}$, by doing so it also prevents SR Ca$^{2+}$ release and the consequent activation of Na$^{+}$/Ca$^{2+}$ exchange current and $I_{C_{a}}$. Thus, the nifedipine-sensitive current may include currents that are Ca$^{2+}$ activated in addition to $I_{C_{a}}$. Terracciano and MacLeod\textsuperscript{33} used a clever approach in guinea pig and rat ventricular myocytes by integrating $I_{C_{a}}$ and Na$^{+}$/Ca$^{2+}$ exchange over a full contraction-relaxation cycle, knowing that in the steady state, Ca$^{2+}$ entry must equal Ca$^{2+}$ efflux. In this way, whatever Ca$^{2+}$ entered and left via Na$^{+}$/Ca$^{2+}$ exchange during a cardiac cycle would not show on the integral, whereas each Ca$^{2+}$ ion that entered via $I_{C_{a}}$ and left via Na$^{+}$/Ca$^{2+}$ exchange would produce inward movement of 3 charges (2 coming in as Ca$^{2+}$, and 1 entering per Ca$^{2+}$ extruded in exchange for 3 Na$^{+}$). Thus, two thirds of the inward current could be attributed to $I_{C_{a}}$. They found steady-state Ca$^{2+}$ entry via $I_{C_{a}}$ of 4 and 14 μmol/L cytosol in rat and guinea pig AP, respectively, at 0.5 Hz and 22°C. However, these authors could not interpret the time course of $I_{C_{a}}$ during the AP, because $I_{C_{a}}$ and $I_{Na/Ca}$ would be overlapping in time.

Yuan et al\textsuperscript{34} isolated $I_{C_{a}}$ using AP clamp in rabbit and rat ventricular myocytes but under conditions where [Ca$^{2+}$], transients were inhibited by dialysis with 10 mmol/L EGTA. They found integrated Ca$^{2+}$ entry of 20 and 13 μmol/L cytosol during rabbit and rat AP, respectively, and under similar conditions, Grantham and Cannell\textsuperscript{7} reported $\approx$34 μmol/L cytosol during the guinea pig AP (after adjusting their values to the units used here). These values for rabbit and guinea pig are somewhat higher than observed in the present study, but this may be due to lower Ca$^{2+}$-dependent inactivation with EGTA or BAPTA in the cell.

Ca$^{2+}$ can also enter the cell during the cardiac AP via Na$^{+}$/Ca$^{2+}$ exchange, and this would be thermodynamically favored at the very early phase of the AP.\textsuperscript{6} However, no convincing direct measurements of this outward current during an AP have been reported. Under normal physiological conditions, Ca$^{2+}$ entry via this route is likely to be quantitatively small for several reasons. After SR Ca$^{2+}$ release is prevented, Ca$^{2+}$ influx during the AP can still activate a substantial contraction in rabbit ventricle, but if Ca$^{2+}$ channels are also blocked, contractions are abolished.\textsuperscript{6,34} However, if [Na$^{+}$], is elevated by blocking the Na$^{+}$/K$^{+}$-ATPase, Ca$^{2+}$ entry via Na$^{+}$/Ca$^{2+}$ exchange during the AP can increase sufficiently to activate large contractions. What probably limits Ca$^{2+}$ entry via Na$^{+}$/Ca$^{2+}$ exchange during the normal AP is the rise in local Ca$^{2+}$ due to both Ca$^{2+}$ entry via Ca$^{2+}$ channels and release from the SR. Thus, such Ca$^{2+}$ entry via Na$^{+}$/Ca$^{2+}$ exchange is probably limited to the first few milliseconds of the AP. This would constrain the integrated Ca$^{2+}$ influx to be probably much less than 1 μmol/L cytosol, and this is consistent with model calculations.\textsuperscript{35} Grantham and Cannell\textsuperscript{7} reported an upper limit of Ca$^{2+}$ entry via Na$^{+}$/Ca$^{2+}$ exchange to be 30% of the total Ca$^{2+}$ influx during the first 10 ms of the AP, but they overestimated the outward Na$^{+}$/Ca$^{2+}$ exchange current, because $I_{C_{a}}$ and Ca$^{2+}$ transients (and early Na$^{+}$/Ca$^{2+}$ exchange current reversal) were prevented by nifedipine. Thus, it is likely that the integrated $I_{C_{a}}$ reported in the present study is by far the major Ca$^{2+}$ influx during the AP.

Temperature Alters $I_{C_{a}}$ Kinetics but not Total $I_{C_{a}}$ Flux

It is well-known that warming accelerates Ca$^{2+}$ channel activation and inactivation and increases peak $I_{C_{a}}$ for square voltage-clamp pulses.\textsuperscript{28} Our results are consistent with these classic observations, even during the more complex AP waveform. However, the higher peak of $I_{C_{a}}$ along with the faster inactivation at 35°C resulted in a crossover such that there was more Ca$^{2+}$ influx at 25°C for times longer than 6 ms (Figure 4A). This makes it less obvious how the current integral will change with temperature, especially with changes in AP duration and Ca$^{2+}$ transient. Surprisingly, we found that these changes almost counterbalanced each other, such that the total integrated $I_{C_{a}}$ flux during the AP was almost unchanged between 25°C and 35°C. This was true for both steady-state twitches and those when the SR was Ca$^{2+}$ depleted.

During steady-state contraction, Ca$^{2+}$ influx and efflux must be matched. Otherwise, the cell will gradually gain or lose Ca$^{2+}$. If steady-state Ca$^{2+}$ influx during the AP does not change between 25°C and 35°C, the same should be true for Ca$^{2+}$ efflux. Puglisi et al\textsuperscript{40} performed quantitative analysis of Ca$^{2+}$ transport during relaxation at 25°C and 35°C in rabbit ventricular myocytes. Although transport rates and relaxation were much faster at 35°C, the integrated Ca$^{2+}$ extrusion via Na$^{+}$/Ca$^{2+}$ exchange and balance of removal fluxes by Na$^{+}$/Ca$^{2+}$ exchange and SR Ca$^{2+}$ pump were almost the same. Thus, our expectation of flux balance is fulfilled.

Reduction of temperature from 35°C to 25°C normally produces a large increase in developed force (∼500%), referred to as hypothermic inotropy.\textsuperscript{29} The present results indicate that increased Ca$^{2+}$ influx during the AP is unlikely to contribute to this effect. This inotropy may be due more to the slowing of Ca$^{2+}$ removal from the cytosol, a prolonged active state, and increased SR Ca$^{2+}$ content.\textsuperscript{4,25}
Inactivation Under Physiological Conditions

Our results clearly indicate that the overall integrated Ca\(^{2+}\) influx during the AP via \(i_c\) is reduced by 50\% when normal SR Ca\(^{2+}\) release occurs. Similar conclusions were drawn by Trafford et al\(^{16}\) based on experiments in ferret ventricular myocytes with square pulses after caffeine-induced SR Ca\(^{2+}\) depletion. They found that integrated Ca\(^{2+}\) influx declined from 14.8 to 6.7 \(\mu\)mol/L cytosol (after correction for 30\% mitochondrial volume). Terraciano and MacLeod\(^{33}\) also found that steady-state Ca\(^{2+}\) influx during AP clamp in guinea pig ventricular myocytes was increased by 39\% after blocking SR Ca\(^{2+}\) function with thapsigargin. Sipido et al\(^{16}\) also demonstrated inactivation of Ca\(^{2+}\) channels by SR Ca\(^{2+}\) release during long square pulses and further showed that the Ca\(^{2+}\) channels could recover as [Ca\(^{2+}\)]\(_i\) declined. Sham et al\(^{37}\) and Adachi-Akahane et al\(^{17}\) also showed that blocking SR Ca\(^{2+}\) release slowed \(i_c\) inactivation (eg, by 67\%), and they also emphasized the apparent local nature of this effect at the dyadic junction. Thus, regardless of species, temperature, or depolarization waveform, it appears that SR Ca\(^{2+}\) release inhibits \(\approx 50\%\) of Ca\(^{2+}\) entry, which would otherwise occur via \(i_c\).

Inactivation of L-type Ca\(^{2+}\) channels depends on both voltage and Ca\(^{2+}\).\(^{14}\) In the absence of divalent cations, when Na\(^+\) is used as the charge carrier for the Ca\(^{2+}\) channel, the half-time for current decline can be \(\approx 500\) ms (at voltages that would correspond to the AP plateau).\(^{35}\) This purely voltage-dependent inactivation is so much slower than \(i_c\) inactivation under physiological conditions that one may infer that almost all physiological \(i_c\) inactivation is Ca\(^{2+}\)-dependent. This also suggests that altered driving forces due to local Ca\(^{2+}\) accumulation or depletion\(^{19}\) are unlikely to contribute significantly to the decline of \(i_c\) during the pulse.\(^{16}\) In our experiments, at the first postcaffeine AP clamp, when the SR is depleted of Ca\(^{2+}\) (Figures 7 and 9), all Ca\(^{2+}\)-dependent inactivation must be due to Ca\(^{2+}\) entry via the channel. We did not measure how much inactivation occurred during this AP. However, by comparing normalized flux for square pulses with Ca\(^{2+}\) versus Na\(^+\) as charge carrier,\(^{36}\) it can be inferred that Ca\(^{2+}\) entry is responsible for reducing total influx by \(\approx 60\%\) for a 160-ms pulse. Given that the steady-state APs here reduced integrated Ca\(^{2+}\) influx by \(\approx 50\%\), we infer that Ca\(^{2+}\) influx and SR Ca\(^{2+}\) release contribute about equally to \(i_c\) inactivation during the AP.

Thus, it is clear that SR Ca\(^{2+}\) release can limit Ca\(^{2+}\) influx by feedback on the L-type Ca\(^{2+}\) channel. This would serve to limit Ca\(^{2+}\) entry when the SR is already full. Conversely, it would allow for greater SR Ca\(^{2+}\) replenishment when SR Ca\(^{2+}\) release is small (eg, Figure 9).

Local [Ca\(^{2+}\)]\(_i\) Sensed by Channels

From our sequential \(i_c\) traces (Figure 7), it is clear that inactivation induced by SR Ca\(^{2+}\) release starts very rapidly. Indeed, the L-type Ca\(^{2+}\) channel may sense released Ca\(^{2+}\) much before a fluorescent Ca\(^{2+}\) indicator is distributed throughout the bulk cytoplasm. This is an intrinsic advantage of this electrophysiological signal from molecules perfectly positioned to sense the local [Ca\(^{2+}\)] of interest. Furthermore, the present measurements are done without adding exogenous Ca\(^{2+}\) indicator buffers, which could perturb local Ca\(^{2+}\) transients. The amount of Ca\(^{2+}\)-dependent inactivation was used as an indicator of local [Ca\(^{2+}\)]\(_i\), near the L-type Ca\(^{2+}\) channel, which may be located very close to the SR Ca\(^{2+}\) release channel. The rate of change of this local signal (Figure 8) may then be a local indicator of the rate of SR Ca\(^{2+}\) release sensed very near the release channel. Although it is not practical to calibrate these signals with respect to rates of Ca\(^{2+}\) flux, they may provide unique insight into the timing of SR Ca\(^{2+}\) release. During the rabbit ventricular AP, peak SR Ca\(^{2+}\) release appeared to occur at \(\approx 2.5\) and 5 ms after the start of depolarization at 35°C and 25°C, respectively (which coincided with the time of peak \(i_C\)). This indicates very tight functional coupling between the Ca\(^{2+}\) influx and release channels.

Using currents to indicate local [Ca\(^{2+}\)] changes may also help to further characterize the relative locations of SR Ca\(^{2+}\) release channels, L-type Ca\(^{2+}\) channels, Na\(^+\)/Ca\(^{2+}\) exchangers, and Ca\(^{2+}\)-activated Cl\(^-\) channels. Indeed, [Ca\(^{2+}\)], buffering that is sufficient to prevent released Ca\(^{2+}\) from activating Na\(^+\)/Ca\(^{2+}\) exchange does not prevent released Ca\(^{2+}\) from profoundly affecting \(i_c\) inactivation.\(^{17}\) This suggests that the Na\(^+\)/Ca\(^{2+}\) exchanger is not localized discretely as close to the SR Ca\(^{2+}\) release channel as is the L-type Ca\(^{2+}\) channel. Although our main objective with respect to \(i_C\) in the present study was to block it, we did observe that it also sensed high local [Ca\(^{2+}\)], quickly at 35°C. \(i_C\)\(_{Ca}\) reached a peak in \(\approx 20\) ms, similar to the \(i_c\) inactivation. However, we have insufficient data to determine whether this channel senses the same local [Ca\(^{2+}\)], as the Ca\(^{2+}\) channel. Differential [Ca\(^{2+}\)] sensitivities and delays for impact on the currents may also complicate more detailed comparisons of this nature. Nevertheless, it is clear that changes in \(i_c\) inactivation can be a valuable indicator of local [Ca\(^{2+}\)], at the location that might be most critical for understanding excitation-contraction coupling.

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Ca^{2+} Influx Through Ca^{2+} Channels in Rabbit Ventricular Myocytes During Action Potential Clamp: Influence of Temperature
José L. Puglisi, Weilong Yuan, José W. M. Bassani and Donald M. Bers

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