Differential Modulation of L-type Ca\(^{2+}\) Current by SR Ca\(^{2+}\) Release at the T-Tubules and Surface Membrane of Rat Ventricular Myocytes

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Abstract—We have characterized modulation of \(I_{Ca}\) by Ca\(^{2+}\) at the t-tubules (ie, in control cells) and surface sarcolemma (ie, in detubulated cells) of cardiac ventricular myocytes, using the whole-cell patch clamp technique to record \(I_{Ca}\). \(I_{Ca}\) inactivation was significantly slower in detubulated cells than in control cells (27.1±7.8 ms, \(n=22\), versus 16.4±7.9 ms, \(n=22\); \(P<0.05\)). In atrial myocytes, which lack t-tubules, \(I_{Ca}\) inactivation was not changed by the treatment used to produce detubulation. In the presence of ryanodine or BAPTA, or when Ba\(^{2+}\) was used as the charge carrier, the rate of inactivation was not significantly different in control and detubulated cells. Frequency-dependent facilitation occurred in control cells but not in detubulated cells, and was abolished by ryanodine. These results suggest that Ca\(^{2+}\) released from the SR has a greater effect on \(I_{Ca}\) in the t-tubules than at the surface sarcolemma. This does not appear to be due to differences in local Ca\(^{2+}\) release from the SR, because the gain of Ca\(^{2+}\) release was the same in control and detubulated cells. These data suggest that the t-tubules are a key site for the regulation of transsarcolemmal Ca\(^{2+}\) flux by Ca\(^{2+}\) release from the SR; this could play a role in altered Ca\(^{2+}\) homeostasis in pathological conditions. The full text of this article is available online at http://circres.ahajournals.org. (Circ Res. 2004;95:000-000.)

Key Words: transverse tubules ■ calcium channel ■ inactivation ■ facilitation

Calcium influx via the L-type Ca\(^{2+}\) current (\(I_{Ca}\)) is the major trigger for Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) of cardiac ventricular myocytes.\(^1\) This influx activates a cluster of adjacent SR Ca\(^{2+}\) release channels (ryanodine receptors; RyRs); the consequent systolic Ca\(^{2+}\) transient is the spatial and temporal sum of such local Ca\(^{2+}\) releases.\(^2\) The transverse (t-) tubules of ventricular myocytes play an important role in this process. These tubules are invaginations of the sarcolemma that occur at the Z-line, perpendicular to the longitudinal axis of the cell (see review).\(^3\) Functional and immunohistochemical data suggest that \(I_{Ca}\) occurs predominantly in the t-tubules, adjacent to RyRs, which are also located predominantly at the t-tubules.\(^4\)–\(^6\) Thus, it appears that the t-tubules are the major site of Ca\(^{2+}\) entry and hence Ca\(^{2+}\) release in cardiac ventricular myocytes. Conversely, Ca\(^{2+}\) released by the SR can modulate \(I_{Ca}\); this plays an important role in cellular Ca\(^{2+}\) homeostasis, controlling Ca\(^{2+}\) entry via negative feedback.\(^7\)–\(^8\) However, it is unknown whether the efficacy of coupling between SR Ca\(^{2+}\) release and \(I_{Ca}\) is the same in the t-tubule and surface membranes, so that the relative importance of these sites in cellular Ca\(^{2+}\) homeostasis is unknown. We have, therefore, investigated the regulation of \(I_{Ca}\) by Ca\(^{2+}\) released from the SR in normal ventricular myocytes, in which \(I_{Ca}\) triggers Ca\(^{2+}\) release predominantly at the t-tubules, and in myocytes in which the t-tubules have been physically and functionally uncoupled from the surface membrane (detubulated),\(^5\) in which Ca\(^{2+}\) release occurs predominantly at the surface membrane.\(^9\)

Materials and Methods
This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Isolation and Detubulation of Rat Ventricular Myocytes
Myocytes were isolated from theventricles of Wistar rat hearts and detubulated using formamide as described previously.\(^5\) All experiments were performed at room temperature (22 to 25°C).

Recording \(I_{Ca}\)
Most of the experiments described in this article used the whole-cell (ruptured) patch clamp technique\(^10\) using Na\(^{+}\) and K\(^{+}\)-free internal and external solutions to avoid contamination of \(I_{Ca}\) by overlapping ionic currents and to allow us to use a physiological holding potential. An Axopatch 200B (Axon Instruments) voltage clamp amplifier was used, controlled by a Pentium PC connected via a Digidata 1322A A/D converter (Axon Instruments), which was also used for data acquisition and analysis using pClamp software (Axon Instruments). Signals were filtered at 2 kHz using an 8-pole Bessel
low-pass filter before digitization at 10 kHz and storage. Low resistance (1 to 3 MΩ) patch pipettes were used and the junction potential between the pipette solution and the reference electrode was cancelled before obtaining a tight gigaseal (>1 GΩ). Cell membrane capacitance was measured by integrating the capacitance current recorded during a 10-mV hyperpolarizing pulse from −80 mV. Cell capacitance and series resistance were compensated (>70%) so that the maximum voltage error was <3.5 mV. ICa was recorded during a 200-ms test pulse to 0 mV from a holding potential of −80 mV. Trains of depolarizing pulses were applied at 0.1 or 1 Hz.

The experiments shown in Figure 4 were performed using the perforated patch clamp technique to record ICa and the Ca2+ transient simultaneously. Cells were loaded with the Ca2+ indicator Fura2-acetoxymethyl ester (AM, 3 μmol/L; Molecular Probes) for 10 minutes at room temperature. Fura2 fluorescence was elicited by alternate (every 2 ms) illumination with 340 and 380 nm light, obtained using a rotating filter wheel (Cairn Research Ltd) in front of a Xenon excitation lamp. The fluorescence emitted at 510 nm was monitored using a photomultiplier tube (Cairn Research Ltd). The ratio of fluorescence emitted at 510 nm during excitation at 340 nm to that emitted during excitation at 380 nm (R) is a function of [Ca2+], and was converted to [Ca2+] as described in Data Analysis section. The tip of the patch electrode was filled with pipette solution and then back filled with the same solution containing 250 to 400 μg/mL amphotericin-B (Sigma). After seal formation and pipette capacitance compensation, holding potential was set to −40 mV, and 5 mV, 20-ms depolarizing pulses were applied to monitor pore formation. The pipette solution contained 1 mmol/L CaCl2 to ensure cell death on accidental rupture of the membrane. Electrical access was typically obtained within 10 minutes and measurements were made after the capacitance transients became constant. ICa was recorded during a 500-ms test pulse to 0 mV from a holding potential of −40 mV. Trains of depolarizing pulses were applied at 0.2 Hz.

Solutions

Myocytes were studied in a chamber mounted on the stage of an inverted microscope (Nikon Diaphot). Cells were initially superfused with a normal physiological salt solution containing (in mmol/L) NaCl 113, KCl 5, MgSO4 1, CaCl2 1, NaH2PO4 1, Na acetate 20, glucose 10, HEPES 10, and insulin 5 U/L; pH set to 7.4 with NaOH. When ICa was measured using the ruptured whole-cell patch clamp configuration, the pipette solution contained (in mmol/L) CsCl 110, TEACl 20, MgCl2 0.5, Mg-ATP 5, EGTA 5, HEPES 10, and GTPtris 0.4; set to pH 7.2 with CsOH. A fast perfusion system placed close to the cell was used to deliver the external solution, which contained (in mmol/L) 4AP 5, TEACl 130, MgCl2 0.5, HEPES 10, Glucose 10, and CaCl2 1. At least 5 minutes was allowed for cell dialysis by the pipette solution before experiments were initiated.

When ICa was measured using the perforated whole-cell patch clamp configuration, the pipette solution contained (in mmol/L) CsCl 130, NaCl 10, MgCl2 6H2O 1, HEPES 10, and CaCl2 1; pH set to 7.2 using CsOH. CsCl (5 mmol/L) was also added to the normal physiological (extracellular) solution to prevent ICa contamination.

Data Analysis

ICa was measured as the difference between the peak inward current and the current at the end of the depolarizing pulse. Currents are expressed as current density (pA/pF). Because the decay of ICa varied between cell types and experimental conditions, the kinetics of inactivation of ICa were characterized by the time required for the current to decay to 0.37 of the peak amplitude (T1/3). Most currents decayed monoeXponentially, in which case T1/3 is equivalent to the time constant of decay. However in control cells, the decay was biexponential, in which case T1/3 is used as a simple measure to compare the time course of decay in these cells with that in detubulated and treated cells. Frequency-dependent facilitation was analyzed by integrating ICa (pA/ms) during the 200 ms test pulse to obtain total Ca2+ influx during the pulse.

Figure 1. ICa in control and detubulated rat ventricular myocytes. A, ICa elicited at 0 mV in representative control (left) and detubulated (right) myocytes. B, Mean ±SEM cell capacitance (left), ICa density at 0 mV (middle), and time for ICa to decay to 0.37 of peak amplitude (T1/3; right) in control (n=22, black bars) and detubulated (n=22, open bars) myocytes. *P<0.05 between cell types.

[Ca2+]i was calculated from [Ca2+]i=Kdβ(R−Rmin)/(Rmax−R).12 Rmax, Rmin, and β were determined experimentally and Kd taken as 200 mmol/L.13 Traces were averaged (10 to 15 transients); the difference between diastolic and peak [Ca2+]i was taken as [Δ[Ca2+]i], and the maximum rate of change of [Ca2+]i ([d[Ca2+]i/dt]max) was calculated using Origin software. Δ[Ca2+]i/ICa, and (d[Ca2+]i/dt)max/ICa were taken as commonly used and useful approximations of the gain of the Ca2+ release process as defined by Wier et al.14

Statistics

Data are presented as mean±SD in the text and mean±SEM in the figures. Statistical analysis was performed using SigmaStat software. A two-tailed unpaired t test was used to compare data from control and formamide treated cells when normal distribution and equal variance were confirmed, otherwise a nonparametric test (Mann-Whitney rank sum) was used. Paired t tests were used to test the effect of ryanodine or Ba2+ within the same group of cells. A value of P<0.05 was taken as significant.

Results

Detubulation Slows ICa Inactivation

Figure 1A shows recordings of ICa elicited by test pulses from −80 to 0 mV in representative control and detubulated ventricular myocytes. Detubulation decreased ICa density, confirming that this current is concentrated in the t-tubules.3 These traces also show that ICa, recorded from control myocytes showed biphasic inactivation whereas ICa recorded from detubulated myocytes showed monophasic inactivation. Detubulation of ventricular myocytes significantly decreased cell capacitance and ICa density (193±22 pF and −12.6±5.7 pA/pF respectively in control, n=22; 137±34 pF and −5.3±2.2 pA/pF in detubulated cells, n=22; P<0.05; Figure 1B). The time required for the current to decay to 0.37 of its peak amplitude (T1/3) was significantly longer in detubulated cells (27.1±7.8 ms, n=22) than in control cells (16.4±7.9 ms, n=22, P<0.05; Figure 1B).

To ensure that the slowed inactivation of ICa was not due to a direct effect of formamide, the effect of formamide treatment on ICa was determined in atrial cells, which lack t-tubules.9 ICa was recorded in atrial myocytes using the same solutions and experimental conditions as for ventricular myocytes except that the holding potential was set to −40 mV in order to inactivate T-type Ca2+ current. In contrast to
ventricular myocytes, $I_{\text{Ca}}$ elicited at 0 mV showed biphasic inactivation in control and formamide-treated atrial myocytes (Figure 2A), and formamide treatment of atrial cells had no significant effect on cell capacitance, $I_{\text{Ca}}$ density or $T_{0.37}$ (Figure 2B).

Thus it appears that in ventricular myocytes the rate of inactivation of $I_{\text{Ca}}$ is different at the t-tubules and the surface membrane. Subsequent experiments were designed to investigate the mechanism(s) underlying this difference.

$I_{\text{Ca}}$ Inactivation Due to Ca\textsuperscript{2+} Released From the SR

The inactivation phase of $I_{\text{Ca}}$ is due to two mechanisms: voltage-dependent inactivation (VDI) and Ca\textsuperscript{2+}-dependent inactivation (CDI).\cite{1} To investigate whether VDI is altered by detubulation we recorded $I_{\text{Ca}}$ using Ba\textsuperscript{2+} as the charge carrier, to eliminate CDI.\cite{2} $I_{\text{Ca,b}}$ was recorded at −10 mV to compensate for screening of surface charge.\cite{3} The top panel of Figure 3A shows representative currents from control and detubulated ventricular myocytes recorded using Ca\textsuperscript{2+} (red trace) or Ba\textsuperscript{2+} (black trace) as the charge carrier; the currents have been normalized to allow comparison of their time course. Inactivation of $I_{\text{Ca,b}}$ was not significantly different in control and detubulated myocytes ($T_{0.37}$: 72±7 ms, n=7 control, versus 79±15, n=8 detubulated; P>0.05; Figure 3B), suggesting that VDI is the same at the t-tubules and the surface membrane. These results, and the observation that the rapid phase of inactivation, which is due to CDI,\cite{7} is reduced in detubulated myocytes (eg, Figure 3A, top panel), suggest that CDI rather than VDI is reduced in detubulated myocytes.

In ventricular myocytes, CDI is due in part to Ca\textsuperscript{2+} released from the SR.\cite{16,17} The SR inhibitor ryanodine (30 μmol/L) was therefore used to investigate the role of SR Ca\textsuperscript{2+} release in the decrease of CDI observed in detubulated myocytes. Ryanodine significantly slowed inactivation and prolonged $I_{\text{Ca}}$ in control and detubulated myocytes (Figure 3A, middle panels); however, this effect was greater in control cells, so that in the presence of ryanodine, $T_{0.37}$ was not significantly different in control and detubulated myocytes (29.7±4.8 ms, n=13, versus 32.9±8.3, n=13, respectively; P>0.05; Figure 3B; cf, Figure 1B, right). This suggests, in agreement with previous work,\cite{16-20} that Ca\textsuperscript{2+} release from the SR inactivates $I_{\text{Ca}}$; however, this effect is reduced after detubulation, suggesting that SR Ca\textsuperscript{2+} release has a greater effect on $I_{\text{Ca}}$ inactivation in the t-tubules than at the surface membrane.

Including the fast Ca\textsuperscript{2+} chelator BAPTA (10 mmol/L) in the pipette solution had similar effects (Figure 3A, bottom, and 3B).

The decreased effect of SR Ca\textsuperscript{2+} release on $I_{\text{Ca}}$ inactivation in detubulated cells could be explained if $I_{\text{Ca}}$ triggered less SR Ca\textsuperscript{2+} release at the surface membrane. To address this hypothesis, we simultaneously recorded $I_{\text{Ca}}$ and intracellular Ca\textsuperscript{2+}.

Effect of Detubulation on $I_{\text{Ca}}$-Induced Ca\textsuperscript{2+} Release

The left traces in Figure 4A show representative recordings of [Ca\textsuperscript{2+}]; (top) and $I_{\text{Ca}}$ (bottom) from a control myocyte during a pulse to 0 mV. Under these conditions, the majority of
L-type Ca\(^{2+}\) current and Ca\(^{2+}\) release occur at the t-tubule (see Introduction). The right traces in Figure 4A show corresponding recordings from a detubulated cell. In these cells, the amplitude of \(I_{c_a}\) and the Ca\(^{2+}\) transient are reduced, as reported previously, and are determined predominantly by Ca\(^{2+}\) entry and release at the surface membrane. The decay of the Ca\(^{2+}\) transient is also slower because Na\(^{+}\)-Ca\(^{2+}\) exchange occurs predominantly in the t-tubules.

Figure 4B shows mean data from 14 control and 16 detubulated cells. Figure 4B, a, shows that \(I_{c_a}\) density was significantly reduced (left) and \(T_{33}\) (right) for \(I_{c_a}\) inactivation significantly prolonged (right) in detubulated cells, as in the previous set of experiments (cf, Figure 1). \(\Delta[Ca^{2+}]\) and \(d[Ca^{2+}]/dt_{\text{max}}\) were significantly smaller after detubulation (Figure 4B, b), although the time to \(d[Ca^{2+}]/dt_{\text{max}}\) normalized to \(\Delta[Ca^{2+}]\) were the same in the two cell types (Figure 4B, c).

**Effect of Detubulation on \(I_{c_a}\) Facilitation**

To investigate this idea further, we studied the response of control and detubulated myocytes to an increase in stimulation rate, which causes frequency-dependent facilitation (FDF) of \(I_{c_a}\) that is dependent, in part, on SR Ca\(^{2+}\) release. FDF in a representative control cell is shown in Figure 5A (top, left) as increased amplitude and slowed inactivation of \(I_{c_a}\) during the fourth voltage clamp pulse from 80 to 0 mV after an increase in stimulation rate from 0.1 to 1 Hz. The SR inhibitor ryanodine (30 μmol/L) slowed the time course of inactivation, and abolished FDF (Figure 5A, bottom left). The right traces in Figure 5A show that FDF was absent in detubulated cells in either the absence (top) or presence (bottom) of ryanodine. Figure 5B shows that \(I_{c_a}\), expressed as


Fraction of \( I_{c2} \) Remaining 20 ms After Its Peak, and Proportion of CDI in Control and Detubulated Myocytes

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Detubulated</th>
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<tbody>
<tr>
<td>( I_{c2Ba} )*</td>
<td>0.86±0.02</td>
<td>0.89±0.02</td>
</tr>
<tr>
<td>( I_{c2Ba,R} )*</td>
<td>0.54±0.03</td>
<td>0.56±0.02</td>
</tr>
<tr>
<td>( I_{c2Ca} )</td>
<td>0.33±0.02</td>
<td>0.53±0.02</td>
</tr>
<tr>
<td>CDI/Total Inac, %†</td>
<td>62</td>
<td>40</td>
</tr>
<tr>
<td>SR CDI/Total CDI, %‡</td>
<td>40</td>
<td>8</td>
</tr>
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</table>

\( I_{c2Ba} \) indicates Ba as charge carrier; \( I_{c2Ba,R} \) as charge carrier and 30 \( \mu \)mol/L ryanodine; \( I_{c2Ca} \), Ca as charge carrier.

*Mean±SEM from data in Figure 3. †Calculated as \( (I_{c2Ba}-I_{c2Ca})/I_{c2Ba} \)×100. ‡Calculated as \( (I_{c2Ba,R}-I_{c2Ca})/(I_{c2Ba,R}-I_{c2Ca}) \)×100.

the integral of the current, increased significantly in control cells when stimulation rate was increased but not in detubulated cells (\( I_{c2} / I_{c2Ba} = 1.34±0.23 \), \( n = 11 \), and 0.97±0.08, \( n = 13 \), respectively), nor was there any significant change in either control or detubulated cells in the presence of ryanodine (\( I_{c2} / I_{c2Ba} = 0.96±0.06 \) and 0.98±0.09, respectively). Inclusion of BAPTA in the patch pipette, or use of Ba\(^{2+}\) as the charge carrier, also abolished FDF (not shown). These data support the idea that the effect of SR Ca\(^{2+}\) release on \( I_{c2} \) is smaller at the surface membrane than in the t-tubules.

Quantification of CDI

The principal findings of this study are summarized in the Table. To quantify inactivation of \( I_{c2} \), we measured the fraction of current remaining 20 ms after its peak (\( I_{c2To} \)). This time was chosen because the peak of SR Ca\(^{2+}\) release occurs \( \approx 5 \) ms after peak \( I_{c2} \), with a time to 90% decay of \( \approx 45 \) ms.

When Ba\(^{2+}\) is used as the charge carrier, \( I_{c2} \) inactives almost exclusively by VDI, and \( I_{c2Ba} \) was not significantly different between control and detubulated myocytes (Table). When Ca\(^{2+}\) is used as the charge carrier, \( I_{c2} \) inactives by VDI and CDI. Thus the difference between \( I_{c2} \) and \( I_{c2Ca} \) represents the current inactivated by CDI. By normalizing to \( I_{c2Ba} \) (ie, \( \{I_{c2Ba}-I_{c2Ca}\}/I_{c2Ba} \)×100), we estimated total CDI to be \( \approx 62\% \) in control and \( \approx 40\% \) in detubulated myocytes (Table).

To separate SR-induced CDI from total CDI, we used a similar analysis, comparing \( I_{c2Ca} \) in the absence and presence of ryanodine. The difference current between \( I_{c2} \) and \( I_{c2Ca} \) in the presence of ryanodine represents the fraction of \( I_{c2} \) inactivated by SR CDI. By normalizing to total CDI (ie, \( \{I_{c2Ba} - I_{c2Ca}\}/I_{c2Ba} \)×100), we estimate the SR CDI to be \( \approx 40\% \) of total CDI in control myocytes and \( \approx 8\% \) in detubulated myocytes (table 1). Correcting for the 10% of cells that remain non-detubulated following formamide treatment, we can calculate that \( \approx 20\% \) of \( I_{c2} \) is located at the surface membrane (see Discussion) and thus that SR-dependent CDI at the t-tubules is \( \approx 50\% \), and \( \approx 5\% \) at the surface membrane.

Discussion

Experimental Approach

The method used to detubulate rat ventricular myocytes has been described and validated previously. The observed effects are unlikely to be due to formamide treatment per se because no changes were observed in atrial cells (Figure 2 and Brette et al).9

The decrease in \( I_{c2} \) and cell capacitance after formamide treatment, and correction for the presence of 10% nondetubulated myocytes (assessed as in Despa et al), indicate that \( \approx 80\% \) of \( I_{c2} \) is within the t-tubules, so that the density of \( I_{c2} \) in the t-tubules is \( \approx 6 \) fold higher than in the surface membrane. Thus, Ca\(^{2+}\) signaling in control cells is dominated by the t-tubules, whereas Ca\(^{2+}\) signaling in detubulated cells occurs predominantly at the cell surface.

It is well known that there is bidirectional cross-talk between \( I_{c2} \) and RyRs in cardiac myocytes.1 This local Ca\(^{2+}\) signaling occurs in a restricted diffusion space; the use of a low concentration of slow Ca\(^{2+}\) buffer (EGTA)4 as in the present study allows Ca\(^{2+}\) in the bulk cytosol to be “clamped” (indicated by the absence of cell contraction) while permitting Ca\(^{2+}\) in the dyadic space to change (indicated by the effect of ryanodine on \( I_{c2} \)), whereas a faster Ca\(^{2+}\) chelator (BAPTA) inhibits this local signaling, slowing the rate of inactivation of \( I_{c2} \) to the same level in control and detubulated myocytes (Figure 3). This local Ca\(^{2+}\) signaling has been observed in numerous studies recording \( I_{c2} \) using EGTA to buffer bulk Ca\(^{2+}\) exchange current, which is concentrated in the t-tubules.21,22 Such contamination could alter the shape of \( I_{c2} \), particularly from the physiological holding potential used (–80 mV), which is necessary to elicit FDF.7

Modulation of \( I_{c2} \) by SR Ca\(^{2+}\) Release Is Smaller at Surface Sarcolemma Than T-Tubule

Ca\(^{2+}\) entering the cell through the L-type Ca\(^{2+}\) channel, and Ca\(^{2+}\) released from the SR, appear to bind to calmodulin (CaM) that is prebound to the Ca\(^{2+}\) channel, causing channel inactivation.25 Figure 3A shows such Ca\(^{2+}\)-dependent inactivation; inhibition of SR Ca\(^{2+}\) release by ryanodine slowed the time course of inactivation in control cells. In detubulated cells, the time course of inactivation of \( I_{c2} \) was slower than in control cells; this is probably due to lack of feedback of Ca\(^{2+}\) released from the SR rather than to smaller Ca\(^{2+}\) influx, because \( T_{0.37} \) was little affected by ryanodine in detubulated cells, and was the same in control and detubulated cells in the presence of ryanodine (Figure 1B) despite differences in the amplitude of \( I_{c2} \). It is also unlikely that this prolongation of \( T_{0.37} \) occurs because of the solutions used for cell dialysis, because although \( T_{0.37} \) was slower in both cell types compared with ruptured patch experiments, probably due to the difference in holding potential, the ratio of \( T_{0.37} \) in detubulated:control myocytes in perforated patch (1.53) was similar to that in the ruptured patch experiments (1.65).

The idea that Ca\(^{2+}\) released from the SR has less effect on inactivation of \( I_{c2} \) in detubulated cells is supported by the observations that FDF did not occur in control cells in the
presence of ryanodine (showing that it is due to SR Ca\(^{2+}\) release), that it did not occur in detubulated cells and that ryanodine had no effect on the response of \(I_{\text{Ca}}\) to increasing stimulation rate in detubulated cells. FDF may be a consequence of Ca\(^{2+}\) binding to CaM on the Ca\(^{2+}\) channel,\(^{25}\) phosphorylation of the channel after activation of Ca\(^{2+}\)/calmodulin–dependent protein kinase II (CaMKII)\(^{26,27}\) or to less Ca\(^{2+}\)-dependent inactivation of \(I_{\text{Ca}}\).\(^{18}\)

**\(I_{\text{Ca}}\)-Induced SR Ca\(^{2+}\) Release Is the Same at the T-Tubules and Surface Membrane**

Although SR Ca\(^{2+}\) release had a greater effect on \(I_{\text{Ca}}\) at the t-tubules than at the surface membrane, the gain of the Ca\(^{2+}\) release process was not significantly altered by detubulation. Our experimental conditions precluded analysis of gain as first defined by Wier et al.;\(^{14}\) instead, we used two methods widely accepted as useful approximations:\(^1\) \(\Delta [\text{Ca}^{2+}] / I_{\text{Ca}}\) and \((\text{d}[\text{Ca}^{2+}] / \text{d}t)_{\text{max}} / I_{\text{Ca}}\). \(\Delta [\text{Ca}^{2+}] / I_{\text{Ca}}\), and hence \(\Delta [\text{Ca}^{2+}] / I_{\text{Ca}}\), might be overestimated in detubulated myocytes because of the spatially and temporally inhomogeneous Ca\(^{2+}\) transient,\(^{22,28}\) so we also used \(\text{d}[\text{Ca}^{2+}] / \text{d}t)_{\text{max}} \). However, neither method showed a significant change in gain, nor was the time to dCa\(^{2+}\)/dt\(_{\text{max}}\) altered after detubulation. This suggests that coupling of Ca\(^{2+}\) release to Ca\(^{2+}\) influx is the same in control and detubulated cells, and hence at the t-tubules and surface membrane; differences in such coupling would be expected to alter these measures.\(^{29}\) These data agree with previous work showing that the rate of rise of Ca\(^{2+}\) is the same at the cell periphery after detubulation as at the cell periphery and center of control myocytes using confocal microscopy.\(^{23,28}\)

Thus, it is unlikely that the smaller effect of SR Ca\(^{2+}\) release on \(I_{\text{Ca}}\) at the surface membrane is due to less local Ca\(^{2+}\) release, because feed-forward (\(I_{\text{Ca}}\) to SR Ca\(^{2+}\) release) is the same but feedback (SR Ca\(^{2+}\) release to \(I_{\text{Ca}}\)) is smaller at the surface membrane than in the t-tubules. A possible explanation for this dyadic rectification is considered next.

**Possible Mechanisms for Differential Modulation of \(I_{\text{Ca}}\) by SR Ca\(^{2+}\) Release**

There are a number of possible explanations for why \(I_{\text{Ca}}\) is affected more by SR Ca\(^{2+}\) release at the t-tubules than at the surface membrane: (1) Ca\(^{2+}\)-dependent modulation of \(I_{\text{Ca}}\) depends on local Ca\(^{2+}\) entry and local Ca\(^{2+}\) release;\(^{16}\) the present data could be explained if either is different in detubulated cells. It is unlikely that differences in local Ca\(^{2+}\) entry can account for the present observations, because in the presence of ryanodine, the rate of inactivation of \(I_{\text{Ca}}\) was the same in control and detubulated cells (Figure 3). It is also unlikely that differences in local Ca\(^{2+}\) release are responsible, because this appears to be the same at the t-tubules and surface membrane (above), consistent with previous work showing that SR Ca\(^{2+}\) content (assessed using caffeine) is not altered by detubulation.\(^{22,28}\) (2) Ca\(^{2+}\) entering the cytoplasm via \(I_{\text{Ca}}\) and from the SR enters a restricted diffusion space in which high [Ca\(^{2+}\)] occurs before Ca\(^{2+}\) diffuses to the bulk cytosol.\(^1\) The present data could be explained if the rise of Ca\(^{2+}\) in this space, and sensed by the Ca\(^{2+}\) channel, were lower at the surface sarcolemma. This could occur if the dyadic space is less restricted at the surface membrane than at the t-tubules, allowing Ca\(^{2+}\) released from the SR to diffuse away rapidly, or if RyR and \(I_{\text{Ca}}\) are less well colocalized at the surface membrane; either could result in a rise of Ca\(^{2+}\) around the Ca\(^{2+}\) channel that is too small or brief to affect \(I_{\text{Ca}}\). However, this seems unlikely because electron microscopy has shown no difference in the size of the dyadic space at the surface sarcolemma and t-tubules.\(^{30}\) and immunocytochemistry has shown similar colocalization of \(I_{\text{Ca}}\) and RyR\(^{1,6}\) at the two sites. In addition, any differences in the geometry and/or structure of the dyadic cleft would be expected to alter the gain of Ca\(^{2+}\) release and the rate of rise of Ca\(^{2+}\),\(^{29}\) both of which appear to be unaltered after detubulation. (3) The sensitivity of \(I_{\text{Ca}}\) to Ca\(^{2+}\) could be different at the 2 sites. This could be due to different channel isoforms; although several different isoforms have been found in ventricular myocytes,\(^{31}\) we are unaware of any evidence that they are differentially located between the t-tubule and surface membranes. Alternatively, CaM or CaMKII may regulate \(I_{\text{Ca}}\) more effectively at the t-tubules as a consequence of concentration of these proteins at the t-tubules\(^{26}\) or closer colocalization with the channel. This could explain why \(I_{\text{Ca}}\)-induced SR Ca\(^{2+}\) release is the same at both sites; whereas the feedback is different. We have recently shown such localized regulation of \(I_{\text{Ca}}\) by protein kinase A at the t-tubules.\(^{28}\)

**Physiological Significance**

Previous work has shown that Na\(^+\)-Ca\(^{2+}\) exchange is concentrated in the t-tubules and is more sensitive to Ca\(^{2+}\) released from the SR than to bulk cytoplasmic Ca\(^{2+}\).\(^{8,22,32}\) The present work shows that \(I_{\text{Ca}}\) is concentrated in the t-tubules and is regulated more effectively by SR Ca\(^{2+}\) release at the t-tubules than at the surface membrane. These data suggest that Ca\(^{2+}\) released from the SR will stimulate Ca\(^{2+}\) efflux via Na\(^+\)-Ca\(^{2+}\) exchange, and inhibit Ca\(^{2+}\) influx via \(I_{\text{Ca}}\), most effectively at the t-tubules. Thus, the t-tubules appear to be the primary site at which changes in SR Ca\(^{2+}\) release bring about compensatory changes in transsarcolemmal Ca\(^{2+}\) flux\(^8\) and are therefore central to cellular Ca\(^{2+}\) homeostasis.\(^{22}\) This may also be important during development and in pathological conditions (see review);\(^3\) for example, during heart failure, t-tubule density decreases\(^3\) and FDF is blunted\(^{33,34}\); this could be explained by the present work.

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Differential Modulation of L-type Ca\textsuperscript{2+} Current by SR Ca\textsuperscript{2+} Release at the T-Tubules and Surface Membrane of Rat Ventricular Myocytes

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