Quantification of Calcium Entry at the T-Tubules and Surface Membrane in Rat Ventricular Myocytes

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ABSTRACT The action potential of cardiac ventricular myocytes is characterized by its long duration, mainly due to Ca flux through L-type Ca channels. Ca entry also serves to trigger the release of Ca from the sarcoplasmic reticulum. The aim of this study was to investigate the role of cell membrane invaginations called transverse (T)-tubules in determining Ca influx and action potential duration in cardiac ventricular myocytes. We used the whole cell patch clamp technique to record electrophysiological activity in intact rat ventricular myocytes (i.e., from the T-tubules and surface sarcolemma) and in detubulated myocytes (i.e., from the surface sarcolemma only). Action potentials were significantly shorter in detubulated cells than in control cells. In contrast, resting membrane potential and action potential amplitude were similar in control and detubulated myocytes. Experiments under voltage clamp using action potential waveforms were used to quantify Ca entry via the Ca current. Ca entry after detubulation was reduced by ~60%, a value similar to the decrease in action potential duration. We calculated that Ca influx at the T-tubules is 1.3 times that at the cell surface (4.9 vs. 3.8 μmol/L cytosol, respectively) during a square voltage clamp pulse. In contrast, during a cardiac action potential, Ca entry at the T-tubules is 2.2 times that at the cell surface (3.0 vs. 1.4 μmol/L cytosol, respectively). However, more Ca entry occurs per μm² of junctional membrane at the cell surface than in the T-tubules (in nM/m²: 1.43 vs. 1.06 during a cardiac action potential). This difference is unlikely to be due to a difference in the number of Ca channels/junction at each site because we estimate that the same number of Ca channels is present at cell surface and T-tubule junctions (~35). This study provides the first evidence that the T-tubules are a key site for the regulation of action potential duration in ventricular cardiac myocytes. Our data also provide the first direct measurements of T-tubular Ca influx, which are consistent with the idea that cardiac excitation-contraction coupling largely occurs at the T-tubule dyadic clefts.

INTRODUCTION

During the cardiac action potential (AP), Ca influx via individual L-type Ca channels activates a cluster of adjacent sarcoplasmic reticulum (SR) Ca release channels (ryanodine receptors; RyRs); the consequent systolic Ca transient is the spatial and temporal sum of such local Ca releases (1,2). It is now generally accepted that L-type Ca current (I_{Ca}) is the major trigger for SR Ca release (3–5), and that alternative pathways (Na/Ca exchange, T-type Ca current) are weak triggers under physiological conditions (6,7). The rise of intracellular Ca leads to contraction of the cardiomyocyte; relaxation is brought about by Ca extrusion from the cell and resequestration into intracellular stores, via Ca ATPases and Na/Ca exchange (8). I_{Ca} also helps to load the SR with Ca for subsequent release (9): the contribution of the late phase of I_{Ca} to SR Ca loading has been demonstrated using action potential waveforms in voltage clamp experiments on guinea-pig ventricular myocytes (10), and the substantial role of I_{Ca} in SR Ca loading has been demonstrated in previous work from Eisner’s laboratory (reviewed in 11). Ca is thus both a second messenger (above) and a charge carrier: I_{Ca} is a depolarizing current and therefore determines the shape of the action potential.

Although I_{Ca} is present on the sarcolemma of cardiac myocytes, it has become increasingly clear that it is not uniformly distributed on the cardiac cell membrane. The sarcolemma of mammalian ventricular myocytes contains invaginations called transverse (T)-tubules (see 12 for review). T-tubules occur perpendicularly to the longitudinal axis of the cell at intervals of ~1.8–2 μm (13,14). They are located at the Z-lines and have a mean diameter of ~250 nm (14,15). Several studies have shown that I_{Ca} is located predominantly in the T-tubules in ventricular myocytes (see 12 for review). For example, immunocytochemistry has shown that in ventricular cells staining of L-type Ca channels occurs primarily at the T-tubules in rabbit (16), guinea-pig (17), and rat (18) myocytes. These data are supported by work investigating the functional localization of I_{Ca}: we have developed a technique to disrupt the T-tubules of rat ventricular myocytes (detubulation) (19). After osmotic shock, the T-tubules seal off within the cell and hence are physically and electrically uncoupled from cell surface membrane (20). By comparing currents from detubulated and control myocytes it is possible to estimate the proportion of current within the T-tubules. We have found ~80% of I_{Ca} within the T-tubules (21). This concentration of I_{Ca} at the T-tubules (and its colocalization with RyRs) allows spatial and temporal synchronization of Ca release throughout the cell, ensuring rapid and synchronous contraction (20,22,23). Although we have previously characterized the role of I_{Ca} at the T-tubules and cell surface...
in triggering SR Ca release (21), they are no quantitative data about Ca entry at the two sites. Furthermore, no information is available on the role of T-tubules in shaping the AP.

In this study, we have therefore investigated the role of the T-tubules in determining action potential configuration. We have also examined Ca entry in control and detubulated myocytes under voltage clamp using square and action potential waveforms, to quantify Ca entry at the T-tubule and surface membranes.

MATERIALS AND METHODS

Isolation of rat ventricular myocytes

Male Wistar rats (250–300 g) were killed humanely by cervical dislocation after stunning, and the heart rapidly removed, in accordance with the United Kingdom Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986. The heart was mounted on a Langendorff apparatus and perfused retrogradely with a HEPES-based isolation solution containing (in mmol/L): 113 NaCl, 5.4 KCl, 0.4 NaH2PO4, 1.4 MgCl2, 0.75 CaCl2,10 HEPES, 10 glucose, 20 taurine, and 10 creatine (pH 7.3 with NaOH). When the coronary circulation had cleared of blood, perfusion was continued with Ca-free isolation solution (in which CaCl2 was replaced with 0.1 mmol/L EGTA) for 5 min, followed by perfusion for a further 7–10 min with isolation solution containing 0.8 mg/ml collagenase (type IV; Worthington Biochemical, Lakewood, NJ), and 0.08 mg/ml protease (type XIV; Sigma, St. Louis, MO). The ventricles were then excised from the heart, minced, and gently shaken at 37°C in collagenase-containing isolation solution supplemented with 1% bovine serum albumin. Cells were filtered from this solution at 5 min intervals and resuspended in isolation solution containing 0.75 mmol/L Ca.

Detubulation of rat ventricular myocytes

Detubulation was induced by osmotic shock as described previously (19). Briefly, 1.5 mol/L formamide was added to the cell suspension for 15–20 min, before returning the cells to the standard solution. Detubulation occurs because of the osmotic shock produced by formamide withdrawal.

Electrophysiological recordings

Myocytes were studied in a chamber mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). Cells were initially superfused with a normal physiological salt solution containing (in mmol/L): 113 NaCl, 5 KCl, 1 MgSO4, 1 CaCl2, 1 Na2HPO4, 20 Na acetate, 10 glucose, 10 HEPES, and 5 U/L insulin, pH set to 7.4 with NaOH. All experiments were performed at room temperature (22–25°C).

Membrane potential and currents were recorded using the whole-cell configuration of the patch clamp technique (24). An Axopatch 200B (Axon Instruments, Union City, CA) 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 2–10 kHz using an 8-pole Bessel low pass filter (21). Signals were filtered at 2–10 kHz using an 8-pole Bessel low pass filter (21). Action potentials were evoked by 2.5 ms subthreshold current steps. Membrane potential and currents were recorded during a 10-mV hyperpolarizing pulse from −80 mV. Electrotonic capacitance and series resistance were compensated (> 80%) so that the maximum voltage error was <5 mV. ICa was elicited by either a rectangular step (150 ms, −80 mV) or a holding potential of −80 mV or a rectangular step (150 ms, −80 mV) or a representing action potential waveform. The action potential waveform was the average of the APs recorded in the current clamp experiments in control and detubulated myocytes (n = 10 of each cell type, see Fig. 1A). Trains of depolarizing pulses were applied at 0.1 Hz.

Data analysis

Action potential amplitude was measured as the difference between the overshoot and the resting membrane potential. Membrane potentials were corrected by −11 mV to compensate for liquid junction potentials between the external and pipette solutions. APD was measured as the duration from the overshoot to three different percentages of repolarization (25: APD25; 50: APD50; 90: APD90). 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). Time to peak ICa was measured from the start of the depolarizing pulse. Because the decay of ICa varied between cell types and experimental conditions, the kinetics of inactivation of ICa was characterized by the time required for the current to decay to 0.37 of the peak amplitude (τ0.37) (21). ICa was also analyzed by integrating ICa during the test pulse to obtain total Ca influx during the pulse. Ca entry is expressed as charge density (fC/pF) and as cytosolic [Ca2+] using estimates of surface to volume ratios for control and detubulated cardiac myocytes (25).

Chemicals

All solutions were prepared using ultrapure water supplied by a Milli-Q system (Millipore, Watford, UK). All solution constituents were reagent grade and purchased from Sigma (St. Louis, MO).

FIGURE 1 Action potentials are shorter after detubulation. (A) Mean action potential obtained in 10 control (left) and 10 detubulated (right) rat ventricular myocytes. (B) The mean ratio between resting potential (RP), action potential amplitude (Amp), APD25, APD50, and APD90 in detubulated and control myocytes.
Statistics
Data are presented as mean ± SE. Statistical analysis was performed using SigmaStat software. A two-tailed unpaired t-test was used to compare data from control and formamide treated cells, after confirmation of normal distribution and equal variance. Friedman Repeated Measures Analysis of Variance on Ranks and Student-Newman-Keuls Method were used to test the effect of multiple voltage waveforms within the same group of cells. \( P < 0.05 \) was taken as significant.

RESULTS
Detubulation shortens the action potential
Fig. 1 A shows action potentials elicited by brief current pulses at 0.1 Hz in control and detubulated ventricular myocytes. Each trace shows the mean of the APs recorded from 10 control (left) and 10 detubulated (right) myocytes; these mean APs were used as representative action potentials for subsequent voltage clamp studies. The principal effect of detubulation was to shorten the action potential, shown by a significant reduction of APD\(_{25}\), APD\(_{50}\), and APD\(_{90}\) (Table 1). In contrast, resting membrane potential and action potential amplitude were unchanged (−81.2 ± 0.9 mV vs. −81.0 ± 1.2 mV; NS, and 114 ± 4 mV vs. 108 ± 3 mV; NS; \( n = 10 \) myocytes of each type, Table 1). Fig. 1 B shows the detubulated/control ratios for these variables, showing that the effect of detubulation appears most marked for APD\(_{50}\), the point at which \( I_{\text{Ca}} \) contributes most to the action potential (26). To investigate this further, we recorded \( I_{\text{Ca}} \) using action potential waveforms in voltage clamp experiments on control and detubulated myocytes.

\( I_{\text{Ca}} \) elicited by voltage clamp using square and action potential waveforms
\( I_{\text{Ca}} \) was elicited at 0.1 Hz in each cell type using three different voltage command waveforms: a square pulse, the control action potential, and the detubulated action potential (Fig. 2, top panel).

Typical results for control myocytes are shown in the middle panel of Fig. 2. Peak \( I_{\text{Ca}} \) was slightly, but significantly, smaller when the control AP waveform was used, compared to the square pulse and detubulated AP waveform (Fig. 3 A). However the major effects were on \( I_{\text{Ca}} \) kinetics:

\( \delta I_{\text{Ca}} \) during a square pulse, control AP waveform and detubulated waveform in control and detubulated myocytes. The top panel shows the voltage waveform applied to cardiac myocytes (square pulse, left; control action potential, middle; detubulated action potential, right). The middle panel shows representative example of \( I_{\text{Ca}} \) measured in the same control rat ventricular myocyte under the conditions shown directly above (cell capacitance, 210 pF). The lower panel shows representative example of \( I_{\text{Ca}} \) measured in the same detubulated rat ventricular myocyte under the conditions shown on the top panel (cell capacitance, 110 pF).

Figure 2

TABLE 1  Action potential characteristics of control and detubulated myocytes

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Detubulated</th>
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<tbody>
<tr>
<td>Resting potential (mV)</td>
<td>81.2 ± 0.9</td>
<td>81.0 ± 1.2</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>114 ± 4</td>
<td>108 ± 3</td>
</tr>
<tr>
<td>APD(_{25}) (ms)</td>
<td>11.9 ± 2.1</td>
<td>6.7 ± 0.9*</td>
</tr>
<tr>
<td>APD(_{50}) (ms)</td>
<td>31.6 ± 5.8</td>
<td>13.5 ± 1.7*</td>
</tr>
<tr>
<td>APD(_{90}) (ms)</td>
<td>61.0 ± 9.8</td>
<td>30.7 ± 3.4*</td>
</tr>
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</table>

Values are mean ± SE from 10 cells in each type; APD, action potential duration.

\*\( P < 0.05 \).
voltage waveform used; these data were used, therefore, to quantify Ca entry across the T-tubule and surface membranes when using different waveforms.

**Ca entry during voltage clamp using action potential waveforms**

$I_{\text{Ca}}$ was integrated, and the integral normalized to cell capacitance, to quantify Ca entry. Fig. 4 A shows that Ca influx was significantly smaller in detubulated cells than in control cells when using a given waveform, but was larger during square pulse stimulation, compared to AP waveforms, in both cell types. Fig. 4 B shows the ratio of Ca entry/peak $I_{\text{Ca}}$ density, showing that a given peak $I_{\text{Ca}}$ produced more Ca influx in detubulated myocytes than in control myocytes during a square pulse, but that this difference was absent when using AP waveforms. The ratio of net entry of positive charge when using the detubulated AP for detubulated myocytes versus control AP for a control myocyte was 0.421 ($8.3 \pm 1.4 \text{ pC}$ vs. 23.5 $\pm 4.2 \text{ pC}$, $n = 13$ and 12, respectively). This is similar to the ratio of APD$_{50}$ for detubulated versus control myocytes (0.427, Fig. 1 B). Thus the reduction in Ca entry after detubulation is compatible with the reduction in APD shown in Fig. 1, and with the idea

**FIGURE 3** $I_{\text{Ca}}$ characteristics during square pulse, control AP waveform, and detubulated waveform in control and detubulated myocytes. (A) Mean (± SE) $I_{\text{Ca}}$ density in control (solid bars) and detubulated (open bars) rat ventricular myocytes during square pulse (Square, left), control AP waveform (AP Ctl, middle), and detubulated AP waveform (AP Det, right). (B) Mean (± SE) time to peak $I_{\text{Ca}}$ in control (solid bars) and detubulated (open bars) rat ventricular myocytes during square pulse (Square, left), control AP waveform (AP Ctl, middle), and detubulated AP waveform (AP Det, right). (C) Mean (± SE) time to decline to 37% of peak $I_{\text{Ca}}$ (T$_{0.37}$) in control (solid bars) and detubulated (open bars) rat ventricular myocytes during square pulse (Square, left), control AP waveform (AP Ctl, middle), and detubulated AP waveform (AP Det, right). Data are from 12 control and 13 detubulated myocytes. *$P < 0.05$ between cell types, #$P < 0.05$ versus square pulse, and †$P < 0.05$ versus AP Ctl.

**FIGURE 4** Ca entry during $I_{\text{Ca}}$ is smaller after detubulation. (A) Mean (± SE) Ca entry during $I_{\text{Ca}}$ in control (solid bars) and detubulated (open bars) rat ventricular myocytes during square pulse (Square, left), control AP waveform (AP Ctl, middle), and detubulated AP waveform (AP Det, right). (B) Ratio Ca entry/peak $I_{\text{Ca}}$ for control (solid bars) and detubulated (open bars) rat ventricular myocytes during square pulse (Square, left), control AP waveform (AP Ctl, middle), and detubulated AP waveform (AP Det, right). (C) Mean (± SE) ratio for control (solid bars) and detubulated (open bars) myocytes when cells are stimulated with AP of their own type (APpair) and the other type (APinv). Data are from 12 control and 13 detubulated myocytes. *$P < 0.05$ between cell types, #$P < 0.05$ versus square pulse, and †$P < 0.05$ versus AP Ctl.
that the role of the T-tubules in shaping AP configuration is
mainly due to the localization of $I_{\text{Ca}}$ is these invaginations.
Conversely, AP waveform can influence $I_{\text{Ca}}$, and therefore
Ca entry. We examined this by calculating the ratio of Ca
entry during different voltage waveforms (Fig. 4C). The
ratio of Ca entry is reduced when using AP waveforms
(either control or detubulated) compared to square pulse,
although AP voltage waveform (control versus detubulated)
has little effect upon Ca entry in either cell type, as the ratio is
$\sim 1$ (Fig. 4C, right). This suggests that reduction of APD has
little effect upon Ca entry via $I_{\text{Ca}}$, therefore the decrease in
Ca entry after detubulation is mainly due to a decrease in $I_{\text{Ca}}$
rather than a decrease in APD.

**Quantification of Ca entry at the surface membrane and T-tubules**

The main findings of this study are summarized in Table 2. Ca fluxes and $I_{\text{Ca}}$ in the T-tubules were calculated as the
difference in whole-cell Ca entry between control and detubu-
lated myocytes. These Ca fluxes and $I_{\text{Ca}}$ were divided by the
difference in membrane capacitance between control and
detubulated cells, to derive the Ca flux and $I_{\text{Ca}}$ density in the
T-tubules. The data from detubulated myocytes have been
corrected by 10% to account for the presence of nondetubu-
lated myocytes (25).

In rat ventricular myocytes, Ca entry is greater when using
the square voltage waveform than when using the AP wave-
form (Table 2). Ca entry at the T-tubules represents 53% and
65% of total during a square pulse and AP waveform, re-
spectively. These values are less than the percentage of $I_{\text{Ca}}$ at
the T-tubules (75%).

These Ca influx measurements were further converted to
changes in [Ca]. Integrated fluxes were converted to molar
quantity (by dividing by $zF$) and normalized to cell volume.
Surface/volume ratios (5.1 and 3.4 pF/pL in control and
detubulated myocytes from our recent study (25)) give
a volume of 36.4 ± 2.2 pL for control ($n = 14$) and 39.22 ±
2.3 pL for detubulated myocytes ($n = 13$); these values are
not significantly different. Fig. 5 shows the results of these
calculations with [Ca] in μmol/L cytosol (assuming the
fraction of nonmitochondrial cell volume to be 0.65 L
cytosol/L cell (28)). Ca influx at the T-tubules is 1.3 times
that at the cell surface (4.9 vs. 3.8 μmol/L cytosol, respec-
tively) during a square pulse. In contrast, during an AP, Ca
entry at the T-tubules is 2.2 times that at the cell surface
(3.0 vs. 1.4 μmol/L cytosol, respectively, calculated by the
difference between average of control and detubulated AP; Fig. 5, A–C). The percentage of Ca influx at the T-tubules is
also smaller than the percentage of $I_{\text{Ca}}$ density (Table 2).

We also estimated the junctional Ca flux at the two sites.
In rat ventricular myocytes, 7.7% of the surface sarcolemma,
and 48% of the T-tubules, is junctional (29). Assuming that
1 μF represents 1 cm² (30), we calculated that junctions
represent 978 μm² at the cell surface and 2832 μm² in the
T-tubules (Table 3). Thus Ca influx per μm² of junction is
2.2 higher at the cell surface than in the T-tubules (3.89 vs.

| TABLE 2 Distribution of mean cell capacitance, $I_{\text{Ca}}$, and Ca entry in the external sarcolemma and T-tubules |
|-----------------|-----------|----------|----------|----------|
|                  | Surface area | $I_{\text{Ca}}$ SP | Ca entry SP | Ca entry AP |
|                  | (pF)        | (pA/pF)  | (fC/pF)  | (fC/pF)  |
| Total SL         | 186        | 10.3     | 215      | 108      |
| (control cells)  |            |          |          |          |
| External SL      | 127        | 3.8      | 149      | 55       |
| (detubulated cells)* |          |          |          |          |
| T-tubules (calculated) | 59   | 24.3     | 357      | 222      |
| % in T-tubules   | 32         | 75       | 53       | 65       |
| Density T-tub/ext SL | 6.4 | 2.4      | 4        |

*These values have corrected by 10% for the presence of nondetubulated myocytes.

FIGURE 5 Relative distribution of Ca entry between the T-tubules and
external sarcolemma. (A) Mean Ca entry during square pulse (solid bars)
and AP waveform (open bars) at the total sarcolemma (Total SL, left),
surface sarcolemma (Surface SL, middle), and T-tubules (right) during
action potential stimulation. Ca fluxes are converted to [Ca$^{2+}$] in μmol/L
cytosol (see text for details). (B) The relative distribution of Ca entry
during square pulse (solid bars) and AP waveform (open bars) at the surface
sarcolemma (Surface SL, left) and the T-tubules (right). (C) The ratio
between the Ca entry at surface sarcolemma and in the T-tubules during
square pulse (Square, left) and AP waveform (AP, right).

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TABLE 3  Junctional Ca entry at the surface sarcolemma and T-tubules

<table>
<thead>
<tr>
<th></th>
<th>External SL</th>
<th>T-tubules</th>
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<tbody>
<tr>
<td>Total surface area (pF)</td>
<td>127</td>
<td>59</td>
</tr>
<tr>
<td>Junctional surface area (μm²)*</td>
<td>978</td>
<td>2832</td>
</tr>
<tr>
<td>Ca entry SP (μM)</td>
<td>3.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Ca entry AP (μM)</td>
<td>1.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Ca entry per μm² junction (nM) SP</td>
<td>3.89</td>
<td>1.73</td>
</tr>
<tr>
<td>Ca entry per μm² junction (nM) AP</td>
<td>1.43</td>
<td>1.06</td>
</tr>
</tbody>
</table>

SP, square pulse; AP, action potential; SL, sarcolemma.
*These values have been calculated assuming that junctional sarcolemma forms 7.7% of the surface SL and 48% of the t-tubule membrane.

1.73 nmol/L cytosol/μm², respectively) during a square pulse, and 1.3 times higher during an AP (1.43 vs. 1.06 nmol/L cytosol/μm², respectively; Table 3). To investigate whether the greater Ca entry per μm² of cell surface, compared to the T-tubules, is due to a difference in the number of Ca channels at each junction, we estimated the total number of Ca channels, using the equation \( N = \frac{I_{Ca}}{i_{Ca} \times p} \), where \( p \) is the probability of channel opening (0.05, see Discussion).

From these values, we estimate that 191,600 functional Ca channels are present in rat myocytes, with 48,200 channels at the cell surface and 143,400 at the T-tubules (Table 4). Given that 90% of Ca channels are junctional (18), we estimate that a typical junction in rat ventricular myocytes contains \( \sim 35 \) Ca channels at both the cell surface and T-tubules (Table 4).

**DISCUSSION**

This study provides the first quantitative description of Ca entry at the T-tubules and cell surface of cardiac ventricular myocytes.

**Experimental approach**

The method used to detubulate rat ventricular myocytes has been described and validated previously (19,20). Notably, this procedure has no effect on cell capacitance, \( I_{Ca} \), or the AP in atrial myocytes, which lack T-tubules (20). This method enables investigation of the physiological function of surface and T-tubule membranes in rat ventricular myocytes. Using this technique, we have shown previously that L-type Ca current, Na/Ca exchange and Na-K pump currents are located predominantly in the T-tubules (21,25). In contrast, K currents are evenly distributed between the surface sarcolemma and T-tubules (34). We have also shown that the T-tubules are essential for spatial and temporal synchronization of Ca release throughout the cell (20,23,35).

We chose to use Na- and K-free experimental solutions to record \( I_{Ca} \) because this enabled us to use a physiological resting potential (near −80 mV for step and AP waveforms) without contamination by other currents, such as Na current, Na/Ca exchanger current, and K currents, allowing us to quantify Ca entry via \( I_{Ca} \) only. The use of a physiological holding potential is important since depolarized holding potentials (e.g., −40 mV) are closer to the activation threshold of \( I_{Ca} \) and can therefore interfere with Ca channel availability and gating (see 32 for review). However, we might have slightly underestimated Ca entry because of the lack of Na/Ca exchange activity, although Ca entry via this route in cardiac myocytes is small compared to \( I_{Ca} \) (8). Inclusion of a low concentration of a slow Ca buffer (5 mmol/L EGTA) in the pipette solution allows Ca to be “clamped” (indicated by the absence of cell contraction) while permitting Ca in the dyadic space to change (27). Therefore, Ca entry via \( I_{Ca} \) measured in this study is close to Ca entry during normal excitation-contraction coupling.

**Role of the T-tubules in action potential shape**

The main effect of loss of the T-tubules was to decrease APD. This shortening is unlikely to be due to differences in K currents because they are evenly distributed between surface membrane and T-tubules (34), consistent with the absence of changes in the resting membrane potential.
(mainly due to $I_{K1}$ in cardiac myocytes, 31). Reduction of APD is also unlikely to be due to a change in Na current because: i), Na current causes only a small entry of positive charge because it is very brief (36); and ii), AP amplitude, which is mainly due to Na current, is the same in control and detubulated myocytes, compatible with uniform distribution of Na current at the cell surface (23). It is therefore more likely that the decrease in APD is due to less $I_{Ca}$ and Na/Ca exchange current, which carry positive charge into the cell and are concentrated at the T-tubules (21,25). Given the results from this study, it is expected that the AP in the T-tubules of cardiac myocytes will be longer than at the cell surface. Calculation from APD50 in detubulated (surface membrane) and control (total membrane) myocytes gives a value of 73.8 ms for APD50 in the T-tubules (30% of membrane), ~5.5 times longer than at the cell surface. This challenging speculation of course requires experimental confirmation, but to date no electrophysiological technique has enabled recording of electrical activity of the T-tubules only. The action potential is shaped by ionic currents; conversely the form of the action potential influences ionic currents. This study shows that a shorter action potential enhances the magnitude and reduces the time to peak and $T_{0.37}$ of $I_{Ca}$ (Fig. 3). This is similar to previous work showing that rapid early repolarization of the AP is crucial in shaping $I_{Ca}$ (37,38).

**Ca entry at the T-tubules versus cell surface membrane**

Our data show that Ca entry during a square pulse is larger than during an AP waveform (Fig. 4). This is consistent with a previous report in rat ventricular myocytes using a similar approach (39), and highlights the caution required when interpreting results obtained using square pulses to calculate Ca flux. Our calculated Ca entry is similar to previous work using an AP waveform in rat myocytes (~120 fC/pF (40); ~4 μmol/L cytosol (41)). In contrast, Yuan et al. (39) found values that are somewhat higher than observed in this study (~14 μmol/L cytosol), but this may be due to higher external [Ca] and lower Ca-dependent inactivation. It is unlikely that the temperature used to perform our experiments (room temperature) altered the quantification of Ca entry because temperature (25°C vs. 35°C) has been shown to alter $I_{Ca}$ kinetics but not total $I_{Ca}$ flux in rabbit ventricular myocytes (42).

We found that Ca entry at the T-tubules is larger than at surface sarclemma, although not to the extent of $I_{Ca}$ density (~75%). This can be explained by reduced Ca-dependent inactivation of the Ca channels present at the cell surface (21), which will prolong $I_{Ca}$. However, this difference in Ca-dependent inactivation of $I_{Ca}$ at the T-tubules and cell surface was less marked during the AP waveform (Fig. 3 C).

Interestingly, Ca entry after detubulation is reduced by ~60%, a value close to the density of Na/Ca exchanger present in the T-tubules (63%, (25)). Since Ca entry via $I_{Ca}$ is extruded by the Na+/Ca2+ exchanger during a normal Ca cycle in cardiac myocyte (11), this can explain how SR Ca2+ load remains constant after detubulation (19,23,35). The relative difference between $I_{Ca}$ density and Ca entry at the T-tubule and surface membranes (Table 2) also suggests a different role for $I_{Ca}$ at the two sites: the large, rapidly inactivating $I_{Ca}$ in the T-tubules will form an effective trigger for SR Ca release (9), whereas the more slowly inactivating $I_{Ca}$, and relatively large Ca entry (for the density of $I_{Ca}$) at the cell surface will be effective in loading the SR with Ca2+ that can be released in response to a subsequent stimulus (9,43).

This work suggests that Ca entry per μm² of junctional membrane is greater at the cell surface than in the T-tubules (Table 3). When normalized to the number of junctions present (Table 4), calculated from available electron microscopy data (33), the data suggest that Ca entry is 1.13 nmol/L cytosol/junction at the cell surface versus 0.85 nmol/L cytosol/junction at the T-tubules during an action potential. However our calculation of the number of junctions may be overestimated because, to the best of our knowledge, there is currently no information about the mean distance between junctions but only the minimum distance between them (33). Similarly, our calculation of the number of Ca channels is speculative, since it depends critically on $i_{Ca}$ (the unitary current) and $p$ (the probability of channel opening). Experimental values are quite disparate, ranging from 0.15 to 0.4 pA for $i_{Ca}$ and 0.015 to 0.08 for $p$ (at 0 mV, e.g., see (44–48) and for review (31)); this probably reflects differences in species and experimental conditions between studies. We have therefore used midrange values which are classically used for computer modeling of cardiac excitation-contraction coupling ($i_{Ca} = 0.2$ pA and $p = 0.05$, see 49,50). These values give us a total Ca channel number (Table 4) that is within the range observed experimentally by others (from 28,000 (48) to 300,000 (47)). It is also important to note that we used similar parameters for cell surface and T-tubule Ca channels and this might not be the case, although no experimental data from single Ca channel recording at the T-tubules are available. We estimated that ~35 Ca channels are present at each junction in rat ventricular myocytes (independent of the subcellular location of the junction). This is somewhat higher than described by Bers (10–25 Ca channels, (31)), although rat ventricular myocytes tend to have more feet (or ryanodine receptors, 267 (33)) per junction than other species (e.g., 60 in dog, 128 in mouse; see 33). Thus our values give a ryanodine receptor/Ca channel ratio of ~7, which is in the range of other species (4–10; see 31). These considerations therefore suggest that a similar number of Ca channels are present at each junction at the cell surface and T-tubules. Thus it appears likely that greater Ca entry/Ca channel at the cell surface, rather than a greater number of Ca channels, accounts for the differential Ca influx at the two sites (Table 3).

This differential Ca entry might also have implications for the gain of SR Ca release; however previous work has
suggested that the gain of SR Ca release is similar at the surface and T-tubule membranes (21), so that the different Ca dependent inactivation at the two sites (above) is unlikely to be due to differences in local Ca release. The extensive T-tubule system therefore allows synchronous Ca release within the cell (see above). Ca entry at the cell surface provides Ca\(^{2+}\) for the SR which can then diffuse within the SR to be available for subsequent release at the T-tubules. This requires further investigation, although it has been recently shown that Ca diffuses very quickly within the SR (51,52). Such balance between Ca entry at the cell surface and T-tubules might also be important during development and in pathological conditions in which T-tubule density changes (see 12 for review); it has, for example, been reported to decrease during heart failure (53–55).

In conclusion, our study provides the first evidence that the T-tubules are a key site for the regulation of action potential duration in ventricular cardiac myocytes. Our data also provide the first direct measurements of T-tubular Ca influx, which are consistent with the idea that cardiac excitation-contraction coupling largely takes place at the T-tubule dyadic clefts. The quantification of local Ca entry within the T-tubules and at junctional membrane may also have important implications for modeling cardiac cell function and for understanding cellular Ca cycling and suggests that the key role of Ca entry may be different at the T-tubule and surface membranes.

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