Ca\(^{2+}\) waves during triggered propagated contractions in intact trabeculae

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Miura, Masahito, Penelope A. Boyden, and Henk E. D. J. ter Keurs. Ca\(^{2+}\) waves during triggered propagated contractions in intact trabeculae. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H266–H276, 1998.—Triggered propagated contractions (TPCs) are initiated near the damaged ends of trabeculae (D) and have been observed in both rat ventricular (10, 19) and human atrial trabeculae (9).

AFTERCONTRACTIONS ARISE in damaged regions of cardiac muscle and propagate into the neighboring myocardium. Propagating contractions, which can be measured as waves of sarcomere shortening, have been denoted as triggered propagated contractions (TPCs) and have been observed in both rat ventricular (10, 19) and human atrial trabeculae (9).

Similar to the mechanism proposed for Ca\(^{2+}\) waves in a single isolated myocyte (17), the propagation mechanism of a TPC is thought to be consistent with a model of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from sarcoplasmic reticulum (SR) mediated by Ca\(^{2+}\) diffusion to adjacent SR for the following reasons. First, TPCs do not require an intact sarcolemma (8). In addition, Gd\(^{3+}\), a stretch-activated channel blocker, affects neither initiation nor propagation velocity of a TPC in intact trabeculae, suggesting that stretch activation of ion fluxes are not involved in TPC propagation (32). Second, TPCs are abolished by agents that interfere with SR Ca\(^{2+}\) loading or release, such as ryanodine and caffeine (11), similar to findings with Ca\(^{2+}\) waves in single myocytes (17).

Third, TPCs propagate along the undamaged parts of the trabeculae at constant velocities that vary from 0.1 to 15 mm/s depending on SR Ca\(^{2+}\) loading (10, 19). These propagation velocities are too fast for simple diffusion of Ca\(^{2+}\) alone (see Ref. 2, discussion) and slower than conduction velocities of electrical signals reported for normal ventricular muscle (1 m/s). Finally, computer simulation studies using a model of CICR and Ca\(^{2+}\) diffusion predict propagation velocities of TPCs that are in agreement with the observed experimental data (2).

Therefore, if we assume that a CICR model describes the propagation mechanism of TPCs, it is important to know how fast Ca\(^{2+}\) diffuses through cells as well as from cell to cell within trabeculae. The equivalent diffusion coefficient (D\(_{eq}\)) in water (D\(_{eq}\)) for Ca\(^{2+}\) is 750 \(\times 10^{-8}\) cm\(^2\)/s (30); D\(_{eq}\) in myoplasm (D\(_{myop}\)) for Ca\(^{2+}\) is unknown but should be lower than that in pure water because of the intracellular milieu, which has an ionic strength of 0.15—0.20 M, and the presence of large protein moieties (22). In a multicellular preparation, Ca\(^{2+}\) must diffuse from cell to cell through gap junctions (GJ) s, which respond dynamically to a variety of regulatory factors (24), such that D\(_{eq}\) in trabecula (D\(_{tra}\)) for Ca\(^{2+}\) should be affected by the agents that alter the permeability of gap junctions (P\(_{GJ}\)). Recently, we demonstrated that octanol and heptanol, which decrease open probability of GJ channels in a nonspecific manner (26), reduce the propagation velocity, triggering rate, and force of TPCs (31). These observations are consistent with the idea that Ca\(^{2+}\) diffuses from cell to cell through GJs for the propagation of a TPC. However, the direct effects of octanol or heptanol on P\(_{GJ}\) in intact cardiac muscle have not yet been evaluated.

With regard to Ca\(^{2+}\) dynamics within trabeculae during TPCs, regional increases in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) are thought to underlie the TPCs and are assumed to be similar to the Ca\(^{2+}\) waves in single myocytes (16-18). Furthermore, Ca\(^{2+}\) sparks and waves occur at a subcellular level (20) and Ca\(^{2+}\) sparks precede or lead to Ca\(^{2+}\) waves in myocytes (6). In contrast, little is known about the spatial and temporal changes in [Ca\(^{2+}\)]\(_i\) in multicellular cardiac trabeculae.

Therefore, in this study we have tested 1) whether a regional increase in [Ca\(^{2+}\)]\(_i\) propagates along trabeculae during a TPC in multicellular preparations (>1 mm). Furthermore, we determined 2) the diffusion properties for fura 2 to evaluate how Ca\(^{2+}\) ions diffuse through cells as well as from cell to cell within trabeculae and 3) the effects of octanol on fura 2 diffusion properties. Subsequently, we could then evaluate to
what extent Ca\(^{2+}\) diffusion through the myoplasm and the GJs is affected by octanol under our experimental conditions. Preliminary data have recently been published (31).

**METHODS**

**Dissection and Mounting of Rat Ventricular Trabeculae**

Lewis Brown Norway rats (250–300 g) were anesthetized with diethyl ether. The hearts were excised, and the coronary arteries were immediately perfused via the aorta with Krebs-Henseleit (KH) solution modified by adding 15 mM KCl. After the arrest of the heart, long, thin trabeculae (n = 27, length = 2.6 ± 1.2 mm, width = 260 ± 150 µm, thickness = 110 ± 29 µm) were dissected from 27 rat right ventricles and were mounted horizontally between a force transducer and a micromanipulator in a perfusion bath located on the stage of an inverted microscope (Nikon). Preparations were superfused with bicarbonate-buffered KH solution containing (in mM) 120 NaCl, 5 KCl, 1.2 MgCl\(_2\), 1.2 Na\(_2\)SO\(_4\), 2.0 NaH\(_2\)PO\(_4\), 19 NaHCO\(_3\), 10 glucose, and CaCl\(_2\) as specified in Fura 2 loading. All solutions were in equilibrium with 95% O\(_2\)-5% CO\(_2\) such that pH was 7.4.

**Experimental Equipment**

Figure 1A shows the experimental setup. Light from a 150-W xenon arc lamp (model 6255, Oriel, Stratford, CT) was filtered using band-pass filters (Melles Griot, Irvine, CA). The epifluorescence from trabeculae was collected by the objective lens, passed through a 150-W xenon arc lamp (model 6255, Oriel, Stratford, CT) was filtered using band-pass filters (Melles Griot, Irvine, CA) and then projected onto a photomultiplier tube (PMT; PMT-R2693 with a C1053-01 socket, Hamamatsu). The PMT signal was filtered at 100 Hz (3 dB), fed into an analog-to-digital converter (2801A Data-Translation, Marlborough, MA), and stored in a personal computer (Gateway 2000, North Sioux City, SD). Alternatively, the fluorescent image of the trabecula was recorded by a charge-coupled device camera coupled to a two-stage image intensifier (IIC; model C330, General Scanning, Water- town, MA) through a 510- to 560-nm band-pass filter (Nikon). Images from the camera were recorded directly with a videocassette recorder (VCR; EV-S7000 NTSC, Sony, Tokyo, Japan) for later analysis. The VCR allowed reliable display of single-time-code video frames. Concurrently, the force of the muscle was measured using a modified silicon semiconductor strain gauge (model AE-801, Sensoron, Horten, Norway). Sarcomere length (SL) was measured using laser diffraction by illuminating the muscle with a He-Ne laser (Spectraphysics, Eugene, OR) described in detail elsewhere (27). Briefly, the intensity distribution of the first-order diffraction pattern was scanned by a photodiode array (Reticon 256 EC, Sunnyvale, CA) and SL was calculated from the median of the intensity distribution. Trabeculae were stimulated at 0.5 Hz through two parallel platinum electrodes in the bath using 5-ms pulses 50% above threshold.

**Fura 2 Loading and Analysis of [Ca\(^{2+}\)]**

Fura 2 loading. The free acid form of fura 2 was microinjected electrophoretically into trabeculae, as described previously (1). In brief, the tip of the microelectrode was filled with 2 mM fura 2 pentapotassium salt (Molecular Probes, Eugene, OR) and the remainder of the electrode was back filled with 4 mM KCl. With this microelectrode solution, the tip resistances ranged from 180 to 250 MΩ when measured using the standard KH solution. Intracellular membrane potentials were measured using an intracellular amplifier (Intra 767, World Precision Instruments, Sarasota, FL) and were between -40 and -80 mV under standard conditions [SL = 2.1 µm, extracellular Ca\(^{2+}\) concn (Ca\(^{2+}\)o) = 0.7 mM, 26°C]. After stability was achieved, 5–10 nA of negative current was passed for 10–20 min. During the injection period, the fura 2 diffusion from the impalement site into the adjacent cells via GJs as previously described (1). On completion of injection and removal of the microelectrode, the trabecula was stimulated at 1 Hz for 30–60 min until uniform loading with fura 2 was obtained. Under such conditions, changes in the fluorescence ratio of fura 2 determined using the IIC or PMT were superimposable, as shown in Fig. 2A, even when the trabecula was moved over several hundred micrometers, showing that fura 2 loading was uniform.

To induce TPCs, bath temperature was lowered to 20–22°C and trains of electrical stimuli (n = 15) were applied at

Fig. 1. A: simplified diagram shows experimental setup used for measurement of fluorescence signals, force (F), and sarcomere length (SL). Xe, xenon arc lamp; PMT, photomultiplier tube; IIC, charge-coupled device (CCD) camera coupled to 2-stage image intensifier; PC, personal computer, VCR, video cassette recorder. B: original SL and stress tracings of last stimulated twitch of a train of 15 twitches at 2 Hz and a subsequent triggered propagated contraction (TPC). SL was measured at 2 sites (A and B) 270 µm apart. During TPC, sarcomere shortening transient (arrowheads) occurred earlier at site A than site B. Experiment was performed at 20.8°C and 1.5 mM extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)o]). C: in vitro calibration curve. Applied [Ca\(^{2+}\)] was plotted against ratio of fluorescent intensity at 340 and 380 nm (340/380) calculated from signal of PMT after subtraction of autofluorescence. Kd, effective dissociation constant; Rmin, ratio at zero [Ca\(^{2+}\)]; Rmax, ratio at a saturating [Ca\(^{2+}\)].
Fig. 2. A: superimposed ratio transients (360/380 or 340/380) measured at 3 positions along trabecula (at 0-, 210-, and 650-µm displacement) calculated either from signal of PMT (top) or from signals at central 20 × 20 pixels of IIC (bottom) during same electrically stimulated twitch (0.5-Hz stimulation rate, [Ca\(^{2+}\)]\(_o\) 0.7 mM, and 26°C). B: relationships of ratios determined by IIC vs. PMT at the 3 different positions on trabeculae (0 µm, top; 210 µm, middle; 650 µm, bottom) calculated from A. Note that at each position along trabecula, ratios determined by the 2 methods were correlated linearly. r, Correlation coefficient.

[Ca\(^{2+}\)], of 0.3 mM and SL of 2.10 µm. [Ca\(^{2+}\)], was then increased in steps of 0.2 mM until TPCs appeared (10, 19). The measurement of [Ca\(^{2+}\)], was started only when TPCs were reproducible (Fig. 1B).

Analysis of signal from PMT. [Ca\(^{2+}\)] was determined using the following equation (13) (after subtraction of the autofluorescence of the muscle)

\[
[\text{Ca}^{2+}] = K_d \cdot \beta \cdot (R - R_{\text{min}})/(R_{\text{max}} - R)
\]  

(1)

where \(K_d\) is the effective dissociation constant, \(R\) is the ratio of the fluorescence at 340-nm excitation to that at 380-nm excitation (340/380), \(R_{\text{min}}\) is \(R\) at zero [Ca\(^{2+}\)], \(R_{\text{max}}\) is \(R\) at a saturating [Ca\(^{2+}\)], and \(\beta\) is the ratio of fluorescence value for Ca\(^{2+}\)-free dye to fluorescence value for Ca\(^{2+}\)-bound dye at 380-nm excitation. Values for \(K_d, R_{\text{min}}, R_{\text{max}},\) and \(\beta\) were determined using in vitro calibrations (Fig. 1C) as previously described (1). Previously, we reported that there is a good correlation between in vitro and in vivo calibrations when free Mg\(^{2+}\) is 1 mM in the solutions, mimicking the intracellular milieu (1). Solutions for calibration were prepared using a calcium calibration buffer kit (Molecular Probes) and contained different known free [Ca\(^{2+}\)], 1 mM free Mg\(^{2+}\), 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 100 mM KCl, 10 mM 3-(N-morpholino)propanesulfonic acid, and 1 mM fura 2 (pH 7.2). Calibration was performed by placing 100 µl of solutions of different known free [Ca\(^{2+}\)] into chambers on the microscope stage. Each solution was illuminated with 340-, 360-, and 380-nm excitation light, and fluorescence was recorded and stored in the computer for later analysis. The background fluorescence was determined by illumination of a solution with zero free Ca\(^{2+}\) in the absence of fura 2. Values for \(K_d, R_{\text{min}}, R_{\text{max}},\) and \(\beta\) were calculated from the curve describing the relationship between the ratio and pCa. For these experiments the \(R_{\text{min}}\) and \(R_{\text{max}}\) were 0.152 and 4.60, respectively, and \(K_d\) and \(\beta\) were 361 mM and 9.18, respectively. This is in agreement with previous data from this laboratory (1).

Analysis of image from IIC. The fluorescence data of each video frame were digitized with a 8-bit analog-to-digital converter and stored in a frame buffer memory of 512 × 480 pixels (Coreco). Therefore, in our optical system, 1 pixel of memory corresponded to 2.87 × 2.87 µm of the image. For analysis of the image, a sampling region of interest (ROI) was set horizontally along the long axis of the fluorescence image of trabecula. The horizontal length of the ROI was always 512 pixels (1,470 µm), and its vertical width was 20 pixels (57.4 µm). To obtain intensity profiles of the fluorescence (F\(_i\)) along the long axes of trabeculae, we calculated an average intensity value from each vertical line of pixels within the ROI. To eliminate high-frequency noise from the intensity profile, we used the low-pass finite impulse response filter of MATLAB (Math Works, Natick, MA), which was a Hamming-windowed, linear-phase filter, and set the cutoff frequency of the filter at 5 pixels (14.4 µm). After subtraction of the autofluorescence of the muscle, we calculated the ratio of the fluorescence at 360-nm to that at 380-nm excitation (360/380) at each point on the average intensity profiles obtained from the images at 360- and 380-nm excitation light.

The area of investigation in the muscle is large (>1 mm) using a ×10 objective lens, and the aperture of the xenon lamp was smaller than that of the objective lens, such that the excitation light onto the muscle yielded a nonuniform distribution of light on the muscle within the field of view. Therefore, we corrected for the effects of this nonuniform illumination as follows. Changes in fluorescence of trabeculae during electrically stimulated twitches (26°C, 0.5 Hz, [Ca\(^{2+}\)]\(_o\) = 0.7 mM) were determined using both the IIC and PMT at several sites along the long axis of the trabeculae, and the values obtained at these sites were compared (Fig. 2A). For this comparison, we first defined an ROI along the long axis of the muscle image recorded by the IIC. We then compared the intensity ratio (360/380) obtained from images at the central 20 × 20 pixels (57.4 × 57.4 µm) to the ratio (340/380) obtained using the PMT at three different sites. The ratio changes at the central regions of the IIC were superimposable (Fig. 2A) and linearly correlated with the PMT ratio changes despite the movement of the trabeculae (Fig. 2B). Therefore, [Ca\(^{2+}\)] transients were uniform throughout the central millimeter of the muscle during twitches. Moreover, the calculated ratios (360/380) at each sampling point along the 512 × 20 pixel array, with the muscle in a stationary position, correlated linearly with the ratio (340/380 or 360/380) calculated from PMT recordings (r = 0.96–0.99) during the twitch; however, the slope of the relationship varied at sites along the pixel array. Thus for each trabeculae we calculated [Ca\(^{2+}\)] at each sampling point after the induction of TPCs using the regression line derived from the relationship between the PMT and IIC ratios determined at the same
determine the effects of octanol on (Table 1) using 10 nA of negative current for 10 min. To salt was microinjected electrophoretically into trabeculae D trabeculae set at SL of 2.10 µm with [Ca2+], that was changing. Therefore, the time resolution of the images is a limitation of our measured velocities. Furthermore, we used only data from the centrally located 250 pixels (719 µm) of the intensity profiles in the determination of velocity. Thus the upper limit of the measurable velocity of Ca2+ waves using our experimental setup was ~7 mm/s. Results were obtained from reproducible Ca2+ waves that were induced in eight rat right ventricular trabeculae (length = 2.1 ± 0.35 mm, width = 170 ± 19 µm, thickness = 96 ± 18 µm) at [Ca2+], of 0.73 ± 0.30 mM and temperature of 21.5 ± 0.9°C.

Analysis of Deq for Fura 2

For this subset of experiments, we calculated three types of Deq for fura 2 along the trabecula, in myocytes, and in solution (Dtrab, Dmyop, and Dsol). All the settings of the IIC and the microscope were kept constant during the recordings of trabeculae set at 5 L of 2.10 µm with [Ca2+], of 0.7 mM.

Measurement of Dtrab for fura 2. Fura 2 pentapotassium salt was microinjected electrophoretically into trabeculae (Table 1) using 10 nA of negative current for 10 min. To determine the effects of octanol on Dtrab fura 2 was injected after a 1- or 3-h superfusion of trabeculae with KH solution containing 100 µM 1-octanol (Table 1). Trabeculae were constantly stimulated at 0.5 Hz except for the fura 2 injection periods. Epifluorescence of the trabeculae obtained using the IIC (see Analysis of Image from IIC) was recorded with the

Table 1. Diffusion coefficients for fura 2 in rat cardiac trabeculae

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Control</th>
<th>1 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dtrab Trabeculae</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>Length, mm</td>
<td>3.5 ± 2.1</td>
<td>2.5 ± 0.31</td>
<td>2.1 ± 0.39</td>
</tr>
<tr>
<td>Width, µm</td>
<td>200 ± 64</td>
<td>290 ± 23</td>
<td>320 ± 16</td>
</tr>
<tr>
<td>Thickness, µm</td>
<td>100 ± 28</td>
<td>97 ± 23</td>
<td>120 ± 40</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>26.1 ± 0.3</td>
<td>26.3 ± 0.3</td>
<td>26.0 ± 0.3</td>
</tr>
<tr>
<td>Dmyop Trabeculae</td>
<td>n = 3</td>
<td>n = 3</td>
<td>♦</td>
</tr>
<tr>
<td>Length, mm</td>
<td>2.4 ± 0.35</td>
<td>1.7 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>Width, µm</td>
<td>280 ± 12</td>
<td>230 ± 170</td>
<td></td>
</tr>
<tr>
<td>Thickness, µm</td>
<td>140 ± 26</td>
<td>94 ± 8</td>
<td></td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>25.9 ± 0.1</td>
<td>26.0 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

1-Octanol (100 µM)

Dsol × 10⁶ cm²/s | 359 ± 145 |
Dmyop × 10⁶ cm²/s | 42.5 ± 20.8 | 32.3 ± 5.67 | 39.7 ± 16.8 |
Dtrab × 10⁶ cm²/s | 21.0 ± 7.29 | 12.7 ± 3.45† | 7.07 ± 1.47† |
Pij × 10⁶ cm²/s | 4.15 | 2.10 | 0.86 |

Values are means ± SD; n, no. of trabeculae. D, diffusion coefficient; Dtrab, Dmyop, and Dsol, D for fura 2 in trabecula, myoplasm, and water, respectively; Pij, calculated permeability of gap junctions. Differences between Dmyop of different groups were not significant. *P < 0.05, 1 h vs. control; †P < 0.05, 3 h vs. control; ♦ same trabeculae as 1-h group.

VCR at the completion of fura 2 injection and thereafter every 10 min for 60 min.

To determine Dtrab for fura 2 in the absence and presence of octanol, we set an ROI of 512 × 21 ± 8 pixels horizontally along the long axis of the fluorescence image of trabeculae and calculated Fsol using the procedure described in Analysis of Image from IIC. To improve the ratio of signal to noise, we calculated an average value of each horizontally corresponding pixel of Fsol from 25 sequential frames (0.83 s) and obtained an averaged intensity profile of the fluorescence (Fav) every 10 min (Fig. 3A). Fav was symmetrical with respect to the site of implanation and spread at comparable velocity in both directions in the absence and presence of octanol. To eliminate the effect of uneven intensity of illumination, the fluorescence from the solution containing 1 µM fura 2 pentapotassium salt excited by 360-nm light was recorded after the measurement of Dsol. To do this, we set an ROI of 512 × 20 pixels along the image of the fluorescing solution and calculated an average intensity profile of fluorescence (Fsol) from each vertical line of pixels within the ROI. We then determined a normalized intensity profile of the fluorescence (Fnorm) by dividing Fsol by the spatially corresponding values of the Fsol (Fig. 3B).
where \( k \) is a constant related to the amount of the fura 2 deposited in the cell, \( t \) is time, and \( x \) is the distance from the center of the intensity profile. Because \( D_{eq} \) equals \( D_{trab} \) for our measurement, spanning 500–1,000 µm along the trabecula, \( D_{eq} \) is \( D_{myop} \) for our measurement of changes of the fluorescence profile over distances <100 µm. For all data, we avoided using peak and far-edge values of fluorescence, because the peak region signals often saturated the IIC and the far-edge signals showed noise caused by low-excitation light. From the fitted data, \( D_{trab} \) was calculated (Fig. 3C). This procedure was repeated for the profiles obtained every 10 min. \( D_{trab} \) depended in a linear manner on the time of measurement (Fig. 3D). Therefore, the effects of octanol we observed were not caused by a progressive decrease of \( D_{trab} \) during the sampling period. Accordingly, calculated \( D_{trab} \) will reflect the diffusion coefficients 1.5 h (or 3.5 h) after the first exposure of the muscle to octanol. \( D_{myop} \) was calculated from the time course of \( D_{trab} \) (Fig. 3D). There was no significant difference in the shapes of trabeculae, temperature, or the remaining force among the groups (Table 1).

Measurement of \( D_{myop} \) for fura 2. Fura 2 was microinjected 11 times into three trabeculae (Table 1) using 7 nA of negative current for 10–20 s. To study the effects of octanol on \( D_{myop} \), fura 2 was microinjected 6 times into trabeculae after 1 h of superfusion of the muscle with 100 µM 1-octanol and 11 times into the same trabeculae (Table 1) after a 3-h superfusion with the same solution. The epifluorescence from the trabeculae was recorded, using the procedure described in Analysis of image from IIC, for 30 s immediately after the completion of each fura 2 injection.

To determine \( D_{myop} \) from these image profiles, a narrow ROI of 512 × 3 pixels (1,472 × 8.63 µm) was set through the spot of injected fura 2 along the long axis of the trabeculae and \( F_{s} \) values were determined. \( F_{av} \) was then calculated (Fig. 4A) from eight sequential frames (0.27 s) obtained every 5 s as described in Measurement of \( D_{trab} \) for fura 2. We calculated \( F_{norm} \) (Fig. 4B) and fitted data to Eq. 2 using the procedure described for our measurement of \( D_{trab} \) (Fig. 4C). \( D_{myop} \) was calculated directly from the time course of \( D_{myop} \) (Fig. 4D). There were no significant differences in the shapes of trabeculae, temperature, or the force development between the groups (Table 1).

Often the profiles of \( F_{av} \) showed clear asymmetries after a 3-h exposure to octanol (Fig. 4E). That is, it appeared that fura 2 diffusion away from the impalement site with different velocities in opposite directions. We further observed that, after octanol exposure, fura 2 diffused very slowly across regions that were ~100 µm apart bordering the injected cell. The border regions are indicated by the gray bars in Fig. 4E. In these cases we calculated \( D_{myop} \) using the diffusion toward the direction indicated by arrows into the gray bars.

Measurement of \( D_{sol} \) for fura 2. A droplet of fura 2 solution (300-µm diameter) was placed in a stationary flow bath and image profiles of the droplet excited by 360-nm light were recorded for 30 s at room temperature (20°C). To determine \( D_{sol} \) for fura 2, an ROI of 512 × 20 pixels was set horizontally through the fura 2 droplet. The \( F_{av} \) was calculated from \( F_{s} \) obtained from eight sequential frames (0.27 s) every 5 s. With \( F_{av} \) and \( D_{sol} \) was then determined at every 5 s for 30 s using the procedure described above. The contribution of convective fluid motion caused by illumination of the fluid was ignored.

Calculation of \( P_{GJ} \). \( P_{GJ} \) was calculated using the following equations (7, 12)

\[
L/D_{trab} = L_{myoc}/D_{myop} + L_{GJ}/D_{GJ} \tag{3}
\]

\[
P_{GJ} = D_{GJ}/L_{GJ} \tag{4}
\]

where \( L_{myoc} \) is the length of one myocyte, \( L_{GJ} \) is the dimension of \( GJ \) s, and \( L \) is the total length of \( L_{myoc} \) and \( L_{GJ} \). For the calculation of \( P_{GJ} \), we used 100 µm for \( L_{myoc} \).
Statistics
All averaged values are expressed as means ± SD. Single factor analysis of variance was used to detect significant difference (P < 0.05).

RESULTS
Diffusion of Fura 2
Diffusion of fura 2 in free solution was ≈10 times faster than that in the myoplasm (Table 1). In addition, diffusion through the cytosol of single cells was twice as fast as diffusion through the whole trabecula. Octanol did not reduce the diffusion of fura 2 through the cytosol but reduced the diffusion through the trabecula twofold and threefold after 1 and 3 h of superfusion, respectively (Table 1). A brief injection of fura 2 caused an oval spot of fluorescence with the long axis parallel to the muscle axis; the length of the spot was <100 µm. It was striking that, after a 3-h superfusion with octanol, F_r and after a similar brief injection of fura 2 often showed a clear asymmetrical pattern along muscle (Fig. 4E). We assume that in these cases the microelectrode had been positioned in the muscle near the end of the cell and that diffusion toward the cell center was faster than that toward adjacent cells. Accordingly, for the three cases shown in Fig. 4E, we calculated the diffusion coefficient using the diffusion of fura 2 in both directions. The calculated diffusion coefficient toward the direction indicated by arrows was 43.9 ± 30.3 × 10^{-8} cm²/s, whereas the diffusion coefficient in the opposite direction across the regions including the gray bars was 6.99 ± 2.56 × 10^{-8} cm²/s. The latter value is similar to the value of the D_trab obtained after a 3-h superfusion with octanol (Table 1). It should be noted that in Fig. 4E the distance between the gray bars was 100–150 µm, i.e., similar to the length of a single myocyte.

We are not aware of data to compare our D_gj measures directly with those of previous studies. Therefore, we calculated the P_gj from D_trab and D_myoc using Eqs. 3 and 4 (see METHODS). It follows from Eqs. 3 and 4 that

\[ P_{gj} = \frac{D_{trab} \cdot D_{myoc}}{L \cdot (D_{myoc} - D_{myoc} \cdot D_{trab})} \]  

(5)

where P_gj does not require an assumption about the linear dimension of the GJ s (L_gj ) and thus can directly be compared with results from studies on cell pairs. When we calculated P_gj for fura 2 based on our average values of D_trab and D_myoc, we found that P_gj was reduced from 4.15 × 10^{-5} cm²/s in control to 2.10 × 10^{-5} cm²/s and 0.86 × 10^{-5} cm²/s after 1 and 3 h of superfusion with 100 µM 1-octanol, respectively (Table 1). Our control calculated P_gj for fura 2 pentapotassium salt (mol wt 832) is in close agreement with data presented by Dr. Irisawa’s group (15) depicting the relationship between the P_gj of a solute and its molecular weight. They showed that there is an inverse linear relation between log(P_gj) and molecular weight (see Fig. 3 in Ref. 15), where P_gj values range from 7 × 10^{-5} cm/s (mol wt 559, lissamine rhodamine B-200) to 770 × 10^{-5} cm/s (mol wt 39, K+).

The conductance of GJ s (g_gj ) at a voltage difference across the GJ s of 0 mV can now be calculated using P_gj and the following equation (12)

\[ g_{gj} = F^2 \cdot P_{gj} \cdot A \cdot (C_{out} - C_{in})/RT \]  

(6)

where F is the Faraday constant, C_{ox} is the concentration of fura 2 within the cell in which fura 2 had been injected (M_0), C_{in} is the concentration of fura 2 within the neighboring cell (M_1) of M_0, R is the area of intercalated disk between M_0 and M_1, and T is absolute temperature. The calculated g_{gj} is ≈3.3 nS. When we calculate the conductance of a single GJ channel on the assumption that open probability of the GJ channel is 0.43% and that GJ channel density is 2.3 × 10^4 µm⁻² in rat ventricular muscle (15), the conductance of a single GJ channel for fura 2 is ≈0.15 pS. Using the relationship between P_gj and molecular weight of the solute, this value would predict a unitary conductance for K^+ of ~28 pS in trabeculae, which is in the same order of magnitude as the reported unitary conductance of GJ channels for K^+ (~50 pS) (24).

Ca²⁺ Waves During TPCs
Figure 1B shows a typical TPC in the form of a traveling sarcomere shortening wave accompanied by an aftercontraction (10, 19). Although several studies have implied that such TPCs are related to traveling Ca²⁺ waves in trabeculae (10, 19), there has been no direct proof of this. In fact, in a previous publication (31) we only hypothesized that octanol affected TPC propagation via its effect on P_gj (see Diffusion of Fura 2), thereby reducing the likelihood of propagating Ca²⁺ waves. Therefore, in the next series of experiments, we determined the spatial and temporal changes in [Ca²⁺] in intact trabeculae during a TPC.

Figure 5 shows a typical example of the global [Ca²⁺] and the force development during the last electrically stimulated twitch of a train and a subsequent TPC in one trabecula at 22.1°C and [Ca²⁺] of 0.7 mM. Figure 5A shows the change in [Ca²⁺], calculated from a PMT signal. When the muscle was stimulated at 2 Hz, the Ca²⁺ transient shortened progressively so that the total duration after 15 stimuli was ~0.37 s. Figure 5B shows the force records made while fluorescence was recorded, first using excitation with UV light at 340 nm followed by excitation at 380 nm. The arrows indicate an increase in [Ca²⁺], (Fig. 5A) and the occurrence of an aftercontraction (Fig. 5B) observed during a TPC. The global measurement obtained with the PMT shows that the amplitude of the Ca²⁺ transient during the TPC is small (10%) compared with that of the preceding stimulated twitch and lasts twice as long as the Ca²⁺ transient of the twitch. The force transient of the TPC is also small in amplitude (14%) and lasts longer (40%) than that of the preceding stimulated twitch. The force transient of the TPC lasts slightly longer than the concomitant Ca²⁺ transient. In this study, all imaging data were accepted for analysis when the stresses
recorded at both excitation wavelengths were identical as shown in Fig. 5.

To determine the spatial and temporal changes in \([\text{Ca}^{2+}]_i\) during the TPC, we analyzed the images recorded by the IIC directly after a PMT recording. Figure 6 shows the last stimulated \([\text{Ca}^{2+}]_i\) transient and a cytosolic \([\text{Ca}^{2+}]_i\) wave during the TPC in the same trabeculae as presented in Fig. 5. In this three-dimensional representation the abscissa is time, the ordinate is \([\text{Ca}^{2+}]_i\), and the z-axis is the position of each pixel along the long axis of the trabecula. After the end of a series of stimulated \([\text{Ca}^{2+}]_i\) transients, a smaller \([\text{Ca}^{2+}]_i\) transient was observed to move as a wave from position A (indicated by * in Fig. 6) toward position B. The \([\text{Ca}^{2+}]_i\) waves appeared to travel at constant velocity along the trabeculae similar to properties of TPCs we have previously described (Refs. 10, 19; Fig. 1B).

To calculate the velocity of the \([\text{Ca}^{2+}]_i\) wave in Fig. 6, we selected the peak of the \([\text{Ca}^{2+}]_i\) transient during the \([\text{Ca}^{2+}]_i\) wave at each pixel along the trabeculae and plotted the time of the peak point against the position of the peak (Fig. 7A). Regression analysis showed a linear relationship. The slope of the fitted line was taken as the velocity of the \([\text{Ca}^{2+}]_i\) wave occurring during the TPC. This analysis shows that the \([\text{Ca}^{2+}]_i\) wave propagates along the trabecula at a constant velocity, and the calculated velocity of the \([\text{Ca}^{2+}]_i\) wave was 2.27 mm/s (Fig. 7A). The analysis of 10 reproducible \([\text{Ca}^{2+}]_i\) waves in 8 trabeculae shows that the velocity of the traveling \([\text{Ca}^{2+}]_i\) waves was 1.69 ± 1.48 mm/s (range 0.34–5.47 mm/s).

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![Fig. 5](http://apjhep.physiology.org/)

**Fig. 5.** Global intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) calculated from fluorescence signals recorded by PMT (A) together with force records (normalized for cross-sectional area of muscle) recorded at 340- and 380-nm excitation wavelength (B) during last electrically stimulated twitch and a subsequent TPC. Arrows indicate an increase in [Ca\(^{2+}\)]\(_i\), and an aftercontraction during which a TPC was observed. s, Moment of electrical stimulation.

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![Fig. 6](http://apjhep.physiology.org/)

**Fig. 6.** Regional [Ca\(^{2+}\)]\(_i\), calculated from images by IIC during last electrically stimulated twitch and a TPC a few minutes after recording of PMT shown in Fig. 5. Abscissa, time; ordinate, [Ca\(^{2+}\)]\(_i\); z-axis is position along long axis of trabecula. After end of clearly uniform stimulated Ca\(^{2+}\) transient, a smaller Ca\(^{2+}\) transient appeared to move from A (*) toward B (for explanation see text).

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![Fig. 7](http://apjhep.physiology.org/)

**Fig. 7.** A: relationship between moment at which [Ca\(^{2+}\)]\(_i\) during Ca\(^{2+}\) wave (Fig. 6) was maximal against position of maximal point along trabecula. Relationship was fitted by linear regression \((r = 0.971)\); slope of fitted line was taken as velocity of Ca\(^{2+}\) wave B: peak [Ca\(^{2+}\), during Ca\(^{2+}\) wave (solid line) and diastolic [Ca\(^{2+}\)]\(_i\), between last stimulated Ca\(^{2+}\) transient and Ca\(^{2+}\) wave (dotted line) as a function of position along trabecula. C: average [Ca\(^{2+}\)]\(_i\) in a 600-µm-long region of interest (ROI) along trabecula (calculated from Fig. 6). Solid line shows average of stimulated Ca\(^{2+}\) transients during twitch and average of Ca\(^{2+}\) transient during Ca\(^{2+}\) wave (TPC) after correction for time required for Ca\(^{2+}\) wave to move from pixel to pixel as calculated from Fig. 6. Dotted line shows same trace of global change in [Ca\(^{2+}\)]\(_i\), as presented in Fig. 5, recorded with PMT from a region of ~1.00 µm that overlapped with ROI of IIC.
mm/s, n = 10) at [Ca^{2+}]_o of 0.73 ± 0.30 mM and a temperature of 21.5 ± 0.9°C. Figure 7B shows that the [Ca^{2+}] at the peak of the Ca^{2+} wave and the diastolic [Ca^{2+}], between the last stimulated Ca^{2+} transient and the Ca^{2+} wave at each pixel position along the trabeculae were almost constant.

The average time course of Ca^{2+} transients during the Ca^{2+} wave was obtained by using our observation that Ca^{2+} waves traveled at constant velocity. Therefore, we could shift the time axis of the Ca^{2+} transient of every pixel according to the time required for the Ca^{2+} wave to move from a pixel to pixel. After this correction, the Ca^{2+} transients of all corresponding pixels (n = 250) were averaged. The solid line in Fig. 7C shows the average of the stimulated Ca^{2+} transients in all pixels during the twitch as well as the average of regional Ca^{2+} transients during the Ca^{2+} wave after the shift of the time axis calculated from the imaging data of Fig. 6. The peak of the averaged regional Ca^{2+} wave reached 40% of that of the stimulated Ca^{2+} transient, whereas its duration was slightly longer than that of the stimulated Ca^{2+} transient itself. The time course of decline of the “regional” Ca^{2+} wave was identical to that of the electrically induced transient, although the time constant of decline of Ca^{2+} was shorter in the stimulated Ca^{2+} transient (144 ms) than in the regional Ca^{2+} wave (235 ms) because of the difference in their peak Ca^{2+} levels (4). The rise of the regional Ca^{2+} wave lasted ∼0.15 s longer than that of the electrically induced transient, accounting for the difference in duration of the two transients. The dotted line in Fig. 7C shows the same record of the global Ca^{2+} transient, measured with the PMT, as presented in Fig. 5A. The twofold longer duration of the global Ca^{2+} transient (dotted line) during the TPC is presumably caused by the displacement of the Ca^{2+} wave during the TPC along the segment of muscle, which was observed by the PMT.

It follows from Figs. 5 and 6 that when the Ca^{2+} wave started during the late relaxation of the twitch (as we have shown previously (19)), the site of origin of the wave must have been located ∼700 μm from position A toward the tricuspid valve, as was indeed the distance between position A and the insertion of this trabecula in the AV ring.

In summary, a sequential, spatial, and temporal change in [Ca^{2+}]_i (a traveling Ca^{2+} wave) underlies a TPC in thin right ventricular trabeculae. These waves start at one of the damaged ends of the trabeculae.

**DISCUSSION**

To our knowledge, this is the first study to provide a quantitative assessment of propagating Ca^{2+} waves in multicellular cardiac preparations. As we discuss here, both the presence and properties of Ca^{2+} waves during TPCs are consistent with a previously proposed model of propagation, i.e., CICR from the SR mediated by diffusion of Ca^{2+} along the cell. Our previous observation that TPCs are delayed by octanol is consistent with the conclusion from this study that the permeability of GJs is drastically reduced by octanol, such that the propagation process for TPCs is delayed because of slower diffusion of Ca^{2+} from cell to cell.

**Diffusion of Fura 2 Within Trabeculae**

D_{sol} for fura 2 is in good agreement with the diffusion coefficient for fura 2 in aqueous solution (390 ± 0.6 × 10^{-8} cm^2/s) measured previously (28) and is one-half of the D_{sol} for Ca^{2+} (750 × 10^{-8} cm^2/s; Ref. 30). D_{myo}, for fura 2 calculated within 30 s after the injection into trabeculae under drug-free conditions is in good agreement with the diffusion of fura 2 measured in isolated single myocytes (31.9 ± 18.5 × 10^{-8} cm^2/s, 21°C; Ref. 5), barnacle muscle fibers (39 × 10^{-8} cm^2/s, 20°C; Ref. 28), and frog skeletal muscle (36 × 10^{-8} cm^2/s, 16°C; Ref. 3). Diffusion of fura 2 (D_{trab}) calculated for 1 h after the injection of the probe into trabeculae illustrates that an additional diffusion barrier was present along trabeculae, because D_{trab}, was nearly one-half of D_{myo}. We assume that this barrier is formed by the GJs. Several lines of evidence support this assumption. First, the diffusion coefficient of the GJs (D_{GJ}) can be calculated from D_{myo} and D_{trab} treating the trabeculae as a planar diffusion sheet. The permeability of GJs follows from D_{GJ} and is similar to P_{GJ} determined for molecules of a similar size (15). Second, our calculated g_{GJ}, based on reasonable assumptions regarding GJ density and open probability, was similar to g_{GJ} measured in lipid bilayers (24). Third, when we observed fura 2 diffusion directly after injection of fura 2 into a cell, cell diffusion was clearly rapid and unaffected by octanol (see RESULTS).

The asymmetry of short-term diffusion of fura 2 shown in Fig. 4E is also consistent with our interpretation of an effect of octanol on GJs for the following reason. In these cases fura 2 diffusion faster in one direction than another (see RESULTS). We propose a simple explanation for this observation, i.e., impalement with a microelectrode occurs at a random position within the impaled cell. Obviously, the microelectrode is more likely to impale the cell near one of the two ends than in the center. Hence, we assume that fura 2 diffusion was slow on the side of the fluorescence profile when it had been injected near the edge of a cell. Asymmetrical diffusion was visible in muscles that had not been treated with octanol (data not shown) but became pronounced after exposure to octanol (Fig. 4E). Calculation of the diffusion coefficient for the three cases shown in Fig. 4E suggests that diffusion across the regions including the gray bars reflects a barrier with diffusion properties similar to those of the barrier that exists during diffusion along the whole trabecula. In addition, the distance between the gray bars was similar to the length of a single myocyte. Thus we assume that GJs were located at the regions indicated by the gray bars and that the inhibitory effect of octanol on diffusion of fura 2 is caused by the effect of octanol on GJs. It is reasonable to assume that the reduction of P_{GJ} by octanol is caused by the reduction of the open probability of GJ channels, as has been described by others (26).
The formation of an oval spot of fluorescence after injection of fura 2 with the long axis parallel to the muscle axis means that diffusion of fura 2 is faster longitudinal than transverse to myocardial fiber orientation, suggesting anisotropy of the GJ distribution. This nonuniform distribution of fura 2 is consistent with the report that velocity of electrical conduction is greater longitudinal than transverse to myocardial fiber orientation (23). This report also shows that heptanol slows the conduction velocity transverse to fiber orientation to a greater degree than longitudinal. However, in this study we did not compare the effect of octanol on PK in the longitudinal direction with that in the transverse direction quantitatively, because we did not measure the effect of octanol on PK calculated from the transverse diffusion of fura 2.

In summary, by using diffusion characteristics of fura 2, we conclude that octanol, in a concentration known to affect TPC propagation velocity (31), reduces PK, thus supporting the concept that calcium ions need to diffuse from cell to cell through GJs for propagation of a TPC through normal myocardium.

**Ca**\(^{2+}\) Waves

It is simple to show that the maximal rate of displacement of a Ca\(^{2+}\) front by diffusion (starting with the same Ca\(^{2+}\) concn gradient as for fura 2 in this experiment) is \(\sim 20 \mu\text{m} / \text{s}\) (Fig. 4), assuming that Ca\(^{2+}\) diffusion is twice as fast as fura 2 diffusion. This means that simple diffusion of Ca\(^{2+}\) cannot be responsible for the velocity of TPCs, which is 5–500 times higher (10, 19). For this reason, we have previously proposed CICR as a propagation mechanism underlying TPCs (2). Although it is reasonable to assume that TPCs are caused by traveling Ca\(^{2+}\) waves (10, 19), there has been no direct proof of this. In fact, in a previous publication (31) we only hypothesized that octanol affected TPC propagation via its PK effect (see Diffusion of Fura 2 Within Trabeculae), which would then reduce the likelihood of propagating Ca\(^{2+}\) waves.

Comparison between global and regional measurement. The global Ca\(^{2+}\) transient (Figs. 5 and 7) during the TPCs showed a smaller maximal amplitude and longer duration than the regional Ca\(^{2+}\) transients (Figs. 6 and 7). This difference can be explained in a straightforward manner because the PMT averages the fluorescence intensity from an area of the muscle that is \(\sim 1 \text{mm} \times 1 \text{mm}\) long. The Ca\(^{2+}\) wave travels at 2.27 mm/s and lasts \(\sim 0.40 \text{s}\). Therefore, the PMT is expected to "see" a Ca\(^{2+}\) wave that lasts \(\sim 0.84 \text{s} (1/2.27 + \text{duration of the wave itself})\), as is indeed the case. For the same reason, the maximal amplitude of the global Ca\(^{2+}\) wave seen by the PMT is expected to be almost one-half of that of the regional Ca\(^{2+}\) wave, as is the case.

Comparison with Ca\(^{2+}\) waves in single myocytes. The Ca\(^{2+}\) waves during TPCs in trabeculae propagate faster (0.34–5.47 mm/s) than the reported Ca\(^{2+}\) waves in isolated single myocytes (\(\sim 0.1 \text{mm/s}\); Refs. 16–18). In our previous studies, the velocity of the TPCs varied depending on the [Ca\(^{2+}\)]\(_i\), the number and frequency of the electrical stimuli (10, 19), and the presence or absence of Ca\(^{2+}\) channel agonists and antagonists (11). These observations are consistent with the assumption that Ca\(^{2+}\) loading of the cell is the main determinant of the velocity of the TPCs. Thus we assume that the Ca\(^{2+}\) waves during TPCs propagate along trabeculae faster than the Ca\(^{2+}\) waves in single myocytes, because both the myoplasm and the SR are probably more loaded with Ca\(^{2+}\) in trabeculae than in myocytes because of the preceding repetitive stimulation and thereby CICR is probably facilitated.

The duration of the Ca\(^{2+}\) transient (\(\sim 0.40 \text{s}\)) during Ca\(^{2+}\) waves in trabeculae was similar to that in single myocytes (0.35–0.45 s), whereas the region of elevated [Ca\(^{2+}\)]\(_i\) (900 \(\mu\text{m}\)) during Ca\(^{2+}\) waves in trabeculae was larger than that reported in single myocytes (30–50 \(\mu\text{m}\); Refs. 16, 18). This observation is consistent with the prediction that the faster a Ca\(^{2+}\) wave propagates along a trabecula, the larger the region of the elevated [Ca\(^{2+}\)]\(_i\) (2).

Comparison with TPCs. Similar to the TPCs (10, 19), the propagation velocity of the Ca\(^{2+}\) waves was constant along the trabeculae (Fig. 7A) and varied under the conditions of this study from 0.34 to 5.47 mm/s (mean 1.69 \pm 1.48 mm/s). We were unable to measure Ca\(^{2+}\) waves with a velocity >7 mm/s in our experimental setup (see METHODS). The duration of the Ca\(^{2+}\) transients seen in this study during Ca\(^{2+}\) waves was \(\sim 0.40 \text{s}\) (Fig. 7C), which is similar to the duration of the SL shortening waves observed during TPCs (10, 19). Furthermore, our results are consistent with the observation that TPCs are initiated at or near the (damaged) ends of the trabeculae (19). These similarities in behavior suggest that Ca\(^{2+}\) waves underlie TPCs in trabeculae.

Comparison with CICR computer model of Ca\(^{2+}\) wave propagation. Propagation of the Ca\(^{2+}\) waves was clearly faster than the expected maximal rate of displacement of a diffusion front of Ca\(^{2+}\) (20 \(\mu\text{m/s}\), see above). The presence of Ca\(^{2+}\) waves during TPCs is consistent with the hypothesis that TPCs propagate along the trabeculae by CICR mediated by Ca\(^{2+}\) diffusion to adjacent SR. This hypothesis is supported by the observation that the time course of the Ca\(^{2+}\) wave is similar to that of the electrically evoked transient, suggesting that the Ca\(^{2+}\) wave and twitch transient share underlying mechanisms. In particular, our observation is consistent with the report that the decline of Ca\(^{2+}\) during both the Ca\(^{2+}\) transients is mainly caused by uptake by SR and that the rate of uptake depends on the [Ca\(^{2+}\)]\(_i\) (4).

The duration of the regional Ca\(^{2+}\) transients observed during TPCs was longer and the amplitude was smaller than the result of the computer-simulated model of CICR (2). The computer model, however, was developed on the basis of the time course of [Ca\(^{2+}\)]\(_i\), as estimated from aequorin. The aequorin signal is proportional to (Ca\(^{2+}\)]\(_i\))^2, so the signal tends to underestimate the diastolic Ca\(^{2+}\) level. This then would tend to curtail the transient and to overestimate the diastolic [Ca\(^{2+}\)] if no allowance for this relationship is made. Accordingly, later studies (17), including this study, where more sensitive fluorescent probes have been
used, have reported substantially lower diastolic [Ca$^{2+}$]$_i$ and substantially longer [Ca$^{2+}$]$_i$ transients during the stimulated twitch (Fig. 2A). Even with these fluorescent probes, reported [Ca$^{2+}$], values should be considered with caution because it has been recently reported that the calculated subsarcolemmal [Ca$^{2+}$], reaches a higher peak and falls more quickly than the bulk [Ca$^{2+}$], during spontaneous Ca$^{2+}$ release from the SR (29). This is presumably caused by a barrier to diffusion of Ca$^{2+}$ that separates the bulk cytoplasm from the subsarcolemmal space. This phenomenon adds to the causes for the difference between our data and the computer results, because the [Ca$^{2+}$]$_i$ measurement using fura 2 is thought to reflect the [Ca$^{2+}$], in the bulk cytoplasm. On the other hand, parameter values used for previous simulation (2) were obtained at low temperatures, e.g., the troponin Ca$^{2+}$ binding sites and troponin Ca$^{2+}$-Mg$^{2+}$ binding sites at 4°C (14) and the SR pump rate at 15°C (21). The difference between the temperature in our experiments and that used in the computer model may have reduced differences between observations in this study and the results from the computer simulation. Still, it is clear that with the present quantitative data in hand it is worthwhile to model propagating Ca$^{2+}$ waves on the basis of CICR coupled by Ca$^{2+}$ diffusion again.

Limitation of resolution of fluorescence measurement. Injection of fura 2 into single cells for the measurement of D$_{myos}$ probably allowed resolution within a few micrometers, such that transport from cell to cell (via GJs) could be monitored. In contrast, we cannot expect to resolve events at this level when Ca$^{2+}$ waves pass through the trabeculae. Trabeculae were ~90-µm thick, so that Ca$^{2+}$ waves passing through the four cells above and below the plane of focus contributed to the wave observed by the ICC. This would blur the observed and below the plane of focus contributed to the wave propagation velocity of TPCs, reduced only the P$_{GJs}$ in trabeculae under our experimental conditions. These observations support the hypothesis that TPCs are caused by CICR from the SR mediated by diffusion of Ca$^{2+}$ through cell and from cell to cell through GJs.

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