Ca\(^{2+}\) Waves During Triggered Propagated Contractions in Intact Trabeculae

Determinants of the Velocity of Propagation

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Abstract—During triggered propagated contractions, Ca\(^{2+}\) waves travel along cardiac trabeculae with a constant velocity (\(V_{\text{prop}}\)) ranging from 0.34 to 5.47 mm/s. To explore the determinants of \(V_{\text{prop}}\), we studied (1) the relationship between [Ca\(^{2+}\)]\(i\) and \(V_{\text{prop}}\) and (2) the effect of low concentrations of caffeine on \(V_{\text{prop}}\). Trabeculae were dissected from the right ventricle of rat hearts. [Ca\(^{2+}\)]\(i\) was measured using electrophoretically injected fura-2 and an image-intensified CCD camera. Force was measured using a silicon strain gauge, and sarcomere length was measured using laser diffraction techniques. After induction of reproducible Ca\(^{2+}\) waves by trains of electrical stimuli (2.5 Hz) at 21.9±0.2°C, the number of stimuli or [Ca\(^{2+}\)]\(i\) was varied in 9 trabeculae. In 5 trabeculae, the effects of caffeine (0.1 to 1.0 mmol/L) at [Ca\(^{2+}\)]\(i\) of 2.2±0.3 mmol/L were determined. All images were recorded under stable conditions of wave propagation. The increment in [Ca\(^{2+}\)]\(i\) during the last electrically stimulated transient (ΔCa\(_T\)) and [Ca\(^{2+}\)]\(i\), just before onset of the Ca\(^{2+}\) waves (Ca\(_0\)) were used to estimate the Ca\(^{2+}\) loading of the sarcoplasmic reticulum (SR) and the myoplasm, respectively. The ratio (ΔCa\(_{\text{Myo}}\)/ΔCa\(_{\text{SR}}\)) of the [Ca\(^{2+}\)]\(i\) increment during the waves (ΔCa\(_W\)) to ΔCa\(_T\) was used to estimate the probability of opening of the SR-Ca\(^{2+}\) release channel during wave propagation. As a result of an increase in the number of stimuli or [Ca\(^{2+}\)]\(i\), \(V_{\text{prop}}\) increased in proportion to (1) ΔCa\(_T\) (\(r=0.82\)); (2) Ca\(_0\) (\(r=0.88\)); (3) ΔCa\(_{\text{Myo}}\) (\(r=0.85\)); and (4) ΔCa\(_{\text{Myo}}\)/ΔCa\(_T\) (\(r=0.74\)). The addition of caffeine (≤0.3 mmol/L) increased \(V_{\text{prop}}\) for any ΔCa\(_T\) and any ΔCa\(_{\text{Myo}}\), revealing an increased sensitivity of \(V_{\text{prop}}\) to ΔCa\(_T\) and ΔCa\(_{\text{Myo}}\). In contrast, caffeine had little effect on the relationship between \(V_{\text{prop}}\) and Ca\(_0\) and no effect on that between \(V_{\text{prop}}\) and ΔCa\(_{\text{Myo}}\)/ΔCa\(_T\). These results suggest that both the cellular Ca\(^{2+}\) loading and open probability of the SR-Ca\(^{2+}\) release channels determine the velocity of propagation of Ca\(^{2+}\) waves. (Circ Res. 1999;84:1459-1468.)

Key Words: rat cardiac trabeculae ■ triggered propagated contraction ■ Ca\(^{2+}\) wave ■ caffeine

Local aftercontractions starting from damaged regions of muscle propagate along cardiac trabeculae.\(^1\) The propagated contractions, which can be measured as waves of sarcomere shortening in both rat ventricular\(^2\) and human atrial trabeculae,\(^3\) have been denoted as triggered propagated contractions (TPCs). Recently, regional increases in [Ca\(^{2+}\)]\(i\) have been observed to travel along rat cardiac trabeculae during TPCs and denoted as Ca\(^{2+}\) waves.\(^4\) These Ca\(^{2+}\) waves travel along trabeculae at the same velocities as TPCs, ranging from 0.34 to 5.47 mm/s.

Several studies have suggested that the propagation mechanism of TPCs is consistent with a model of Ca\(^{2+}\)--induced Ca\(^{2+}\) release (CICR) from sarcoplasmic reticulum (SR) mediated by Ca\(^{2+}\) diffusion to adjacent SR.\(^1,4-6\) The velocity of propagation (\(V_{\text{prop}}\)) of TPCs varies depending on the [Ca\(^{2+}\)]\(i\), the number and frequency of the electrical stimuli,\(^1,2\) and the presence or absence of Ca\(^{2+}\) channel agonists and antagonists.\(^7\) These observations are consistent with the assumption that Ca\(^{2+}\) loading of the cell (SR Ca\(^{2+}\) content and/or cytosolic Ca\(^{2+}\)) is a main determinant of \(V_{\text{prop}}\). Therefore, we propose that the Ca\(^{2+}\) level in the myoplasm and/or SR can determine \(V_{\text{prop}}\) via modulation of CICR. Furthermore, computer simulation of CICR and Ca\(^{2+}\) diffusion supports the hypothesis that \(V_{\text{prop}}\) of Ca\(^{2+}\) waves (or TPCs) will be altered depending on the combined effects of an increase in (1) the diastolic Ca\(^{2+}\) content; (2) the rate of rise of the Ca\(^{2+}\) release; and (3) the amount of Ca\(^{2+}\) released by the SR.\(^8\)

At a low concentration (0.3 mmol/L), caffeine has been shown to increase \(V_{\text{prop}}\) and force.\(^7\) In addition, 0.5 mmol/L caffeine can increase the amplitude of delayed afterdepolarizations and cause triggered activity.\(^9-12\) Recently, caffeine has been shown to enhance the release of Ca\(^{2+}\) from the SR by activating a cardiac SR-Ca\(^{2+}\) release channel (RyR) incorporated into planar phospholipid bilayers.\(^13-16\) This activation of the channel leads to net shift of Ca\(^{2+}\) from SR to myoplasm, subsequently modulating Ca\(^{2+}\) loading level of the...
myoplasm and the SR. Thus, we hypothesize that the effect of caffeine on $V_{\text{prop}}$ of TPCs is secondary to drug modulation of CICR due to the changes in $\text{Ca}^{2+}$ levels in the myoplasm and SR.

Therefore, in the present study, we investigated (1) the relationship between \([\text{Ca}^{2+}]_i\) and $V_{\text{prop}}$ of $\text{Ca}^{2+}$ waves to evaluate how $\text{Ca}^{2+}$ levels in myoplasm and SR can affect $V_{\text{prop}}$ and (2) the effect of caffeine on these relationships to evaluate whether $V_{\text{prop}}$ is sensitive to changes in the probability of opening of the SR-$\text{Ca}^{2+}$ release channel (P$_R$) of RyR.

### Materials and Methods

#### Dissection and Mounting of Rat Ventricular Trabeculae

The experiments in the present study were conducted in accordance with the principles outlined in the most recent Guide to the Care and Use of Experimental Animals by the Canadian Council on Animal Care. Lewis Brown Norway rats (Harlan Sprague-Dawley Inc, Indianapolis, Ind; 250 to 300 g) were anesthetized with diethyl ether. Twelve hearts were excised, and the coronary arteries were immediately perfused via the aorta with a Krebs-Henseleit (K-H) solution modified by adding 15 mmol/L KCl. After arrest of the heart, trabeculae (n=12, length 2.07±0.09 mm, width 207±37 $\mu$m, thickness 101±24 $\mu$m) were dissected from the right ventricle and mounted horizontally between a force transducer and a micromanipulator in a perfusion bath located on the stage of an inverted microscope (Nikon). Trabeculae were stimulated at 0.5 Hz through a ultrator in a perfusion bath located on the stage of an inverted microscope (Nikon). Trabeculae were stimulated at 0.5 Hz for 30 to 60 minutes until fura-2 had diffused uniformly throughout the preparation. The epifluorescence of fura-2 from the trabeculae at excitation wavelengths of 360 and 380 nm was recorded by a CCD camera (Scanning Inc) through a 510- to 560-nm bandpass filter. The images were recorded with a videocassette recorder (VCR) for offline analysis. The results were in equilibrium with 95% O$_2$ and 5% CO$_2$; pH was 7.4.

#### Fura-2 Loading and Measurement of Fluorescence \([\text{Ca}^{2+}]_i\)

In the trabeculae as measured previously. Briefly, fura-2 pentapotassium salt was microinjected electrophoretically into one cell and allowed to spread throughout the trabeculae via gap junctions. After the injection, the trabeculae were stimulated at 1 Hz for 30 to 60 minutes until fura-2 had diffused uniformly throughout the preparation. The epifluorescence of fura-2 from the trabeculae at excitation wavelengths of 340 and 380 nm was measured at 1 Hz by a photomultiplier tube (PMT) (PMT-RT263 with a C1053-01 socket, Hamamatsu). The signal from the PMT was stored in a personal computer through an analog-digital converter. Alternatively, the fluorescence image of the trabeculae at excitation wavelengths of 360 and 380 nm was recorded by a CCD camera coupled to a 2-stage image intensifier (IIC; model C330, General Scanning Inc) through a 510- to 560-nm bandpass filter. The images were recorded with a videocassette recorder (VCR) for offline analysis. The force of the muscle was measured using a modified silicon semiconductor strain gauge. Sarcomere length (SL) was measured using laser diffraction techniques.

#### Analysis of the Signal From the PMT \([\text{Ca}^{2+}]_i\)

The ratio of the fluorescence at 360-nm excitation to that at 380-nm excitation (360/380) was calculated for each sampling point after the induction of TPCs using the regression line derived from the relationship between PMT and the IIC ratio determined at the same sampling point. To avoid noise caused by low-excitation light intensity on the far edges of the profile of fluorescence, we calculated \([\text{Ca}^{2+}]_i\), only at the regions of the centrally located 250 pixels (719 $\mu$m) of the profiles along the trabeculae.

To calculate $V_{\text{prop}}$, we identified the peak of a $\text{Ca}^{2+}$ transient during the $\text{Ca}^{2+}$ wave at each pixel along trabeculae and plotted the time of the maximum against the position of the peak. $V_{\text{prop}}$ was calculated from the slope of the fitted line to the plot, when regression analysis showed a linear relationship ($r$=0.9), as described previously.

### Experimental Protocol

To induce a $\text{Ca}^{2+}$ wave (or TPC), bath temperature was lowered to 20°C to 23°C, and trains of electrical stimuli at 2.5 Hz were applied for 10 seconds at \([\text{Ca}^{2+}]_o\) of 0.3 mmol/L. SL was set to 2.10±0.09 mm, $\text{Ca}^{2+}$ bound dye at 380-nm excitation. Because we have previously reported a good correlation between in vitro and in vivo calibrations with free Mg$_2^{2+}$ was 1 mmol/L, in the solutions mimicking the intracellular milieu, values for $K_d$, $R_{\text{max}}$, $R_{\text{min}}$, and $\beta$ were determined using in vitro calibrations. $R_{\text{max}}$ and $R_{\text{max}}$ were 0.152 and 4.60, and $K_d$ and $\beta$ were 361 nmol/L and 9.18, respectively. This is in agreement with previous data from our laboratory.

#### Analysis of an Image From the IIC

Fura-2 fluorescence images recorded at 30 frames per second on the VCR were analyzed as previously described. Briefly, fluorescence data of each video frame were digitized with an 8-bit analog-digital converter and stored in a frame buffer memory of 512×480 pixels (Coreco Inc). Therefore, in our optical system, one pixel corresponded to 2.9×2.9 $\mu$m in the image plane. For the analysis of the image, a region of interest (ROI) was set horizontally along the long axis of the fluorescence image of trabeculae. The length of the ROI was always 512 pixels (1470 $\mu$m) whereas its width was 20 pixels (57.4 $\mu$m). To obtain intensity profiles of the fluorescence along the long axis of trabeculae, we calculated an average intensity value from each transverse line of pixels within the ROI. To eliminate high-frequency noise from the intensity profile, we used a low-pass finite impulse response filter (MATLAB) with a cutoff frequency of 5 pixels (14.4 $\mu$m). After subtraction of autofluorescence, we calculated the ratio of the fluorescence at 360-nm to that at 380-nm excitation (360/380) at each point on the intensity profiles obtained from the images at 360- and 380-nm excitation light. To correct for the effects of nonuniform illumination of excitation light, we calculated \([\text{Ca}^{2+}]_i\), at each sampling point after the induction of TPCs using the regression line derived from the relationship between PMT and the IIC ratio determined at the same sampling point. In addition, to avoid noise caused by low-excitation light intensity on the far edges of the profile of fluorescence, we calculated \([\text{Ca}^{2+}]_i\), only at the regions of the centrally located 250 pixels (719 $\mu$m) of the profiles along the trabeculae.

Thus, we eventually analyzed 23 reproducible $\text{Ca}^{2+}$ waves (or TPCs) triggered by serial trains of stimuli varied by ≤10%. We regarded such $\text{Ca}^{2+}$ waves as reproducible; reproducible conditions lasted at least 30 minutes. In the present study, we induced reproducible $\text{Ca}^{2+}$ waves in 12 trabeculae. To change $\text{Ca}^{2+}$ loading of the muscle, we varied \([\text{Ca}^{2+}]_o\), and/or duration of the train of electrical stimuli in 9 trabeculae. The measurement of \([\text{Ca}^{2+}]_i\), was started again when $\text{Ca}^{2+}$ waves (or TPCs) reached a new steady state. Thus, we eventually analyzed 23 reproducible $\text{Ca}^{2+}$ waves (i$\text{[Ca}^{2+}]_i$), 2.2±0.3 mmol/L, temperature 21.9±0.2°C).

The effect of caffeine on $\text{Ca}^{2+}$ waves was studied using 7 reproducible $\text{Ca}^{2+}$ waves elicited in 5 trabeculae (length 2.19±0.19 mm, width 228.57 $\mu$m, thickness 101±4 $\mu$m, \([\text{Ca}^{2+}]_o\), 2.4±0.3 mmol/L, temperature 21.6±0.3°C, 10-second electrical stimulation at 2.5 Hz). When the $\text{Ca}^{2+}$ waves were reproducible, trabeculae were superfused with K-H solution containing varied concentrations (0.1 to 1.0 mmol/L) of caffeine (Sigma). After superfusion with caffeine, force development induced by trains of electrical stimuli was monitored and reached a new steady-state level within 5 minutes; measurement of \([\text{Ca}^{2+}]_i\), was then begun. All measurements made in the presence of caffeine were completed within 30 minutes, and trabeculae were then superfused with caffeine-free K-H solution. After washout, we confirmed that the $\text{Ca}^{2+}$ waves were still reproducible.

#### Data Analysis

To assess $\text{Ca}^{2+}$ loading of myoplasm and SR, we calculated the following parameters (see Figure 1). First, we estimated the SR $\text{Ca}^{2+}$ loading from the increment in \([\text{Ca}^{2+}]_i\), during the last stimulated
twitch of the trains (ΔCa1), i.e., the difference between a peak of a Ca2+ transient during the twitch and the minimal [Ca2+]i, preceding the last twitch. Second, we measured the diastolic [Ca2+]i (CaD) just before a Ca2+ wave, i.e., the minimal [Ca2+]i observed between the last twitch and a subsequent TPC. Third, the amount of Ca2+ released during the wave was estimated from the increment in [Ca2+]i, during a Ca2+ wave (ΔCa1), i.e., the difference between the peak of a Ca2+ wave (CaW) and CaD. Using these parameters, we calculated ΔCa1/ΔCaD, the amount of Ca2+ released during the wave normalized for the SR Ca2+ content. We assumed that this parameter reflects the released fraction of Ca2+ inside SR and corresponds to the probability of Ca2+ release from SR during the wave (see Discussion). When obtained with use of the PMT (Figure 1), we will refer to these parameters as global changes in [Ca2+]i ([ΔCa1], gΔCa1, gCaD, and gΔCaD). When obtained from images recorded by the IIC, we first calculated these parameters at each pixel position along trabeculae and then averaged the values obtained at each pixel position. In that case, we will refer to the averaged values as regional changes in [Ca2+]i, (rΔCa1, rCaD, and rΔCaD). Moreover, we measured developed force during the last twitch (F1) and that during a TPC (FTPC).

Statistics

All averaged values were expressed as mean ± SEM. Single-factor ANOVA, unless stated otherwise, was used to detect significant differences (P < 0.05).

Results

Figures 1 and 2 show clearly that Ca2+ waves are accelerated by Ca2+ loading.4 To change Ca2+ loading of the muscle, we increased [Ca2+]o, or the duration of the train of electrical stimuli after the induction of reproducible Ca2+ waves. Figure 1 shows global [Ca2+]i, and force development during the last 2 electrically stimulated twitches of a train at 2.5-Hz stimulation. With a longer period of stimulation, all parameters of global [Ca2+]i ([ΔCa1], gΔCa1, gCaD, and gΔCaD) increased by 19%, 20%, and 127%, respectively, and parameters of force (F1 and FTPC) increased by 6% and 37%, respectively. In Figure 2, regional changes in [Ca2+]i, during comparable TPCs were determined directly after the recordings in Figure 1. With a longer period of stimulation, Vprop of the Ca2+ wave increased from 2.92 to 4.40 mm/s. The parameters of regional [Ca2+]i, (rΔCa1, rCaD, and rΔCaD) also increased in proportion to each other (Figure 3). Ca2+ loading of the muscle increased the amplitude of TPCs and accelerated their propagation. As a result, TPCs already did occur in between the twitches during the stimulus train (Figure 1), which might cause nonuniformity of the Ca2+ release process during the (last) twitch that preceded the TPC, which was analyzed here. The nonuniformity of Ca2+ transients after the last stimulus along the analyzed region appeared to be small. The maximal difference in peak amplitude of rΔCa1 was < 40 nmol/L whereas rCaD differed < 20 nmol/L between both ends of the ROI (data not shown). Hence, we believed that activation of the ROI was sufficiently uniform to permit evaluation of the factors that dictate the rate of propagation of the Ca2+ waves occurring after the twitch.

Data obtained from 23 reproducible Ca2+ waves from 12 trabeculae showed that Vprop correlated strongly with rΔCa1 (Figure 3A), rCaD (Figure 3B), and rΔCaD (Figure 3C) as well as rCaD/rΔCa1 (Figure 3D). In 15 Ca2+ waves from 8 trabeculae, Vprop also correlated linearly with F1 (r = 0.70) and FTPC (r = 0.67) (data not shown), as we have described previously.7 The increase of cytosolic Ca2+ correlated with ΔCa1 (Figure 3) and F1 (not shown). F1 is proportional to the amplitude of rapid cooling contractures (H. Banijamali, H.E.D.J. ter Keurs, unpublished observations, 1994) so that one may conclude that Ca2+ loading in our experiments led to proportional increases in cytosolic Ca2+ and in the SR Ca2+ content (reflected by rΔCa1) of the muscle.

It was striking that rCaD/rΔCa1 under drug-free conditions increased linearly with both rΔCa1 and rCaD (Figure 3). The increase of this ratio suggests that an increase of the Ca2+ loading of the muscle increases the probability of opening of the SR-Ca2+ channels during the wave. Ca2+ wave propagation accelerated with an increase in the latter parameter (rΔCaD/rΔCa1; Figure 3D). Regional measurements showed that acceleration of the Ca2+ wave was not the cause of the
A larger amplitude of the transient owing to faster wave propagation, as would be observed in the recordings with the PMT, which collected the fluorescence from a larger region in the muscle.4

To modify the kinetics of net Ca\textsuperscript{2+} transport from the myoplasm to the SR, 5 trabeculae were superfused with caffeine (0.1 to 1.0 mmol/L) after the induction of a reproducible Ca\textsuperscript{2+} wave. Figure 4 shows an example of the global [Ca\textsuperscript{2+}]i and the force development during the last 2 stimulated twitches in a train and a subsequent TPC in the absence and in the presence of caffeine. The addition of 0.3 mmol/L caffeine decreased the amplitude of g\Delta Ca\textsubscript{T} by 47% while

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**Figure 2.** Effect of changes in the duration of electrical stimuli on regional changes in [Ca\textsuperscript{2+}]. The regional [Ca\textsuperscript{2+}], calculated from the images by the IIC during the last electrically stimulated twitch and a TPC a few minutes after the recording of the PMT shown in Figure 1. Abscissa represents time; ordinate, [Ca\textsuperscript{2+}]; and z-axis, the position along the long axis of the trabecula. s indicates moment of electrical stimulation (experiment 970110). A, Electrical stimulation for 5 seconds. After the end of the clearly uniform stimulated Ca\textsuperscript{2+} transient, a small Ca\textsuperscript{2+} transient was observed to move as a wave from position A (*) toward position B at the calculated V\textsubscript{prop} of 2.92 mm/s. B, Electrical stimulation for 15 seconds. V\textsubscript{prop} increased to 4.40 mm/s. In addition, r\Delta Ca\textsubscript{T} increased from 555 to 615 nmol/L, rCa\textsubscript{D} increased from 165 to 245 nmol/L, and r\Delta Ca\textsubscript{W} increased from 157 to 245 nmol/L.

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**Figure 3.** Relationships between V\textsubscript{prop} and the regional changes in [Ca\textsuperscript{2+}] under control conditions (n=23). V\textsubscript{prop} was tightly and linearly correlated with r\Delta Ca\textsubscript{T} (A), rCa\textsubscript{D} (B), r\Delta Ca\textsubscript{W} (C), and r\Delta Ca\textsubscript{W}/r\Delta Ca\textsubscript{T} (D). Note that all Ca\textsuperscript{2+} parameters correlate strongly and linearly, indicating that the Ca\textsuperscript{2+} loading of the muscle caused a proportional increase of rCa\textsubscript{D}, r\Delta Ca\textsubscript{W}, and r\Delta Ca\textsubscript{T}. In addition, r\Delta Ca\textsubscript{W}/r\Delta Ca\textsubscript{T} increased in proportion with the Ca\textsuperscript{2+} loading of the muscle (for further explanation, see text).
gCaW increased by 51% and 91%, respectively. FT also decreased (27%) whereas FTPC increased (113%). In the presence of 1.0 mmol/L caffeine, we could neither detect an increase in 
\[\text{Ca}^{2+}\]i nor an aftercontraction following the train of electrical stimuli. gDcAT and FT decreased by 77% and 55%, respectively. The diastolic diffraction pattern was uniform and stationary whereas twitch force during the train stayed constant. These observations suggested that random spontaneous sarcomere contractions occurred only rarely in the presence of 1 mmol/L caffeine.

Figure 5. Effects of caffeine on regional changes in 
\[\text{Ca}^{2+}\]i in the trabecula of Figure 4. The regional 
\[\text{Ca}^{2+}\]i calculated from the images by the IIC dur-
ning the last electrically stimulated twitch and a TPC a few minutes after the recording of the PMT shown in Figure 4. In these 3-dimensional represen-
tations, the axes are the same as those in Fig-
ure 2. After the end of the clearly uniform stimu-
lated 
\[\text{Ca}^{2+}\] transient, a small 
\[\text{Ca}^{2+}\] transient appeared to move from A (*) toward B (for expla-
nation, see text). s indicates moment of electrical stimulation (experiment 970214). A, Before the addition of caffeine. The calculated Vprop was 1.85 mm/s. B, After the addition of 0.3 mmol/L caffeine. Vprop increased to 6.39 mm/s. rCaW increased from 66 to 142 nmol/L whereas rDcAT decreased from 616 to 351 nmol/L.
and in the presence of 0.3 mmol/L caffeine (Figure 5B). The addition of 0.3 mmol/L caffeine increased the calculated $V_{\text{prop}}$ from 1.86 to 6.39 mm/s. $\Delta \text{Ca}_{\text{T}}$ decreased by 43%, but both $\text{rCa}_{\text{D}}$ and $\Delta \text{Ca}_{\text{W}}$ increased by 40% and 115%, respectively.

Figure 6 shows the effects of caffeine on global and regional changes in $[\text{Ca}^{2+}]$, and $V_{\text{prop}}$. The twitch $\text{Ca}^{2+}$ transient declines to $\approx 25\%$ of the control value whereas diastolic $\text{Ca}^{2+}$ increased monotonically even at 1 mmol/L caffeine. This observation would be expected if caffeine eliminates contribution of the SR to the twitch, leaving only $\text{Ca}^{2+}$ transport across the sarcolemma to supply and remove $\text{Ca}^{2+}$. $\text{Ca}^{2+}$ waves and aftercontractions increased and accelerated with caffeine up to 0.3 mmol/L, but they always disappeared after the addition of 1.0 mmol/L caffeine (n=3). Thus, we measured $\Delta \text{Ca}_{\text{T}}$, $\text{Ca}_{\text{D}}$, and $\Delta \text{Ca}_{\text{W}}$ at caffeine concentrations of 0, 0.1, 0.3, and 0.5 mmol/L (n=7, 3, 4, and 4, respectively) and measured $\text{gCa}_{\text{T}}$ at 1.0 mmol/L caffeine (n=3). At these concentrations, $\Delta \text{Ca}_{\text{T}}$ decreased and $\text{Ca}_{\text{D}}$ increased; $\Delta \text{Ca}_{\text{W}}$ increased at 0.1 mmol/L caffeine but then decreased at 0.5 mmol/L caffeine. Measurement of regional $[\text{Ca}^{2+}]$, changes in 2 of 7 muscles tested at 0.3 mmol/L caffeine and in all 4 muscles tested at 0.5 mmol/L caffeine became inaccurate, because $V_{\text{prop}}$ became too fast to be calculated from video frames obtained at 30 per second. Thus, we calculated $\Delta \text{Ca}_{\text{T}}$, $\text{Ca}_{\text{D}}$, $\Delta \text{Ca}_{\text{W}}$, and $V_{\text{prop}}$ in control, 0.1 mmol/L, and 0.3 mmol/L caffeine (n=7, 3, 4, and 5, respectively). At these concentrations, $\Delta \text{Ca}_{\text{T}}$ decreased significantly whereas $V_{\text{prop}}$ increased in the presence of caffeine; $\Delta \text{Ca}_{\text{W}}$ increased at 0.1 mmol/L caffeine.

$F_{\text{T}}$ also decreased to $72.8\pm3.3\%$ ($P<0.005$) and $62.0\pm3.5\%$ ($P<0.0005$) of control $F_{\text{T}}$ in the presence of 0.1 and 0.3 mmol/L caffeine whereas $F_{\text{T\text{PC}}}$ increased to $147\pm11.2\%$ ($P<0.05$) and $201\pm21.5\%$ ($P<0.01$) of control $F_{\text{T\text{PC}}}$, respectively (unpaired $t$ test with unequal variation). The data obtained in each muscle in the presence of the drug were compared, for this analysis, with the data from the same muscle in the drug-free state ($[\text{Ca}^{2+}]_o$ 2.1±0.2, temperature 21.8±0.2).

The data in Figure 6 suggest that caffeine decreases $\Delta \text{Ca}_{\text{T}}$ while it increases $\Delta \text{Ca}_{\text{W}}$ at 0.1 mmol/L. This would be consistent with the effect of caffeine to cause an increase of the probability of opening of the SR-$\text{Ca}^{2+}$ channels, increasing $\text{Ca}^{2+}$ leak from the SR.13–17 The expectation that $\Delta \text{Ca}_{\text{W}}/\Delta \text{Ca}_{\text{T}}$ reflects the probability of opening of the SR-$\text{Ca}^{2+}$ channels is indeed met by observation of the effect of caffeine. Figure 7A shows that caffeine (0.1 to 0.5 mmol/L) increased both global and regional $\Delta \text{Ca}_{\text{W}}/\Delta \text{Ca}_{\text{T}}$ significantly. Changes in force development ($F_{\text{T\text{PC}}}/F_{\text{T}}$) in the same muscles in the presence of caffeine increased significantly with increased caffeine concentration (Figure 7B).

Figure 8 shows the effects of caffeine on the relationship between $V_{\text{prop}}$ and regional changes in $[\text{Ca}^{2+}]$, of 8 $\text{Ca}^{2+}$ waves from 5 trabeculae. For reference, control data (Figure 3) in the absence of caffeine are reproduced as open circles (○). In the presence of caffeine, there remains a reasonable relationship between $V_{\text{prop}}$ and $\Delta \text{Ca}_{\text{T}}$ (Figure 8A) and $\Delta \text{Ca}_{\text{W}}$ (Figure 8C). However, if we assume that $\Delta \text{Ca}_{\text{T}}$ reflects the SR $\text{Ca}^{2+}$ content in both drug-free and drug-containing solutions, then we are struck by the observation that the small $\Delta \text{Ca}_{\text{T}}$ in caffeine are associated with waves with higher $V_{\text{prop}}$ (leftward shift only). This suggests to us that caffeine renders $V_{\text{prop}}$ of $\text{Ca}^{2+}$ waves more sensitive to SR $\text{Ca}^{2+}$ content. It appears that in the presence of caffeine, $\text{Ca}^{2+}$ waves (r$\Delta \text{Ca}_{\text{W}}$) similar in size to control waves propagate faster (parallel shift upward only), again suggesting that the higher $V_{\text{prop}}$ reflects an increased sensitivity to the SR $\text{Ca}^{2+}$ content.

In contrast, caffeine has little effect on the relationships between $V_{\text{prop}}$ and r$\text{Ca}_{\text{D}}$ (Figure 8B) or r$\Delta \text{Ca}_{\text{D}}/\Delta \text{Ca}_{\text{T}}$ (Figure 8D) seen in control, although an increase of $V_{\text{prop}}$ correlated with increased r$\Delta \text{Ca}_{\text{D}}/\Delta \text{Ca}_{\text{T}}$ in the presence of caffeine. The relationship between $V_{\text{prop}}$ and $F_{\text{T\text{PC}}}/F_{\text{T}}$ is not altered by caffeine (data not shown). Clearly, Figure 8D shows that the rate of propagation increases in proportion to the increase in fractional $\text{Ca}^{2+}$ release during a wave (reflected by r$\Delta \text{Ca}_{\text{D}}$/}
Thus, caffeine increases fractional Ca$^{2+}$ release (increased $\Delta\text{Ca}_W/\Delta\text{Ca}_T$) with a commensurate increase of $V_{\text{prop}}$.

As stated, we assumed that the probability of opening of the SR-Ca$^{2+}$ channels would be reflected by the amount of Ca$^{2+}$ released from the SR relative to the SR Ca$^{2+}$ content ($\Delta\text{Ca}_W/\Delta\text{Ca}_T$). The observation that both the cellular Ca$^{2+}$ loading (as reflected by $\text{rCa}_T$, $\text{rCa}_D$, and $\text{rCa}_W$) and caffeine$^{13-16}$ increased $\Delta\text{Ca}_W/\Delta\text{Ca}_T$ (Figure 9) is consistent with this prediction. In control drug-free conditions, increases in $\Delta\text{Ca}_T$, $\text{rCa}_D$, and $\text{rCa}_W$ resulted in a concomitant increase in $\Delta\text{Ca}_W/\Delta\text{Ca}_T$. Caffeine increased $\Delta\text{Ca}_W/\Delta\text{Ca}_T$ 2-fold (Figure 9B and 9C). Intriguingly, caffeine shifted the rela-

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**Figure 7.** Effects of caffeine on global (C) and regional (D) $\Delta\text{Ca}_W/\Delta\text{Ca}_T$ (A) and $F_{\text{TPC}}/F_T$ (B). The global and regional $\Delta\text{Ca}_W/\Delta\text{Ca}_T$ and $F_{\text{TPC}}/F_T$ increased significantly ($P<0.005$) in the presence of caffeine.

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**Figure 8.** Effects of caffeine on the relationships between $V_{\text{prop}}$ and regional changes in [Ca$^{2+}$] in the absence of caffeine (C) and after the addition of 0.1 mmol/L (●) and 0.3 mmol/L of caffeine (■). After the addition of caffeine (0.1 and 0.3 mmol/L), the relationships between $V_{\text{prop}}$ and $\Delta\text{Ca}_T$ (A), $\text{rCa}_D$ (B), and $\Delta\text{Ca}_W$ (C) moved upward.

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**Figure 9.** Relationships between $\Delta\text{Ca}_W/\Delta\text{Ca}_T$ and regional changes in [Ca$^{2+}$]$\text{rCa}_W/\text{rCa}_T$ in the absence of caffeine (C) and after the addition of 0.1 mmol/L (●) and 0.3 mmol/L of caffeine (■). After the addition of caffeine (0.1 and 0.3 mmol/L), the relationships between $V_{\text{prop}}$ and $\Delta\text{Ca}_T$ (A), $\text{rCa}_D$ (B), and $\Delta\text{Ca}_W$ (C) moved upward.
since it has been shown that TPCs are abolished by agents that interfere with SR Ca\(^{2+}\) loading or release, such as ryanodine and caffeine\(^7\) (Figure 6). Thus, we assumed that ∆Ca\(_{\text{SR}}\) reflects the SR Ca\(^{2+}\) content available for release, and that ∆Ca\(_{\text{cyt}}\) reflects the amount of Ca\(^{2+}\) released from the SR during the Ca\(^{2+}\) wave. It follows that the released fraction of the SR Ca\(^{2+}\) content during the wave (∆Ca\(_{\text{cyt}}\)/∆Ca\(_{\text{SR}}\)) reflects the probability of opening of SR-Ca\(^{2+}\) release channel.

It has been reported that the P\(_o\) of cardiac RyR incorporated into planar phospholipid bilayers can be modulated by intraluminal SR Ca\(^{2+}\)\(^{16,27–30}\) as well as by cytosolic Ca\(^{2+}\)\(^{31,32}\). These studies have shown that elevation of [Ca\(^{2+}\)]\(_i\) on either the luminal or cytosolic side increases P\(_o\) of RyR. The relationships between r∆Ca\(_{\text{SR}}\)/r∆Ca\(_{\text{SR}}\) and r∆Ca\(_{\text{SR}}\) on the one hand (Figure 9A) and rCa\(_{\text{cyt}}\) on the other hand (Figure 9B and 9C) suggest that under drug-free conditions, SR Ca\(^{2+}\) content and cytosolic Ca\(^{2+}\) during a Ca\(^{2+}\) wave indeed determine the released fraction of Ca\(^{2+}\) from inside the SR, possibly by modulating the probability of opening of the SR-Ca\(^{2+}\) channels. If so, our observations are consistent with the features of RyR measured using phospholipid bilayers,\(^{16,27–32}\)

It follows from the tight correlations between V\(_{\text{prop}}\) and r∆Ca\(_{\text{SR}}\), rCa\(_{\text{cyt}}\), r∆Ca\(_{\text{SR}}\), and r∆Ca\(_{\text{SR}}\)/r∆Ca\(_{\text{SR}}\) described in Figure 3 that V\(_{\text{prop}}\) is facilitated under conditions of a higher SR Ca\(^{2+}\) content, a higher diastolic [Ca\(^{2+}\)], and a larger amount of Ca\(^{2+}\) released from SR and possibly P\(_o\). These findings were predicted by computer simulation of Ca\(^{2+}\) waves using a model of CICR and Ca\(^{2+}\) diffusion.\(^8\) These observations are conceptually summarized in Figure 10, assuming that Ca\(^{2+}\) waves (or TPCs) travel along trabeculae, owing to the combination of CICR and Ca\(^{2+}\) diffusion.\(^{1,4–6}\) The simplest explanation of the observations is that SR Ca\(^{2+}\) content and cytosolic Ca\(^{2+}\) increase P\(_o\) of RyR. The latter effect would increase fractional Ca\(^{2+}\) release from the SR during a Ca\(^{2+}\) wave (r∆Ca\(_{\text{cyt}}\)/r∆Ca\(_{\text{SR}}\)), thus decreasing the time needed to release Ca\(^{2+}\) from adjacent release sites during propagation of the Ca\(^{2+}\) wave. We cannot prove from these data that the SR Ca\(^{2+}\) content influences P\(_o\) as has been suggested by lipid bilayer studies,\(^{16,27–30}\) but our data are certainly consistent with this hypothesis.

An increase in diastolic Ca\(^{2+}\) decreases the buffering capacity for Ca\(^{2+}\)\(^{33–36}\) because of the increased Ca\(^{2+}\) binding to ligand proteins, as shown in Figure 10. With reduced buffering capacity for Ca\(^{2+}\), Ca\(^{2+}\) will diffuse faster,\(^37\) and Ca\(^{2+}\) release in adjacent SR will be “induced” earlier.

**Effect of Caffeine**

Caffeine has been reported to enhance the sensitivity of the myofilaments to Ca\(^{2+}\) and inhibit net Ca\(^{2+}\) uptake by the SR.\(^{39,40}\) Recently, it has been concluded from studies using cardiac RyR incorporated into planar lipid bilayers that caffeine can increase the sensitivity of RyR to Ca\(^{2+}\) and increase P\(_o\) of RyR.\(^{13,14,16,28}\) The increase in P\(_o\) of RyR can enhance the release of Ca\(^{2+}\) from SR and result in an increase in [Ca\(^{2+}\)]. On the other hand, the increase in P\(_o\) reduces SR Ca\(^{2+}\) content and eventually depletes the SR of Ca\(^{2+}\), depending on the concentration of caffeine. The observation that caffeine produced an increase in Ca\(_{\text{cyt}}\) and a decrease in ∆Ca\(_{\text{SR}}\) (or F\(_T\)) (Figure 6A and 6B) is consistent with this concept.
With caffeine at 1.0 mmol/L, Ca\(^{2+}\) waves (and TPCs) disappeared, probably as a result of Ca\(^{2+}\) depletion of the SR. We assume that the decrease in ΔCa\(_T\) (and V\(_F\)) in the presence of caffeine is due to a net decrease in SR Ca\(^{2+}\) content available for release but not due to the preceding spontaneous Ca\(^{2+}\) transient (and aftercontraction) for the following reasons. First, gΔCa\(_T\) in the presence of 1.0 mmol/L caffeine was significantly smaller than that of 0.1 to 0.3 mmol/L caffeine (Figure 6A), although spontaneous Ca\(^{2+}\) transient (and aftercontractions) had already disappeared at 1.0 mmol/L caffeine. Second, in the presence of 0.1 and 0.3 mmol/L caffeine, the developed force triggered by the last stimulus of a train changed by <10% of that triggered by the first one of the train, which was not preceded by aftercontractions. Therefore, our observations are consistent with the effect of caffeine on RyR observed within lipid bilayers.13–16,29

In the presence of caffeine, the relationships between V\(_{prop}\) and rCa\(_0\) (Figure 8B) and rCa\(_{A0}/r\Delta Ca\(_T\) (Figure 8D) were almost similar to control. This means that caffeine has little effect on the mechanisms that link rCa\(_0\) and rCa\(_{A0}/r\Delta Ca\(_T\) to V\(_{prop}\). In contrast, the relationships between V\(_{prop}\) and rCa\(_T\) (Figure 8A) and rCa\(_{A0}\) (Figure 8C) suggest that caffeine makes V\(_{prop}\) much more sensitive to the SR Ca\(^{2+}\) content. This means that caffeine has a substantial effect on the pathway(s) that couple(s) rΔCa\(_T\) or rΔCa\(_{A0}\) to V\(_{prop}\). As suggested in Figure 10, caffeine increases P\(_c\), of RyR similar to SR Ca\(^{2+}\) content and cytosolic Ca\(^{2+}\). The latter effect of caffeine would increase fractional Ca\(^{2+}\) release from the SR during a Ca\(^{2+}\) wave (rΔCa\(_{A0}/r\Delta Ca\(_T\)), as shown in Figures 7 and 9, and would decrease the time needed to release Ca\(^{2+}\) from adjacent release sites during propagation of the Ca\(^{2+}\) wave. Hence, for any level of SR Ca\(^{2+}\) loading, V\(_{prop}\) would increase, owing to caffeine (Figure 8A). V\(_{prop}\) would also increase, even if the amplitude of the Ca\(^{2+}\) propagating transient would be constant, as is shown by Figure 8C.

**Limitations of the Study**

It is well known that the unitary SR Ca\(^{2+}\) release event in both cardiac cells and muscle consists of Ca\(^{2+}\) sparks,41 Ca\(^{2+}\) sparks have been described to underlie propagating Ca\(^{2+}\) waves41,42 at low levels of cellular loading (Ca\(_o\) ≈50% of the EC\(_{50}\)). Such Ca\(^{2+}\) waves travel a distance of only a few sarcomeres,41,42 Although it is tempting to assume that the microscopic waves observed in that study could consist of an avalanche of propagating Ca\(^{2+}\) sparks, our methods do not permit statements to this effect for the following reasons. First, we used fura-2, which does not exhibit a high enough photon efficiency to permit visualization of sparks. Second, the use of conventional microscopy precludes evaluation of events, which occur at a submicron scale. Hence, the fundamental question whether Ca\(^{2+}\) release by RyR (Ca\(^{2+}\) sparks) together with diffusion of Ca\(^{2+}\) from terminal cisterna to terminal cisterna (2 μm) causes Ca\(^{2+}\) waves as observed in the present study has to await exploration using probes such as confocal microscopy. This approach is important because theoretical modeling of propagation of Ca\(^{2+}\) waves has put the challenging constraint on the model that Ca\(^{2+}\) release from the SR has to occur in a fraction of a millisecond to permit V\(_{prop}\) to reach values of several millimeters per second. Even though it has been shown that opening of RyR exhibits rapid kinetics,43 the capacity of the channel to Ca\(^{2+}\) release on a submillisecond timescale remains to be proven.

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Ca\textsuperscript{2+} Waves During Triggered Propagated Contractions in Intact Trabeculae: Determinants of the Velocity of Propagation
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