Cellular Origins of the Transient Inward Current in Cardiac Myocytes
Role of Fluctuations and Waves of Elevated Intracellular Calcium

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Activation of the transient inward current (I\textsubscript{\texttau}) by a rise in intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) is believed to be responsible for generating triggered cardiac arrhythmias. In this study, the cellular basis of the rise in [Ca\textsuperscript{2+}]\textsubscript{i} that activates I\textsubscript{\texttau} and aftercontractions in single rat ventricular myocytes was examined. [Ca\textsuperscript{2+}]\textsubscript{i} was measured both indirectly by cell contraction and directly with fura-2. Under conditions that caused steady-state [Ca\textsuperscript{2+}]\textsubscript{i} to increase (i.e., calcium overload) membrane repolarization after a voltage-clamp depolarization resulted in the appearance of I\textsubscript{\texttau} that was similar in many respects to that observed in multicellular preparations. This I\textsubscript{\texttau} occurred at the same time that [Ca\textsuperscript{2+}]\textsubscript{i} spontaneously increased and preceded the aftercontraction by 60–90 msec. However, I\textsubscript{\texttau} recorded from a single cell was variable in time course and amplitude (unlike that observed in multicellular preparations). Examination of cell contraction and digital imaging of fura-2 fluorescence showed that I\textsubscript{\texttau} was often associated with propagating regions of increased [Ca\textsuperscript{2+}]\textsubscript{i}, which arose from discrete sites of origin within the cell. Apparently synchronous aftercontractions could also be associated with multiple propagating waves of [Ca\textsuperscript{2+}]\textsubscript{i}. The variation in the time course and amplitude of I\textsubscript{\texttau} in single cells appeared to be due to changes in the location and number of sites of origin for the waves of [Ca\textsuperscript{2+}]\textsubscript{i}. After the first aftercontraction and I\textsubscript{\texttau}, desynchronization of the sites of origin of increased [Ca\textsuperscript{2+}]\textsubscript{i} occurred, and this resulted in a decrease in the amplitude of I\textsubscript{\texttau} and an increase in its duration. We conclude that the variability seen in single cells arises from changes in the pattern of spontaneous Ca\textsuperscript{2+} release. Such phenomena will seriously complicate interpretation of multicellular data, even when [Ca\textsuperscript{2+}]\textsubscript{i} is measured directly. (Circulation Research 1989;65:115-126)

The arrhythmogenic transient inward current (I\textsubscript{\texttau}) has been demonstrated in many cardiac preparations including sheep and calf Purkinje fibers,\textsuperscript{1-3} papillary muscle,\textsuperscript{4,5} and rat and guinea pig ventricular muscle cells.\textsuperscript{6-8} The appearance of I\textsubscript{\texttau} is accompanied by the development of spontaneous fluctuations of membrane current, tension,\textsuperscript{9,10} and [Ca\textsuperscript{2+}]\textsubscript{i}.\textsuperscript{11-13} These events appear to arise from the spontaneous release of Ca\textsuperscript{2+} by the sarcoplasmic reticulum.\textsuperscript{11-13,14} which occurs when resting [Ca\textsuperscript{2+}]\textsubscript{i} becomes elevated.\textsuperscript{12,15} Nevertheless, the changes in [Ca\textsuperscript{2+}]\textsubscript{i} that may underlie I\textsubscript{\texttau} activation have not been characterized at the cellular level.

Analysis of current and tension fluctuations during calcium overload has led to the suggestion that I\textsubscript{\texttau} may arise from synchronous activation of Ca\textsuperscript{2+}-activated current sources (i.e., electrogenic transport proteins or ion channels).\textsuperscript{3,6,9,10,16} This suggestion is consistent with aequorin-light recordings from voltage-clamped Purkinje fibers.\textsuperscript{17} However, direct evidence for the synchronization of spontaneous Ca\textsuperscript{2+} release activating I\textsubscript{\texttau} is lacking. Recent observations of localized waves of sarcomere shortening within a single heart cell (which become more prominent under conditions of calcium overload)\textsuperscript{10,19} suggest that subcellular heterogeneity of [Ca\textsuperscript{2+}]\textsubscript{i} may play an important role in the genesis of I\textsubscript{\texttau}. In this study, we have measured [Ca\textsuperscript{2+}]\textsubscript{i} with the fluorescent calcium indicator, fura-2, and cell con-
traction in voltage-clamped cardiac myocytes to characterize the cellular events that lead to activation of \( I_N \). Preliminary communication of some of the results has been made.8,12

**Materials and Methods**

**Cell Preparation**

Single ventricular myocytes were prepared from rat hearts by the method of Powell et al.,20 which was modified to include collagenase (1 mg/ml; Type II, Worthington Biochemical, Freehold, New Jersey) and hyaluronidase (0.25 mg/ml; Type 1-S, Sigma Chemical, St. Louis, Missouri) in the enzyme digestion solution. Isolated single cells were stored at 37°C in a physiological salt solution containing (mM) NaCl 140, KCl 4, MgCl\(_2\) 1, CaCl\(_2\) 1, glucose 10, and HEPES 10 (Sigma Chemical), pH 7.4. The isolated cells were rod-shaped with clear striations and sharp edges. In the present experiments, only cells that were quiescent under control conditions were used (25–50% of the isolated cells).

**Experimental Methods**

All experiments were performed at 32–35°C in an experimental chamber that was mounted on the stage of an inverted microscope.21 Cells were initially superfused with the physiological salt solution described above. Cells were voltage-clamped by a single microelectrode technique22 with a List EPC-7 voltage clamp (Medical Systems, Greensvale, New York). The patch pipette (1–3 MΩ resistance) contained (mM) potassium glutamate 140, KCl 10, MgCl\(_2\) 1, adenosine triphosphate (K\(_2\) salt) 3, EGTA 10 (μM) (K\(_2\) salt, Sigma Chemical), and PIPES 3 (Sigma Chemical), pH 7.2. To induce calcium overload in the cells, the potassium chloride concentration in the superfusing solution was lowered from 4.0 mM to 0.4 mM to inhibit the sodium pump (which increases intracellular Na\(^+\) and, therefore, [Ca\(^{2+}\)], by means of the Na-Ca exchange mechanism).23

[Ca\(^{2+}\)], was measured by including 35 μM of the K\(^+\) salt of fura-2 (Calbiochem, La Jolla, California) in the patch pipette solution.24,25 The cell was illuminated sequentially with 340 nm and 380 nm light from an epifluorescence illuminator. Fura-2 fluorescence (centered at 505 nm with an interference filter, 40 nm full width at half-maximal transmission) was either measured from a 10 μm diameter region of the cell with a photomultiplier tube or recorded as fluorescence images of the cell with an ISIT video camera (Dage MTI, Michigan City, Indiana).12,24 In both cases, background fluorescence was measured after gigahertz seal formation (but before cell loading with fura-2). After breaking the membrane patch under the pipette, constituents of the pipette solution, including fura-2, reached a steady-state level over a period of about 5–10 minutes. Electrophysiological and photomultiplier data were recorded on FM tape, and video information was recorded with a videocassette recorder (model NV-8500, Panasonic) for off-line analysis. Timing information was recorded on both FM and videotape to allow for the identification of specific experimental events in the separate recordings.

**Data Analysis**

Cell movement was recorded with a video camera (vidicon camera tube) and measured with a video edge tracking system (Instrumentation for Physiology and Medicine, San Diego, California). Video images were captured with 8-bit resolution with a microcomputer equipped with a PC Vision board (Imaging Technology, Woburn, Massachusetts). Filtering and contrast enhancement of video images were performed with commercial software (Imagepro, Media Cybernetics, Silver Spring, Maryland). Current and photomultiplier fluorescence data were digitized with a computer data acquisition system consisting of a computer equipped with an analog to digital converter (Data Translation, Marlboro, Massachusetts). Errors in the timing of the length measurements produced by the video dimension analyzer were corrected. Fluctuation analysis of cell shortening data was performed with a Cooley-Tukey fast Fourier algorithm on single 10-second bins of data sampled at 50 Hz and filtered at 20 Hz with an 8-pole Bessel filter (Frequency Devices, Haverhill, Massachusetts). Power spectra were calculated from the square of the Fourier transform.26

**Two-Wavelength Measurements of [Ca\(^{2+}\)]**

In each cell, [Ca\(^{2+}\)], was calculated by determining the ratio of fura-2 fluorescence during illumination with 340 nm and 380 nm light. The ratiometric signal depends on [Ca\(^{2+}\)], and is independent of indicator concentration (or cell thickness) and can be calibrated by use of an in vitro method.24,27,28 For [Ca\(^{2+}\)], transients that were constant from beat to beat, fluorescence intensity was recorded during a depolarization while the myocyte was illuminated with 340 nm light. The wavelength of the illuminating light was then changed to 380 nm, and the depolarization was repeated. The fluorescence intensity recorded during 340 nm illumination was then divided by that recorded during 380 nm illumination after subtraction of appropriate background fluorescence signals. The fluorescence ratio was converted to [Ca\(^{2+}\)], with an in vitro calibration curve (Figure 2D).24

**Single-Wavelength Measurements of [Ca\(^{2+}\)]**

The application of the ratiometric method described above relied upon the fact that the stimulated [Ca\(^{2+}\)], transient in the cardiac myocyte is a stereotyped event (see Figure 2A). Some changes in [Ca\(^{2+}\)], however, may be unique events. When such changes in [Ca\(^{2+}\)], occur amidst stereotyped changes, it is possible to use the ratiometric fluorescence data to calibrate a single-wavelength measurement of [Ca\(^{2+}\)] provided that the dye concentration does not change between measurements. This procedure
Figure 1. Current and cell length changes during calcium overload. Tracings in panels A and B are (top to bottom) membrane potential, current, and cell shortening. Panel A: Control. The cell was superfused with a 4 mM potassium chloride solution. Voltage-clamp depolarizations were delivered every 4 seconds. Panel B: Calcium overload. Extracellular potassium chloride concentration was decreased to 0.4 mM to inhibit the sodium pump and increase intracellular calcium. The dashed line indicates resting cell length under control conditions. Panel C: Synchronization of cell shortening. The top tracing shows membrane potential. Cell shortening for the left side (L1) and the right side (L2) of the cell are displayed separately and are measured independently by assuming that the patch electrode was stationary and was a point of no cell motion. The bottom tracing (L1 - aL2) is the difference record of cell shortening between L1 and L2, where a is a scaling factor to make maximal L1 and L2 equal. The dashed line indicates where L1 - aL2 = 0. A flat tracing for L1 - aL2 indicates that contraction occurs simultaneously on both sides of the cell; a biphasic waveform indicates a difference in the timing of cell shortening.

Results

Figure 1A shows the effects of a depolarizing voltage-clamp pulse on membrane current and cell shortening in a rat ventricular myocyte under control conditions. Depolarizations to +5 mV produced contractions that were characterized by synchronous sarcomere shortening throughout the cell. After relaxation at the holding potential, cell length did not change nor were fluctuations in holding current observed. The effects of decreasing extracellular K+ concentration from 4.0 mM to 0.4 mM (to inhibit the sodium pump) are shown in Figure 1B. As tonic shortening of the cell developed, voltage-clamp depolarizations produced a twitch of smaller amplitude than was observed during the control period. After repolarization, spontaneous aftercontractions, which could be as large as the twitch (see also Berlin et al20), and transient inward current fluctuations were observed at the holding potential (~44 mV). Additional aftercontractions and current fluctuations could also be observed, but they became progressively smaller in amplitude and longer in duration. These events are similar to the...
A. 

Panel A: Tracings show (from top to bottom) membrane potential, current, and fura-2 fluorescence at 505 nm measured from a 10 μm diameter spot on the cell. The cell was illuminated with 380 nm and 340 nm wavelength light as indicated. The fluorescence at 505 nm is expressed as a fraction of the fura-2 fluorescence measured before a voltage clamp depolarization. The fluorescence calibration bars show a 50% decrease and increase in fura-2 fluorescence during 380 nm and 340 nm illumination, respectively. Panel B: [Ca$^{2+}$], transient calculated by the ratiometric method. The calcium transient was determined by dividing the fluorescence transient produced during illumination with 340 nm light with a corresponding transient during 380 nm illumination (both marked with an asterisk in panel A) and comparing the ratio of fluorescence intensities to the in vitro calibration curve shown in panel D of this figure (see “Materials and Methods”). Panel C: [Ca$^{2+}$], transient calculated by single-wavelength calibration. The same fluorescence transient recorded during illumination with 380 nm light (marked with asterisk) was converted directly to [Ca$^{2+}$], as outlined in “Materials and Methods”. Panel D: Calibration curve. The in vitro calibration was performed as outlined by Cannell et al. The calibration solution contained (mM) KCl 140, MgCl$_2$ 1, K$_2$EGTA 5, fura-2, potassium salt 20 (μM), PIPES 20, 7.2 pH (35° C) with CaCl$_2$ to set the calcium concentration to desired levels. Panel E: Relation between fura-2 fluorescence during 380 nm illumination and Ca$^{2+}$ concentration. The line drawn through the data points was calculated from the equation for fura-2 fluorescence at a single illumination wavelength:

$$\text{Fluorescence} = k(S_{\text{bound}}[\text{fura}] + S_{\text{free}}[\text{fura} - \text{Ca}])$$

where $S_{\text{bound}}S_{\text{free}}$ is 1.75, total dye concentration is 30 μM, and $k$ is a constant. $S_{\text{bound}}$ and $S_{\text{free}}$ are instrument-specific proportionality coefficients for the bound and free forms of fura.$^{27}$

effects of decreasing extracellular K$^+$ in other cardiac preparations.$^{2-4,30}$

Fluctuations of [Ca$^{2+}$] during calcium overload occur at the holding potential$^{11-13}$ and are thought to be synchronized by membrane repolarization to give rise to an aftercontraction and $I_n$. A simple test for synchronization of mechanical activity is to measure cell shortening independently in each half of the myocyte during a contraction. This measurement was possible because the center of the cell was fixed by the immobile pipette used to voltage-clamp the cell. In Figure 1C, the motion of the left side of the cell is displayed as tracing $L_1$, and the motion on the right side of the cell is displayed as tracing $L_2$. The difference in the timing of shortening in each half of the cell is displayed as
(L_2 - \alpha L_2), where \alpha is a scale factor arising from the difference in L_1 and L_2 at rest. The length-difference record \(L_2 - \alpha L_2\) was flat for the contraction elicited by the voltage-clamp depolarization, as would be expected if the sarcomere shortening during the twitch was synchronous throughout the cell. The difference trace also shows that the first aftercontraction occurred at virtually the same time on both sides of the cell. With each succeeding aftercontraction, however, shortening at opposite ends of the cell became more desynchronized as demonstrated by the increasingly biphasic length-difference record.

Inspection of the contraction patterns in the videotape record of the experiment shown in Figure 1 revealed that the apparently desynchronized aftercontractions resulted from the development of a focus of sarcomere shortening on the left side of the cell, which then propagated throughout the cell. Similar localized sarcomere shortening, accompanied by an inward fluctuation of the holding current, was observed even in the absence of voltage-clamp depolarizations (data not shown).

We examined the time course of changes in intracellular \(\text{Ca}^{2+}\) during calcium overload to investigate how changes in intracellular calcium at the level of the single cell are associated with \(I_{\text{IN}}\). The potassium salt of fura-2 was injected into single ventricular myocytes from the electrode that was used to voltage-clamp the cell as described in “Materials and Methods.” Under control conditions, fluctuations in \([\text{Ca}^{2+}]_i\) were not observed when the cell was held at -74 mV. Figure 2A shows the effect of 300-msec depolarizations from -74 to -4 mV when the cell was illuminated with 380 nm and 340 nm light. Depolarizations produced a transient decrease in fura-2 fluorescence during illumination with 380 nm light and a similar increase in fluorescence during illumination with 340 nm light (Figure 2A). These changes in fluorescence levels are consistent with an increase in \([\text{Ca}^{2+}]_i\).3,24,27 The fluorescence transients were also reproducible from beat-to-beat as would be expected if the normal \([\text{Ca}^{2+}]_i\) transient was a stereotyped event. Figure 2B shows the \([\text{Ca}^{2+}]_i\) transient calculated ratiometrically (see “Materials and Methods”) from the individual fluorescence records marked with an asterisk (*). On depolarization, \([\text{Ca}^{2+}]_i\) increased from 75 nM to 500 nM. During the 300-msec depolarization, \([\text{Ca}^{2+}]_i\) declined toward a steady level above the resting level, and only after repolarization did \([\text{Ca}^{2+}]_i\) again return to 75 nM.

Figure 3 shows fluorescence signals in the same cell during calcium overload. Prominent spontaneous changes in fluorescence intensity were observed at the holding potential that were consistent with increases in \([\text{Ca}^{2+}]_i\). Ratiometric measurements of the resting \([\text{Ca}^{2+}]_i\), at this time indicated that \([\text{Ca}^{2+}]_i\) was about 150 nM (a doubling of the resting level compared with control). This observation is consistent with our earlier results showing that spontaneous fluctuations of fluorescence in unclamped myocytes were associated with higher levels of \([\text{Ca}^{2+}]_i\) than found in quiescent cells.12 In addition, the elevated level of resting \([\text{Ca}^{2+}]_i\) is consistent with the decrease in resting cell length observed during calcium overload in Figure 1.

The single-wavelength method (see “Materials and Methods”) was used to estimate \([\text{Ca}^{2+}]_i\), during the spontaneous fluctuations in fluorescence shown in Figure 3. The changes in \([\text{Ca}^{2+}]_i\), produced by a 600-msec depolarization from -94 to -34 mV are shown during calcium overload. During the depolarization, \([\text{Ca}^{2+}]_i\), transiently decreased from its peak level before beginning a secondary slow increase. This secondary increase in \([\text{Ca}^{2+}]_i\), may be analogous to tonic shortening observed in Purkinje fibers1,30 and ventricular muscle preparations during calcium overload.4,5 On repolarization, calcium returned to resting levels briefly before \([\text{Ca}^{2+}]_i\), increased spontaneously to a peak level approximately 70% of the maximal \([\text{Ca}^{2+}]_i\), observed during the stimulated \([\text{Ca}^{2+}]_i\), transient. This is consistent with the observation that the aftercontraction can be almost as large as the stimulated contraction.

Examination of the changes in membrane current and \([\text{Ca}^{2+}]_i\), gave no indication of a delay between the increase in \([\text{Ca}^{2+}]_i\), and \(I_{\text{IN}}\) (n=9). Thus, increases in \([\text{Ca}^{2+}]_i\), seemed to be able to rapidly activate \(I_{\text{IN}}\). The peak of the aftercontraction, however, occurred 60-90 msec after the peak of \(I_{\text{IN}}\).

Figure 3C shows that spontaneous increases in \([\text{Ca}^{2+}]_i\), that occur just before a depolarization can affect the size of the stimulated \([\text{Ca}^{2+}]_i\), transient. When the depolarization was given at the peak of the spontaneous transient, only a small increase in \([\text{Ca}^{2+}]_i\), was observed after depolarization. If the depolarization was given near the end of the spontaneous transient, the size of the stimulated \([\text{Ca}^{2+}]_i\), transient was reduced. Similar results have been reported in papillary muscle preparations32 and are consistent with the finding that stimulated twitch is reduced when the stimulus occurs during an aftercontraction in calcium-overloaded cardiac muscle.29,33,34

There are a number of differences between the \(I_{\text{IN}}\) we observe in single cells and those observed in multicellular preparations. The width of \(I_{\text{IN}}\) in these cells was 100–300 msec (versus approximately 500 msec in papillary muscle). A similar duration of \(I_{\text{IN}}\) has been reported in single guinea pig cardiac myocytes.8 The time to peak \(I_{\text{IN}}\) after membrane repolarization can also be extremely variable in single cells but is quite constant in multicellular preparations.2

Figure 4 demonstrates the beat-to-beat variability of \(I_{\text{IN}}\) in a single cell. The four current traces on the right-hand side of Figure 4 were recorded after consecutive depolarizations of a cell that had been superfused in a K^+-depleted solution for 5 minutes. The delay from repolarization to the initiation of the inward current fluctuations showed significant variability between depolarizations. The most prominent difference, however, was the marked variation...
in the time course and shape of the current fluctuations (Figure 4). Unlike the inward current fluctuations shown in Figure 1B, the shape of the current could have a complex waveform. The current tracing on the left is the average of currents from 32 consecutive depolarizations. The duration of the average $I_{\text{n}}$ was approximately 400 msec, very similar to the duration of $I_{\text{n}}$ reported in several heart muscle preparations. This result suggests that $I_{\text{n}}$ measured in the multicellular preparation could reflect the summation of current fluctuations originating from a large number of cells that individually do not have the same time course of $I_{\text{n}}$ activation.
To investigate the variability in timing and shape of 

of elevated \( [\text{Ca}^{2+}] \), fluctuations more completely, fura-2 fluorescence was recorded with a video camera whose incident light was filtered at 505 nm. After a 100-msec depolarization, local regions of elevated calcium could be observed within the cell. Figure 5A (left panel) shows a fura-2 fluorescence image of a myocyte recorded at the holding potential, 1.5 seconds after a depolarizing clamp pulse. The dark bands are regions of elevated \( [\text{Ca}^{2+}] \), within the cell. Figure 5A (right panel) also shows a drawing that represents the cell and the location of the voltage-clamp electrode. The regions of elevated \( [\text{Ca}^{2+}] \), are shown as cross-hatched bands. These regions of elevated \( [\text{Ca}^{2+}] \), propagated along the axis of the cell in the direction indicated by the arrows at a rate of approximately 100 \( \mu \text{m/sec} \) as a wave of elevated \( [\text{Ca}^{2+}] \). At this time, fluctuations in membrane current and propagating regions of localized sarcomere shortening in the cell were observed.

The local regions of elevated \( [\text{Ca}^{2+}] \), in Figure 5A arose from a single site of origin (or focus) at the lower right end of the myocyte before propagating throughout the remainder of the cell. The drawing labeled number 8 in Figure 5B shows the location of this site of origin. Figure 5B shows the sites of origin of elevated \( [\text{Ca}^{2+}] \), that arose after each of 12 consecutive depolarizations. The number and location of the sites of origin within the cell were quite variable and could change after a depolarization. Nevertheless, these sites tended to dominate the initiation of spontaneous increases in \( [\text{Ca}^{2+}] \), until the next depolarization (which in effect reset the sites of origin). In addition, when more than one site of origin was present, separate waves of elevated \( [\text{Ca}^{2+}] \), would propagate from each origin. An example of multiple waves of elevated \( [\text{Ca}^{2+}] \), is shown in Figure 5C. Three waves of increased \( [\text{Ca}^{2+}] \), originated from separate locations (drawing number 6 in Figure 5B). Waves "a" and "b", which were moving in generally the same direction, merged to form a single propagating wave; however, waves "c" and "d", which were moving in opposite directions, collided, and further propagation stopped. When multiple waves of elevated \( [\text{Ca}^{2+}] \), propagated to a single region within the cell, waves would either merge to form a single wave front or collide and prevent further wave propagation. Separate waves of elevated \( [\text{Ca}^{2+}] \), did not propagate through each other.

Changes in the number and location of foci for spontaneous \( [\text{Ca}^{2+}] \) release would also be expected to affect the pattern of sarcomere shortening within a cell. Figure 6A shows cell shortening observed when a spontaneous wave of increased \( [\text{Ca}^{2+}] \), originated from a single site of origin after a voltage-clamp pulse. Cell length was measured independently on different sides of the cell as in Figure 1C. Before the depolarization, spontaneous shortening was observed on one side of the cell (\( L_{L2} \)). However, a 100-msec depolarization from \(-70\) to \(0\) mV caused cell shortening to occur simultaneously as evidenced by the difference tracing (\( L_{L1} - \alpha L_{L2} \)) equaling zero by the end of the depolarization. After repolarization, spontaneous shortening in one half of the cell (\( L_{L1} \)) preceded shortening in the remainder of the cell (note the biphasic nature of \( L_{L1} - \alpha L_{L2} \)). This pattern of cell shortening was consistent with a single wave of increased \( [\text{Ca}^{2+}] \), propagating through the cell. Figure 6B shows cell shortening observed in the presence of multiple sites of origin of increased \( [\text{Ca}^{2+}] \). The voltage-clamp depolarization produced simultaneous shortening on both sides of the cell. The first aftercontraction also occurred simultaneously on both sides of the cell as demonstrated by the relatively flat difference record. Note, however, that succeeding aftercontractions showed a marked difference in the timing of spontaneous shortening in each half of the cell. This desynchronization of spontaneous cell shortening is similar to that shown in Figure 1C. Thus, changes in the number of sites of origin for elevated \( [\text{Ca}^{2+}] \), do affect the pattern of spontaneous cell shortening within a cell. In addi-
Local regions of elevated \([Ca^{2+}]_i\), in a calcium overloaded myocyte. Panel A: Fura-2 fluorescence image. The left section of the panel is a fura-2 fluorescence image of a cell recorded during illumination with 380 nm light. The dark bands running through the cell are regions of elevated \([Ca^{2+}]_i\). A contrast enhancement algorithm was applied to the digitized video frame to clarify the decrease in fluorescence intensity, which indicates an increase in \([Ca^{2+}]_i\). The right section of panel A is a pictorial representation of the cell showing the placement of the clamping electrode. The areas of increased calcium are depicted with cross hatching, and the arrow shows the direction of propagation of the wave of increased \([Ca^{2+}]_i\). This image was recorded when the cell had been at the holding potential (−70 mV) for 1.5 seconds after a 200 msec depolarization to 0 mV. Panel B: Changes in the sites of origin of elevated \([Ca^{2+}]_i\). The 12 pictorial representations of the cell (as in panel A) show the location of the sites of origin for spontaneous increases in \([Ca^{2+}]_i\) that followed consecutive depolarizations. Each site of origin is depicted by a solid dot. Note that both the number and location of the foci change after each depolarization. Panel C: Image of cell during propagation of multiple waves of increased \([Ca^{2+}]_i\). The left section of the panel is a fura-2 fluorescence image of the cell 800 msec after the end of the voltage-clamp depolarization. The right section of the panel is a pictorial representation of the cell. Regions of increased \([Ca^{2+}]_i\) are depicted by cross-hatched bands. Three waves of elevated \([Ca^{2+}]_i\) originate from three sites of origin. Note that wave "a" will merge with wave "b" to continue propagation until they collide with wave "c". Arrows point in direction of wave propagation.

Thus far, we have demonstrated that spontaneous aftercontractions can be associated with localized increases in \([Ca^{2+}]_i\). During prolonged depolarizations, spontaneous fluctuations in cell length can become extremely rapid. Figure 7 shows power spectra of spontaneous fluctuations of cell length. The six tracings show that the frequency of spon-
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Panel A: Cell shortening that occurs with a depolarization and the subsequent spontaneous increase in \([\text{Ca}^{2+}]_i\) arising from a single focus. Panel B: Cell shortening that occurs when two foci of increased \([\text{Ca}^{2+}]_i\) occur simultaneously on different sides of the cell.

Figure 6. Cell contraction during calcium overload with single and multiple foci for spontaneous calcium release. (Measurements taken from the same cell shown in Figure 5.) Cell shortening was measured independently on the left (L_1) and right (L_2) ends of the cell as in Figure 1C. The horizontal dashed line in the bottom tracings shows where \(L_1 - aL_2 = 0\).

Panel A: Cell shortening that occurs with a depolarization and the subsequent spontaneous increase in \([\text{Ca}^{2+}]_i\) that arises from a single focus. Panel B: Cell shortening that occurs when two foci of increased \([\text{Ca}^{2+}]_i\) occur simultaneously on different sides of the cell.

FIGURE 7. Effect of membrane potential on spontaneous fluctuations of cell length. Power spectra of fluctuations in cell length were calculated as described in "Materials and Methods." The cell was depolarized to various potentials for 15-second periods, and data were collected for the last 10 seconds. Membrane potential is indicated at the right of each spectrum. Note that spectral power shifts to higher frequencies at more depolarized potentials.

Discussion

Increases in \([\text{Ca}^{2+}]_i\) Underlie I_{\text{f}}

In this study, spontaneous changes in \([\text{Ca}^{2+}]_i\), cell contraction, and I_{\text{f}} have been examined under conditions of calcium overload in single rat cardiac cells. Studies in cardiac Purkinje fibers and ventricular muscle have shown that, under conditions that lead to an increase in \([\text{Ca}^{2+}]_i\), spontaneous fluctuations in tension and \([\text{Ca}^{2+}]_i\) are observed. Fluctuations in membrane potential, and membrane current in voltage-clamped preparations, can also be observed under similar conditions. Similarly, when resting calcium became elevated in the present experiments, spontaneous cell shortening was evident, which propagated as a wave of sarcomere shortening through the cell. Similar waves of contractile activity have been previously reported in a variety of cardiac ventricular cells.18,19 Digital imaging of fura-2 fluorescence from these cells showed that underlying the wave of sarcomere shortening is a localized increase in \([\text{Ca}^{2+}]_i\) that propagates within the cell. During the spontaneous increase in \([\text{Ca}^{2+}]_i\), fluctuations in holding current also appeared. In all cases, the localized increase in \([\text{Ca}^{2+}]_i\) preceded or occurred at the same time as the other electrical and mechanical events. This finding reinforces the conclusion that the transient rise in \([\text{Ca}^{2+}]_i\) is the underlying cause of the spontaneous fluctuations in current observed during calcium overload.

Under these conditions, a depolarizing voltage-clamp pulse was followed by a spontaneous aftercontraction and an inward deflection in holding current, similar to the I_{\text{f}} reported in multicellular preparations. In our experiments, the peak of the aftercontraction followed the peak current by 60–90
A similar delay has been reported in guinea pig myocytes\(^2\) and in multicellular preparations.\(^2\) When fura-2 fluorescence was measured instead of cell shortening, the peak of the spontaneous rise of \([\text{Ca}^{2+}]\); after membrane repolarization did not lag behind the peak of the inward current fluctuation. The present results demonstrate that the spatial and temporal properties of the spontaneous rise in \([\text{Ca}^{2+}]\), can be quite complex, even at the level of the single cell. The nonuniform nature of these spontaneous oscillations of \([\text{Ca}^{2+}]\), confound detailed analysis of the relation between the rise in \([\text{Ca}^{2+}]\); and \(I_n\) activation.

(Figure 3B shows a stimulated \([\text{Ca}^{2+}]\), transient followed by a spontaneous increase in \([\text{Ca}^{2+}]\). In this experiment, \([\text{Ca}^{2+}]\), was measured from only a small portion \(10 \mu\text{m}^2\) diameter of the cell with a photomultiplier tube. Because \([\text{Ca}^{2+}]\), changes uniformly, this measurement will be an accurate measure of spatial mean \([\text{Ca}^{2+}]\),. On the other hand, the spontaneous increase in \([\text{Ca}^{2+}]\), that follows repolarization may be marked by large spatial inhomogeneities in \([\text{Ca}^{2+}]\). If this is the case, the spatial mean \([\text{Ca}^{2+}]\), of the cell during spontaneous increases in \([\text{Ca}^{2+}]\), would not have been accurately measured.)

We have not investigated the ionic basis for \(I_n\) in these experiments; however, our results suggest that considerable care should be taken in interpreting the results of any such investigation. At positive potentials, \(I_n\) and the related oscillations in cell length (and \([\text{Ca}^{2+}]\),) can increase in frequency to nearly 10 Hz in rat ventricular myocytes. At these high frequencies, spatial nonuniformity of \([\text{Ca}^{2+}]\), combined with the lag between peak \([\text{Ca}^{2+}]\), and peak contraction makes it difficult to use tension or length oscillations as an accurate indicator of mean \([\text{Ca}^{2+}]\). In addition, there is no a priori reason to assume that the calcium dependence of \(I_n\) activation is linear so that the relation between spatial averaged \([\text{Ca}^{2+}]\), and membrane current may not be unique.

**Variability of \(I_n\) in Single Cells**

In many cells, the timing and magnitude of \(I_n\) showed marked variability, which was also apparent in the aftercontraction and the underlying increase in \([\text{Ca}^{2+}]\). Significant variability in the size of spontaneous calcium transients during calcium overload has also been reported in ventricular muscle\(^{26}\) and Purkinje fibers injected with the bioluminescent protein, aequorin.\(^{28}\) Variability could arise in multicellular preparations because spontaneous \([\text{Ca}^{2+}]\), transients might occur in different cells at slightly different times. The data in this paper do not address this possibility, but it is clear that spontaneous \([\text{Ca}^{2+}]\), transients do show variability within a single cell. This variability may occur because spontaneous increases in \([\text{Ca}^{2+}]\), can be initiated from focal regions (sites of origin) within the cell whose number and location can change with time. An explanation for this observation is that total intracellular calcium load changes either throughout the entire cell or within local regions of the cell on a beat-to-beat basis. If membrane depolarization changes the relative distribution of sarcoplasmic reticulum calcium, then it is quite possible that the foci for spontaneous calcium release could also change.

**Effect of Spontaneous [Ca\(^{2+}\)], Release on Stimulated [Ca\(^{2+}\)], Transients**

In calcium-overloaded cardiac muscle preparations, the size of the stimulated twitch is reduced when the stimulus occurs during an aftercontraction.\(^{29,33,34}\) Allen et al\(^32\) further showed that the size of the aequorin light transient in papillary muscle was decreased when a stimulus was given during a spontaneous increase of diastolic \([\text{Ca}^{2+}]\). These experiments, however, could not determine how the \([\text{Ca}^{2+}]\), transient was affected at the cellular level. The present results demonstrate that the size of the \([\text{Ca}^{2+}]\), transient in a single cell is decreased when the stimulus occurs during a spontaneous elevation in \([\text{Ca}^{2+}]\). This decrease in the size of the \([\text{Ca}^{2+}]\), transient could occur by means of two mechanisms. The spontaneous elevation of \([\text{Ca}^{2+}]\) could inactivate a calcium-induced sarcoplasmic reticulum calcium release mechanism.\(^36\) An alternative explanation is that the decrease in the stimulated \([\text{Ca}^{2+}]\), transient is due to a transient depletion of sarcoplasmic reticulum \([\text{Ca}^{2+}]\) after spontaneous \([\text{Ca}^{2+}]\), release. This second proposal is consistent with our data that shows the depression of the stimulated transient by a spontaneous \([\text{Ca}^{2+}]\), oscillation is minimized as the separation between the spontaneous and stimulated \([\text{Ca}^{2+}]\), transients is increased.

An additional factor in the decrease in twitch force during calcium overload could be the presence of a large degree of inhomogeneity in the size of the \([\text{Ca}^{2+}]\), transient between cells in a multicellular preparation. Such inhomogeneity would arise if spontaneous \([\text{Ca}^{2+}]\), oscillations decreased the stimulated \([\text{Ca}^{2+}]\), transient in some cells. Cell-to-cell variation in the size of the stimulated \([\text{Ca}^{2+}]\), transient would allow cells with a greater degree of activation to shorten against the remaining cells. The presence of such local cell shortening within a muscle fiber would lead to less force generation than if uniform activation of contraction occurred.\(^32,39,40\) Thus, the presence of spontaneous oscillations of \([\text{Ca}^{2+}]\), would decrease force development by decreasing the average size of the \([\text{Ca}^{2+}]\), transient as well as increasing the variability of the \([\text{Ca}^{2+}]\), transient between cells.

**Waves of Increased [Ca\(^{2+}\)]\), and \(I_n\)**

The spontaneous increase in \([\text{Ca}^{2+}]\), responsible for \(I_n\) and the increase in \([\text{Ca}^{2+}]\), underlying waves of sarcomere shortening in unstimulated cells appear to reflect the same underlying phenomenon. In the present study and previously,\(^12\) we have shown that spontaneous waves of increased \([\text{Ca}^{2+}]\), and cell shortening are observed in unstimulated cells when resting \([\text{Ca}^{2+}]\), is elevated. Maneuvers that increase the level of intracellular \([\text{Ca}^{2+}]\) loading increase the
frequency of spontaneous waves. After a voltage-clamp depolarization, propagated waves of increased \([\text{Ca}^{2+}]_i\) appeared to underlie the aftercontraction and \(I_{\text{f}}\). Thus, spontaneous increases in \([\text{Ca}^{2+}]_i\), both in quiescent cells and after stimulation, can be associated with a wave-like phenomenon. These waves of increased \([\text{Ca}^{2+}]_i\) have been attributed to spontaneous release of \(\text{Ca}^{2+}\) from the sarcoplasmic reticulum.41–44

There are some differences between the spontaneous \([\text{Ca}^{2+}]_i\), transient responsible for \(I_{\text{f}}\) and the wave of sarcomere shortening observed in unstimulated cells. The first \(I_{\text{f}}\) after a voltage-clamp pulse was typically of greater amplitude and shorter duration than successive current fluctuations. Similarly, the first aftercontraction was larger and of shorter duration than succeeding spontaneous fluctuations in cell shortening. This first aftercontraction often resulted from sarcomere shortening occurring synchronously at both ends of the cell. Fura-2 fluorescence imaging also showed that when spontaneous oscillations in \([\text{Ca}^{2+}]_i\) arose from multiple foci, cell shortening could occur synchronously at both ends of the cell. Thus, the increase in \([\text{Ca}^{2+}]_i\), that underlies \(I_{\text{f}}\) can occur simultaneously in several areas of the cell and lead to an apparent synchronization of electrical and mechanical activity. In the extreme, this result suggests that \(I_{\text{f}}\) can even occur with complete synchronization of \(\text{Ca}^{2+}\) release (i.e., no wave-like propagation of increased \([\text{Ca}^{2+}]_i\)). This point, however, needs additional study.

After the first aftercontraction that followed membrane repolarization, additional spontaneous fluctuations in cell length were often present. These additional fluctuations in cell length were associated with a progressive desynchronization of cell shortening so that the pattern of sarcomere shortening became similar to the propagated wave of sarcomere shortening observed in the absence of membrane depolarization. These results are consistent with the proposal that, after membrane potential changes, spontaneous \(\text{Ca}^{2+}\) release from the sarcoplasmic reticulum can be transiently synchronized in multiple regions of the cell.3,6,9,10,16

Conclusion

Under conditions of calcium overload, changes in membrane potential produce \(I_{\text{f}}\) and aftercontractions, which are similar to those reported in multicellular cardiac muscle preparations. The aftercontraction can result from synchronous shortening at both ends of a cell. The underlying increase in \([\text{Ca}^{2+}]_i\), however, is observed as propagated waves of increased \([\text{Ca}^{2+}]_i\) that arise from focal regions within the cell. The apparent synchronization of cell shortening can come about because more than one focus of \(\text{Ca}^{2+}\) release can occur simultaneously in different areas of the cell. The simultaneous increase in \([\text{Ca}^{2+}]_i\) in many regions of the cell could be the cellular manifestation of the synchronization of spontaneous sarcoplasmic reticulum calcium release, which has been proposed to underlie \(I_{\text{f}}\). In addition, variability in the activation of \(I_{\text{f}}\) and cell shortening are, at least in part, due to an underlying variability in spontaneous increases of \([\text{Ca}^{2+}]_i\).

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