Behavior of Ca\textsuperscript{2+} waves in multicellular preparations from guinea pig ventricle

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Submitted 26 April 2004; accepted in final form 7 August 2004

Kurebayashi, Nagomi, Haruyo Yamashita, Yuji Nakazato, Hiroyuki Daida, and Yasuo Ogawa. Behavior of Ca\textsuperscript{2+} waves in multicellular preparations from guinea pig ventricle. Am J Physiol Cell Physiol 287: C1646–C1656, 2004. First published August 11, 2004; doi:10.1152/ajpcell.00200.2004.—Ca\textsuperscript{2+} waves have been implicated in Ca\textsuperscript{2+} overload-induced cardiac arrhythmias. To deepen understanding of the behavior of Ca\textsuperscript{2+} waves in a multicellular system, consecutive two-dimensional Ca\textsuperscript{2+} transient elevation of cytosolic Ca\textsuperscript{2+} cell. These results are consistent with the conclusion that the loading inhibitor reversibly prolonged the wave intervals. In Na\textsuperscript{+} later, their amplitudes and velocities remaining unchanged. A SERCA no Ca\textsuperscript{2+} 287: C1646–C1656, 2004. First published August 11, 2004. Am J Physiol Cell Physiol

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IN EXCITATION-CONTRACTION coupling in mammalian cardiac muscle, Ca\textsuperscript{2+} influx through activated dihydropyridine receptors triggers the opening of ryanodine receptors in the sarcoplasmic reticulum (SR) of muscle cells, resulting in a transient elevation of cytosolic Ca\textsuperscript{2+} sufficient for muscular contraction. In recent years, the development of confocal fluorescence microscopy with high spatial resolution has led to the identification of “Ca\textsuperscript{2+} sparks,” spontaneously or electrically triggered and localized Ca\textsuperscript{2+} release events from SR (4, 20). Ca\textsuperscript{2+} sparks are believed to be “elementary events” of cardiac excitation-contraction coupling in the sense that whole cell calcium release can be plausibly reconstructed as a summation of these events. In the Ca\textsuperscript{2+}-overloaded condition, however, Ca\textsuperscript{2+} is spontaneously released from SR and propagates throughout the cell in a wave, the “Ca\textsuperscript{2+} wave.” The Ca\textsuperscript{2+} wave may lead to aftercontraction and delayed afterdepolarization (DAD; see Refs. 5 and 24), which is probably caused by enhanced extrusion of Ca\textsuperscript{2+} via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange reaction. The elevated Ca\textsuperscript{2+} might also activate Cl\textsuperscript{−} efflux through Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels (31, 35). The DAD can trigger an extrasystolic action potential when it reaches the threshold. Thus Ca\textsuperscript{2+} waves could be a candidate for the mechanism underlying cardiac arrhythmia (28).

Ca\textsuperscript{2+} waves have been studied extensively, primarily with enzymatically isolated single cardiac cells (6, 19, 28), and have been shown able to induce action potential (5, 24). Those preparations, however, lack cell-to-cell interactions through gap junctions, which have critical roles in the conduction of cellular excitation and generation of arrhythmia. To date, only a limited number of groups have investigated Ca\textsuperscript{2+} waves in multicellular cardiac muscle preparations (1, 13, 18, 23, 26, 33). Wier and colleagues (33) injected fluo 3 in rat trabeculae and showed that Ca\textsuperscript{2+} waves did not propagate beyond cells (33). Kaneko et al. (13) monitored Ca\textsuperscript{2+} waves on the outer surface of cardiac cells in Langendorff-perfused rat heart. They classified waves into three different categories on the basis of resting fluorescence intensity, which probably reflected viability of the cells, and concluded that Ca\textsuperscript{2+}-overloaded waves showed a high probability of propagation whereas the other types of Ca\textsuperscript{2+} waves were less likely to propagate. These reports, although informative, were carried out mostly with rat heart muscles. It is well known that there are marked differences among animal species in Ca\textsuperscript{2+} homeostasis in heart muscle. Contribution of Ca\textsuperscript{2+} influx and SR Ca\textsuperscript{2+} release to the Ca\textsuperscript{2+} transients differs in magnitude among animals; SR Ca\textsuperscript{2+} release is the greatest in rat and mouse ventricular muscles, among those including guinea pig and human hearts (3). Other properties specific to rat heart are also reported (3). This makes results with hearts from animals other than rat of great interest.

It is interesting to know whether Ca\textsuperscript{2+} waves propagate across the cell boundary, because if so the possibility of spontaneous activity/DAD would be greatly increased by the intercellular propagation. In this study, we took real-time images of Ca\textsuperscript{2+} waves in intact ventricular papillary muscles from guinea pigs to examine whether cell-to-cell propagation of Ca\textsuperscript{2+} waves could be observed and how they behaved in multicellular tissue. We monitored Ca\textsuperscript{2+} waves induced by high-frequency electrical stimulation, which caused temporal Ca\textsuperscript{2+} overload and often an aftercontraction. In addition, Ca\textsuperscript{2+} waves in damaged muscles, where they occurred spontaneous-
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MATERIALS AND METHODS

Preparation. All experiments were carried out in accordance with Juntendo University Ethics Committee guidelines. Guinea pigs were anesthetized with an intraperitoneal injection of pentobarbital sodium. The heart was removed quickly from the chest and then perfused via the aorta with high-K\(^{+}\) (25 mM KCl added) Krebs solution. Papillary muscle bundles (0.5–1.5 mm in diameter, ~3 mm in length) were excised from left and right ventricles after the muscle tendon and basal portion of the bundle had been secured with silk thread in the high-K\(^{+}\) solution.

Confocal imaging of CA\(^{2+}\) waves. The muscle bundles were incubated with a CA\(^{2+}\) indicator (fluor 3-AM or rhod 2-AM) at 10 \mu\text{M} for 80 min at room temperature in normal Krebs solution. After the dye was washed out, the muscle bundle was connected to hooks in a chamber with thread (14). The chamber, the bottom of which was made of a coverslip, was placed on the stage of an inverted microscope. The bundle was stretched to ~110% of its slack length and gently pushed toward the bottom with a Plexiglas plate so that the lower surface of the bundle was several micrometers above the bottom. Through this gap, cells at the lower surface were allowed access to the bathing solution. The chamber was perfused with Krebs solution at a rate of 2 ml/min. Experiments were carried out at 25–27\(^{\circ}\)C.

The bundles were viewed with a confocal laser scanning microscope system (Oz system; Noran Instruments) equipped with an Argon Krypton Ion Laser System (488 and 568 nm excitation). Fluor 3 was excited at 488 nm, and fluorescence was measured at wavelengths of >500 nm, whereas rhod 2 was excited at 568 nm and fluorescence of >590 nm was detected. To observe multiple cells on the bundle, a x10 or x20 objective lens was used. The z-axis resolution as estimated by imaging fluorescent beads (Noran Instruments) was 5 and 15 \mu\text{m} for the x20 and x10 objective lenses, respectively. CA\(^{2+}\) images sometimes included waves from cells at the second layer when the focused plane was close to the interface of the stacked cells. However, even in such cases, we were able to identify overlapping CA\(^{2+}\) waves from the second layer cells because their fluorescence intensity was much lower than those of the surface layer cells.

Intact muscle preparations were usually conditioned by stimulating at 0.5 Hz for >5 min in normal Krebs solution by a pair of platinum-plate electrodes (25 mm in length) using rectangular current pulses (1 ms, 1.5 threshold voltage). For induction of waves in an intact muscle preparation, the muscle was stimulated at a higher frequency, 2–3 Hz, for 3–5 min, and then the stimulation was stopped. Because CA\(^{2+}\) waves occurred after the train of stimulation, acquisitions of fluorescent images were started 3–4 s before the cessation of a series of stimulations. In typical experiments, each image was taken with 256 \times 240 pixels every 8.3 ms, and eight images were averaged to get a single image; this averaged image was obtained every 67 ms. These 500 averaged images were taken continuously for one measurement of 33 s. During the 33-s measurements, fluorescence intensity of fluor 3 decreased by ~10% because of quenching, as determined from peak fluorescence intensity at 0.5 Hz stimulation. In addition, fluorescence intensity gradually decreased with time without any illumination of laser light, probably because dye was diffusing through the gap junction from surface cells to those at deeper regions. The change in intensity was 10–20%/h.

Damaged muscles, which showed spontaneous and recurrent CA\(^{2+}\) waves for a long period (>15 min) at rest, were obtained by either of the following treatments. 1) A muscle was cut at one end (mechanical injury), and cells near the cut end then showed spontaneous CA\(^{2+}\) waves. 2) A muscle was stimulated at 2 Hz for ~30 min without perfusing Krebs solution (overload and anoxia). 3) A muscle was first incubated in CA\(^{2+}\)-free Krebs solution for ~30 min and then challenged with normal Krebs solution (CA\(^{2+}\) paradox). Data were obtained 15–45 min after these treatments. In a specimen subjected to one of these treatments, damaged cells showed varied viability with different magnitudes of impairment.

In this study, we did not use any reagents to immobilize muscle bundles. The muscle bundle moved quite a bit in response to electrical stimulation and often moved out of the field of view and/or out of focus. Therefore, the CA\(^{2+}\) transient signals on electrical stimulation suffered from considerable movement artifacts. However, CA\(^{2+}\) waves that occurred after the series of stimulations were analyzable because the movements caused by the CA\(^{2+}\) waves, if any, did not affect the results.

Solutions. Normal Krebs solution contained (in mM): 120 NaCl, 5 KCl, 25 NaHCO\(_3\), 1 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\), 1 MgCl\(_2\), and 10 glucose. For low-Na\(\sim\) (20 mM Na\(^{+}\)) solutions, NaCl was replaced with the equimolar CaCl\(_2\); these solutions were saturated with 95% O\(_2\)-5% CO\(_2\). In some experiments, a HEPES-buffered solution was used instead (in mM): 146 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, and 5 HEPES, pH adjusted to 7.4 with NaOH. For a Na\(^{+}\)-free/CA\(^{2+}\)-free (0Na\(^{+}\)/0Ca\(^{2+}\)) solution, CaCl\(_2\) was omitted. NaCl was replaced with equimolar LiCl, and pH was adjusted to 7.4 with LiOH. Each HEPES-buffered solution was saturated with 100% O\(_2\). When the effects of acidic pH were determined (the last part of RESULTS), a bicarbonate-buffered Na\(^{+}\)-free/CA\(^{2+}\) free (bicarbonate-buffered 0Na\(^{+}\)/0Ca\(^{2+}\)) solution was used in which all Na\(^{+}\) in normal Krebs solution was replaced with equimolar Li\(^+\) and CaCl\(_2\) was omitted. This solution was saturated either with 95% O\(_2\)-5% CO\(_2\) (pH 7.4) or 70% O\(_2\)-30% CO\(_2\) (pH 6.7). Cyclopiazonic acid (CPA) was dissolved in DMSO and added to modified Krebs solutions at a final DMSO concentration of 0.1%.

Fluo 3-AM and rhod 2-AM were obtained from Dojindo (Kumamoto, Japan) and Molecular Probes (Eugene, OR), respectively. The CA\(^{2+}\) signals from fluo 3 and rhod 2 showed the same time course of CA\(^{2+}\) transient in double-stained muscle cells (data not shown), although F\(_{max}\)/F\(_{0}\) values were somewhat different. Therefore, all the data of CA\(^{2+}\) waves were analyzed similarly.

Data analysis. A series of the image data obtained with the Noran Oz system was converted to stacks of 500 TIF images for analysis by National Institutes of Health Image software. Time-based scan images were obtained along lines drawn on the stacks of images with “Reslice” command and were used to determine wave amplitudes, velocities, and intervals. All averaged values were expressed as means \pm SD. Statistical analysis was performed using ANOVA for multiple comparisons and Student’s t-test. P values <0.05 were considered to be significant.

RESULTS

In guinea pig papillary muscle, intact muscle cells showed low background fluorescence and no obvious change at rest, but responded to stimulation with a marked increase in fluorescence intensity as CA\(^{2+}\) transient. Most results in this study were initially obtained with these intact preparations to characterize the CA\(^{2+}\) waves. Later experiments with damaged cells were performed to understand the pathological implications.

Figure 1 shows typical experiments. The specimen was first stimulated for 5 min at 1 Hz and then kept unstimulated for >30 s (A–C). A series of two-dimensional CA\(^{2+}\) images of fluorescence changes at the last 2–3 s of stimulation and ~30 s of unstimulated periods was acquired for each determination, as described in MATERIALS AND METHODS. During 1 Hz stimulation, the muscle cells showed an increase in CA\(^{2+}\) signal in response to each electrical stimulation, and, after the cessation...
of stimulation, the fluorescence intensities gradually and monotonically decreased without Ca$^{2+}$/H$\text{11001}$ waves (Fig. 1B). The fluorescence signal taken from a wider field of view (Fig. 1A) that contained $\sim 20$ cells also showed a monotonic decrease (Fig. 1C). Similar determinations with the initial 2 Hz stimulation followed in succession (Fig. 1, D–F). Stimulation at 2 Hz also caused fluorescent Ca$^{2+}$/H$\text{11001}$ transient in individual cells (Fig. 1E). A remarkable difference, however, was observed after the cessation of electrical stimulation. After 2 Hz stimulation, Ca$^{2+}$/H$\text{11001}$ waves or Ca$^{2+}$/H$\text{11001}$ oscillations were intermittently observed (Fig. 1, D and E; please refer to the Supplemental Material for this article to view renderings).\(^1\) A time course of the Ca$^{2+}$/H$\text{11001}$ signal taken from the entire field of view that corresponds to an ensemble signal from $\sim 20$ cells showed a hump overlaying a gradual decrease in the Ca$^{2+}$/H$\text{11001}$ concentration after cessation of electrical stimulation, resembling a profile of “aftercontraction” (Fig. 1F). The following descriptions were focused on the Ca$^{2+}$/H$\text{11001}$ waves during the period of nonstimulation after the train of stimulations at a specified frequency.

To analyze Ca$^{2+}$/H$\text{11001}$ waves more systematically, we obtained time-based scan images from the already-acquired stack of images (see MATERIALS AND METHODS). A time-based scan image taken along a broken line drawn on the four cells (a–d) contoured in Fig. 2A is shown in Fig. 2B. The horizontal axis of Fig. 2B corresponds to the location along the broken line as shown at the top of Fig. 2B, whereas the vertical axis corresponds to time during image sampling. Fluorescence transients of the four cells synchronously increased during field stimulation. During the following period of nonstimulation, however, the increase in fluorescence was asynchronous. On closer examination, those fluorescence increases occurred at localized points in each cell, then propagated within the cell in the Ca$^{2+}$/H$\text{11001}$ wave, and finally stopped at the cell boundary. The origins of the waves were not necessarily fixed within the individual cells although some appeared to have fixed origins. In some cells, Ca$^{2+}$/H$\text{11001}$ waves originated at multiple points as reported on rat cardiac muscle (2) (see the third wave in cell b of Fig. 2B).

\(^1\) Supplemental data for this article may be found at http://ajpcell.physiology.org/cgi/content/full/00200.2004/DC1/.
Direction of intracellular propagation of Ca\(^{2+}\) waves along the longitudinal axis was variable among cells (bidirectional) under this condition.

In Fig. 2B, all Ca\(^{2+}\) waves appeared to stop at the cell boundary. We were interested in whether this is a general feature of waves in guinea pig ventricular muscle. Similar determinations were carried out with 15 preparations. With 178 cells, 497 out of a total of 502 Ca\(^{2+}\) waves observed definitely stopped at the cell boundary. Only 1% of total waves observed appeared to propagate across the boundary, although some of them might, by chance, have appeared to do so. Therefore, it can be concluded that Ca\(^{2+}\) waves did not propagate from cell to cell in intact guinea pig papillary muscle cells.

It has been reported that amplitude, velocity, and frequency of Ca\(^{2+}\) waves depend on Ca\(^{2+}\) content in SR and/or intracellular Ca\(^{2+}\) concentration (1, 13, 21). Because total Ca\(^{2+}\) content of muscle cells was gradually decreasing during rest in guinea pig ventricle (30), it is interesting to know how these parameters of Ca\(^{2+}\) waves change during the resting period. Therefore, we determined peak amplitudes and velocities of Ca\(^{2+}\) waves after cessation of stimulation. The time courses of fluorescence intensities of fluo 3 were determined at points

Fig. 2. Ca\(^{2+}\) waves in multicellular cardiac preparation. A: image of the fluo 3-loaded papillary muscles. Regions enclosed by white lines are contours of 4 cells aligned transversely. B: time-based scan image obtained along a broken line drawn on the 4 cells in A. Note that all Ca\(^{2+}\) waves stopped at the cell boundary. C: time-based scan images of 4 consecutive Ca\(^{2+}\) waves with expanded time scale, which were taken from B. Note Nos. 1–4 indicate the first to the fourth Ca\(^{2+}\) waves. D: time course of fluorescence intensity in each cell. Plots of fluorescence intensities (a–d) were obtained from points indicated by arrows in cells a–d in B. E: comparison of amplitudes of the first, second, third, and fourth Ca\(^{2+}\) waves after cessation of stimulation. Ca\(^{2+}\) signal intensities of the second and later waves were normalized to those of the first waves in individual cells. Values are means ± SD of 30 cells. F: comparison of velocities of the first, second, third, and fourth waves. Values are means ± SD of 30 cells. There was no significant difference detected using ANOVA for multigroup comparisons.
Fig. 3. Wave intervals after cessation of stimulation. A: time-based scan image obtained from a line transverse to cells in guinea pig papillary muscle (vertical white solid line on left). B: scheme for determination of wave intervals. Intervals were determined as depicted. C: wave interval vs. wave-number relationship. Curves are least-squares fit to Eq. 1. Different symbols indicate different cells.

indicated with arrows in individual cells in Fig. 2B and are shown in Fig. 2D. The peak amplitudes of Ca\(^{2+}\) waves looked very similar. Similar determinations were carried out, and the average peak fluorescence intensities of Ca\(^{2+}\) waves are shown in Fig. 2E: they decreased by a small difference with time although the difference was not significant by ANOVA for multigroup comparisons. We can conclude that these decreases were minor, taking bleaching of the dye during the measurements (10% decrease during 33 s) into consideration. Almost the same amplitudes of Ca\(^{2+}\) waves up to the fourth were also obtained with fluo 5F, which had a lower affinity for Ca\(^{2+}\) (\(K_D = 2.3\ \mu M\); data not shown).

Wave velocities examined in individual cells were obtained by expanding the time axis of the time-based scan image and determining the slopes of ridges of Ca\(^{2+}\) signal bands (Fig. 2C). With cells where four to five waves occurred during 30 s, the average velocities of the first, second, third, and fourth waves were 131 ± 29, 122 ± 26, 119 ± 33, and 126 ± 38 μm/s (30 cells from 5 preparations), respectively. The first waves propagated significantly faster than the second and third ones when tested by paired t-test (\(P < 0.05\)); the difference of ~7–9%, however, was not very large. In fact, it was not significant by ANOVA for multigroup comparison. Therefore, we may conclude that the peak amplitude or velocity of Ca\(^{2+}\) waves does not dramatically change during the period of determination.

The time-based scan image shown in Fig. 2B indicates that the wave interval gradually increased with time. For extensive analysis of wave intervals in each cell, time-based scan images were acquired along the line oriented with a shorter axis of cells as shown by a white line in Fig. 3A, left, thus allowing examination of a large number of cells at once (Fig. 3A, right, also see Figs. 4 and 5). Figure 3B explains how the wave number was defined and how the wave intervals between two consecutive waves were determined. The relationships between wave number and wave interval in individual cells is plotted in Fig. 3C. The wave intervals increased more as waves occurred later in each cell. On closer examination, the wave interval between two adjacent Ca\(^{2+}\) waves, one after the other, increased with a constant ratio as described by the following geometric progression equation:

\[
a_n = a_1 \times r^{n-1}
\]

where \(a_n\) is an interval between \((n-1)\)th and \(n\)th Ca\(^{2+}\) waves, \(a_1\) is an interval between the last Ca\(^{2+}\) transient and the initial Ca\(^{2+}\) wave, \(r\) (must be >1) is the increasing ratio of wave interval, and \(n\) is the ordinal number of the Ca\(^{2+}\) wave. The fit of data in each cell to Eq. 1 was strikingly good, with an average value of the least square of the fit, \(R^2\), of 0.971 ± 0.037 (\(n = 32\)) in cells that showed more than three waves in normal Krebs solution. In cells that showed more than two waves within 30 s, values for \(a_1\) and \(r\) were 0.75–7 s and 1.3–4.2, respectively. Some cells showed no wave or only one after stimulation, where \(a_1\) or \(r\) is infinitely large. Therefore the first interval (\(a_1\)) and the increasing ratio (\(r\)) values were variable from cell to cell, even in the same preparation. Similar results were obtained with 15 other preparations.

The above results showed that wave amplitudes or velocities changed only slightly. Wave intervals, in contrast, increased one after the other. These findings suggest that, in each cell, the amount of Ca\(^{2+}\) released from SR at wave occurrence is almost constant and that a wave can be triggered when the SR loading reaches a threshold level. Because Ca\(^{2+}\) waves appeared when the background Ca\(^{2+}\) concentration was decreasing (Figs. 1 and 2), the ambient cytoplasmic Ca\(^{2+}\) concentration cannot have been a critical factor. To confirm this idea, we perturbed Ca\(^{2+}\) homeostasis in cardiac cells by modulating Ca\(^{2+}\) uptake by SR and Ca\(^{2+}\) extrusion by Na\(^+\)/Ca\(^{2+}\) exchanger and examined how wave intervals were affected by them.

In Fig. 4A, Ca\(^{2+}\) wave images were obtained after high-frequency stimulation in the absence and presence of CPA, a SERCA inhibitor. Because intensive inhibition of SERCA...
activity by CPA resulted in no occurrence of Ca\(^{2+}\) waves (data not shown), the determination in Fig. 4B was carried out 10 min after addition of 10 \(\mu\)M CPA, where SERCA inhibition was partial. With this preparation, in the absence of CPA, eight cells showed two or more Ca\(^{2+}\) waves, whereas in the presence of CPA two out of the eight cells showed no wave, one showed only one, and the other cells showed two waves. In the presence of CPA, Eq. 1 was still effective, but both \(a_1\) and \(r\) were increased (Fig. 4B). With the five cells that showed two waves in the presence of CPA, the averages of \(a_1\) increased by

![Fig. 4. Effect of cyclopiazonic acid (CPA), a SERCA inhibitor, on occurrence of Ca\(^{2+}\) waves. A: time-based scan images after cessation of high-frequency stimulation in the presence (b) and absence (a and c) of 10 \(\mu\)M CPA. Image c was obtained after washout of CPA. The muscle fiber was loaded with rhod 2 and observed with a \(\times 20\) objective. B: wave intervals in the same cell (indicated by arrows in A) before, during, and after treatment of CPA.](image)

![Fig. 5. Effect of lowering Na\(^{+}/Ca^{2+}\) exchange activity on Ca\(^{2+}\) wave intervals. A: time-based scan images in the presence of 26 mM Na\(^{+}\) solution. The muscle had been stimulated at 0.5 Hz for 4 min, and then the stimulation was stopped. B: wave intervals in individual cells in the presence of 26 mM Na\(^{+}\). Different symbols indicate different cells. C: time-based scan image obtained in 0Na\(^{+}/0Ca^{2+}\) solution. Before the acquisition of Ca\(^{2+}\) images, the muscle had been stimulated at 2 Hz for 5 min in normal Krebs solution and then incubated in 0Na\(^{+}/0Ca^{2+}\) solution under a resting condition for 10 min. D: wave intervals in individual cells. For this experiment, waves were numbered in order of occurrence during acquisition (from left to right in C). Muscles were loaded with fluo 3. Different symbols indicate different cells.](image)
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70%, from 4.3 ± 1.4 to 7.4 ± 3.3 s by addition of CPA, whereas r increased by ~50%, from 2.1 ± 0.5 to 3.1 ± 0.6. This means that average intervals increased 2.5-fold (1.7 × 1.5 = 2.55). After washout of CPA, the two parameters were partially reversed (Fig. 4, Ac and B). Wave velocity, on the other hand, was slightly but significantly decreased, from 135 ± 15 (n = 26) to 124 ± 26 (n = 13) μm/s, by addition of CPA. Similar results were obtained with three other preparations. Effect of CPA on Ca\(^{2+}\) wave amplitudes was not examined because fluorescence intensity of fluo 3 itself decreased with CPA. Similar results were obtained with three other preparations: recovery with eventual disappearance of Ca\(^{2+}\) waves after gradual prolongation or final cell death with sustained elevated cytoplasmic Ca\(^{2+}\) after more frequent Ca\(^{2+}\) waves in several subsequent tens of minutes. Cells showing spontaneous Ca\(^{2+}\) waves are thus considered to be in a state between alive and dead. Interestingly, in the partially damaged muscles, wave propagation often appeared to be unidirectional, with waves traveling from a damaged focus to an intact region (Fig. 6). We examined whether these waves propagate in adjacent cells. Figure 6Aa–Ac shows the case in “Ca\(^{2+}\) paradox” (see MATERIALS AND METHODS), where most Ca\(^{2+}\) waves (marked by white arrowheads) in c traveled from left to right along longitudinal cellular axes, with only a few exceptions (marked by white arrows) in the reverse direction (please refer to the Supplemental Material for this article to view renderings). To know whether those waves actually propagate across the cell boundary under these conditions, time-based scan images from myocytes marked 1–3 in Fig. 6A were obtained (Fig. 6Aa–Ag). These images showed that the majority of Ca\(^{2+}\) waves propagated rightward and stopped at the cell boundary (Fig. 6, Aa and Ag). Only waves marked with asterisks seemed to propagate across the cell boundary. However, this may be fortuitous because wave frequencies in individual cells were almost regular, although with distinct rates. The Ca\(^{2+}\) wave marked by the arrow in Fig. 6Ag was probably an exceptional case of propagation: it appears that the Ca\(^{2+}\) wave from the right propagated across the cell boundary and stopped when it collided with another wave from the left. Figure 6B shows another example of Ca\(^{2+}\) waves at a region partially damaged by “overload and anoxia” (see MATERIALS AND METHODS). In this muscle, the damage was more heterogeneous (see white cells and their surrounding cells) than that seen in Fig. 6A. In this case, unidirectional Ca\(^{2+}\) waves were also seen in four longitudinally adjacent cells. A time-based scan image revealed that Ca\(^{2+}\) waves propagated leftward, in the direction of decreasing frequency of Ca\(^{2+}\) waves. Similar results were obtained with mechanically damaged muscle (Fig. 6C). We analyzed the direction of propagation of these spontaneous waves to confirm the relationship between the direction and wave frequency. Among 44 cases in 6 preparations that showed spontaneous waves in two longitudinally neighboring cells, 31 cases showed wave propagation in the direction of less frequency, 4 cases in the reverse direction, and 9 cases in either direction. These results indicate that there is a clear tendency in the direction of wave propagation in cells in damaged regions.

To examine whether Ca\(^{2+}\) waves in one cell actually do not propagate into the next cell in these regions, we determined the latency period between two successive waves in longitudinally adjacent cells where unidirectional waves were observed. As shown in Fig. 7A, a period between the arrival of one wave at the cell boundary in the cell and the occurrence of a new wave in the next cell was determined. Figure 7B shows a histogram for the latency period. Because of the similar frequency of events at each bin of the period throughout 0–0.8 s, it is concluded that wave occurrences in one cell were not perturbed by Ca\(^{2+}\) waves from neighboring cells. Therefore, these results suggest that very few Ca\(^{2+}\) waves show cell-to-cell propagation, even in regions of unidirectional wave flow.

To know what the cause is for the unidirectional propagation of waves, we carried out some preliminary experiments. It has been reported that, at damaged regions, cytoplasmic pH is more acidic with accumulation of lactic acid (8, 10, 11, 25); also, H\(_2\)O\(_2\) or free radical has been shown to be produced during ischemia and reperfusion (27, 34). It is therefore pos-
sible that a gradient in one of these factors might underlie the one-way waves. However, an addition of butyrate (20 mM), which has been reported to make intracellular pH acidic (8), slightly lowered wave frequency in 0Na+/0Ca2+ solution, although it prolonged the decay time of Ca2+/H11001 waves significantly. Similar results were obtained in an acidic bicarbonate-buffered 0Na+/0Ca2+ solution (pH of 6.7), which was made by bubbling with 30% CO2-70% O2 (data not shown). Lactate (20 mM) or H2O2 (300 μM) also did not increase wave frequency. These results suggest that those factors cannot be the cause of the unidirectional Ca2+ waves. Next, the effects of Ca2+ influx from the outside and extrusion by the Na+/Ca2+ exchanger were examined. When muscles showing spontaneous Ca2+ waves were incubated in Ca2+-free solution, frequency of the waves gradually decreased, although it took more time than intact muscle did. At this point, the direction of waves became two way (see Fig. 6A). The one-way Ca2+ waves were also converted to two way if the muscles were incubated in 0Na+/0Ca2+ solution for >10 min, although Ca2+ waves of high frequencies were often converted to a sustained elevation of

Fig. 6. Spontaneous Ca2+ waves in partially damaged muscles under resting condition; 500 consecutive images were taken every 67 ms without electrical stimulations. A: Ca2+ waves in a muscle subjected to treatment of Ca2+ paradox. a–c: Determination of direction of wave propagation; b was taken at 266 ms after a; c, Ca2+ waves during the first 133 ms are shown in green, and those during the next 133 ms are in red. Note that, in most cells, waves proceeded from left to right (arrowheads), and in only a few cells from right to left (arrows). *Wave initiated within the second 133 ms; d, contour of some cells in a–c; e–g, time-based scan images obtained from lines drawn on adjoining cells (e, f, and g correspond to lines 1, 2, and 3 in d, respectively). Vertical bars above e–g mark the cell boundary. Asterisks in e indicate waves that appeared to propagate across the cell boundary. Arrow in g indicates a wave that appeared to propagate beyond the cell boundary. Note also that this wave showed an exceptional 2-way propagation; h, time-based scan image in Ca2+-free Krebs solution that was taken 5 min after e. B and C: Ca2+ waves in cells damaged by anoxia (B) and cutting (C). a, Images of the damaged muscles. Contoured cells showed spontaneous Ca2+ waves. Bright (white) regions are dead cells that showed neither waves nor electrical activity; b, time-based scan image obtained from a line on adjoining cells. *Waves that appeared to propagate over the cell boundary. Vertical lines indicate the cell boundary. Please refer to the Supplemtal Material for this article (published online at the American Journal of Physiology-Cell Physiology web site) to view a video of Aa–Ac.
cytoplasmic Ca$^{2+}$ in 0Na$^+$/0Ca$^{2+}$ solution with highly damaged/overloaded muscles (data not shown). Taken together, these results suggest that the gradient in the increased cytoplasmic Ca$^{2+}$ may be the underlying mechanism for spontaneous and unidirectional Ca$^{2+}$ waves in a partly damaged region of muscles.

**DISCUSSION**

In this investigation of Ca$^{2+}$ waves in guinea pig papillary muscle, the type of preparation used has the following advantages over single-cell preparations in the study of cardiac muscle function, and led to the conclusions summarized below. 1) Many pieces of information from a large number of individual cells can be obtained at once, and each of them can be easily integrated into the behavior of a mass of muscle. The data in Figs. 1–4 show clearly that the Ca$^{2+}$ signals underlying the aftercontraction, which is considered to be a prelude to triggered arrhythmias, consist of asynchronous Ca$^{2+}$ waves in individual cells. 2) Cells composing papillary muscles turned out to be miscellaneous in their intrinsic properties related to Ca$^{2+}$ waves and independent of each other (Figs. 1, 3, and 5). This independence of each cell is also the case under such pathological conditions, as shown in Figs. 6 and 7. 3) Only these multicellular preparations allow us to also investigate the interaction between cells under pathological conditions. One-way propagation of waves with partially damaged cells was observed, for which the gradient of cytoplasmic Ca$^{2+}$ concentration may be the underlying cause (Fig. 6). 4) Ca$^{2+}$ waves scarcely propagated across the cell boundary, not only of intact cells but also of partially impaired cells (Figs. 2 and 6).

In view of the well-known marked species specificity in Ca$^{2+}$ homeostasis in heart muscle, the results of this study are valuable because they were obtained with guinea pig ventricular muscles, whereas the previous results (1, 13, 18, 26) were obtained exclusively with rat and mouse heart. There are significant differences in Ca$^{2+}$ homeostasis in ventricular muscles between rat/mouse and other animals, including guinea pig (3, 29, 30). Postrest contraction is a noticeable example. After a period of rest after steady-state stimulation, the first contraction of rat muscle is largest, followed by decremental ones (negative staircase), whereas guinea pig ventricular muscle shows a positive staircase, being well explained by the change in Ca$^{2+}$ stored in SR during rest (3, 17).

This study has revealed some differences in the properties of Ca$^{2+}$ waves between rat and guinea pig ventricular muscles. First, when the muscle was maintained at rest, no Ca$^{2+}$ wave occurred in guinea pig papillary muscle. In rat ventricular muscle, spontaneous Ca$^{2+}$ waves were observed at room temperature (1, 13, 18, 22, 33). Second, Ca$^{2+}$ wave intervals dramatically changed after high-frequency stimulation in guinea pig ventricle, whereas in rat ventricular muscle, frequency of Ca$^{2+}$ waves remained nearly constant (13). These results can be explained by a large contribution of Na$^+$/Ca$^{2+}$ exchange reaction in guinea pig ventricle.

In intact guinea pig ventricular muscle, Ca$^{2+}$ waves seldom propagated beyond the cell boundary. Although a similar conclusion was reached in previous studies with rat hearts, the reported probability with rat heart (3–13% for normal and >20% for Ca$^{2+}$ overloaded; see Ref. 13) was much greater than that with guinea pig heart (<1%). The reason for this difference is not clear. Because the most important molecule involved in intercellular communication is the gap junction channel, one possibility would be some differences in its number and/or properties.

The luminal Ca$^{2+}$ levels, cytoplasmic Ca$^{2+}$ concentrations, and Ca$^{2+}$ sensitivity of the Ca$^{2+}$-releasing channels were proposed to be important in the occurrence of Ca$^{2+}$ waves (1, 13, 21). In this study, we showed that Ca$^{2+}$ waves were induced after a series of high-frequency stimulations under the condition where the cytoplasmic Ca$^{2+}$ concentration was gradually decreasing with time (Fig. 1). Wave amplitudes and velocities were not changed much, although the interval between Ca$^{2+}$ waves gradually prolonged (Figs. 2 and 3). These results suggest that the loading level of SR was similar when a spontaneous Ca$^{2+}$ wave occurred, indicating that the luminal Ca$^{2+}$ level is critically important for induction of Ca$^{2+}$ waves, as proposed previously (12). The effects of inhibition of SERCA (Fig. 4) and the reduction in the Na$^+$/Ca$^{2+}$ exchange rate (Fig. 5) on wave intervals further support this conclusion. Ca$^{2+}$ that has entered via the L-type Ca$^{2+}$ channel upon electrical stimulation is partly accumulated in the SR by Ca$^{2+}$-ATPase, is partly extruded out of cells by the Na$^+$/Ca$^{2+}$ exchanger, and partly stays in cytoplasm during the subsequent
resting period. When the Ca$^{2+}$ loading level in SR reaches the threshold in a cell, spontaneous Ca$^{2+}$ release occurs. Because SR throughout the entire cell should be loaded with Ca$^{2+}$ to a similar extent when the wave occurs, Ca$^{2+}$ release would be easily propagated as waves. Released Ca$^{2+}$, in turn, is partly taken up to SR by SERCA activity and partly extruded by Na$^{+}$/Ca$^{2+}$ exchange reaction from the cell. This extrusion of Ca$^{2+}$ by the Na$^{+}$/Ca$^{2+}$ exchange reaction results in reduction in the cytoplasmic Ca$^{2+}$ level, which leads to reduction of the Ca$^{2+}$ uptake rate in SR and in turn to prolongation of wave intervals. Amplitude and velocity of the next Ca$^{2+}$ wave, however, remain unaffected.

Time-based analysis of consecutive two-dimensional Ca$^{2+}$ images of the multicellular preparation showed that Ca$^{2+}$ wave intervals were variable among cells, even in the same preparation (Fig. 3, A–C). This variation was also seen in the absence of Na$^{+}$/Ca$^{2+}$ exchange activity (Fig. 5). These results suggest that variations among cells in the ability of Ca$^{2+}$ handling must be intrinsic properties of the plasma membrane, SR, and/or other systems that directly affect Ca$^{2+}$ movements or indirectly do so by changing critical modulatory factors, such as ATP concentration, pH, and so on. Cells in multicellular preparations appear to be rather miscellaneous in their characteristics of Ca$^{2+}$ homeostasis and independent of neighboring cells.

It is interesting that Ca$^{2+}$ waves often show one-way propagation in a neighboring area in a partly damaged region. This result indicates that Ca$^{2+}$ waves apparently travel along gradients of some components that tissue degradation has caused. It should be noted, however, that Ca$^{2+}$ waves seldom propagated across the cellular boundary. Because cells are independent in terms of wave frequency and because cell-to-cell propagation of Ca$^{2+}$ waves was rarely observed, destruction of cell border or gap junction cannot be the reason. Our preliminary results excluded the possibility of changes in pH or H$_2$O$_2$ level but suggested the gradient of the cytoplasmic Ca$^{2+}$ concentration as a candidate. It is quite probable that some Ca$^{2+}$ flow into a neighboring cell during Ca$^{2+}$ waves through gap junction channels. This Ca$^{2+}$ influx may contribute to accelerating Ca$^{2+}$ loading of SR, but it would not induce the Ca$^{2+}$ wave if the luminal Ca$^{2+}$ level did not reach the critical level. A possibility of the opening of the L-type Ca$^{2+}$ channel is less likely because nifedipine was not effective in protecting against Ca$^{2+}$ paradox (9) or spontaneous Ca$^{2+}$ waves in our experiments (data not shown), although a possibility of other Na$^{+}$/Ca$^{2+}$ exchange pathways (7, 16) cannot be excluded. Inhibition of the Na$^{+}$/Ca$^{2+}$ exchanger, which extrudes Ca$^{2+}$, is another likely explanation for the increase in cytosplasmic Ca$^{2+}$. A factor of resting membrane potential must also be considered as a reason for the reduced Na$^{+}$/Ca$^{2+}$ exchange rate. A more depolarized membrane potential, which is expected in damaged cells, would be unfavorable to Ca$^{2+}$ extrusion by the Na$^{+}$/Ca$^{2+}$ exchanger. The electrotonic spatial distribution of depolarized membrane potential at the damaged region would help form the gradient of cytosplasmic Ca$^{2+}$ concentration.

The decrease in or loss of the conductance of gap junction channels might be a cause for infrequently occurring cell-to-cell propagation of Ca$^{2+}$ waves in temporarily Ca$^{2+}$-overloaded intact muscles or partially damaged muscles, because their permeability is reported to be decreased by high intracellular Ca$^{2+}$ or low pH (3, 32). In intact muscles, the possibility of inactivation of the gap junction is excluded because whole muscle bundles ~3 mm long were excited by a localized stimulation with a small electrode of ~50 × 20 µm in size (data not shown). Based on this and previous observations (32), it seems reasonable to assume that functional gap junctions were well maintained in the experiments shown in Figs. 1–5. In partially damaged muscles, it was difficult to estimate the function of gap junctions from electrical conductivity because the cells themselves had already become less responsive to field electrical stimulation. The conductance of gap junctions might be somewhat lowered, since the previous report showed that an exposure of calf ventricular muscle to a highly toxic dose of ouabain gradually decreased the conduction to ~60% of the initial after 90 min (32). However, the reduced conductance of the gap junction, if any, may not be the main reason for the scarce cell-to-cell propagation, because occurrence of the unidirectional wave is well explained by ionic or electronic conductivity of gap junctions, as described above. In either event, it would be interesting to investigate how the increased basal Ca$^{2+}$ or long-lasting occurrence of Ca$^{2+}$ waves modulates gap junctions.

In conclusion, we concentrated on whether Ca$^{2+}$ waves propagate beyond a cell boundary and found that their cell-to-cell propagation was infrequent not only in intact cardiac muscles but also in partially damaged ones. During the course of the experiments, we also found unidirectional Ca$^{2+}$ waves in partially damaged muscles. Because this phenomenon may be involved in the prognosis for a partially injured heart, the relation between Ca$^{2+}$ waves and spread of damage in a multicellular preparation requires further investigation.

ACKNOWLEDGMENTS

We are grateful to Professor Emeritus Hiroshi Yamaguchi, Department of Cardiology, Juntendo University School of Medicine, for arranging our collaboration on this study. We thank Mei Ling and Yuriko Yotsui for helpful assistance.

GRANTS

This work was supported in part by Grants-in-Aid to Y. Ogawa (no. 11470026) and N. Kurebayashi (no. 13670096) and a High Technology Research Center Grant for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

DO Ca\(^{2+}\) WAVES PROPAGATE ACROSS THE CELL BOUNDARY?


