Intracellular Calcium Transients and Arrhythmia in Isolated Heart Cells


Intracellular calcium ([Ca$^{2+}$]$_i$) elevation may mediate cardiac arrhythmias. However, direct measurement of the rapid alterations of [Ca$^{2+}$]$_i$ on a beat-to-beat basis using fast temporal resolution and without signal averaging in the spontaneously beating in vivo heart is lacking. Furthermore, data from an isolated spontaneously beating myocyte preparation that develops arrhythmia similar to that in the in vivo heart are unavailable. We measured rapid changes of [Ca$^{2+}$]$_i$, with fast temporal resolution in isolated spontaneously beating neonatal rat ventricular myocytes with cell-to-cell communication and characterized the interrelation between [Ca$^{2+}$]$_i$ and arrhythmia. An elevated extracellular calcium ([Ca$^{2+}$]$_o$) concentration of 10.8 mM induced premature beats, a rapid beating rate (tachyarrhythmia), and chaotic or fibrillatory beating activity in a small group of myocytes. [Ca$^{2+}$]$_i$, levels during systole increased from the nanomolar to micromolar concentration range before arrhythmia development. Spontaneous oscillations of [Ca$^{2+}$]$_i$, during diastole could evoke a spontaneous tachyarrhythmia. In the presence of [Ca$^{2+}$]$_i$, elevation, a spontaneous tachyarrhythmia could induce severe [Ca$^{2+}$]$_i$ overload. Reduction of [Ca$^{2+}$]$_i$, with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid AM (5 μM) in the presence of 10.8 mM [Ca$^{2+}$]$_o$, reversed the arrhythmia. In single ventricular myocytes superfused with 10.8 mM [Ca$^{2+}$]$_o$, oscillations of membrane potential characteristic of transient inward current occurred that were prevented by ryanodine (0.1 μM), an inhibitor of Ca$^{2+}$ flux across the sarcoplasmic reticulum. This study characterizes 1) an isolated multicellular myocyte model of arrhythmia similar to that evident in in vivo hearts, 2) elevation of [Ca$^{2+}$]$_i$, with systolic [Ca$^{2+}$]$_i$, levels of 1–3 μM and diastolic [Ca$^{2+}$]$_i$, oscillations before the initiation of arrhythmia, 3) tachyarrhythmia as a cause of severe [Ca$^{2+}$]$_i$, overload, which may be important in the perpetuation and degeneration of arrhythmias, and 4) reversal of arrhythmia with reduction of [Ca$^{2+}$]$_i$. The results in the isolated myocyte model may have relevance to the generation and perpetuation of certain cardiac arrhythmias associated with calcium overload. (Circulation Research 1991;69:810–819)

Fabianto and Fabianto$^{1–3}$ detected localized areas of cyclic contractions and asynchrony within parts of rat ventricular fibers with disrupted sarclemmal membranes and also recorded spontaneous beating activity in collagenase-trypsin-digested single rat ventricular myocytes superfused with calcium concentrations as low as 0.05 mM. Chiesi et al$^4$ reported phasic contractile activity in adult rat ventricular myocytes with electrochemically shunted membranes superfused with calcium concentrations as low as 1×10$^{-7}$ M. Electron probe x-ray microanalysis of these cells showed dissipation of transmembrane gradients to sodium and potassium that was indicative of membrane disruption. The spontaneous waves of cyclic contractions were attributed to the oscillatory release and reuptake of calcium by the sarcoplasmic reticulum.$^{1–4}$ Repetitive contractile waves have also been found in single ventricular myocytes$^{5–7}$ in association with a localized increase in intracellular calcium ([Ca$^{2+}$]). Oscillations in [Ca$^{2+}$], and in tension as well as fluctuations of membrane potential and membrane current have been reported in association with conditions increasing [Ca$^{2+}$] in ventricular muscle, in Purkinje fibers, and in single cells.$^{8–13}$

Increased [Ca$^{2+}$]$_i$ concentrations have been recorded during metabolic inhibition,$^{14}$ acute myocardial ischemia,$^{15–17}$ reperfusion of ischemic myocardium,$^{18}$ and sympathomimetic amine stimulation.$^{19}$

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Several calcium-dependent electrophysiological alterations such as slow response action potentials, afterpotentials, cell-to-cell uncoupling, and injury current have been linked to the development of ventricular arrhythmias. Procedures designed to inhibit Ca\(^{2+}\) fluxes across the sarcolemma and sarcoplasmic reticulum were found to reduce the incidence of ventricular arrhythmias in heart during acute myocardial ischemia and on subsequent reperfusion. Therefore, one hypothesis has been that Ca\(^{2+}\) elevation may be critical in the initiation of selected arrhythmias.

In considering the above hypothesis, it is pertinent to note that direct measurement of the rapid alterations of [Ca\(^{2+}\)], on a beat-to-beat basis using fast temporal resolution and without signal averaging in an isolated multicellular myocyte preparation developing arrhythmia is not available. Previous studies have used single or disrupted cardiac myocytes to study the relation between alterations in [Ca\(^{2+}\)], excitation-contraction coupling, and arrhythmias. Although studies using the single myocyte provide valuable information with respect to electrophysiological changes, characterization of arrhythmia manifested by premature beats, tachyarrhythmia, and fibrillatory beating activity is not possible since cell-to-cell communication is lacking. For example, the response of quiescent single myocytes to high extracellular calcium ([Ca\(^{2+}\)]\(_{j}\)) is asynchronous intracellular contractile movements. Since single quiescent myocytes do not exhibit spontaneous beating, measurements of [Ca\(^{2+}\)]\(_{j}\) reflect only diastolic content; moreover, the influence of arrhythmia on spontaneous [Ca\(^{2+}\)], transients cannot be defined. Other limitations of previous studies include the use of fluorescent calcium indicators, such as aequorin and 1,2-bis(2-aminophenoxy)ethane-N\(_2\),N\(_2\),N\(_\text{'}\),N\(_\text{'}\)-tetraacetic acid (BAPTA), that are either insensitive to measure small spontaneous oscillations of diastolic calcium that may evoke arrhythmia or chelate [Ca\(^{2+}\)], and impair ventricular function. In view of several different substrates (metabolic inhibition, ischemia, reperfusion, and catecholamine stimulation) that may cause arrhythmia, it is not possible that any one study using any one experimental model can resolve a single mechanism and/or the role of calcium in the genesis of all arrhythmias. However, common to each of the above-mentioned substrates is the accumulation of [Ca\(^{2+}\)].

In the present study, [Ca\(^{2+}\)]\(_{j}\) was measured from a group of five to 10 myocytes using a microprocessor-controlled spectrofluorometer with a temporal resolution of 2 msec and the fluorescent calcium indicator, fura 2-AM, which is sensitive to measure [Ca\(^{2+}\)], on a beat-to-beat basis. The objectives of the present study were to 1) directly measure rapid alterations of [Ca\(^{2+}\)], on a beat-to-beat basis in a spontaneously beating isolated myocyte preparation with cell-to-cell communication, thereby characterizing a model of arrhythmia, 2) assess whether arrhythmia is preceded by an increase in spontaneous beating rate or an elevation of [Ca\(^{2+}\)], and characterize the concentration of [Ca\(^{2+}\)], at which arrhythmia occurs, 3) define the role of tachyarrhythmia in producing [Ca\(^{2+}\)], overload and the magnitude of the calcium increase, and 4) define whether reduction of [Ca\(^{2+}\)] is associated with reversal of arrhythmia.

The study resulted in several new findings. First, we characterized an isolated multicellular myocyte model with cell-to-cell communication that develops an arrhythmia profile, including fibrillatory beating activity, similar to that in whole hearts. Second, we demonstrated reversibility of the spontaneous arrhythmia with reduction of [Ca\(^{2+}\)], using BAPTA. Third, we showed that in the presence of [Ca\(^{2+}\)] elevation, a rapid tachyarrhythmia caused severe [Ca\(^{2+}\)], overload. A rapid tachyarrhythmia may be of critical importance in elevating [Ca\(^{2+}\)], perpetuating arrhythmia, and/or inducing degeneration of an arrhythmia into fibrillatory activity. Fourth, we obtained evidence that diastolic oscillations of internal calcium may induce arrhythmia before any alteration in basal beating rate.

**Materials and Methods**

**Experimental Protocol**

Neonatal rat myocardial cells were cultured by a modification of the method of Harary and Farley as described by Buja and coworkers. Hearts were isolated from 2-3-day-old rats, atra were removed, and the ventricles were minced in a HEPES-buffered balanced salt solution. The myocardial cells were dispersed by incubation in a pancreatin (60 mg/100 ml, GIBCO Laboratories, Lawrence, Mass.) and collagenase type II (6,000–6,400 units/ml, Cooper Biomedical, Freehold, N.J.) solution at 37°C for 20 minutes. The initial supernatant was removed and discarded. The mince was incubated in fresh pancreatin-collagenase for 20 minutes at 37°C. These steps were repeated four times. The cell suspensions from each digestion were combined and centrifuged. The pellets were suspended in culture medium consisting of 68% Dulbecco’s modified Eagle’s culture medium, 17% medium 199 with Earle’s salts, and 15% serum (10% horse serum and 5% fetal calf serum) with antibiotics (10,000 units/ml penicillin and 10,000 μg/ml streptomycin). Myocytes were separated from nonmyocytic cells using a Percoll differential separation gradient.

At 3 days after dispersion, the myocytes had attached to the plates and formed multicellular colonies of spontaneously beating cells. These spontaneously beating myocytes grown on laminin-coated glass coverslips were incubated with medium 199 containing 3.0 μM fura 2-AM (Boehringer Diagnostics) for 30 minutes at 37°C and then placed in fura-free medium 199 for 30 minutes of equilibration. This procedure allows for intracellular uptake of fura 2-AM followed by release of free fura 2 by the action of intracellular esterases. After 30 minutes of equilibration, the beating rate was similar to that present.
before the addition of the fluorescent indicator. The coverslips were mounted in Sykes-Moore chambers (Belco Glass Co.), placed on a heated stage (37°C) of an inverted Nikon microscope equipped with ultraviolet optics, and coupled by quartz bifurcated fiberoptics to a Fluoroprobe 1000 microspectrophotometer (Tracor Northern, Middleton, Wis.). A 10-segment chopper wheel operating at 3,600 rpm provided alternating 340- and 380-nm excitation produced by a 150-W xenon lamp and two monochromators. Fluorescence emission maximum at 510 nm was directed into a photomultiplier tube, and photon counting data were processed using a multichannel scaler and a TN 6500 controller. Fura 2 binds with calcium in a 1:1 ratio and, on binding with calcium, shifts its excitation maximum from 380 to 340 nm, while the emission maximum remains stable at ~510 nm. [Ca2+]d, estimated from a group of approximately five to 10 cells by dividing the fura 2 emission at 340-nm excitation by that obtained at 380-nm excitation after background autofluorescence subtraction, thus yielding fura 2 ratios (340/380). The background autofluorescence was measured in myocytes before the addition of fura 2-AM. The myocyte preparation used for measurement of the background autofluorescence was obtained from the same culture as that used for experimental study. It is recognized that absolute quantitation of the fura 2 ratios is complicated by several factors, including potential compartmentalization and alterations in the properties of the dye in cells as compared with cell-free suspension. In experiments in this study, samples were calibrated to assess the contribution of calcium-insensitive fura 2 to the total fluorescence signal as recommended by Scanlon et al. The maximum fluorescence ratio was determined at the end of an experimental protocol, using the method of Peeters et al. by treating the cells with the nonfluorescent ionophore, 4-bromo-A23187 (20 μM, Calbiochem, La Jolla, Calif.). The minimum fluorescence ratio was determined by treatment with 5 mM EGTA. [Ca2+]d was calculated according to the formula of Grynkiewicz et al using a Kd of 225 nM and a fluorescence ratio of SF2/Sb2, where SF2 and Sb2 represent the emission intensities at 380-nm excitation at saturating and calcium-free conditions, respectively, corrected for autofluorescence. Three pieces of evidence are presented arguing against any significant compartmentalization or sequesteration of fura 2 dye in intracellular organelles. First, Williford et al reported intramitochondrial accumulation of fura 2 fluorescence in isolated guinea pig myocytes but not in isolated rat ventricular myocytes. Second, in work in our laboratory, digital imaging of fura 2 fluorescence showed uniform distribution of [Ca2+]d in neonatal rat ventricular myocytes. Third, permeabilization of the sarcolemma with 5 μM digitonin caused loss of ~90% of fura 2 dye. Addition of a further 10 μM digitonin produced no further loss of the fura 2 dye. At the concentrations of digitonin used (15 μM), we are not certain that there was complete lysis of intracellular organelles. Judged from the loss of ~90% of the fura 2 fluorescence, we would argue against any significant compartmentalization of fura dye in intracellular organelles. However, we cannot exclude the possibility that some compartmentalization of fura occurred in intracellular organelles.

Myocytes were superfused in a modified Tyrode’s buffer containing (mM) NaCl 143, KCl 5.9, MgSO4 1.18, CaCl2 1.8, glucose 10, and HEPES 10, pH 7.3. The [Ca2+]d concentration was increased (from 0.25 or 1.8 to 10.8 mM) or increased and then decreased (from 10.8 to 0.25 mM), and [Ca2+]d transients from a group of approximately five to 10 cells were recorded during spontaneous myocyte contractions. The optimal time required for stabilization of the [Ca2+]d transient at each [Ca2+] d level was ~10 minutes. Ventricular myocyte beating rate and rhythm were recorded on videotape and played back on a video motion analysis detector. A photodiode placed directly over the cell of interest recorded the rate and rhythm. Since cell-to-cell communication was present, the myocyte under study was in contact with the adjacent cells.

Whole-cell calcium currents were measured under voltage-clamp technique from single cells. For technical reasons, the electrophysiological data could only be obtained in single cells at a stage before cell-to-cell communication (at 2 days in culture); [Ca2+]d transients were measured in myocytes with cell-to-cell communication (at 3 days in culture). Patch pipettes were prepared by a two-stage pull and were fire-polished. The resistance of the pipettes ranged from 3 to 5 MΩ. The indifferent electrode was an Ag/AgCl plug connected to the bath via a 200 mM KCl bridge. Ca2+ currents were obtained from a holding potential of ~80 mV in response to depolarization steps in 10-mV increments up to +60 mV. The extracellular solution contained (mM) tetraethylammonium chloride 140, CaCl2 2, MgCl2 2, KCl 5.9, glucose 10, HEPES 10, and 4-aminopyridine 5.0, pH adjusted to 7.3 with KOH. The intracellular pipette solution contained (mM) CsCl 140, BAPTA 5, MgCl2 2.5, and HEPES 10, pH adjusted to 7.2 with CsOH. The above extracellular solution eliminated the Na+ and K+ currents present in these cells. The pipettes were made with 8161 glass (Garner glass). To detect the development of the transient inward current, depolarizing voltage-clamp pulses were delivered to 0 mV every 5 seconds from the holding potential of ~40 mV, and membrane current fluctuations were evaluated from single myocytes. The extracellular solution was modified Tyrode’s buffer (as described), and the patch pipette contained (mM) potassium aspartate 140, KCl 10, MgCl2 1, ATP 3, and 10 HEPES, along with 10 μM EGTA, pH adjusted to 7.3.

Statistical Analysis

Data were expressed as the mean ± SEM. All analysis of variance F tests are one-tailed tests and were con-
sidered significant at \( p < 0.05 \). Duncan’s multiple-range tests were performed at the 0.05 level of significance.

**Results**

Isolated neonatal rat ventricular myocytes perfused with a \([Ca^{2+}]_o\) of 1.8 mM (control) demonstrated spontaneous, regular beating activity and regular \([Ca^{2+}]_i\) transients (Figure 1). In the example shown, each beat was accompanied by an increase in 340-nm and a decrease in 380-nm wavelength fluorescence intensity signal (top panel). \([Ca^{2+}]_i\) was estimated by dividing the fura 2 fluorescence at 340-nm illumination by that obtained at 380-nm illumination after background autofluorescence subtraction. This division gives the fura 2 ratio (middle panel) that was converted into \([Ca^{2+}]_i\), concentration (bottom panel). Thus, the data are presented as fura 2 ratios with estimates of absolute \([Ca^{2+}]_i\), values in some experiments (Figure 1) obtained by application of calibration factors derived from cellular recordings. Cellular calibrations yielded a maximum fluorescence ratio of 4.12±0.2, a minimum fluorescence ratio of 0.52±0.05, and S2/S02 of 4.3±0.5. The calculated maximal and minimal levels of the \([Ca^{2+}]_i\) transients were 445±38 and 115±16 nM, respectively (n=15). These values are comparable to the \([Ca^{2+}]_i\) transients reported in isolated cardiac cells.1,4,27,28,34

**Elevation of Extracellular Calcium**

Progressive elevation of the \([Ca^{2+}]_o\) from either 0.25 mM (low) or 1.8 mM (normal) to 10.8 mM was associated with an increase of both the maximal and minimal levels of the \([Ca^{2+}]_i\) transients (n=6 to 15 experiments) (Figure 2, top panel). The relations between \([Ca^{2+}]_i\), concentration and the maximal and minimal levels of the \([Ca^{2+}]_i\) transients, using polynomial regression analysis, were \( r=0.99 \). In each case, they were indicative of sigmoidal dose–response curves.

The peak calcium current (n=3) at voltages positive to \(-20 \text{ mV}\) showed an increase with elevation of \([Ca^{2+}]_o\) from 1.8 to 10.8 mM (Figure 2, bottom panel). The peak inward calcium current occurred between \(-10\) and 0 mV with control, whereas with increased \([Ca^{2+}]_o\), the peak current shifted rightward (from 0 to +10 mV).

At \([Ca^{2+}]_o\) of 1.8 mM, the mean beating rate was 105±5/min (n=15), and the rhythm remained regular. At \([Ca^{2+}]_o\) of 5.4 mM (n=12), \([Ca^{2+}]_i\) levels were elevated, and the regular beating rate was interrupted by premature beats or periods of marked rapid beating rate (tachyarrhythmia) (Figure 3, top panel). Similar findings were noted with periodicity of the \([Ca^{2+}]_i\) transients (Figure 3, middle panel). At \([Ca^{2+}]_o\) of 10.8 mM (n=12), \([Ca^{2+}]_i\) levels were further elevated (Figure 2, top panel). \([Ca^{2+}]_i\), oscillations occurred during the decay period of the \([Ca^{2+}]_i\) transient and could induce a tachyarrhythmia. Chotic or fibrillatory beating activity (Figure 3, top panel) occurred and was associated with chaotic \([Ca^{2+}]_i\) fluctuations as shown in Figure 3, middle panel. The variation of the amplitude of cell motion (Figure 3, top panel) in the myocytes superfused with different levels of \([Ca^{2+}]_o\) probably reflects limited sensitivity of the photodiode placed on the cell to record motion. Variations of the apparent amplitude of motion appear to be related to methodological factors and do not directly relate to the magnitude of the calcium transients.

To further test the relation between \([Ca^{2+}]_i\), levels and arrhythmia, we investigated the effect of reducing \([Ca^{2+}]_o\), on beating activity. Reduction of the \([Ca^{2+}]_o\) concentration from 10.8 to 0.25 or 0.5 mM decreased the levels of \([Ca^{2+}]_i\) (Figure 3, middle panel) and abolished the arrhythmia (n=8/8 experiments, Figure 3, top panel). However, since the increase and subsequent decrease of \([Ca^{2+}]_i\), occurred in concert with

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** In this typical preparation of cultured neonatal rat cardiac myocytes, each beat was accompanied by an increase in emission at an excitation of 340-nm and a decrease in emission at an excitation of 380-nm wavelength fluorescence intensity signal (top panel). \([Ca^{2+}]_i\) was estimated by dividing the fura 2 fluorescence at 340-nm illumination by that obtained at 380-nm illumination after background autofluorescence subtraction. This division gives the fura 2 ratio (middle panel) that was converted into \([Ca^{2+}]_i\), concentration (bottom panel) in some experiments.
elevation and subsequent reduction of [Ca\(^{2+}\)]_o, a plausible alternative hypothesis is that arrhythmia was related to some mechanism by which high [Ca\(^{2+}\)]_o perturbs the cell membrane at its outer surface. To determine whether arrhythmia development was a manifestation of an elevated [Ca\(^{2+}\)]_o or [Ca\(^{2+}\)]_i, we used the probe BAPTA-AM. BAPTA-AM does not bind to [Ca\(^{2+}\)]_o. It is membrane permeant, and on entry into the cell, it is deesterified with resultant chelation of [Ca\(^{2+}\)]. With elevated [Ca\(^{2+}\)]_i of 10.8 mM, BAPTA-AM (5 μM) decreased (n=5) the elevated maximal levels of [Ca\(^{2+}\)]_o from 1,628±350 to 236±27 nM and abolished the arrhythmia (example in Figure 3, bottom panel).

Whether the elevation of [Ca\(^{2+}\)]_o preceded the abnormality in beating rate or was a consequence of increased beating rate is a key question. In the example shown in Figure 4, top panel, the [Ca\(^{2+}\)]_o concentration of 1.8 mM was associated with a beating rate of 60/min and a maximal level of [Ca\(^{2+}\)]_o of ~700 nM. Elevating the [Ca\(^{2+}\)]_i concentration from 1.8 mM directly to 10.8 mM was associated with an immediate increase in the maximal level of [Ca\(^{2+}\)]_o to ~1.5–2.0 μM. However, the beating rate remained unchanged. With such [Ca\(^{2+}\)]_o elevation, [Ca\(^{2+}\)]_o oscillations occurred, which interrupted the decay processes of the [Ca\(^{2+}\)]_o transients (Figure 4, top panel) and were able to initiate a spontaneous tachyarrhythmia (fourth beat with [Ca\(^{2+}\)]_o of 10.8 mM, rate increase from 60 to 240 beats/min, Figure 4, top panel). Thus, when [Ca\(^{2+}\)]_o had increased from nanomolar to micromolar concentrations, arrhythmia occurred. The occurrence of a spontaneous tachyarrhythmia resulted in an immediate increase (almost doubling) in both the maximal and minimal levels of [Ca\(^{2+}\)]_o (Figure 4, top panel). The minimal level of [Ca\(^{2+}\)]_o, approximated that of the maximal level of [Ca\(^{2+}\)]_o, evident under control conditions when cells were superfused with [Ca\(^{2+}\)]_o of 1.8 mM. The effect of arrhythmia on [Ca\(^{2+}\)]_o is further evident in the example shown in Figure 4, bottom panel. Alteration in the interval between beats changes the minimal [Ca\(^{2+}\)]_o level. For example, a premature beat (second beat of Figure 4, bottom panel) impairs the complete decay of the [Ca\(^{2+}\)]_o transient to its expected baseline level. Thus, the minimal [Ca\(^{2+}\)]_o is reset at a higher level. Consequently, the point of takeoff of the premature beat occurs from a higher level. However, the maximal level of the [Ca\(^{2+}\)]_o transient associated with the premature
The electrocardiographic (EKG) was not altered. A longer interbeat interval (fifth beat of Figure 4, bottom panel) was associated with a more complete decay of the [Ca$^{2+}$] transient. Thus, the minimal 340/380 ratio was lower. Therefore, the minimal level of the [Ca$^{2+}$], is not constant but dependent on the interbeat interval.

**Electrophysiological Alterations**

Increased [Ca$^{2+}$] has been shown to depolarize the isolated neonatal rat heart cell by way of an inward current flowing through a Ca$^{2+}$-activated cation channel, thereby mediating enhanced pacemaker activity. To detect the development of the transient inward current, depolarizing voltage-clamp pulses to 0 mV were delivered every 5 seconds from the holding potential of −40 mV, and membrane current fluctuations were evaluated in single ventricular myocytes. When the [Ca$^{2+}$], was elevated from 1.8 to 10.8 mM, membrane repolarization after a voltage-clamp depolarization was associated with fluctuations of membrane potential (in five of five experiments) manifested by transient inward deflections in the holding potential characteristic of transient inward current (Figure 5). Ryanodine (0.1 μM), which inhibits the influx of calcium across the sarcoplasmic reticulum, prevented (in four of four experiments) the transient inward current fluctuations (Figure 5).

**Discussion**

The isolated, cultured, spontaneously beating neonatal rat ventricular myocyte preparation with cell-to-cell communication responds to an increased [Ca$^{2+}$], concentration with development of premature beats, tachyarrhythmia, and chaotic fibrillatory beating activity, an arrhythmia profile similar to that recorded from the isolated or in vivo heart. Therefore, we characterize an isolated multicellular myocyte model of spontaneous arrhythmia that allows for evaluation of the interrelation between [Ca$^{2+}$], transients and arrhythmia. One limitation of the neonatal

**Figure 3.** Top panel: An example of an experiment with analysis of cell motion showing a beating rate of 120/min with [Ca$^{2+}$], of 1.8 mM, a regular fast rhythm at a rate of 240 beats/min (tachyarrhythmia) with [Ca$^{2+}$], of 5.4 mM, irregular fibrillatory beating activity at [Ca$^{2+}$], of 10.8 mM, and reversion to regular and slow beating activity with [Ca$^{2+}$], of 0.25 mM. Note time scale of 10 mm/sec. The variation of the amplitude of cell motion in the myocytes superfused with different levels of extracellular calcium probably reflects limited sensitivity of the photodiode placed on the cell to record motion. Variations of the apparent amplitude of motion appear to be related to methodological factors and do not directly relate to the magnitude of the calcium transients. Middle panel: Recordings from another experiment. As the [Ca$^{2+}$], was elevated from 1.8 to 10.8 mM, there was an increase in the [Ca$^{2+}$], level and in the rate of [Ca$^{2+}$], transients. At 5.4 mM [Ca$^{2+}$], rapid and regular [Ca$^{2+}$], transients (equivalent of tachyarrhythmia) are present; at 10.8 mM, chaotic [Ca$^{2+}$], fluctuations (equivalent of fibrillatory beating activity) are noted. Reduction of the [Ca$^{2+}$], to 0.25 mM reduced the [Ca$^{2+}$], transients and abolished the arrhythmia, resulting in regular [Ca$^{2+}$], transients. Please note difference in time scales. Bottom panel: Recordings showing that elevation of [Ca$^{2+}$], from 1.8 to 10.8 mM increased the [Ca$^{2+}$], and evoked rapid and irregular [Ca$^{2+}$], transients. Addition of 1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetracetic acid (BAPTA)-AM (5 μM) markedly decreased the elevated [Ca$^{2+}$], and reversed the arrhythmic [Ca$^{2+}$], transients despite the continued presence of an [Ca$^{2+}$], of 10.8 mM. Please note difference in time scales.
model is that it has an immature sarcoplasmic reticulum.36 However, this preparation is responsive to ryanodine, a pharmacological inhibitor of calcium fluxes across the sarcoplasmic reticulum, as shown in the present study. The neonatal myocyte does not dedifferentiate in culture and has normal β-adrenergic receptor subtypes and responsiveness.37

Spontaneous beating activity and arrhythmia are associated with rapid and large changes of [Ca²⁺], during systole and diastole on a beat-to-beat basis in myocardial aggregates, as demonstrated in the present study. Accurate evaluation of these rapid alterations of [Ca²⁺], and characterization of the interrelations between [Ca²⁺] and arrhythmia, therefore, require measurement of [Ca²⁺] with a high degree of temporal resolution and utilization of a spontaneous beating cardiac preparation. In our study, we used a microprocessor-controlled microspectrofluorimeter with a temporal resolution of 2 msec and the fluorescent indicator fura 2. These methods are sensitive enough to determine systolic and diastolic [Ca²⁺]. We acknowledge that fura 2 does produce some buffering of [Ca²⁺], but so does every fluorescent calcium indicator. The ability to measure [Ca²⁺], from a small group of approximately five to 10 cells enables estimation of [Ca²⁺] at or near the site of initiation of the arrhythmia. Using the intervention of increasing [Ca²⁺], concentration, we were able to demonstrate an increase in [Ca²⁺], during systole and diastole before alteration in inherent beating rate; the increase in [Ca²⁺], was followed by the onset of small [Ca²⁺], oscillations during diastole, from which a spontaneous arrhythmia could occur. In the spontaneously beating heart or cellular preparation, a major question is whether there is simply an increase in beating rate with development of tachyarrhythmia or whether one can show a specific calcium-mediated mechanism, such as [Ca²⁺], oscillation, directly resulting in a rapid beating rate. In this isolated beating preparation, we resolve this issue. [Ca²⁺], elevation was also associated with the development of chaotic or fibrillatory beating activity in the multicellular preparation, an arrhythmia profile similar to the fibrillatory beating activity found in vivo hearts. Thus, the isolated myocyte preparation with cell-to-cell communication may represent a useful model for the study of mechanisms of arrhythmogenesis.

Arrhythmia occurred when the [Ca²⁺], concentration during systole reached levels of 1–3 μM. These values may be relevant, since similar values have been recorded in isolated hearts subjected to acute myocardial ischemia.15 The development of calcium-mediated arrhythmia is not peculiar to the isolated neonatal myocyte, since similar arrhythmia occurs in adult ventricular myocardium. In the in vivo heart, the effect of elevated [Ca²⁺], has been proposed to be mediated by a direct or indirect action of Ca²⁺ on sympathetic nerves.38,39 The experiments described herein provide evidence for a direct action of Ca²⁺ in cardiac myocytes on arrhythmia development, since the perfusion medium was free of catecholamines and the myocytes were bereft of neural innervation. Koretsune and Marban40 showed an unchanged [Ca²⁺], level at the initiation of ventricular fibrillation but elevated levels after the development of arrhythmia induced by burst-pacing in isolated beating

**Figure 4.** Top panel: Recording showing that increasing the [Ca²⁺], from 1.8 to 10.8 mM increased the maximal level of [Ca²⁺], from −700 nM to −1.5–2.0 μM before any change in the beating rate. With elevation of the [Ca²⁺], level, [Ca²⁺], oscillations interrupted the decay process of the [Ca²⁺], transients and could initiate a spontaneous tachyarrhythmia (fourth beat with [Ca²⁺], of 10.8 mM). Bottom panel: Recordings showing that, at [Ca²⁺], of 5.4 mM, the premature occurrence of a [Ca²⁺], transient (second beat) abbreviates the complete decay of the preceding [Ca²⁺], transient, thereby elevating the minimal level of [Ca²⁺],. The maximal level of [Ca²⁺], is not elevated. As the decay of the transient is prolonged (fourth to fifth beat), the minimal level of [Ca²⁺], returns to a lower level.
These authors proposed that elevation of [Ca\textsuperscript{2+}] was unlikely to be the initiator but, rather, was the result of the arrhythmia. However, in the latter study, [Ca\textsuperscript{2+}] was measured by spectral shifts over 1–10 minutes of the probe, 5F-BAPTA, as detected by nuclear magnetic resonance spectroscopy. Since the latter technique has relatively poor temporal and spatial resolution, it might not detect changes of [Ca\textsuperscript{2+}], or oscillations of [Ca\textsuperscript{2+}], at the initiation of arrhythmia. The present study emphasizes the importance of measuring changes of [Ca\textsuperscript{2+}], in real time and with a high degree of temporal resolution to resolve the potential role of small changes in [Ca\textsuperscript{2+}], that may directly mediate arrhythmia. A recent report\textsuperscript{41} shows that diastolic calcium was increased before the development of ventricular fibrillation in isolated rat or hamster hearts perfused with high calcium, catecholamines, or digoxin.

Further evidence of the role of [Ca\textsuperscript{2+}] in arrhythmogenesis was provided by the observation that reduction of the elevated [Ca\textsuperscript{2+}] level could result in reversal of the arrhythmia. Reducing [Ca\textsuperscript{2+}], concentration from 10.8 to 0.25 mM decreased the elevated levels of [Ca\textsuperscript{2+}], and abolished the arrhythmia. Since the increase and subsequent decrease of [Ca\textsuperscript{2+}], occurred in concert with elevation and subsequent reduction of [Ca\textsuperscript{2+}], it is possible that arrhythmia was related to some mechanism by which high [Ca\textsuperscript{2+}], perturbs the cell membrane at its outer surface. Alternatively, it may have been caused by elevation of another ion, such as the intracellular accumulation of sodium. To exclude the latter possibilities, we used BAPTA-AM, which allows manipulation of [Ca\textsuperscript{2+}], without alteration of [Ca\textsuperscript{2+}]. It is known from voltage-clamp procedures that chelation of [Ca\textsuperscript{2+}], does not decrease and may actually increase the slow calcium current. With an elevated [Ca\textsuperscript{2+}], of 10.8 mM, BAPTA-AM decreased the elevated maximal levels of [Ca\textsuperscript{2+}], and abolished the arrhythmia. The latter finding provides additional evidence that it is the elevation of [Ca\textsuperscript{2+}], rather than [Ca\textsuperscript{2+}], that mediates the arrhythmia in this experimental model.

In the setting of [Ca\textsuperscript{2+}], elevation, the onset of spontaneous tachyarrhythmia caused an immediate and marked increase in [Ca\textsuperscript{2+}] levels. The magnitude of the increase of [Ca\textsuperscript{2+}], with tachyarrhythmia was such that [Ca\textsuperscript{2+}] concentrations during systole and diastole almost doubled, and values during diastole were similar to those present during systole under control conditions. Although [Ca\textsuperscript{2+}], elevation has been previously reported to occur in response to an increase in beating rate, both the magnitude of the increase of [Ca\textsuperscript{2+}], and the association with a spontaneous increase in beating rate have not been charac-

![Figure 5](https://circres.ahajournals.org/)

**FIGURE 5.** Tracings representing membrane potential and membrane current. In the top left panel, with [Ca\textsuperscript{2+}], of 1.8 mM, there are no current fluctuations. In the top right panel, with an elevated [Ca\textsuperscript{2+}], of 10.8 mM, transient inward current (TI) deflection occurred at holding potential of −40 mV. In the bottom panel with [Ca\textsuperscript{2+}], of 10.8 mM plus addition of 0.1 μM ryanodine (which inhibits Ca\textsuperscript{2+} fluxes across the sarcoplasmic reticulum), TI current deflections are abolished.
terized. The relevance of our findings of the marked increase of \([\text{Ca}^{2+}]_i\), with arrhythmia development is that \([\text{Ca}^{2+}]_i\) may be able to perpetuate arrhythmia. In the in vivo heart, ventricular premature beats and tachycardia may degenerate into ventricular fibrillation. One postulate from our findings is that induction of \([\text{Ca}^{2+}]_i\) elevation by the tachyarrhythmia could represent a mechanism whereby ventricular tachycardia degenerates into ventricular fibrillation in the in vivo heart. Furthermore, repeated bouts of marked \([\text{Ca}^{2+}]_i\), elevation with tachyarrhythmia may result in postarrhythmic reversible contractile dysfunction, as is reported to occur with the stunned myocardium. Stefenelli et al.\(^{41}\) recently reported that ventricular premature beats led to an increase in \([\text{Ca}^{2+}]_i\), and could initiate ventricular fibrillation in isolated perfused rat or hamster hearts subjected to catecholamine stimulation, high calcium, or digoxin.

Spontaneous tachyarrhythmia was associated with increases in both the minimal and maximal levels of \([\text{Ca}^{2+}]_i\). Since calcium influx occurs during the plateau phase of each cardiac action potential, a faster beating rate results in a greater increase in the rate of calcium uptake by cardiac tissue.\(^{32}\) In addition, with a faster beating rate, intracellular sodium increases, and this may predispose to \([\text{Ca}^{2+}]_i\), elevation via a \(\text{Na}^+\text{-Ca}^{2+}\) exchange antiport system. The increased \([\text{Ca}^{2+}]_i\), loading at high \([\text{Ca}^{2+}]_i\), and the increased rate of beating probably limits the capacity of the sarcoplasmic reticulum to take up \(\text{Ca}^{2+}\) because it is overloaded. This eventually results in oscillatory \(\text{Ca}^{2+}\) release from the sarcoplasmic reticulum.

Increased \([\text{Ca}^{2+}]_i\), has been shown to depolarize the isolated neonatal rat heart cell by way of a nonspecific transient inward current flowing through a \(\text{Ca}^{2+}\)-activated cation channel,\(^{35}\) thereby mediating enhanced pacemaker activity. In the present study, membrane repolarization after a voltage-clamp depolarization in single cardiac myocytes was associated with fluctuations of membrane potential (manifested by transient inward deflections in the holding potential) characteristic of transient inward current that was inhibited with ryanodine (an agent that blocks calcium fluxes across sarcoplasmic reticulum). Cannell and coworkers\(^{43}\) have previously reported that ryanodine blocks \([\text{Ca}^{2+}]_i\), oscillations in heart muscle. Recently, Berlin et al.\(^{37}\) have observed that the occurrence of transient inward current in single rat ventricular myocytes with \([\text{Ca}^{2+}]_i\), overload was often associated with propagating regions of increased \([\text{Ca}^{2+}]_i\), that arose from discrete sites of origin within the cell, consistent with the sarcoplasmic reticulum. Both \([\text{Ca}^{2+}]_i\), oscillations and spontaneous contractile waves are considered to result from the premature release and sequestration of \(\text{Ca}^{2+}\) from intracellular stores, specifically sarcoplasmic reticulum.\(^{1,11}\) In our study, elevated \([\text{Ca}^{2+}]_i\), increased \([\text{Ca}^{2+}]_i\), could evoke oscillations of internal calcium, and induced tachyarrhythmia and fibrillatory beating activity. \([\text{Ca}^{2+}]_i\), elevation, by inducing premature oscillations of \(\text{Ca}^{2+}\) from the sarcoplasmic reticulum, may evoke depolarizing afterpotentials and result in triggered automaticity.\(^{44}\) Our findings suggest an arrhythmogenic mechanism consistent with the above proposal. It should be noted that one limitation of our study is that measurement of the transient inward current was made in single cells at a stage before cell-to-cell communication (2 days in culture), whereas the \([\text{Ca}^{2+}]_i\), measurements were obtained in a small group of myocytes with cell-to-cell communication (3 days in culture). Accordingly, we cannot exclude the possibility that arrhythmia was caused by a mechanism other than the arrhythmogenic transient inward current. Elevation of \([\text{Ca}^{2+}]_i\), has been proposed to cause cell-to-cell uncoupling, which is another mechanism that may mediate arrhythmia. A recent preliminary study\(^{45}\) in a neonatal rat ventricular myocyte preparation (with cell-to-cell communication) exposed to concentrations of \([\text{Ca}^{2+}]_i\), >7 mM reported failure of transfer of fluorescent dye between asynchronously beating cells, suggestive of cell-to-cell uncoupling. Our study shows that \([\text{Ca}^{2+}]_i\), elevation induces both a rapid increase in beating activity that is regular and also causes chaotic fibrillatory beating activity. Cell-to-cell uncoupling is less likely to mediate the regular and rapid tachyarrhythmia but may cause each cell to beat independently of the other, resulting in chaotic beating activity. Thus, oscillatory afterpotentials could mediate a rapid increase in beating rate, and cell-to-cell uncoupling could cause asynchronous beating activity. Therefore, both mechanisms could play a role in the generation of the arrhythmias described in our study. Our findings showing that reduction of \([\text{Ca}^{2+}]_i\), with BAPTA decreases the rapid beating rate and the chaotic beating activity assumes greater importance, since it addresses the potential cause of the arrhythmia.

The present study provides additional and new evidence supporting the hypotheses that 1) \([\text{Ca}^{2+}]_i\), elevation may play an important role in arrhythmia development and 2) arrhythmia induces an abrupt and immediate increase in \([\text{Ca}^{2+}]_i\), levels that can perpetuate arrhythmia. These results may have relevance to certain arrhythmias associated with calcium overload, although other types of arrhythmias may be produced by different mechanisms.

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