Modulation of Ca\textsuperscript{2+} Release in Cultured Neonatal Rat Cardiac Myocytes

Insight From Subcellular Release Patterns Revealed by Confocal Microscopy

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**Abstract** It is well established that in heart muscle the influx of Ca\textsuperscript{2+} through Ca\textsuperscript{2+} channels during the action potential is the main trigger for Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR), but intact cardiac tissue and single myocytes are also known to exhibit spontaneous Ca\textsuperscript{2+} release from the SR under a variety of circumstances. Although conditions favoring spontaneous activity have been examined extensively, mechanisms modulating or regulating spontaneous as well as triggered Ca\textsuperscript{2+} release are still largely unknown. Using the high spatial and temporal resolution of laser-scanning confocal microscopy, we investigated subcellular aspects of spontaneous and triggered Ca\textsuperscript{2+} release in isolated rat neonatal myocytes loaded with the Ca\textsuperscript{2+}-sensitive fluorescent dye fura 2. Three distinct patterns of spontaneous Ca\textsuperscript{2+} release were identified: (1) a homogeneous Ca\textsuperscript{2+} release, presumably corresponding to Ca\textsuperscript{2+} release during a spontaneous action potential, (2) a focal or spatially restricted Ca\textsuperscript{2+} release with no or only limited subcellular propagation, and (3) a Ca\textsuperscript{2+} release propagating as a wave throughout the entire cell. Pharmacologic tools that interfere with the SR revealed that all release types were critically dependent on the Ca\textsuperscript{2+} release and uptake function of the SR. From our results we conclude that the probability, extent, and pattern of Ca\textsuperscript{2+} release are modulated on the subcellular level. The observed spectrum of release patterns can be explained by a space- and time-dependent variability in the spontaneous Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release mechanism within an individual myocyte. Presumably, this variability depends on the existence of subcellular functional elements of the SR. The actual degree of positive feedback may be modulated locally by the Ca\textsuperscript{2+}-loading state of each SR element. *(Circ Res. 1994;74:979-990.)*

**Key Words** • confocal Ca\textsuperscript{2+} imaging • modulation of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release • heart muscle

In mammalian cardiac myocytes, the release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR) is the principal link between electrical excitation of the sarcolemma and mechanical activation of the myofilaments (for reviews see References 1 and 2). Under physiological conditions, the Ca\textsuperscript{2+} release from the SR is triggered by Ca\textsuperscript{2+} influx via Ca\textsuperscript{2+} channels of the sarcolemma.\textsuperscript{3} This signal transduction mechanism is called Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR)\textsuperscript{4} (for review see Reference 6). The increase of [Ca\textsuperscript{2+}]\textsubscript{i} is reversed by the combined activity of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange of the plasma membrane and the sarcoplasmic and sarcolemmal Ca\textsuperscript{2+} pumps. A signaling system with such a substantial amplification of the initial trigger is expected to show some spontaneous activity. This tendency for spontaneous activity may result from occasional openings of SR Ca\textsuperscript{2+}-release channels even under resting conditions.\textsuperscript{7} Indeed, spontaneous Ca\textsuperscript{2+} release is known to occur under various conditions of cellular "Ca\textsuperscript{2+} overload" and has pathophysiological implications for arrhythmias (eg, during intoxication with cardiac glycosides).\textsuperscript{8} The arrhythmogenic activity of cardiotonic steroids has been attributed to delayed afterdepolarizations (for review see References 9 and 10). These afterdepolarizations result from transient inward currents activated by spontaneous Ca\textsuperscript{2+} release under conditions of Ca\textsuperscript{2+} overload.\textsuperscript{11,12} However, the term Ca\textsuperscript{2+} overload of the cell or SR is not yet clearly defined experimentally. It has been used as a descriptive term to explain phenomena induced by conditions that are believed to increase the cellular and SR Ca\textsuperscript{2+} content.

More than a decade ago, spontaneous activity was observed indirectly by analyzing scattered light intensity fluctuations in intact cardiac tissue.\textsuperscript{13,14} After the development of video-imaging techniques for direct intracellular Ca\textsuperscript{2+} measurements in single cells, spatial aspects of triggered and spontaneous Ca\textsuperscript{2+} release from the SR have been investigated (eg, see References 15 and 16). Generally, two types of Ca\textsuperscript{2+} release were distinguished: (1) A spatially homogeneous Ca\textsuperscript{2+} release that simultaneously developed within the entire cell was usually triggered by Ca\textsuperscript{2+} current (during an action potential or a voltage-clamp depolarization).\textsuperscript{17} (2) A Ca\textsuperscript{2+} signal propagating as a wave throughout the entire cell was regularly observed in Ca\textsuperscript{2+}-overloaded cardiac myocytes.\textsuperscript{15,18}

We used laser-scanning confocal microscopy to investigate patterns of Ca\textsuperscript{2+} signals in three different experimental settings: (1) Ca\textsuperscript{2+} release triggered by Ca\textsuperscript{2+} current in voltage-clamped single cardiac myocytes, (2) spontaneous Ca\textsuperscript{2+} release in the absence of Ca\textsuperscript{2+}-overload conditions, and (3) spontaneous Ca\textsuperscript{2+} release under conditions expected to induce Ca\textsuperscript{2+} overload. It is proposed that the different patterns of spontaneous Ca\textsuperscript{2+} release may be explained by a CICR mechanism.
exhibiting a variable degree of positive feedback. The Ca\(^{2+}\)-loading state of the SR may determine the propagation distance of the resulting Ca\(^{2+}\) wave by modulating the degree of positive feedback. The analysis of the observed Ca\(^{2+}\)-release patterns also suggests that the positive feedback does not remain constant within a single cell but can change in space and time.

Materials and Methods

Cell Preparation

Myocytes were isolated and cultured from neonatal 2-day-old Wistar rats according to a method described previously.\(^{19}\) Briefly, after enzymatic isolation a single droplet of cell suspension was transferred onto a glass coverslip placed in a small Petri dish. All dishes were stored at 37°C in a CO\(_2\) incubator (2% CO\(_2\)). Cells used for the experiments were between 1 and 5 days in culture. No age-dependent differences in the experimental results could be detected. All experiments were performed at room temperature (20°C to 23°C).

Solutions

Unless stated otherwise, the experiments were carried out in a superfusion solution containing (mmol/L) NaCl 140, KCl 4, CaCl\(_2\) 2, MgCl\(_2\) 1, glucose 10, and HEPES/NaOH 10 adjusted to pH 7.4. Caffeine (Sigma Chemie), ryanodine (Penick), and thapsigargin (Calbiochem AG) were added to the external solution in the required amounts from concentrated stock solutions. The pipette filling solution contained (mmol/L) cesium aspartate 120, NaCl 5, MgCl\(_2\) 1, Mg\(^{2+}\)-ATP 5, K\(_2\)-fluoro 3 0,1, and HEPES/CSOH 10 adjusted to pH 7.2. Mg\(^{2+}\)-ATP was purchased from Fluka AG, and K\(_2\)-fluoro and fluo 3 acetoxy methyl ester (fluo 3-AM) were obtained from Molecular Probes. Rapid (>500 milliseconds) changes of external solution were performed using a multibarreled pipette with a common opening.\(^{20}\)

Loading of the Ca\(^{2+}\) Indicator Fluo 3

For voltage-clamp experiments, the Ca\(^{2+}\) indicator was dialyzed into the cell via the patch pipette after rupture of the membrane patch. The cells were allowed to load with fluo 3 for at least 2 minutes. In all other cases, the cells were loaded with the ester form of the dye (fluo 3-AM). For this purpose, the cells were incubated at 20°C to 23°C with 1 to 2 mmol/L fluo 3-AM (diluted from a 5 mmol/L stock solution of fluo 3-AM in dimethyl sulfoxide (Fluka AG) with 25% pluronic F-127 (Molecular Probes). After a loading period of 20 minutes, the solution was exchanged with a dye-free solution, and the cells were allowed to deesterify the indicator for an additional 20 minutes. The low ester concentration, together with the low temperature during the loading process, was chosen to minimize problems arising from compartmentalization of the indicator.\(^{21,22}\)

Confocal Ca\(^{2+}\) Measurements and Image Analysis

The equipment for confocal fluorescence measurements has been described in detail recently.\(^{23}\) Briefly, the confocal imaging system (MRC-600, BioRad, Glattbrugg, Switzerland) with an Argon-ion laser (available excitation wavelengths, 488 and 514 nm) was attached to an inverted microscope (Nikon TMD, Diaphot). This setup was shielded by a Faraday cage and mounted on an air-driven vibration-isolated table. In all experiments, the 488-nm line of the laser was used to excite the Ca\(^{2+}\) indicator fluo 3, and the emitted fluorescence was collected at wavelengths of >515 nm. Image acquisition and analysis were performed as described previously.\(^{24}\) Briefly, two different modes of the confocal microscope were used for fluorescence imaging. Two-dimensional confocal images were acquired by scanning an image of 192×192 pixels at the highest possible rate. This procedure resulted in an image-acquisition rate of ~8 frames per second. The distortion of moving waves in these images was negligible. It takes ~125 milliseconds to scan an image corresponding to ~125 μm. The wavelength of a Ca\(^{2+}\) wave was ~15 μm and was therefore scanned in 15 milliseconds. During this time period, the wave propagates ~1 μm (propagation velocity, ~65 μm/s). Therefore, the wavelength was overestimated or underestimated by ~6%, depending on the direction of wave propagation. The images were stored on an S-VHS videotape for later off-line analysis (VHR-6850H HQ, Sony). The resulting nonlinearity of the gray scale images was corrected with software running on an Apple computer.

For high temporal resolution, the line-scan mode of the setup was used (see Fig 1A). A single line selected from the confocal image was scanned repeatedly (up to a frequency of 250 Hz). The acquired lines were arranged successively in a top-down order to build up the line-scan image. Cells with detectable motion artifacts were excluded from the study. For image sequences and line plots, local inhomogeneities of the fluo 3 concentration were accounted for by calculating fluo 3 pseudoratios (ie, fluorescence normalization with the resting fluorescence [F/F\(_{rest}\)]), as suggested when fluo 3 was initially introduced.\(^{24}\) Analysis of the confocal sequences was performed in several steps. Each frame in an image sequence was digitized from the videotape (editing recorder BR-S810E, JVC) with a frame grabber card (RasterOps 24STV) installed in an Apple Macintosh IIfx computer (Industrade Inc). The images were stored on a magneto-optical disk (REQ-650, Pinnacle Micro Inc). For long-time storage, a digital audiotape (DAT, DA-7, APS Inc, Independence, Mo) was used. Final analysis and visualization of the acquired data were performed with an image-processing software package (NIH Image). Three-dimensional surface plots of the confocal data were generated on a SPARCstation (SUN Microsystems Inc) running a data-analysis package (PV-WAVE, Moor Data, Regensdorf). All images were reproduced with a slide maker (Agfa PCR II, ComputerGraphix AG).

Results

Ca\(^{2+}\) Current Triggers Homogeneous Ca\(^{2+}\) Release

During physiological excitation-contraction coupling (EC coupling), Ca\(^{2+}\) release from the SR is triggered by influx of Ca\(^{2+}\) through Ca\(^{2+}\) channels (for review see Reference 1) and Na\(^+\)-Ca\(^{2+}\) exchange after activation of Na\(^+\) current.\(^{25,26}\) To characterize this type of release, Ca\(^{2+}\) currents were elicited by depolarizing voltage-clamp pulses. Single neonatal cardiac cells were held at a membrane potential of ~50 mV to inactivate the Na\(^+\) current, and voltage steps to +5 mV were applied to elicit Ca\(^{2+}\) currents.

Since three-dimensional surface plots are frequently used to present data in the present study, Fig 1A illustrates the method of data acquisition and processing. Briefly, a single line within the cell was chosen from the cell image (Fig 1A, left) and repeatedly scanned with a frequency of up to 250 Hz. The recorded lines were successively ordered to build up the line-scan image representing one spatial dimension (x) and time (t) running downward (Fig 1A, center). To facilitate the interpretation of line-scan images, we calculated three-dimensional surface plots by encoding the fluorescence intensity in the height of each pixel (i; Fig 1A, right). For the line plots, the same data were processed with a different technique. To compensate for inhomogeneities in the fluo 3 distribution, the fluorescence was normalized with the resting fluorescence (see “Materials and Methods” for details).

The fluo 3 fluorescence transient resulting from a voltage-clamp depolarization is shown in Fig 1B and 1C.
From the three-dimensional surface plot, it is obvious that the onset of the Ca²⁺ transient was simultaneous within the entire scanned line (at least within the temporal resolution of the line scan). A line plot of the time-dependent fluorescence changes is shown in Fig 1C. Here, the fluo 3 ratio measured in two different regions of the cell is illustrated (labeled 1 and 2 in Fig 1B). This type of Ca²⁺-release pattern was defined as a homogeneous Ca²⁺ release, because the Ca²⁺ transients peaked at the same time in all subcellular regions and the signals exhibited a similar time course.

The apparent "broadening" of the cell in this and some of the following examples is due to the fact that the fluorescence levels of the cell borders were indistinguishable from the background fluorescence and therefore seemed to blend into the background. Thus, during a transient increase in fluorescence, peripheral subcellular regions appear to broaden the cell in the line-scan image.

**Spontaneous Ca²⁺ Release in the Absence of Ca²⁺ Overload**

In solutions with a low [Ca²⁺] (2 mmol/L), few cells exhibited spontaneous activity, with Ca²⁺ transients closely resembling those of voltage-clamped cells. The homogeneous and simultaneous release observed in these cells resulted in signals with spatial and temporal characteristics identical to those in Fig 1 (not shown). This similarity suggests that spontaneous action potentials triggered the Ca²⁺ transients. However, other cells from the same batch showed a completely
different pattern of spontaneous Ca\(^{2+}\) release. The cell in Fig 2 exhibited Ca\(^{2+}\) transients that did not propagate through the entire cell but remained subcellularly localized. The line plots enable direct comparison of the signals recorded from different regions of the scanned line. Each Ca\(^{2+}\) transient that remained spatially restricted contributed to the whole-line fluorescence (and presumably also to the whole-cell fluorescence) to a different degree. Since no signal characteristic for homogeneous Ca\(^{2+}\) release was found, we attributed these properties to a focal release with spatially restricted or limited propagation. This result indicates that spontaneous Ca\(^{2+}\) release from the SR also occurred without experimental conditions inducing Ca\(^{2+}\) overload. Since the precise Ca\(^{2+}\) load in our cells was unknown, we could not decide whether the cells exhibiting focal release were actually Ca\(^{2+}\)-overloaded or not. However, we could obtain information about the influence of the loading state by comparing release patterns under different Ca\(^{2+}\)-loading protocols. Therefore, in the next set of experiments, we exposed the cells to conditions favoring Ca\(^{2+}\) overload by elevating [Ca\(^{2+}\)].

**Ca\(^{2+}\)-Overload Conditions Induce Different Ca\(^{2+}\)-Release Patterns**

After increasing [Ca\(^{2+}\)] to 10 mmol/L, >80% of the cells became spontaneously active. A sequence of nine confocal images separated by 125 milliseconds was recorded from a cardiac myocyte exhibiting spontaneous wave-type Ca\(^{2+}\) release (Fig 3A). The Ca\(^{2+}\) transient originated in a focal Ca\(^{2+}\) release and subsequently propagated through the entire cell. Waves exhibiting the same properties were also recorded at higher temporal resolution in the line-scan mode of the confocal microscope (Fig 3B and 3C). Line plots of the fluo 3 signal reveal that the Ca\(^{2+}\) transient appeared at three different regions within the cell in a successive order. The dotted line connects the peaks of the transients. The propagation velocity determined from the sequence was -65 μm/s.

In ~60% of the spontaneously active myocytes, the Ca\(^{2+}\) release had properties strikingly different from the wave-type pattern. In contrast to the propagation of Ca\(^{2+}\) along the entire cell as a Ca\(^{2+}\) wave, the Ca\(^{2+}\) transients remained spatially restricted (Fig 4A). The limited propagation of this focal type of Ca\(^{2+}\) release is even more evident in the line scan (Fig 4B and 4C). Three different domains in this cell showed focal Ca\(^{2+}\) release independent from one another. The absence of a temporal correlation between the localized Ca\(^{2+}\) transients in this cell is also apparent from the line plot.

Are focal- and wave-type Ca\(^{2+}\) release different entities, or is there a common mechanism responsible for the two patterns? The following findings suggest that both phenomena are manifestations of the same process with different functional states and are not determined by the ultrastructure of a particular myocyte. In ~30% of all myocytes exhibiting focal Ca\(^{2+}\) release with limited propagation, the release pattern occasionally changed to the wave-type Ca\(^{2+}\) release after a period of focal activity. In the example shown in Fig 5, the Ca\(^{2+}\)
transients were spatially restricted and not correlated during the first few seconds, properties characteristic for focal-type Ca\(^{2+}\) release with limited propagation. After this initial period, a large Ca\(^{2+}\) transient did not remain focal but propagated along the entire line, typical for a wave-type Ca\(^{2+}\) release. Immediately after the Ca\(^{2+}\) wave, spontaneous activity was suppressed for a variable time, after which focal activity resumed. The apparently spontaneous transition from focal to wave-type Ca\(^{2+}\) release, and vice versa, confirms that an individual cell can exhibit either one of the two release patterns and can intermittently change from one to the other. No obvious increase of cytosolic resting [Ca\(^{2+}\)] preceded the spontaneous transitions, suggesting that the Ca\(^{2+}\) release itself may be regulated or modulated by the Ca\(^{2+}\)-loading state of the SR.

Since Ca\(^{2+}\)-release properties of neonatal myocytes in culture are not well characterized, we could not rule out the possibility that some Ca\(^{2+}\) transients were the result of Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores other than the SR, e.g., from inositol trisphosphate-sensitive Ca\(^{2+}\) stores found in heart cells of different species (see Reference 27). To test this possibility, we applied pharmacologic tools known to exhibit well-defined effects on Ca\(^{2+}\) stores. These tools should help to identify the type of Ca\(^{2+}\) store and the basic mechanisms underlying the different patterns of Ca\(^{2+}\) release.

**Ryanodine and Caffeine Suppress Ca\(^{2+}\) Release**

We used ryanodine for the identification of a ryanodine-sensitive Ca\(^{2+}\) store responsible for the spontane-
ous activity of cardiac cells. This substance is known to suppress Ca\(^{2+}\) release from the SR.\(^1\) Ryanodine (10 \(\mu\)mol/L) completely abolished spontaneous activity in neonatal myocytes within 6 minutes (Fig 6A). Before complete cessation of spontaneous Ca\(^{2+}\) release, the Ca\(^{2+}\) transients gradually declined in frequency and amplitude. These findings indicate that the spontaneous activity in neonatal cardiac cells was dependent on a ryanodine-sensitive Ca\(^{2+}\) store, most likely the SR.

For an additional characterization of spontaneous Ca\(^{2+}\) release, we investigated the effect of caffeine (1) on resting [Ca\(^{2+}\)], and (2) on Ca\(^{2+}\) release in the absence and presence of ryanodine (Fig 6B and 6C). Rapid application (\(t_{1/2}, <500\) milliseconds) of 20 mmol/L caffeine to a resting cell resulted in a homogeneous Ca\(^{2+}\) release from the SR resembling a Ca\(^{2+}\) transient triggered by Ca\(^{2+}\) current. After application of caffeine, [Ca\(^{2+}\)], increased rapidly and recovered slowly to the baseline despite the continued presence of caffeine (data not shown). Exposing a myocyte to 20 mmol/L caffeine during a period of frequent spontaneous Ca\(^{2+}\) release resulted in a similar transient, which was followed by a suppression of spontaneous activity during the presence of this compound (Fig 6B). After caffeine removal, spontaneous activity of the cells resumed with a variable delay. Repetitive brief exposures of a quiescent myocyte to caffeine evoked Ca\(^{2+}\) transients of uniform shape and amplitude (Fig 6C, left). After adding 10 \(\mu\)mol/L ryanodine for 1 minute, the train of caffeine applications was repeated. The amplitude of the Ca\(^{2+}\) transient was reduced threefold and subsequently decreased further until caffeine was no longer
Thapsigargin Inhibits Spontaneous and Caffeine-Induced Ca\(^{2+}\) Release

After release from the SR, Ca\(^{2+}\) is either removed from the cell by Na\(^+-\)Ca\(^{2+}\) exchange or pumped back into the lumen of the SR by a Ca\(^{2+}\) pump. In cardiac muscle, the application of thapsigargin (a SERCA inhibitor) prevents further spontaneous Ca\(^{2+}\) release and Ca\(^{2+}\) uptake into the SR. This results in a decrease in the amplitude of subsequent Ca\(^{2+}\) transients and a decrease in the frequency of Ca\(^{2+}\) release events. The lack of Ca\(^{2+}\) uptake into the SR also indicates that Ca\(^{2+}\) stores are depleted. Therefore, thapsigargin is a useful tool for investigating the role of Ca\(^{2+}\) release in cardiac muscle function.
muscle, thapsigargin is a specific inhibitor of the SR Ca\(^{2+}\) pump and prevents refilling of the SR.\(^{29,30}\) The effect of 150 nmol/L thapsigargin on spontaneous activity is shown in Fig 7A. Two minutes after the application of thapsigargin, no spontaneous activity could be monitored. Surprisingly, thapsigargin had a more graded effect on caffeine-induced Ca\(^{2+}\) release (Fig 7B). Despite the presence of thapsigargin, Ca\(^{2+}\) transients could still be evoked with caffeine, although with a lower amplitude and rate of relaxation. The finding that Ca\(^{2+}\) release can still be triggered by caffeine suggests that the Ca\(^{2+}\) store continues to contain some residual Ca\(^{2+}\) even after 2 minutes in thapsigargin. However, the reduced SR Ca\(^{2+}\) load appears to dramatically reduce the probability for spontaneous Ca\(^{2+}\) release, suggesting a modulatory role for [Ca\(^{2+}\)] in the lumen of the SR.

In summary, these results indicate that the Ca\(^{2+}\) store that is responsible for spontaneous Ca\(^{2+}\) release is ryanodine, caffeine, and thapsigargin sensitive. Since each of the three pharmacologic tools was able to completely suppress spontaneous Ca\(^{2+}\) release, we conclude that the SR and the CICR were the essential elements for the Ca\(^{2+}\) transients and all Ca\(^{2+}\)-release patterns we observed in the neonatal myocytes.

**Discussion**

In the present study, we report on properties of Ca\(^{2+}\)-release signals recorded with laser-scanning confocal microscopy in cultured neonatal rat cardiac myocytes. This technique offers a high spatial and temporal resolution and enabled us to investigate aspects of Ca\(^{2+}\) signaling on the subcellular level. Surprisingly, we found a variety of subcellular patterns of Ca\(^{2+}\) release not only among different spontaneously active cardiac myocytes but also within individual cells. Often, the Ca\(^{2+}\) transients were spatially restricted, but sometimes they propagated as Ca\(^{2+}\) waves over an intermediate distance or throughout the entire cell, most likely driven by the CICR mechanism.\(^{4,5,15,31,32}\) Since spontaneous transitions between different patterns were frequently found (in the time domain of seconds), the prevalence for a particular release pattern cannot be an inherent property of an individual myocyte and does not depend directly on the extracellular conditions. This finding is in contrast to the behavior of some nonexcitable cells. For example, the actual pattern of Ca\(^{2+}\) oscillations in insulinoma cells is believed to represent a characteristic property of a particular cell and has been called a “Ca\(^{2+}\) fingerprint.”\(^{33}\) The variability we found implies that the properties of the SR are spatially nonuniform on the subcellular level and change with time.

Since the propagation of subcellular Ca\(^{2+}\) waves depends on the regenerative nature of the CICR, the observed variability of Ca\(^{2+}\) wave propagation suggests that the degree of positive feedback (gain) inherent in the regenerative CICR is not constant at the subcellular level (see Reference 32).

**Variability of Positive Feedback in CICR**

The hypothesis of a variable gain has already been proposed on a supracellular level on the basis of a number of experimental observations. Several studies with voltage-clamped cardiac myocytes have revealed a close correlation between L-type Ca\(^{2+}\) current and the amount of Ca\(^{2+}\) released from the SR during EC coupling.\(^{3,34}\) When SR Ca\(^{2+}\) release in isolated myocytes was triggered by flash photolysis of caged Ca\(^{2+}\), the mechanical activity was related to the light energy.\(^{35}\) From these studies, it can be concluded that the degree of positive feedback in CICR has to be quite low. In contrast, other experimental results obtained under apparently similar conditions have indicated that the positive feedback has to be rather large. For example, in analogous voltage-clamp experiments, several authors found a considerably higher amplification of the Ca\(^{2+}\) release at voltages between −50 and −20 mV than at more positive potentials, although the release was still graded.\(^{36,37}\) Based on these and related observations, differences in the positive feedback were proposed to depend on membrane voltage.\(^{30}\) Furthermore, video-imaging systems for monitoring [Ca\(^{2+}\)] revealed the existence of Ca\(^{2+}\) waves in Ca\(^{2+}\)-overloaded cardiac myocytes.\(^{15,16}\) The waves were frequently initiated by a focal release in the periphery of the cell but then always propagated throughout the entire cell,\(^{18}\) again suggesting the presence of a large positive feedback. Recently, the observation of waves was confirmed in rat ventricular myocytes with confocal microscopy.\(^{31}\) It was then quite unexpected that in myocytes without Ca\(^{2+}\) overload, local contractions could be activated that did not propagate along the cell as a Ca\(^{2+}\) wave and did not
result in contraction of the entire cell, indicating a much lower positive feedback under these conditions. On the basis of this surprising finding, it was proposed that the degree of Ca\textsuperscript{2+} overload of the myocyte could be responsible for the variability in positive feedback observed under different experimental conditions.

**Feedback of CICR Is Also Variable on the Subcellular Level**

Present concepts of feedback modulation in cardiac Ca\textsuperscript{2+} signal transduction imply that each cell behaves as a single functional unit able to exhibit a different degree of positive feedback. However, the results presented in the present study suggest a more complex view of the processes modulating the function of the SR and the gain of CICR.

Can the degree of SR Ca\textsuperscript{2+} load explain the different Ca\textsuperscript{2+}-release patterns and the subcellular variability of positive feedback we observe? Or are several Ca\textsuperscript{2+}-release pools or mechanisms involved? We have to realize that there is no straightforward definition of Ca\textsuperscript{2+} overload in cardiac myocytes, because it is difficult to quantify the Ca\textsuperscript{2+} load experimentally. In particular, the SR Ca\textsuperscript{2+} content has not yet been assessed under different Ca\textsuperscript{2+}-loading conditions (but see Reference 39). But from our results, it becomes apparent that the Ca\textsuperscript{2+}-release pattern of a cardiac myocyte is not only determined by the extracellular conditions. We found focal Ca\textsuperscript{2+} release without propagation when the cells were in experimental solutions that promote Ca\textsuperscript{2+} overload of the cells. Furthermore, the coexistence of both release patterns (ie, focal and wave-type Ca\textsuperscript{2+} release) in a single cell indicated a complex modulation of CICR, resulting in a continuous spectrum of Ca\textsuperscript{2+}-release types. Transitions between different release patterns were not preceded by obvious changes in resting [Ca\textsuperscript{2+}]. Increasing the Ca\textsuperscript{2+} load appeared to increase the probability for propagated Ca\textsuperscript{2+} release while the spatially limited release type simultaneously became less frequent. This finding is in agreement with recent observations in adult rat ventricular myocytes. Propagation of locally activated Ca\textsuperscript{2+} release was found to depend on the Ca\textsuperscript{2+} load of the SR and was not associated with a higher level of diastolic [Ca\textsuperscript{2+}]. The notion of a variable positive feedback on the subcellular level is also supported by our observation of spontaneous Ca\textsuperscript{2+} release under conditions of a “physiological” [Ca\textsuperscript{2+}]. Although transitions appeared to occur in a random fashion, switching between several fairly stable patterns of Ca\textsuperscript{2+} release is not necessarily a probabilistic process. A similar behavior has also been observed “on the verge of chaos” in other complex systems.

The hypothesis of a modulatory effect of intraluminal [Ca\textsuperscript{2+}] in cardiac myocytes is also consistent with the reported modulation of SR Ca\textsuperscript{2+} release in skeletal muscle SR vesicles. Additional evidence for the existence of a modulatory effect of intraluminal [Ca\textsuperscript{2+}] in cardiac myocytes comes from experiments on single SR-release channels incorporated into lipid bilayers and from our experiments. The inhibitory effect of thapsigargin on spontaneous Ca\textsuperscript{2+} release appeared to be mediated by a reduced luminal SR Ca\textsuperscript{2+} content, since thapsigargin has no direct effect on the SR release channel in cardiac myocytes.

How can the modulatory effect of intraluminal [Ca\textsuperscript{2+}] be mediated? Are there additional processes affecting the variability of the gain in CICR? Without taking into account spatial aspects, the basic mechanisms that modulate the overall gain of the SR generally can be divided into two categories: (1) mechanisms affecting the Ca\textsuperscript{2+}-release channel and (2) processes acting on the SR Ca\textsuperscript{2+} pump.

Besides a possible direct effect of intraluminal Ca\textsuperscript{2+} on the Ca\textsuperscript{2+}-release channel, Ca\textsuperscript{2+} may also act via a mechanism involving the sarcoplasmic Ca\textsuperscript{2+}-binding protein calsequestrin. In response to different levels of intraluminal [Ca\textsuperscript{2+}], calsequestrin may interact with the SR membrane–bound protein triadin. Triadin itself is believed to mediate an interaction between calsequestrin and the ryanodine receptor Ca\textsuperscript{2+}-release channel. This interaction may either increase the open probability of the release channel directly or increase the Ca\textsuperscript{2+} sensitivity. Additional mechanisms possibly controlling the gain of CICR on the level of the single release channel are described as follows: (1) Recently, the effect of the [Mg\textsuperscript{2+}], on cardiac and skeletal muscle EC coupling has been reviewed, but neither the exact level nor the kinetic changes of [Mg\textsuperscript{2+}], are known at present. Therefore, it is difficult to evaluate the role of [Mg\textsuperscript{2+}], for the Ca\textsuperscript{2+}-release pattern observed in the cardiac myocytes. (2) Recent biayer studies on reconstituted SR Ca\textsuperscript{2+}-release channels have indicated a marked increase of the Ca\textsuperscript{2+} sensitivity after phosphorylation of the channel protein, but at present the functional significance of these findings is not yet clear, and the process of phosphorylation and dephosphorylation may be too slow to explain the rapid switching between the focal and wave-type Ca\textsuperscript{2+}-release patterns we have observed.

The biochemical equivalent for the observed changes in positive feedback may also correspond to modifications of SR Ca\textsuperscript{2+}-pump function. The SR Ca\textsuperscript{2+} pump represents an element of negative feedback in the CICR mechanism. By removing Ca\textsuperscript{2+} from the cytosol, it can prevent the spread of the regenerative Ca\textsuperscript{2+} waves. The pump may be directly modulated by intraluminal [Ca\textsuperscript{2+}] (ie, the sarcoplasmic membrane [Ca\textsuperscript{2+}] gradient) or by phosphorylation of the pump itself (or by phosphorylation of regulatory proteins like phospholamban).

A possible participation of Ca\textsuperscript{2+} stores other than the SR was investigated with three different pharmacologic tools. Each of these compounds exhibits different and well-defined actions on SR Ca\textsuperscript{2+} release (caffeine and ryanodine) or Ca\textsuperscript{2+} uptake (thapsigargin). The sensitivity of Ca\textsuperscript{2+} release for these compounds indicates that all release patterns depend on a functional SR in terms of both its release and uptake properties. Taking these results together, the spatiotemporal complexity of the Ca\textsuperscript{2+}-release machinery is presumably a functional characteristic of the SR itself and related to the subcellular functional organization of this Ca\textsuperscript{2+} store. Thus, this system is far more complicated than the simplified view of a myocyte reacting as a single unit suggests.

**The Subcellular SR Network Has Microscopic Functional Elements**

We therefore propose that the SR in neonatal cardiac myocytes is a highly organized network of microscopic functional elements and that the degree of positive
feedback (gain) is determined on the level of these elements. Each of these elements has components capable of producing positive feedback (Ca\(^{2+}\)-release channels) but also able to generate negative feedback (ie, SR Ca\(^{2+}\) pumps). As outlined above, both components may be under modulation by the intraluminal [Ca\(^{2+}\)] in each SR element. As a consequence, the overall gain of CICR is the sum of both processes, resulting in a smoothly graded positive or negative feedback. During progressive Ca\(^{2+}\) loading of a particular element, the degree of positive feedback through the release channel would increase while the negative feedback provided by the Ca\(^{2+}\) pump would simultaneously decrease.

During Ca\(^{2+}\) uptake, the subcellular SR elements reach the threshold for spontaneous Ca\(^{2+}\) release with different delays. As a consequence of this diversity, the SR element reaching the threshold for spontaneous Ca\(^{2+}\) release first acts as a subcellular pacemaker and initially releases Ca\(^{2+}\) in a spatially restricted way.

It should be mentioned that different mechanisms for “spontaneous” Ca\(^{2+}\) release can be imagined. Ca\(^{2+}\) release may be initiated by an increase in the open probability of a single SR-release channel.43 This notion would characterize the spontaneous Ca\(^{2+}\) release as an inherent property of the subcellular SR network. Alternatively, Ca\(^{2+}\) release may only apparently be spontaneous but in fact may be triggered by Ca\(^{2+}\) from the cytosolic side. The appearance of propagating waves can more easily be reconciled with the latter mechanism but does not exclude the presence of the first mechanism (or both at the same time).

Are the distinct Ca\(^{2+}\)-release patterns in neonatal cardiac myocytes the result of differences in the particular morphology of the SR in developing cells? Several studies have revealed a close similarity of the ultrastructure with respect to the SR network in cultured neonatal and freshly isolated adult rat ventricular myocytes. It has been reported that mammalian myocardial cells in culture reconstruct an adultlike myotopic SR network around bundles of myofilaments.51 The neonatal cultured myocytes also possess intact T-tubule–SR junctions (diadic couplings) that are morphometrically similar to those found in adult cells.52 Although the structure of the SR network is certainly important for Ca\(^{2+}\) signaling, our results cannot be explained on morphological grounds alone, since we observed different patterns in individual cells including transitions between focal and propagating Ca\(^{2+}\)-release types. These transitions most likely reflect changes of the functional state of the SR.

How can the loading state of the SR elements modulate the propagation pattern of the ensuing Ca\(^{2+}\) wave? The initial focal Ca\(^{2+}\) release reaches neighboring SR elements by diffusion. If the positive feedback of these elements is large (ie, if their loading state is high), further propagation of the Ca\(^{2+}\) wave is supported. However, wave propagation can fail and terminate locally when this process is interrupted by one or few neighboring SR elements exhibiting low positive or even negative feedback (through the Ca\(^{2+}\) pump). The released Ca\(^{2+}\) may be removed from the cytosol close to the focus of release, because the spread of Ca\(^{2+}\) is prevented by Ca\(^{2+}\) uptake into surrounding SR elements. The corresponding microscopic patterns are either focal Ca\(^{2+}\)-release events without noticeable propagation or short waves that do not propagate throughout the entire cell.

When larger subcellular regions exhibit differences in CICR feedback, more complex phenomena can be observed during Ca\(^{2+}\) wave propagation in cardiac myocytes. These phenomena include subcellular spiral waves of Ca\(^{2+}\) that exhibit a drifting core.52 Core drift of spiral waves in excitable media is known to occur along regions with low positive feedback.53 Thus, from these studies in adult cardiac myocytes, it can be concluded that regions with low and high positive feedback (gain) can even coexist in a single cell, again indicating the existence of functional elements in the SR network.

A similar functional compartmentalization of the SR has recently been proposed in a mathematical model to explain several experimental observations of CICR and EC coupling in cardiac cells.54 In particular, this model could account for the apparent discrepancy between graded Ca\(^{2+}\) release (low gain) and spontaneous Ca\(^{2+}\) release with wave propagation (high gain). Although “common-pool” models could not be used to simulate the available data, a mathematical equivalent of a highly structured SR (“cluster-bomb” model) was able to explain the apparent discrepancy. We thus speculate that the functional SR elements we observed in the present study (ie, focal Ca\(^{2+}\) release) reflect the spatial organization of the SR network and possibly correspond to the proposed clusters. It should be pointed out that neither the “clusters” nor the functional “elements” proposed in the present study have a defined morphological correlate.

**Functional Compartmentation of the SR May Also Be Important During EC Coupling**

The observed spatial complexity of the SR network is not only crucial for the behavior of the SR during Ca\(^{2+}\) overload but may also have important implications during EC coupling. For example, it has been proposed that SR elements located in the subsarcolemmal space and in proximity to the Ca\(^{2+}\) entry sites (ie, Ca\(^{2+}\) channels) may sense a higher [Ca\(^{2+}\)] than SR elements located in the bulk cytosol and thus exhibit apparent high gain.55,56,57 Furthermore, Ca\(^{2+}\) uptake into the SR may also attenuate spurious influx or accidental release of Ca\(^{2+}\) and thus produce the amount of negative feedback necessary to stabilize the CICR mechanism.57

Consistent with this view, two functionally different Ca\(^{2+}\) stores have been observed in cultured atrial cells66,60 and were assigned to subsarcolemmal and central SR compartments, respectively.

In conclusion, the variability of Ca\(^{2+}\)-release patterns and Ca\(^{2+}\) wave propagation features suggests the existence of a spatially complex subcellular Ca\(^{2+}\)-signaling network. Different structures of the network can be characterized on the basis of morphological (diads) and mathematical (cluster-bomb theory56) criteria as well as on functional grounds (eg, focal release versus wave-type release; subsarcolemmal and central SR compartment55). The complex interaction of subcellular functional elements has to be considered to understand cardiac EC coupling and is also likely to be relevant to other intracellular signal transduction processes.

**Note added in proof:** While this manuscript was being reviewed, elementary Ca\(^{2+}\) release events were also
reported in adult rat ventricular myocytes (Science. 1993;262:740-744).

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