Luminal Ca\(^{2+}\) content regulates intracellular Ca\(^{2+}\) release in subepicardial myocytes of intact beating mouse hearts: effect of exogenous buffers

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Kornyeyev D, Reyes M, Escobar AL. Luminal Ca\(^{2+}\) content regulates intracellular Ca\(^{2+}\) release in subepicardial myocytes of intact beating mouse hearts: effect of exogenous buffers. Am J Physiol Heart Circ Physiol 298: H2138–H2153, 2010. First published April 9, 2010; doi:10.1152/ajpheart.00885.2009.—Ca\(^{2+}\)-induced Ca\(^{2+}\) release tightly controls the function of ventricular cardiac myocytes under normal and pathological conditions. Two major factors contributing to the regulation of Ca\(^{2+}\) release are the cytosolic free Ca\(^{2+}\) concentration and sarcoplasmic reticulum (SR) Ca\(^{2+}\) content. We hypothesized that the amount of Ca\(^{2+}\) released from the SR during each heart beat strongly defines the refractoriness of Ca\(^{2+}\) release. To test this hypothesis, EGTA AM, a high-affinity, slow-association rate Ca\(^{2+}\) chelator, was used as a tool to modify luminal SR Ca\(^{2+}\) content. An analysis of the cytosolic and luminal SR Ca\(^{2+}\) dynamics recorded from the epicardial layer of intact mouse hearts indicated that the presence of EGTA reduced the diastolic SR free Ca\(^{2+}\) concentration and fraction of SR Ca\(^{2+}\) depletion during each beat. In addition, this maneuver shortened the refractory period and accelerated the restitution of Ca\(^{2+}\) release. As a consequence of the accelerated restitution, the frequency dependence of Ca\(^{2+}\) release in subepicardial myocytes was modulated by the free Ca\(^{2+}\) concentration (28, 36) and by the rate of change of free Ca\(^{2+}\) in the cytosolic face of RyR2 (20, 25). The luminal protein calsequestrin (CSQ)\(_2\), which is a Ca\(^{2+}\)-binding protein inside the SR, has been also shown to play an important role in the luminal control of Ca\(^{2+}\) release (23, 24, 26, 42, 44). Therefore, one would expect that the characteristics of Ca\(^{2+}\) release can be modified by introducing an exogenous buffer. Indeed, Terentyev et al. (48) demonstrated that low-affinity Ca\(^{2+}\) buffers are able to regulate Ca\(^{2+}\) release from the SR by affecting the amplitude and regularity of spontaneous Ca\(^{2+}\)-release events in isolated ventricular myocytes.

Despite the accumulation of experimental data, several problems remain unsolved. For example, the role of luminal Ca\(^{2+}\) content in controlling the magnitude and dynamics of Ca\(^{2+}\) release is still poorly understood. Our progress in finding an answer to this question depends on the ability to assess intra-SR Ca\(^{2+}\) dynamics. The measurement of intra-SR Ca\(^{2+}\) with fluorescent dyes is a relatively new technique previously used only in isolated cells (38, 45). As a result, Ca\(^{2+}\) dynamics in the SR have not yet been characterized in the intact heart.

We applied the pulsed local field fluorescence (PLFF) technique (33) to assess cytosolic and SR luminal Ca\(^{2+}\) dynamics in the epicardial layer of intact beating mouse hearts. EGTA AM was used as a high-affinity, slow-binding Ca\(^{2+}\) chelator to study the effect of Ca\(^{2+}\) buffering on intracellular Ca\(^{2+}\) dynamics. EGTA allows Ca\(^{2+}\) released to the cytosol through RyR2 to diffuse to distances greater than other Ca\(^{2+}\) chelators like BAPTA, a high-affinity, fast-binding Ca\(^{2+}\) buffer (47). Therefore, unlike BAPTA, EGTA does not prevent the activation of neighboring RyR2\(_s\) (30), allowing the occurrence of regenerative Ca\(^{2+}\) release from the SR. Although the presence of EGTA is expected to reduce cytoplasmic Ca\(^{2+}\) transients by chelating Ca\(^{2+}\), an exogenous buffer may also affect the Ca\(^{2+}\) release process itself.

We hypothesized that the refractoriness of Ca\(^{2+}\) release depends on the amount of Ca\(^{2+}\) released from the SR during each heart beat. Therefore, we proposed that EGTA cannot only decrease Ca\(^{2+}\) release from the SR but also reduce the refractoriness of Ca\(^{2+}\) release due to a smaller degree of depletion of Ca\(^{2+}\) stores during each beat. The modification of the refractoriness of Ca\(^{2+}\) release will affect the development of Ca\(^{2+}\) alternans, which is potentially arrhythmogenic and is characterized by cyclic beat-to-beat variations in the amplitude of Ca\(^{2+}\) transients.

The main goal of this work was to define the mechanism of action of an exogenous buffer (EGTA AM) on cytosolic and intra-SR Ca\(^{2+}\) dynamics and on the refractoriness of Ca\(^{2+}\) release in the epicardial layer of an intact beating heart. The results described in this report imply that the luminal Ca\(^{2+}\) level plays a significant role in controlling SR Ca\(^{2+}\) release in the beating heart.

METHODS

Heart Preparation

Hearts were removed from young Swiss Webster mice (male, age: 3–7 wk old). The aorta was cannulated and connected to a horizontal Langendorff apparatus for constant perfusion with normal Tyrode solution (2 mM CaCl\(_2\), 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl\(_2\), 10 mM NaHCO\(_3\), 2.5 mM KH\(_2\)PO\(_4\), and 1.2 mM CaCl\(_2\)).
0.33 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM HEPES, and 10 mM glucose; pH 7.4). The temperature of the solution outside the heart was controlled with a Peltier unit. All of the solutions were equilibrated with 100% O<sub>2</sub>. This protocol was approved by the Institutional Animal Care and Use Committee of the University of California (Merced, CA).

Fluorophore Loading

Mag-fluo-4 AM (Invitrogen) was used to measure changes in the intra-SR Ca<sup>2+</sup> concentration. The dye was dissolved in 45–60 μl DMSO with 2.5% Pluronic and added to 1 ml normal Tyrode solution. Perfusion with dye started after the spontaneous heart rate became regular (within 10 min after cannulation). After 1 h of perfusion at room temperature (21–23°C), the solution was switched to normal Tyrode solution and the temperature was increased to 37°C within 10 min. The temperature increase induced the washing out of mag-fluo-4 from the cytosol, allowing us to measure intra-SR Ca<sup>2+</sup> signals [see Shannon et al. (45) for additional information on this technique]. In most cases, a downward fluorescence signal reflecting depletion of the SR was apparent even before the heart was warmed up, although some minor upward (cystosolic) component was still present. This upward component completely disappeared within 10–20 min after the temperature reached 37°C. After mag-fluo-4 was removed from the cytosol, a sufficient amount of dye remained inside the SR to generate detectable signals for at least 2 h.

Rhod-2 AM (Invitrogen) was used to measure Ca<sup>2+</sup> signals in the cytosol. Di-8-ANEPPS (Invitrogen) was used to measure the membrane potential in the epicardial layer of the hearts. These dyes were prepared and loaded in the same way as mag-fluo-4 AM. The time of loading was 25–35 min.

Optical Setup

A modified version of our custom-made PLFF microscopy setup (33) was used to measure Ca<sup>2+</sup> signals from the intact heart. A simplified scheme of the optical apparatus is shown in the Supplemental Material (Supplemental Fig. 1). Briefly, two solid-state YAG lasers were used as an illuminating source. A MGL-50B-1 CW Nd:YAG laser (Enlight Technologies, Branchburg, NJ) was used for the excitation of rhodamine-based dyes with green light (532 nm). For the excitation of fluorescein-based dyes, blue light (473 nm) was obtained from a MBL-10-3 CW Nd:YAG laser (Enlight Technologies). Both lasers were time multiplexed by two ferroelectric modulators (50075 Oriel optical shutters, Newport, Stratford, CT) and then optically mixed with ultrafast dichroic mirrors.

The excitation light pulses were focused by a standard microscope objective (×40, numerical aperture: 0.45) into a small multimode optical fiber for the transmission of the excitation light to the epicardial layers. Emitted light was carried back through the same fiber, filtered to eliminate the reflected excitation component, and then focused on an avalanche photodiode, which was connected to an integrating current-to-voltage converter controlled by a digital signal processor (DSP 320, Texas Instruments). Headstage units and their corresponding high-voltage power supply were manufactured by IonOptix (Milton, MA). Fluorescence signals were digitized at a sampling frequency of 5 MHz and filtered to a bandwidth of 500 kHz. The acquisition system was controlled by an Athlon-based PC with a custom-designed, G-based software program (LabView).

One end of the optical fiber was gently placed on the tissue. This effectively allowed synchronous movement of the end of the fiber together with the heart surface. Such procedures considerably attenuated the motion artifacts generated by the beating hearts. The motion of the hearts was noticeably decreased upon the addition of EGTA AM (Invitrogen) to the solution used to load the hearts with dye. The final concentration of EGTA AM in the loading solution varied from 35.5 to 211 μM in different experiments. After the pacemaker cells of the sinoatrial and atrioventricular nodes were electrically ablated using an ophthalmic bipolar pencil (Mentor Ophthalmics, Santa Barbara, CA), hearts were continuously paced at various rates by an electrical stimulator (ISOSTIM A320R, World Precision Instruments, Sarasota, FL) controlled by a PC.

Drugs and Chemicals

All reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Statistical Analysis

Data are expressed as means ± SD; N indicates the number of independent experiments in each separate series. The total amount of animals used in this study was 60 animals. Statistical significance was tested using ANOVA. Differences were considered to be significant if P values were <0.05.

Mathematical Modeling of EGTA Treatment

A diffusion-reaction theoretical model was developed to provide a conceptual framework to increase the rigorousness of our interpretation. To simulate the myoplasmic Ca<sup>2+</sup> transient during a twitch, a multicompartment unidimensional diffusion model was used (8, 9, 40, 48, 54). A detailed description of this model is provided in the Supplemental Material. One innovative aspect of the model is that it incorporates the published Markovian scheme of RyR2 gating (43). Single RyR2 gating is regulated by both cytosolic and luminal (i.e., inside the SR) Ca<sup>2+</sup> levels. The RyR2-CSQ interaction is thought to be involved in the luminal Ca<sup>2+</sup> regulation of RyR2. AP-like depolarizations activated an influx of Ca<sup>2+</sup> across the surface membrane, elevating the free Ca<sup>2+</sup> level in the diadic space (space between the T-tubule and SR membranes). This Ca<sup>2+</sup> influx activates RyR2 channels, initiating the Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release.

Here, we present the details of the model, which allowed us to predict how an exogenous Ca<sup>2+</sup> buffer (EGTA) may impact sarco-(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) activity and, ultimately, intracellular Ca<sup>2+</sup> dynamics. Local changes in cytosolic and luminal Ca<sup>2+</sup> concentrations were simulated using a one-dimensional diffusion-reaction model of Ca<sup>2+</sup> dynamics in the myoplasm and SR luminal spaces derived from our previous models of Ca<sup>2+</sup> handling (9, 48, 54). The geometry of the model consist of two adjacent sections, representing the myoplasm and SR luminal spaces of a sarcomere, all surrounded by an extracellular compartment. Each of these sections was sliced into a number (N) of diffusionally connected subcompartments. Ca<sup>2+</sup> exchange between the cytosolic and luminal sections occurs only via RyRs (located inside the SR membrane between the first cytosolic and luminal compartments) and SERCA pumps (placed between the cytosolic and luminal compartments in the central region of the model). SR Ca<sup>2+</sup> efflux is governed by the Ca<sup>2+</sup> gradient across the SR membrane and by the SR Ca<sup>2+-</sup>release channel’s P<sub>o</sub>, as described by a Markov model. The model accurately predicts 1) the measured rates of Ca<sup>2+</sup>-dependent activation, deactivation, adaptation, and inactivation; 2) the existence of modal gating; and 3) luminal regulation by the Ca<sup>2+</sup>–CSQ complex. The basic features of the Markovian scheme describing RyR2 kinetic behavior are the four Ca<sup>2+</sup>-binding sites for activation, the three open states for different kinetic modes, the one Ca<sup>2+</sup>-dependent inactivation site, and the luminal Ca<sup>2+</sup> regulation through the interaction between Ca<sup>2+</sup>–CSQ and the RyR channel. Under steady-state conditions, the opening of single RyR channels occurs in bursts. These bursts fall into two categories and are temporally clustered into distinct modes of RyR channel gating [i.e., high P<sub>o</sub> and low P<sub>o</sub> (14, 51)]. In addition, the luminal regulation stabilizes the channel in the open state and mimics the luminal regulatory effects described by Györke and Györke (21).

1 Supplemental Material for this article is available online at the American Journal of Physiology-Heart and Circulatory Physiology website.
Ca\(^{2+}\) influx to the SR was simulated by integrating kinetic models for SERCA identical to the ones we have previously described (48).

Ca\(^{2+}\) buffering inside the SR by CSQ was modeled as for a low-affinity, high-capacity Ca\(^{2+}\)-binding protein. An allosteric model (34) was used to describe the interaction between Ca\(^{2+}\) and CSQ. Myoplasmic Ca\(^{2+}\) buffering was modeled by including several mobile buffers (i.e., ATP, EGTA, and a Ca\(^{2+}\) indicator) and one fixed buffer (troponin).

Extracellular Ca\(^{2+}\) influx is controlled by a voltage-dependent Ca\(^{2+}\) channel modeled as a sarcolemmal Hodgkin-Huxley-type channel (41) located between the first cytosolic compartment and the extracellular medium. The channel also has voltage- and Ca\(^{2+}\)-dependent inactivation that is activated by a controlled membrane potential change. Ca\(^{2+}\) extrusion from the cytosol was modeled through a Na\(^{+}\)/Ca\(^{2+}\) exchanger transport mechanism (41). The parameters used for the simulation are shown in Supplemental Table 1. Finally, all the equations were numerically integrated using a finite-difference approximation (Euler method). The initial values for the state variables were calculated using inversion of the Q-matrix procedure (10). The boundary conditions were established to satisfy the following equation:

\[
\frac{\partial [\text{Ca}^{2+}](t,0)}{\partial t} = J_{\text{in}}^{\text{Ca}}(t,0) - J_{\text{out}}^{\text{Ca}}(t,0)
\]

where \(J_{\text{in}}^{\text{Ca}}\) is the net Ca\(^{2+}\) influx and \(J_{\text{out}}^{\text{Ca}}\) is the net Ca\(^{2+}\) efflux from compartment 0 at all times and \(t\) is time. In addition, a Neumann's boundary condition (49) was implemented in the last cytosolic and luminal compartments. Finally, the code was written in G language (National Instruments), and simulations were run in a 36-node Athlon-MP-based cluster under the control of Linux. The differential equations were numerically integrated with time increments of 4 \(\mu\)s to generate a 20-s trace.

RESULTS

Ca\(^{2+}\) Dynamics in the Cytosol and SR Lumen

Measuring Ca\(^{2+}\) signals from intracellular organelles has always been a major challenge in cardiovascular physiology (6, 9, 22). Furthermore, up until now, it was unfeasible to evaluate the luminal Ca\(^{2+}\) dynamics at the whole organ level. Here, SR luminal Ca\(^{2+}\) dynamics at the subepicardial layer of an intact beating mouse heart were characterized using the fluorescent dye mag-fluo-4 AM. After the dye was effectively washed out of the cytosol at 37°C, the signal declined sharply in response to an electrical stimulation pulse and then slowly recovered to diastolic levels (Fig. 1A). The signal detected from the hearts loaded with rhod-2 AM changed in the opposite direction (Fig. 1D).

To verify that the changes in the fluorescence signals observed in our experiments were due to changes in intra-SR and cytosolic Ca\(^{2+}\) content, Ca\(^{2+}\) release was modified by perfusing the hearts with Tyrode solution containing 20 mM caffeine, which causes the fast release of Ca\(^{2+}\) from the SR (Fig. 1, B and E). The application of caffeine for 10 s led to a decrease in both the diastolic level of mag-fluo-4 fluorescence and in the

Fig. 1. Changes in fluorescence from the sarcoplasmic reticulum (SR) lumen (A–C) and cytosol (D–F) measured with the Ca\(^{2+}\)-sensitive dyes rhod-2 AM and mag-fluo-4 AM, respectively. Representative traces of luminal (A) and cytosolic (D) Ca\(^{2+}\) transients are shown. Diastolic (resting) levels of mag-fluo-4 (B) and rhod-2 (E) fluorescence changed in different directions in response to the application of caffeine. An analogous effect was observed in the presence of ryanodine. Changes in mag-fluo-4 (C) and rhod-2 (F) fluorescence reflect the release of Ca\(^{2+}\) from the SR and accumulation of Ca\(^{2+}\) in the cytosol, respectively. Data are means ± SD. All measurements were conducted at 4 Hz and 37°C.
amplitude of Ca\textsuperscript{2+} transients [down to 92 ± 0.05% (N = 4, \( P = 0.012 \)) and 51 ± 0.21% (N = 4, \( P = 0.003 \)) of the initial values, respectively]. The amplitude of Ca\textsuperscript{2+} transients recorded with rhod-2 also decreased in the presence of caffeine [down to 18 ± 0.09% of the initial value (N = 4, \( P < 0.001 \))], whereas the diastolic level of rhod-2 fluorescence increased [up to 135 ± 0.09% (N = 4, \( P < 0.001 \))], reflecting the accumulation of Ca\textsuperscript{2+} in the cytosol (Fig. 1E). These data imply that mag-fluo-4, unlike rhod-2, detects the changes of free Ca\textsuperscript{2+} in a compartment that loses Ca\textsuperscript{2+} during caffeine pulses.

Another piece of evidence confirming the intra-SR origin of the mag-fluo-4 fluorescence was obtained in the experiments with 25 \( \mu \text{M} \) ryanodine, an alkaloid that binds specifically to RyR2 Ca\textsuperscript{2+}-release channels located in the membranes of the SR and locks the channels in a long-dwell time subconductance state (50). The diastolic level of Ca\textsuperscript{2+} in the SR lumen slowly decreased in the presence of ryanodine (Fig. 1C). No additional decline in the fluorescence was observed when 20 mM caffeine pulses were applied after treatment with ryanodine (data not shown). Electrically induced changes in the mag-fluo-4 fluorescence signal disappeared almost completely after 10 min of ryanodine application (Supplemental Fig. 2). This experiment illustrates the time course of fractional binding of ryanodine to the RyR2 in an intact preparation and how ryanodine induces the accumulation of Ca\textsuperscript{2+} in the cytosol (Fig. 1F). Since the results described above are in accordance with the mode of action of ryanodine, they support the assumption that rhod-2 and mag-fluo-4 fluorescence detect changes in cytosolic and intra-SR Ca\textsuperscript{2+}, respectively.

Similar results were obtained in the presence of EGTA (Supplemental Figs. 2 and 3). Electrically stimulated mouse hearts still showed changes in the rhod-2 signal after 10 min of perfusion with ryanodine (Supplemental Fig. 3C). These remaining fluorescence transients can be attributed to the influx of external Ca\textsuperscript{2+} during the cardiac AP via voltage-dependent Ca\textsuperscript{2+} channels located in the sarcolemma. The difference in the amplitude between transients obtained in the presence and absence of ryanodine illustrates the magnitude of Ca\textsuperscript{2+} release from the SR in subepicardial cardiac myocytes. When an identical electrical stimulation protocol was used in a mag-fluo-4-loaded mouse heart, the fluorescence signal did not show any evident changes (i.e., AP-induced SR depletion) at the same time of perfusion with ryanodine (Supplemental Fig. 2C). This clearly indicates that mag-fluo-4 is located in a different ryanodine-sensitive intracellular compartment.

**Thermodynamics of Intra-SR Ca\textsuperscript{2+} Dynamics**

Rodents are homeothermic animals in which Ca\textsuperscript{2+} transport is sensitive to temperature. For example, the time course of relaxation of intracellular Ca\textsuperscript{2+} transients involves several temperature-dependent Ca\textsuperscript{2+} removal mechanisms [Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, SR Ca\textsuperscript{2+} uptake through the SERCA pump, uptake of Ca\textsuperscript{2+} by the mitochondria, etc., and cytosolic buffering processes (mobile buffers, e.g., ATP, calmodulin, etc.) as well as fixed buffers (e.g., troponin C, etc.)]. Temperature can strongly modify the kinetics of all the transport mechanisms mentioned above. However, the relaxation phase of SR Ca\textsuperscript{2+} transients (depletion) is mainly governed by Ca\textsuperscript{2+} uptake by the SERCA pump and by the binding of Ca\textsuperscript{2+} to CSQ. Since the SERCA pump needs to hydrolyze ATP to transport Ca\textsuperscript{2+}, measuring the temperature dependence of the luminal Ca\textsuperscript{2+} relaxation dynamics will allow us to examine the thermodynamic properties of the SERCA pump in vivo. Thus, to further understand the relationship between cytosolic and SR luminal Ca\textsuperscript{2+} dynamics, we studied the temperature dependence of these processes.

The increase in temperature noticeably accelerated the dynamics of Ca\textsuperscript{2+} in both the cytosol and SR (Fig. 2). The time to peak for rhod-2 signals (Fig. 2A) decreased from 28 ± 3 ms at 21°C to 19 ± 4 ms at 37°C (N = 4, \( P = 0.013 \)). The time to nadir for mag-fluo-4 signals decreased from 34 ± 5 ms at 21°C to 22 ± 3 ms at 37°C (N = 4, \( P = 0.007 \)). There were no statistical differences between the time to peak (rhod-2, cytosol) and time to nadir (mag-fluo-4, SR lumen) at any given temperature (\( P = 0.098 \) for 21°C and \( P = 0.234 \) for 37°C).

As shown in Fig. 2, the higher temperature was associated with a faster recovery of the Ca\textsuperscript{2+} signal to its diastolic level. The decay time constant decreased from 162 ± 35 ms at 21°C to 92 ± 4 ms at 37°C for the cytosolic signal (N = 4, \( P = 0.007 \)). For the luminal signal, the magnitudes of the decay time constant were 120 ± 15 and 74 ± 7 ms at 21 and 37°C, respectively (N = 4, \( P = 0.001 \)). Temperature coefficients (Q10 values) calculated on the basis of these data were not statistically different between cytosolic (1.42 ± 0.21) and luminal (1.35 ± 0.12) signals (N = 4, \( P = 0.593 \)).
The similarity of the temperature dependence of the relaxation of Ca\(^{2+}\)/H\(^{1+}\) transients in the cytosol and within the SR suggests that both processes are governed by the same Ca\(^{2+}\) transport mechanism.

**Effect of EGTA on Cytosolic and Luminal Ca\(^{2+}\) Transients**

The gain of Ca\(^{2+}\) release can determine both the time course and amplitude of cytosolic Ca\(^{2+}\) transients and the degree of depletion of the SR. In this study, the gain of Ca\(^{2+}\) release was modified by adding an exogenous cytosolic buffer to the Tyrode solution used to perfuse a mouse heart. Our working hypothesis was that the presence of a slow Ca\(^{2+}\) buffer like EGTA, which has a low association rate constant \([5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}]\) (13), would decrease the gain of Ca\(^{2+}\) release by buffering Ca\(^{2+}\) close to the cytosolic binding sites of the SERCA pump. Since the SERCA pump needs to bind two Ca\(^{2+}\) to be fully activated, a reduction of the free cytosolic Ca\(^{2+}\) concentration will reduce pump activity (5), promoting Ca\(^{2+}\) depletion of the SR. This will finally result in a decrease of the Ca\(^{2+}\) released from the SR. To test this hypothesis, mouse hearts previously loaded with the fluorescent dyes were perfused with Tyrode solution containing 211 \(\mu\)M EGTA AM, and changes in fluorescence were recorded.

Figure 3A shows rhod-2 signals recorded before and after perfusion with the exogenous buffer. EGTA AM induced changes in both diastolic and systolic myoplasmic fluorescence. Average data representing the time course of these changes are shown in Fig. 3B. Diastolic and systolic levels of rhod-2 fluorescence were reduced by 23\% \((P = 0.012)\) and 25\% \((P = 0.010)\), respectively, whereas the amplitude of Ca\(^{2+}\) transients was reduced by 39\% \((P < 0.001);\) Fig. 3C. Moreover, the kinetics of Ca\(^{2+}\) transients were dramatically modified by the buffer. Note the two very distinct components of the relaxation process in hearts paced at 2 Hz after the 30-min load with EGTA AM (Fig. 3D).

The effect of EGTA on the amplitude and kinetics of cytoplasmic Ca\(^{2+}\) transients can be explained by several mechanisms. For example, EGTA may compete with cytosolic Ca\(^{2+}\) buffers (including rhod-2), reducing, in this way, the free Ca\(^{2+}\) concentration during Ca\(^{2+}\) release. A second possibility could be that EGTA competes with the cytosolic Ca\(^{2+}\)-binding sites on RyR2. However, this option seems unlikely due to the slow
association rate constant of EGTA (see the DISCUSSION). Finally, EGTA could decrease the free diastolic Ca\(^{2+}\) in the cytosol (Fig. 3B), thereby decreasing the activity of the SERCA pump, leading to a reduction in the intra-SR Ca\(^{2+}\) concentration (depletion) and, consequently, to a decrease in the amplitude of Ca\(^{2+}\) transients.

To test this last possibility, we evaluated the intra-SR Ca\(^{2+}\) dynamics by measuring the fluorescence of mag-fluo-4 when hearts were perfused with EGTA AM. A noticeable decline in diastolic and systolic intra-SR Ca\(^{2+}\) levels was observed (Fig. 4A). Figure 4B shows that the diastolic level of mag-fluo-4 fluorescence was decreased by 18% \((P = 0.001)\), implying that the exogenous buffer depleted intra-SR Ca\(^{2+}\). Moreover, the amplitude of Ca\(^{2+}\) release from the SR was dramatically decreased \([by \sim 70\% of the initial value](P < 0.001); Fig. 4C\], which indicates that intra-SR Ca\(^{2+}\) content has a significant effect on Ca\(^{2+}\) release from the SR. The presence of EGTA also slowed down the rate of Ca\(^{2+}\) replenishment of the SR (Fig. 4D). These results support the hypothesis that EGTA decreases the free diastolic Ca\(^{2+}\) concentration in the cytosol, leading to a reduction in the intra-SR Ca\(^{2+}\) concentration (depletion) and, consequently, to a decrease in the amplitude of Ca\(^{2+}\) transients.

**Calibration of the Mag-fluo-4 Signal and the Effect of EGTA on Intra-SR Ca\(^{2+}\) Concentration**

The procedure of in vivo calibration of a Ca\(^{2+}\) dye requires obtaining minimal \((F_{\text{min}})\) and maximal fluorescence \((F_{\text{max}})\) levels corresponding to Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound dye, respectively. We conducted these experiments at a pacing frequency of 4 Hz and temperature of 37°C. \(F_{\text{max}}\) was recorded in the presence of 10 mM CaCl\(_2\) in the extracellular perfusate. In addition to a higher Ca\(^{2+}\) concentration, Na\(^{+}\)/Ca\(^{2+}\) exchanger activity was inhibited by replacing 70 mM NaCl with 70 mM LiCl in the Tyrode solution, allowing the accumulation of Ca\(^{2+}\) inside the cells. An additional 30 mM NaCl was replaced with 30 mM KCl to induce a depolarization of the cell membrane. The final concentrations of NaCl and KCl were equal to 40 and 35.4 mM, respectively. A typical recording is shown in Fig. 5A. Average \(F_{\text{max}}\) calculated from four independent experiments (hearts) was 242 \(\pm\) 20% of the diastolic (resting) value, which was recorded at 4 Hz before the application of high-Ca\(^{2+}\) Tyrode solution. These results imply that intra-SR Ca\(^{2+}\) concentrations do not saturate mag-fluo-4 fluorescence under our experimental conditions, i.e., before the application of high-Ca\(^{2+}\) solution. Note that changes in mag-

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**Fig. 4. Effect of 4-min perfusion with Tyrode solution containing 211 \(\mu\)M EGTA AM on Ca\(^{2+}\) dynamics in the SR lumen measured with mag-fluo-4 AM (A–C).** Measurements were conducted at 37°C at a pacing frequency of 4 Hz. A: representative traces obtained before (thin line) and 4 min after (thick line) perfusion with EGTA AM. B and C: changes in the diastolic (resting) and systolic (nadir) levels of mag-fluo-4 fluorescence (B) and decline in the amplitude of Ca\(^{2+}\) depletion (C) in response to 4-min treatment with 211 \(\mu\)M EGTA AM. The period of treatment with EGTA AM is indicated by the shaded bar. Data are means \(\pm\) SD; \(N\) is the number of independent experiments (hearts). \(P\) values are the results of one-way ANOVA (initial vs. final levels of the parameters). D: typical traces of fluorescence from mouse hearts loaded with mag-fluo-4 AM in the absence (thin line) and presence (thick line) of 211 \(\mu\)M EGTA AM (37°C, 2 Hz). Traces were normalized to the maximum amplitude of the depletion to compare the kinetics.
fluorescence at 518 nm on Ca\textsuperscript{2+} concentration in a cuvette in the presence and absence of albumin (BSA). Measurements were conducted at pH 6.8, 2 mM MgCl\textsubscript{2}, and 37°C. The absence of albumin (BSA). Measurements were conducted at pH 6.8, 2 mM MgCl\textsubscript{2}, and 37°C.

To relate the changes in mag-fluo-4 fluorescence recorded from whole heart preparations to the actual concentrations of Ca\textsuperscript{2+}, we performed in vitro calibrations of the dye. This was performed by dissolving the salt form of the dye with a solution containing 30 mM MOPS (pH 6.8), 100 mM KCl, and 2 mM MgCl\textsubscript{2}. To account for the effects of proteins present in the SR, either 55 or 100 mg/ml BSA (fraction V, U.S. Biological, Swampscott, MA) was added. Calcium Sponge S (BAPTA polystyrene, Invitrogen) was added into the buffer solutions (with or without BSA, 1/10 of the solution volume) to chelate the contaminant Ca\textsuperscript{2+}. After a vigorous mix (vortexing), the solution was centrifuged at 10,000 rpm for 3 min, and the supernatant was carefully collected to be used in the experiments. Finally, mag-fluo-4 tetrapotassium salt (at a final concentration of 0.25 μM) was added. The fluorescence was excited at 473 nm and monitored at 37°C within the range of 475–630 nm using a spectrofluorometer (QuantaMaster 40, Photon Technology, Birmingham, NJ). The dependence of the maximum amplitude of fluorescence at 518 nm on the Ca\textsuperscript{2+} concentration was studied to determine the apparent dissociation constant (K\textsubscript{d}).

In absence of BSA, the average measured K\textsubscript{d} was 49 μM. These data correlate well with the numbers reported in Ref. 17 (70 and 81 μM), which were obtained at 22°C in the absence and presence of 1 mM MgCl\textsubscript{2}, respectively. The presence of BSA caused a significant shift of K\textsubscript{d} values toward higher Ca\textsuperscript{2+} concentrations. K\textsubscript{d} values of 940 and 4,471 μM were obtained in the presence of 55 and 100 mg/ml BSA, respectively. This effect was analogous to that observed for fluo-3 in the presence of aldolase (16).

The fluorescence values obtained in vitro were normalized using the diastolic fluorescence measured on intact hearts at 4 Hz and 37°C as a reference level (Fig. 5B). Minimal and maximal levels of fluorescence were adjusted to F\textsubscript{min} and F\textsubscript{max} obtained in vivo (54% and 242% of the diastolic level at 4 Hz, respectively). The fitting curve for the samples with 100 mg/ml BSA was used to estimate intra-SR free Ca\textsuperscript{2+} concentrations before and during the application of EGTA AM. As shown in Fig. 5C, 4-min perfusion with 211 μM EGTA AM caused a decrease in the diastolic free Ca\textsuperscript{2+} inside the SR from 1,381 ± 128 to 760 ± 245 μM. It should be noted that such estimations provide only approximate levels of intra-SR Ca\textsuperscript{2+} under the assumption that the obtained F\textsubscript{max} and F\textsubscript{min} values were close to the true values. Error sources include dye deterioration and washing out of the cells as well as variations in pH and SR protein concentrations.

**Mathematical Modeling of EGTA Treatment**

The experiments shown in Figs. 3D and 4D demonstrated that EGTA has a differential kinetic effect between fluorescent signals measured in the cytosol and SR. While EGTA accelerates the relaxation of cytosolic Ca\textsuperscript{2+} transients, the same buffer produced a slowness of SR Ca\textsuperscript{2+} uptake. To address this and other experimental observations, we used a mathematical model to simulate, in silico, the effect of EGTA treatment on free Ca\textsuperscript{2+} content in the SR and on the kinetics of Ca\textsuperscript{2+} transients.
The results of the calculations implied that the diastolic and systolic free Ca\(^{2+}\) concentration declines in both the SR (Fig. 6A) and cytosol (Fig. 6D) after the addition of 400 \(\mu M\) EGTA in the cytosol. The amplitude of intra-SR (Fig. 6B) and cytosolic (Fig. 6E) Ca\(^{2+}\) transients also decreased. In the presence of EGTA, the relaxation kinetics of Ca\(^{2+}\) transients were slowed in the SR compartment (Fig. 6C) and accelerated in the cytosol (Fig. 6F). The decrease in the intra-SR Ca\(^{2+}\) concentration as well as the slowness of SR Ca\(^{2+}\) replenishment were due to the effects of EGTA in the cytosol and not a direct effect of EGTA in the SR. EGTA induced a decrease in the diastolic free Ca\(^{2+}\) concentration in the cytosol that promoted a decrease in SERCA pump activity that finally led to a reduction in the rate of SR Ca\(^{2+}\) uptake and to a decrease in the SR diastolic Ca\(^{2+}\) concentration. The results of these simulations are in agreement with the experimental data described above.

**Effect of EGTA on the Kinetics of Cytosolic and Luminal Ca\(^{2+}\) Transients**

To further evaluate our working hypothesis and explore the effect of EGTA on the Ca\(^{2+}\) dynamics of cytosolic and intra-SR Ca\(^{2+}\) transients, we conducted experiments at several EGTA AM concentrations in the loading solution. A summary of the results is shown in Fig. 7. In response to the elevation of EGTA AM concentration, a decrease in the time to peak and time to nadir was observed for both cytosolic and luminal SR signals (Fig. 7A). However, the effect of 211 \(\mu M\) EGTA AM on the time to peak was much larger than the effect on the time to nadir [53% (\(P = 0.003\)) vs. 29% (\(P = 0.018\)), respectively]. This suggests that the effect of EGTA on the releasing phase of the cytosolic Ca\(^{2+}\) transient is mostly due to the competition between EGTA and cytosolic Ca\(^{2+}\) buffers and not to the release mechanism itself. The presence of EGTA also considerably modified the time course of the relaxation of cytosolic Ca\(^{2+}\) transients. Two distinct components were observed in the presence of the buffer: an initial phase accelerated by EGTA and a delayed (or second) phase, which was slowed down by the presence of the buffer (Fig. 3D). Consequently, hearts not treated with EGTA AM reached the diastolic levels of free Ca\(^{2+}\) in shorter times.

Interestingly, the effect of increasing the concentration of EGTA AM on the time course of relaxation of SR luminal Ca\(^{2+}\) transients was dramatically different from that observed for cytosolic Ca\(^{2+}\) transients. The relaxation of the intra-SR

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**Fig. 6. Results of the mathematical model that simulates changes in SR Ca\(^{2+}\) content (A–C) and in the cytosol (D–F) in response to the addition of an exogenous buffer (EGTA). A: effect of EGTA on free diastolic and systolic Ca\(^{2+}\) concentrations upon the addition of 400 \(\mu M\) EGTA in the cytosolic compartments. B and C: effects of EGTA on the amplitude and kinetics of Ca\(^{2+}\) transients in the SR. D and E: concentrations of rhod-2 bound to Ca\(^{2+}\) in the cytosol during diastole and systole as well as the changes and amplitude (difference between systole and diastole) when 400 \(\mu M\) EGTA was added to the cytosol. F: effect of EGTA on the kinetics of the fluorescent transient measured with rhod-2 in the cytosol. See the Supplemental Material for details.**
signal was found to be slower in EGTA-treated hearts. As shown in Fig. 7, the fastest time constant obtained as a result of increasing the EGTA AM concentration in the loading solution, $\tau_1$, increased for intra-SR Ca$^{2+}$ depletion by 142% ($P = 0.007$) in response to the elevation of EGTA AM concentration, whereas $\tau_1$, calculated for cytosolic Ca$^{2+}$ transients, decreased by 91% ($P < 0.001$) in the presence of the buffer. The data for SR luminal Ca$^{2+}$ transients (depletion) are in accordance with the previous observations that the kinetics of relaxation of cytosolic Ca$^{2+}$ concentration during reuptake depend on the cytosolic Ca$^{2+}$ level (5). The lower the level of free Ca$^{2+}$ concentration in the cytosol, the slower will be the uptake kinetics. This is fully consistent with our working hypothesis (i.e., EGTA affects intra-SR Ca$^{2+}$ loading).

**Time Course of SR Ca$^{2+}$ Replenishment**

**Restitution of Ca$^{2+}$ depletions.** To understand the relationship between SR Ca$^{2+}$ replenishment and Ca$^{2+}$ release from this intracellular store, we used a time restitution protocol to evaluate the kinetics of both processes. The restitution of Ca$^{2+}$ transients in the cytosol and SR lumen (depletion) was explored by applying an extrasystolic stimulus in hearts paced at 2 Hz at 37°C. The extrasystolic stimulus was generated by interspersing an extrastimulus, which was delayed with respect to the basal pacemaker stimulation (or stimulation frequency, 2 Hz).

Superimposed traces of cytosolic and SR lumen Ca$^{2+}$ transients obtained as a result of applying extrasystolic stimuli at several delayed times between regular pacemaker stimulus pulses are shown in Fig. 8, A and B, respectively. Changes in

![Fig. 7](http://ajpheart.physiology.org/)

Fig. 7. Changes in the kinetics of Ca$^{2+}$ transients in the cytosol (●) and Ca$^{2+}$ depletion in the SR lumen (○) as a result of increasing the EGTA AM concentration in the loading solution. A and B: time to peak (A) and faster time constant (B) obtained as a result of the fitting with a two-exponential decay function. Measurements were conducted at 37°C and at a pacing frequency of 2 Hz. Data are means ± SD; $N$ is the number of independent experiments (4 for each combination of dye and EGTA AM concentration, total number: 32). $P$ values are results of one-way ANOVA (no EGTA AM vs. different concentrations of EGTA AM).

![Fig. 8](http://ajpheart.physiology.org/)

Fig. 8. Typical traces obtained during the measurement of restitution of Ca$^{2+}$ transients in the cytosol (A) and Ca$^{2+}$ depletions in the SR lumen (B). Changes in cytosolic and SR Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_{cyt}$ and [Ca$^{2+}$]$_{SR}$, respectively) were normalized to the amplitude of the first peak, and the normalized traces were then superimposed. C: comparison of the time courses of restitution of Ca$^{2+}$ release in the cytosol and Ca$^{2+}$ depletion in the SR ($N = 8$, 4 heart preparations for each dye). Recordings were conducted at 37°C and at a pacing frequency of 2 Hz. No EGTA was added into the loading solution.
the amplitude of the signals after an extrasystolic pulse were normalized to the amplitude of the previous Ca\(^{2+}\) transient/depletion trace measured in control hearts, where no exogenous EGTA AM was applied. In both cases, we observed that the shorter the time between electrical stimuli, the smaller the amplitude of the Ca\(^{2+}\) transient/depletion after the second pulse. Overall, the results of the restitution experiments are shown in Fig. 8C. No significant differences were found between the restitution of Ca\(^{2+}\) transients in the cytoplasm and in the lumen of the SR [exponential time constant of restitution: 97 ± 13 and 107 ± 14 ms for cytosolic and luminal Ca\(^{2+}\) transients, respectively (N = 4, P = 0.329)]. This suggests that the restitutions of cytosolic and luminal Ca\(^{2+}\) transients in the absence of exogenous buffer reflect kinetics of the same process (time-dependent recovery of Ca\(^{2+}\) release) monitored from two different sides (the cytosol and SR lumen).

To evaluate how an increase in the cytosolic concentration of EGTA AM modified the relationship between SR Ca\(^{2+}\) replenishment and Ca\(^{2+}\) release from this intracellular store, we then performed restitution experiments in the presence of 211 μM EGTA AM. A typical family of traces of Ca\(^{2+}\) transients in the cytosol and SR lumen (depletion) was obtained when the same protocol as shown in Fig. 8 was applied in the presence of the exogenous buffer, as shown in Fig. 9, A and B. As in Fig. 8, the extrasystolic pulse induced a smaller than regular Ca\(^{2+}\) transient. This decrease in the amplitude of the Ca\(^{2+}\) transient evoked by the extrasystolic stimulus could be due to either an inability of the SR to release Ca\(^{2+}\) or a reduction in the influx of Ca\(^{2+}\) through the plasma membrane. To discriminate between these two possibilities, we performed experiments to find out if the refractoriness of Ca\(^{2+}\) transients was associated with the refractoriness of the AP. Figure 9C shows the restitution of optically recorded AP in hearts perfused with 211 μM EGTA AM. Interestingly, the restitution of the epicardial AP was much faster than that of Ca\(^{2+}\) transients in the cytosol and SR lumen (depletion). The amplitude of the AP was fully recovered after ~100 ms, whereas the amplitude of the Ca\(^{2+}\) transient/depletion was not fully recovered at that time.

However, the refractoriness of Ca\(^{2+}\) transients in the cytosol and SR lumen could still depend on the recovery from inactivation of voltage-activated Ca\(^{2+}\) channels located in the plasma membrane. This hypothesis was evaluated by measuring cytosolic Ca\(^{2+}\) transients in hearts where SR Ca\(^{2+}\) release was completely abolished with 25 μM ryanodine (Fig. 9D). In the presence of ryanodine, Ca\(^{2+}\) transients reflected Ca\(^{2+}\) influx through the plasma membrane. The recovery of the amplitude of these transients during a restitution protocol was also faster than that of Ca\(^{2+}\) transients produced by Ca\(^{2+}\) released from the SR.

The comparison of the data collected for the various processes mentioned above is shown in Fig. 10A. This plot revealed that the kinetics of the recovery of AP and Ca\(^{2+}\) influx

![Fig. 9. Typical traces of Ca\(^{2+}\) transients in the cytosol (A), Ca\(^{2+}\) depletions in the SR lumen (B), action potential (AP; C), and Ca\(^{2+}\) changes in the cytosol during the AP (D; measured in the presence of 25 μM ryanodine) when a restitution protocol was applied. The concentration of EGTA AM in loading solution was 211 μM. The changes in cytosolic and SR Ca\(^{2+}\) concentrations and AP were normalized to the amplitude of the first peak (or nadir in the case of intra-SR Ca\(^{2+}\) transients), and the normalized traces were then superimposed. Recordings were conducted at 37°C and at a pacing frequency of 2 Hz.](http://ajpheart.physiology.org/)
in the presence of ryanodine) through the plasmalemma are noticeably faster than that of the intracellular Ca\textsuperscript{2+} release (see averaged values of time constants in Fig. 10B). This indicates that the time course of the recovery from refractoriness of cytosolic and SR Ca\textsuperscript{2+} transients is mostly defined by the release process from the SR and not by Ca\textsuperscript{2+} entering through the plasma membrane.

**Effect of EGTA AM.** Evaluation of the time course of restitution is an adequate way to measure the refractoriness of the different steps involved in the Ca\textsuperscript{2+}-release process. We performed experiments using several concentrations of EGTA AM in the loading solution to determine how an increase in the Ca\textsuperscript{2+} buffering capacity can modify the refractoriness of cytosolic and SR Ca\textsuperscript{2+} transients. Figure 11 shows that an increase in the EGTA AM concentration led to faster restitution kinetics of both cytosolic and SR Ca\textsuperscript{2+} transients. Time to 50% of peak and nadir recoveries for cytosolic and SR Ca\textsuperscript{2+} transients were 33% and 60% lower after treatment with 211 μM EGTA AM compared with control values (P < 0.001; Fig. 11, B and D, respectively). This suggests that reducing Ca\textsuperscript{2+} release (less SR Ca\textsuperscript{2+} depletion) during every beat can accelerate the restitution of Ca\textsuperscript{2+} release. Interestingly, the presence of EGTA AM also accelerated the restitution of the AP (Fig. 11E) from an average time constant of 28 ± 4 ms (control group) to 15 ± 1 ms (EGTA AM-treated group, P = 0.002). However, as shown in Fig. 10B, even in the presence of EGTA AM, the restitution of the AP was much faster than the restitution of intracellular Ca\textsuperscript{2+} release.

**Ca\textsuperscript{2+} Alternans in the Presence of EGTA AM**

Cytoplasmic Ca\textsuperscript{2+} alternans is an abnormality in Ca\textsuperscript{2+} handling characterized by alternating large and small amplitudes of Ca\textsuperscript{2+} transients. Alternans constitutes an important phenomenon that is considered to be a sign of cardiac dysfunction (46). Although this type of event has been characterized for both isolated cells and intact preparations of various species (7, 29), there is no evidence that alternans are present in mouse ventricles working at physiological temperatures and heart rates.

We proposed that the acceleration of the restitution of Ca\textsuperscript{2+} release in the presence of EGTA AM will affect the frequency dependence of Ca\textsuperscript{2+} alternans. Examples of Ca\textsuperscript{2+} alternans are shown in Fig. 12, A and B. Alternans was induced by pacing with a high stimulation frequency, i.e., 14 Hz. Treatment with 71 μM EGTA AM resulted in a diminished amplitude of Ca\textsuperscript{2+} alternans. The frequency dependence of Ca\textsuperscript{2+} alternans is shown in Fig. 12C. This plot demonstrates that, for tachycardic heart rates (higher than 13 Hz, 780 beats/min), the presence of EGTA AM dramatically reduced the amplitude of Ca\textsuperscript{2+} alternans, most likely due to the acceleration of the restitution of the Ca\textsuperscript{2+} transients. This suggests that the smaller the degree of SR Ca\textsuperscript{2+} depletion during each beat, the smaller the amplitude of Ca\textsuperscript{2+} alternans will be.
DISCUSSION

Changes in the Luminal SR Ca\(^{2+}\) Content Can Be Detected Using Mag-fluo-4 AM

In this report, we show, for the first time, the regulation of luminal SR Ca\(^{2+}\) dynamics in an intact beating heart. Luminal SR Ca\(^{2+}\) dynamics were assessed with the low-affinity dye mag-fluo-4. After perfusion of the heart with mag-fluo-4 AM, a temperature cycle procedure was used to promote the wash-out of the dye from the cytosol to differentially stain the lumen of the SR. The results are consistent with data obtained by Shannon et al. (45) with fluo 5N, a low-affinity dye comparable to mag-fluo-4, implying that dyes of this type can be effectively wash out from the cytosol at 37°C. In that study,
confocal measurements were used to show that the remaining fluorescence signals originated from the SR. Our experimental results in the presence of caffeine and a high concentration of ryanodine (Fig. 1 and Supplemental Fig. 2) are in agreement with that previous report and confirm the luminal origin of the fluorescence signal measured when the hearts were loaded with a fluorescein-based dye (mag-fluo-4 AM) at room temperature (21°C) and then warmed up to 37°C.

The addition of ryanodine in the Tyrode solution led not only to the inhibition of field-induced changes in the mag-fluo-4 signal but also to a decline in the diastolic level of Ca²⁺ within the SR (Fig. 1C and Supplemental Fig. 2, B and C). This can be interpreted as a depletion of the Ca²⁺ store due to ryanodine-induced locking of the Ca²⁺ release channel in a long-open dwell time subconducting state (1, 14). In accordance with this suggestion, the diastolic level of free Ca²⁺ in the cytosol, measured with rhod-2, increased when ryanodine was applied (Fig. 1F and Supplemental Fig. 3B). Caffeine-induced changes in the diastolic level also had different directions in the case of mag-fluo-4 and rhod-2 fluorescence (Fig. 1, B and E). All of the above-discussed results allowed us to conclude that the PLFF technique (33) in combination with the fluorescent dye mag-fluo-4 AM can be applied to successfully track luminal SR Ca²⁺ dynamics in intact beating mouse hearts. Furthermore, the similar temperature dependency of both cytosolic and intra-SR Ca²⁺ dynamics (Fig. 2) indicates that we obtained an equivalent (and specular) view of the same process from the cytoplasm and lumen of the SR.

Ca²⁺ Buffering by EGTA Modifies Cytosolic and Luminal SR Ca²⁺ Dynamics

As shown in Fig. 3, the presence of the Ca²⁺ buffer EGTA produced a reduction in the amplitude of diastolic and systolic levels of myoplasmic free Ca²⁺ at the intact heart level. This reduction was similar to the one reported by Diaz et al. (11) for isolated ventricular cardiac myocytes. The decrease in the amplitude was associated with profound changes in the kinetics (Fig. 3D) and restitution of Ca²⁺ transients measured using the fluorescent dyes located in the cytosol (Fig. 11). Therefore, our data on intact whole hearts are in accordance with those obtained for isolated rat myocytes using photolysis of “caged” EGTA (NP-EGTA) to modify the caffeine response (11).

The kinetics of relaxation of cytosolic Ca²⁺ transients were also dramatically modified by the presence of the Ca²⁺ buffer. The initial relaxation phase was significantly accelerated in the presence of EGTA AM. Similar effects have been observed in skeletal muscle (35, 39) and cardiac myocytes (11). These authors concluded that the increase in the rate of relaxation was due to the chelating kinetics of the exogenous buffer. Surprisingly, the presence of EGTA AM revealed a slow component induced changes in the diastolic level also had different directions in the case of mag-fluo-4 and rhod-2 fluorescence (Fig. 1, B and E). All of the above-discussed results allowed us to conclude that the PLFF technique (33) in combination with the fluorescent dye mag-fluo-4 AM can be applied to successfully track luminal SR Ca²⁺ dynamics in intact beating mouse hearts. Furthermore, the similar temperature dependency of both cytosolic and intra-SR Ca²⁺ dynamics (Fig. 2) indicates that we obtained an equivalent (and specular) view of the same process from the cytoplasm and lumen of the SR.

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The data shown in Fig. 4 are in accordance with the possibility that Ca²⁺ transport mediated by the SERCA pump is impaired in the presence of EGTA AM. Both the diastolic free Ca²⁺ concentration and amplitude of Ca²⁺ transients (depletion) inside the SR were significantly reduced. Interestingly, in contrast to what happened in the cytosol, the kinetics of the relaxation of Ca²⁺ transients in the SR lumen were slower in the presence of EGTA AM (Fig. 7). These results support two hypotheses. First, the buffering effect of EGTA in the SR
lumen is minimal because the high free Ca\(^{2+}\) concentration in this compartment (between 100 \(\mu\)M and 2 mM) saturates a high-affinity buffer like EGTA (\(K_d = 50\) nM). Second, the presence of EGTA in the cytosolic compartment of ventricular cells slows down Ca\(^{2+}\) transport into the SR. This reduction of the rate of uptake viewed from inside the SR indicates that the SR is reloading Ca\(^{2+}\) more slowly because the fraction of Ca\(^{2+}\) released from the SR is smaller [intraluminal control of the SERCA pump turnover (53)] and because the free Ca\(^{2+}\) concentration in the cytosol is lower in the presence of EGTA. This latter condition will decrease the probability of the cytosolic Ca\(^{2+}\)-binding sites of the SERCA pump to be occupied and can reduce the final turnover of the pump (5). The slowness of replenishment of the SR with Ca\(^{2+}\) is expected to be translated into a reduction of the free Ca\(^{2+}\) inside the SR, which will lead to a reduction of the gain of the Ca\(^{2+}\)-release process. Finally, the comparison of the relaxation kinetics of cytosolic and intra-SR transients (Fig. 7) suggests that the speed up induced by EGTA AM in the kinetics of the cytosolic Ca\(^{2+}\) transients is mostly an effect of competition between rhod-2 and EGTA AM and not the effect of EGTA AM on the Ca\(^{2+}\)-release process. These results are in complete agreement with predictions obtained by numerically integrating the diffusion-reaction model presented in this report (Fig. 6 and Supplemental Material).

**Effect of Ca\(^{2+}\) Buffering on the Restitution of Ca\(^{2+}\) Transients and AP in an Intact Beating Heart**

This report describes in detail the restitution of the Ca\(^{2+}\)-release process in the epicardial layer of an intact beating murine heart. The refractoriness of the Ca\(^{2+}\)-release process is a central phenomenon in whole heart cardiac dynamics. Stroke volume and cardiac efficiency are direct functions of myocardial contractility. The changes in cardiac contractility in response to the increase in heart rate will depend on how fast the Ca\(^{2+}\)-release process can be restituted. Additionally, the kinetics of restitution of SR Ca\(^{2+}\) release provide direct experimental evidence on how the heart will respond during an anomalous spontaneous extrasystolic episode.

The time courses of the restitution of cytosolic and luminal SR Ca\(^{2+}\) transients were not significantly different (Fig. 8). However, our experiments revealed that the addition of EGTA AM accelerated the restitution process of the SR more than cytosolic Ca\(^{2+}\) transients (Fig. 11). Additionally, it was demonstrated that the restitutions of the AP as well as the Ca\(^{2+}\) influx via voltage-dependent Ca\(^{2+}\) channels are faster than the restitution of Ca\(^{2+}\) transients generated by the release of Ca\(^{2+}\) from the SR (Figs. 9 and 10). These findings indicate that Ca\(^{2+}\) release from the SR is a rate-limiting process regulating cardiac contractility.

Interestingly, the addition of EGTA AM not only accelerated the restitution process in the SR more than the restitution of cytosolic Ca\(^{2+}\) transients (Figs. 10 and 11) but also led to an acceleration of AP restitution (Fig. 11E). Nevertheless, the AP was still restituted much faster than Ca\(^{2+}\) release from the SR.

There are several reasons why increasing the Ca\(^{2+}\) buffering capacity of the myoplasm with an exogenous buffer can modify myoplasmic Ca\(^{2+}\) dynamics. Those include 1) the ability of an exogenous buffer to bind Ca\(^{2+}\) permeating through L-type Ca\(^{2+}\) channels in the plasma membrane that may prevent these ions from activating the RyR2, 2) the attenuation of the feedthrough mechanism of Ca\(^{2+}\) release from the SR through the same or neighboring RyR2s, and 3) the acceleration of the diffusion of Ca\(^{2+}\) from the dyadic space. Although all these processes can alter luminal SR Ca\(^{2+}\) dynamics because they modify Ca\(^{2+}\) release from this compartment, the possibility that EGTA AM can act through any of these three proposed mechanisms is unlikely due to its very slow association rate constant [10.2 ± 0.3 s\(^{-1}\)]

Another possibility is that EGTA AM affects the restitution of Ca\(^{2+}\) transients by modifying the gain of the Ca\(^{2+}\)-induced Ca\(^{2+}\)-release process. This might happen due to a faster relaxation of myoplasmic Ca\(^{2+}\) transients (reducing Ca\(^{2+}\)-induced Ca\(^{2+}\)-dependent inactivation of RyR2) and/or due to a lesser degree of beat-to-beat SR Ca\(^{2+}\) depletion. Our results suggest that the most likely mechanism by which an addition of this intracellular exogenous buffer modifies the restitution of intracellular Ca\(^{2+}\) transients is by decreasing the gain of the Ca\(^{2+}\)-release process via reducing the free diastolic Ca\(^{2+}\) concentration inside the SR and, consequently, diminishing the relative fraction of beat-to-beat Ca\(^{2+}\) depletion.

**Effect of Ca\(^{2+}\) Buffering on the Frequency Dependence of Ca\(^{2+}\) Release**

Tachycardia is an increase in the heart rate that can induce several physiopathological responses. One example is T-wave alternans. T-wave alternans is observed as alternating beat-to-beat changes in the T wave of the ECG and constitutes an important arrhythmogenic mechanism that can lead to sudden cardiac death (37). T-wave alternans is likely to increase with tachycardia and is thought to be associated with abnormalities in intracellular Ca\(^{2+}\) handling and/or cellular metabolism (7, 18, 27). Despite years of studies and debates, the mechanistic links among T-wave alternans, tachycardia, intracellular Ca\(^{2+}\) handling, and cellular metabolism are still unclear.

Our data showed that treatment with EGTA AM resulted in the acceleration of the restitution of Ca\(^{2+}\) release (Fig. 11) and attenuation of Ca\(^{2+}\) alternans (Fig. 12). Wan and coworkers (52) were able to demonstrate that endocardial cells isolated from guinea pig ventricles are more prone to show alternans than epicardial cells due to their reduced capacity to uptake Ca\(^{2+}\) into the SR. Here, we show that the relaxation kinetics of intra-SR Ca\(^{2+}\) transients were slower in mouse hearts treated with EGTA AM than in control hearts (Fig. 7). In addition, the intra-SR diastolic Ca\(^{2+}\) concentration declined in the presence of the buffer (Fig. 4). If the development of Ca\(^{2+}\) alternans depends only on the efficiency of Ca\(^{2+}\) uptake, the alternans would be more pronounced after treatment with EGTA AM. However, the effect of EGTA was the opposite (Fig. 12). This implies that another factor, namely, the difference between the diastolic and systolic level of intra-SR Ca\(^{2+}\) (the degree of Ca\(^{2+}\) depletion), may play an important role in the restitution of Ca\(^{2+}\) release and, consequently, in the generation of cardiac alternans. At the molecular level, this could be explained by the dynamic regulation of RyR2 activity through the Ca\(^{2+}\)-binding protein CSQ2 (23).

**Conclusions**

The changes in intracellular Ca\(^{2+}\) dynamics induced by EGTA AM indicate that an exogenous buffer significantly
modifies Ca\textsuperscript{2+} release from the SR. The acceleration of the restitution of Ca\textsuperscript{2+} transients in the presence of EGTA supports our hypothesis that the amplitude of Ca\textsuperscript{2+} release (the degree of intra-SR Ca\textsuperscript{2+} depletion during each heart beat) is a major factor regulating the refractoriness of Ca\textsuperscript{2+} transients and that the refractoriness defines the frequency dependency of Ca\textsuperscript{2+} alternans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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