Cross-signaling between L-type Ca\textsuperscript{2+} Channels and Ryanodine Receptors in Rat Ventricular Myocytes

SATOMI ADACHI-AKAHANE, LARS CLEEMANN, and MARTIN MORAD

From the Institute for Cardiovascular Sciences and Department of Pharmacology, Georgetown University Medical Center, Washington, DC 20007

INTRODUCTION

Ca\textsuperscript{2+}-influx through the Ca\textsuperscript{2+} channel is the primary pathway for triggering Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR)\textsuperscript{1} in rat cardiac myocytes (Beuckelmann and Wier, 1988; Nábauer et al., 1989; Niggli and Lederer, 1990; Cleemann and Morad, 1991). Though Ca\textsuperscript{2+} influx via the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger has been reported to trigger Ca\textsuperscript{2+} release in guinea-pig (Leblanc and Hume, 1990; Lipp and Niggli, 1994), this is not the case in rat ventricular myocytes (Sham et al., 1992, 1995). In rat myocytes, Ca\textsuperscript{2+} influx via the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger could trigger Ca\textsuperscript{2+} release only when the cells were dialyzed with Na\textsuperscript{+} concentrations in excess of 10 mM and depolarized to voltages positive to 80 mV. However, the efficacy of the exchanger-triggered Ca\textsuperscript{2+} release was 1/20 to 1/160 of that of the Ca\textsuperscript{2+} channel current (Sham et al., 1995). These findings suggested that Ca\textsuperscript{2+}-mediated signaling between rat dihydropyridine (DHP)- and ryanodine receptors is significantly more efficient than between the exchanger and ryanodine receptors.

If, in fact, such effective Ca\textsuperscript{2+} signaling does exist between the DHP- and ryanodine receptors, then Ca\textsuperscript{2+} release via the ryanodine receptor might also control the inactivation of the Ca\textsuperscript{2+} channel, especially since the inactivation of the L-type Ca\textsuperscript{2+} channel has a major Ca\textsuperscript{2+}-dependent component (Eckert and Chad, 1984; Kass and Sanguinetti, 1984; McDonald et al., 1994). Consistent with this idea, ryanodine (1–10 μM), which abolishes Ca\textsuperscript{2+} release from the SR, has been reported to slow the rate of inactivation of I\textsubscript{Ca} while suppressing I\textsubscript{Ca}-triggered contraction (Mitchell et al., 1984) and intracellular Ca\textsuperscript{2+} transients (Ca\textsubscript{2+}-transients) (Callewaert et al., 1988) in rat ventricular myocytes.

To probe the functional proximity of the DHP- and ryanodine receptors and the extent to which such Ca\textsuperscript{2+}-mediated cross-regulation may take place between the

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\textsuperscript{1}Abbreviations used in this paper: [Ca\textsuperscript{2+}], intracellular Ca\textsuperscript{2+} activity; Ca\textsubscript{2+}-transients, intracellular Ca\textsuperscript{2+} transients; DHP, dihydropyridine; SR, sarcoplasmic reticulum.

A preliminary report of these results has been previously published (Adachi-Akahane, S., L. Cleemann, and M. Morad. 1995. Heart Vessels. 9[Suppl.]:163–166).

Address correspondence to Dr. Martin Morad, Institute for Cardiovascular Sciences & Department of Pharmacology, Georgetown University Medical Center, 3900 Reservoir Road, NW, Washington, DC 20007. Fax: 202-687-8458.
two channels, we dialyzed the myocytes with very high concentrations of Ca\(^{2+}\) buffers (2 mM Fura-2 plus 14 mM EGTA or 10 mM BAPTA) to reduce the diffusion distance for Ca\(^{2+}\). Since Ca\(^{2+}\) buffers are used generally in the lowest possible concentration in order not to interfere with Ca\(^{2+}\) signaling, it is critical to point out that the aim of this communication was the exact opposite, i.e., to use large concentrations of Ca\(^{2+}\) buffers and to examine the extent to which Ca\(^{2+}\) signaling remains intact. In such myocytes, we found that even though the transient rise in global myoplasmic Ca\(^{2+}\) concentrations and contraction or activation of the Na\(^+\)-Ca\(^{2+}\) exchanger were strongly suppressed, neither the ability of the SR to re-accumulate and release Ca\(^{2+}\), nor the precise Ca\(^{2+}\)-mediated cross-signaling between the Ca\(^{2+}\) channel and the ryanodine receptor were significantly altered. Our data, thus, suggest that Ca\(^{2+}\) in microdomains between the DHP- and ryanodine receptors, rather than in global myoplasmic space, controls the gating of the Ca\(^{2+}\) channel and the ryanodine receptor. It is likely that the Na\(^+\)-Ca\(^{2+}\) exchanger is excluded from such microdomains.

**METHODS**

**Single Ventricular Myocytes**

Adult rat ventricular myocytes were isolated according to the method described by Mitra and Morad (1985). Briefly, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.), hearts were excised quickly, perfused at 7 ml/min in a Langendorff apparatus first with Ca\(^{2+}\)-free Tyrode solution composed of (in mM) NaCl, 137; KCl, 5.4; HEPES, 10; MgCl\(_2\), 1; glucose, 10, pH 7.3, at 37°C for 8 min, then with Ca\(^{2+}\)-free Tyrode's solution containing collagenase (0.5-0.6 U/ml) and protease (0.55 U/ml) for 15 min, finally with Tyrode's solution containing 0.2 mM CaCl\(_2\) for 8 min. The ventricle of the digested heart was then cut into several sections and subjected to gentle agitation to dissociate cells. The freshly dissociated cells were stored at room temperature in Tyrode's solution containing 0.2 mM CaCl\(_2\) and were used for up to 10 h after isolation.

**Current Recording**

Ca\(^{2+}\)-current was measured in the whole cell configuration of the patch-clamp technique (Hamill et al., 1981) using a DAGON 8900 amplifier (Dagan Co., Minneapolis, MN). The patch electrodes, made of borosilicate glass capillaries, were fire-polished to have resistance of 1.5 to 3 M\(\Omega\) when filled with the internal solution composed of (in mM) CsCl, 100; tetraethylammonium chloride (TEA-Cl), 30; HEPES, 10; MgATP, 5; LiGTP, 0.1; 5'AMP, 0.2; CsFura-2, 2, titrated to pH 7.4 with CsOH. A second series of the experiments were carried out with additional 14 mM EGTA in the internal solution while, in a third series, 10 mM BAPTA was used for up to 10 h after isolation.

The current recording experiments were carried out with additional 14 mM EGTA in the internal solution while, in a third series, 10 mM BAPTA was used for up to 10 h after isolation.

**Calculation of the Intracellular Ca\(^{2+}\) Activity, Total Fura-2 Concentration, and Concentration of Fura-2 Bound to Ca\(^{2+}\)**

The data collected with dual wavelength excitation of Fura-2 were analyzed to determine not only the intracellular Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)), but also the diffusion of Fura-2 into the cell and the amount of Ca\(^{2+}\) binding to Fura-2. This required an extension of the commonly used ratiometric method (Gryniewicz et al., 1985):
The ratiometric determination of $[\text{Ca}^{2+}]$, is based on the law of mass action:

$$K_a = \frac{[\text{Ca}^{2+}]}{[\text{Fura-2}]} \cdot [\text{CaFura-2}],$$  \hspace{1cm} (6)

where [Fura-2] and [CaFura-2] are, respectively, the concentrations of Fura-2 in its free form and complexed form with Ca$^{2+}$ and on the linear properties ($A_{11}, A_{12}, A_{21},$ and $A_{22}$) of the experimental setup:

$$F_{410} = A_{11} \cdot [\text{Fura-2}] + A_{12} \cdot [\text{CaFura-2}] + F_{410,bg},$$  \hspace{1cm} (7)

$$F_{335} = A_{31} \cdot [\text{Fura-2}] + A_{32} \cdot [\text{CaFura-2}] + F_{335,bg}.$$  \hspace{1cm} (8)

Ratiometric measurements require only determination of the proportions of the instrument constants (three independent values corresponding to Eqs. 3, 4, and 5), but one additional calibration measurement is required if [Fura-2] and [CaFura-2] are also to be determined. In the present study we obtained this measurement by assuming that the total Fura-2 concentration in the fully equilibrated cell ($[\text{Fura-2}]_{\text{tot,eq}}$) was equal to 2 mM included in the patch pipette and derived the following equations for the determination of [Fura-2] and [CaFura-2] during the equilibration process:

$$[\text{Fura-2}] = [\text{Fura-2}]_{\text{tot,eq}}: \frac{F_{410} \cdot F_{335,Ca} - F_{335} \cdot F_{410,eq}}{F_{410,eq} \cdot (F_{335,eq} - F_{335,EGTA}) + F_{335,eq} \cdot (F_{410,eq} - F_{410,Ca})}. \hspace{1cm} (9)$$

The calculation indicated by the above equations were performed using a custom made computer program FURA2N written in QuickBasic which operated on the raw data files ($F_{410}, F_{335}$) in pCLAMP format and produced calibrated files ([Ca$^{2+}$], [Fura-2], [CaFura-2], [Fura-2]$_{\text{tot}} = [\text{Fura-2}] + [\text{CaFura-2}]$), either in the same format or in ASCII format. This program was also used to differentiate records, perform exponential analysis, and extract various averaged values.

**Tests and Precautions**

In these experiments we aimed to measure the various experimental parameters after the cells were fully equilibrated with Ca$^{2+}$ buffer, but before run-down of the Ca$^{2+}$ current had occurred. To facilitate quick equilibration, we used patch pipettes with relatively low resistance (1.5-3 MΩ). The equilibration process was monitored at 10s intervals for 8–10 min after membrane rupture and before the start of the experimental protocols. To prevent or delay run-down of the Ca$^{2+}$ current the cells were dialyzed with 200 μM AMP. Even with these precautions it was recognized that equilibration was not complete, that some run-down was unavoidable, and that only the very best experiments (<1/4) produced intervals which approximated our intended experimental conditions. Our pool of raw data was screened, therefore, based on the following requirements: (a) the estimated equilibration with Ca$^{2+}$ buffers was >50% complete (see Figs. 1 and 2); (b) Ca$^{2+}$ current was >1 nA and continued to remain steady; (c) Ca$^{2+}$ release from the SR by diffusion of 2 mM Fura-2 into rat ventricular myocytes. In F the buildup of Fura-2 is measured versus time, and the points are approximated by monoequational functions which are forced to reach a saturating level equal to that of the dialyzing solution (2,000 μM). The quantities in the other panels are measured versus the concentration of Fura-2. Filled and open circles correspond to two different cells. Inset show sample traces recorded 1, 5, and 9 min after rupture of the membrane in the cell plotted with open circles. The dashed curve in the inset of B indicates an interval where electronic detection of the cell length failed during rapid contraction. In these experiments the strongest contractions were typically 10–15% of the cell length. The membrane potential was clamped from a holding potential of −60 mV to a test potential of 0 mV for 200 ms. The dialyzing solution contained 10 μM cAMP.
the Ca\(^{2+}\) current at 0 mV was >50 \(\mu\)M and approximated at least to as much as 60-70\% of the Ca\(^{2+}\) released by caffeine.

Numerical results are given as "mean \(\pm\) SEM (n =)," where SEM is the standard error of the mean and n is the number of experiments which tested the intervention in question. In many cases the results were normalized as ratios to improve SEM.

**Materials**

Collagenase (type A) was purchased from Boehringer-Mannheim (Indianapolis, IL); Protease (type XIV, pronase E) and MgATP were purchased from Sigma Chemical Co. (St. Louis, MO); thapsigargin and tetrodotoxin were purchased from Calbiochem (La Jolla, CA), and KsFura-2 salt was purchased from Molecular Probes, Inc. (Eugene, OR).

**RESULTS**

Diffusion of Fura-2 into Ventricular Myocytes

To investigate the effects of different concentrations of Ca\(^{2+}\)-buffers on various parameters of E-C coupling, we continuously monitored \(I_{\text{Ca}}\), Ca\(^{2+}\)-transients, and contractions as 2 mM Fura-2 slowly diffused from the patch pipette into the myocyte. The insets in Fig. 1 show that within 5 min of the diffusion of Fura-2 contractions were greatly reduced (B) and Ca\(^{2+}\)-transients became significantly smaller (C).

**FIGURE 1.** F shows the time course of the buildup of Fura-2 in two cells (open and closed circles). It should be noted that the total concentration of Fura-2 ([Fura-2]\(_{\text{tot}}\) = [CaFura-2] + [Fura-2]) remained constant during each test pulse (Fig. 1 F, inset, representative traces at 1, 5, and 9 min). The flat traces of Fig. 1 F, inset, represent the cancellation of upward ([CaFura-2]; Eq. 8) and downward ([Fura-2]; Eq. 9) deflections at fluorescence intensities of 410 and 335 nm (measured every 0.8 ms) as an independent check on the calibration procedure.

The equilibration process in the two illustrated cells was approximated by single exponentials with time constants of 8.5 and 12 min, approaching asymptotically the 2,000 \(\mu\)M concentrations of the dialyzing solution. This allowed the measured parameters (\(I_{\text{Ca}}\) amplitude, \(A_{\text{contraction}}\), \(A_{[\text{Ca}^{2+}]_i}\), and \(A_{[\text{Ca-Fura2}]}\)) during the diffusion of Fura-2 to be plotted with respect to the total intracellular Fura-2 concentration.

As the Fura-2 concentration exceeded 1,000 \(\mu\)M in 6-8 min (B) cell shortening was completely abolished (B) and the Ca\(^{2+}\)-transients decreased to <50 nM (C). On the other hand, the change in intracellular Ca\(^{2+}\) concentration bound to Fura-2 (\(A_{[\text{Ca-Fura2}]}\)) increased up to 100 \(\mu\)M as [Fura-2]\(_{\text{tot}}\) rose up to 0.4 mM, but stayed relatively constant with [Fura-2]\(_{\text{tot}}\) in excess of 0.4 mM. The initial dashed parts of the curves correspond to the previously published data recorded with lower concentrations of Fura-2 (0.4 mM; Cleemann and Morad, 1991). The curves in panel D suggest that Fura-2, when added in millimolar concentrations, becomes the dominant intracellular Ca\(^{2+}\) buffer and binds most of the intracellular Ca\(^{2+}\), making it possible to estimate the net amount of Ca\(^{2+}\), which is either released from the SR or enters through the surface membrane. The gradual decline in \(A_{[\text{Ca-Fura2}]}\) paralleled the rundown of \(I_{\text{Ca}}\) (A) such that the saturating titration of the fixed intracellular Ca\(^{2+}\) buffers with 300-500 \(\mu\)M Fura-2 was seen most clearly by plotting the ratio of \(A_{\text{Ca-Fura2}}\) and \(I_{\text{Ca}}\) (E).

In this initial series of experiments, using only 10 \(\mu\)M cAMP in the dialyzing solutions, it was recognized that serious problems resulted from both rundown of \(I_{\text{Ca}}\) before near complete equilibration with Fura-2, and the ability of the SR to compete for uptake of Ca\(^{2+}\) with larger concentrations of buffers, and activation of strong cell shortening in the initial periods of cell dialysis. To alleviate this situation we increased the concentration of cAMP to 200 \(\mu\)M in all subsequent experiments and allowed the cells to rest 3-6 min while Fura-2 or other Ca\(^{2+}\) buffers reached a concentration sufficient to block cell shortening upon activation of \(I_{\text{Ca}}\). This yielded the improvements illustrated in Fig. 2. Both \(I_{\text{Ca}}\) (Fig. 2 A) and the amount of Ca\(^{2+}\) binding to...
Fura-2 (B) declined more slowly, while as in Fig. 1, Ca\(^{2+}\) released normalized for magnitude of \(I_{\text{Ca}}\) stayed constant (C). The average values for \(\Delta[\text{Ca-Fura2}]\) measured 8–20 min after rupture of the membrane in cells dialyzed with 200 \(\mu\text{M}\) cAMP, was 141 ± 18 \(\mu\text{M}\) (mean ± SEM, \(n = 6\)). These results show that the amount of Ca\(^{2+}\) released from the SR by \(I_{\text{Ca}}\) is of comparable magnitude in different experiments, and is affected little by the presence of a high concentration of Ca\(^{2+}\) buffer (2 mM Fura-2) even when Ca\(_t\)-transients triggered by \(I_{\text{Ca}}\) were greatly reduced and the cell shortening was abolished.

Based on the electrode resistance, cell volume and molecular weight of Fura-2, the diffusion time constant for Fura-2 was estimated to be ~7–8 min (Pusch and Neher, 1988) as compared to the measured time constants which typically ranged from 8 to 12 min (Figs. 1F and 2D). Thus, all the experiments described below were performed only after an 8–10 min equilibration period.

**Caffeine-induced Ca\(^{2+}\) Release and the Kinetics of \(I_{\text{Ca}}\)**

Since caffeine is known to release Ca\(^{2+}\) from the SR by enhancing the open probability of the ryanodine receptor (Sitsapesan and Williams, 1990) and to cause subsequent depletion of the SR, we applied caffeine rapidly at precisely controlled times before or during the activation of \(I_{\text{Ca}}\).

Fig. 3A shows \(I_{\text{Ca}}\), intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), and time derivative of rise of [Ca\(^{2+}\)], (d[Ca\(^{2+}\)]/dt) in myocytes dialyzed with 2 mM Fura-2. Depolarizing test pulses to -10 mV from the holding potential of -60 mV fully activated \(I_{\text{Ca}}\), which triggered rapid but small Ca\(_t\)-transients (a rise from 30 to 80 nM in trace a). After a control period of applying depolarizing test pulses at 10-s intervals, rapid application of 5 mM caffeine induced Ca\(_t\)-transients rising from a resting value of 30 to a peak value of 115 nM. The subsequent depolarizing test pulse, given in the presence of caffeine, activated \(I_{\text{Ca}}\), which inactivated slowly compared to control \(I_{\text{Ca}}\) and triggered a much smaller Ca\(_t\)-transient of ~18 nM (Fig. 3A, trace b) compared to the control value of 47 nM (Fig. 3A, trace a). \(I_{\text{Ca}}\) recorded in the presence of caffeine in Fig. 3A trace b inactivated more slowly compared to the control value in Fig. 3A and B, trace a (the rate of inactivation \(1/\tau = 1/(8.1 \text{ ms}) = 125 \text{ s}^{-1} \) [a]) vs. \(1/(17 \text{ ms}) = 60 \text{ s}^{-1} \) [b]). There were no noticeable changes in the rate of activation of \(I_{\text{Ca}}\) (Fig. 3B). Ca\(_t\)-transients triggered by \(I_{\text{Ca}}\) 10 s after wash out of caffeine had partially recovered (Fig. 3A, trace c). Generally 20–30 s were required for the full recovery of Ca\(_t\)-transients.

Since large concentrations of EGTA are often routinely used in the studies of cardiac Ca\(^{2+}\) current kinetics, we tested if this compound was as effective in buffering [Ca\(^{2+}\)], as has been assumed. Dialysis of the myocytes with 14 mM EGTA in addition to 2 mM Fura-2 further suppressed and abbreviated the duration of the Ca\(_t\)-transients (Fig. 4A). Brief rise in [Ca\(^{2+}\)], triggered by \(I_{\text{Ca}}\) or caffeine decayed rapidly, as compared to the Ca\(_t\)-transients obtained with Fura-2 alone (compare Figs. 3 and 4). The superimposed traces of Ca\(_t\)-transients, d[Ca\(^{2+}\)]/dt and the normalized \(I_{\text{Ca}}\) recorded just before (trace a), during (trace b), and after caffeine exposure (trace c) are shown in Fig. 4B. Even though very small and phasic Fura 2 signals were recorded in cells dialyzed with 2 mM Fura-2 plus 14 mM EGTA, nevertheless, the rate of inactivation of \(I_{\text{Ca}}\) after exposure to caffeine slowed significantly (Fig. 4B, trace a; \(1/\tau = 150 \text{ s}^{-1} \) vs. trace b; 40 s\(^{-1}\)) in a manner similar...
FIGURE 4. Effect of rapid application of caffeine (5 mM) on $I_{\text{Ca}}$ and $[\text{Ca}^{2+}]_{i}$ in cells dialyzed with 2 mM Fura-2 plus 14 mM EGTA. The upper part of A shows the protocol where voltage clamp depolarizations were applied before (a), during (b), and after (c) the exposure to caffeine. The traces below are $[\text{Ca}^{2+}]_{i}$, its derivative $(d[\text{Ca}^{2+}]_{i}/dt)$, and the membrane current. B shows the events during the three test pulses superimposed and with expanded time scale. The membrane currents ($I_{\text{Ca}}$) were normalized to yield the same maximal downward deflection.

Table I supports the notion that the inactivation kinetics of $I_{\text{Ca}}$ and its modulation by the SR release were essentially the same in solutions containing either 2 mM Fura-2 alone or with 2 mM Fura-2 plus 14 mM EGTA. For instance the amplitude and time constant for $I_{\text{Ca}}$ under control conditions were 1.96 ± 0.34 nA and 9.8 ± 1.2 ms, respectively, in myocytes dialyzed with 2 mM Fura-2 ($n = 9$) and 1.93 ± 0.47 nA and 16.3 ± 4.1 ms, with the addition of 14 mM EGTA ($n = 7$).

To explore further this insensitivity to intracellular $\text{Ca}^{2+}$ buffers we conducted experiments where 10 mM BAPTA was dialyzed into myocytes through the patch pipettes. In such experiments, the intracellular $\text{Ca}^{2+}$ activity was not monitored with Fura-2 but the free $\text{Ca}^{2+}$ concentration of the internal solution was buffered to 30 nM by addition of Ca$^{2+}$ (Fabiato, 1988). The results, summarized in Table I, suggest that the inactivation kinetics of $I_{\text{Ca}}$ continued to slow significantly when caffeine was applied before activation of $I_{\text{Ca}}$. Close inspection of Table I reveals minor effects of increasing buffer concentrations. For instance, the control value for the time constants of inactivation of $I_{\text{Ca}}$ is 9.8 ± 1.2 ms with 2 mM Fura-2 and is increased to 16.3 ± 4.1 ms with addition of 14 mM EGTA and to 19.2 ± 1.8 ms with 10 mM BAPTA. This trend is supported by the observation that the time constant in the presence of caffeine is increased by a factor of 2.16 ± 0.11 with 2 mM Fura-2 and 2.16 ± 0.05 with the addition of 14 mM EGTA, but only by a factor 1.98 ± 0.07 when 10 mM BAPTA is used. Thus it appears that the buffering of $\text{Ca}^{2+}$ by 10 mM BAPTA is sufficiently strong to remove some of the inactivation of $I_{\text{Ca}}$, which is otherwise dependent on SR release. It should be noted, however, that as the buffering capacity and the speed of $\text{Ca}^{2+}$ buffering is increase, it is likely that the $\text{Ca}^{2+}$ con-
Figure 5. Simulation of the buffering of intracellular Ca\(^{2+}\) achieved by dialysis of 2 mM Fura-2 and 14 mM EGTA. It is assumed that the endogenous buffers are insignificant compared to the added buffers and that the source (Release in B) corresponds to Ca\(^{2+}\), which is released either quickly by depolarization (160 pM released in 20 ms, A) or relatively slowly by caffeine (200 pM in 160 ms, B). C-F show the time course of the nominal Ca\(^{2+}\) activity as measured with Fura-2 (\(K_{d,Fura} / [Ca-Fura2] / [Fura-2]\)). In C (fast release) and D (slow release) the Ca\(^{2+}\) is buffered only by 2 mM Fura (\(K_{d,Fura} = 220 \text{ nm}, k_{22} = 84 \text{ s}^{-1}\), Grynkiewicz et al., 1985; Jackson et al., 1987) whereas in E (fast release) and F (slow release) 14 mM of EGTA (\(K_{d,EGTA} = 200 \text{ nM}, k_{23} = 0.5 \text{ s}^{-1}\), Bennett and Bagshaw, 1986) is added. The calculations were performed by stepwise integration using the following rate constants for equilibration of Ca\(^{2+}\) between its three different states (inset): \(t_{21} = k_{12}, a = 0.7636 \times 10^6 \text{ s}^{-1}, k_{21} = k_{32} \cdot [EGTA] / K_{d,EGTA} = 3,500 \text{ s}^{-1}\). In the linear limit, when most of the Fura-2 and EGTA is found in free form ([Ca-Fura2] << [Fura-2] ~ 2mM, [CaEGTA] << [EGTA] ~ 14 mM), the process is characterized by two time constants which are the roots of the equation: \(t_{2} \cdot (k_{23} \cdot k_{32} + k_{21} \cdot k_{32}) - t_{1} \cdot (k_{23} + k_{21} + k_{32} + k_{31}) + 1 = 0\) (Smith et al., 1984). In the present case the faster rate constant \(t_{21}\) is approximately equal to \(1/(k_{21} + k_{22}) = 1.25 \text{ ms}\) and is the life time of released Ca\(^{2+}\) ions before they are bound by the buffers. The slower time constant, here: \(t_{ex} = 1 / (k_{23} \cdot k_{32} + k_{21} \cdot k_{31}) \cdot t_{21} = 230 \text{ ms}\), is the time constant for equilibration between the two buffers.

Figure 7 summarizes the normalized results of such comparisons at various times in nine ventricular myocytes. The effects of caffeine on the amplitude of \(I_{Ca}\), its rate of inactivation \((1/\tau)\), and the accompanying \(\Delta [Ca^{2+}]\), and \(d[Ca^{2+}] / dt\) were plotted as functions of the delay \((t)\) between the onset of caffeine-pulse and the activation of \(I_{Ca}\). Only when the onset of caffeine-induced transient occurred <50 ms before the activation of \(I_{Ca}\) did the rate of inactivation of \(I_{Ca}\) accelerate with concomitant increase in \(\Delta [Ca^{2+}]\) and \(d[Ca^{2+}] / dt\). These three parameters were fit by the following equation:

\[
y = (A_i - A_f) \cdot \exp(-t/\tau) + A_f, \tag{10}
\]

where \(A_i\) and \(A_f\) are, respectively, the initial \((t = 0)\) and final \((t = \infty)\) values of the three parameters, and \(\tau\) is a time constant of exponential decay. The derived constants are listed in Table II. In spite of some scatter of the data, it is clear that the rate of inactivation of \(I_{Ca}\) \((1/\tau)\), \(\Delta [Ca^{2+}]\), and \(d[Ca^{2+}] / dt\) are governed by a single exponential. The data also suggest a trend to-
TABLE I

Effect of Ca2+ Buffers on Caffeine-induced Changes of Ca2+ Currents and their Accompanying Ca2+ Transients

<table>
<thead>
<tr>
<th></th>
<th>2 mM Fura-2</th>
<th>2 mM Fura-2 + 14 mM EGTA</th>
<th>10 mM Bapta</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ca2+ current, $I_{Ca}$ [nA]</td>
<td>1.96 ± 0.34 (9)</td>
<td>1.0 ± 0.47 (7)</td>
<td>3.70 ± 0.45 (7)</td>
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<td>Time constant, $\tau$, [ms]</td>
<td>9.8 ± 1.2 (9)</td>
<td>16.3 ± 4.1 (7)</td>
<td>19.2 ± 1.8 (7)</td>
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<td>Ca2+ transient, $\Delta$[Ca2+]i, [nM]</td>
<td>47 ± 7 (9)</td>
<td>49 (2)</td>
<td></td>
</tr>
<tr>
<td><strong>Caffeine</strong></td>
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<tr>
<td>Ca2+ current, $I_{Ca}$ [nA]</td>
<td>2.06 ± 0.38 (9)</td>
<td>2.24 ± 0.59 (7)</td>
<td>3.83 ± 0.45 (7)</td>
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<td>ratio</td>
<td>1.03 ± 0.04 (9)</td>
<td>1.12 ± 0.07 (7)</td>
<td>1.04 ± 0.02 (7)</td>
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<tr>
<td>Time constant, $\tau$, [ms]</td>
<td>20.5 ± 1.9 (9)</td>
<td>35 ± 8 (7)</td>
<td>32 ± 5 (7)</td>
</tr>
<tr>
<td>Ca2+ transient, $\Delta$[Ca2+]i, [nM]</td>
<td>14.7 ± 2.9 (9)</td>
<td>10.0 (2)</td>
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<tr>
<td>ratio</td>
<td>0.33 ± 0.06 (9)</td>
<td>0.29 (2)</td>
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<tr>
<td><strong>Wash</strong></td>
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<tr>
<td>Ca2+ current, $I_{Ca}$ [nA]</td>
<td>2.17 ± 0.49 (6)</td>
<td>2.15 ± 0.40 (5)</td>
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<tr>
<td>ratio</td>
<td>1.09 ± 0.03 (6)</td>
<td>1.10 ± 0.05 (5)</td>
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<tr>
<td>Time constant, $\tau$, [ms]</td>
<td>12.2 ± 1.1 (6)</td>
<td>14.1 ± 2.7 (5)</td>
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<tr>
<td>ratio</td>
<td>1.33 ± 0.10 (6)</td>
<td>1.34 ± 0.14 (5)</td>
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</table>

All values are means ± SEM with the number of experiments in parenthesis (n). Ratios are calculated relative to the control condition with the same Ca2+ buffer. The effects of caffeine included in this table were measured 400-2,000 ms after the application of the drug. The table only includes results from experiments where $I_{Ca}$ was measured under this condition. Ca2+ transients were not measured when 10 mM BAPTA was used as buffer.

ward $1/\tau$ for $I_{Ca}$ falling faster than $\Delta$[Ca2+]i, and d[Ca2+]i /dt. The trend, however, was not statistically significant nor was a consistent finding in the results from individual cells. The amplitude of $I_{Ca}$ in response to caffeine exposure was best described by two exponentials $\exp[-t/48] + 1.15 \cdot \exp(-t/283)]$. $I_{Ca}$ was smaller than control when the interval between the onset of the caffeine-pulse and $I_{Ca}$ was <500 (minimum value occurring at $t = 100$ ms) but was significantly larger ($109 ± 3.1\%$, SEM, $n = 8$, $P < 0.05$) at intervals >500 ms when combining the pool of cells dialyzed with 2 mM Fura 2 and 2 mM Fura 2 plus 14 mM EGTA. The initial suppression of $I_{Ca}$ is consistent with the idea that $I_{Ca}$, before its activation, may be inactivated by Ca2+ release from the SR. The subsequent recovery of $I_{Ca}$, however, occurs even while the global intracellular Ca2+ activity was significantly elevated (100–120 nM, Figs. 3 and 6). This suggests that the local build-up of Ca2+, which serve to inactivate Ca2+ channels near the release sites, dissipates rapidly, shortly after caffeine triggered Ca2+ release due to the diffusion or uptake of Ca2+ by Ca-ATPase and Ca2+ buffers (Balke et al., 1994).

Ca2+ Release Dependence of Inactivation of the Ca2+ Channel

Fig. 8 shows a linear correlation between the rate of inactivation of $I_{Ca}$ as a function of either $\Delta$[Ca2+]i (correlation coefficient: $r = 0.885$) or d[Ca2+]i /dt ($r = 0.781$). Filled circles correspond to measurements during exposure to caffeine while open circles correspond to the first depolarization after removal of caffeine. The values are normalized relative to the last depolarization before application of caffeine. It appears that modulation of the rate of inactivation of $I_{Ca}$ by caffeine is mediated primarily through the magnitude of Ca2+ released from the SR. The y-axis intercept for $1/\tau$ (0.272 ± 0.035 for $\Delta$[Ca2+]i and 0.288 ± 0.049 for d[Ca2+]i /dt) corresponds roughly to a condition where no Ca2+ is released from the SR. This suggests that 72 ± 4% (>67%, $P = 0.05$) of the rate of inactivation of $I_{Ca}$ might be determined by Ca2+ released from the SR. Setting aside the possible contribution to the time constant of inactivation of $I_{Ca}$ from a purely voltage-dependent process, the results of Fig. 8 suggest that Ca2+ influx through the Ca2+ channel contributes only ~35% of its inactivation. This may seem somewhat surprising since the Ca2+-sensing site of the channel is thought to be located in the immediate vicinity of the inner mouth of the Ca2+ channel (Imredy and Yue, 1994; de Leon et al., 1995). On the other hand, it is possible that the ryanodine receptors, in their ability to inactivate the Ca2+ channel, may overcome the disadvantage of distance either by their greater abundance (about nine for each DHP receptor; Wibo et al., 1991) or larger single channel conductance (135 pS; Tinker and Williams, 1992 vs. 6.9 pS for the DHP receptor; Rose et al., 1992).

Caffeine-induced Na+-Ca2+ Exchange Current in Myocytes Dialyzed with Low and High Concentrations of Fura-2

As shown in Fig. 9, in myocytes dialyzed with only 0.2 mM Fura-2, caffeine-induced Ca2+ transient activated a small (50-100 pA) slowly decaying Ni2+-sensitive inward current representing the extrusion of Ca2+ by the Na+-Ca2+ exchanger (Callessa et al., 1989). $I_{Ca}$ activated after the onset of caffeine-pulse inactivated more
FIGURE 6. The effects of the caffeine exposure depended on its timing relative to the voltage clamp depolarizations. Caffeine (5 mM) was applied 850 (A), 250 (B), and 50 ms (C) before the activation of $I_{ac}$. $I_{ac}$-transients and $I_{ca}$ were measured before (a), during (b), and after (c) application of caffeine. The cell was dialyzed with Fura-2 (2 mM). Each panel shows on the left side from top to bottom the timing of exposure to caffeine, the voltage clamp protocol, $[Ca^{2+}]_i$, and the membrane current and, on the right side, the normalized membrane currents with expanded time base. In A (middle), the inset shows the enlarged current at -60 mV during caffeine exposure.

slowly compared to the control current in a manner similar to the myocytes dialedyzed with 2 mM Fura-2 (compare trace a with traces b and c in A; see also B where $I_{ca}$ is normalized). In this series of experiments, the caffeine-induced Na$^+$-Ca$^{2+}$ exchange currents could be observed in all of the myocytes with 0.2 mM Fura-2 ($n = 4$). In contrast, in all myocytes dialedyzed with 2 mM Fura-2 (Fig. 6 A, trace b), caffeine failed to activate the Na$^+$-Ca$^{2+}$ exchange currents even though caffeine continued to alter the kinetics of $I_{ca}$ after the release of Ca$^{2+}$.

It may be argued that the Na$^+$-Ca$^{2+}$ exchange molecule requires certain level of basal Ca$^{2+}$ activity to be activated. To check on this possibility, we carried a series of experiments where the myoplasmic $[Ca^{2+}]_i$, was buffered at 80-100 nM range in the presence of 2 mM Fura-2 by addition of Ca$^{2+}$ (Fabiato, 1988). In such myocytes caffeine still failed to induce $I_{Na,Ca}$ ($n = 22$), even though it consistently modified the inactivation kinetics of $I_{ca}$ (data not shown). These findings support the notion that Na$^+$-Ca$^{2+}$ exchange currents in rat ventricular myocytes are activated by the elevation of the global myoplasmic Ca$^{2+}$ concentrations which under our experimental conditions are effectively buffered by high concentration of Ca$^{2+}$-buffers.

We examined the species variability of this finding and found that even in guinea-pig ventricular myocytes where the exchanger has been reported to trigger Ca$^{2+}$ release (Leblanc and Hume, 1990), the application of 5 mM caffeine, which consistently activated the Na$^+$-
## Table II

Parameters Obtained from the Curve Fitting of the Data Points Using the Eq. 10

<table>
<thead>
<tr>
<th></th>
<th>Initial value (Ai)</th>
<th>Final value (Ai)</th>
<th>Time constant (τ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of inactivation</td>
<td>1.13 ± 0.11</td>
<td>0.48 ± 0.04</td>
<td>69 ± 26 ms</td>
</tr>
<tr>
<td>Δ[Ca( ^{2+} )]_i</td>
<td>1.42 ± 0.16</td>
<td>0.28 ± 0.08</td>
<td>146 ± 42 ms</td>
</tr>
<tr>
<td>d[Ca( ^{2+} )]/dt</td>
<td>1.23 ± 0.16</td>
<td>0.27 ± 0.06</td>
<td>129 ± 34 ms</td>
</tr>
</tbody>
</table>

Ca\( ^{2+} \) exchange current in cells dialyzed with 0.2 mM Fura-2 (free [Ca\( ^{2+} \)]_i = 80 nM, \( n = 5 \)) failed to activate an inward exchange current, in cells dialyzed with 2 mM Fura-2 (\( n = 5 \)).

### Effects of Caffeine on the Inactivation of Ba\( ^{2+} \)-current through the Ca\( ^{2+} \) Channel

To distinguish the role of released Ca\( ^{2+} \) from that of Ca\( ^{2+} \) entering through the Ca\( ^{2+} \) channel on the inactivation of Ca\( ^{2+} \) channel, we conducted a series of experiments using Ba\( ^{2+} \) as the charge carrier through the Ca\( ^{2+} \) channel. In these experiments, caffeine was used to release Ca\( ^{2+} \) from the fully loaded SR. The Ca\( ^{2+} \) content of the SR was maintained at control level by applying a train of 8–10 depolarizing pulses at 5-s intervals in the presence of 2 mM Ca\( ^{2+} \) before and immediately after short periods of Ba\( ^{2+} \) exposure.

Transient replacement of 2 mM Ca\( ^{2+} \) (Fig. 10 A) by 2 mM Ba\( ^{2+} \) (Fig. 10, B and C) in the external solution produced slowly inactivating membrane currents through the Ca\( ^{2+} \) channel. Entry of Ba\( ^{2+} \) through the DHP-sensitive Ca\( ^{2+} \) channel, as previously reported, failed to trigger the release of Ca\( ^{2+} \) from the SR (Näbauer et al., 1989). Influx of Ba\( ^{2+} \), nevertheless, caused changes in the Fura-2 fluorescence intensities, which reflect the binding of Ba\( ^{2+} \) to Fura-2. Though Ba\( ^{2+} \) has 6 times lower affinity for Fura-2 (\( K_a = 1,200 \) nM) than Ca\( ^{2+} \) (Kwan and Putney, 1990), it is estimated that in myocytes dialyzed with 2 mM Fura-2 a great proportion of Ba\( ^{2+} \) ions entering the cell will bind to Fura-2. The changes in fluorescence intensities were thus used to calculate \( -Δ[Fura-2] \) from Eq. 8 and to obtain the concentration of the divalent metal ions (Ca\( ^{2+} \) as well as Ba\( ^{2+} \)) bound to Fura-2.

Fig. 10 also shows the effects of caffeine-induced Ca\( ^{2+} \)-release on Ca\( ^{2+} \) channel when 2 mM Ca\( ^{2+} \) (Fig. 10 A) or 2 mM Ba\( ^{2+} \) (Fig. 10 B) were the charge carriers through the channel. Depletion of Ca\( ^{2+} \) stores by caffeine applied 500 ms before activation of Iba had little or no effect on the rate of its inactivation (Fig. 10 B, right), in contrast to when Ca\( ^{2+} \) was the charge carrier through the Ca\( ^{2+} \) channel (Fig. 10 A, right). On the other hand, release of Ca\( ^{2+} \) by caffeine simultaneous with the activation of Iba caused a relatively rapid rise in the fluorescence signal, and greatly enhanced the rate of inactivation of Iba (Fig. 10 C) in a manner similar to when Ca\( ^{2+} \) was the charge carrier through the channel (Fig. 6 C). The rates of inactivation of Iba in traces a, b, and c of Fig. 10 C were 16 s\(^{-1}\), 56 s\(^{-1}\), and 17 s\(^{-1}\), respectively. It should be noted that this enhancement of the rate of inactivation is comparable in magnitude to the effect illustrated in Figs. 6–8, but occurred only when Ba\( ^{2+} \) permeated through the channel.

![Figure 8](https://example.com/figure8.png)
The combined effect of caffeine and $I_{Ba}$ produces a fluorescence signal (Fig. 10 C, trace b) which has somewhat faster kinetics than those elicited by either of these interventions alone (respectively, trace b in panel B and traces a in panels B and C). The possibility that caffeine sensitizes the ryanodine receptors (Rousseau et al., 1988) to release Ca$^{2+}$ in response to residual contaminants of Ca$^{2+}$ may, in part, be responsible for this observation.

Amplification Factor

Comparison of the fluorescence signals produced by Ca$^{2+}$ and Ba$^{2+}$ currents was used to quantify the SR Ca$^{2+}$ release relative to the Ca$^{2+}$ entry through the Ca$^{2+}$ channel. This “amplification factor” or “gain” of the release system was estimated based on the idea that Ca$^{2+}$ entering through the Ca$^{2+}$ channel triggers the release of Ca$^{2+}$ from the SR. Thus Fura-2 binds Ca$^{2+}$ from both sources, while Ba$^{2+}$ entering through the Ca$^{2+}$ channel binds to Fura-2 without triggering Ca$^{2+}$-release from the SR. The amplification factor ($A$) was given by the following equation:

$$A + 1 = \frac{\Delta [\text{Ca-Fura2}]}{\Delta [\text{Ba-Fura2}]} / \frac{Q_{Ca}}{Q_{Ba}},$$

where $\Delta [\text{Ca-Fura2}]$ and $\Delta [\text{Ba-Fura2}]$ represent the change in the concentration of Fura-2 which was bound to Ca$^{2+}$ and Ba$^{2+}$, respectively, and $Q_{Ca}$ and $Q_{Ba}$ represent the amount of charge carried by Ca$^{2+}$ and Ba$^{2+}$, respectively. $Q_{Ca}$ was calculated by integration of $I_{Ca}$ from the time of depolarization until $\Delta [\text{Ca-Fura2}]$ reached 90% of its final value. $Q_{Ba}$ was obtained by integrating $I_{Ba}$ for its duration.

An average value of $17.6 \pm 1.1$ ($n = 4$, mean $\pm$ SEM) was obtained, suggesting that one Ca$^{2+}$ entering through the DHP-receptor may induce the release of $\sim 16-19$ Ca$^{2+}$ from the SR.

Depletion of the SR Ca$^{2+}$ by Thapsigargin and the Rate of Inactivation of $I_{Ca}$

To probe the role of Ca$^{2+}$ released from the SR in the Ca$^{2+}$-dependent inactivation process of Ca$^{2+}$ channel vis-a-vis the possible direct effects of caffeine, the Ca$^{2+}$ content of the SR was depleted using thapsigargin, a potent inhibitor of SR Ca-ATPase. (Lytton et al., 1991; Janczewski and Lakatta, 1993). In myocytes dialyzed with 2 mM Fura-2, thapsigargin gradually suppressed $C_{Ca}$-transients triggered by $I_{Ca}$ (Fig. 11, A and B) and slightly increased the basal myoplasmic [Ca$^{2+}$]. As the magnitude of $C_{Ca}$-transients decreased, the rate of inactivation of $I_{Ca}$ also slowed. Fig. 11 (lower panels) shows that even though the amount of Ca$^{2+}$ influx through the Ca$^{2+}$ channel increased after thapsigargin treatment, the rate of inactivation of $I_{Ca}$ decreased. The rate of inactivation of $I_{Ca}$ during the treatment were linearly correlated to the magnitude of the $I_{Ca}$-gated Ca$^{2+}$ release ($\Delta [\text{Ca}^{2+}]$) ($r = 0.98478$) and $d[\text{Ca}^{2+}] / dt$ ($r = 0.95292$) in the same way as observed in Fig. 8.

Thapsigargin also abolished the caffeine-induced Ca$^{2+}$ release and its modulation of the inactivation kinetics of $I_{Ca}$ (Fig. 12). The caffeine-induced release was nearly completely abolished after 6-min exposure to thapsigargin (Fig. 12 B), and $I_{Ca}$-triggered release decreased to <20% of control (Fig. 12 A). Yet under these conditions caffeine still caused a significant increase in the release triggered by $I_{Ca}$ thereby accelerating the inactivation (compare traces a and b, Fig. 12 B). This finding resembles the effect described in Fig. 6 C but implies also that caffeine can potentiate $I_{Ca}$-triggered release under conditions where it does not by it-
self cause a regenerative release. Only after 15 min of incubation in thapsigargin did caffeine fail completely to trigger Ca\textsubscript{2+}-transients or alter the kinetics of I\textsubscript{Ca} (Fig. 12 C, compare traces a and b). At this time Ca\textsubscript{2+}-transients triggered by I\textsubscript{Ca} were reduced from 46.1 to 6.4 nM while Q\textsubscript{Ca} had increased from 28 to 59 pC. The amplification factor derived from these numbers [(46.1 nM/28 pC)/(6.4 nM/58 pC) = 14.1] is in fair agreement with the estimate based on the Ba\textsuperscript{2+} current flux (17.6 ± 1.1).

These results suggest that considerable time is required before the SR is emptied completely of its releasable Ca\textsuperscript{2+} by thapsigargin. At incubation times in excess of 15 min, however, the results fully support the notion that the caffeine-induced modification of the rate of inactivation of Ca\textsuperscript{2+} channel was entirely dependent on the Ca\textsuperscript{2+} content of the SR.

**Prolongation of I\textsubscript{Ca} by Conditioning Pulses**

Another procedure used to manipulate the Ca\textsuperscript{2+} content of the SR was the application of a single or multiple conditioning pulses before the activation of I\textsubscript{Ca}. Fig. 13 A shows that application of a conditioning pulse 400 ms before the test pulse slightly slowed the rate of inactivation of I\textsubscript{Ca}. Although such conditioning pulses decreased the subsequent Ca\textsubscript{2+}-transients triggered by I\textsubscript{Ca}, they did not suppress them as effectively as did 5 mM
When Ca$^{2+}$ release was abolished with thapsigargin, the application of a conditioning pulse did not alter the rate of inactivation of I$_{Ca}$ (Fig. 13 A, see normalized I$_{Ca}$ traces in right panel).

A train of conditioning pulses, when applied 800 ms before the activation of test I$_{Ca}$, significantly slowed the rate of inactivation of the subsequent I$_{Ca}$ (Fig. 13 B, trace b and upper right) and decreased its accompanying Ca$^{2+}$-transient. The triple pulses were not more effective than the single conditioning pulse, probably because the 800 ms of the waiting period, introduced to allow nearly complete restitution of I$_{Ca}$, was also sufficient for partial reloading of the SR. On the other hand, the second (b2) and third (b3) closely spaced conditioning pulses activated markedly smaller I$_{Ca}$ and Ca$^{2+}$-gated Ca$^{2+}$-transients. Nevertheless, these Ca$^{2+}$ currents inactivated significantly slower than that observed with the first conditioning pulse (b1). In thapsigargin-treated myocytes, the application of triple conditioning pulses had no effects on the rate of inactivation of I$_{Ca}$ accompanying the test or conditioning pulses (Fig. 13 B, thapsigargin).

**DISCUSSION**

The major finding of this study is that I$_{Ca}$-triggered Ca$^{2+}$ release from the SR is an important feed-back signal in determining the rate of inactivation of the L-type Ca$^{2+}$ channel, and thereby the Ca$^{2+}$ release mechanism itself, even in highly Ca$^{2+}$-buffered myocytes. The resistance of such a Ca$^{2+}$ signaling mechanism to changes in myoplasmic Ca$^{2+}$ concentrations implies that the global myoplasmic Ca$^{2+}$ concentrations have only a minor role in modulation of Ca$^{2+}$ signaling between DHP- and ryanodine receptors. Thus, signaling between the two sets of receptors most likely occurs through microdomains of Ca$^{2+}$ surrounding the DHP- and ryanodine-receptor complex. These Ca$^{2+}$ microdomains seem to exclude functionally the Ca$^{2+}$ transported by the Na$^{+}$-Ca$^{2+}$ exchanger (Fig. 14). These conclusions, in part, rest on the assumption that Ca$^{2+}$ buffers are capable of blocking Ca$^{2+}$ diffusion over distances larger than ~10-50 nm.

**Effectiveness of Ca$^{2+}$ Buffer**

Intracellular Ca$^{2+}$ indicator dyes have often been used in the lowest possible concentrations to minimize interference with E-C coupling (Cannell et al., 1987; Cleemann and Morad, 1991; Berlin and Konishi, 1993), while in other experiments large concentrations of EGTA have been commonly used to keep [Ca$^{2+}$] constant. The present results demonstrate to what extent these practices are justified.

As 2 mM Fura-2 diffused into a cell, its buffering effect was evident by complete block of contraction (Fig. 1 B) and Na-Ca exchange current (Fig. 6 A vs. Fig. 9 A), suppression of Ca$^{2+}$-transients to ~50 nM (Table I), and the saturating amount of Ca$^{2+}$ bound to Fura-2 (Fig. 1 D). As expected from its in vitro kinetics the inclusion of 14 mM EGTA into the dialysate further suppressed the tonic, but not the transient, component of rise in
[Ca$^{2+}$]$_i$, measured with Fura-2 (Fig. 4 vs. Fig. 3). This behavior is thought to reflect the transfer of Ca$^{2+}$ from the fast acting Fura-2 to the slower, but ultimately more effective, EGTA (Fig. 5). The time constant for diffusion of Fura-2 from the patch pipette into the cell was ~10 min (Figs. 1 and 2), so that equilibration of the dye was >50% complete when measurements were started at 8–10 min into cell dialysis. From these results we conclude that the dialyzed Ca$^{2+}$ buffers had strong and predictable effects and that they were present inside the cells in concentrations ranging from 50 to 100% of their nominal values.

It is possible then to estimate not only the buffering of the bulk intracellular Ca$^{2+}$ concentration, but also the time and distance a calcium ion may move by diffusion before it is bound by the added Ca$^{2+}$ buffers. The theoretical life time of a freely diffusing calcium ion can be calculated from Eq. 12:

$$\tau_{\text{life}} = \frac{1}{\{Fura\} / (K_{d,Fura} \cdot t_{\text{off,Fura}}) + \{\text{EGTA}\} / (K_{d,\text{EGTA}} \cdot t_{\text{off,EGTA}})}$$

(for details, see legend of Fig. 5). Based on in vitro assays of the buffers (Jackson et al., 1987; Smith et al., 1984), the life time of Ca$^{2+}$ was estimated to be ~1.3–2.6 μs when dialyzing with 1–2 mM Fura-2 alone and 1.25–2.5 μs with the addition of 7–14 mM EGTA. The modest improvement with addition of high concentrations of EGTA is due to its slower kinetics (Smith et al., 1984).

From the life time estimates ($\tau_{\text{life}} = 1.25–2.5$ μs) and diffusion constant for Ca$^{2+}$ ($D = 10^{-6}$ cm$^2$/s), we estimated that the average Ca$^{2+}$ ion may diffuse approximately $\sqrt{D} \cdot \tau_{\text{life}} = 11–16$ nm before being bound to the buffers and that the vast majority of calcium ions will be bound before they have diffused 3 space constants, or about 50 nm. This calculation would explain why the diffusion of released Ca$^{2+}$ from the ryanodine receptors to the contractile filaments (0.5–1.0 μm) is effectively intercepted by the buffers, while Ca ions traversing much shorter distances, between the DHP- and ryanodine receptors remain unaffected (Stern, 1992a, Fig. 14).

EGTA in a concentration of 10–15 mM has been often used to suppress possible effects of Ca$^{2+}$ released from the SR on the gating kinetics of Ca$^{2+}$ channel (Tihan et al., 1994). However, our data clearly demonstrated that 14 mM EGTA even with addition of 2 mM Fura-2 does not abolish I$_{ca}$-trigged Ca$^{2+}$ release and its effect on the inactivation kinetics of I$_{ca}$ (Fig. 4, Table 1). This suggests that higher concentration of Ca$^{2+}$ buffers will be required to eliminate the effect of SR
Ca$^{2+}$-release on the inactivation of the Ca$^{2+}$ channel. Indeed we found that >10 mM BAPTA was required to suppress noticeably this kind of coupling between the two sets of receptors. As BAPTA and Fura-2 bind Ca$^{2+}$ with similar kinetics, it is estimated that 10 mM BAPTA would impose $\tau_{\text{life}} = 0.25$–0.5 $\mu$s, producing an effective diffusion distance of ~5–7 nm (A2 in Fig. 14) for Ca$^{2+}$ released from the ryanodine receptor to reach the inactivation site of the DHP receptor. These distances are of roughly the same scale as the dimensions of DHP- and ryanodine receptors and the width of the dyadic cleft space. On the other hand, it is possible that the added Ca$^{2+}$ buffers do not gain complete access to, or diffuse freely within, the dyadic cleft. While this would be one of the distinguishing properties of the Ca$^{2+}$ $\mu$-domains, it would also mean that their exact dimensions are not yet known and that it would be premature to conclude whether they encompass a single DHP-receptor communicating with one or more ryanodine receptors, or perhaps an entire t-SR junction with lateral dimension on the scale of 100 nm. For a more detailed evaluation it would be necessary to consider the detailed structure of this junction (Sun et al., 1995), the diffusion of the added Ca$^{2+}$ buffers (Stern, 1992a; Smith et al., 1996), as well as the presence of endogenous, immobile Ca$^{2+}$ buffers (Langer and Peskoff, 1996).
The arrows suggest that the DHP receptor and Ryanodine receptors are coupled by exchange of Ca²⁺ signals (A1 and A2) which are more important than the autoregulatory Ca²⁺ signals (A3 and A4) or the Ca²⁺ fluxes to the Na⁺-Ca²⁺ exchanger (A5) and their possible blockage by Fura-2, EGTA, or BAPTA. This schematic is not meant to imply actual stoichiometry between DHP and ryanodine receptors and their communicating via one microdomain of Ca²⁺.

**Modulation of Rate of Inactivation of Ica by Ca²⁺-release from the SR**

Caffeine had two effects on Ca²⁺-release triggered by Ica (Figs. 6 and 7). When caffeine was applied at intervals shorter than 50 ms before the activation of Ica, Ca²⁺-transients triggered by Ica were enhanced (Figs. 6C and 7). This may result from the increased Ca²⁺-sensitivity of the ryanodine-receptor to Ca²⁺ in the presence of caffeine (Rousseau et al., 1988) and the positive feedback process inherent in the Ca²⁺-induced Ca²⁺-release mechanism (Fabiato, 1985; O’Neill and Eisner, 1990; Sitgespesan and Williams, 1990). On the other hand, when caffeine-transient occurred more than 300 ms before the activation of Ica, Ica-gated Ca²⁺-transients were significantly reduced, presumably because Ca²⁺ stores that were depleted of Ca²⁺ by caffeine remained empty. Thus caffeine could be used as an effective tool to modify the magnitude of Ica-gated Ca²⁺-transients by changing the timing between the application of caffeine-pulse and activation of Ica.

Comparison of the kinetics of inactivation of Ica with the rate of rise of [Ca²⁺], suggested that the higher rates of release of Ca²⁺ were accompanied by faster rates of inactivation of Ica and vice versa (Figs. 6, 7, and 8). It was somewhat unexpected that the rise in myoplasmic Ca²⁺ concentration did not enhance the rate of inactivation (Figs. 5A, 6A, and B, and 4). This finding suggests that alterations of the global myoplasmic Ca²⁺ concentrations do not regulate the rate of inactivation of Ca²⁺ channel. On the other hand, the linear relationship between the rate of inactivation of Ica (1/τ) and d[Ca²⁺]/dt of Fig. 8 suggests that ~65-75% of inactivation of Ca²⁺ channel may be dependent on the release of Ca²⁺ from the ryanodine-receptor (A2 arrow of the schematic of Fig. 14). Similar linear correlations between rate of inactivation of Ica and d[Ca²⁺]/dt were obtained as thapsigargin SR-depleting effect was taking place. These findings do not exclude the inactivation of the DHP receptor by its own unitary Ca²⁺ current (A4 arrow of Fig. 14), but it suggests that this process, in cells with high levels of cAMP, is responsible for only 25–35% of Ca²⁺-induced inactivation of Ica.

Partial depletion of the SR with single (Fig. 13A) or multiple (Fig. 13B) conditioning pulses produced a decrease in the rate of inactivation of Ica. The abolishment of such modifications in the rate of inactivation of Ica by thapsigargin supported the idea that the beat-dependent change in Ica kinetics are, in part, caused by the decrease of Ca²⁺-release from the SR. A causal relationship of this nature was suggested by Tseng (1988). The treatment with thapsigargin makes it possible to dissect the component of the restitution process of Ica which depends on voltage but not Ca²⁺ (Fig. 13).

The result with caffeine, thapsigargin, and conditioning pulses all indicate that Ca²⁺-dependent inactivation of Ica is mediated primarily by Ca²⁺ released from the SR (65–75%, see A2 arrow in Fig. 14), is less dependent on its own Ca²⁺ influx (25–35%, A4 arrow in Fig. 14), and is independent of the global myoplasmic Ca²⁺ concentrations.

At first sight these findings might appear to contradict published results. For instance, it has been amply demonstrated that various intracellular Ca²⁺ buffers (e.g. 20 mM EGTA) slows the inactivation of Ica in cultured guinea-pig atrial myocytes (Bechem and Pott, 1985). The open probability of single L-type Ca²⁺ channels in cell attached patches of guinea-pig ventricular myocytes was modulated by modest changes in the intracellular Ca²⁺ activity (Hirano and Hiraoka, 1994). Both Ica and Ib carried by L-type Ca²⁺ channels reconstituted in planar lipid bilayers inactivate faster when the Ca²⁺ activity at the “intracellular” surface is raised to 10 μM (Haack and Rosenberg, 1994). The α₁ subunit of the cardiac Ca²⁺ channel exhibits the characteristics of Ca²⁺ dependent inactivation when expressed, in high or low density, in Xenopus oocytes (Neely et al., 1994). And, finally, in a cell attached patch the influx of Ca²⁺ through one cell attached patch can facilitate the inactivation of an adjacent channel (Imredy and Yue, 1992). Thus there is little doubt that the inactivation of individual Ca²⁺ channels is directly influenced both by Ca²⁺ in cytoplasmic space entering through the channel itself or one of its neighbors. In this light, the questions raised by the present results assume a quanti-
tative nature. Why is the inactivation of $I_{Ca}$ in the present study determined primarily, but not exclusively, by the Ca$^{2+}$ which is released from the SR?

In part the explanation may be that the SR release is unusually large in our experiments both because the SR is abundant in adult rat ventricular myocytes and because it is stimulated to accumulate Ca$^{2+}$ by 200 μM cyclic AMP. Conversely, the Ca$^{2+}$ channels reconstituted in planar lipid membranes, cell attached patch or cloned in *Xenopus* oocytes (Hirano and Hiraoka, 1994; Neely et al., 1994) most probably were not affected by nearby Ca$^{2+}$ release, consistent with relatively slow inactivation kinetics reported in such studies (>100 ms). Similarly, it is likely that Ca$^{2+}$ channels in surface membrane (Imredy and Yue, 1992, 1994; de Leon et al., 1995) or in cultured myoballs (Bechem and Pott, 1985) may have properties different than those which are concentrated in the dyadic junctions of the transverse tubules, where they initiate and modulate the Ca$^{2+}$ release in adult myocytes (Morad and Gleemann, 1987; Wibo et al., 1991).

Still it may be questioned how a specific Ca$^{2+}$ channel can be less sensitive to its own Ca$^{2+}$ flux (arrow A4, Fig. 14) than to Ca$^{2+}$ transported by more distant ryanodine receptors (arrow A2, Fig. 14). Perhaps the processes are somewhat similar to those which make the ryanodine receptor responsive to $I_{Ca}$ (arrow A1) but not to its own release (arrow A3). Stern (1992b) proposed that the single channel events of the ryanodine receptor may be timed in such a way as to induce Ca$^{2+}$ release only when the activating site is occupied by Ca$^{2+}$ and that the released Ca$^{2+}$ is dissipated by diffusion before it can reopen a randomly closed channel. In an analogous manner it is possible that a bursting Ca$^{2+}$ channel is not prone to Ca$^{2+}$-induced inactivation when it is open but only when it is closed. Another possibility is that the Ca$^{2+}$ ions entering through the Ca$^{2+}$ channel and released from the SR are distinct because either they are separated by a physical barrier or may be chemically different. As an example of an inconspicuous chemical ligand or cofactor it may be suggested that the calcium ions from the two sources may be hydrated to different degrees. For instance, Calcium ions which pass through the Ca$^{2+}$ channel may be partially dehydrated and diffuse over some distance before they regain a configuration suitable for binding at the inactivation site of the channel.

Yet another explanation may be proposed based solely on the magnitude of the Ca$^{2+}$ fluxes and the geometry within the t-SR junction. This is consistent with the ryanodine receptors being both more abundant and having larger unitary Ca$^{2+}$ fluxes than the DHP receptors (Wibo et al., 1991; Tinker and Williams, 1994). From structural data (Langer and Pescoff, 1996) and the effective diffusion distance in experiments with 10 mM BAPTA, it is estimated that the inactivation site is roughly 10 nm from the release site (A2, Fig. 14). Since the Ca$^{2+}$ concentration near a point source is expected to vary inversely with the distance (Stern, 1992b) it is now possible to estimate the distance from the inner opening of the Ca$^{2+}$ channel to the inactivation site by considering that the Ca$^{2+}$ release is 17.6 (amplification factor) times larger than $I_{Ca}$ but only 2–3 (2.5) times more effective in causing inactivation. The result is 10 nm 2.5/17.6 = 1.4 nm (A4, Fig. 14). Such a distance is well within the range of molecular dimensions of channels and is sufficiently small to allow diffusion of Ca$^{2+}$ even in the presence of about 100 mM BAPTA.

**Suppression of $I_{Ba}$ by the Release of Ca$^{2+}$ from the SR**

When Ba$^{2+}$ was used as a charge carrier through the Ca$^{2+}$ channel, the slowly inactivating Ba$^{2+}$-current ($I_{Ba}$) caused a rise in the fluorescence signal, even though it did not trigger Ca$^{2+}$-release from ryanodine-receptors (Näbauer et al., 1989). $I_{Ba}$-induced increase in fluorescence signal can be attributed to direct binding of Ba$^{2+}$ to Fura-2 (Kwan and Putney, 1990). $I_{Ba}$ activated simultaneously with the release of Ca$^{2+}$ by caffeine decayed more rapidly than control $I_{Ca}$ (time constant of 18 ms; Fig. 10 C, middle trace). It is likely, therefore, that the rate of inactivation of $I_{Ba}$ measured during caffeine-induced Ca$^{2+}$ release (Fig. 10 C) represent the rate of rise of the Ca$^{2+}$ concentration in the vicinity of the sarcolemmal Ca$^{2+}$ channel. This finding is consistent with the idea that elevation of Ca$^{2+}$ in a microdomain adjacent to Ca$^{2+}$ channel strongly modulates the gating kinetics of Ca$^{2+}$ channel.

**Na$^{+}$-Ca$^{2+}$ Exchange Current Is Suppressed in Ca$^{2+}$-buffered Myocytes**

Dialysis of myocytes with high concentrations of Ca$^{2+}$-buffers, while having little or no effect on $I_{Ca}$-induced Ca$^{2+}$ release, abolished the inward Na$^{+}$-Ca$^{2+}$ exchange current ($I_{NaCa}$) (Figs. 3, 4, 6, 10, and 12) generally observed upon rapid release of Ca$^{2+}$ by caffeine in myocytes dialyzed with 0.2 mM Fura-2 (Figs. 9 and 14; Callewaert et al., 1989). Under our experimental conditions, based on the stoichiometry of 3Na$^{+}$:1Ca$^{2+}$, the calculated reversal potentials for $I_{NaCa}$ ranged from 152 to 198 mV in myocytes dialyzed with 0.2 mM Fura-2 ([Na$^{+}$]o = 137 mM, [Na$^{+}$]i = 1 mM, [Ca$^{2+}$]o = 2 mM, [Ca$^{2+}$]i = 150 nM [basal] or 900 nM [during caffeine-pulse]) and from 119 to 136 mV in myocytes dialyzed with 2 mM Fura-2 ([Ca$^{2+}$]i = 40 nM [basal] or 80 nM [during caffeine-pulse]).

Despite the strong driving force for $I_{NaCa}$ to extrude Ca$^{2+}$ at the holding potentials of -60 to -80 mV, high concentration of Ca$^{2+}$ buffer interfered with the activation of $I_{NaCa}$ by caffeine triggered Ca$^{2+}$ release. A possi-
ble explanation for these findings is that the Na\textsuperscript+-Ca\textsuperscript{2+} exchangers, though concentrated in the transverse tubular membrane (Frank et al., 1992), may be located at greater distances from the ryanodine receptors than DHP-receptors. In considering the functional evidence for the location of the Na\textsuperscript+-Ca\textsuperscript{2+} exchanger within the t-tubules, t-SR junctions or microdomains of Ca\textsuperscript{2+}, it may be recalled that the exchanger has fairly low affinity for Ca\textsuperscript{2+} (\(K_d = 5 \mu\text{M}\); Hilgemann et al., 1992) and therefore may be relatively more sensitive to exogenous Ca\textsuperscript{2+} buffers.

**Cross Talk between DHP- and Ryanodine Receptors**

The present study showed that 2 mM Fura-2, even with addition of 14 mM EGTA, did not significantly reduce Ca\textsuperscript{2+} released from the SR (Figs. 1 D and 2 D), while abolishing contraction (Figs. 1 B and 2 B) and markedly reducing the global myoplasmic intracellular Ca\textsuperscript{2+} concentration (Figs. 1 C and 2 C). Thus, the Ca\textsuperscript{2+}-uptake system appears to continue to reload the SR, and \(I_{\text{Ca}}\) continues to trigger Ca\textsuperscript{2+} release from the SR even in the presence of high concentration of Ca\textsuperscript{2+} buffers. It should be noted that, in the present study, myocytes were dialyzed with high concentration of CAMP (200 \mu M) to maximize \(I_{\text{Ca}}\) and re-uptake of Ca\textsuperscript{2+} by Ca-ATPase activity through the respective cAMP-dependent protein kinase-induced phosphorylation of Ca\textsuperscript{2+} channels and phospholamban. If the myocytes were dialyzed with even higher concentration of Ca\textsuperscript{2+} buffers, however, the ability of Ca-ATPase to compete for sequestration of Ca\textsuperscript{2+} with exogenous buffers could be severely compromised, leading secondarily to depletion of SR Ca\textsuperscript{2+} content, making it difficult to quantify Ca\textsuperscript{2+} signaling between the two sets of receptors.

The present results indicate that two types of Ca\textsuperscript{2+} signals persist in the presence of high concentrations of Ca\textsuperscript{2+} buffers. One signal is transmitted by Ca\textsuperscript{2+} which enters through the DHP-sensitive sarcolemmal Ca\textsuperscript{2+} channel and triggers the release of Ca\textsuperscript{2+} from the SR, and the other is carried by Ca\textsuperscript{2+} released from the ryanodine receptor (Ca\textsuperscript{2+}-release channel) accelerating the inactivation of the Ca\textsuperscript{2+} channel. Quantifying the equivalent Ca\textsuperscript{2+} and Ba\textsuperscript{2+} charge moving through the channel, we estimated that Ca\textsuperscript{2+}-release is 16–19 times larger than the entry of Ca\textsuperscript{2+} through the Ca\textsuperscript{2+}-channel as \(I_{\text{Ca}}\) (Fig. 10). This supports previous estimates (Callewaert et al., 1989) but does not rely on the assumption that SR release should be completely abolished by ryanodine. Instead it rests on more easily verifiable assumptions that Ba\textsuperscript{2+} current does not deplete the Ca\textsuperscript{2+} contents of the SR (Näbauer et al., 1989), and that Fura-2 is present inside the cell in sufficient amounts to be the dominant Ca\textsuperscript{2+} buffer (Fig. 1).

Ca\textsuperscript{2+} signaling in the opposite direction was quantified by a linear regression (Fig. 8) which showed that the rate of inactivation of \(I_{\text{Ca}}\) was 3 to 4 times larger in the presence than in the absence of the SR release. This suggests that Ca\textsuperscript{2+} ions entering through the L-type Ca\textsuperscript{2+}-channel, thought to play a major role in the Ca\textsuperscript{2+}-mediated inactivation of the channel, are, in fact, less important than Ca\textsuperscript{2+} released from the SR. Even if the Ca\textsuperscript{2+} buffers were as effective as those measured in free aqueous solution, it is still likely that the dimensions of the Ca\textsuperscript{2+} microdomains are sufficiently small as to allow Ca\textsuperscript{2+} to reach its destination before it is captured by the buffers. On the other hand, if molecular distances are significantly larger, then Ca\textsuperscript{2+} transported by various molecular entities may be effectively buffered by the high concentration of buffers. For instance, Ca\textsuperscript{2+} released from the SR is clearly buffered before reaching the myofilaments or the Na\textsuperscript+-Ca\textsuperscript{2+} exchanger, and Ca\textsuperscript{2+}-influx via the Na\textsuperscript+-Ca\textsuperscript{2+} exchanger does not appear to reach the Ca\textsuperscript{2+} sensing site on the ryanodine receptor. The distance-argument does not explain, however why the Ca\textsuperscript{2+} entering via the DHP-receptor is less effective than Ca\textsuperscript{2+} released from the SR in promoting inactivation of Ca\textsuperscript{2+} channel. In other words, how are the Ca\textsuperscript{2+} released from the SR and Ca\textsuperscript{2+} entering through the DHP-receptor distinguished in Ca\textsuperscript{2+} signaling? Irrespective of the molecular mechanisms involved in recognition between various sources of Ca\textsuperscript{2+}, it is clear that cross-signaling between the two sets of Ca\textsuperscript{2+} transporting channels occurs in a confined space on sub-millisecond time scale.

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