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

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ABSTRACT Calcium-mediated cross-signaling between the dihydropyridine (DHP) receptor, ryanodine receptor, and Na^+-Ca^{2+} exchanger was examined in single rat ventricular myocytes where the diffusion distance of Ca^{2+} was limited to <50 nm by dialysis with high concentrations of Ca^{2+} buffers. Dialysis of the cell with 2 mM Ca^{2+}-indicator dye, Fura-2, or 2 mM Fura-2 plus 14 mM EGTA decreased the magnitude of I_{Ca}-triggered intracellular Ca^{2+} transients (Ca_i-transients) from 500 to 20–100 nM and completely abolished contraction, even though the amount of Ca^{2+} released from the sarcoplasmic reticulum remained constant (≈140 μM). Inactivation kinetics of I_{Ca} in highly Ca^{2+}-buffered cells was retarded when Ca^{2+} stores of the sarcoplasmic reticulum (SR) were depleted by caffeine applied 500 ms before activation of I_{Ca}, while inactivation was accelerated if caffeine-induced release coincided with the activation of I_{Ca}. Quantitative analysis of these data indicate that the rate of inactivation of I_{Ca} was linearly related to SR Ca^{2+}-release and reduced by >67% when release was absent. Thapsigargin, abolishing SR release, suppressed the effect of caffeine on the inactivation kinetics of I_{Ca}. Caffeine-triggered Ca^{2+}-release, in the absence of Ca^{2+} entry through the Ca^{2+} channel (using Ba^{2+} as a charge carrier), caused rapid inactivation of the slowly decaying Ba^{2+} current. Since Ba^{2+} does not release Ca^{2+} but binds to Fura-2, it was possible to calibrate the fluorescence signals in terms of equivalent cation charge. Using this procedure, the amplification factor of I_{Ca}-induced Ca^{2+} release was found to be 17.6 ± 1.1 (n = 4). The Na^+-Ca^{2+} exchange current, activated by caffeine-induced Ca^{2+} release, was measured consistently in myocytes dialyzed with 0.2 but not with 2 mM Fura-2. Our results quantify Ca^{2+} signaling in cardiomyocytes and suggest the existence of a Ca^{2+} microdomain which includes the DHP/ryanodine receptors complex, but excludes the Na^+-Ca^{2+} exchanger. This microdomain appears to be fairly inaccessible to high concentrations of Ca^{2+} buffers.

KEY WORDS: Ca^{2+} channel inactivation • ryanodine receptor • ventricular myocytes

INTRODUCTION

Ca^{2+}-influx through the Ca^{2+} channel is the primary pathway for triggering Ca^{2+} release from the sarcoplasmic reticulum (SR)[1] in rat cardiac myocytes (Beuckelmann and Wier, 1988; Näbauer et al., 1989; Niggli and Lederer, 1990; Cleemann and Morad, 1991). Though Ca^{2+} influx via the Na^+-Ca^{2+} exchanger has been reported to trigger Ca^{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^{2+} influx via the Na^+-Ca^{2+} exchanger could trigger Ca^{2+} release only when the cells were dialyzed with Na^+ concentrations in excess of 10 mM and depolarized to voltages positive to 80 mV. However, the efficacy of the exchanger-triggered Ca^{2+} release was 1/20 to 1/160 of that of the Ca^{2+} channel current (Sham et al., 1995). These findings suggested that Ca^{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^{2+} signaling does exist between the DHP- and ryanodine receptors, then Ca^{2+} released via the ryanodine receptor might also control the inactivation of the Ca^{2+} channel, especially since the inactivation of the L-type Ca^{2+} channel has a major Ca^{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^{2+} release from the SR, has been reported to slow the rate of inactivation of I_{Ca} while suppressing I_{Ca}-triggered contraction (Mitchell et al., 1984) and intracellular Ca transients (Ca_i-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^{2+}-mediated cross-regulation may take place between the

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[1] *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular Ca^{2+} activity; Ca_i-transients, intracellular Ca^{2+} transients; DHP, dihydropyridine; SR, sarcoplasmic reticulum.

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 DAGAN 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 MΩ when filled with the internal solution composed of (in mM) CsCl, 100; tetraethylammonium chloride (TEA-Cl), 30; HEPES, 10; MgATP, 5; LiGTP, 0.1; cAMP, 0.2; K_5Fura-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 as the only Ca^{2+} buffer. In some experiments, CsCl in the internal solution was replaced with TEA-Cl. Cells were perfused with Tyrode's solution containing 2 mM $CaCl_2$. Outward K^+-currents were suppressed by replacing KCl with CsCl and TEA-Cl in the internal solution, and inward rectifier K^+-current was suppressed by either addition of Ba^{2+} (0.1 mM) to or omission of K^+ from the external solutions. Na^+-current was mostly suppressed by addition of 3 μM tetrodotoxin in the external solution and by including high concentration (200 μM) of cAMP in the internal solution (Schubert et al., 1989). Myocytes were dialyzed with 200 μM cAMP not only to enhance I_{Ca} but also to fully activate Ca-ATPase activity through phosphorylation of phospholamban.

Generation of voltage-clamp protocols and acquisition of data were carried out using pCLAMP software (version 5.5-1; Axon Instruments, Inc., Foster City, CA). The leak currents were digitally subtracted by the P/N method (N = 5–6). The series resistance was 1.5–3 times the pipette resistance and was electronically compensated through the amplifier. Sampling frequency was 0.5–2.0 kHz, and current signals were filtered at 10 kHz before digitalization and storage. Data in most Figs. (1, 3, 6, 9–13) are shown without leak subtraction to demonstrate that the cells had low and stable leak current.

Drugs were dissolved in the external Tyrode's solution and applied rapidly using a concentration-clamp device (Cleemann and Morad, 1991).

All the experiments were performed at room temperature (22–25°C).

Intracellular Ca^{2+} Activity

The intracellular calcium activity, and in some experiments contraction (Fig. 1), were measured according to the method described earlier (Cleemann and Morad, 1991). Ventricular myocytes were dialyzed with either 2 mM Fura-2 or 2 mM Fura-2 plus 14 mM EGTA or 10 mM BAPTA via the patch-clamp pipettes. Ultraviolet light used for excitation of Fura-2 originated from 100 W mercury arc lamp with an ellipsoidal reflector. The beam was split into two using a mirror vibrating at 1,200 Hz. The wavelengths of the two beams were defined with interference filters (410 and 335 nm, 20-nm bandwidth). The fluorescent light passed through a wide-band interference filter (510 nm, 70-nm bandwidth) and was detected with a photomultiplier. The signal from the photomultiplier was demultiplexed (Cleemann and Morad, 1992), yielding two signals corresponding to the two wavelengths of excitation. These signals were acquired simultaneously with the whole-cell currents using pCLAMP software.

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 (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \cdot B \cdot (R_{EGTA} - R) / (R - R_{Ca}) , \quad (1)$$

$$R = (F_{410} - F_{410,bg}) / (F_{335} - F_{335,bg}) , \quad (2)$$

$$R_{Ca} = F_{410,Ca} / F_{335,Ca} , \quad (3)$$

$$R_{EGTA} = F_{410,EGTA} / F_{335,EGTA} , \quad (4)$$

$$B = F_{335,EGTA} / F_{335,Ca} , \quad (5)$$

K_d = 220 nM (Grynkiewicz et al., 1985) is the dissociation constant for Fura-2, while F_{410} and F_{335} are the fluorescence intensities measured respectively with excitation at 410 and 335 nm. The background fluorescences ($F_{410,bg}$ and $F_{335,bg}$) were measured after making a giga-seal just before rupture of the membrane. Calibration measurements were performed with samples of 50 μM Fura-2 either saturated with 5 mM Ca^{2+} ($F_{410,Ca}$ and $F_{335,Ca}$) or in free form with 10 mM EGTA effectively binding any trace Ca^{2+} contaminants ($F_{410,EGTA}$ and $F_{335,EGTA}$).

The ratiometric determination of $[Ca^{2+}]_i$ is based on the law of mass action:

$$K_d = [Ca^{2+}]_i \cdot [\text{Fura-2}] / [\text{CaFura-2}] , \quad (6)$$

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:

$$F_{410} = A_{11} \cdot [\text{Fura-2}] + A_{12} \cdot [\text{CaFura-2}] + F_{410,bg} ,$$

$$F_{335} = A_{21} \cdot [\text{Fura-2}] + A_{22} \cdot [\text{CaFura-2}] + F_{335,bg} . \quad (7)$$

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 ($[\text{Fura-2}]_{tot,eq}$) 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:

$$[\text{Fura-2}] = [\text{Fura-2}]_{tot,eq} \cdot \frac{F_{410} \cdot F_{335,Ca} - F_{335} \cdot F_{410,Ca}}{F_{410,eq} \cdot (F_{335,Ca} - F_{335,EGTA}) + F_{335,eq} \cdot (F_{410,EGTA} - F_{410,Ca})} , \quad (8)$$

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

The fluorescence intensities of the fully equilibrated cells ($F_{410,eq}$ and $F_{335,eq}$) were measured at the end of experiments lasting >20 min. In some shorter experiments the fully equilibrated state was estimated by mono-exponential extrapolation (Fig. 1).

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 ($[Ca^{2+}]_i$, [Fura-2], [CaFura-2], $[\text{Fura-2}]_{tot}$ = [Fura-2] + [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 10-s 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 maintain values >50% of its initial magnitude; (*c*) Ca^{2+} released from the SR by

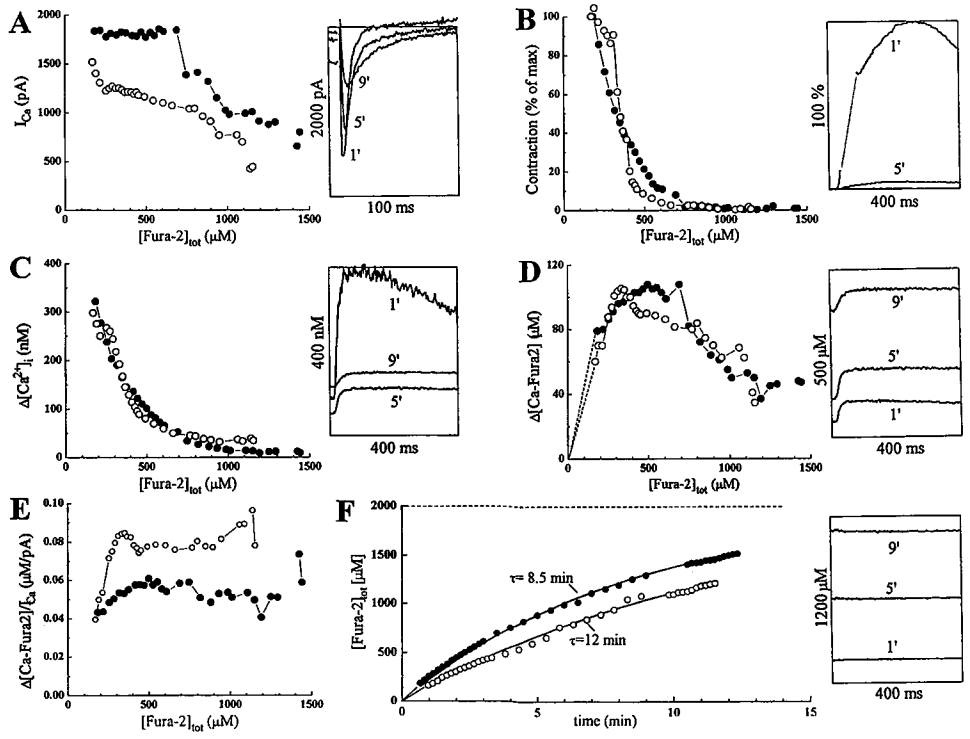

FIGURE 1. Measurements of I_{Ca} (*A*), cell shortening (*B*), intracellular Ca^{2+} activity ($[Ca^{2+}]_i$ from Eq. 1; *C*), change in Ca^{2+} bound to Fura-2 (Δ[Ca-Fura2] from Eq. 9; *D*), the same quantity normalized with respect to I_{Ca} (Δ[Ca-Fura2]/I_{Ca}; *E*), and the total intracellular concentration of Fura-2 ($[\text{Fura-2}]_{tot}$ = [Fura-2] +[Ca-Fura2] from Eqs. 8 and 9; *F*) during 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 mono-exponential 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. Insets 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 μM and approximated at least to as much as 60–70% of the Ca^{2+} released by caffeine.

Numerical results are given as "mean ± 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 K_5Fura-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_{Ca}, Ca_i-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_i-transients became significantly smaller (*C*).

Fig. 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 ($[Fura\text{-}2]_{tot}$ = [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 μM concentrations of the dialyzing solution. This allowed the measured parameters (I_{Ca} amplitude, Δcontraction, $\Delta[Ca^{2+}]_i$, and Δ[Ca-Fura-2]) 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 μM in 6–8 min (*F*) cell shortening was completely abolished (*B*) and the Ca_i-transients decreased to <50 nM (*C*). On the other hand, the change in intracellular Ca^{2+} concentration bound to Fura-2 (Δ[Ca-Fura2]) increased up to 100 μM as $[Fura\text{-}2]_{tot}$ rose up to 0.4 mM, but stayed relatively constant with $[Fura\text{-}2]_{tot}$ 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 Δ[Ca-Fura2] paralleled the run-down of I_{Ca} (*A*) such that the saturating titration of the fixed intracellular Ca^{2+} buffers with 300–500 μM Fura-2 was seen most clearly by plotting the ratio of ΔCaFura-2 and I_{Ca} (*E*).

In this initial series of experiments, using only 10 μM cAMP in the dialyzing solutions, it was recognized that serious problems resulted from both run-down of I_{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 μM 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_{Ca}. This yielded the improvements illustrated in Fig. 2. Both I_{Ca} (Fig. 2 *A*) and the amount of Ca^{2+} binding to

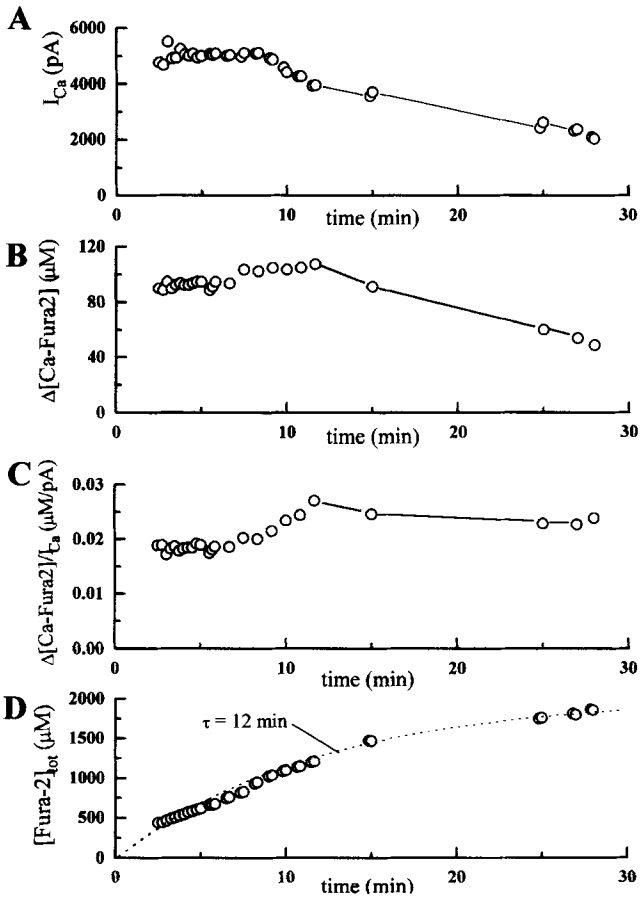

FIGURE 2. Dialysis of a rat ventricular cell with 2 mM Fura-2 and 200 μM cAMP. Changes in I_{Ca} amplitude (*A*), Δ[Ca-Fura2] (*B*), Δ[Ca-Fura2]/I_{Ca} (*C*), and $[Fura\text{-}2]_{tot}$ (*D*) were measured for 28 min after initiation of dialysis.

Fura-2 (*B*) declined more slowly, while as in Fig. 1, Ca^{2+} released normalized for magnitude of I_{Ca} stayed constant (*C*). The average values for Δ[Ca-Fura2] measured 8–20 min after rupture of the membrane in cells dialyzed with 200 μM cAMP, was 141 ± 18 μM (mean ± SEM, $n = 6$). These results show that the amount of Ca^{2+} released from the SR by I_{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_i-transients triggered by I_{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. 1 *F* and 2 *D*). 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_{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_{Ca}.

Fig. 3 *A* shows I_{Ca}, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and time derivative of rise of $[Ca^{2+}]_i$ ($d[Ca^{2+}]_i/dt$) in myocytes dialyzed with 2 mM Fura-2. Depolarizing test pulses to −10 mV from the holding potential of −60 mV fully activated I_{Ca}, which triggered rapid but small Ca_i-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_i-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_{Ca}, which inactivated slowly compared to control I_{Ca}, and triggered a much smaller Ca_i-transient of ~18 nM (Fig. 3 *A*, trace *b*) compared to the control value of 47 nM (Fig. 3 *A*, trace *a*). I_{Ca} recorded in the presence of caffeine in Fig. 3 *A* trace *b* inactivated more slowly compared to the control value in Fig.3, *A* and *B*, trace *a* (the rate of inactivation $1/\tau = 1/(8.1\text{ ms}) = 125\ s^{-1}$ [*a*] vs. $1/(17\text{ ms}) = 60\ s^{-1}$ [*b*]). There were no noticeable changes in the rate of activation of I_{Ca} (Fig. 3 *B*). Ca_i-transients triggered by I_{Ca} 10 s after wash out of caffeine had partially recovered (Fig. 3 *A*, trace *c*). Generally 20–30 s were required for the full recovery of Ca_i-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 $[Ca^{2+}]_i$ 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_i-transients (Fig.4 *A*). Brief rise in $[Ca^{2+}]_i$ triggered by I_{Ca} or caffeine decayed rapidly, as compared to the Ca_i-transients obtained with Fura-2 alone (compare Figs. 3 and 4). The superimposed traces of Ca_i-transients, $d[Ca^{2+}]_i/dt$ and the normalized I_{Ca} recorded just before (trace *a*), during (trace *b*), and after caffeine exposure (trace *c*) are shown in Fig. 4 *B*. 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_{Ca} after exposure to caffeine slowed significantly (Fig. 4 *B*, trace *a*; $1/\tau = 150\ s^{-1}$ vs. trace *b*; $40\ s^{-1}$) in a manner similar

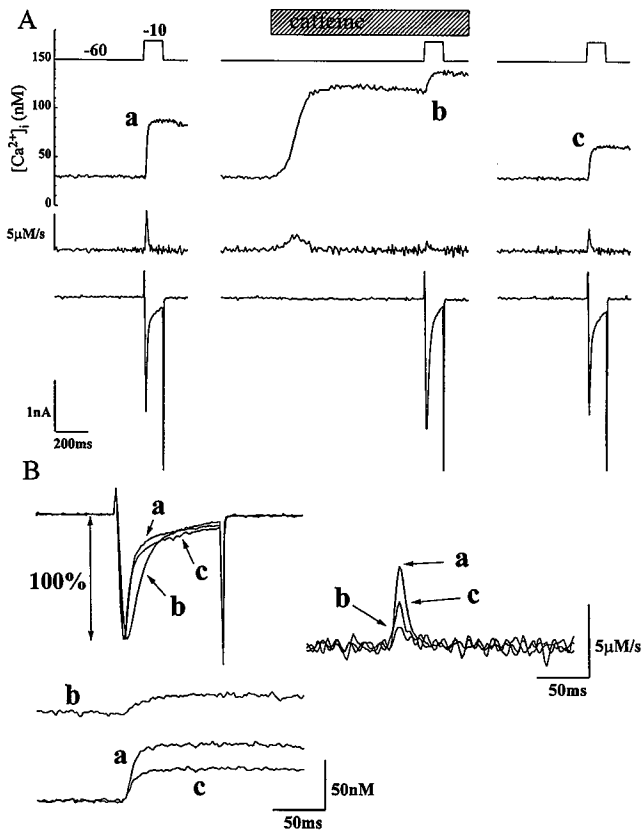

FIGURE 3. Effect of rapid application of caffeine (5 mM) on I_{Ca} and $[Ca^{2+}]_i$ in cells dialyzed with 2 mM Fura-2. The upper part of *A* shows the protocol where voltage clamp depolarizations are applied before (*a*), during (*b*), and after (*c*) the exposure to caffeine as indicated by horizontal shaded bar. The traces below are $[Ca^{2+}]_i$, its derivative ($d[Ca^{2+}]_i/dt$), and the membrane current. Each panel shows only three of a sequence of about eight voltage clamp depolarizations. Thus exposure to caffeine was preceded by 2 or more depolarizations to verify the stability of the amplitude of I_{Ca} and Ca_i-transients and was followed by 4 or more to allow washout and complete re-equilibration. *B* shows the events during the three test pulses (*a*, *b*, and *c*) superimposed and with expanded time scale. The membrane currents (I_{Ca}) were normalized to yield the same maximal downward deflection.

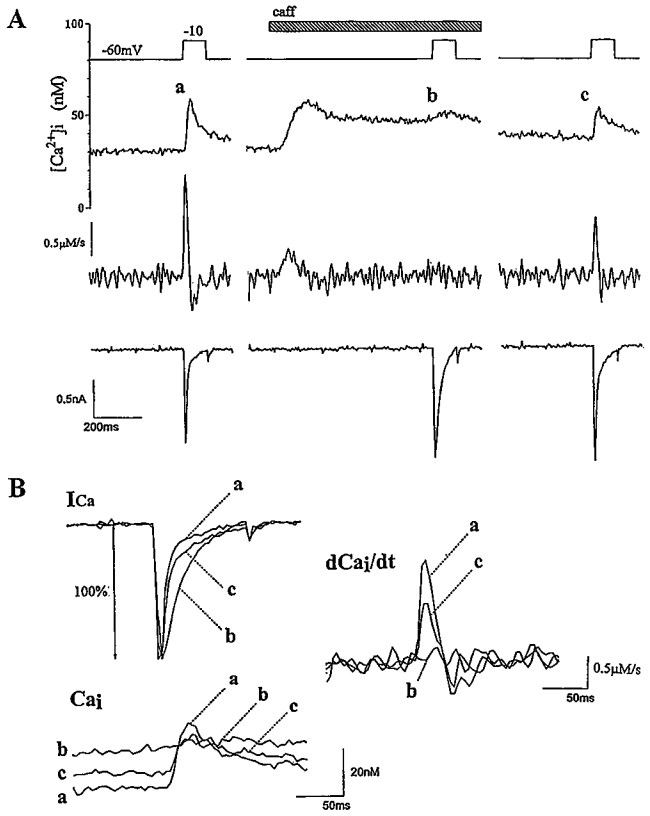

FIGURE 4. Effect of rapid application of caffeine (5 mM) on I_{Ca} and $[Ca^{2+}]_i$ in cells dialyzed with 2 mM Fura-2 plus 14 mM EGTA. The upper part of *A* shows the protocol where voltage clamp depolarizations were applied before (*a*), during (*b*), and after (*c*) the exposure to caffeine. The traces below are $[Ca^{2+}]_i$, its derivative ($d[Ca^{2+}]_i/dt$), and the membrane current. *B* shows the events during the three test pulses superimposed and with expanded time scale. The membrane currents (I_{Ca}) were normalized to yield the same maximal downward deflection.

to those dialyzed with 2 mM Fura-2 alone (Fig.3 *B*, traces *a* and *b*). The average values for the resting $[Ca^{2+}]_i$, peak Ca_i-transients triggered by I_{Ca} and by caffeine alone in myocytes dialyzed with 2 mM Fura-2 were 36 ± 4, 46 ± 5, and 78 ± 14 nM, respectively (mean ± SEM, n = 13). In cells dialyzed with both Fura-2 (2 mM) and EGTA (14 mM), the corresponding values were 33 ± 6, 32 ± 9, and 24 ± 8 nM, respectively (n = 6).

As the affinities of Ca^{2+} buffers in the myoplasmic milieu might be lower than in vitro (Konishi et al., 1988), we compared the magnitude and time course of the Ca_i-transients in Figs. 3 and 4 with the results of a numerical analysis (Fig. 5). The modeling was based on the in vitro kinetics of Fura-2 and EGTA. With Fura-2 alone, Ca_i-transients were well maintained and Ca^{2+} release triggered by caffeine was larger but slower than that triggered by I_{Ca} (compare Fig. 5, *C* and *D* with traces *a* and *b* of Fig. 3 *A*). Adding the buffering effect of 14 mM EGTA to the model, the same triggering signals (Fig. 5, *A* and *B*) produce rapidly decaying Ca_i-transients. Interestingly, the caffeine triggered release was no longer larger than those induced by I_{Ca} (compare Fig. 5, *E* and *F* with traces *a* and *b* of Fig. 4 *A*). In this model the addition of EGTA produces a phasic Ca_i-transient because the released Ca^{2+} is first bound primarily to Fura-2 and thereafter is transferred almost exclusively to EGTA which has the slower kinetics but the higher buffering capacity. The time constant of this transfer was ~100 ms when determined from trace *a* in Fig. 4 *A* and 230 ms when calculated from the in vitro properties of the dye (see legend of Fig. 5). These observations suggest that the kinetic properties of the buffers within the cell are not substantially different from the values determined in vitro, and they explain why the addition of 14 mM EGTA to the Fura-2 internal solution produced only a small suppression of Ca_i-transients triggered by I_{Ca} or caffeine and did not alter the amount of released Ca^{2+} as indicated by the inactivation kinetics of I_{Ca}.

Table I supports the notion that the inactivation kinetics of I_{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_{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 Ca^{2+} buffers we conducted experiments where 10 mM BAPTA was dialyzed into myocytes through the patch pipettes. In such experiments, the intracellular Ca^{2+} activity was not monitored with Fura-2 but the free 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_{Ca} continued to slow significantly when caffeine was applied before activation of I_{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_{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 Ca^{2+} by 10 mM BAPTA is sufficiently strong to remove some of the inactivation of I_{Ca}, which is otherwise dependent on SR release. It should be noted, however, that as the buffering capacity and the speed of Ca^{2+}buffering is increase, it is likely that the Ca^{2+} con-

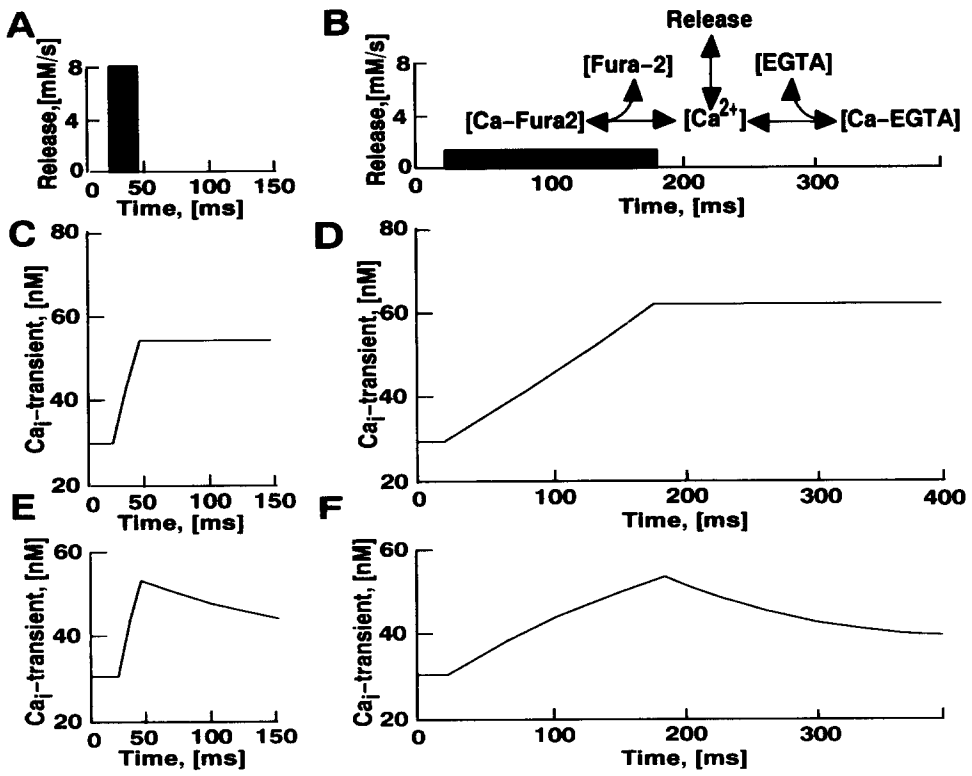

FIGURE 5. Simulation of the buffering of intracellular Ca^{2+} achieved by dialysis of 2 mM Fura-2 and 14 mM EGTA. It is assumed that the endogenous buffers are insignificant compared to the added buffers and that the source (*Release* in *B*) corresponds to Ca^{2+}, which is released either quickly by depolarization (160 μM released in 20 ms, *A*) or relatively slowly by caffeine (200 μM 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 nm, k_{12} = 84 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 nM, k_{32} = 0.5 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*): $k_{21} = k_{12} \cdot [\text{Fura-2}]/K_{d,Fura} = 0.7636 \times 10^6\ s^{-1}$, $k_{23} = k_{32} \cdot [\text{EGTA}]/K_{d,EGTA} = 3{,}500\ s^{-1}$. 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: $t^2 \cdot (k_{12} \cdot k_{23} + k_{32} \cdot k_{21} + k_{12} \cdot k_{32}) - t^2 \cdot (k_{12} + k_{21} + k_{23} + k_{32}) + 1 = 0$ (Smith et al., 1984). In the present case the faster rate constant t_{life} is approximately equal to $1/(k_{21} + k_{23})$ = 1.25 ms and is the life time of released Ca^{2+} ions before they are bound by the buffers. The slower time constant, here: $t_{ex} \cong 1/[(k_{12} \cdot k_{23} + k_{32} \cdot k_{21}) \cdot t_{life}]$ = 230 ms, is the time constant for equilibration between the two buffers.

tent of the SR eventually will be compromised. Since our experimental approach takes advantage of the effects of Ca^{2+} release on I_{Ca}, it was essential to maintain a stable releaseable Ca^{2+} pool; therefore higher buffer concentrations were not attempted.

To examine whether the change in the rate of inactivation of I_{Ca} was dependent on the amount of Ca^{2+} released from the SR, we manipulated the Ca^{2+} content of the SR by applying the caffeine-pulse (duration 5 s) at various times before the activation of I_{Ca}. If I_{Ca} were measured when caffeine-induced Ca_i-transient had already fully developed, the inactivation of I_{Ca} slowed with little change in its amplitude (Fig. 6 *A*). If I_{Ca} were activated during the rising phase of the caffeine-induced Ca_i-transient, the amplitude of I_{Ca} was strongly suppressed (Fig. 6 *B*). Application of caffeine just before the activation of I_{Ca} augmented I_{Ca}-induced Ca_i-transients and the rate of inactivation of I_{Ca} but suppressed its amplitude. In Fig. 6 *C*, for instance, $d[Ca^{2+}]_i/dt$ increased from 1.0 μM/s to 1.6 μM/s (traces *a* and *b*), while the rate of inactivation of I_{Ca} was enhanced from 70 s^{-1} to 100 s^{-1}.

These results suggest that both the rate of inactivation of I_{Ca} and its accompanying release of Ca^{2+} may be either enhanced or reduced depending on the timing of the test pulse relative to the application of caffeine. Fig. 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 $\Delta[Ca^{2+}]_i$, and $d[Ca^{2+}]_i/dt$ 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 $\Delta[Ca^{2+}]_i$ and $d[Ca^{2+}]_i/dt$. These three parameters were fit by the following equation:

$$y = (A_i - A_f) \cdot \exp(-t/\tau) + A_f, \qquad (10)$$

where A_i and A_f are, respectively, the initial (t = 0) and final (t = infinity) values of the three parameters, and τ 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} ($1/\tau$), $\Delta[Ca^{2+}]_i$, and $d[Ca^{2+}]_i/dt$ are governed by a single exponential. The data also suggest a trend to-

TABLE I

Effect of Ca^{2+} Buffers on Caffeine-induced Changes of Ca^{2+} Currents and their Accompanying Ca^{2+} Transients

	2 mM Fura-2	2 mM Fura-2 + 14 m M EGTA	10 mM Bapta
Control			
Ca^{2+} current, I_{Ca}, [nA]	1.96 ± 0.34 (9)	1.0 ± 0.47 (7)	3.70 ± 0.45 (7)
Time constant, τ, [ms]	9.8 ± 1.2 (9)	16.3 ± 4.1 (7)	19.2 ± 1.8 (7)
Ca_i-transient, $\Delta[Ca^{2+}]_i$, [nM]	47 ± 7 (9)	43 (2)	
Caffeine			
Ca^{2+} current, I_{Ca}, [nA]	2.06 ± 0.38 (9)	2.24 ± 0.59 (7)	3.83 ± 0.45 (7)
ratio	1.03 ± 0.04 (9)	1.12 ± 0.07 (7)	1.04 ± 0.02 (7)
Time constant, τ [ms]	20.5 ± 1.9 (9)	35 ± 8 (7)	32 ± 5 (7)
ratio	2.16 ± 0.11 (9)	2.16 ± 0.05 (7)	1.98 ± 0.07 (7)
Ca_i-transient, $\Delta[Ca^{2+}]_i$, [nM]	14.7 ± 2.9 (9)	10.0 (2)	
ratio	0.33 ± 0.06 (9)	0.23 (2)	
Wash			
Ca^{2+} current, I_{Ca}, [nA]	2.17 ± 0.49 (6)	2.15 ± 0.40 (5)	
ratio	1.09 ± 0.03 (6)	1.10 ± 0.05 (5)	
Time constant, τ, [ms]	12.2 ± 1.1 (6)	14.1 ± 2.7 (5)	
ratio	1.33 ± 0.10 (6)	1.34 ± 0.14 (5)	

All values are means ± SEM with the number of experiments in parenthesis (n). Ratios are calculated relative to the control condition with the same Ca^{2+} 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. Ca_i-transients were not measured when 10 mM BAPTA was used as buffer.

ward $1/\tau$ for I_{Ca} falling faster than $\Delta[Ca^{2+}]_i$ and $d[Ca^{2+}]_i/dt$. The trend, however, was not statistically significant nor was it 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 $\{\exp[-t/48] + 1.15 \cdot [1 - \exp(t/283)]\}$. 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 Ca^{2+} released by caffeine from the SR. The subsequent recovery of I_{Ca}, however, occurs even while the global intracellular Ca^{2+} activity was significantly elevated (100–120 nM, Figs. 3 and 6). This suggests that the local build-up of Ca^{2+}, which serve to inactivate Ca^{2+} channels near the release sites, dissipates rapidly, shortly after caffeine triggered Ca^{2+} release due to the diffusion or uptake of Ca^{2+} by Ca-ATPase and Ca^{2+} buffers (Balke et al., 1994).

Ca^{2+} Release Dependence of Inactivation of the Ca^{2+} Channel

Fig. 8 shows a linear correlation between the rate of inactivation of I_{Ca} as a function of either $\Delta[Ca^{2+}]_i$ (correlation coefficient: r = 0.885) or $d[Ca^{2+}]_i/dt$ (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 Ca^{2+} released from the SR. The y-axis intercept for $1/\tau$ (0.272 ± 0.035 for $\Delta[Ca^{2+}]_i$ and 0.288 ± 0.049 for $d[Ca^{2+}]_i/dt$) corresponds roughly to a condition where no Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} influx through the Ca^{2+} channel contributes only ~35% of its inactivation. This may seem somewhat surprising since the Ca^{2+}-sensing site of the channel is thought to be located in the immediate vicinity of the inner mouth of the Ca^{2+} 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 Ca^{2+} 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^+-Ca^{2+} 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 Ca^{2+} transient activated a small (50–100 pA) slowly decaying Ni^{2+}-sensitive inward current representing the extrusion of Ca^{2+} by the Na^+-Ca^{2+} exchanger (Callewaert et al., 1989). I_{Ca} activated after the onset of caffeine-pulse inactivated more

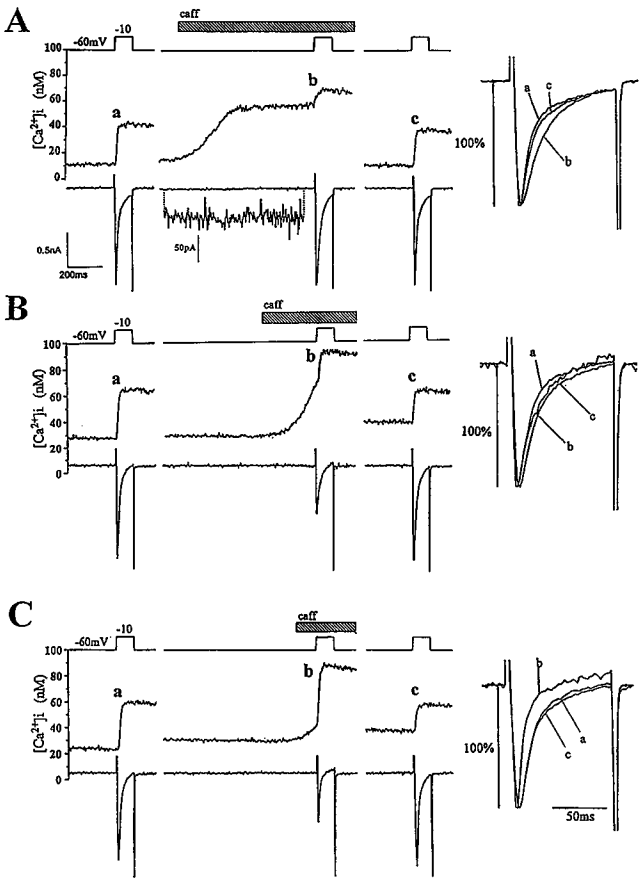

FIGURE 6. The effects of the caffeine exposure depended on its timing relative to the voltage clamp depolarizations. Caffeine (5 mM) was applied 850 (*A*), 250 (*B*), and 50 ms (*C*) before the activation of I_{Ca}. Ca_i-transients and I_{Ca} were measured before (*a*), during (*b*), and after (*c*) 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.

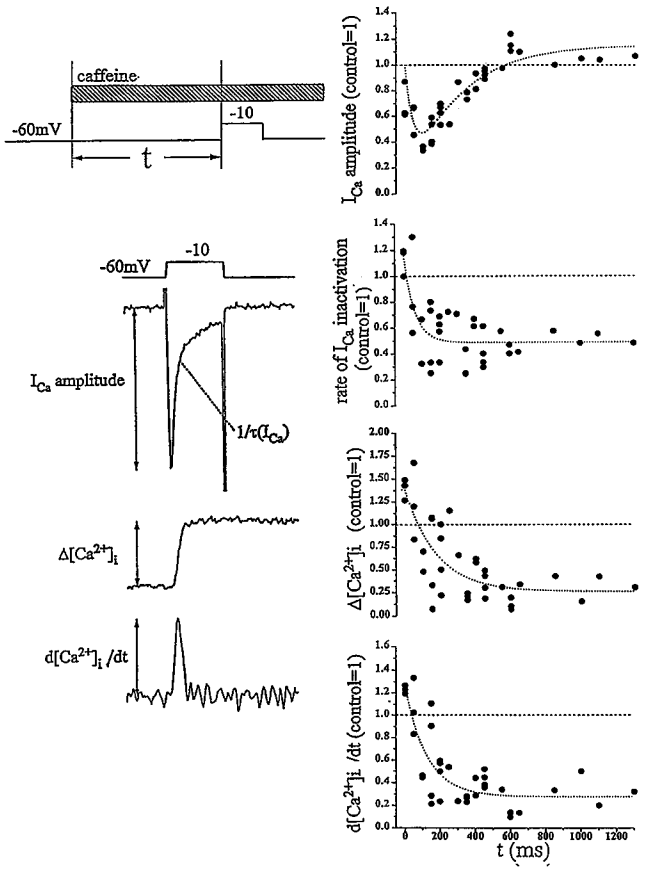

FIGURE 7. The effects of caffeine (5 mM) applied at various times before depolarization on the amplitude of I_{Ca}, the rate of inactivation of I_{Ca} ($1/\tau$), $\Delta[Ca^{2+}]_i$ and $d[Ca^{2+}]_i/dt$. The time (t) was measured from the first indication of caffeine-induced Ca^{2+} release to the onset of depolarization. In individual experiments the time from the application of caffeine to the first indication of caffeine-induced release ranged from 50 to 200 ms and an additional 110 to 220 ms elapsed before $d[Ca^{2+}]_i/dt$ fell to 80% of the control value. The experiments were performed as shown in Fig. 6. The results were normalized relative to the control value recorded before exposure to caffeine in order to pool results obtained with nine different cells. In each panel, the dashed line indicates the control level. The plots in *B* were fitted to two exponential functions $\{\exp[-t/48] + 1.15 \cdot \{\exp(-t/283)]\}$. The plots in panels *C*, *D*, and *E* were fitted to single exponential function (Eq. 10).

slowly compared to the control current in a manner similar to the myocytes dialyzed 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 dialyzed with 0.2 mM Fura-2 ($n = 4$). In contrast, in all myocytes dialyzed 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 out 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 $I_{Na\text{-}Ca}$ ($n = 22$), 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

	Initial value (A_i)	Final value (A_f)	Time constant (τ)
Rate of inactivation	1.13 ± 0.11	0.48 ± 0.04	69 ± 26 ms
$\Delta[Ca^{2+}]_i$	1.42 ± 0.16	0.28 ± 0.08	146 ± 42 ms
$d[Ca^{2+}]_i/dt$	1.23 ± 0.16	0.27 ± 0.06	129 ± 34 ms

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_d = 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 $-\Delta$[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 I_{Ba} 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

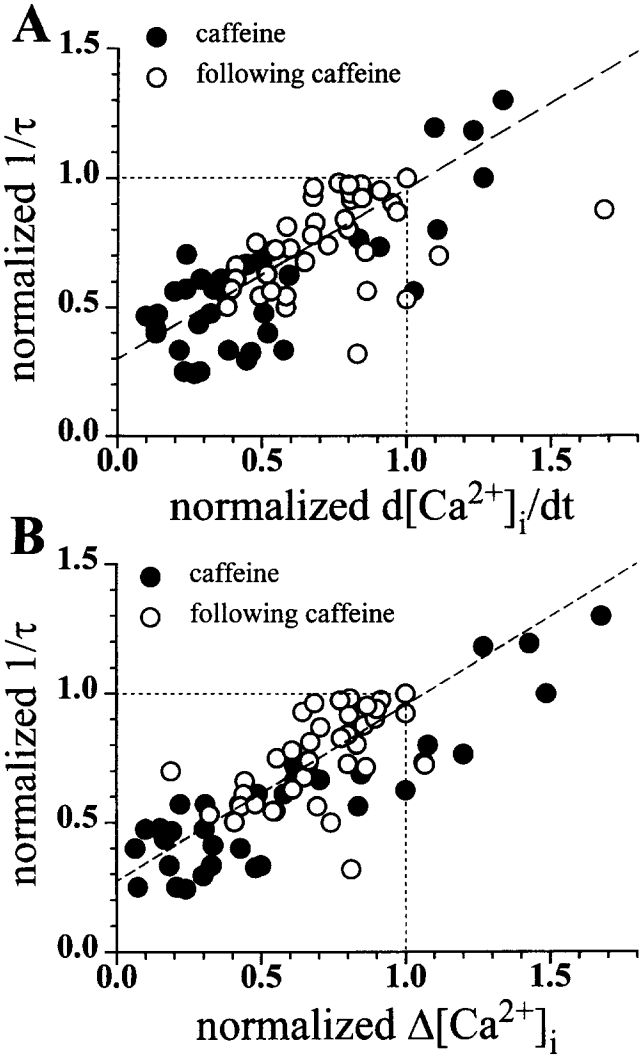

FIGURE 8. The correlation between the normalized rate of inactivation of I_{Ca} ($1/\tau$) and $d[Ca^{2+}]_i/dt$ (*A*) and that between $1/\tau$ and $\Delta[Ca^{2+}]_i$ (*B*). Filled circles correspond to the measurements in the presence of caffeine shown also in Fig. 7, while open circles correspond to the first depolarization after washout. The data were fitted to linear relations based on the least square regression analysis giving the following equation and the correlation coefficients: $1/\tau = 0.272 + 0.534 \cdot \Delta[Ca^{2+}]_i$, $r = 0.885$ (*A*) and $1/\tau = 0.288 + 0.582 \cdot d[Ca^{2+}]_i/dt$, $r = 0.781$ (*B*).

with the activation of I_{Ba} caused a relatively rapid rise in the fluorescence signal, and greatly enhanced the rate of inactivation of I_{Ba} (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 I_{Ba} 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.

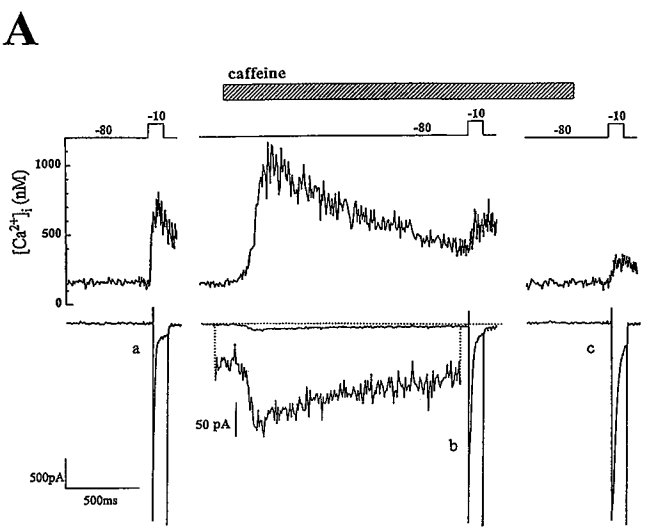

FIGURE 9. In myocytes dialyzed with the low concentration of Ca^{2+} buffer (0.2 mM Fura-2), caffeine (5 mM) not only prolonged I_{Ca} but also induced Na^+-Ca^{2+} exchange like current. The myocyte was clamped at holding potential of −80 mV and was activated by giving command pulses to −10 mV every 10 s. The $[Ca^{2+}]_i$ and current traces recorded before (*a*), during (*b*), and after (*c*) caffeine-application are shown in *A*. An inset shows the currents during caffeine exposure enlarged 10 times. The superimposed current traces are shown in *B*. The internal solution includes 1 mM Na^+.

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:

$$A + 1 = \frac{\Delta[\text{Ca-Fura2}] / Q_{Ca}}{\Delta[\text{Ba-Fura2}] / Q_{Ba}}, \qquad (11)$$

where Δ[Ca-Fura2] and Δ[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 Δ[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 ± 1.1 (n = 4, mean ± SEM) was obtained, suggesting that one Ca^{2+} entering through the DHP-receptor may induce the release of ~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 Ca_i -transients triggered by I_{Ca} (Fig. 11, *A* and *B*) and slightly increased the basal myoplasmic $[Ca^{2+}]_i$. As the magnitude of Ca_i -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 ($\Delta[Ca^{2+}]_i$) (r = 0.98478) and $d[Ca^{2+}]_i/dt$ (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-

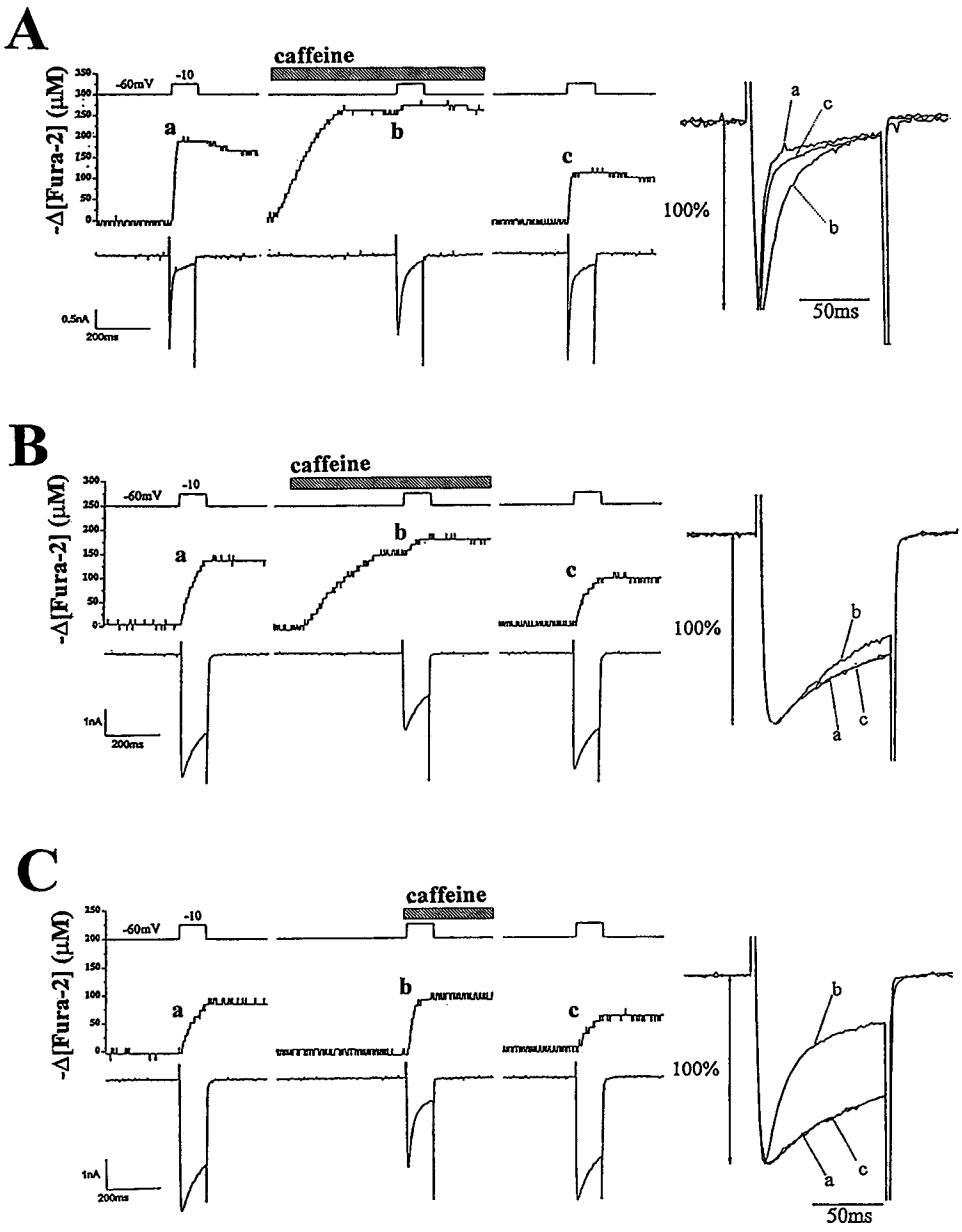

FIGURE 10. Comparison of the effects of 5 mM caffeine on inward current carried either by Ca^{2+} (*A*) or Ba^{2+} (*B* and *C*). In *A* and *B*, caffeine was applied 450 ms before the activation of the current, and in *C* it was applied 10 ms before the activation of I_{Ca}. The currents were recorded before (*a*), during (*b*), and after (*c*) exposure to caffeine. Notice that unlike Figs. 3, 4, and 6, which have generally the same layout, this figure does not show the intracellular Ca^{2+} activity ($[Ca^{2+}]_i$) but instead the negative value of the change in the concentration of free Fura-2 ($-\Delta$[Fura-2]) which is equivalent to the change in the concentration of Fura-2 bound to metal ions. The basal [Fura-2] levels for traces *a*, *b*, and *c* were 663.6, 681.5, and 707.9 μM (in *A*); 690.5, 612.7, and 571.6 μM (in *B*); and 817.1, 753.9, and 734.1 μM (in *C*), respectively. The cell was exposed to Ba^{2+} only during the time required to record the shown traces. Before and after it was perfused with Ca^{2+}-containing solution to allow loading of the SR.

self cause a regenerative release. Only after 15 min of incubation in thapsigargin did caffeine fail completely to trigger Ca_i-transients or alter the kinetics of I_{Ca} (Fig. 12 *C*, compare traces *a* and *b*). At this time Ca_i-transients triggered by I_{Ca} were reduced from 46.1 to 6.4 nM while Q_{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) − 1 = 14.1] is in fair agreement with the estimate based on the Ba^{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^{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^{2+} channel was entirely dependent on the Ca^{2+} content of the SR.

Prolongation of I_{Ca} by Conditioning Pulses

Another procedure used to manipulate the Ca^{2+} content of the SR was the application of a single or multiple conditioning pulses before the activation of I_{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_{Ca}. Although such conditioning pulses decreased the subsequent Ca_i-transients triggered by I_{Ca}, they did not suppress them as effectively as did 5 mM

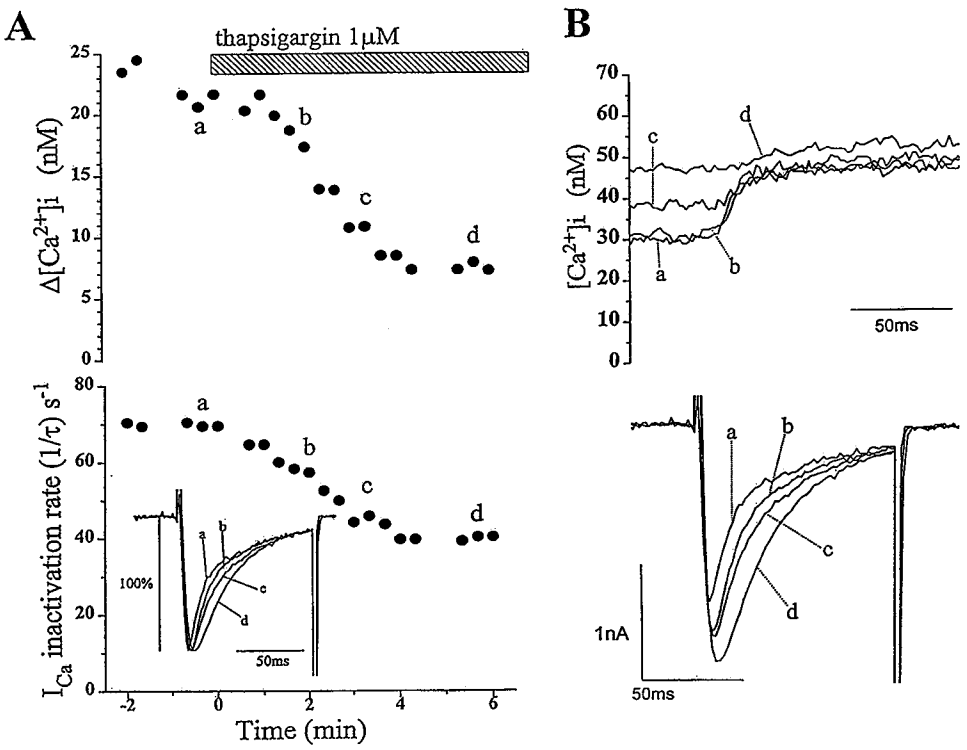

FIGURE 11. Thapsigargin (1 μM) gradually abolished Ca_i-transients triggered by I_{Ca} and decreased the rate of inactivation of I_{Ca}. *A* shows the time course of the effect of thapsigargin on $\Delta[Ca^{2+}]_i$ (*top*) and on I_{Ca} amplitude (*bottom*). The inset is the superimposed normalized current traces recorded at the times indicated in *A*. Intracellular Ca^{2+} activity and I_{Ca} recorded at the corresponding times in *A* are superimposed in *B*.

caffeine. 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_i-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 I_{Ca}-gated Ca_i-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 $[Ca^{2+}]_i$ 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_i-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

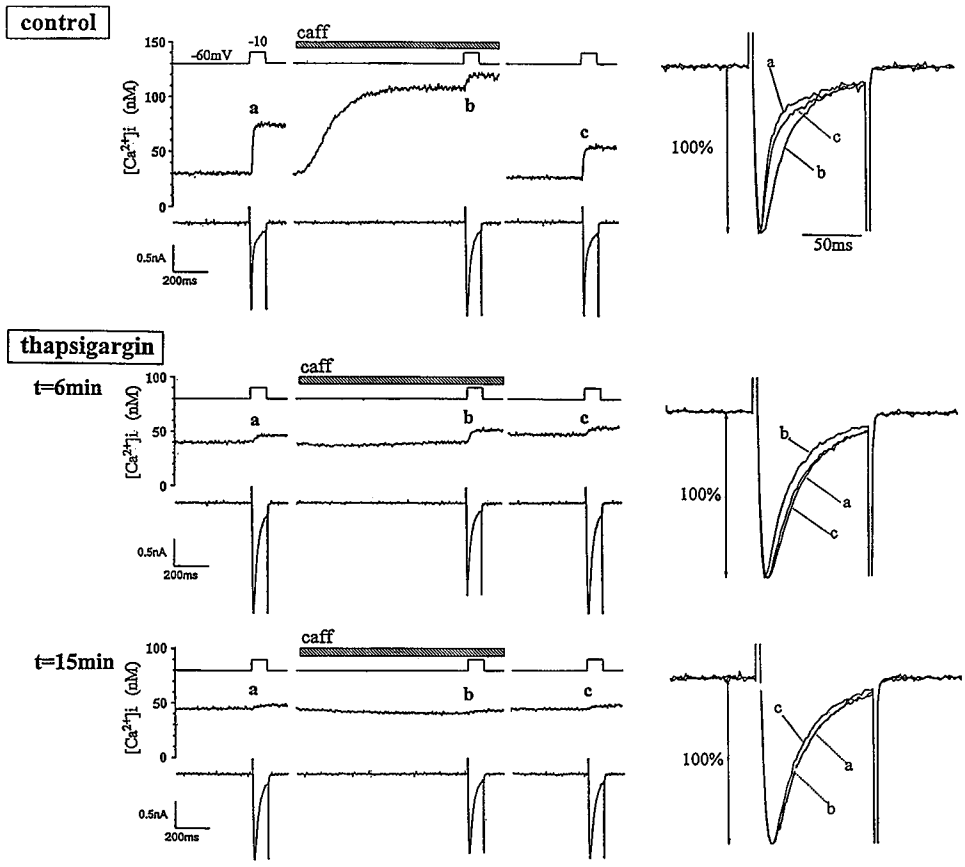

FIGURE 12. The effect of caffeine on I_{Ca} recorded before (*A*) and 6 (*B*) and 15 min (*C*) after application of thapsigargin (1 μM). Pretreatment with thapsigargin abolished the prolongation of I_{Ca} by caffeine. Only the direct suppressive effect of caffeine on I_{Ca} could be observed.

$[Ca^{2+}]_i$, 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 *F* and 2 *D*) 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 predicable 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:

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

(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 (τ_{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 $\sqrt{D} \cdot \tau_{life}$ = 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, 1992*a*, 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 (Tiaho 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}-triggered Ca^{2+} release and its effect on the inactivation kinetics of I_{Ca} (Fig. 4, Table I). This suggests that higher concentration of Ca^{2+} buffers will be required to eliminate the effect of SR

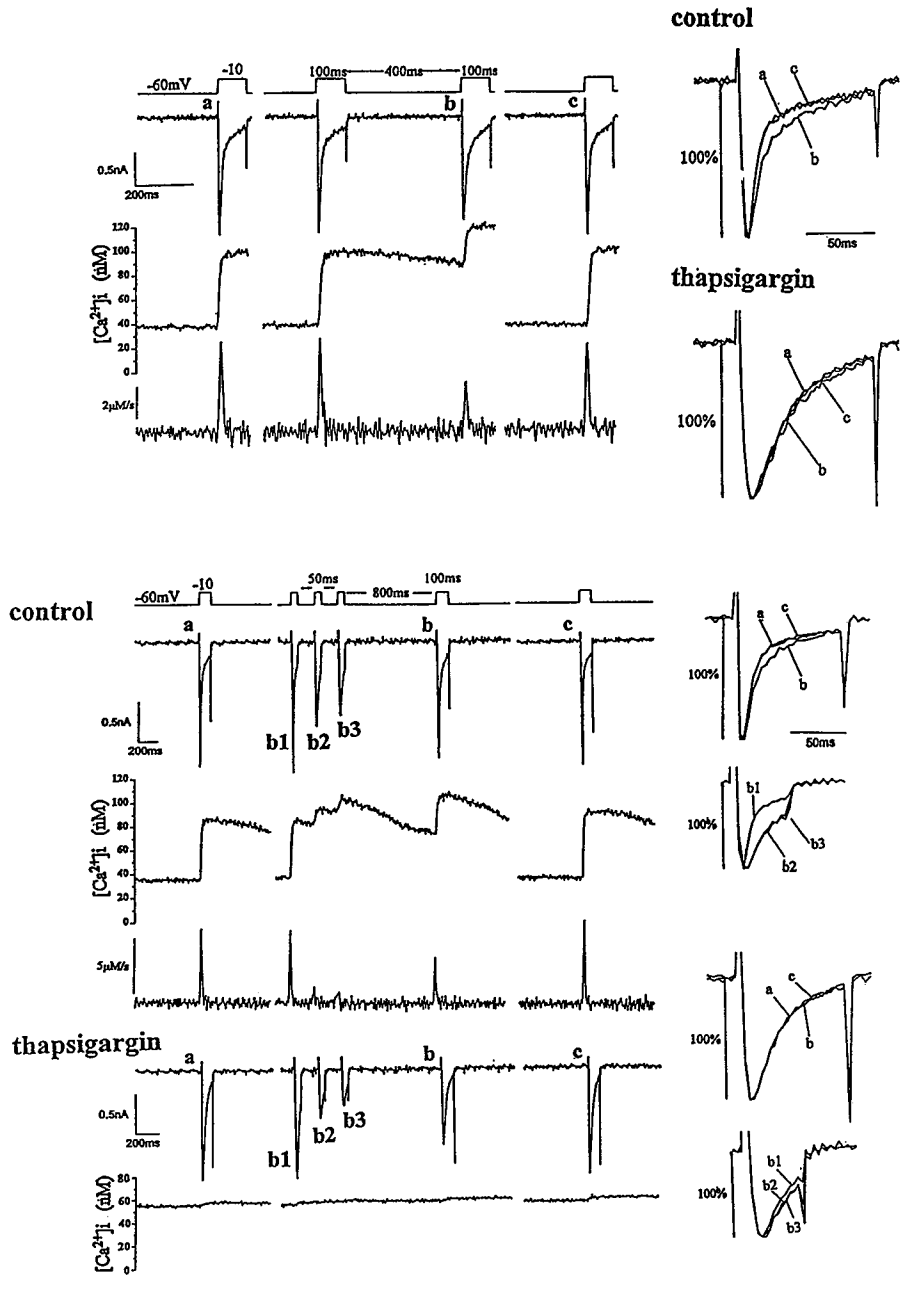

FIGURE 13. The effect of preconditioning pulses on I_{Ca} and Ca^{2+} release. (*A*) The effect of single pre-pulse. The depolarizing pulse protocol is shown on the top of the panel. The left side of panel shows I_{Ca}, $[Ca^{2+}]_i$, and $d[Ca^{2+}]_i/dt$ recorded as a control (*a*), 400 ms after the conditioning pulse (*b*), 10 s later during recovery (*c*) whereas the right panel shows the superimposed normalized I_{Ca} traces, before (*control*) and after exposure to thapsigargin. (*B*) The effect of triple conditioning pulses. The timing of the depolarizations is shown at the top of the figure. The upper right panel shows the superimposed normalized I_{Ca} traces (*a*, *b*, and *c*). In addition, three of I_{Ca} traces evoked by conditioning pulses (*b1*, *b2*, *b3*) were also normalized and superimposed (*lower right*).

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 $\tau_{life} = 0.25$–0.5 μs, 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+} μ-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, 1992*a*; Smith et al., 1996), as well as the presence of endogenous, immobile Ca^{2+} buffers (Langer and Peskoff, 1996).

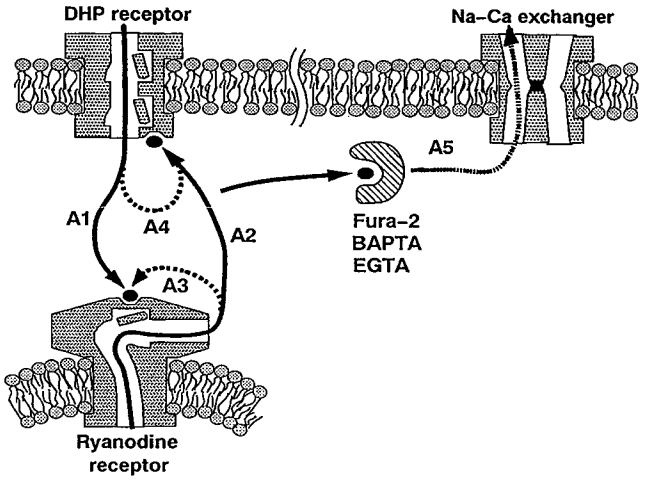

FIGURE 14. Calcium signals and their interception by Ca^{2+} buffers. The arrows suggest that the DHP receptor and Ryanodine receptors are coupled by exchange of Ca^{2+} signals (*A1* and *A2*) which are more important than the autoregulatory Ca^{2+} signals (*A3* and *A4*) or the Ca^{2+} fluxes to the Na^+-Ca^{2+} 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 Ca^{2+}.

Modulation of Rate of Inactivation of I_{Ca} by Ca^{2+}-release from the SR

Caffeine had two effects on Ca^{2+}-release triggered by I_{Ca} (Figs. 6 and 7). When caffeine was applied at intervals shorter than 50 ms before the activation of I_{Ca}, Ca_i-transients triggered by I_{Ca} were enhanced (Figs. 6 *C* and 7). This may result from the increased Ca^{2+}-sensitivity of the ryanodine-receptor to Ca^{2+} in the presence of caffeine (Rousseau et al., 1988) and the positive feedback process inherent in the Ca^{2+}-induced Ca^{2+}-release mechanism (Fabiato, 1985; O'Neill and Eisner, 1990; Sitsapesan and Williams, 1990). On the other hand, when caffeine-transient occurred more than 300 ms before the activation of I_{Ca}, I_{Ca}-gated Ca_i-transients were significantly reduced, presumably because Ca^{2+} stores that were depleted of Ca^{2+} by caffeine remained empty. Thus caffeine could be used as an effective tool to modify the magnitude of I_{Ca}-gated Ca_i-transients by changing the timing between the application of caffeine-pulse and activation of I_{Ca}.

Comparison of the kinetics of inactivation of I_{Ca} with the rate of rise of $[Ca^{2+}]_i$ suggested that the higher rates of release of Ca^{2+} were accompanied by faster rates of inactivation of I_{Ca} and vice versa (Figs. 6, 7, and 8). It was somewhat unexpected that the rise in myoplasmic Ca^{2+} concentration did not enhance the rate of inactivation (Figs. 3 *A*, 6 *A* and *B*, and 4). This finding suggests that alterations of the global myoplasmic Ca^{2+} concentrations do not regulate the rate of inactivation of Ca^{2+} channel. On the other hand, the linear relationship between the rate of inactivation of I_{Ca} ($1/\tau$) and $\Delta[Ca^{2+}]_i$ as well as $d[Ca^{2+}]_i/dt$ of Fig. 8 suggests that ~65–75% of inactivation of Ca^{2+} channel may be dependent on the release of Ca^{2+} from the ryanodine-receptor (*A2 arrow* of the schematic of Fig. 14). Similar linear correlations between rate of inactivation of I_{Ca} and $\Delta[Ca^{2+}]_i$ 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 Ca^{2+} 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 Ca^{2+}-induced inactivation of I_{Ca}.

Partial depletion of the SR with single (Fig. 13 *A*) or multiple (Fig. 13 *B*) conditioning pulses produced a decrease in the rate of inactivation of I_{Ca}. The abolishment of such modifications in the rate of inactivation of I_{Ca} by thapsigargin supported the idea that the beat-dependent change in I_{Ca} kinetics are, in part, caused by the decrease of Ca^{2+}-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 I_{Ca} which depends on voltage but not Ca^{2+} (Fig. 13).

The result with caffeine, thapsigargin, and conditioning pulses all indicate that Ca^{2+}-dependent inactivation of I_{Ca} is mediated primarily by Ca^{2+} released from the SR (65–75%, see *A2 arrow* in Fig. 14), is less dependent on its own Ca^{2+} influx (25–35%, *A4 arrow* in Fig. 14), and is independent of the global myoplasmic Ca^{2+} concentrations.

At first sight these findings might appear to contradict published results. For instance, it has been amply demonstrated that various intracellular Ca^{2+} buffers (e.g. 20 mM EGTA) slows the inactivation of I_{Ca} in cultured guinea-pig atrial myoballs (Bechem and Pott, 1985). The open probability of single L-type Ca^{2+} channels in cell attached patches of guinea-pig ventricular myocytes was modulated by modest changes in the intracellular Ca^{2+} activity (Hirano and Hiraoka, 1994). Both I_{Ca} and I_{Ba} carried by L-type Ca^{2+} channels reconstituted in planar lipid bilayers inactivate faster when the Ca^{2+} activity at the "intracellular" surface is raised to 10 μM (Haack and Rosenberg, 1994). The α_1 subunit of the cardiac Ca^{2+} channel exhibits the characteristics of Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} channels is directly influenced both by Ca^{2+} 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 Cleemann, 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 (1992*b*) 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, 1992*b*) 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 simultaneous with the release of Ca^{2+} by caffeine decayed more rapidly than control I_{Ba} (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 ($I_{Na\text{-}Ca}$) (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 $I_{Na\text{-}Ca}$ 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 $I_{Na\text{-}Ca}$ to extrude Ca^{2+} at the holding potentials of −60 to −80 mV, high concentration of Ca^{2+} buffer interfered with the activation of $I_{Na\text{-}Ca}$ by caffeine triggered Ca^{2+} release. A possi-

ble explanation for these findings is that the Na^+-Ca^{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^+-Ca^{2+} exchanger within the t-tubules, t-SR junctions or microdomains of Ca^{2+}, it may be recalled that the exchanger has fairly low affinity for Ca^{2+} (K_d = 5 μM; Hilgemann et al., 1992) and therefore may be relatively more sensitive to exogenous Ca^{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^{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^{2+} concentration (Figs. 1 *C* and 2 *C*). Thus, the Ca^{2+}-uptake system appears to continue to reload the SR, and I_{Ca} continues to trigger Ca^{2+} release from the SR even in the presence of high concentration of Ca^{2+} buffers. It should be noted that, in the present study, myocytes were dialyzed with high concentration of cAMP (200 μM) to maximize I_{Ca} and re-uptake of Ca^{2+} by Ca-ATPase activity through the respective cAMP-dependent protein kinase-induced phosphorylation of Ca^{2+} channels and phospholamban. If the myocytes were dialyzed with even higher concentration of Ca^{2+} buffers, however, the ability of Ca-ATPase to compete for sequestration of Ca^{2+} with exogenous buffers could be severely compromised, leading secondarily to depletion of SR Ca^{2+} content, making it difficult to quantify Ca^{2+} signaling between the two sets of receptors.

The present results indicate that two types of Ca^{2+} signals persist in the presence of high concentrations of Ca^{2+} buffers. One signal is transmitted by Ca^{2+} which enters through the DHP-sensitive sarcolemmal Ca^{2+} channel and triggers the release of Ca^{2+} from the SR, and the other is carried by Ca^{2+} released from the ryanodine receptor (Ca^{2+}-release channel) accelerating the inactivation of the Ca^{2+} channel. Quantifying the equivalent Ca^{2+} and Ba^{2+} charge moving through the channel, we estimated that Ca^{2+}-release is 16–19 times larger than the entry of Ca^{2+} through the Ca^{2+} channel as I_{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^{2+} current does not deplete the Ca^{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^{2+} buffer (Fig. 1).

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

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