# The Effects of Exogenous Calcium Buffers on the Systolic Calcium Transient in Rat Ventricular Myocytes

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ABSTRACT The aim of this work was to characterize the effects that two commonly used "caged" calcium buffers (NP-EGTA and nitr-5) have on the amplitude and time course of decay of the calcium transient. We made quantitative measurements of both free and total calcium using the measured buffering properties of the cell. Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with fluo-3 in rat ventricular myocytes. Incorporation of the buffer NP-EGTA decreased both the amplitude and rate of decay of the caffeine response. The slowing could be quantitatively accounted for by the measured increased buffering. These effects were removed by photolysis of NP-EGTA. Similar results were obtained with nitr-5 except that the effects were not completely removed by photolysis. This was shown to be due to the persistence of a component of the increased buffering after photolysis. Both buffers decreased the amplitude of the systolic calcium transient. However, although nitr-5 produced a simple slowing of the decay, NP-EGTA resulted in an initial rapid phase of decay. This rapid phase of decay is attributed to calcium binding to NP-EGTA. This work represents the first quantitative analysis of the effects that extra buffering by a fast and a slow calcium chelator may have on the calcium transient.

## INTRODUCTION

Many biological processes are controlled by changes of the free intracellular calcium concentration, $[Ca^{2+}]_i$. These result from fluxes of calcium ions across cellular membranes, in particular, the surface membrane and that of the endoplasmic or sarcoplasmic reticulum. The magnitude and kinetics of the changes of $[Ca^{2+}]_i$ depend, however, not only on the underlying fluxes, but also on the calcium buffering properties of the cytoplasm (Bers and Berlin, 1995). In addition to endogenous buffers, fluorescent indicators that are used to measure $[Ca^{2+}]_i$ have been reported to have effects on calcium buffering (Noble and Powell, 1991; Berlin and Konishi, 1993). The buffering produced by exogenous buffers has also been used deliberately to study calcium sparks (Song et al., 1998). Manipulation of free $[Ca^{2+}]_i$ by photolabile "caged" calcium compounds has become a very common tool to study different calcium-regulated mechanisms in cardiac muscle (Gurney et al., 1989; Lipp and Niggli, 1998; Lipp et al., 1996; Niggli and Lederer, 1991; Näbauer et al., 1989; Valdeolmillos et al., 1989; Patel et al., 1995). Although it is very important, for the quantitative interpretation of the signal obtained, to know the possible effects that such exogenous $Ca^{2+}$ buffers may have on the time course and magnitude of the physiological events under study, no reports are yet available regarding the effects that caged-calcium compounds may have on the cytoplasmic buffering capacity of the myocytes and, therefore, on the calcium transient.

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We have recently described a method to determine the cytoplasmic buffering properties for $Ca^{2+}$ and the time course of changes in sarcoplasmic reticulum (SR) calcium during systole (Trafford et al., 1999). We also exploit the fact that the degree of buffering in a cell can be changed rapidly by photolysis of caged calcium buffers. The results show that, although calcium buffers produce the expected slowing of the rate of decay of the caffeine response and increase in the cytoplasmic calcium buffering capacity, the effect on the systolic calcium transient depends on the kinetics of the caged-calcium compound used. Although extra buffering by nitr-5 (a fast calcium buffer) decreases the amplitude and rate of decay of the calcium transient, NP-EGTA (which has a slower binding rate for calcium) (Ellis-Davies and Kaplan, 1994; Ellis-Davies et al., 1996) has more complicated effects.

## MATERIALS AND METHODS

Cardiac ventricular myocytes were isolated (from rats killed by stunning and cervical dislocation) using a collagenase-protease digestion technique as previously described (Eisner et al., 1989).

### $[Ca^{2+}]_i$ measurements

This was achieved by loading the cells with the penta-acetoxy-methyl (AM) ester form of the calcium-fluorescent indicator fluo-3 (10 $\mu$M) for 5 min. Fluorescence was excited at 488 nm and measured between 520 and 620 nm. Fluorescence measurements were calibrated in terms of absolute values of $[Ca^{2+}]_i$ as described previously (Trafford et al., 1999). Briefly, when recording was finished, the cell was damaged with the patch pipette. This produced an immediate rise of $[Ca^{2+}]_i$ and fluorescence (F) followed by a slow decay. The initial peak fluorescence is taken to be the fluorescence at saturating $[Ca^{2+}]_i$ ($F_{max}$). Because the fluorescence of fluo-3 is negligible in the absence of calcium, this results in the expression,

$$[Ca^{2+}]_i = F \cdot K_d/(F_{max} - F),$$

where $K_d$ was taken as 400 nM.

## Manipulation of $[Ca^{2+}]_i$

To study the effects of exogenous buffers on the calcium transient, the cells were loaded with the AM-forms of one of the caged-calcium compounds NP-EGTA (5 $\mu$M) or nitr-5 (10 $\mu$M) for 10 and 5 min, respectively. Control superfusion solution was then added to excess to stop the loading process. This solution contained (in mM): NaCl, 134; KCl, 4; $MgCl_2$, 1.2; HEPES, 10; glucose, 11; $CaCl_2$, 1–5; titrated to pH 7.4 with NaOH. Photolysis of the caged-calcium compounds with an intense flash (250–360 J) of ultraviolet light (~350–360 nm [Ellis-Davies and Kaplan, 1994; Adams et al., 1988]) was achieved by using a xenon-flash photolysis unit (Cairn, Sittingbourne, UK). The light from the flash lamp passed through a beamsplitter where it was combined with the fluo-3 excitation light. The beamsplitter transmitted 80% of the flash and reflected 20% of the fluo-3 light to the microscope. The dichroic mirror under the objective (part 72100 Chroma Technology, Battleboro, VT) reflected both lights to the objective while allowing the light emitted from fluo-3 to pass through it. Flash photolysis allowed rapid changes of the extra buffering capacity added by the caged compounds, thus allowing control and test measurements to be performed on the same cell. All our postflash records were taken after complete photolysis of the caged compound under study, in which case further flashes did not generate any increase of $[Ca^{2+}]_i$.

## Perforated patch recording

Voltage clamp was imposed using the perforated patch technique with amphotericin-B (Horn and Marty, 1988). Microelectrodes (made from borosilicate glass) had resistances of 2.5–3.0 MΩ when filled with (mM): $KCH_3O_2S$, 125; KCl, 20; NaCl, 12; HEPES, 10; $MgCl_2$, 5; $K_2EGTA$, 0.1; titrated to pH 7.2 with NaOH. Amphotericin-B was dissolved in DMSO and added to the pipette filling solution to a final concentration of 240 $\mu g.ml^{-1}$. All the experiments were performed at room temperature (23°C). During the patch clamp experiments, 4-aminopyridine (5mM) and $BaCl_2$ (0.1 mM) were added to the bathing solution to block interfering outward currents.

## Buffering curves

Changes of $[Ca^{2+}]_I$ were converted to changes of total $[Ca^{2+}]_i$ using the cytoplasmic calcium buffering properties of the cell obtained as previously described (Trafford et al., 1999). Briefly, the transient increase of cytoplasmic free calcium resulting from the application of caffeine (10 mM) generates an inward electrogenic current as calcium is extruded from the cell via the sodium/calcium exchanger. This electrogenic current, which can be measured under voltage clamp, gives a direct measure of the rate at which calcium is being pumped out of the cell by sodium–calcium exchange. To calculate the total rate of calcium removal from the cytoplasm, it is necessary to correct for that fraction of calcium that is removed from the cytoplasm by nonelectrogenic mechanisms. The major such mechanism is the sarcolemmal Ca-ATPase (Choi and Eisner, 1999) but there may also be a contribution from mitochondrial sequestration. As in previous work, we have corrected for the nonelectrogenic calcium removal by measuring the rate constant of decay of the rise of $[Ca^{2+}]_i$ evoked by 10 mM caffeine. This was measured under both control conditions ($k_{cont}$) and with sodium–calcium exchange inhibited with 10 mM $Ni^{2+}$ ($k_{Ni}$). Multiplying a measured sodium-calcium exchange flux by $k_{cont}/(k_{cont} - k_{Ni})$ gives the corrected total flux. It should also be noted that the magnitude of this correction factor is constant over the range of $[Ca^{2+}]_i$ encountered (Choi et al., 2000). The calculated total calcium efflux gives a measure of the change of total calcium, which produces a measured change of free calcium and therefore allows construction of a buffering curve (see Fig. 2 *B*).

In our previous paper (Trafford et al., 1999), we used the free acid form of fluo-3 to measure $[Ca^{2+}]_i$ and buffering properties. In the present paper, the AM ester was used. We have therefore checked to see whether the different method of loading the fluorescent indicator affects the measurements. We found that the mean value for the maximum buffering ($B_{max}$) was 128 ± 18 $\mu$mol/l cell volume in acid loaded and 162 ± 15 $\mu$mol/l cell volume in AM loaded. The $K_d$ was 526 ± 125 nM ($n = 8$) and 447 ± 38 nM ($n = 10$), respectively. Neither of these parameters is significantly different between free acid and AM loaded cells ($p > 0.05$).

## Statistics

All values are presented as the mean ± SEM of $n$ experiments. Statistical significance was assessed using paired or unpaired $t$-test at the level of 0.05.

# RESULTS

## The effects of calcium buffers on the caffeine response

### *The effects of NP-EGTA on the caffeine response*

Figure 1 shows the effects of the caged-calcium chelator NP-EGTA on the caffeine response. NP-EGTA was added as the AM ester before experimenting on the cells. This compound initially buffers calcium with a $K_d$ of 80 nM (Ellis-Davies and Kaplan, 1994). However, photolysis by illumination with ultraviolet light irreversibly cleaves the chelator, thus yielding photoproducts with a much lower calcium affinity ($K_d$ of 1 mM), which therefore do not buffer calcium appreciably at the $[Ca^{2+}]_i$ tested in these experiments. Figure 1 *A* shows a caffeine response from a cell that had not been loaded with NP-EGTA (*left trace*, control). The middle trace (preflash) is from a cell loaded with NP-EGTA. Compared to the control cell, the transient increase of $[Ca^{2+}]_i$ produced by caffeine is dramatically reduced. In addition, the decay of the $[Ca^{2+}]_i$ transient is also slower than in control. This latter effect is more obvious in the normalized traces of Fig. 1 *B*. To see whether the difference between the caffeine responses in the cells illustrated as control and preflash is due to the extra calcium buffering, the caffeine response in the right panel (postflash) was obtained after photolyzing NP-EGTA. This resulted in an increase in the magnitude and rate of decay of the caffeine response to levels similar to those found in unloaded cells. On average, both the amplitude and rate of decay of the caffeine response were significantly less in NP-EGTA-loaded cells than in control (see Fig. 1 *C* and Table 1). After photolysis, the amplitude and rate of decay increased to levels similar to those in control cells. In other words, the effects of NP-EGTA on the caffeine response are removed by photolysis and are therefore due to calcium buffering rather than nonspecific effects. The concentration of NP-EGTA we tested (5 $\mu$M) did not show any significant effects on the diastolic level of calcium (86.7 ± 8.89 nM; $n = 11$) in comparison with the control cells (85.4 ± 7.4 nM; $n = 8$).

Subsequent experiments were designed to obtain quantitative measurements of changes of cytoplasmic calcium

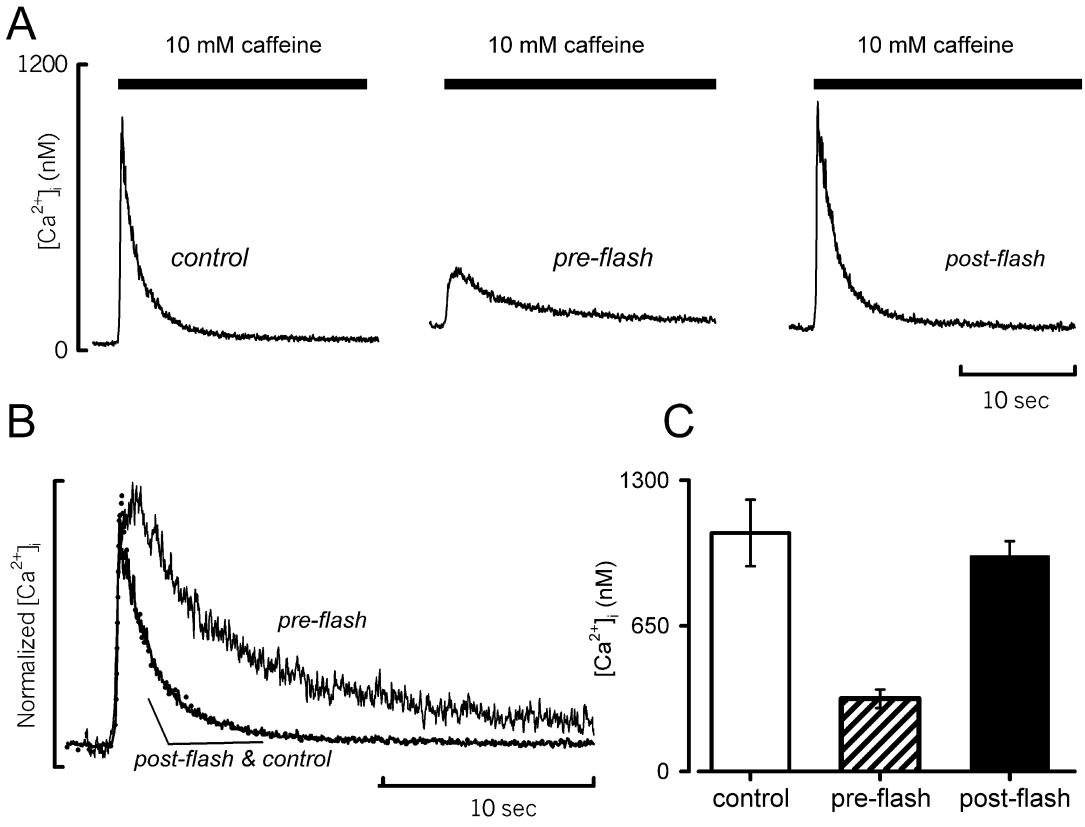


FIGURE 1 The effects of loading with NP-EGTA on the caffeine response. (*A*) Original data. The bars above the traces show when caffeine (10 mM) was applied. The left-hand panel was obtained from a control cell (not loaded with NP-EGTA). A second cell loaded with NP-EGTA was used for preflash and postflash traces. The preflash (*middle trace*) was obtained before and the postflash (*right trace*) after photolysis. (*B*) Superimposed traces normalized to the same amplitude. Note that the record from the control cell (*continuous line*) is indistinguishable from that after photolysis (*dotted line*). (*C*) Histogram of the mean amplitude of the caffeine response (from Table 1) in the three conditions (bars show the mean ± SEM for 12 control cells and 11 pre- and postflash).

buffering and relate these to the differences in calcium handling. The experiment illustrated in Fig. 2 *A* shows another caffeine response. Again, photolysis increased both the magnitude of the caffeine-evoked calcium transient and the rate of decay. This was accompanied by an increase in both the magnitude of the Na-calcium exchange current and its integral. The fact that the integral was increased by photolysis indicates that the total amount of calcium pumped out of the cell and presumably stored in the SR was decreased by the exogenous calcium buffer. On average, in

**TABLE 1 Effects of exogenous buffers (NP-EGTA and nitr-5) on the Ca transient**

| | | NP-EGTA | | nitr-5 | |
|---|---|---|---|---|---|
| | Control | Pre flash | Post flash | Pre flash | Post flash |
| Caffeine amplitude (nM) | 1064 ± 149 (12) | 262 ± 40 (11)** | 960 ± 68 (11) | 422 ± 50 (17)** | 741 ± 98 (17)* |
| Caffeine halftime (sec) | 1.68 ± 0.22 (9) | 16.37 ± 2.39 (11)** | 1.79 ± 0.38 (11) | 8.13 ± 1.19 (16)** | 2.79 ± 0.35 (16)* |
| Systolic amplitude (nM) | 519 ± 79 (12) | 236 ± 24 (13)** | 534 ± 63 (13) | 218 ± 18 (14)** | 356 ± 29 (14)* |
| Systolic halftime (sec) | 0.18 ± 0.02 (9) | — | 0.16 ± 0.02 (21) | 0.89 ± 0.05 (7)** | 0.54 ± 0.05 (7)** |
| $B_{max}$ ($\mu$mol/l) | 162 ± 15 (10) | 829 ± 79 (10)** | 218 ± 32 (10) | 754 ± 151 (8)** | 345 ± 53 (8)* |
| $K_d$ ($\mu$M) | 0.45 ± 0.04 (10) | 0.25 ± 0.03 (10)** | 0.53 ± 0.13 (10) | 1.56 ± 0.86 (8) | 0.40 ± 0.08 (8) |

The amplitude and rate of decay were measured both during stimulation (systolic parameters) and during application of 10 mM caffeine (caffeine parameters).

Control values correspond to cells only loaded with fluo-3 recorded the same days as the caged-compound loaded cells.

The values represent means ± SEM of (*n*) cells; *$p < 0.05$, **$p < 0.001$ when compared to controls.

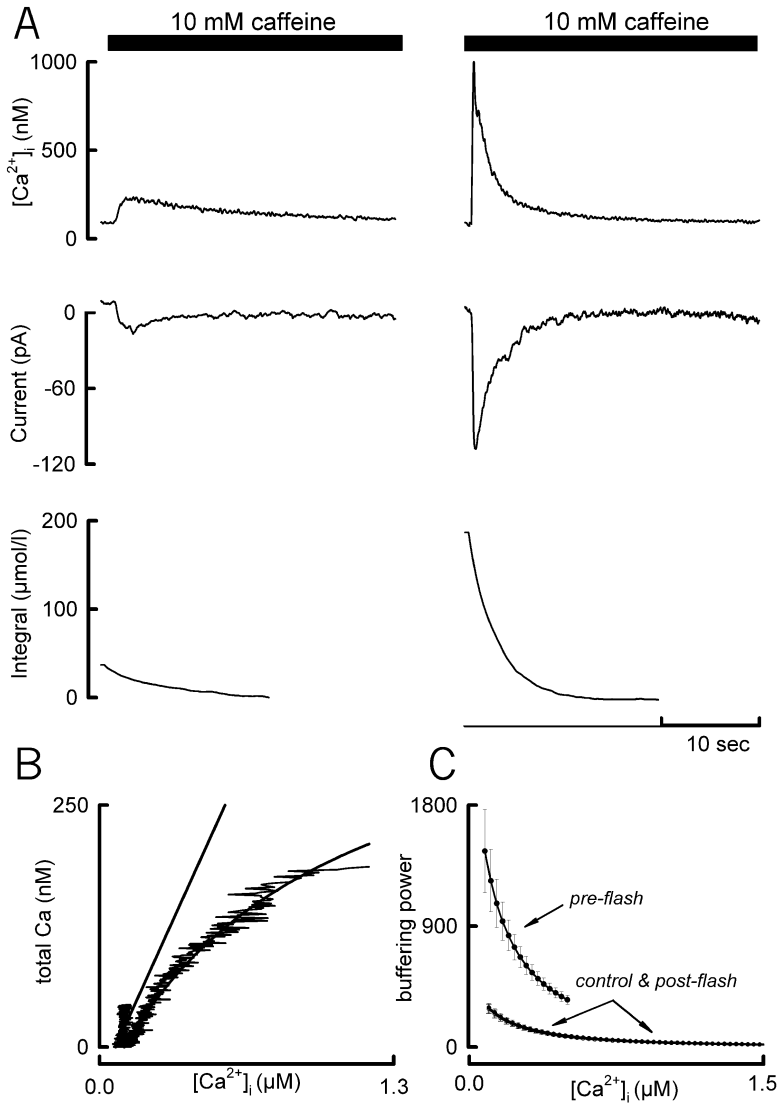


FIGURE 2 The effects of NP-EGTA on caffeine-evoked currents and buffer properties. (*A*) Original data. Traces show (from *top* to *bottom*): $[Ca^{2+}]_i$, membrane current, and integrated current (converted to equivalent calcium movements after correction for nonelectrogenic calcium removal pathways). Caffeine (10 mM) was applied for the period shown by the bars above. Both records were obtained from the same NP-EGTA-loaded cell, the left-hand one before and the right-hand after photolysis. (*B*) Calcium buffering curves obtained from the data of (*A*). (*C*) Buffering power as a function of $[Ca^{2+}]_i$ for 10 control and 11 NP-EGTA- loaded cells.

12 cells, the SR calcium content was 61.7 ± 6 $\mu$mol/l in the preflash condition, a value that was significantly lower ($p < 0.001$) from that measured in postflash photolysis (131.4 ± 13 $\mu$mol/l). Figure 2 *B* shows measurements of calcium buffering before and after photolysis performed in the same cell (Trafford et al., 1999). It is clear that, over the measured range, before photolysis, a much larger increase of total calcium concentration ($Ca_T$) is required to increase free calcium by a certain amount. To present mean data and make comparisons between different conditions, we have calculated the $[Ca^{2+}]_i$-dependence of the buffering power. This is defined as

$$\text{buffering power} = d(Ca_T)/d[Ca^{2+}]_i).$$

If we assume that

$$Ca_T = (B_{max} \cdot [Ca^{2+}]_i)/([Ca^{2+}]_i + K_d), \quad (1)$$

where $B_{max}$ is the maximum binding capacity; then,

$$d(Ca_T)/d[Ca^{2+}]_i = B_{max} \cdot K_d/([Ca^{2+}]_i + K_d)^2. \quad (2)$$

Therefore, for each cell, we calculated $B_{max}$ and $K_d$ by fitting Eq. 1 to data such as that of Fig. 2 *B*. Using Eq. 2, we then calculated the buffer power as a function of $[Ca^{2+}]_i$ for each cell. The data were then averaged at 10 nM intervals, giving the graphs of Fig. 2 *C*.

Figure 2 *C* shows that, as expected for a saturating buffer, the buffer power is greater at low $[Ca^{2+}]_i$. At any given value of $[Ca^{2+}]_i$, the buffer power is greatly increased before compared to after photolysis. It should also be noted that the buffering curves for control cells superimpose on those after photolysis. The mean data (Table 1) show that cells loaded with NP-EGTA have a higher mean maximum calcium binding capacity ($B_{max}$) than do control cells, and

that this increased buffering, like the effect on the caffeine response, is removed by photolysis.

The experiments described above show that addition of NP-EGTA increases the calcium buffering capacity of the cytoplasm and slows the rate constant of decay of $[Ca^{2+}]_i$. Figure 3 addresses the question of whether the measured changes of cytoplasmic calcium buffering can account quantitatively for the observed slowing of the calcium transient. The graph in Fig. 3 *A* shows the rate of decay of free calcium ($-d[Ca^{2+}]_i/dt$) plotted as a function of $[Ca^{2+}]_i$. As expected from the decrease of the rate constant of decay of $[Ca^{2+}]_i$, the slope of this relationship is greatly decreased by NP-EGTA. Figure 3 *B* shows the rate of fall of total calcium ($-d[Ca^{2+}]_T/dt$) as a function of $[Ca^{2+}]_i$. There is much less effect on the slope of this relationship than was the case for the relationship between $-d[Ca^{2+}]_i/dt$ and $[Ca^{2+}]_i$. The curves are easier to distinguish in the expanded plots below, which show the data over the lower range of $[Ca^{2+}]_i$ with fitted straight lines. In this experiment, the addition of NP-EGTA decreased the slope of the relationship between $-d[Ca^{2+}]_i/dt$ and $[Ca^{2+}]_i$ to 3.3% of the control value, whereas that between $-d[Ca^{2+}]_T/dt$ and $[Ca^{2+}]_i$ was only decreased to 83%. On average, the addition of NP-EGTA decreased the slope of the relationship between $-d[Ca^{2+}]_i/dt$, and $[Ca^{2+}]_i$ to 22 ± 7% ($n = 8$).

*The effects of nitr-5 on the caffeine response*

Nitr-5 is a calcium chelator with a very high affinity for calcium ($K_d$ = 145 nM), which can be rapidly modified by flash photolysis, resulting in a product with a lower affinity ($K_d$ = 6 μM) for calcium (Adams et al., 1988; Gurney et al., 1987). Also, because it is synthesized from BAPTA, nitr-5 has very fast kinetics of binding and release of calcium, particularly if compared with NP-EGTA (which is an EGTA derivative). Figure 4 *A* illustrates the effects of nitr-5 on the peak and decay rate of the caffeine-evoked calcium transient. The control (*left trace*) was taken from a control cell that had not been loaded with the caged-calcium compound. The application of caffeine in the preflash (*middle trace*) shows that the extra-buffering by nitr-5 clearly decreases the peak and slows the rate of decay of the calcium transient. The effects of nitr-5 are decreased by flash photolysis as shown by the postflash (*right trace*) (see also summary data in Table 1). However, even after photolysis, the mean value of the amplitude of the calcium transient was still less than in unloaded cells (Table 1). Again, no significant effects were observed on the resting level of calcium among nitr-5-loaded cells (85.9 ± 6 nM; $n = 11$), after flash photolysis (90.4 ± 6 nM; $n = 11$), or the control cells (85.4 ± 7 nM; $n = 8$). Analysis of the half time of

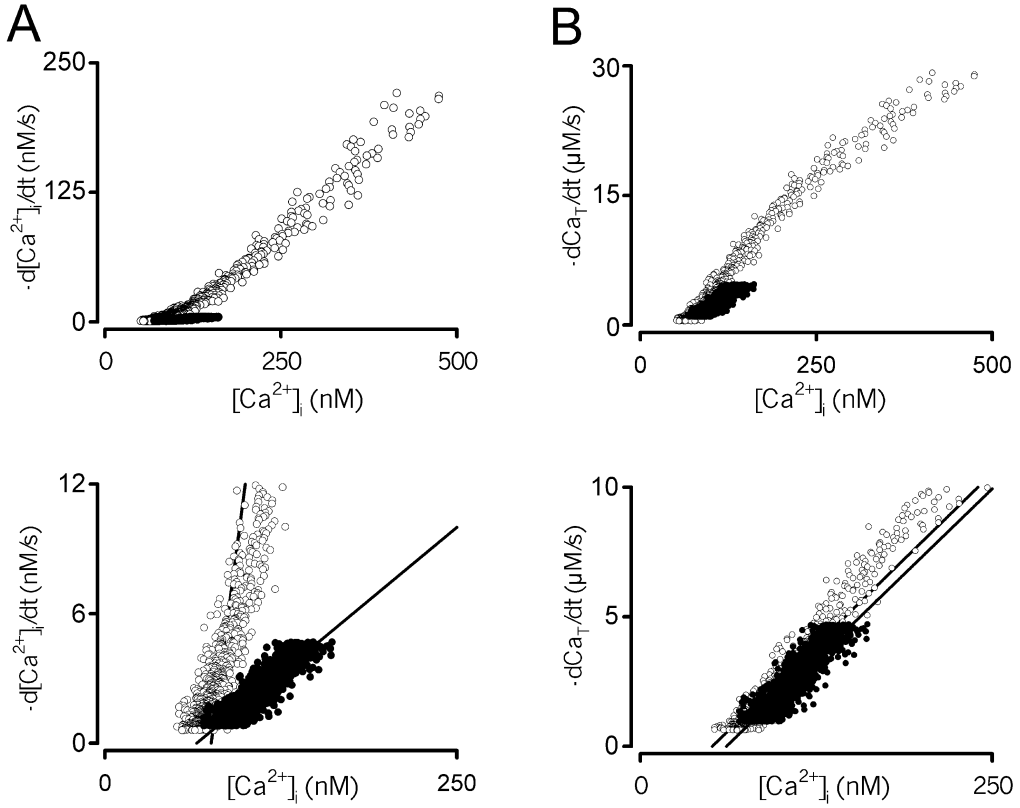


FIGURE 3 The effects of NP-EGTA on the rate of fall of free and total calcium during a caffeine response. (*A*) Rate of fall of free calcium ($-d[Ca^{2+}]_i/dt$) plotted as a function of $[Ca^{2+}]_i$. The solid symbols were obtained before and the open symbols after photolysis. The lower panel shows an expanded version of the lower range of $[Ca^{2+}]_i$. The lines are linear regressions to the data. (*B*) Rate of fall of total calcium ($-dCa_T/dt$) as a function of $[Ca^{2+}]_i$ .

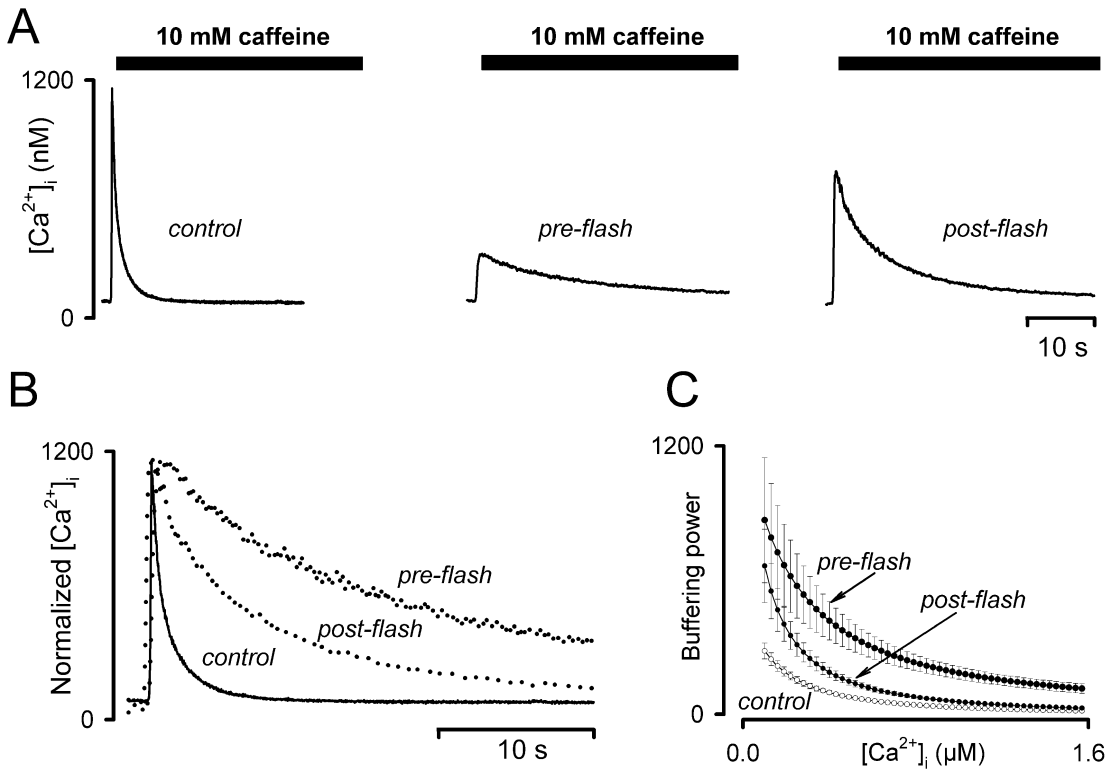


FIGURE 4 The effects of nitr-5 on the caffeine response. (*A*) Original data. Caffeine (10 mM) was applied for the periods shown. The left-hand record was obtained from a control cell. The other two records were from the same cell loaded with nitr-5 and were obtained before (*middle*) or after (*right*) photolysis. (*B*) Data from (*A*) normalized to the same amplitude. (*C*) Buffer power plotted as a function of $[Ca^{2+}]_i$ (mean data from 10 control cells and 8 loaded with nitr-5). From top to bottom, the curves represent preflash, postflash, and control cells.

decay of the caffeine-evoked calcium transient also showed a significant increase in the presence of nitr-5 (8.1 ± 1.2 s; $n = 16$) in comparison to the controls (1.7 ± 0.2 s; $n = 9$; $p < 0.001$). This effect was also not completely removed by flash photolysis (2.8 ± 0.3 s; $n = 16$). This partial effect of photolysis is emphasized by the normalized traces of Fig. 4 *B*. Figure 4 *C* shows the buffering power as a function of $[Ca^{2+}]_i$. It demonstrates that nitr-5 increases the buffering power at any given $[Ca^{2+}]_i$, and that flash photolysis does not completely remove this effect. This is emphasized by the data of Table 1.

## The effects of exogenous buffers on the systolic calcium transient

### *The effects of nitr-5 on the systolic calcium transient*

Experiments such as the one illustrated in Fig. 5 were designed to investigate the effects of adding nitr-5 on the peak and decay rate of the systolic calcium transient. Figure 5 *A* shows that extra-buffering by nitr-5 has a dramatic effect on both the peak and the decay of the calcium transient (*middle trace*, preflash) and that both effects can be removed, at least in part, by flash photolysis (*right trace*, postflash). The normalized average traces in Fig. 5 *B* show that, although photolysis of nitr-5 results in a much larger and faster decaying calcium transient (Table 1) this value is still significantly slower ($p < 0.001$) than expected from a control (Fig. 5 *A*, *left trace*) cell. The partial effects of photolysis are presumably due to the fact (Fig. 4 *C* and Table 1) that the photolyzed form of nitr-5 is still an effective calcium buffer.

Figure 6 presents a quantitative study of the effects of nitr-5 on the rate of decay of both free and total calcium. As would be expected from a much slower decay rate in the presence of nitr-5, the slope of the relationship between $d[Ca^{2+}]_i/dt$ and $[Ca^{2+}]_i$ is much lower during the preflash records compared to those obtained after photolysis (Fig. 6 *A*). This point is emphasized in the expanded data in the bottom panel. In contrast, the relationship between $dCa_T/dt$ and $[Ca^{2+}]_i$ is unaffected by nitr-5. This observation shows that the slowing of the decay of the systolic calcium transient (like that of the caffeine response) can be attributed quantitatively to increased calcium buffering.

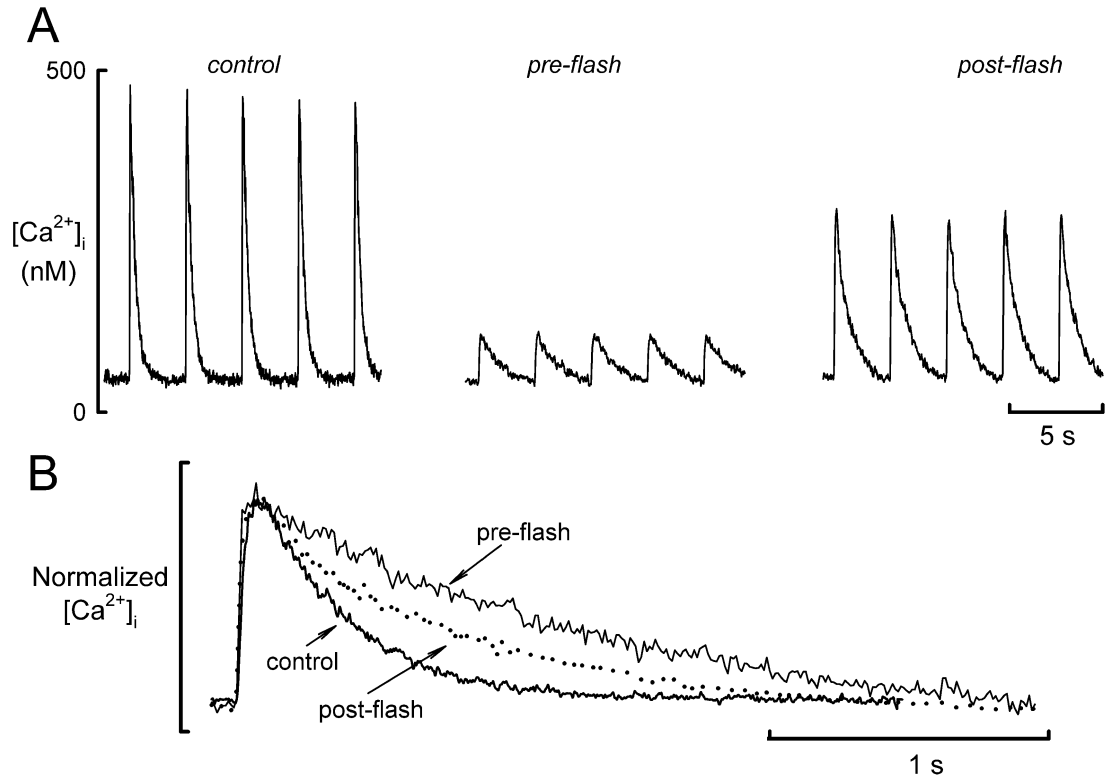


FIGURE 5 The effects of nitr-5 on the systolic calcium transient. (*A*) Original data. Each panel shows the calcium transients produced by 5 (100-ms duration) voltage clamp pulses from −40 to 0 mV every 0.33 Hz. The left-hand record was obtained from a control cell. The other two records were from a cell loaded with nitr-5 and were obtained either before (*middle*) or after (*right*) photolysis. (*B*) Normalized data.

### *The effects of NP-EGTA on the systolic calcium transient*

The experiment illustrated in Fig. 7 compares systolic calcium transients from NP-EGTA-loaded cells before (Fig. 7 *A*, *left trace*) and after photolysis. It is clear that loading with NP-EGTA results in a marked decrease in the amplitude of the systolic calcium transient. This is reversed by photolysis (Fig. 7 *A*, *right trace*). It is also evident that NP-EGTA loading has complicated effects on the kinetics of decay of the systolic calcium transient that are best seen in the superimposed and normalized traces of Fig. 7 *B*. The addition of the caged chelator produces a marked slowing of the final phase of the decay of the calcium transient, as would be expected. However, there is an initial very rapid phase, which is faster than that seen in the control cells. That the effects of NP-EGTA buffering on the decay of the systolic calcium transient are removed by flash photolysis is demonstrated by the fact that the mean half time of decay obtained after flash photolysis (Table 1) was not significantly different from the mean half time measured from control (noncaged compound-loaded) cells.

## DISCUSSION

The results of this paper show that incorporation of exogenous calcium buffers alters the kinetics of both the systolic and caffeine-evoked calcium transient. Previous studies have shown that cytoplasmic calcium buffers produce various effects on cellular calcium handling, including a decrease in both the amplitude and rate of decay of the systolic calcium transient (Brandes et al., 1993; Kirschenlohr et al., 1988; Berlin and Konishi, 1993). The major advance of the present paper is to make simultaneous quantitative measurements of both free and total calcium as well as sarcolemmal calcium fluxes.

### The effects of exogenous buffers on the caffeine response

The caffeine response is a useful probe of cell function because, after the calcium is released from the SR, the subsequent kinetics depend only on sarcolemmal fluxes and intracellular calcium buffering. The results show that the amplitude and rate of decay of this response are decreased by both nitr-5 and NP-EGTA. These effects are accompanied by an increase of intracellular calcium buffering power as measured directly. One difference between the two compounds is that, although the effects of NP-EGTA are removed completely by photolysis, those of nitr-5 only partly disappear. This difference correlates with the effects on the buffering power. The increase of buffering produced by

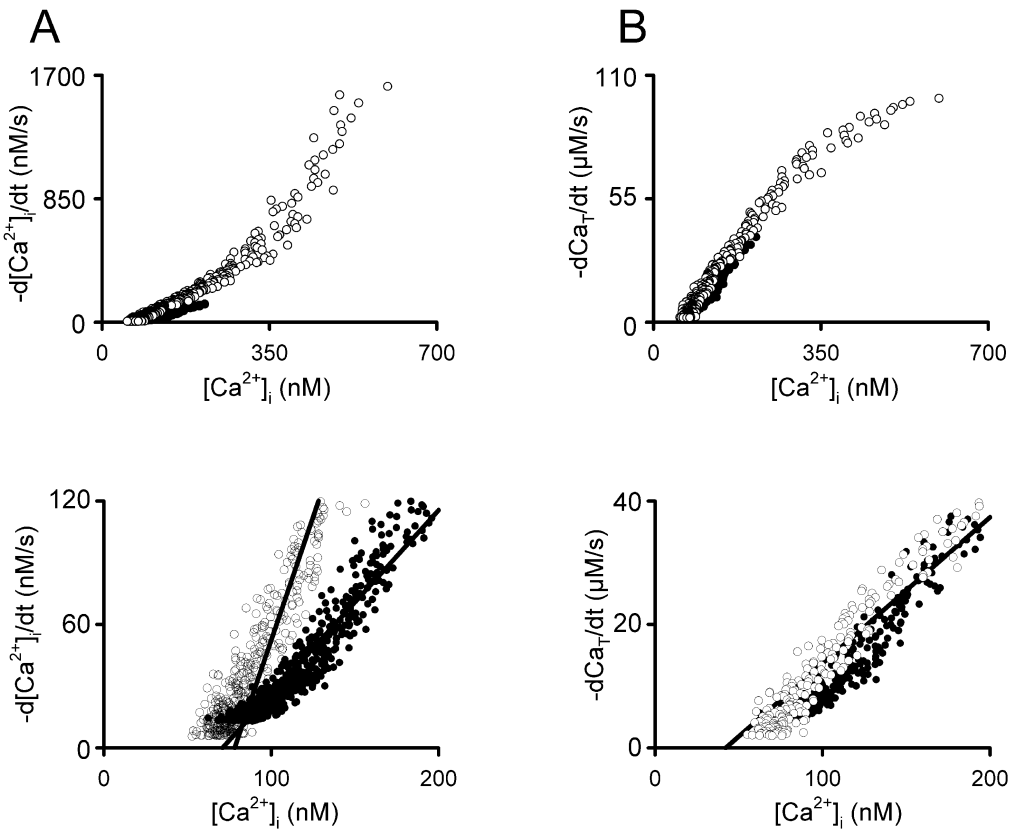


FIGURE 6 The effects of nitr-5 on the rate of fall of free and total calcium. (*A*) Free calcium. The data show $-d[Ca^{2+}]_i/dt$ plotted as a function of $[Ca^{2+}]_i$. The closed symbols were obtained before and the open after photolysis. The lower panel shows an expanded version of the data at lower $[Ca^{2+}]_i$. (*B*) Total calcium. The data show $-dCa_T/dt$ as a function of $[Ca^{2+}]_i$. The straight lines are linear regressions to the data.

NP-EGTA is reversed by photolysis. In contrast, although photolysis decreases the buffering produced by loading with nitr-5, it is still greater than in control (unloaded) cells. This difference presumably reflects the difference in the affinity for calcium of the photolysed products. That for photolyzed NP-EGTA is 1 mM (Ellis-Davies and Kaplan, 1994), a value that is much greater than the ranges of calcium encountered in the cytoplasm. The photolyzed form of NP-EGTA will not, therefore, contribute significantly to calcium buffering. In contrast, the $K_d$ for photolyzed nitr-5 is much lower (about 6 $\mu$M), and some buffering is therefore to be expected in our loading conditions. This remaining buffering by the photolysed form of nitr-5 has previously been reported (Patel et al., 1995).

Both caged calcium compounds decreased the rate of decay of the caffeine-evoked increase of $[Ca^{2+}]_i$. This could arise simply as a function of increased buffering leading to a lower rise of free calcium and thence of less activation of the sodium–calcium exchange. Alternatively, it could be due to some other effects of the compounds. That this is unlikely is supported by the observation that, at least for NP-EGTA, the effects of the compounds are removed by photolysis. Further evidence that the changes of kinetics are simply due to calcium buffering is provided by the observation that the incorporation of these buffers does not affect the relationship between the rate of fall of total calcium (as calculated from the buffer curves) and $[Ca^{2+}]_i$. One unexplained result is that the SR calcium content, as estimated by the caffeine response, was decreased by the exogenous buffer. Resting calcium was not affected, so the decrease of SR calcium content cannot be the result of decreased uptake from the cytoplasm due to decreased resting calcium. The explanation of the decrease of SR calcium content is therefore unclear.

## The effects of exogenous calcium buffers on the systolic calcium transient

The incorporation of nitr-5 decreased both the amplitude and rate of decay of the systolic calcium transient. This effect was largely reversed by photolysis. The residual effect after photolysis (compared to unloaded cells) is presumably (see above) due to buffering by the photolyzed compound. Exogenous calcium buffers have been found to decrease the amplitude and rate of decay of the systolic

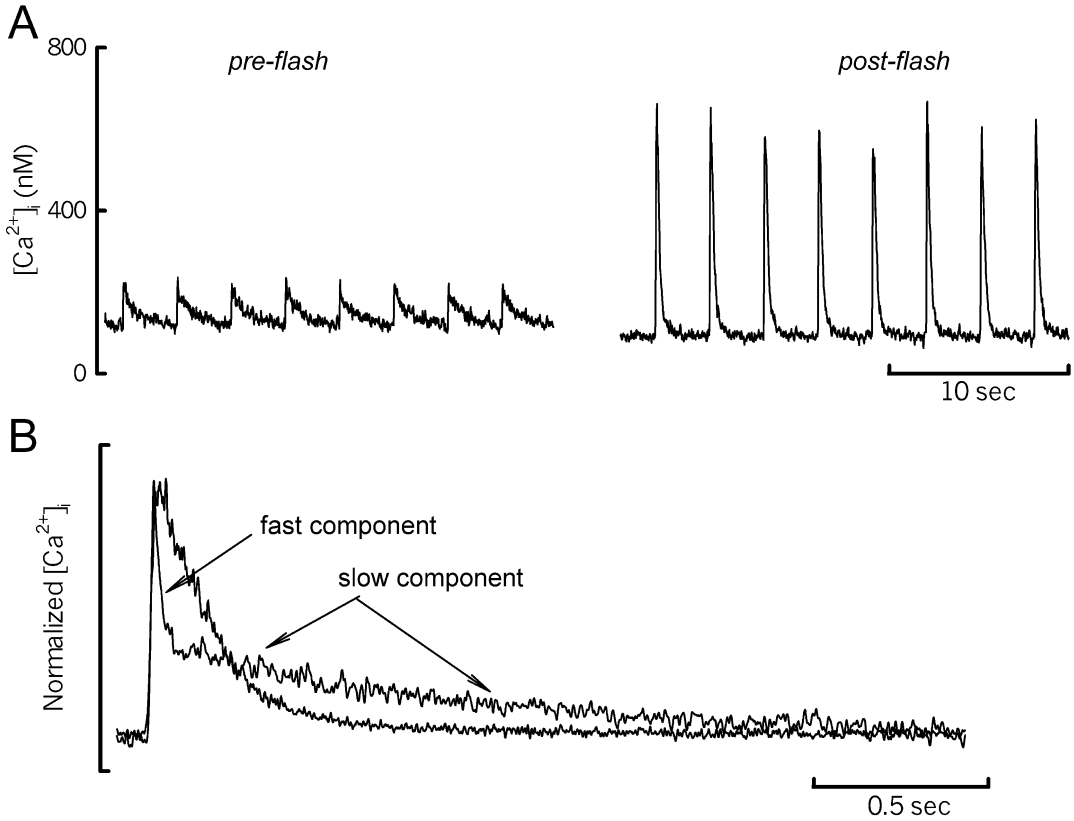


FIGURE 7 The effects of NP-EGTA on the systolic calcium transient. (*A*) Original data. The records show systolic calcium transient resulting from depolarizing (100-ms) pulses from −40 to 0 mV applied at 0.33 Hz in the same cell either before (*left*) or after (*right*) photolysis. (*B*) Normalized traces for the average of 20 systolic transient in each condition.

calcium transient in several previous studies (Backx and ter Keurs, 1993; Berlin and Konishi, 1993). One of these studies (Berlin and Konishi, 1993) concluded that the slowing of the decay might be due to effects of the exogenous buffer (in that case fura-2) on the systems removing calcium from the cytoplasm. That this is not the case in the present work is shown by the fact that the relationship between the rate of decay of total calcium and $[Ca^{2+}]_i$ was unaffected by nitr-5. This again suggests that the effects of nitr-5 are simply those expected from additional cytoplasmic buffering.

More complicated results were produced with NP-EGTA, where the normally monotonic decay of the calcium transient became split into two distinct components. One of these components was faster than control and the other slower. A plausible explanation for these biphasic kinetics is as follows. NP-EGTA is an EGTA-based compound and therefore has slow calcium-binding kinetics (Ellis-Davies et al., 1996). When calcium is released from the SR, it will therefore initially go into the cytoplasm and then, after a delay determined by the time constant of calcium binding, will bind to NP-EGTA. The kinetics of decay of the fast component will therefore represent those of calcium binding to NP-EGTA, whereas the slow component is the rate at which calcium is taken back off the combined buffers and then pumped back into the SR and out of the cell. This explanation is demonstrated by the model of Fig. 8. We assume that, during the systolic calcium transient, a certain quantity of calcium is released from the SR into the cytoplasm. The subsequent behavior is determined by three processes.

1. Binding to intrinsic calcium buffers. We assume that these are fast buffers such that, at any instant, the calcium bound to them is

$$B_{max} \cdot [Ca^{2+}]_i/(K_b + [Ca^{2+}]_i). \quad (1)$$

2. Pumping out of the cell, which we assume to be proportional to $[Ca^{2+}]_i$ (rate $= \alpha \cdot [Ca^{2+}]_i$). Set against this, there is a fixed entry of calcium into the cell of magnitude $l$. Therefore,

$$dCa_T/dt = l - \alpha \cdot [Ca^{2+}]_i. \quad (2)$$

3. Finally, calcium will bind to NP-EGTA according to the reaction,

$$Ca + \text{NP-EGTA} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} Ca \cdot \text{NP-EGTA}.$$

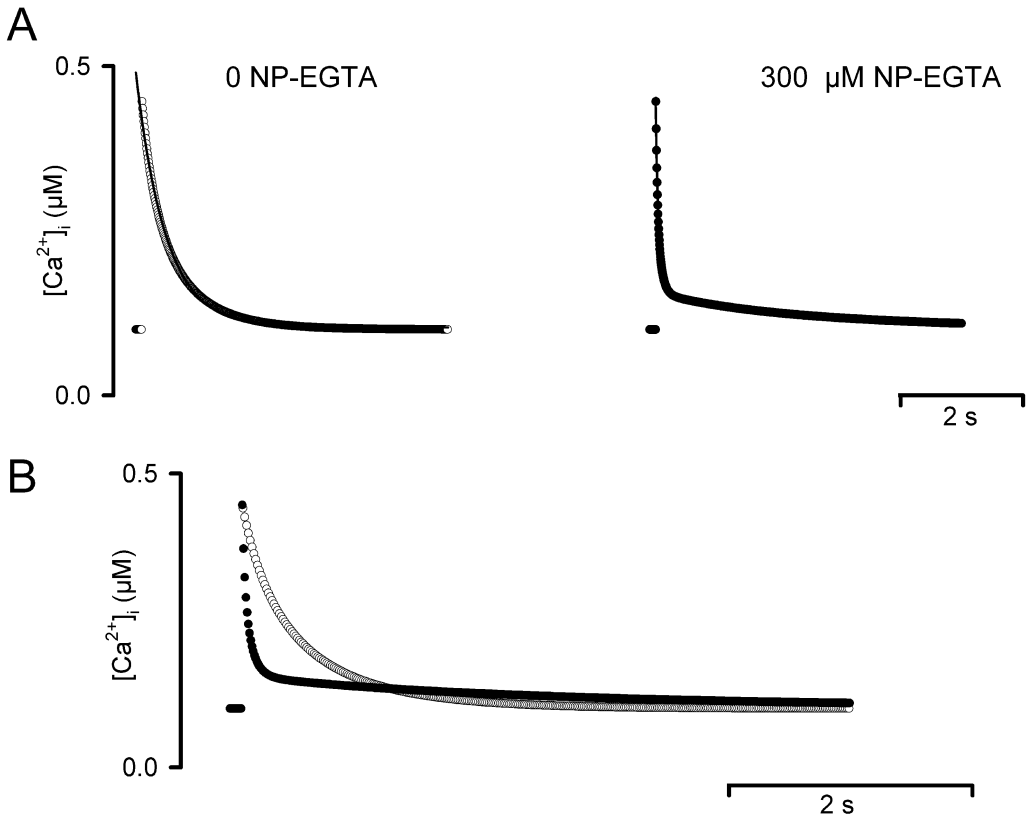


FIGURE 8 Simulation of the effects of NP-EGTA on the calcium transient. See text for a description of the model. (*A*) The left-hand panel shows a simulated transient in the absence of NP-EGTA and the right-hand one in the presence of 300 $\mu$M NP-EGTA. (*B*) Superimposed records. The following parameters were used in the modeling: $B_{max}$, 175 $\mu$mol·l$^{-1}$; $K_b$, 0.59 $\mu$M; $\alpha$, 300 l·s$^{-1}$; $l$, 30 $\mu$mol·s$^{-1}$; $k_f$, 17 $\mu$mol$^{-1}$·s$^{-1}$; $k_r$, 1.36 s$^{-1}$.

The values for all the parameters are given in the legend to Fig. 8. Those for $k_r$ and $k_f$ were obtained from published data (Ellis-Davies et al., 1996) and those for the parameters of the endogenous buffers from our previous work (Trafford et al., 1999). The other parameters were selected to give reasonable control calcium transients. It is obvious that the inclusion of NP-EGTA reproduces the initial fast and subsequent slow components of the decay of the systolic calcium transient. This acceleration of the decay of the calcium transient is very similar to that reported using EGTA (Song et al., 1998). It is also reminiscent of other markedly biphasic calcium transient decays in the literature (Sipido et al., 1998), which may result from the use of Kraft–Brühe solutions containing EGTA.

It is also obvious that NP-EGTA does not produce an acceleration of the initial phase of decay of the caffeine response. One difference between the caffeine and the systolic calcium transient is that the former rises more slowly than the latter. The rate constant of rise of the caffeine response is 9.3 $\pm$ 1.1 s$^{-1}$, whereas that of the systolic calcium transient is 72 $\pm$ 17 s$^{-1}$ ($n$ = 5). Modeling (not shown) confirms that the slower rise (and therefore slower calcium release from the SR) in the caffeine response results in nitr-5 being able to bind a larger fraction of the released calcium immediately thereby decreasing the size of the rapid component.

In summary, the data in this paper provide a quantitative analysis of the effects of two caged calcium buffers on calcium signaling in cardiac cells. The effects of these compounds can be understood in terms of their (measured) effects on cell calcium buffering and their kinetics. The effects that fast and slow exogenous calcium buffer exert on the cytoplasmic calcium buffering, and therefore on the decay of $[Ca^{2+}]_i$, may have implications on the interpretation of data obtained in previous work.

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