

The role of sarcolemmal Ca^{2+} -ATPase in the regulation of resting calcium concentration in rat ventricular myocytes

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1. The aim of this work was to investigate the role of sarcolemmal Ca^{2+} -ATPase in rat ventricular myocytes. We have measured intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) using indo-1. The actions of the ATPase inhibitor carboxyeosin were studied.
2. Carboxyeosin increased resting $[\text{Ca}^{2+}]_i$ and the magnitude of the systolic Ca^{2+} transient and slowed the rate of its relaxation by 5%.
3. Carboxyeosin increased the magnitude of the caffeine-evoked increase in $[\text{Ca}^{2+}]_i$ and slowed its relaxation by 20%. Removal of extracellular Na^+ slowed the rate constant by 80%. When Na^+ was removed in a carboxyeosin-treated cell, the caffeine-evoked increase in $[\text{Ca}^{2+}]_i$ did not decay.
4. Carboxyeosin increased the integral of the Na^+ - Ca^{2+} exchange current activated by caffeine. This is, in part, due to an increase in sarcoplasmic reticulum Ca^{2+} content.
5. When extracellular Na^+ was removed, there was a transient increase in $[\text{Ca}^{2+}]_i$ which then decayed. The rate of this decay was slowed by carboxyeosin by a factor of about four. The residual decay could be abolished with caffeine.
6. In the absence of extracellular Na^+ , increasing extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) elevated $[\text{Ca}^{2+}]_i$. In carboxyeosin-treated cells, $[\text{Ca}^{2+}]_i$ was much more sensitive to $[\text{Ca}^{2+}]_o$.
7. These results demonstrate the role of a carboxyeosin-sensitive Ca^{2+} -ATPase in the control of resting $[\text{Ca}^{2+}]_i$ and the reduction in $[\text{Ca}^{2+}]_i$ following an increase in $[\text{Ca}^{2+}]_i$.

In cardiac cells, as in many other cells, there is a large electrochemical gradient favouring Ca^{2+} entry into the cell from the extracellular fluid. Two sarcolemmal mechanisms are known to extrude Ca^{2+} from the cytoplasm: sarcolemmal Ca^{2+} -ATPase and Na^+ - Ca^{2+} exchange. The role of Na^+ - Ca^{2+} exchange in Ca^{2+} homeostasis has been extensively characterized in cardiac muscle (Chapman & Tunstall, 1981; Allen *et al.* 1984; Crespo *et al.* 1990). In contrast, much less is known about Ca^{2+} -ATPase. A sarcolemmal Ca^{2+} -ATPase has been demonstrated in the heart (Caroni & Carafoli, 1981). It has been suggested that Ca^{2+} -ATPase has a higher affinity and lower maximum velocity than Na^+ - Ca^{2+} exchange and may therefore be more important for control of resting $[\text{Ca}^{2+}]_i$ than for removing larger Ca^{2+} loads produced by stimulation (DiPolo & Beaugé, 1979) although recent work has questioned this functional distinction (Lamont & Eisner, 1996).

Most previous work studying the role of Ca^{2+} -ATPase in cardiac muscle has been performed under conditions that inhibit Na^+ - Ca^{2+} exchange to reveal any potential contribution of ATPase. Specifically, the following observations have led to the suggestion that something other than Na^+ - Ca^{2+} exchange, perhaps sarcolemmal Ca^{2+} -ATPase,

may have a significant functional role in Ca^{2+} homeostasis, at least under experimental conditions. (1) When caffeine is applied to release Ca^{2+} from the sarcoplasmic reticulum (SR), inhibition of Na^+ - Ca^{2+} exchange slows but does not abolish the decay of $[\text{Ca}^{2+}]_i$ (O'Neill *et al.* 1991; Bassani *et al.* 1992; Negretti *et al.* 1993; Bassani *et al.* 1994). It has been suggested that the Na^+ - Ca^{2+} exchange-independent component of the decay of $[\text{Ca}^{2+}]_i$ is due to a combination of sarcolemmal Ca^{2+} -ATPase and mitochondrial sequestration. Separation of mitochondrial and sarcolemmal Ca^{2+} -ATPase components has been achieved by inhibiting the former with uncouplers or the latter by elevating extracellular Ca^{2+} (Bassani *et al.* 1992; Negretti *et al.* 1993). However, these approaches are both rather non-specific. (2) Removal of extracellular Na^+ produces an increase in $[\text{Ca}^{2+}]_i$ as Ca^{2+} enters the cell via Na^+ - Ca^{2+} exchange. However, $[\text{Ca}^{2+}]_i$ then falls to levels which are only slightly elevated above the control. This secondary relaxation of $[\text{Ca}^{2+}]_i$ may reflect the activity of a sarcolemmal Ca^{2+} -ATPase. (3) In the absence of extracellular Na^+ , $[\text{Ca}^{2+}]_i$ can still be regulated and respond to changes in extracellular Ca^{2+} concentration (Lamont & Eisner, 1996) and, again, this may reflect the activity of a sarcolemmal Ca^{2+} -ATPase.

Evidence for the role of sarcolemmal Ca^{2+} -ATPase would be much stronger if there was a specific inhibitor which could be applied to dissect out its contribution. It has been shown that ATPase is specifically inhibited by carboxyeosin (Gatto & Milanik, 1993). In rabbit and ferret ventricular myocytes, carboxyeosin has been used to show that Ca^{2+} -ATPase makes an appreciable contribution to the decay of $[\text{Ca}^{2+}]_i$ during a caffeine-evoked response (Bassani *et al.* 1995).

Using carboxyeosin, in the present paper we show that sarcolemmal Ca^{2+} -ATPase has significant effects on cell function even under physiological conditions. Furthermore it provides the major component of the secondary decay of $[\text{Ca}^{2+}]_i$ following Na^+ removal and is essential for $[\text{Ca}^{2+}]_i$ homeostasis in Na^+ -free solutions.

METHODS

Cell isolation and fluorescence measurement. Ventricular myocytes were isolated from rats which had been killed by cervical dislocation and single ventricular myocytes were isolated as previously described (Eisner *et al.* 1989). Cells were loaded with the acetoxymethyl (AM) ester form of the fluorescent indicator indo-1 in order to measure the intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) as described previously (O'Neill *et al.* 1990).

Carboxyeosin loading. Cells were loaded with 5- (and -6)-carboxyeosin diacetate (succinimidyl ester) from Molecular Probes. A 10 mM stock solution was made in DMSO. This stock solution was added to the control experimental solution (for composition see below) to a final concentration of $20 \mu\text{M}$ and the cells were loaded for 15 min. Cells were then superfused with carboxyeosin-free solution for a further 10 min to allow carboxyeosin de-esterification and to wash off the residual compound in the bath solution. As indicated in the text, in some experiments, recordings were made from the same cell before and after loading with carboxyeosin.

Membrane current measurement. Voltage clamp control was imposed with the perforated patch technique. The suction pipette was filled with (mM): 125 $\text{KCH}_3\text{O}_3\text{S}$, 20 KCl, 12 NaCl, 10 Hepes, 5 MgCl_2 , 0.1 K_2EGTA , adjusted to pH 7.2 with KOH. Five microlitres of amphotericin B (stock 60 mg ml^{-1} in DMSO) was added to 1.25 ml of pipette solution. After forming a seal onto the cell membrane, the membrane potential was held at -80 mV . To compensate for the relatively high access resistance, a switch clamp (Axoclamp-2A, Axon Instruments) was used.

Experimental solutions. The control solution contained (mM): 134 NaCl, 4 KCl, 1 MgCl_2 , 10 Hepes, 11 glucose, 1 CaCl_2 , adjusted to pH 7.4 with NaOH. 4-Aminopyridine (5 mM) and BaCl_2 ($100 \mu\text{M}$) were added to this solution to block K^+ current in perforated patch clamp experiments. The Na^+ -free solution contained (mM): 130 LiCl, 10 Hepes, 11 glucose, 1 MgCl_2 , 4 KCl, 1 CaCl_2 , adjusted to pH 7.4 with KOH. In some experiments a Na^+ -free, Ca^{2+} -free solution was used. In this case Ca^{2+} was removed and EGTA (1 mM) added. All experiments were performed at room temperature (23°C).

Statistics. Statistical comparisons were made using Student's paired *t* test. All data are expressed as means \pm s.e.m. for *n* cells. The rate of decay of $[\text{Ca}^{2+}]_i$ is generally analysed by fitting single exponentials to the indo-1 ratio. This should be regarded as an experimental convenience and not as meaning that the underlying processes are first order.

RESULTS

The effect of carboxyeosin on diastolic and systolic $[\text{Ca}^{2+}]_i$

The first series of experiments was designed to evaluate the role of sarcolemmal Ca^{2+} -ATPase during electrically stimulated contractions. In Fig. 1A, a single rat cardiac myocyte was stimulated every 2 s and $[\text{Ca}^{2+}]_i$ transients were recorded both under control conditions and after loading the cells with $20 \mu\text{M}$ carboxyeosin. Carboxyeosin

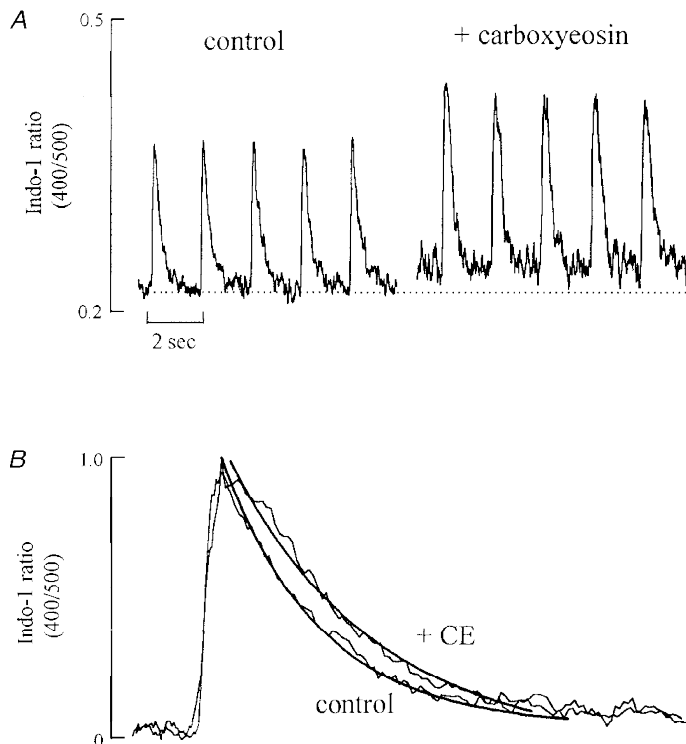


Figure 1. The effect of carboxyeosin on the systolic Ca^{2+} transient

A, specimen records. The cell was stimulated at 0.5 Hz. The first 5 transients were obtained under control conditions. The cell was then exposed to $20 \mu\text{M}$ carboxyeosin for 15 min and, after removing carboxyeosin the remaining Ca^{2+} transients were obtained. The baseline is noisier after loading with carboxyeosin due to a loss of some of the indo-1 signal during the carboxyeosin (CE) loading period.

B, normalized transients obtained from control and after CE loading. In each case 5 transients were averaged. The continuous curves through the data are single exponentials with rate constants of: control, 3.01 s^{-1} ; CE, 2.83 s^{-1} .

Table 1. The effects of carboxyeosin (CE) on the rate constants of decay of systolic and caffeine-activated Ca²⁺ transients

Systolic Ca ²⁺ transient		Caffeine Ca ²⁺ transient		CE-sensitive component		Caffeine-sensitive component		
Control (A)	+ CE (B)	Control (C)	+ CE (D)	Systolic (E) (A - B)	Caffeine (F) (C - D)	Control (G) (A - C)	+ CE (H) (B - D)	(I) (G - H)
2.29 ± 0.18	2.17 ± 0.17	0.313 ± 0.017	0.248 ± 0.017	0.129 ± 0.027	0.065 ± 0.003	1.98 ± 0.18	1.92 ± 0.16	0.06 ± 0.02

All data show the rate constants of decay (in s⁻¹) of the transients. Columns A–D show the rate constants derived directly from the data as indicated. Column E gives the result of subtracting the rate constant of decay of the systolic Ca²⁺ transient in CE from that in its absence. Column F is likewise obtained by subtracting the rate constant of the caffeine-evoked response in CE from that in control. Note that the calculated CE-sensitive component of the systolic decay is greater than that of the caffeine-evoked response (*P* < 0.05; Student's paired *t* test). Column G gives the result of subtracting the rate constant of the caffeine-evoked response from the control and column H gives the result of the same calculation in the presence of CE. Column I gives the difference of the caffeine-sensitive component in control and CE. Note that the calculated caffeine-sensitive component of relaxation is significantly less in CE than control (*P* < 0.05). *n* = 7 cells for all data.

increased both the diastolic and systolic [Ca²⁺]_i. On average the amplitude of the systolic Ca²⁺ transient increased by 21 ± 2% (*n* = 9; *P* < 0.01). Figure 1*B* shows normalized [Ca²⁺]_i transients recorded before and after inhibition of the sarcolemmal Ca²⁺-ATPase by carboxyeosin. The rate constant of decay decreased significantly from 2.42 ± 0.61 to 2.30 ± 0.15 s⁻¹. On average the rate constant in the presence of carboxyeosin was 95.0 ± 0.9% of that in control (*P* < 0.05; *n* = 9).

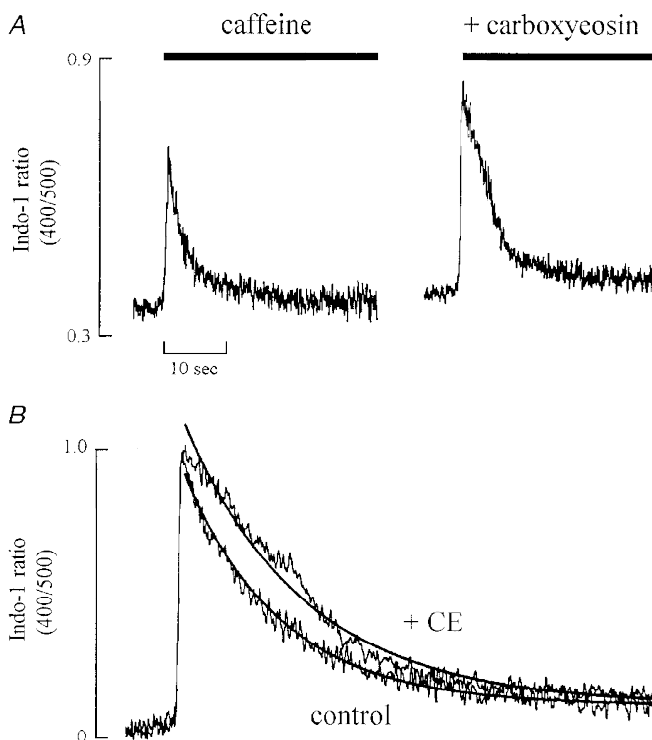
The effect of carboxyeosin on the caffeine-induced calcium transient

The application of caffeine results in an increase in [Ca²⁺]_i which decays over a few seconds (Smith *et al.* 1988). The rate

constant of the decay of this response can be used as a measure of the rate of Ca²⁺ removal from the cytoplasm by processes other than the sarcoplasmic reticulum. To evaluate the fractional contribution of the sarcolemmal Ca²⁺-ATPase to Ca²⁺ removal during this response, we have measured the rate constant of decay of the caffeine-evoked increase in [Ca²⁺]_i before and after inhibition of the sarcolemmal Ca²⁺-ATPase with carboxyeosin. Figure 2*A* shows that carboxyeosin (20 μM) increased the amplitude of the caffeine-induced [Ca²⁺]_i transient. On average the magnitude of the caffeine-evoked response was increased by 27 ± 5% of control (*n* = 9, *P* < 0.01). Figure 2*B* shows the caffeine-evoked responses normalized to the same peak magnitude. Carboxyeosin decreased the rate constant of [Ca²⁺]_i decline

Figure 2. The effect of carboxyeosin on the caffeine-evoked increase in [Ca²⁺]_i

A, original data. Caffeine (10 mM) was applied for the periods shown by the bars. Until 5 s before the application of caffeine, the cell was stimulated at 0.5 Hz. Following the first caffeine-evoked response shown the cell was loaded with carboxyeosin (CE) before obtaining the second response. *B*, normalized records shown on an expanded time scale. The continuous curves through the data are single exponentials with rate constants of: control, 0.31 s⁻¹; CE, 0.25 s⁻¹.



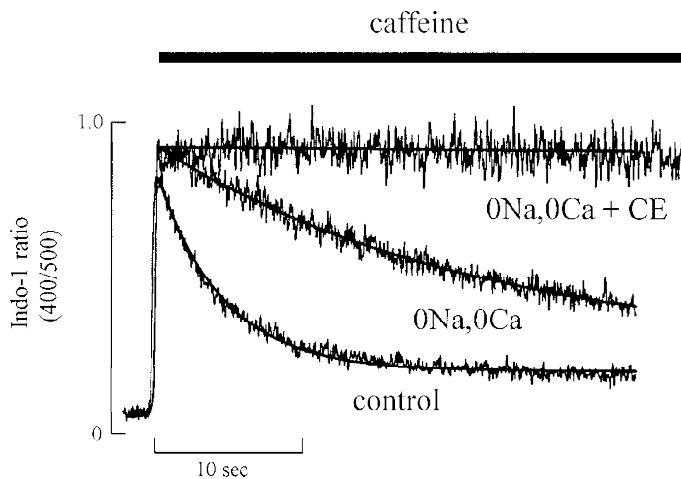


Figure 3. Carboxyeosin (CE) has a greater effect in the absence of Na^+ - Ca^{2+} exchange

Three applications of caffeine (10 mM) (applied for the period shown by the bar) are shown superimposed. Caffeine was applied under the following conditions: control; 0 Na^+ , 0 Ca^{2+} ; 0 Na^+ , 0 Ca^{2+} + CE. The continuous curves through the control and 0 Na^+ , 0 Ca^{2+} data are exponentials with rate constants of 0.36 and 0.079 s^{-1} , respectively.

from 0.31 ± 0.02 to $0.25 \pm 0.01 \text{ s}^{-1}$. On average the rate constant of decay in caffeine in carboxyeosin was $79.8 \pm 0.1\%$ of that in control, a significant slowing ($P < 0.05$; $n = 9$).

If carboxyeosin only affected sarcolemmal Ca^{2+} -ATPase, one might expect that it would produce the same absolute slowing of the systolic Ca^{2+} transient as that of the caffeine-evoked response. The experiments summarized in Table 1 were designed to investigate this directly. They show that carboxyeosin significantly ($P < 0.05$) slowed the rate constant of decay of the systolic Ca^{2+} transient by 0.13 s^{-1} whereas it slowed that of the caffeine-evoked response by a smaller amount (0.065 s^{-1}). Assuming that it is legitimate to subtract rate constants, this suggests that, as well as affecting the sarcolemmal Ca^{2+} -ATPase, carboxyeosin must

be affecting some process which is involved in Ca^{2+} removal from the cytoplasm during the systolic Ca^{2+} transient but not in the caffeine-evoked response, most likely the SR Ca^{2+} -ATPase. Another way to look at this is to measure the caffeine-sensitive component of Ca^{2+} removal: in other words the difference between the rate constant of decay of the systolic Ca^{2+} transient and the caffeine-evoked response. This had a value of 1.98 in the absence and 1.92 in the presence of carboxyeosin. The mean difference in these values (0.064) was significantly different from zero. The implications of this will be considered in Discussion.

The effects of carboxyeosin in Na^+ -free solutions

The effects of carboxyeosin were more prominent if the Na^+ - Ca^{2+} exchange was also inhibited. In the experiment illustrated in Fig. 3, following a control caffeine-evoked

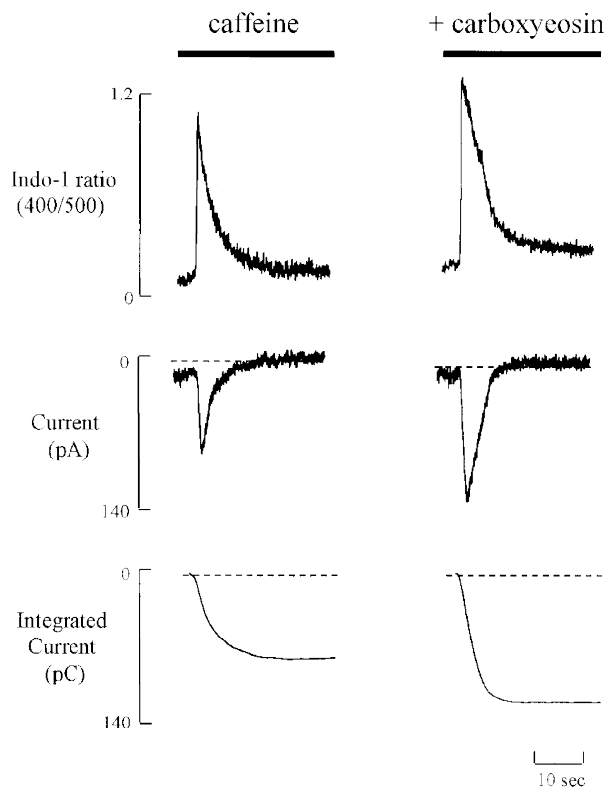


Figure 4. The effect of carboxyeosin on the calcium transient and Na^+ - Ca^{2+} exchange current

Caffeine (10 mM) was applied for the period shown by bars and the calcium transient and Na^+ - Ca^{2+} exchange current were simultaneously measured in perforated-patch clamped single rat ventricular myocyte. The membrane potential was held at -80 mV . The integrated currents before (control, left traces) and after (right traces) the cell was loaded with carboxyeosin were 83 and 119 pC, respectively.

response, Na^+ - Ca^{2+} exchange was inhibited by removing extracellular Na^+ (extracellular Ca^{2+} was also removed in order to prevent the large influx of Ca^{2+} which would otherwise have occurred). This resulted in a slowing of the decay. Finally, the cell was loaded with carboxyeosin and caffeine again applied in the Na^+ -free solution. Under these conditions relaxation did not occur. On average (in 7 cells), the rate of decay of the caffeine-evoked response in Na^+ -free solution was $19.9 \pm 0.5\%$ of that in Na^+ -containing solution whereas the combination of Na^+ -free solution with carboxyeosin decreased the rate to $0.27 \pm 0.09\%$. This suggests that about 80% of Ca^{2+} is removed by Na^+ - Ca^{2+} exchange and the remaining 20% by sarcolemmal Ca^{2+} -ATPase. This is consistent with the result in Fig. 2 in which carboxyeosin alone decreased the rate of $[\text{Ca}^{2+}]_i$ decline during the caffeine-evoked response to 80% of control.

The effects of carboxyeosin on SR Ca^{2+} content

The experiments presented above show that both the systolic Ca^{2+} transient and the Ca^{2+} transient produced by caffeine are increased by carboxyeosin. One possible explanation for this observation is that the SR Ca^{2+} content has increased as a result of the measured rise in diastolic $[\text{Ca}^{2+}]_i$. In order to examine this possibility, we measured the SR Ca^{2+} content by measuring the integral of the Na^+ - Ca^{2+} exchange current during a caffeine-evoked response (Varro *et al.* 1993). The application of caffeine (Fig. 4) results in a transient increase in $[\text{Ca}^{2+}]_i$ which is accompanied by a transient inward Na^+ - Ca^{2+} exchange current. Both the amplitude of the increase in $[\text{Ca}^{2+}]_i$ and of the inward current are increased by exposure to carboxyeosin (Fig. 4). The integrated current is also increased. On average carboxyeosin increased the integrated net current from 87.6 ± 4.8 to 128 ± 7 pC ($n = 8$, $P < 0.05$). This increase is in part due to the fact that, whereas in control only 80% of

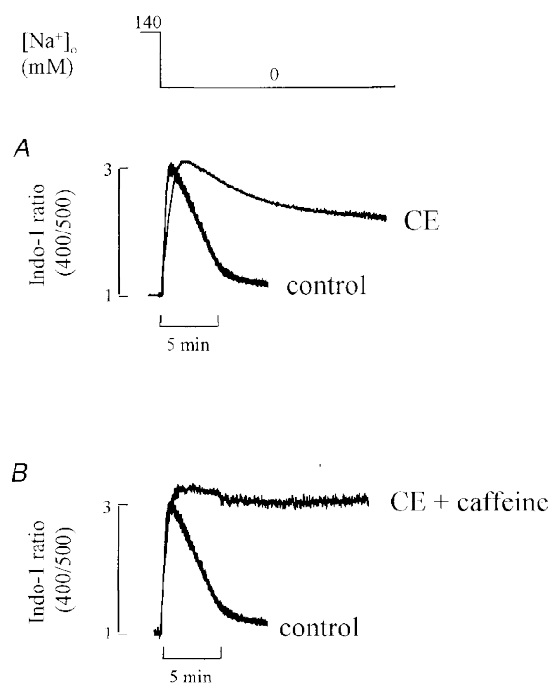
Ca^{2+} is removed by Na^+ - Ca^{2+} exchange (with the remainder being carried by the non-electrogenic Ca^{2+} -ATPase), in the presence of carboxyeosin all the Ca^{2+} is transported by Na^+ - Ca^{2+} exchange. We can correct for this by dividing the control data by 0.8 to estimate the amount of charge which would have been transferred under control conditions if all the Ca^{2+} was removed via Na^+ - Ca^{2+} exchange. This gives a value of 109 ± 6 pC. This is still significantly ($P < 0.05$; Student's paired *t* test) less than the value measured in carboxyeosin. We therefore conclude that carboxyeosin increased the SR Ca^{2+} content.

Regulation of diastolic $[\text{Ca}^{2+}]_i$ by sarcolemmal Ca^{2+} -ATPase in the absence of Na^+ - Ca^{2+} exchange

The experiment in Fig. 1 showed that carboxyeosin increased diastolic $[\text{Ca}^{2+}]_i$. This suggests that sarcolemmal Ca^{2+} -ATPase plays a role in regulating the resting level of $[\text{Ca}^{2+}]_i$. In subsequent experiments we looked directly at the role of Ca^{2+} -ATPase by inhibiting Na^+ - Ca^{2+} exchange. This approach increases the importance of Ca^{2+} -ATPase in Ca^{2+} homeostasis. In the experiment shown in Fig. 5A, extracellular Na^+ was removed first under control conditions and then after loading with carboxyeosin. In agreement with previous work (Allen *et al.* 1984; Kim *et al.* 1987), the Na^+ removal under control conditions displays an initial increase in $[\text{Ca}^{2+}]_i$ which then decays to a lower level which is still greater than the resting level. The initial increase in $[\text{Ca}^{2+}]_i$ is due to Ca^{2+} entry on 'reverse' Na^+ - Ca^{2+} exchange. However, the factors responsible for the subsequent decay of $[\text{Ca}^{2+}]_i$ to and regulation at the steady-state level are uncertain, both mitochondria and sarcolemmal Ca^{2+} -ATPase having been suggested to contribute (Allen *et al.* 1983, 1984; Chapman, 1986; Lamont & Eisner, 1996). We have therefore investigated the possibility that Ca^{2+} -ATPase may play a role in determining both the relaxation of $[\text{Ca}^{2+}]_i$ in Na^+ -free

Figure 5. The effects of carboxyeosin on the Na^+ -withdrawal response

Extracellular Na^+ was removed (replaced by Li^+) for the period shown at the top. Before removing Na^+ , the cell was stimulated at 0.5 Hz. *A*, comparison of Na^+ removal under control conditions with that in the presence of carboxyeosin (CE). *B*, comparison of Na^+ removal under control conditions with that in the combined presence of carboxyeosin and caffeine (10 mM).



solution and the regulation of steady-state $[Ca^{2+}]_i$. Loading with carboxyeosin markedly slowed the rate of $[Ca^{2+}]_i$ decay produced by Na^+ -free solution and resulted in a new steady-state $[Ca^{2+}]_i$ level which was higher than that under control conditions. On average, carboxyeosin increased the half-time of $[Ca^{2+}]_i$ decay from 124 ± 17 to 465 ± 36 s ($n = 13$, $P < 0.05$). This result implies that sarcolemmal Ca^{2+} -ATPase contributes more than 70% to $[Ca^{2+}]_i$ removal showing that it is the main mechanism for Ca^{2+} removal when the Na^+ - Ca^{2+} exchanger is inactive. However, carboxyeosin did not completely abolish $[Ca^{2+}]_i$ decay suggesting that other mechanisms also contribute to Ca^{2+} removal. We tested the possibility that the carboxyeosin-insensitive component of decay of $[Ca^{2+}]_i$ results from uptake of Ca^{2+} into the SR. This was done by applying 10 mM caffeine at the same time as Na^+ removal. As Fig. 5B shows, in the presence of caffeine, carboxyeosin almost abolished $[Ca^{2+}]_i$ decay suggesting that part of the Ca^{2+} can be taken up by the SR while the remainder is removed by the sarcolemmal Ca^{2+} -ATPase.

The effect of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ in the absence of Na^+ - Ca^{2+} exchange

In the experiments described in the previous section, we examined the effects of carboxyeosin on the ability of the cell to recover from increases in $[Ca^{2+}]_i$ produced by extracellular Na^+ removal. In subsequent experiments, we looked at the regulation of steady-state $[Ca^{2+}]_i$ in Na^+ -free solutions and, in particular, at the effects of changing $[Ca^{2+}]_o$. In the absence of extracellular Na^+ , the effect of changing $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ was tested in 11 rat ventricular myocytes before and after inhibition of the sarcolemmal Ca^{2+} -ATPase. To prevent Ca^{2+} influx via Na^+ - Ca^{2+} exchange,

cells were pre-exposed to Ca^{2+} -free solution for 10–30 s before removing extracellular Na^+ and Ca^{2+} . The effects of varying extracellular Ca^{2+} in Na^+ -free solutions are shown in Fig. 6 which shows the change in $[Ca^{2+}]_i$ when $[Ca^{2+}]_o$ was raised to 0.3, 0.6 and 1 mM. Over this range of $[Ca^{2+}]_o$, the greater the $[Ca^{2+}]_o$, the greater $[Ca^{2+}]_i$. As $[Ca^{2+}]_o$ was increased first to 0.3 mM and then to 0.6 mM, oscillations in $[Ca^{2+}]_i$ developed due to release of Ca^{2+} from the SR. Increasing $[Ca^{2+}]_o$ to lower levels produced no measurable increase in $[Ca^{2+}]_i$. This is demonstrated in Fig. 7A. In control (Na^+ -containing) solution changes in $[Ca^{2+}]_o$ had smaller effects on $[Ca^{2+}]_i$ (not shown; cf. Díaz *et al.* 1997). In a Na^+ -free solution, increasing $[Ca^{2+}]_o$ from zero to low levels such as 0.1 mM had no measurable effect on $[Ca^{2+}]_i$. In 21 of 23 cells tested, no measurable increase in $[Ca^{2+}]_i$ was produced by increasing $[Ca^{2+}]_o$ from 0 to 0.1 mM. In contrast, after treatment with carboxyeosin, the same increase in $[Ca^{2+}]_o$ produced a large increase in $[Ca^{2+}]_i$ to a level larger than that of the normal systolic Ca^{2+} transient (Fig. 7A).

To test whether Ca^{2+} release from the SR is responsible for the increase in $[Ca^{2+}]_i$ seen in Fig. 7A similar experiments were performed in the presence of 10 mM caffeine to deplete the SR. The result, illustrated in Fig. 7B, is identical to that in the absence of caffeine. It therefore appears that the changes in $[Ca^{2+}]_i$ produced by changing $[Ca^{2+}]_o$ do not involve significant contributions from the SR.

DISCUSSION

The experiments described in this paper allow quantification of the contribution of sarcolemmal Ca^{2+} -ATPase to Ca^{2+} removal from the cytoplasm under a variety of conditions.

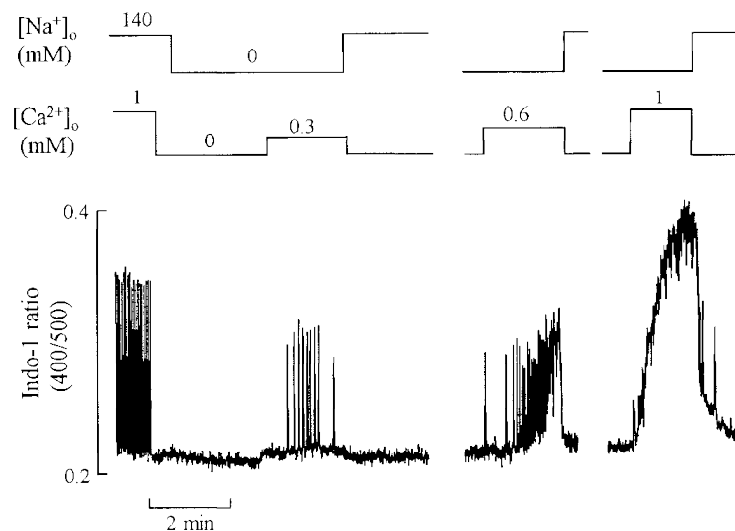


Figure 6. The effects of changing $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ in the absence of Na^+ - Ca^{2+} exchange

The cell was initially stimulated at 0.5 Hz. After stopping stimulation, extracellular Ca^{2+} was removed (1 mM EGTA) for 30 s and then extracellular Na^+ removed (replaced by Li^+) for 2 min. Following this, $[Ca^{2+}]_o$ was increased to 0.3, 0.6 and 1 mM as shown. During the breaks in the record, extracellular Na^+ and Ca^{2+} were removed as shown in the first part of the record.

We have examined the effects of carboxyeosin on the rate of decay of both the caffeine-evoked and the systolic Ca^{2+} transient, as well as on the regulation of diastolic or resting $[\text{Ca}^{2+}]_i$. For simplicity, we will consider the effects on the caffeine-evoked response first.

The effects of carboxyeosin on the caffeine-evoked increase in $[\text{Ca}^{2+}]_i$

Before considering the data, it is important to assess the specificity of carboxyeosin. At concentrations which inhibit sarcolemmal Ca^{2+} -ATPase, carboxyeosin does not inhibit Na^+ - Ca^{2+} exchange (Gatto *et al.* 1994). Furthermore, although carboxyeosin may inhibit the Na^+ - K^+ pump, its inhibition potency is lower than that for sarcolemmal Ca^{2+} -ATPase (Skou & Esmann, 1981; Gatto & Milanik, 1993). It is therefore reasonable to assume that the effects of carboxyeosin on the rate of decay of the caffeine-evoked response are due to inhibition of sarcolemmal Ca^{2+} -ATPase.

The present experiments show that carboxyeosin slows the rate of decay of the caffeine-evoked response by 20%

compared with control. This compares with a reduction of 80% produced by removal of extracellular Na^+ . This is consistent with the hypothesis that the decay of the caffeine-evoked response can be entirely accounted for by two components: (1) a carboxyeosin-sensitive component, presumably sarcolemmal Ca^{2+} -ATPase; (2) Na^+ - Ca^{2+} exchange. This is confirmed by the observation that the rate of decay of the caffeine-evoked response is decreased to less than 1% in the combined presence of both carboxyeosin and a Na^+ -free solution. The fact that relaxation of $[\text{Ca}^{2+}]_i$ is abolished in Na^+ -free solution containing carboxyeosin is inconsistent with a role for mitochondria in Ca^{2+} removal. This result differs from that found in ferret myocytes where, in order to completely inhibit the decay of $[\text{Ca}^{2+}]_i$, mitochondrial Ca^{2+} uptake had to be inhibited, as well as applying carboxyeosin and Na^+ -free solutions (Bassani *et al.* 1995). In summary, the present results suggest that, in rat ventricular myocytes, a carboxyeosin-sensitive sarcolemmal Ca^{2+} -ATPase makes a contribution to Ca^{2+} removal from the cell which is equal to about 25% of that made by Na^+ - Ca^{2+}

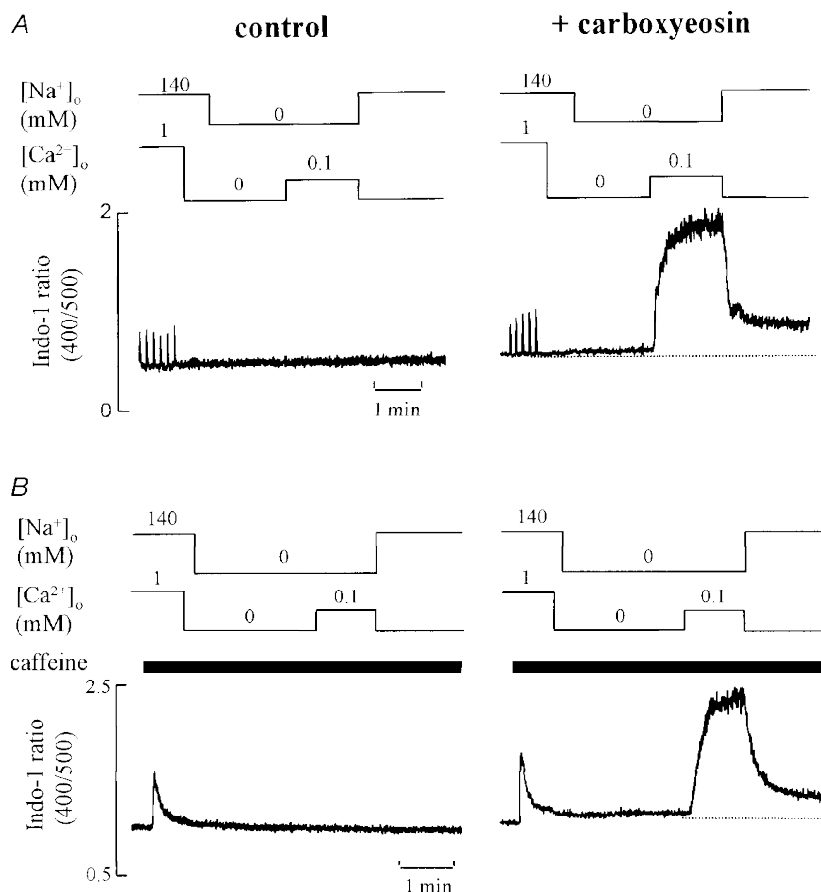


Figure 7. The effects of a small increase in $[\text{Ca}^{2+}]_o$ on $[\text{Ca}^{2+}]_i$ in Na^+ -free solution in the presence and absence of carboxyeosin

A, the cell was initially stimulated at 0.1 Hz. Extracellular Ca^{2+} and Na^+ were removed. Extracellular Ca^{2+} was then increased from 0 to 0.1 mM for the period shown. In the right-hand panel the same protocol was repeated on this cell except that the cell had been loaded with carboxyeosin. *B*, the same protocol as in *A* except that caffeine (10 mM) was applied (for the period shown by the bars) immediately before removing extracellular Ca^{2+} in order to empty the SR Ca^{2+} store.

exchange. The contribution of 80% made by $\text{Na}^+-\text{Ca}^{2+}$ exchange to the decay of $[\text{Ca}^{2+}]_i$ in rat ventricular myocytes is identical to that estimated by us in a previous study (Díaz *et al.* 1997), not very different from the ~87% reported by Bassani *et al.* (1994) but higher than the 66% reported by Varro *et al.* (1993). Presumably the precise contribution depends on the exact experimental conditions.

As well as decreasing the rate of decay of the caffeine-evoked response, carboxyeosin produced a significant increase (27%) in its magnitude. This suggests that it increases the SR Ca^{2+} content. This would be expected if carboxyeosin has inhibited one of the mechanisms of Ca^{2+} removal from the cell. This will have two effects on SR Ca^{2+} content. (1) It will increase resting $[\text{Ca}^{2+}]_i$ (as shown directly in Fig. 1) thereby increasing the activation of SR Ca^{2+} -ATPase and hence the SR Ca^{2+} content. (2) During the systolic Ca^{2+} transient, SR Ca^{2+} -ATPase effectively competes with the combined effects of the sarcolemmal $\text{Na}^+-\text{Ca}^{2+}$ exchange and Ca^{2+} -ATPase. Inhibition of the sarcolemmal Ca^{2+} -ATPase will therefore result in a larger fraction of the Ca^{2+} being taken back into the SR thereby also increasing the SR Ca^{2+} content. In other experiments we measured the SR Ca^{2+} content directly by integrating the caffeine-evoked $\text{Na}^+-\text{Ca}^{2+}$ exchange current. This integral increased by 46%. As mentioned above, part of this increase in integral is due to the fact that, whereas under control conditions only 80% of the Ca^{2+} is removed from the cell by $\text{Na}^+-\text{Ca}^{2+}$ exchange (with the remainder being pumped by Ca^{2+} -ATPase and not therefore producing current), in the presence of carboxyeosin all the Ca^{2+} is removed by $\text{Na}^+-\text{Ca}^{2+}$ exchange. However, even after this is corrected for, we still find a 17% increase in SR Ca^{2+} content. This result suggests that, in the intact cell, sarcolemmal Ca^{2+} -ATPase acts to decrease SR Ca^{2+} content.

The effects of carboxyeosin on the systolic Ca^{2+} transient

We found that carboxyeosin increased the magnitude of the systolic Ca^{2+} transient to 120% of its control value. This presumably results from the increase in SR Ca^{2+} content suggested by the increase of the caffeine-evoked response. In ferret cells no significant effect was found on the magnitude of the systolic Ca^{2+} (Bassani *et al.* 1995). In addition carboxyeosin decreased the rate constant of decay of the Ca^{2+} transient by about 5% similar to the effect in ferret cells (Bassani *et al.* 1995). In the ferret cells this slowing of the rate of decay was tentatively attributed to inhibition of SR Ca^{2+} -ATPase by carboxyeosin. The present data allow us to distinguish the idea that the slowing results from (i) inhibition of the sarcolemmal Ca^{2+} -ATPase *versus* (ii) an effect on the SR Ca^{2+} -ATPase. The simplest explanation of this slowing of the decay is that it represents the contribution of the sarcolemmal Ca^{2+} -ATPase to normal relaxation. In relation to Table 1, we have produced two arguments suggesting that the situation may be more complicated. (1) The slowing of the systolic Ca^{2+} transient

produced by CE is more than would be expected from the contribution of the sarcolemmal Ca^{2+} -ATPase as assessed by the effects of CE on the caffeine-evoked response. (2) The caffeine-sensitive component of decay of the systolic Ca^{2+} transient is affected by CE. The slowing of the caffeine-sensitive component could be due to either (i) a direct effect of carboxyeosin on the SR Ca^{2+} -ATPase or (ii) an effect secondary to the increase in SR Ca^{2+} content. The present data do not allow us to discriminate between these alternatives. It should, however, also be noted that the above arguments depend on subtracting the rate constant of decay of the indo-1 ratio obtained in one condition from that in another. This is tantamount to assuming that Ca^{2+} is controlled by independent, first-order mechanisms (and also to ignoring any non-linearity between $[\text{Ca}^{2+}]_i$ and the indo-1 ratio). As far as the use of carboxyeosin is concerned, it should be noted that any effect on SR Ca^{2+} -ATPase is only a very small fraction and will be insignificant in many experimental purposes. For example, the slowing of the caffeine-sensitive component by CE is 0.06 s^{-1} compared with a mean initial value of the caffeine-sensitive component of 1.98 s^{-1} . This is therefore only a 3% effect.

The role of sarcolemmal Ca^{2+} -ATPase in recovery of $[\text{Ca}^{2+}]_i$ during the Na^+ -withdrawal response

Previous work (Allen *et al.* 1983) has shown that, when $\text{Na}^+-\text{Ca}^{2+}$ exchange is inhibited, resting $[\text{Ca}^{2+}]_i$ is still regulated but at a higher level than normal. This suggests that the other mechanisms, such as sarcolemmal Ca^{2+} -ATPase, by themselves are incapable of keeping resting $[\text{Ca}^{2+}]_i$ at the normal level. In the present work, we have been able to inhibit Ca^{2+} -ATPase to ask the converse question. Can $\text{Na}^+-\text{Ca}^{2+}$ exchange alone maintain the low resting $[\text{Ca}^{2+}]_i$? The data show that carboxyeosin increases resting $[\text{Ca}^{2+}]_i$ and that, therefore, in addition to $\text{Na}^+-\text{Ca}^{2+}$ exchange, a functional sarcolemmal Ca^{2+} -ATPase is required to maintain resting $[\text{Ca}^{2+}]_i$ at the normal low resting level. Previous work on rabbit and ferret myocytes found no effect of carboxyeosin on resting $[\text{Ca}^{2+}]_i$ (Bassani *et al.* 1995). It is possible that the greater effect in the rat reflects the fact that $\text{Na}^+-\text{Ca}^{2+}$ exchange plays a larger role than does the sarcolemmal Ca^{2+} -ATPase in Ca^{2+} extrusion in these species compared with the rat (Bassani *et al.* 1994).

Removal of extracellular Na^+ results in entry of Ca^{2+} into the cell on reverse $\text{Na}^+-\text{Ca}^{2+}$ exchange (accompanied by Na^+ efflux). This results in a large increase in $[\text{Ca}^{2+}]_i$ which then decays to a steady-state level (Fig. 5). This spontaneous decay of $[\text{Ca}^{2+}]_i$ suggests a significant contribution of other mechanisms to Ca^{2+} removal when the $\text{Na}^+-\text{Ca}^{2+}$ exchange is inhibited. Previous studies have shown that the recovery of $[\text{Ca}^{2+}]_i$ during Na^+ withdrawal is an energy-dependent process (Allen *et al.* 1983) but could not distinguish between the effects of mitochondrial Ca^{2+} sequestration and sarcolemmal Ca^{2+} -ATPase. Using carboxyeosin, we have shown that sarcolemmal Ca^{2+} -ATPase is the major mechanism

responsible for restoring diastolic $[\text{Ca}^{2+}]_i$ to a relatively low level when the Na^+ - Ca^{2+} exchange is inhibited. As seen in Fig. 6A, when sarcolemmal Ca^{2+} -ATPase is inhibited by carboxyeosin, the half-time of $[\text{Ca}^{2+}]_i$ decay is prolonged approximately fourfold. In other words, more than 70% of the Ca^{2+} is removed from the cytoplasm by the sarcolemmal Ca^{2+} -ATPase. The fact that the remaining decrease in Ca^{2+} is inhibited by caffeine suggests that it results from uptake by the SR. It is worth noting that the caffeine-sensitive fraction of recovery of $[\text{Ca}^{2+}]_i$ is only about 50% of that due to the sarcolemmal Ca^{2+} -ATPase showing that, under these conditions, sarcolemmal Ca^{2+} -ATPase is more effective than is accumulation by the SR. This probably reflects the fact that the SR Ca^{2+} content is near the maximum level and therefore the ability of the SR to accumulate Ca^{2+} will be much diminished compared with that during systole. This inhibition of $[\text{Ca}^{2+}]_i$ decay by carboxyeosin emphasizes the important role of sarcolemmal Ca^{2+} -ATPase in the control of diastolic $[\text{Ca}^{2+}]_i$ at least when Na^+ - Ca^{2+} exchange is inhibited. The abolition of $[\text{Ca}^{2+}]_i$ decay by carboxyeosin plus caffeine during the Na^+ withdrawal response emphasizes, again, that mitochondria make little contribution to Ca^{2+} removal in rat ventricular myocytes.

The role of sarcolemmal Ca^{2+} -ATPase in the control of resting $[\text{Ca}^{2+}]_i$

The work described above has examined the role of sarcolemmal Ca^{2+} -ATPase and other mechanisms in dealing with increases in $[\text{Ca}^{2+}]_i$ produced either by electrical stimulation or caffeine. Other experiments showed the role of Ca^{2+} -ATPase in the regulation of resting $[\text{Ca}^{2+}]_i$. When extracellular Ca^{2+} was increased over a low concentration range, resting Ca^{2+} could be controlled at approximately normal levels even in the absence of extracellular Na^+ (i.e. Na^+ - Ca^{2+} exchange inhibited). If, however, the sarcolemmal Ca^{2+} -ATPase was also inhibited then even modest increases in $[\text{Ca}^{2+}]_o$ (from 0 to 0.1 mM) resulted in large increases in resting $[\text{Ca}^{2+}]_i$. This result shows that Ca^{2+} -ATPase alone can regulate $[\text{Ca}^{2+}]_i$ at a steady level. The experiments in Na^+ -free solutions identify Ca^{2+} -ATPase as the mechanism responsible for removing Ca^{2+} . As mentioned in the Introduction, it has been suggested that Ca^{2+} -ATPase is a low capacity, high affinity system responsible more for the control of resting $[\text{Ca}^{2+}]_i$ than for removing large Ca^{2+} loads. However, we have shown previously that the relative contribution of the two Ca^{2+} extrusion systems, i.e. sarcolemmal Ca^{2+} -ATPase and Na^+ - Ca^{2+} exchange, does not change as $[\text{Ca}^{2+}]_i$ declines (Lamont & Eisner, 1996). In agreement with this, inhibition of sarcolemmal Ca^{2+} -ATPase with carboxyeosin simply decreases the rate constant of decay of the exponential fall of $[\text{Ca}^{2+}]_i$. In contrast, if Ca^{2+} -ATPase was most important at low $[\text{Ca}^{2+}]_i$ one would expect the initial part of the decay of a caffeine-evoked response to carboxyeosin to be less sensitive than the final component.

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