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Ca^{2+} ENTRY THROUGH Na^+–Ca^{2+} EXCHANGE CAN TRIGGER Ca^{2+} RELEASE FROM Ca^{2+} STORES IN Na^+-LOADED GUINEA-PIG CORONARY MYOCYTES

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SUMMARY

1. The ionized cytosolic calcium concentration ($[Ca^{2+}]_i$) was monitored in voltage-clamped coronary myocytes at 36 °C and 2·5 mM $[Ca^{2+}]_o$ using the Ca^{2+} indicator indo-1. $[Ca^{2+}]_i$ was transiently increased by fast application of 10 mM caffeine, and the mechanisms involved in decay of $[Ca^{2+}]_i$ were analysed.

2. Resting $[Ca^{2+}]_i$ was 166 ± 62 nM (mean ± S.D.). Caffeine increased $[Ca^{2+}]_i$ within 1–2 s to 1618 ± 490 nM. In the continuous presence of caffeine $[Ca^{2+}]_i$ fell close to resting values with a half-decay time of $5{\cdot}0 \pm 1{\cdot}6$ s. Wash-out of caffeine induced an undershoot of $[Ca^{2+}]_i$ to 105 ± 30 nM. When caffeine was applied repetitively the $[Ca^{2+}]_i$ transients were of reduced amplitude indicating that the store had lost a part of releasable Ca^{2+}.

3. After a 1 s caffeine application $[Ca^{2+}]_i$ decayed with a half-time of $2{\cdot}3 \pm 0{\cdot}8$ s to the undershoot of 112 ± 57 nM. The decay of $[Ca^{2+}]_i$ was largely prevented by 3 mM $[La^{3+}]_o$; after wash-out of La^{3+} $[Ca^{2+}]_i$ fell to the resting value without an undershoot. The results demonstrate that La^{3+}-sensitive Ca^{2+} extrusion contributes to the decay of the $[Ca^{2+}]_i$ transient and to the undershoot.

4. With 10 mM $[Na^+]_i$, sodium removal from the bath incremented $[Ca^{2+}]_i$ in three out of ten cells by 71 ± 11 nM; in the other cells $[Ca^{2+}]_i$ did not change. In the absence of extracellular sodium the decay of $[Ca^{2+}]_i$ after wash-out of caffeine was not retarded.

5. To stimulate Na^+–Ca^{2+} exchange, cells were dialysed with pipette solution containing 150 mM NaCl. Elevation of $[Na^+]_i$ had no significant effect on the resting $[Ca^{2+}]_i$ (180 ± 47 nM) or on the caffeine-induced $[Ca^{2+}]_i$ transients (peak 1614 ± 530 nM, half-time of decay 3 s, undershoot 107 ± 40 nM).

6. With 150 mM $[Na^+]_i$, sodium removal resulted in an increase of $[Ca^{2+}]_i$, although responses varied in amplitude (from 130 to 2300 nM) and rate of rise. In the absence of sodium $[Ca^{2+}]_i$ remained elevated. After a 1 s caffeine application the undershoot of $[Ca^{2+}]_i$ was abolished in sodium-free solution. When caffeine was applied in sodium-free solution, the $[Ca^{2+}]_i$ transient decayed to a sustained level and the following caffeine response was attenuated.

7. With 150 mM $[Na^+]_i$, the effects of sodium removal were strongly suppressed by a preceding depletion of the Ca^{2+} stores with caffeine. Ryanodine pretreatment

abolished the caffeine-induced $[Ca^{2+}]_i$ transients and reduced $[Ca^{2+}]_i$ response due to sodium removal. The ryanodine-insensitive component of the $[Ca^{2+}]_i$ transient may originate directly from Ca^{2+} influx through Na^+–Ca^{2+} exchange, the ryanodine-sensitive one from sarcoplasmic reticulum (SR) Ca^{2+} release triggered by Ca^{2+} influx through Na^+–Ca^{2+} exchange.

8. The results suggest that the Na^+–Ca^{2+} exchanger is of minor importance for the $[Ca^{2+}]_i$ transients of guinea-pig coronary myocytes as long as $[Na^+]_i$ is at the physiological level of 10 mM. When stimulated by 150 mM $[Na^+]_i$, Ca^{2+} influx through Na^+–Ca^{2+} exchange can trigger Ca^{2+} release from the SR which, thus, amplifies the Ca^{2+} signals according to the SR Ca^{2+} load.

INTRODUCTION

A steady balance of cellular calcium requires that the Ca^{2+} that enters from extracellular space is extruded again. In vascular myocytes, Ca^{2+} is extruded against a large electrochemical gradient by two Ca^{2+} mechanisms; by a plasmalemmal ATP-driven calmodulin-regulated Ca^{2+}-ATPase (PMCa, Missiaen *et al.* 1992; for coronary muscle, Grover & Samson, 1986) and by an electrogenic Na^+–Ca^{2+} exchanger (Sheu & Blaustein, 1992). Whereas the Ca^{2+} extrusion through the Na^+–Ca^{2+} exchange has been well established for cardiac myocytes, in vascular myocytes its functional role is much less clear (Sheu & Blaustein, 1992).

The importance of Na^+–Ca^{2+} exchange for vascular smooth muscle was suggested by the early studies of Reuter, Blaustein & Haeusler (1973) who reported tonic contractions upon removal of sodium from the superfusate. However, it could not be ruled out that sodium removal resulted in a membrane depolarization activating Ca^{2+} entry through potential-dependent Ca^{2+} channels (van Breemen, Aaronson & Loutzenheiser, 1979) or in a presynaptic release of a neurotransmitter that induced Ca^{2+} influx or Ca^{2+} release (Rembold, Richard & Chen, 1992). An additional problem may arise from the different types of smooth muscle tissue. Na^+–Ca^{2+} exchange has been shown to modify force and $[Ca^{2+}]_i$ in aortic tissue. However, sodium removal was without effect on force or $^{45}Ca^{2+}$ efflux in the coronary arteries (Ito, Kitamura & Kuriyama, 1979; Ito, Kajiwara, Kitamura & Kuriyama, 1982).

Some of the experimental problems can be overcome when $[Ca^{2+}]_i$ is measured by microfluometry in isolated myocytes (myocytes cultured from aorta: Vigne, Breittmayer, Duval, Frelin & Lazdunski, 1988; Blaustein *et al.* 1991). A further advance was achieved when $[Ca^{2+}]_i$ was measured in the same cell that was voltage clamped; the results of those studies provided convincing evidence for electrogenic Na^+–Ca^{2+} exchange for myocytes isolated from ureter (Aaronson & Benham, 1989) but not from guinea-pig urinary bladder (Ganitkevich & Isenberg, 1991). Thus, it seems that the importance of Na^+–Ca^{2+} exchange for the cellular Ca^{2+} balance cannot be extrapolated and has to be evaluated in each respective tissue.

The ideas about the functional role of the Na^+–Ca^{2+} exchanger in vascular tissues have changed over the years (Sheu & Blaustein, 1992). Initially, it was proposed that sodium removal activates contraction directly due to Ca^{2+} influx through the Na^+–Ca^{2+} exchanger. Since sodium removal did not always activate contraction, Ca^{2+} inflowing through Na^+–Ca^{2+} exchange was suggested to be sequestered into the sarcoplasmic reticulum (SR), the greater extent of SR Ca^{2+} load providing a more

intense release of activator Ca^{2+} during application of an agonist (e.g. angiotensin) or caffeine (Ashida & Blaustein, 1987). The hypothesis of SR Ca^{2+} load being controlled by Na^+–Ca^{2+} exchange is tested in this study with caffeine as a tool to release Ca^{2+}.

It has been suggested that the functional importance of Na^+–Ca^{2+} exchange increases with the elevation of $[Na^+]_i$ and many experiments were done at elevated $[Na^+]_i$, i.e. after inhibition of the Na^+,K^+-ATPase with ouabain (Blaustein *et al.* 1991; Mulvany, Aalkjaer & Jensen, 1991). Thus, in this study the isolated myocytes were dialysed with solutions containing 150 mM $[Na^+]_i$ in order to saturate the intracellular Na^+ binding sites of the Na^+–Ca^{2+} exchanger (dissociation constant (K_D) = 28 mM, Smith, Lyu & Smith, 1991).

There are only a few reports where $[Ca^{2+}]_i$ was measured in single vascular myocytes (Benham, 1989; Stehno-Bittel & Sturek, 1992); thus, evaluation of the changes in $[Ca^{2+}]_i$ induced by caffeine is required. The $[Ca^{2+}]_i$ measurements were performed at a constant holding potential of −50 mV which is close to the resting membrane potential of these cells (Kitamura & Kuriyama, 1979). A possible depolarization and activation of Ca^{2+} influx through L-type Ca^{2+} channels was, thus, prevented in the present experiments. The results suggested that the caffeine-sensitive intracellular Ca^{2+} stores can modify the effects of Ca^{2+} influx through Na^+–Ca^{2+} exchange on $[Ca^{2+}]_i$ either by amplifying it by Ca^{2+} release or reducing it by Ca^{2+} sequestration.

METHODS

Adult guinea-pigs (300–500 g) were killed by cervical dislocation, and the heart was then removed. The methods of cell isolation and recording of whole-cell membrane currents have been published before (Ganitkevich & Isenberg, 1990). Briefly, cells were voltage clamped with patch electrodes with resistances of 3–5 MΩ. Whole-cell membrane currents were measured with an RK-300 amplifier (Biologic, Echirolles, France), filtered at 1 kHz and stored on an IBM-compatible host computer. Following the establishment of the whole-cell mode, at least 2 min of loading with 100 μM indo-1 was allowed before starting the experiment. For microfluospectroscopy, the cells were illuminated at 340 nm through a 100 × oil immersion objective (Nikon, fluor) with a 75 W xenon lamp. Emitted light in bands from 395 to 425 nm and 450 to 490 nm was collected and amplified by a pair of photomultipliers (Hamamatsu Photonics, Japan). After filtering at 20 Hz the fluorescence ratio 410/470 was delivered on-line by an analog divider (Burr Brown DIV100). The background fluorescence was subtracted electronically in the cell-attached mode. $[Ca^{2+}]_i$ was evaluated off-line, using the intracellular calibration procedure described previously (Ganitkevich & Isenberg, 1991). The results are presented as both fluorescent ratio (410/470) and calibrated $[Ca^{2+}]_i$.

The myocytes were continuously superfused with a physiological salt solution preheated to 36 °C, composed of (mM): 150 NaCl, 2·5 $CaCl_2$, 1·2 $MgCl_2$, 5·4 KCl, 20 glucose, 5 Hepes, adjusted with NaOH to pH 7·4. Na^+-free extracellular solution contained 150 mM Li^+ or 150 mM *N*-methyl-glucosamine$^+$ (NMG^+) instead of 150 mM Na^+ and pH was adjusted with KOH. No differences were observed between effects of Li^+ and NMG^+ as sodium substitutes. The actual cation substituting Na^+ is mentioned in the legend to each figure. The pipettes were filled with an intracellular solution containing (mM): 140 KCl, 2 Na_2ATP, 3 $MgCl_2$, 10 Hepes, 0·1 indo-1 (pentapotassium salt), adjusted with NaOH to pH 7·2. Final sodium concentration was approximately 10 mM. In a series of experiments, intracellular solution contained 140 mM NaCl instead of 140 mM KCl; final sodium concentration was approximately 150 mM. The method of the fast caffeine application (Ganitkevich & Isenberg, 1992) could complete an extracellular solution change within approximately 0·5 s.

The myocytes from the coronary artery have a small volume. The advantage of this is that cell dialysis was fast (*ca* 3 min as indicated by experiments with a reversed K^+ gradient). Also, the speed of indo-1 loading allowed measurements after a 2 min period of loading. A problem of the small cells, however, was the run-down of the $[Ca^{2+}]_i$ transients which limited the experimental

time to a 5 min period; probably, during this time, some unknown constituents of the cell were washed out.

All experiments were performed at 36 °C. When appropriate, the results are expressed as means ± S.D. of the mean. Statistical significance was evaluated with Student's paired t test ($P \leqslant 0{\cdot}05$).

RESULTS

Resting $[Ca^{2+}]_i$

On average, $[Ca^{2+}]_i$ was 166 ± 62 nM ($n = 25$) when measured at a holding potential of -50 mV 3–4 min after the start of cell dialysis with the electrode solution containing 140 mM KCl, 10 mM NaCl and 100 μM indo-1. More negative holding potentials (-60 or -90 mV) did not modify $[Ca^{2+}]_i$. Eight cells showed an initial $[Ca^{2+}]_i$ of approximately 300 nM; however, $[Ca^{2+}]_i$ fell within a further 2 min to the above value at which time the experiments were started. At the holding potential of -50 mV, spontaneous transient outward currents (STOCs) of variable amplitude and frequency were usually recorded. Changes in $[Ca^{2+}]_i$ related to the STOCs will be considered elsewhere.

Caffeine-induced $[Ca^{2+}]_i$ *transients*

Fast application of 10 mM caffeine increased $[Ca^{2+}]_i$ within 1–2 s to 1618 ± 490 nM ($n = 25$). In the constant presence of caffeine, $[Ca^{2+}]_i$ fell; the half-decay time was $5{\cdot}0 \pm 1{\cdot}6$ s on average (range 4–8 s, $n = 17$). Within less than 30 s $[Ca^{2+}]_i$ returned close to the resting level. When caffeine was washed out, $[Ca^{2+}]_i$ always fell below the resting value, i.e. undershoot of $[Ca^{2+}]_i$ occurred. The $[Ca^{2+}]_i$ during this undershoot was 105 ± 60 nM ($n = 14$).

Figure 1 shows a typical caffeine-induced $[Ca^{2+}]_i$ transient. Upon application of caffeine $[Ca^{2+}]_i$ increased from the resting level of 180 nM to the peak of 1550 nM which was reached within 2 s. Despite the continuous presence of caffeine, $[Ca^{2+}]_i$ decayed, the half-time of the decay being 5 s. When caffeine was washed out, $[Ca^{2+}]_i$ fell to an undershoot of 80 nM.

The lower panel shows the changes in membrane current induced by caffeine. Before application, there were irregular STOCs, which were abolished during and after the caffeine exposure. During the $[Ca^{2+}]_i$ transient, an outward calcium-activated K^+ current ($I_{K,Ca}$) appeared at the holding potential of -50 mV. $I_{K,Ca}$ was transient and disappeared within 4 s. The time course of $I_{K,Ca}$ was always faster than the time course of the $[Ca^{2+}]_i$ transient. We attribute the outward current to the Ca^{2+} activation of plasmalemmal Ca^{2+}-activated K^+ channels and the difference in the time course of $I_{K,Ca}$ and $[Ca^{2+}]_i$ to a dissociation between the subsarcolemmal and global $[Ca^{2+}]_i$. Details of such a comparison will be considered elsewhere.

In the absence of caffeine $[Ca^{2+}]_i$ decayed faster than in its continuous presence (Fig. 2*A*). When caffeine was applied for *ca* 1 s $[Ca^{2+}]_i$ decayed from its peak with a half-time of $2{\cdot}3 \pm 0{\cdot}8$ s (range 1–3 s, $n = 28$). The undershoot of $[Ca^{2+}]_i$ after a 1 s caffeine exposure (112 ± 57 nM) was not significantly different from the undershoot after a long caffeine exposure.

Caffeine deprives the store of releasable Ca^{2+}

Figure 2*A* shows the experiment when caffeine was applied three times to the same cell. The first 1 s application induced a $[Ca^{2+}]_i$ transient which peaked to 1290 nM.

After 20 s caffeine was applied for a second time. The second $[Ca^{2+}]_i$ transient peaked to 730 nM or *ca* 60% of the first response. This suggests that 60% of the Ca^{2+} available for the first $[Ca^{2+}]_i$ transient was taken back into the caffeine-sensitive store and that the other 40% was extruded into the extracellular space. This idea is

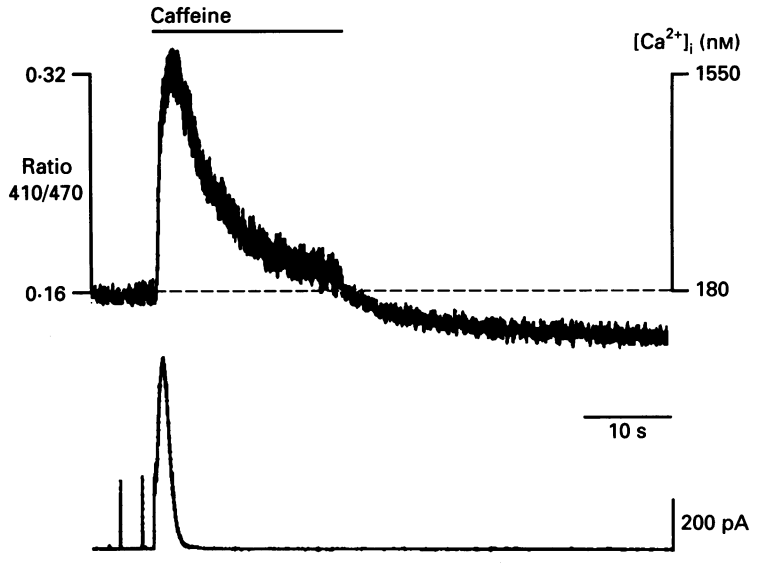

Fig. 1. Caffeine-induced increase in $[Ca^{2+}]_i$ (upper trace) and Ca^{2+}-activated K^+ current ($I_{K,Ca}$, lower trace). Caffeine application (10 mM) for 20 s induced an $I_{K,Ca}$ that peaked within less than 1 s and fell within 4 s. It also induced a transient rise of $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ transient, from 180 to 1550 nM) that peaked within 2 s and then decayed. Upon wash-out of caffeine, $[Ca^{2+}]_i$ fell to an undershoot of 80 nM. The duration of caffeine application is indicated above the traces. Note the on-line pen-recording shows the ratio of fluorescence at 410 to 470 nm, thus the $[Ca^{2+}]_i$ scale is non-linear. The enhanced noise of the $[Ca^{2+}]_i$ trace is due to quenching of indo-1 fluorescence by caffeine.

supported by the results obtained in the continuous presence of caffeine. Figure 2*A* (second exposure) shows that the $[Ca^{2+}]_i$ transient fell with a half-decay time of approximately 4 s. After a 10 s exposure time caffeine was washed out, and an undershoot to 80 nM developed. The third caffeine application induced a $[Ca^{2+}]_i$ transient that peaked to 350 nM as if the preceding long caffeine exposure had reduced the releasable Ca^{2+} to *ca* 20% of the Ca^{2+} that was available for the first response to caffeine.

The idea that the continuous presence of caffeine depletes the store is supported by the results of Fig. 2*B*. The first 1 s caffeine application induced a transient which peaked from 190 to 1820 nM. After caffeine wash-out $[Ca^{2+}]_i$ fell with a half-time of *ca* 2 s. After 8 s caffeine was applied a second time; the second $[Ca^{2+}]_i$ transient peaked to 860 nM (*ca* 50% of the first). In the continuous presence of caffeine, $[Ca^{2+}]_i$ decayed with a half-time of 5 s. Upon wash-out, $[Ca^{2+}]_i$ fell to an undershoot of 90 nM. After a further 6 s, caffeine was applied for the third time, and $[Ca^{2+}]_i$ peaked to 290 nM only (*ca* 15%).

The results suggest that the Ca^{2+} stores were largely Ca^{2+} depleted when $[Ca^{2+}]_i$ decayed in the presence of caffeine but only partially deprived when $[Ca^{2+}]_i$ fell in the

absence of caffeine. The finding suggests that Ca^{2+} reuptake and Ca^{2+} extrusion can compete for the released Ca^{2+}. In the continuous presence of caffeine the SR is leaky, Ca^{2+} extrusion is favoured and the extruded Ca^{2+} is lost for the subsequent Ca^{2+} release.

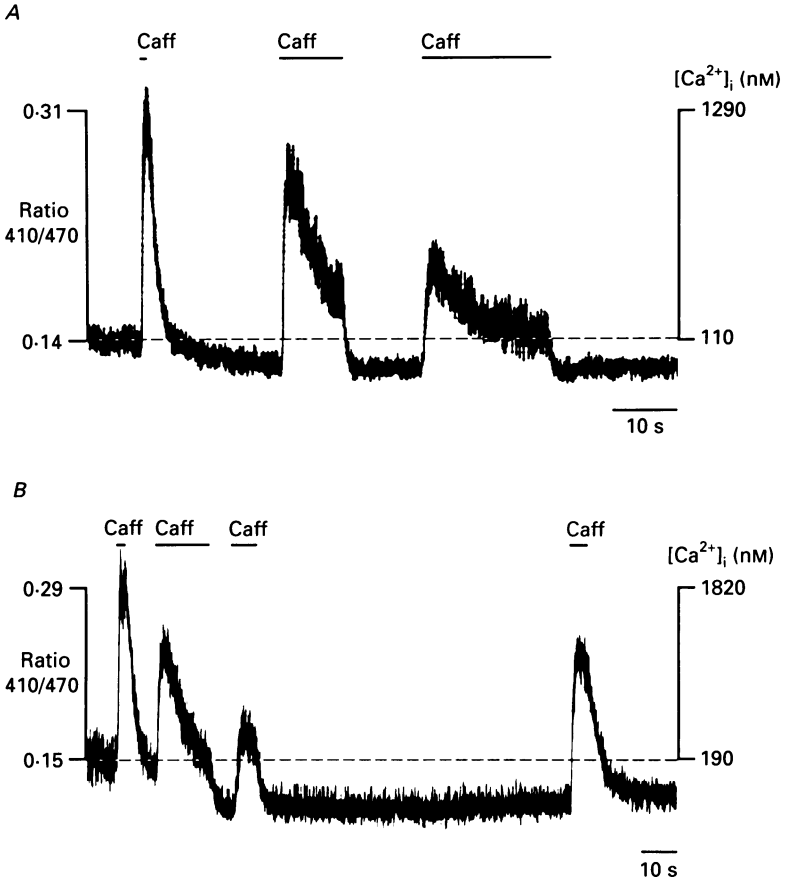

Fig. 2. Caffeine deprives the store of releasable Ca^{2+}. *A*, upon repetitive caffeine (Caff) applications, the peak of the $[Ca^{2+}]_i$ transient becomes attenuated. The attenuation is larger after long caffeine exposures than after short ones. *B*, after an 80 s rest period, the peak of the caffeine-induced $[Ca^{2+}]_i$ transient partially recovered, indicating refilling of the Ca^{2+} store. The caffeine was applied for the time indicated above each trace.

The undershoot of $[Ca^{2+}]_i$ after wash-out of caffeine

When caffeine was washed out after a long exposure time, $[Ca^{2+}]_i$ fell to values lower than the resting level. This undershoot was similar to that described recently for urinary bladder myocytes where it had been attributed to the low intraluminal $[Ca^{2+}]$ stimulating the reuptake of Ca^{2+} by Ca^{2+}-ATPase in the sarcoplasmic reticulum (SERCa) (Ganitkevich & Isenberg, 1992). In coronary myocytes the $[Ca^{2+}]_i$ transients triggered by depolarization and I_{Ca} were small, and the decay rate of depolarization-induced $[Ca^{2+}]_i$ transients could not be used to demonstrate that stimulated Ca^{2+} reuptake through SERCa is responsible for the undershoot.

Both undershoot and the amplitude of caffeine-induced $[Ca^{2+}]_i$ transients (i.e. the amount of releasable Ca^{2+}) slowly recovered when caffeine was not applied for longer periods of time. When 80 s after the third caffeine-induced $[Ca^{2+}]_i$ transient, the caffeine was applied again (Fig. 2*B*), it induced a $[Ca^{2+}]_i$ transient with a peak that had recovered from 290 to 750 nM, but was still smaller than the first one (1820 nM). Complete restoration of the amount of releasable Ca^{2+} was not observed within a 4 min period. Restoration was most probably superimposed on the development of run-down phenomena. Recovery of the caffeine transients was not facilitated by more negative holding potentials (−90 mV), suggesting the functional absence of sarcolemmal Ca^{2+} channels controlled by SR Ca^{2+} load (Pacaud & Bolton, 1991) in this preparation.

Modification of the $[Ca^{2+}]_i$ decay by lanthanum

Lanthanum (La^{3+}) is known to block the plasmalemmal Ca^{2+} fluxes. Thus, it was used to evaluate the importance of Ca^{2+} extrusion for the decay of the caffeine-induced $[Ca^{2+}]_i$ transients. Unfortunately, solution containing both 10 mM caffeine and 3 mM La^{3+} precipitated, thus La^{3+} was applied during the wash-out of caffeine. With the assumption that La^{3+} blocked the transmembrane Ca^{2+} movement, the decay of $[Ca^{2+}]_i$ should reflect the Ca^{2+} reuptake by SERCa.

In the experiment shown in Fig. 3*A* the caffeine-induced $[Ca^{2+}]_i$ transient peaked within 1 s to 1660 nM. Then, simultaneously with the wash-out of caffeine, 3 mM La^{3+} was added. During the exposure to La^{3+}, the decay of $[Ca^{2+}]_i$ was considerably slowed down (Fig. 3*A*, $n = 5$) or even prevented (Fig. 3*B*, $n = 1$). Wash-out of La^{3+} resulted in a decay of $[Ca^{2+}]_i$, however, without an undershoot ($n = 6$). As a control, a short caffeine exposure without La^{3+} was tested when $[Ca^{2+}]_i$ reached the resting values (Fig. 3*A* and *B*). In the absence of La^{3+}, the caffeine-induced $[Ca^{2+}]_i$ transient always decayed faster than in its presence.

The results show that the loss of caffeine-releasable Ca^{2+} can be attenuated by block of Ca^{2+} extrusion with La^{3+}. They further show that the block of Ca^{2+} extrusion by La^{3+} reduces the rate of $[Ca^{2+}]_i$ decay to very low levels. The latter result may be unexpected since the Ca^{2+} reuptake by SERCa should be undisturbed by extracellular La^{3+}. However, the effect results from the high initial level of $[Ca^{2+}]_i$. Pumping of Ca^{2+} by SERCa occurs into a leaky SR: the Ca^{2+}-activated SR Ca^{2+} release channels do not close as long as $[Ca^{2+}]_i$ remains high. Thus, the result gives a demonstration how Ca^{2+} extrusion and Ca^{2+} reuptake are interconnected. Due to this interaction through Ca^{2+}, a quantitative separation of the two process is not possible on the basis of present experiments.

Does Ca^{2+} efflux through Na^+–Ca^{2+} exchange contribute to the decay of caffeine-induced $[Ca^{2+}]_i$ transient?

Substitution of extracellular Na^+ by Li^+, or by the less permeable cation *N*-methyl-glucosamine$^+$ is thought to shift the reversal potential of the Na^+–Ca^{2+} exchanger to strongly negative potentials, thereby reversing the exchanger from the Ca^{2+} efflux into the Ca^{2+} influx mode. Sodium removal from the extracellular solution was tested in ten cells. In seven cells, sodium removal did not have any significant effect on

$[Ca^{2+}]_i$. In three cells, sodium removal incremented $[Ca^{2+}]_i$ by 71 ± 11 nM within 15–20 s (Fig. 4*A*). In all cells membrane current at -50 mV was not modified by Na^+ removal.

The possibility of contribution of Na^+–Ca^{2+} exchange to the decay of the caffeine-induced $[Ca^{2+}]_i$ transients was tested with paired-application experiments in five

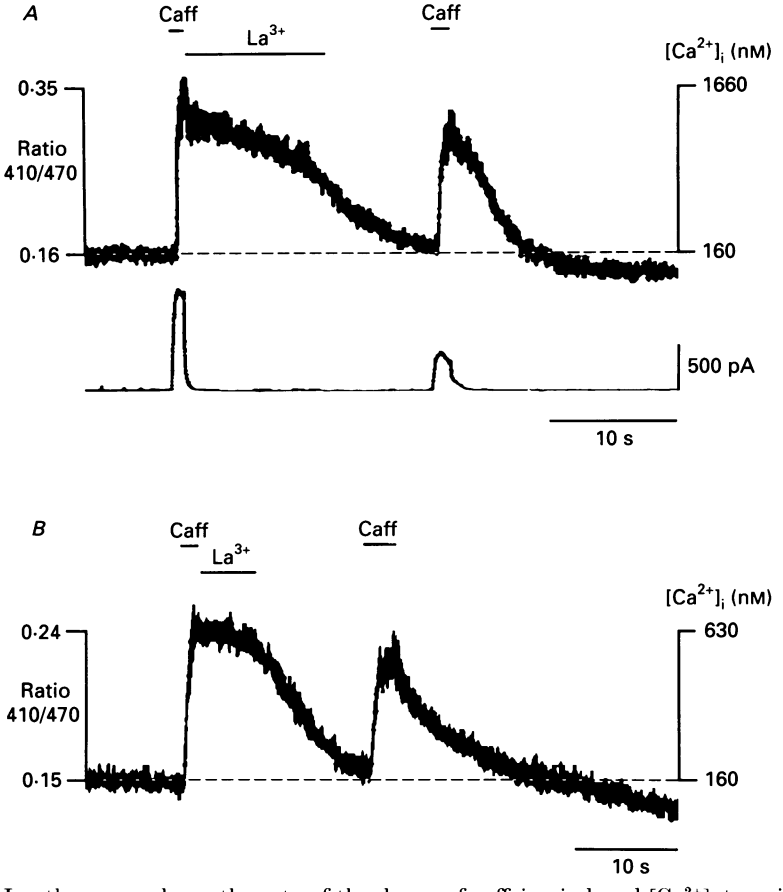

Fig. 3. Lanthanum reduces the rate of the decay of caffeine-induced $[Ca^{2+}]_i$ transient. *A*, after a 2 s caffeine application, wash-out of caffeine occurred simultaneously with the application of 3 mM La^{3+}, and the rate of $[Ca^{2+}]_i$ decay is very much retarded. Upon wash-out of La^{3+}, $[Ca^{2+}]_i$ returns to the baseline without an undershoot. For comparison, a caffeine transient was recorded a second time in the absence of La^{3+}. *B*, experiment where 3 mM La^{3+} nearly prevented the decay of $[Ca^{2+}]_i$ after wash-out of caffeine. The duration of application of corresponding solution is indicated above each trace. Note, La^{3+} blocks $I_{K,Ca}$, indicating the interaction of La^{3+} with the membrane.

myocytes. In Fig. 4*B*, the first caffeine response peaked within 2 s to 1240 nM. Caffeine was then washed out by application of the Na^+-free solution to the cell. In the absence of $[Na^+]_o$, $[Ca^{2+}]_i$ fell with the half-time of 2 s to an undershoot (Fig. 4*B*). Then, the short caffeine application was repeated. The decay of $[Ca^{2+}]_i$ was very similar in the absence (first transient) and presence of 150 mM $[Na^+]_o$ (second

transient, Fig. 4*B*). The absence of a significant retardation of the $[Ca^{2+}]_i$ decay in Na^+-free solution suggests that Ca^{2+} efflux through Na^+–Ca^{2+} exchange does not significantly contribute to the Ca^{2+} extrusion during the decay of the caffeine-induced $[Ca^{2+}]_i$ transients.

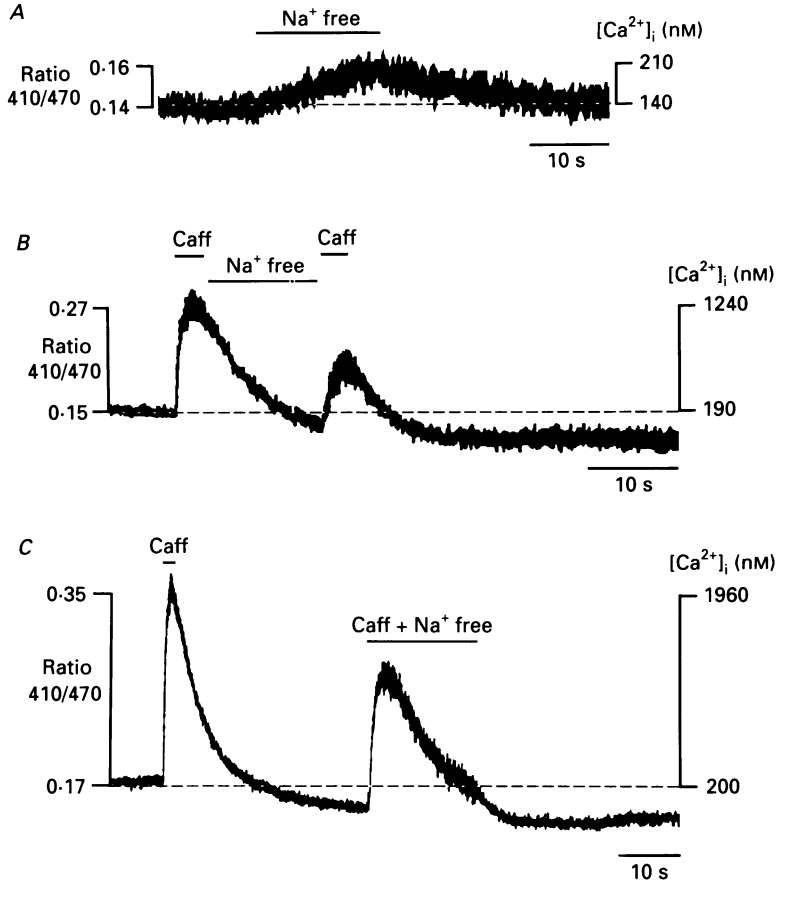

Fig. 4. Effects of extracellular sodium removal in cells dialysed with electrode solution containing 10 mM $[Na^+]_i$. *A*, substitution of 150 mM $[Na^+]_o$ by 150 mM $[Li^+]_o$ induced a small increase in $[Ca^{2+}]_i$. This effect was recorded in only three of ten cells. *B*, sodium removal did not change the half-time of the $[Ca^{2+}]_i$ decay (150 mM Na^+ replaced by 150 mM NMG^+). *C*, application of a sodium-free caffeine-containing solution induced a $[Ca^{2+}]_i$ transient. The application time of sodium-free solution is labelled above each trace.

In four cells, the effect of sodium removal was tested on the decay of $[Ca^{2+}]_i$ in the continuous presence of caffeine. The decay of $[Ca^{2+}]_i$ was not retarded (half-time *ca* 5 s) and reached the resting level within 20 s (Fig. 4*C*). After removal of caffeine and return to 150 mM $[Na^+]_o$, the undershoot developed. Again, the results suggest that Ca^{2+} efflux through the Na^+–Ca^{2+} exchanger is not required for the La^{3+}-sensitive Ca^{2+} extrusion in the presence of caffeine.

$[Ca^{2+}]_i$ transients due to sodium removal at elevated $[Na^+]_i$

The driving force for Ca^{2+} influx through the Na^+–Ca^{2+} exchange can be increased by elevation of $[Na^+]_i$, for example by dialysing of electrode solutions that contained 150 mM NaCl. At a holding potential of −50 mV, dialysis of this solution reversed

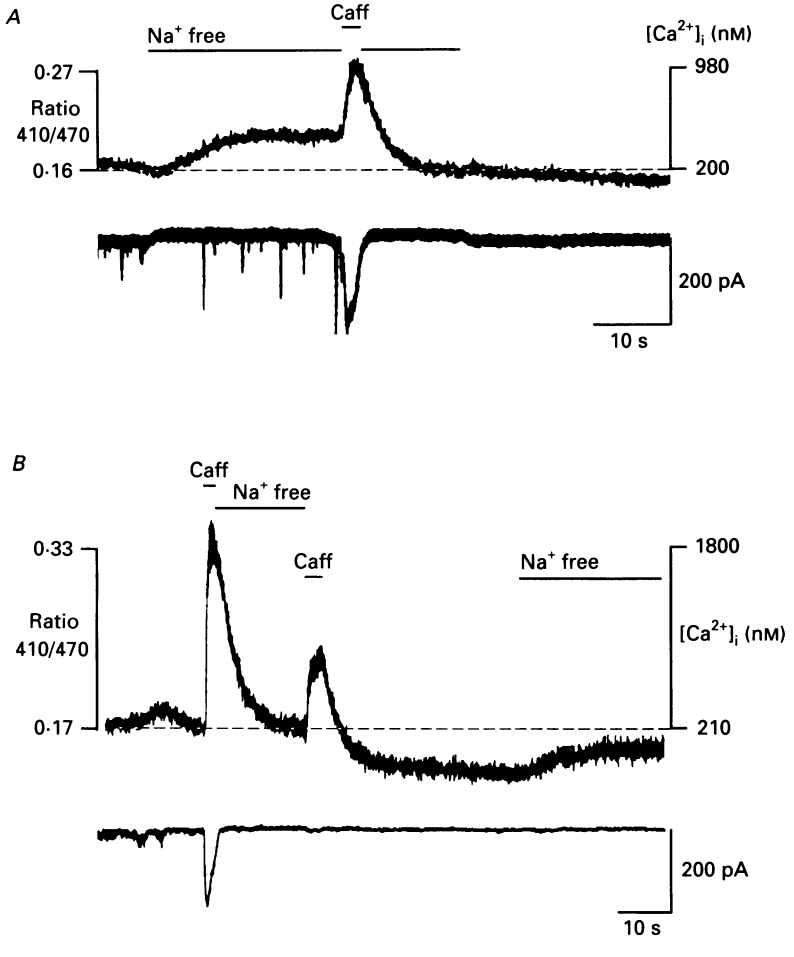

Fig. 5. Contribution of Na^+–Ca^{2+} exchange to the $[Ca^{2+}]_i$ signals in cells dialysed with 150 mM $[Na^+]_i$. Upper traces in *A* and *B* represent $[Ca^{2+}]_i$, lower traces the membrane current recorded at a holding potential of −50 mV. Effective cell dialysis indicated by the reversal of spontaneous outward into inward currents due to the reversed K^+ driving force. *A*, sodium removal (NMG^+ substitution) elevated $[Ca^{2+}]_i$ from 200 nM to a steady value of 420 nM. Following caffeine application for 2 s a $[Ca^{2+}]_i$ transient was induced; upon a wash-out of caffeine $[Ca^{2+}]_i$ fell in the absence of $[Na^+]_o$ to 200 nM. *B*, the $[Ca^{2+}]_i$ decay in the absence of caffeine was not modified by sodium removal (Li^+ substitution). The second caffeine response had an attenuated peak. The time of application of the corresponding solution is labelled above each $[Ca^{2+}]_i$ trace. Note, sodium removal induces a small outward current which might be due to the electrogenicity of Na^+–Ca^{2+} exchange. Since the K^+ driving force is inward, the Ca^{2+}-activated K^+ current is negative.

the STOCs within 3 min into spontaneous inward currents, indicating that the potassium gradient had been reversed. With the high-sodium intracellular solution resting $[Ca^{2+}]_i$ was 180 ± 47 nM ($n = 13$) which is not significantly different from the control with 10 mM $[Na^+]_i$.

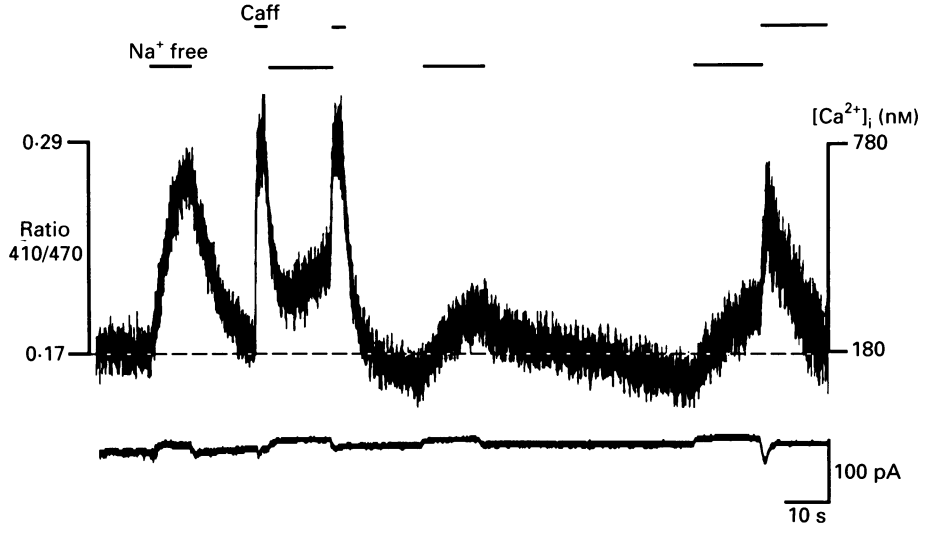

Fig. 6. Interrelation of $[Ca^{2+}]_i$ transients due to extracellular sodium removal and $[Ca^{2+}]_i$ transients due to caffeine (150 mM $[Na^+]_i$, Li^+ substitution). Upper trace, $[Ca^{2+}]_i$; lower trace, membrane current. An initial sodium removal for 10 s reversibly increased $[Ca^{2+}]_i$ to 640 nM. During the decay of the first caffeine-induced $[Ca^{2+}]_i$ transient, sodium was removed a second time; it resulted in the decay of $[Ca^{2+}]_i$ to 290 nM and induced a slow rise in $[Ca^{2+}]_i$. The following caffeine-induced $[Ca^{2+}]_i$ transient remained unaffected. The third sodium removal induced a diminished increment of $[Ca^{2+}]_i$ as if the first $[Ca^{2+}]_i$ transient due to sodium removal depended on release of Ca^{2+} from a store. When the store was deprived by two caffeine treatments the response to sodium removal was attenuated. The time of application of the corresponding solution is indicated above the $[Ca^{2+}]_i$ trace.

With 150 mM $[Na^+]_i$, removal of extracellular sodium induced an increase in $[Ca^{2+}]_i$. The response was variable in amplitude and rate (see below). In experiment illustrated in Fig. 5*A* sodium removal increased $[Ca^{2+}]_i$ from 200 to 420 nM within 15 s with $[Ca^{2+}]_i$ then remaining constant. Simultaneously with the increase in $[Ca^{2+}]_i$, an outward current of approximately 20 pA was recorded (see also Fig. 6) which disappeared upon readmission of extracellular sodium. The current may represent the electrogenicity of the Na^+–Ca^{2+} exchange; however, here it was not further investigated.

Caffeine effect with reversed Na^+ gradient

With 150 mM $[Na^+]_i$ and sodium-free extracellular solution (Fig. 5*A*), the short caffeine application increased $[Ca^{2+}]_i$ from 420 to 980 nM within *ca* 2 s. After caffeine was washed out, $[Ca^{2+}]_i$ fell below the tonic level of 420 nM induced by sodium removal; a resting level of 200 nM was reached within *ca* 10 s (Fig. 5*A*). Similar results were found in four other cells. They suggest that the decay of $[Ca^{2+}]_i$ can occur even at the reversed 0 mM $[Na^+]_o$/150 mM $[Na^+]_i$ gradient, i.e. that the Ca^{2+} reuptake

by SERCa and Ca^{2+} extrusion by plasmalemmal Ca^{2+}-ATPase (PMCa) are powerful enough to overcome the Ca^{2+} influx through the augmented Na^+–Ca^{2+} exchanger. The elimination of the tonic increase in $[Ca^{2+}]_i$ induced by the preceding sodium removal may indicate that stimulation of SERCa and PMCa by caffeine-induced Ca^{2+} release resulted in removal of all Ca^{2+} ions that enter the cell via Na^+–Ca^{2+} exchange. Another explanation could be that the $[Ca^{2+}]_i$ transients due to sodium removal are not only due to Ca^{2+} influx but also have a component of Ca^{2+} release from the caffeine-sensitive store.

Caffeine-induced $[Ca^{2+}]_i$ transients at elevated 150 mM $[Na^+]_i$

With 150 mM $[Na^+]_i$, caffeine induced $[Ca^{2+}]_i$ transients that peaked to 1614 ± 530 nM ($n = 13$, not significantly different to the control with 10 mM $[Na^+]_i$). After a short caffeine application, $[Ca^{2+}]_i$ fell with a half-time of 2 s to an undershoot of 107 ± 40 nM ($n = 10$, not significantly different to control). Sodium removal during the decay phase of a caffeine-induced $[Ca^{2+}]_i$ transient did not significantly modify the half-time of decay; however, the development of an undershoot was usually prevented (Fig. 5*A* and *B*).

In some cells, sodium removal during the $[Ca^{2+}]_i$ decay resulted in a reversal of undershoot, i.e. $[Ca^{2+}]_i$ slowly increased to an elevated level. Figure 6 shows the result where the caffeine-induced $[Ca^{2+}]_i$ transient decayed in a sodium-free solution to 290 nM and not to the resting level of 180 nM. The fall was followed by a slow (within 12 s) secondary increase of $[Ca^{2+}]_i$ to 380 nM. The second caffeine application increased $[Ca^{2+}]_i$ to 780 nM, the peak being similar to that during the first caffeine application. The result supports the view that the decay of the $[Ca^{2+}]_i$ transient induced by a short caffeine application is rate limited by Ca^{2+} reuptake by SERCa but that sodium removal can interfere with this process.

With 150 mM $[Na^+]_i$, the rise of $[Ca^{2+}]_i$ during sodium removal was subject to variability; small increments of 130 nM contrasted with regenerative responses that peaked up to 2400 nM. The high variability may indicate that in addition to Ca^{2+} influx through the Na^+–Ca^{2+} exchanger other mechanisms are also involved in the effects of sodium removal on $[Ca^{2+}]_i$.

Interference of sodium removal and caffeine-induced $[Ca^{2+}]_i$ transients

The $[Ca^{2+}]_i$ signals due to Ca^{2+} influx through Na^+–Ca^{2+} exchange could be modified by the SR: either attenuated by Ca^{2+} sequestration or amplified by Ca^{2+}-induced Ca^{2+} release. The latter possibility is supported by experiments in which SR Ca^{2+} load was reduced by caffeine. Figure 6 compares the effects of sodium removal under varied conditions. The first sodium removal resulted in a large elevation of $[Ca^{2+}]_i$ (460 nM, peak (640 nM) – resting (180 nM) $[Ca^{2+}]_i$). The second sodium removal was combined with the caffeine removal, with the result that the decay of $[Ca^{2+}]_i$ to the resting level was prevented. The third sodium removal was tested after the second caffeine exposure; it increased $[Ca^{2+}]_i$ from the undershoot (120 nM) to only 250 nm which is equivalent to a small increment of 130 nM (250 – 120 nM). Readdition of 150 mM $[Na^+]_o$ restored the undershoot and during the fourth sodium removal similar elevation of $[Ca^{2+}]_i$ was recorded. At this time, a 12 s caffeine application induced a $[Ca^{2+}]_i$ transient that peaked to only *ca* 400 nM.

In all four cells studied, preceding caffeine application reduced the amplitude of the $[Ca^{2+}]_i$ transients induced by sodium removal. Since caffeine deprives the SR of releasable Ca^{2+}, the attenuation of the $[Ca^{2+}]_i$ increment due to sodium removal could be (i) due to a more efficient pumping of Ca^{2+} into SR by the SERCa or (ii) a diminished contribution of SR Ca^{2+} release which can be triggered by the Ca^{2+} influx through the Na^+–Ca^{2+} exchanger. The strongly attenuated peak of the last caffeine response in Fig. 6 suggests that the SR was deprived of Ca^{2+}; thus, the result supports the latter hypothesis. However, the result is not conclusive, since the preceding short caffeine applications may have left some releasable Ca^{2+} in the store. Thus, sodium removal experiments were performed in the continuous presence of caffeine.

Deprivation of the store with caffeine reduces the $[Ca^{2+}]_i$ transients induced by sodium removal

$[Ca^{2+}]_i$ transients induced by sodium removal ($[Na^+]_i$ = 150 mM) in the absence and presence of 10 mM caffeine are compared in Fig. 7. The first transient in Fig. 7*A* shows that sodium removal incremented $[Ca^{2+}]_i$ from 200 to 820 nM. Upon the return to 150 mM $[Na^+]_o$ $[Ca^{2+}]_i$ returned to the resting level. The following long caffeine application increased $[Ca^{2+}]_i$ from 200 to 1420 nM. In the continuous presence of caffeine, $[Ca^{2+}]_i$ fell, and when sodium was removed $[Ca^{2+}]_i$ was incremented by only *ca* 100 nM, a value low in comparison with the first increment of 620 nM (820 − 200 nM). As it was shown before, the continuous presence of caffeine largely depletes the stores of releasable Ca^{2+}. Thus, the comparison of the $[Ca^{2+}]_i$ transients induced by sodium removal in the absence and presence of caffeine (Fig. 7*A*) strongly suggests that a large part of the $[Ca^{2+}]_i$ transients derives their Ca^{2+} from Ca^{2+} release from the caffeine-sensitive Ca^{2+} store.

Figure 7*B* shows an example where sodium removal triggered a 'synchronized' $[Ca^{2+}]_i$ transient, the amplitude and time course of which resembled the caffeine-induced $[Ca^{2+}]_i$ transients. (A similar synchronized response was recorded in two other of eleven cells, thus it is not a typical response.) In Fig. 7*B*, sodium removal increased $[Ca^{2+}]_i$ within 1 s from 100 to 2400 nM. From the peak, $[Ca^{2+}]_i$ decayed with a half-time of 4 s, despite the absence of $[Na^+]_o$. When $[Ca^{2+}]_i$ had fallen to 180 nM, $[Na^+]_o$ was restored to 150 mM and caffeine was applied simultaneously. The result was a $[Ca^{2+}]_i$ transient that peaked to 1460 nM and decayed with a half-time of 5 s. When, in the continuous presence of caffeine, $[Ca^{2+}]_i$ had fallen to a level of *ca* 180 nM, the effect of sodium removal was tested again. Now the response was small (approximately 160 nM, Fig. 7*B*).

The continuous caffeine application combined with the removal of extracellular sodium induced a $[Ca^{2+}]_i$ transient that peaked rapidly to 2040 nM and decayed not to rest but to a level of 250 nM; this $[Ca^{2+}]_i$ level was almost constant for 35 s (Fig. 7*C*). On average, in three experiments in caffeine-containing sodium-free solution $[Ca^{2+}]_i$ decayed within 30 s to a sustained level which was 232 ± 19 nM above the rest. Simultaneous caffeine removal and readmittance of 150 mM $[Na^+]_o$ resulted in a $[Ca^{2+}]_i$ fall to an undershoot of 80 nM. When, 30 s later, caffeine was applied a second time (150 mM $[Na^+]_o$), a $[Ca^{2+}]_i$ transient of small amplitude was induced (to *ca* 300 nM). The results suggest that the tonic elevation of $[Ca^{2+}]_i$ did not refill the SR.

The continuous caffeine application deprived the SR of releasable Ca^{2+} even if the reversed sodium gradient should have promoted Ca^{2+} influx. The results do not favour the view that the $[Ca^{2+}]_i$ transient due to sodium removal is a direct consequence of Ca^{2+} influx. Instead they rather support the idea that the $[Ca^{2+}]_i$

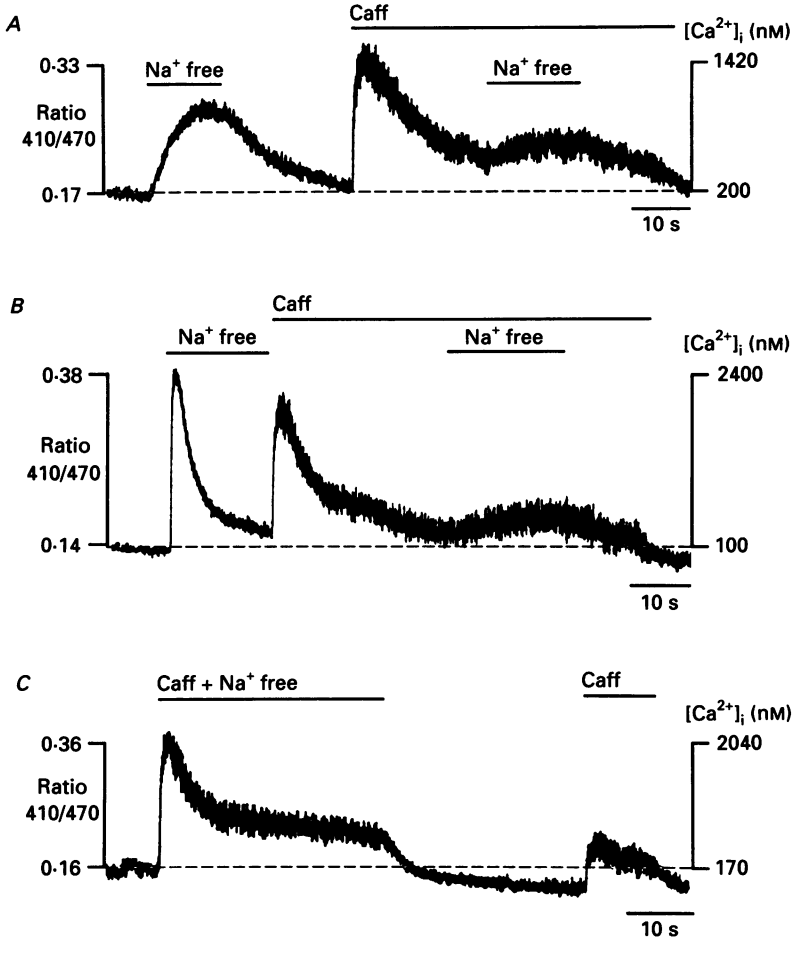

Fig. 7. Comparison of $[Ca^{2+}]_i$ transients due to sodium removal in the absence and presence of caffeine (150 mM $[Na^+]_i$). *A*, the first $[Ca^{2+}]_i$ transient due to sodium removal (NMG^+ substitution) was a reversible increment by 620 nM (from 200 to 820 nM). When sodium removal was repeated in continuous presence of caffeine, the increment was only 100 nM (from 360 to 460 nM). *B*, example where sodium removal (NMG^+ substitution) triggered a large synchronized $[Ca^{2+}]_i$ transient; $[Ca^{2+}]_i$ rose within 1 s to 2400 nM and decayed in the continuous absence of $[Na^+]_o$. When the sodium removal was repeated in the continuous presence of caffeine, $[Ca^{2+}]_i$ rose only by 160 nM. *C*, during simultaneous application of caffeine and removal of sodium (Li^+ substitution) the $[Ca^{2+}]_i$ transient decayed to a tonic $[Ca^{2+}]_i$ level. The following caffeine application induced a small transient suggesting that the Ca^{2+} store was depleted, despite the tonic elevation of $[Ca^{2+}]_i$ during the sodium-free period.

transient induced by sodium removal comprises a large component due to Ca^{2+} release from SR. Most probably, this component is triggered by the Ca^{2+} influx through the Na^+–Ca^{2+} exchanger. The contribution of the Ca^{2+} release varied depending on the degree of SR Ca^{2+} loading and the synchronization of the SR Ca^{2+} release channels.

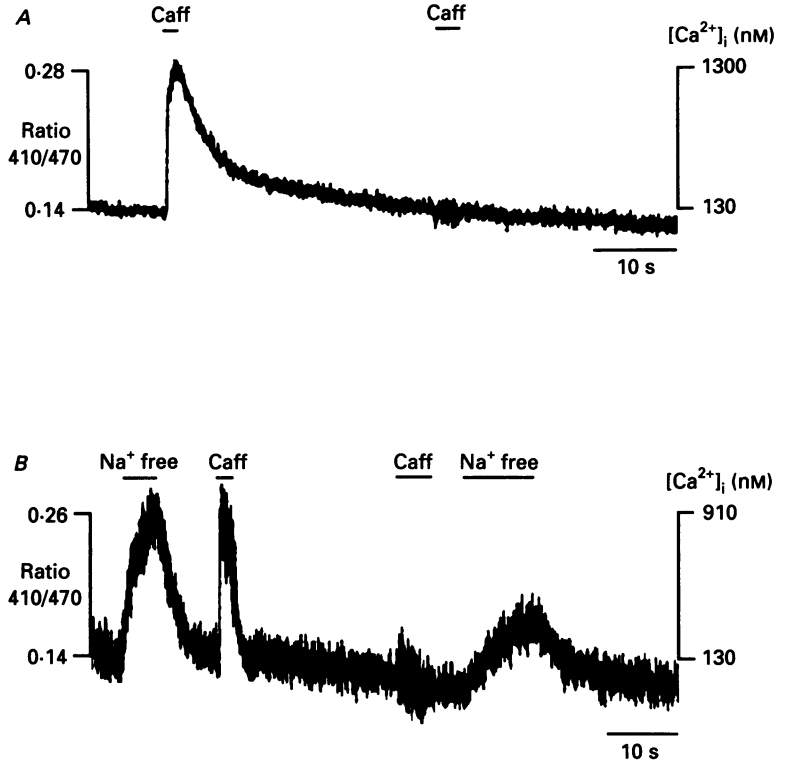

Fig. 8. $[Ca^{2+}]_i$ transients in the presence of 5 μM ryanodine (150 mM $[Na^+]_i$). *A*, ryanodine was applied through the patch pipette for 2 min before the first caffeine application. After a slow decay, the second caffeine application failed to induce a $[Ca^{2+}]_i$ transient. The presence of caffeine in the cell is indicated by an increased noise due to a caffeine quenching of indo-1 fluorescence. *B*, after a 2 min ryanodine loading, the first sodium removal induced a reversible increase in $[Ca^{2+}]_i$ from 130 to 910 nM. A subsequent caffeine application induced a $[Ca^{2+}]_i$ transient with a peak of *ca* 900 nM. The second caffeine application failed to induce a $[Ca^{2+}]_i$ transient suggesting that the SR had been functionally removed. Under these conditions, the second sodium removal incremented $[Ca^{2+}]_i$ to only *ca* 300 nM which may be the direct contribution of Ca^{2+} influx to $[Ca^{2+}]_i$.

Ryanodine suppresses the $[Ca^{2+}]_i$ transients induced by sodium removal

Treatment with ryanodine is another way to deprive the caffeine-sensitive SR of releasable Ca^{2+}. In coronary myocytes, the effect of 5 μM ryanodine included in the pipette solution developed quickly, i.e. within 2–3 min the caffeine-induced $[Ca^{2+}]_i$ transients no longer recovered. Figure 8*A* shows a result obtained with 150 mM $[Na^+]_i$. After a 2 min period of ryanodine loading, the first short caffeine application

induced a $[Ca^{2+}]_i$ transient that peaked within 2 s to 1300 nM and slowly decayed. The second caffeine application, however, completely failed to increase $[Ca^{2+}]_i$ ($n = 7$). The block of the second Ca^{2+} release is compatible with the idea that the ryanodine effects on the Ca^{2+} release channels are use dependent.

In a cell loaded with ryanodine for 2 min, the first sodium removal increased $[Ca^{2+}]_i$ within 5 s from 130 to 910 nM. During the following readdition of 150 mM $[Na^+]_o$, $[Ca^{2+}]_i$ decayed to the resting value (Fig. 8*B*). The subsequent caffeine application induced a $[Ca^{2+}]_i$ transient that peaked to *ca* 900 nM. Then, caffeine was washed out. When the second caffeine application was tested 20 s later, it failed to change $[Ca^{2+}]_i$. Under such a condition where caffeine was unable to raise $[Ca^{2+}]_i$ (suggesting that the caffeine-sensitive Ca^{2+} store was functionally removed by ryanodine) sodium removal could still increase $[Ca^{2+}]_i$: within 10 s the Ca^{2+} reached *ca* 300 nM. Similar results were obtained in four other cells. This residual $[Ca^{2+}]_i$ transient can be attributed to the direct effects of Ca^{2+} influx on $[Ca^{2+}]_i$, under conditions when caffeine-sensitive Ca^{2+} is functionally removed by ryanodine treatment and Ca^{2+} influx through the Na^+–Ca^{2+} exchanger being facilitated by the high $[Na^+]_i$ of 150 mM.

DISCUSSION

In this study, $[Ca^{2+}]_i$ transients in guinea-pig coronary myocytes were induced by rapid application of caffeine or by rapid removal of sodium from the bath. The $[Ca^{2+}]_i$ transients started from a resting level of 166 nM which is somewhat higher than the 120 nM published for urinary bladder myocytes (Ganitkevich & Isenberg, 1991). The caffeine-induced $[Ca^{2+}]_i$ transients peaked within 1–2 s to *ca* 1600 nM. Whereas the peak amplitude is similar, the time to peak is longer than the 0·3 s recently reported for urinary bladder myocytes (Ganitkevich & Isenberg, 1992); the explanation may be that it is more difficult to activate the Ca^{2+} release channels in a 'synchronized' fashion in coronary than in urinary bladder myocytes. The caffeine-induced $[Ca^{2+}]_i$ transients were blocked by a pretreatment with ryanodine as is expected from a caffeine-sensitive store that releases Ca^{2+} through ryanodine receptors. Ca^{2+} activation of ryanodine receptors (Herrmann-Frank, Darling & Meissner, 1991) is the basis for Ca^{2+}-induced Ca^{2+} release which constitutes a positive feedback that may amplify the effects of a small Ca^{2+} influx.

In the present experiments caffeine was supplied to the cell by fast application. A slower bath application limited the synchronization of the Ca^{2+} release and hence the $[Ca^{2+}]_i$ transients (authors' unpublished observations). These different experimental conditions could explain why caffeine was reported to increase $[Ca^{2+}]_i$ only after a long delay and with a time to peak of *ca* 8 s (Stehno-Bittel & Sturek, 1992). When $[Ca^{2+}]_i$ rises along such a slow time course, the effects of Ca^{2+} release superimpose on those of Ca^{2+} reuptake and Ca^{2+} extrusion and as a consequence the $[Ca^{2+}]_i$ transients will underestimate capacity of the caffeine-sensitive Ca^{2+} stores.

The decay of the caffeine-induced $[Ca^{2+}]_i$ transients is due to reuptake of Ca^{2+} by SERCa and to Ca^{2+} extrusion by PMCa, and in certain experimental conditions by the Na^+–Ca^{2+} exchanger in parallel (see below). In the continuous presence of caffeine, Ca^{2+} release channels (ryanodine receptors) remain open and Ca^{2+} reuptake into the leaky SR is impaired. Thus, the decay of $[Ca^{2+}]_i$ in the presence of 10 mM

caffeine is mostly due to Ca^{2+} extrusion. The results show that Ca^{2+} extrusion through PMCa was powerful enough to reduce $[Ca^{2+}]_i$ to the resting level. Due to Ca^{2+} extrusion in the presence of caffeine, the cell was deprived of releasable Ca^{2+} and the following $[Ca^{2+}]_i$ transient was strongly attenuated. After wash-out of caffeine, the SR reloads with Ca^{2+} and a second $[Ca^{2+}]_i$ transient could be induced.

After a 1 s caffeine application, $[Ca^{2+}]_i$ decayed faster than in its continuous presence (half-time of decay 2 s instead of 5 s). The fast decay rate points to the importance of the SERCa for the regulation of $[Ca^{2+}]_i$ in guinea-pig coronary myocytes. Since SERCa is $[Ca^{2+}]_i$-stimulated with a Michaelis–Menten constant (K_m) of 0·6 μM and PMCa with a K_m of 0·9 μM (Grover & Samson, 1986) one expects that both Ca^{2+} ATPases contribute to the decay of $[Ca^{2+}]_i$ under physiological conditions. After wash-out of caffeine $[Ca^{2+}]_i$ fell below the resting value. This low $[Ca^{2+}]_i$ may be attributed to a stimulation of SERCa by intraluminal Ca^{2+} concentrations (Inesi & De Meis, 1989). A similar $[Ca^{2+}]_i$ undershoot after the $[Ca^{2+}]_i$ transients has also been reported recently for urinary bladder myocytes (Ganitkevich & Isenberg, 1992) as well as for sympathetic neurons (Friel & Tsien, 1992).

Under physiological conditions, SERCa and PMCa interact via $[Ca^{2+}]_i$. The importance of PMCa for the decay of caffeine-induced $[Ca^{2+}]_i$ transients is in line with the result that decay was suppressed by lanthanum which is known to block not only the PMCa but also the Na^+–Ca^{2+} exchange (Missiaen *et al.* 1992). Initially the suppression of the $[Ca^{2+}]_i$ decay by lanthanum seems to conflict with the above idea of SERCa being effective in reducing $[Ca^{2+}]_i$. However, SERCa pumps Ca^{2+} into a leaky SR as long as the Ca^{2+}-activated release channels are not closed due to the reduction of $[Ca^{2+}]_i$ for which the operating PMCa is essential. Due to the interaction of $[Ca^{2+}]_i$ with both SERCa and PMCa a quantitative separation of the $[Ca^{2+}]_i$ signal, e.g. into lanthanum-sensitive Ca^{2+} extrusion and lanthanum-insensitive SERCa, is not possible.

The larger part of this study concerned the possible contribution of Na^+–Ca^{2+} exchange to the cellular Ca^{2+} balance. With physiological 10 mM $[Na^+]_i$, sodium removal increased $[Ca^{2+}]_i$ in only three out of ten cells and only by 70 nM. The result is in line with results from multicellular coronary preparations (Ito *et al.* 1979). It suggests that the resting $[Ca^{2+}]_i$ does not significantly depend on the Na^+–Ca^{2+} exchanger as long as $[Na^+]_i$ is at physiological 10 nM. The presence or absence of extracellular sodium modified neither peak nor the decay of the caffeine-induced $[Ca^{2+}]_i$ transient, a result that suggests that the Ca^{2+} load of the intracellular stores is controlled by mechanisms other than the Na^+–Ca^{2+} exchange. Sodium removal was also ineffective during the undershoot of $[Ca^{2+}]_i$, hence these conclusions can be generalized to coronary myocytes with low SR Ca^{2+} load.

It has been suggested that sodium removal does not induce appreciable Ca^{2+} influx unless $[Na^+]_i$ is raised to concentrations above the K_D of the internal Na^+ site (28 mM $[Na^+]_i$, Smith *et al.* 1991). Cell dialysis of solutions containing 150 mM NaCl should have saturated these sites. Our measurements revealed that this elevation of $[Na^+]_i$ did not increase the resting $[Ca^{2+}]_i$ significantly as if the SERCa and PMCa were able to keep the $[Ca^{2+}]_i$ low, independently of Na^+–Ca^{2+} exchange. With 150 mM $[Na^+]_i$, however, sodium removal did increase $[Ca^{2+}]_i$ although the responses varied widely in amplitude and time course. Some myocytes responded to the sodium removal with

a slow rise to a tonic $[Ca^{2+}]_i$ level. In other cells, sodium removal triggered a regenerative phasic response, i.e. the $[Ca^{2+}]_i$ transient rose within 1 s to 2400 nM and decayed rapidly. The results suggested the hypothesis that the $[Ca^{2+}]_i$ transient due to sodium removal comprises a variable component of Ca^{2+}-induced Ca^{2+} release from SR which is triggered by the small Ca^{2+} influx upon extracellular sodium removal. The hypothesis is supported by the result that pretreatment with caffeine, depriving the store of releasable Ca^{2+}, attenuated the $[Ca^{2+}]_i$ transients due to sodium removal. The hypothesis is further supported by the result that ryanodine largely suppressed the $[Ca^{2+}]_i$ transients due to sodium removal; the increment in $[Ca^{2+}]_i$ recorded in ryanodine-treated cells is suggested to represent the Ca^{2+} influx through Na^+–Ca^{2+} exchange stimulated by 150 mM $[Na^+]_i$. In conclusion, $[Ca^{2+}]_i$ signal due to sodium removal is amplified by SR Ca^{2+} release if the store is filled with Ca^{2+}, or vice versa, the Ca^{2+} influx through the $[Na^+]_i$-stimulated Na^+–Ca^{2+} exchange can trigger Ca^{2+} release from the SR. As a consequence, the $[Ca^{2+}]_i$ transients due to sodium removal cannot be used as an indicator for Ca^{2+} influx through Na^+–Ca^{2+} exchange. The functional SR may not only attenuate signals of Ca^{2+} influx as suggested in the literature ('Ca^{2+} sink' by Sheu & Blaustein, 1992; 'superficial buffer barrier' by van Breemen & Saida, 1989). In addition, the SR may also act as a Ca^{2+} source that amplifies the Ca^{2+} influx. Under physiological conditions Ca^{2+} influx through the Na^+–Ca^{2+} exchanger is insignificant. However, Ca^{2+}-induced SR Ca^{2+} release may be triggered by influx of extracellular Ca^{2+} through channels. It may also be triggered by Ca^{2+} released from SR when agonists induce Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores.

Our results suggest that the Na^+–Ca^{2+} exchanger is of minor importance for the $[Ca^{2+}]_i$ transients of guinea-pig coronary myocytes as long as $[Na^+]_i$ is at the physiological 10 mM. Whether this conclusion can be extrapolated to other types of smooth muscle cells has to be tested by future experiments.

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