Dissociation of subsarcolemmal from global cytosolic $[Ca^{2+}]$ in myocytes from guinea-pig coronary artery

Vladimir Ya. Ganitkevich and Gerrit Isenberg *

Department of Physiology, University of Cologne, 50924 Köln, Germany

1. Changes in cytosolic Ca^{2+} concentration ($\Delta[Ca^{2+}]_c$) were measured by global indo-1 fluorescence and compared with changes in subsarcolemmal Ca^{2+} concentration ($\Delta[Ca^{2+}]_{sl}$) indicated by Ca^{2+}-activated K^+ currents ($I_{K(Ca)}$).

2. At −50 mV holding potential, 10 mM caffeine increased both $I_{K(Ca)}$ and $[Ca^{2+}]_c$ without measurable delay. While $I_{K(Ca)}$ peaked within 0·3 ± 0·16 s (mean ± S.D.) and decayed to 50% within 0·4 ± 0·2 s, $\Delta[Ca^{2+}]_c$ peaked within 1·5 ± 0·5 s and decayed to 50% within 5·2 ± 1·0 s. The different time courses support the idea that $[Ca^{2+}]_{sl}$ and $[Ca^{2+}]_c$ deviate.

3. When 10 mM caffeine was applied 20 s after an initial 2 s caffeine application, $I_{K(Ca)}$ was suppressed to 22 ± 5% and $\Delta[Ca^{2+}]_c$ to 40 ± 4%. During the following 1 min caffeine-free period, $I_{K(Ca)}$ recovered to 61 ± 7% while $\Delta[Ca^{2+}]_c$ remained at 40 ± 3%. The differences between $I_{K(Ca)}$ and $\Delta[Ca^{2+}]_c$ suggest that Ca^{2+} deprivation and Ca^{2+} refilling is faster in peripheral than in central sarcoplasmic reticulum (SR).

4. During the loading period of indo-1, a spontaneous $\Delta[Ca^{2+}]_c$ of 30–80 nM appeared both at −50 mV and at more positive potentials. The amplitude of spontaneous $\Delta[Ca^{2+}]_c$ increased with the amplitude, the frequency or the fusion of spontaneous transient outward currents (STOCs).

5. Block of sarcolemmal Ca^{2+} fluxes by 1 mM La^{3+} increased $[Ca^{2+}]_c$ by 250 ± 100 nM and suppressed the spontaneous $\Delta[Ca^{2+}]_c$. However, La^{3+} did not significantly retard the rate of decay of STOCs which may therefore be limited by Ca^{2+} diffusion into the cytosol and not by Ca^{2+} extrusion.

6. The dissociation of $I_{K(Ca)}$ (or STOCs) and $\Delta[Ca^{2+}]_c$ may indicate a Ca^{2+} concentration gradient during Ca^{2+} release directed from the sarcolemma towards the centre of the cell, which later reverses direction.

In vascular smooth muscle cells, the sarcoplasmic reticulum (SR) has both peripheral and central components (see Somlyo & Somlyo, 1992, for review). The peripheral SR surrounds the individual caveolae of the sarcolemma, from which it is separated by a 15–50 nm narrow gap space (Nixon, Mignery & Somlyo, 1994). Presumably, the membranes of the peripheral SR hinder Ca^{2+} diffusion, i.e. Ca^{2+} ions may accumulate in this space during Ca^{2+} influx; model calculations suggest that the Ca^{2+} concentration in this subsarcolemmal space ($[Ca^{2+}]_{sl}$) may exceed the global cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) by several orders of magnitude (Kargacin, 1994).

The idea that $[Ca^{2+}]_{sl}$ deviates from $[Ca^{2+}]_c$ has led to speculations on its functional importance. van Breemen & Saida (1989) postulated that most Ca^{2+} ions provided by influx would be sequestered by Ca^{2+}-ATPase (SERCA) into the peripheral SR (the 'superficial Ca^{2+} barrier hypothesis'). Part of this Ca^{2+} would be released from the peripheral SR into the cytosol, and another part would be released vectorially into the sarcolemma; the high $[Ca^{2+}]_{sl}$ would facilitate Ca^{2+} extrusion (Chen, Cannell & van Breemen, 1992).

Despite these ideas, the deviation of $[Ca^{2+}]_{sl}$ from $[Ca^{2+}]_c$ has not yet been experimentally verified by the use of Ca^{2+} fluorescence signals. It is unlikely that $[Ca^{2+}]_{sl}$ can contribute to the global fluorescence signal originating from a high-affinity Ca^{2+} indicator, because the volume fraction of an individual gap space is as small as 5×10^{-8}, and that of all gap spaces is only *ca* 2×10^{-4}. Even in confocal images with *ca* 1 μm^3 voxel resolution, the gap space

* To whom correspondence should be addressed at the Department of Physiology, Martin Luther University, 06097 Halle, Germany.

contributes only a volume fraction of 10^{-4}, too small for a quantitative resolution. The dissociation between $[Ca^{2+}]_c$ and $[Ca^{2+}]_{sl}$ was suggested by more indirect experiments, in which $[Ca^{2+}]_{sl}$ was monitored by Ca^{2+} activation of sarcolemmal K^+ channels (rabbit portal vein and small intestine: Benham & Bolton, 1986; coronary arteries: Ganitkevich & Shuba, 1988; Stehno-Bittel & Sturek, 1992). Spontaneous Ca^{2+} release from a peripheral SR vesicle into the gap space, generating a 'cloud' of high $[Ca^{2+}]_{sl}$, was considered to activate a cluster of K^+ channels which gave rise to spontaneous transient outward currents (STOCs; Benham & Bolton, 1986). In this paper we demonstrate for the first time that STOCs accompany spontaneous changes in $[Ca^{2+}]_c$. By comparing the time course of STOCs with spontaneous changes in $[Ca^{2+}]_c$ ($\Delta[Ca^{2+}]_c$), the idea of vectorial Ca^{2+} release from the peripheral SR into the gap space can, hopefully, be tested. Pharmacologically, Ca^{2+} release from the SR can be triggered by caffeine. During rapid application of 10 mM caffeine, the cytosolic caffeine concentration ($[\text{caffeine}]_c$) equilibrates within less than 0·5 s (O'Neill, Donoso & Eisner, 1990). This results in a large $\Delta[Ca^{2+}]_c$ and a large, synchronized Ca^{2+}-activated K^+ current ($I_{K(Ca)}$), the large signals suggesting that caffeine releases Ca^{2+} from both peripheral and central SR throughout the entire cell. In this paper, we observed a different time course for $I_{K(Ca)}$ and $\Delta[Ca^{2+}]_c$, suggesting that a radial Ca^{2+} gradient may exist between $[Ca^{2+}]_{sl}$ and $[Ca^{2+}]_c$.

This paper does not calibrate the STOCs or $I_{K(Ca)}$ in terms of $[Ca^{2+}]_{sl}$. This should be commented on because the *in vivo* calibration of fluorescence signals in terms of $\Delta[Ca^{2+}]_c$ is routine. In smooth muscle cells, the cytosol close to the sarcolemma forms a series of narrow spaces, both in between the enfoldings of the sarcolemma (the caveolae) as well as between the sarcolemma and the SR. In these narrow spaces, neither EGTA nor BAPTA interact rapidly enough with Ca^{2+} to 'clamp' $[Ca^{2+}]_{sl}$ when plasmalemmal or SR Ca^{2+} fluxes are active (Stern, 1992; cf. Xiong, Kitamura & Kuriyama, 1992, for inside-out patches). The necessary spatially homogeneous Ca^{2+} distribution is usually achieved by blocking the Ca^{2+} fluxes. Such a block, however, interfered with the K^+ channel activity and our attempts at calibration failed. One of the procedures, depletion of intracellular ATP, reduced the Ca^{2+} sensitivity of the K^+ channels (cf. Robertson, Schubert, Hescheler & Nelson, 1993; Wellner & Isenberg, 1995). 'Functional removal' of the SR, e.g. by blocking the SERCA with thapsigargin, does not block the large contribution of the plasmalemmal Ca^{2+}-ATPase (PMCA; cf. Ganitkevich & Isenberg, 1995) to the $[Ca^{2+}]_{sl}$ heterogeneities. Block of PMCA by La^{3+} (the only established blocker of PMCA) turned out to modify K^+ channel currents (this paper). Finally, the existence of STOCs suggests that subsarcolemmal patches with active Ca^{2+} release and high $[Ca^{2+}]_{sl}$ coexist with others that do not have spontaneous Ca^{2+} release and have low $[Ca^{2+}]_{sl}$; the amplitude of these local subsarcolemmal ionic microheterogeneities (cf. Wendt-Gallitelli, Voigt & Isenberg, 1993) cannot be derived from a $I_{K(Ca)}$ signal calibrated with a homogeneous cytosolic Ca^{2+}–BAPTA mixture.

METHODS

Myocytes were enzymatically isolated from the ramus circumflexus of the left coronary artery. Guinea-pigs (200–400 g) were killed by cervical dislocation. The artery was cut out from the heart and placed for 40–60 min in a nominally Ca^{2+}-free physiological salt solution (PSS, composition below). Then the artery was placed for 20–30 min in 1 ml of this solution complemented with 1 mg ml^{-1} collagenase (0·5–0·7 U mg^{-1}) and 1 mg ml^{-1} elastase (130–200 U mg^{-1}; both from Serva, Heidelberg, Germany). The suspension of isolated cells was obtained by pipetting the tissue with a wide-bore Pasteur pipette. Cells were placed in Ca^{2+}-free PSS and stored at +4 °C before use on the same day. During the experiment, the myocytes were continuously superfused with warm PSS (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.

The voltage-clamp method for measuring membrane currents and the microfluospectroscopy for measuring $[Ca^{2+}]_c$ have been described previously (Ganitkevich & Isenberg, 1993*a*). Briefly, the isolated cells were loaded with 100 μM K_5-indo-1 through patch electrodes of 3–5 MΩ resistance. Whole-cell membrane currents were recorded with a RK-300 amplifier (Biologic, Echirolles, France), filtered at 1 kHz and stored on an IBM-compatible host computer.

For microfluospectroscopy, the cells were excited at 340 nm through a ×100 oil immersion objective (fluor, Nikon) with a 75 W xenon lamp. Emitted light in bands from 395–425 nm and 450–490 nm was collected and amplified by a pair of photomultipliers (Hamamatsu Photonics, Japan). After filtering at 20 Hz, the fluorescence ratio F_{410}/F_{470} was obtained on-line by an analog divider (DIV100, Burr Brown). The background fluorescence was electronically subtracted in the cell-attached mode. $[Ca^{2+}]_c$ was evaluated off-line, using an intracellular calibration procedure.

The pipettes were filled with a K^+ electrode solution containing (mM): 130 KCl, 2 Na_2ATP, 3 $MgCl_2$, 10 Hepes, 0·1 K_5-indo-1; adjusted with NaOH to pH 7·2. Caffeine was applied to cells through a four-barrel pipette as described before (Ganitkevich & Isenberg, 1993*a*, *b*), solution change being completed within *ca* 0·5 s. Dibutyryl-cAMP (dB-cAMP), 8-bromoguanosine-cAMP (8-Br-cAMP) and the inhibitory peptide of cAMP-dependent protein kinase, Type II (PKI) were from Sigma. All experiments were performed at 36 °C. Where possible, results were expressed as means ± S.D. of the mean.

RESULTS

Caffeine-induced I_{KCa} and $\Delta[Ca^{2+}]_c$ follow a different time course

Figure 1*A* compares the effects of 10 mM caffeine on $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$ at a holding potential of −50 mV (close to the resting potential of the coronary myocyte). Caffeine increased both $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$. The increase in $[Ca^{2+}]_c$ delayed $I_{K(Ca)}$ by *ca* 20 ms, as can be expected from the 20 Hz electronic filtering. $I_{K(Ca)}$ peaked within 0·4 s while $\Delta[Ca^{2+}]_c$ peaked 0·9 s after caffeine application ($\Delta[Ca^{2+}]_c$ is defined as the peak $[Ca^{2+}]_c$ of 1300 nM minus the resting $[Ca^{2+}]_c$ of 150 nM). From its peak, $I_{K(Ca)}$ decayed faster than $\Delta[Ca^{2+}]_c$; while $I_{K(Ca)}$ fell to 50% of peak within 0·4 s, $\Delta[Ca^{2+}]_c$ fell to 50% within 1·6 s.

Similar dissociation in the time courses of the two caffeine-induced Ca^{2+} signals (as shown in Fig. 1*A*) was recorded in all twenty-two cells analysed. On average, time to peak was $0{\cdot}5 \pm 0{\cdot}2$ s for $I_{K(Ca)}$ and $1{\cdot}5 \pm 0{\cdot}5$ s for $\Delta[Ca^{2+}]_c$. The half-time of decay was $0{\cdot}4 \pm 0{\cdot}2$ s for $I_{K(Ca)}$ and $5{\cdot}2 \pm 1{\cdot}0$ s for $\Delta[Ca^{2+}]_c$. This difference suggests that the subsarcolemmal $[Ca^{2+}]_{sl}$, indicated by $I_{K(Ca)}$, increases and falls along a faster time course than the $[Ca^{2+}]_c$, as measured by global indo-1 fluorescence.

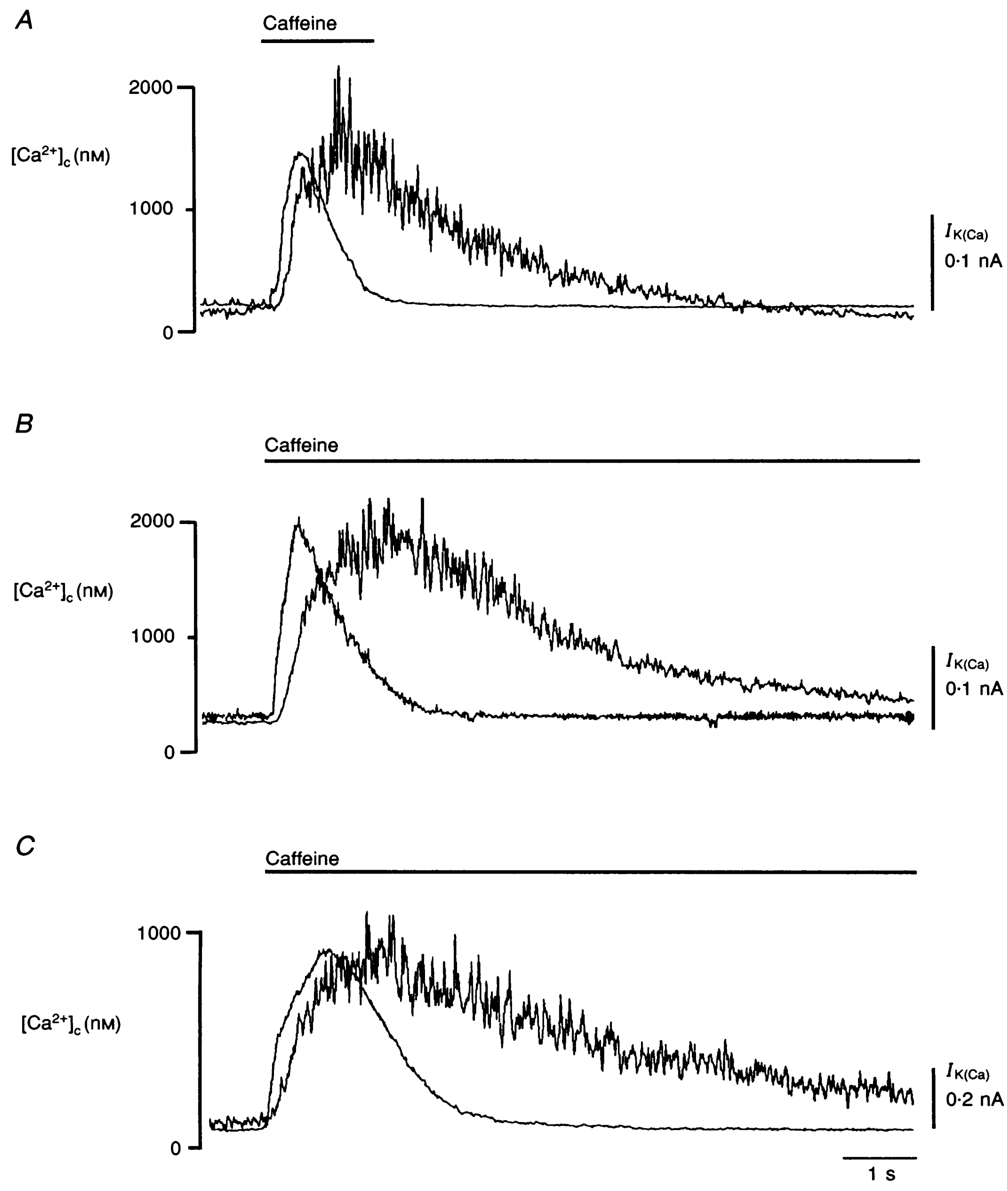

Figure 1. Dissociation of global $\Delta[Ca^{2+}]_c$ (noisy trace) and local subsarcolemmal $\Delta[Ca^{2+}]_{sl}$ ($I_{K(Ca)}$) during caffeine application

Caffeine (10 mM) was rapidly applied through a picospritzer. *A*, 2 s caffeine exposure at a holding potential of −50 mV. $I_{K(Ca)}$ peaked within 0·4 s and $\Delta[Ca^{2+}]_c$ within 0·9 s. While $I_{K(Ca)}$ fell to 50% of the peak within 0·5 s, $\Delta[Ca^{2+}]_c$ decayed to 50% within 1·6 s. *B*, after 30 s caffeine exposure at −50 mV, $I_{K(Ca)}$ peaked within 0·4 s and decayed to 50% within 0·6 s, while the corresponding periods for $\Delta[Ca^{2+}]_c$ were 1·3 and 3·5 s, respectively. *C*, after 30 s caffeine exposure at 0 mV, $I_{K(Ca)}$ peaked within 0·9 s and decayed to 50% within 0·9 s, and $\Delta[Ca^{2+}]_c$ within 1·6 and 4 s, respectively.

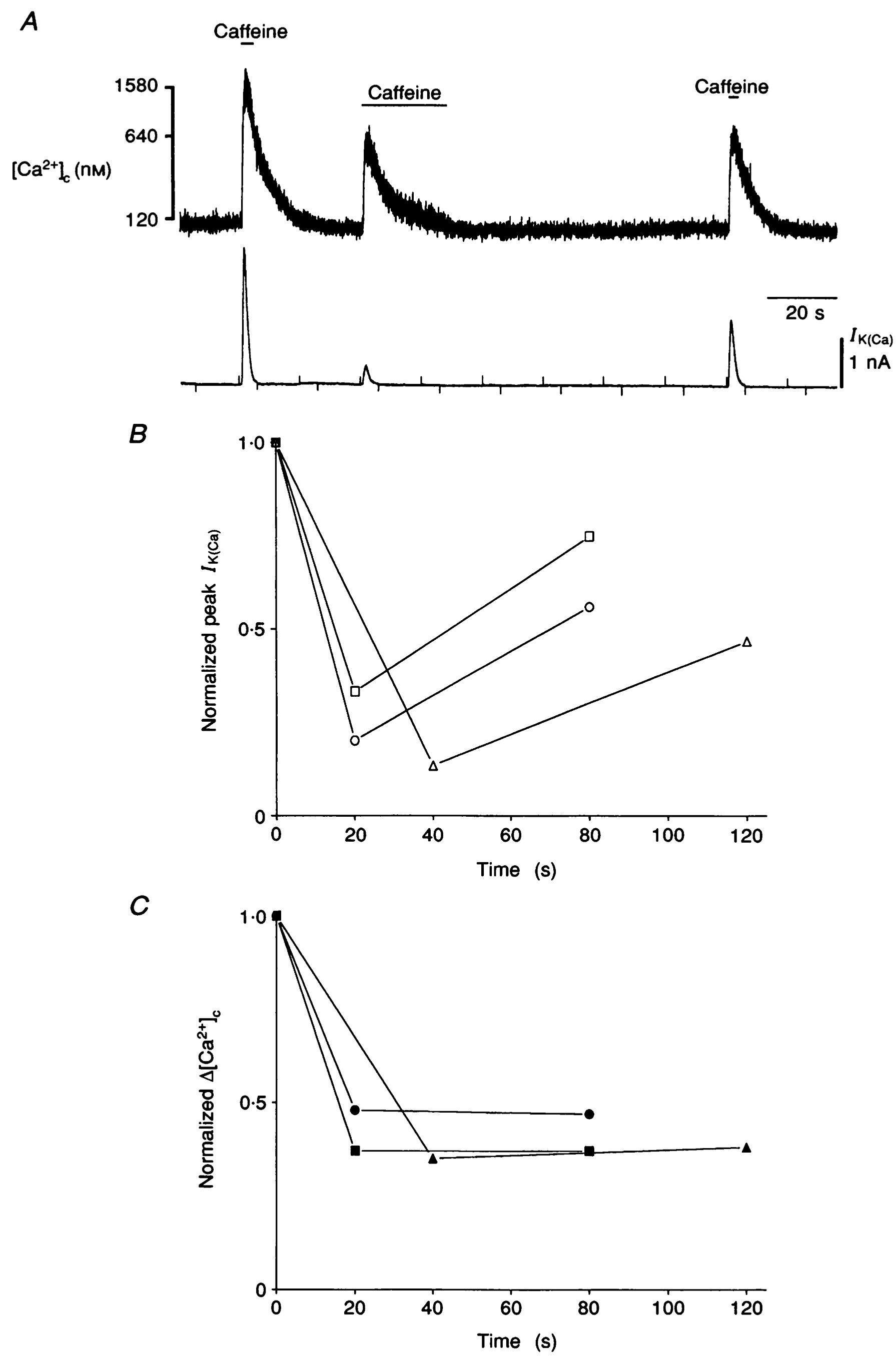

Figure 2. Dissociation of $I_{K(Ca)}$ and $\Delta[Ca^{2+}]_c$ during Ca^{2+} deprivation and refilling of caffeine-sensitive Ca^{2+} stores

Holding potential 0 mV, exposure to 10 mM caffeine is shown by the horizontal bars. *A*, in this on-line pen recording, an initial short 10 mM caffeine application induced a $\Delta[Ca^{2+}]_c$ of 1460 nM and an $I_{K(Ca)}$ of 3 nA. After 30 s washout, a second 20 s caffeine exposure (also at 10 mM) induced a $\Delta[Ca^{2+}]_c$ of 510 nM and a $I_{K(Ca)}$ of 0·4 nA. After a 60 s washout, a third caffeine application (10 mM) induced a $\Delta[Ca^{2+}]_c$ of 630 nM and a $I_{K(Ca)}$ of 1·4 nA. *B* and *C* show the depression and recovery of caffeine-induced $I_{K(Ca)}$ (*B*) and $\Delta[Ca^{2+}]_c$ (*C*) as a function of the period of time after peak of the first caffeine exposure to which the amplitudes are normalized. The change of the signals is attributed to Ca^{2+} depletion and refilling of caffeine-sensitive SR close to the sarcolemma ($I_{K(Ca)}$) or throughout the cell ($\Delta[Ca^{2+}]_c$). Different symbols refer to different experiments.

The more rapid increase in $[Ca^{2+}]_{sl}$ compared with $[Ca^{2+}]_c$ may be due to the diffusion of caffeine. The equilibration of extra- and intracellular caffeine concentrations requires *ca* 0·5 s (O'Neill *et al.* 1990). During wash in, caffeine may have initially activated Ca^{2+} release from peripheral SR. During washout, the caffeine concentration may have fallen below the level necessary for Ca^{2+} release more rapidly in the peripheral than in the central SR. However, in the continuous presence of 10 mM caffeine, $I_{K(Ca)}$ still decayed faster than $[Ca^{2+}]_c$ (Fig. 1*B*). That is, $I_{K(Ca)}$ and $\Delta[Ca^{2+}]_c$ still followed a different time course under conditions in which caffeine is thought to be homogeneously distributed in the cytosol.

Figure 1*C* compares $I_{K(Ca)}$ and $\Delta[Ca^{2+}]_c$ recorded from a cell to which 10 mM caffeine was applied for 30 s at a holding potential of 0 mV. In general, the dissociation in the time courses was similar to those described above. Quantitatively, the less negative membrane potential was associated with a larger amplitude of $I_{K(Ca)}$. In inside-out patches, the apparent K_D of the Ca^{2+}-activated 200 pS K^+ channel was 2·3 μM at 0 mV and 7·7 μM at −50 mV (Markwardt & Isenberg, 1992). Hence, the similarity of $I_{K(Ca)}$ at −50 and 0 mV suggests that the BK channel can 'monitor' the changes of $[Ca^{2+}]_{sl}$ at −50 mV without going into Ca^{2+} saturation.

$[Ca^{2+}]_c$ and $I_{K(Ca)}$ during multiple caffeine applications

Suppression of $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$ by preceding caffeine

A second application of 10 mM caffeine for 20 s, following the first one of 10 mM after a 30 s caffeine-free period, induced signals of reduced amplitude; peak $I_{K(Ca)}$ was suppressed to 15% and $\Delta[Ca^{2+}]_c$ to 50% of the respective amplitudes recorded during the first caffeine exposure (Fig. 2*A*). The result that the preceding caffeine exposure suppressed $I_{K(Ca)}$ to a stronger extent than $\Delta[Ca^{2+}]_c$ was found in all cells tested ($n = 7$); on average, $I_{K(Ca)}$ was 22 ± 5% and $\Delta[Ca^{2+}]_c$ was 40 ± 4% of control. This result is in line with the view that the first caffeine exposure had depleted the SR of releasable Ca^{2+} to a larger extent in the peripheral than in the central SR.

Recovery of $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$

Presumably, after a 30 s exposure to caffeine both central and peripheral SR were depleted of releasable Ca^{2+}. The reloading of the SR with Ca^{2+} was tested by applying caffeine a third time. For a better comparison, we selected those results where the third $\Delta[Ca^{2+}]_c$ was similar to the second one (Fig. 2*A*, after a 90 s caffeine-free period). In comparison with the signals during the second caffeine exposure, $I_{K(Ca)}$ had recovered by a factor of 3, although it was still smaller than $I_{K(Ca)}$ during the first caffeine application. On average, peak $I_{K(Ca)}$ had recovered from 22% to 61 ± 7%, while $\Delta[Ca^{2+}]_c$ remained at the 40 ± 3% that was recorded as the second signal. The dissociation, recovery of $I_{K(Ca)}$ (Fig. 2*B*) and lack of recovery of $\Delta[Ca^{2+}]_c$ (Fig. 2*C*), suggest that the peripheral SR contributed the major part to $I_{K(Ca)}$ while the central SR contributed much less, as if the peripheral SR were filled with Ca^{2+} preferentially, or quicker than, the central SR. With longer recovery times (3 min), $\Delta[Ca^{2+}]_c$ recovered to *ca* 80% of the value obtained with the first caffeine response, i.e. a complete recovery was not always obtained (not shown).

Can the dissociation of caffeine-induced $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$ be attributed to cAMP-dependent K^+ channel phosphorylation?

Since caffeine inhibits phosphodiesterase, it could have caused elevation of cytosolic cAMP concentrations and phosphorylation of the 200 pS K^+ channels by cAMP- or cGMP-dependent protein kinases (cGMP in cerebral arteries, Robertson *et al.* 1993; cAMP in urinary bladder, Wellner & Isenberg, 1995). Such a mechanism could have caused $[Ca^{2+}]_{sl}$ independent dissociation of $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$. In a first series of experiments to investigate this, the cAMP-dependent kinase was stimulated by bath application of 8-Br-cAMP (0·5 mM) or dB-cAMP (0·2 mM) for 3–6 min. The presence of the membrane-permeant cAMP derivatives did not change either the frequency or the amplitude of STOCs ($n = 4$ for 8-Br-cAMP, $n = 8$ for dB-cAMP). Effects of the cAMP derivatives were recorded in only three out of the twelve cells: in these they increased the resting $[Ca^{2+}]_c$ by 50–80 nM and the steady outward current at −50 mV by 25–60 pA. The smooth muscle cells from the coronary artery responded to cAMP derivatives only exceptionally. This result differs from the regular increase in $I_{K(Ca)}$ reported for other smooth muscle cells (e.g. urinary bladder, Wellner & Isenberg, 1995). In the continuous presence of dB-cAMP, the effects of rapid caffeine application (10 mM) on $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$ and the dissociation in the time course between the two signals were not distinct ($n = 6$) from those in the absence of cAMP derivatives (see above).

In a second series of experiments, the cAMP-dependent protein kinase was inhibited by the inhibitory peptide (PKI) that was dialysed from the patch pipette (4 μM) into the cell for 3–4 min. PKI did not modify the frequency or amplitude of STOCs ($n = 6$; see Fig. 3*A*). Further, in the presence of PKI, there was no response to addition of dB-cAMP (Fig. 3*A*). Four minute dialysis of PKI did not reduce the amplitudes of caffeine-induced $\Delta[Ca^{2+}]_c$ or $I_{K(Ca)}$ ($n = 7$). In experiments with multiple caffeine applications (Fig. 3*B*), the second application induced a $\Delta[Ca^{2+}]_c$ that was 54% of the first one, and this value did not change significantly when caffeine was applied a third time after a 50 s caffeine-free period, and a fourth time after a 60 s caffeine-free period. In contrast, the $I_{K(Ca)}$ response to caffeine was 24, 37 and 95% of the first response, respectively. That is, in the presence of the inhibitory peptide, caffeine-induced $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$ recovered along a different time course ($n = 6$), similar to that in the cells where cAMP-dependent protein kinase was not inhibited. The results favour the idea that the dissociation between global $[Ca^{2+}]_c$ and $I_{K(Ca)}$ is due to a dissociation between global and subsarcolemmal $[Ca^{2+}]$ and not due to a changed relationship between $[Ca^{2+}]_{sl}$ and K^+ channel activity.

Peculiarities of the responses to bath-applied 10 mM caffeine

When 10 mM caffeine was bath applied, the complete solution change took *ca* 5 s and responses varied between the individual experiments. Usually, $I_{K(Ca)}$ and $\Delta[Ca^{2+}]_c$ peaked up to 80% of the respective signals obtained by rapid caffeine application, but after variable delays and with a complex time course. Figure 4 shows a representative example. The response started with a small $I_{K(Ca)}$ that peaked within 0·2 s. When $I_{K(Ca)}$ decayed, $\Delta[Ca^{2+}]_c$ increased at a high rate. One second after the first peak, $I_{K(Ca)}$ was close to the baseline and $\Delta[Ca^{2+}]_c$ became almost time independent (600 nM). Then, $I_{K(Ca)}$ started a second rise and when $I_{K(Ca)}$ was close to its peak, $\Delta[Ca^{2+}]_c$ increased again. The final peak of $\Delta[Ca^{2+}]_c$ occurred 2·3 s after the

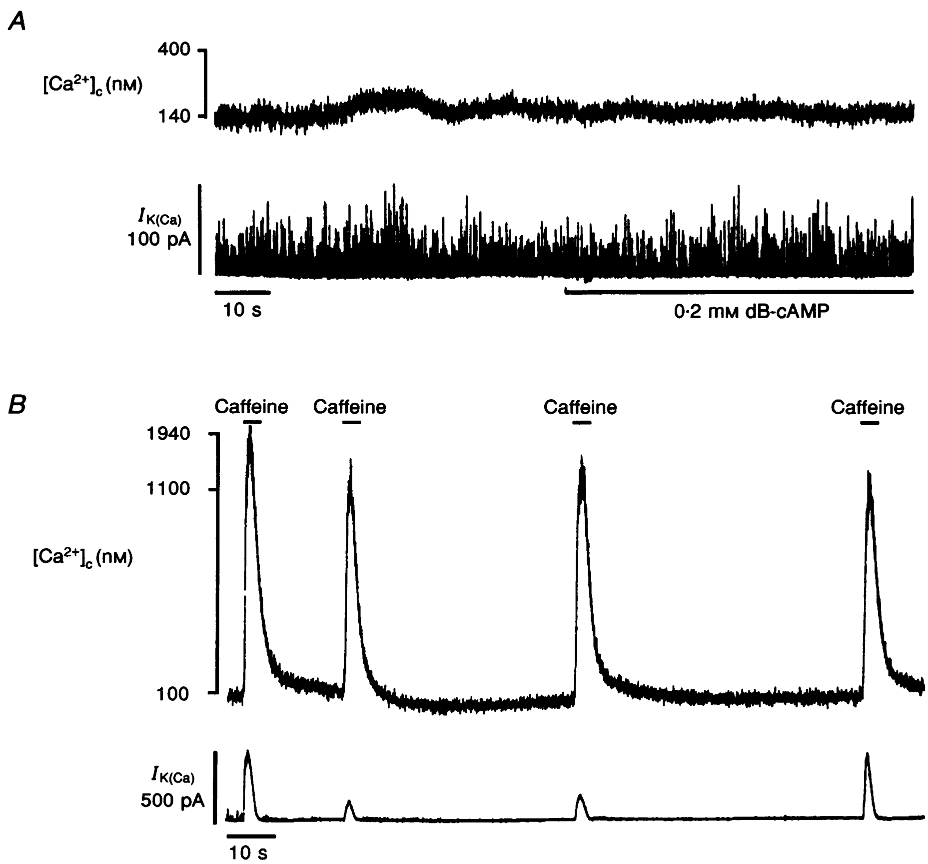

Figure 3. Exogenous cAMP or the inhibitory peptide of cAMP-dependent protein kinase (PKI) do not interfere with STOCs or caffeine-induced $I_{K(Ca)}$

A, STOC activity at −50 mV was not obviously altered by 4 μM PKI, and addition of 0·2 mM dB-cAMP did change their frequency. *B*, PKI (4 μM) was dialysed from the pipette for 4 min before the recordings were started. Events during 4 brief applications of 10 mM caffeine show dissociation between the slow recovery of $\Delta[Ca^{2+}]_c$ and the rapid recovery of $I_{K(Ca)}$, similar to that in the absence of PKI (cf. with Fig. 2). Note the highly non-linear $[Ca^{2+}]_c$ scale.

initial rise, a time that is longer than the 1·5 s time to peak obtained with rapid caffeine application. The results suggest that bath-applied caffeine induced SR Ca^{2+} release from different parts of the cell 'asynchronously', a situation during which simultaneous Ca^{2+} redistribution processes may significantly attenuate $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$ (see Discussion).

$\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$ induced by 1 mM caffeine

Caffeine is thought to induce SR Ca^{2+} release by sensitizing the Ca^{2+} release channel (ryanodine receptor) in such a way that it can be activated by resting $[Ca^{2+}]_c$ (Sitsapesan & Williams, 1990). At low concentrations (1 mM), caffeine may induce Ca^{2+} release locally from those parts of the cell where $[Ca^{2+}]$ is high. The existence of such a spatial $[Ca^{2+}]$ heterogeneity is suggested by two results: $[Ca^{2+}]_c$ increased along a complex time course, and STOCs either did or did not fuse into a synchronized $I_{K(Ca)}$.

When caffeine was rapidly applied at a concentration of 1 mM, three out of seven cells responded with $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$ signals that did not significantly differ from their counterparts induced by 10 mM caffeine. In the other four cells, $[Ca^{2+}]_c$ increased at a low rate to a late peak of *ca* 1000 nM (time to peak, 4 s; see Fig. 5*A*). During the rising phase of $\Delta[Ca^{2+}]_c$, multiple STOCs occurred. After 4 s, caffeine was washed out, $\Delta[Ca^{2+}]_c$ decayed and STOCs were absent. This suppression of STOCs is remarkable because global $[Ca^{2+}]_c$ was still high and could have activated K^+ channels.

Figure 5*B* shows the complex situation during a 15 s application of 1 mM caffeine. Initially, caffeine induced a short-lasting $I_{K(Ca)}$, and simultaneously, $[Ca^{2+}]_c$ increased rapidly by 100 nM. Afterwards, $I_{K(Ca)}$ fell but $\Delta[Ca^{2+}]_c$ continued to increase. One and a half seconds later, STOCs occurred, while $[Ca^{2+}]_c$ was almost constant. Finally, when the STOCs fused to a 'synchronized' $I_{K(Ca)}$, $[Ca^{2+}]_c$ increased more steeply again. The washout of caffeine caused a near-immediate disappearance of $I_{K(Ca)}$ while $[Ca^{2+}]_c$ decayed along a slow time course. Twenty seconds later, when $[Ca^{2+}]_c$ was already below 300 nM, multiple STOCs occurred again.

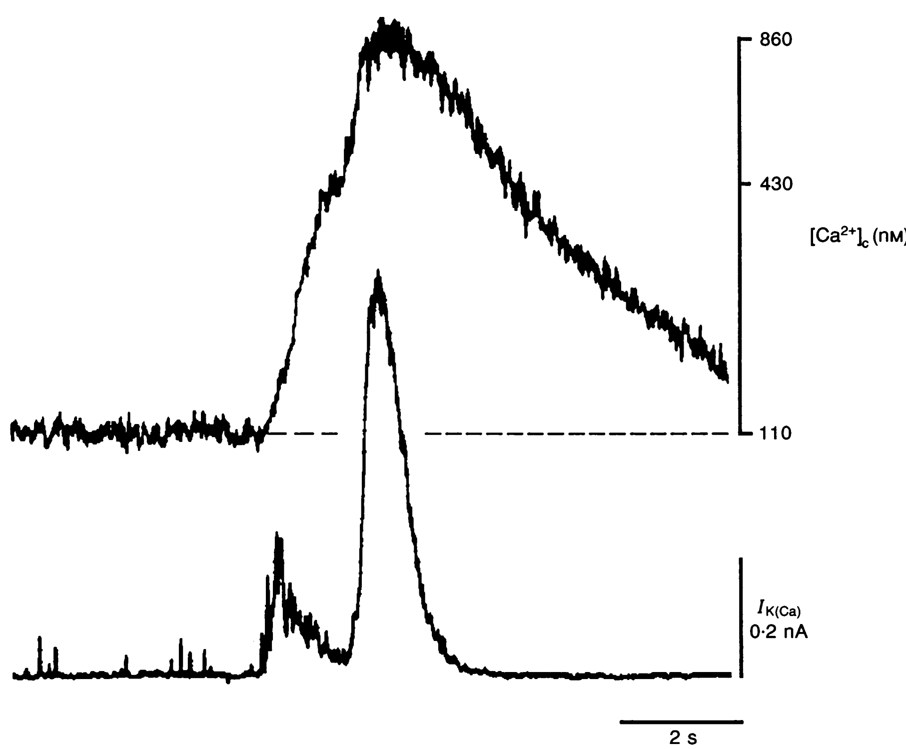

Figure 4. $I_{K(Ca)}$ and $\Delta[Ca^{2+}]_c$ during slow bath application of 10 mM caffeine

Holding potential, −50 mV. $I_{K(Ca)}$ peaked after 2 s and $\Delta[Ca^{2+}]_c$ after 2·3 s. The early hump in $I_{K(Ca)}$ and the several inflexion points in $\Delta[Ca^{2+}]_c$ are interpreted as 'asynchronous' Ca^{2+} release from different parts of the cell due to the slow distribution of caffeine within the cell.

Figure 5C shows STOCs at a holding potential as negative as −50 mV, as had also occurred in approximately 50% of isolated guinea-pig coronary myocytes (Ganitkevich & Shuba, 1988). Caffeine (1 mM) increased the frequency of STOCs. Caffeine also increased $[Ca^{2+}]_c$ within 10 s to a constant value of *ca* 300 nM. $[Ca^{2+}]_c$ increased again up to a final peak of 450 nM 12 s after caffeine application, at the moment where the STOCs fused to an $I_{K(Ca)}$. Generally, the fusion of individual STOCs to a synchronized $I_{K(Ca)}$ correlated with a high rate of increase in $[Ca^{2+}]_c$. However, the occurrence of such a fusion of STOCs was unpredictable, hence a more detailed evaluation was not possible.

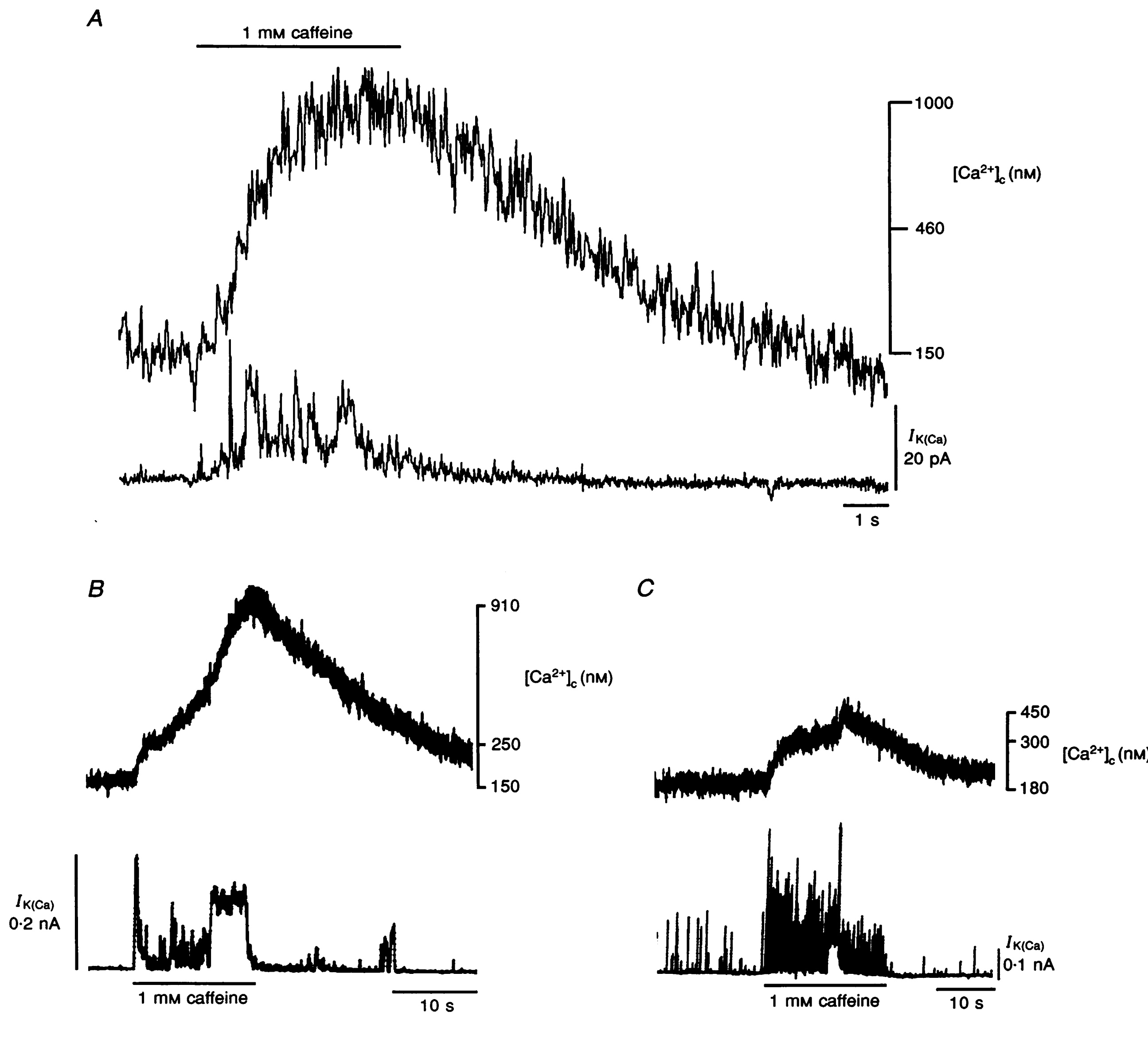

Figure 5. $\Delta[Ca^{2+}]_c$ and spontaneous transient outward currents (STOCs) during application of 1 mM caffeine

Holding potential −30 mV (*A* and *B*) or −50 mV (*C*). *A*, this computer play-back shows that during the increase in $[Ca^{2+}]_c$, multiple STOCs occured. After 4 s, when $\Delta[Ca^{2+}]_c$ decayed, STOCs were absent although $\Delta[Ca^{2+}]_c$ was still elevated. *B* and *C* are on-line pen recordings. In *B*, during a 15 s application of 1 mM caffeine, initial short-lasting $I_{K(Ca)}$ appeared simultaneously with a $\Delta[Ca^{2+}]_c = 100$ nM. During the ensuing decay of $I_{K(Ca)}$, $\Delta[Ca^{2+}]_c$ did not increase further. Enhanced STOCs frequency occurred together with increased $\Delta[Ca^{2+}]_c$ again. When STOCs fused to $I_{K(Ca)}$, $[Ca^{2+}]_c$ increased at a high rate up to 910 nM. During washout of caffeine, $[Ca^{2+}]_c$ decayed and STOCs were almost absent. *C* shows a cell in which STOCs appeared before caffeine application. Caffeine increased the frequency of STOCs, while simultaneously, $\Delta[Ca^{2+}]_c$ increased to 300 nM. When STOCs fused, $[Ca^{2+}]_c$ increased further to 450 nM.

STOCs and spontaneous $\Delta[Ca^{2+}]_c$ in non-stimulated cells

In coronary myocytes clamped to a holding potential of −50 mV, $[Ca^{2+}]_c$ was not absolutely constant, but fluctuated. Usually, the spontaneous $[Ca^{2+}]_c$ fluctuations were most obvious briefly after cell access, i.e. during the period of indo-1 loading. During the ensuing 2–3 min, the amplitude of the fluctuations became smaller, until *ca* 5 min after cell access, $[Ca^{2+}]_c$ seemed to be stable. The spontaneous $[Ca^{2+}]_c$ fluctuations were small or absent in those cells that did not show STOCs; these fluctuations could be resolved if their amplitudes were larger than 30 nM, a situation that occurred in *ca* 50% of the cells. On average, $[Ca^{2+}]_c$ fluctuated by ±40 nM around a mean value of 160 nM (initial 3 min, $n = 11$).

The three panels of Fig. 6 show typical situations to indicate how the appearance of $\Delta[Ca^{2+}]_c$ fluctuations and STOCs correlated. Panel *A* shows records from a cell that generated a single STOC of long duration (0·5 s) and large amplitude (*ca* 0·1 nA) at regular intervals of *ca* 6 s. During each of the large STOCs, $\Delta[Ca^{2+}]_c$ increased by *ca* 100 nM, and during the ensuing intervals, $[Ca^{2+}]_c$ decayed. The traces of Fig. 6*B* illustrate a situation where $[Ca^{2+}]_c$ fell during the periods without STOCs and rapidly increased when one large STOC occurred after this period of silence. The ensuing appearance of STOCs of smaller amplitude did not further increase $[Ca^{2+}]_c$ but stabilized it at the elevated level. In the traces of Fig. 6*C* the STOCs changed spontaneously to a higher frequency and large amplitude, and $[Ca^{2+}]_c$ increased simultaneously. At the end of this burst of STOCs, $[Ca^{2+}]_c$ decayed.

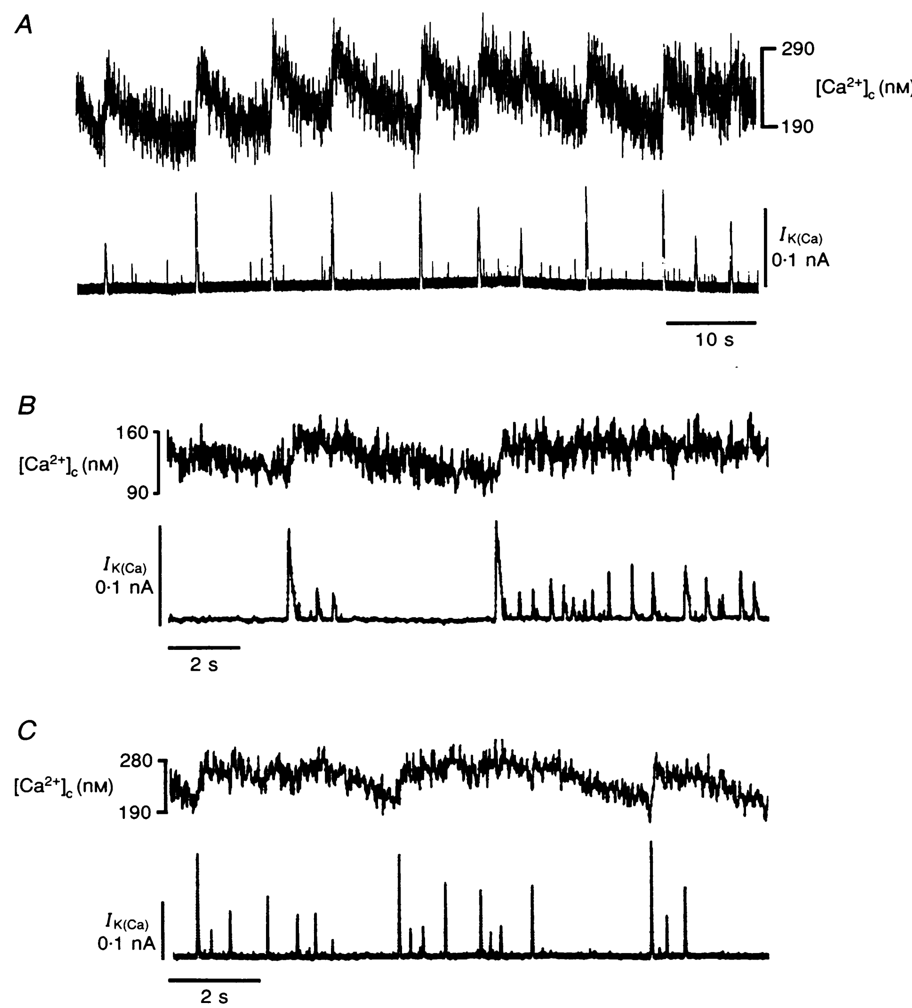

Figure 6. Spontaneous $[Ca^{2+}]_c$ fluctuations in comparison with STOCs

A, *B* and *C* are representative pen recordings. *A*, holding potential, −50 mV. STOCs of large amplitude and long duration appeared ahead of $\Delta[Ca^{2+}]_c \approx 100$ nM. *B*, holding potential, −40 mV. Decay of $[Ca^{2+}]_c$ occurred preferentially during periods in which STOCs were absent. *C*, holding potential, 0 mV. Occurrence of $\Delta[Ca^{2+}]_c \approx 90$ nM was preceded by large STOCs as at negative potentials (*A* and *B*).

Time course of spontaneous $\Delta[Ca^{2+}]_c$ follows time integral of STOCs

As already mentioned, in the coronary myocytes demonstrating STOCs, $[Ca^{2+}]_c$ was not constant but fluctuated by up to 100 nM. These spontaneous $[Ca^{2+}]_c$ fluctuations followed a time course that seemed to be related to the appearance of augmented or fused STOCs. In particular, there was no time delay between the start of an augmented STOC and the increase in $[Ca^{2+}]_c$. This prompted us to fit the time-dependent fluctuations with:

$$\Delta[Ca^{2+}]_c(t) = \alpha\int\{\text{STOC} - \gamma\}dt \approx \alpha\int\{I_{rel}(t) - \gamma\}dt, \quad (1)$$

where α is the proportionality constant (nM pC^{-1}), $I_{rel}(t)$ is the Ca^{2+} release flux, and γ is the redistribution current (pA). Although primarily eqn (1) was used for the purpose of an empirical description, it was sufficiently flexible to fit the data from eleven out of twelve cells. Since eqn (1) nicely fits the data, this may suggest that the events are causally related. Equation (1) postulates that the increase in $[Ca^{2+}]_c$ is a linear function of the time integral of STOCs (proportionality constant α, nM pC^{-1}). In the Discussion, it will be argued that the amplitude and duration of STOCs may reflect the amplitude and duration of the Ca^{2+} release flux, $I_{rel}(t)$, from peripheral SR into the gap space beneath the sarcolemma. Equation (1) approximates the decay of $[Ca^{2+}]_c$ by a single $[Ca^{2+}]_c$-independent first-order process, the several Ca^{2+} redistribution fluxes being summarized by a Ca^{2+} redistribution current, γ (pA).

Figure 7 shows that eqn (1) fits the spontaneous $\Delta[Ca^{2+}]_c$ fluctuations sufficiently well with different patterns of STOCs. The results of panel *A* were recorded at −50 mV. A large STOC induced a $\Delta[Ca^{2+}]_c$ of *ca* 150 nM while the small individual STOCs did not have a counterpart in $[Ca^{2+}]_c$. Panel *B* was recorded at 0 mV. When STOCs occurred in groups, $[Ca^{2+}]_c$ increased by *ca* 90 nM. During the period between the STOCs, $[Ca^{2+}]_c$ returned back to baseline at almost constant rate.

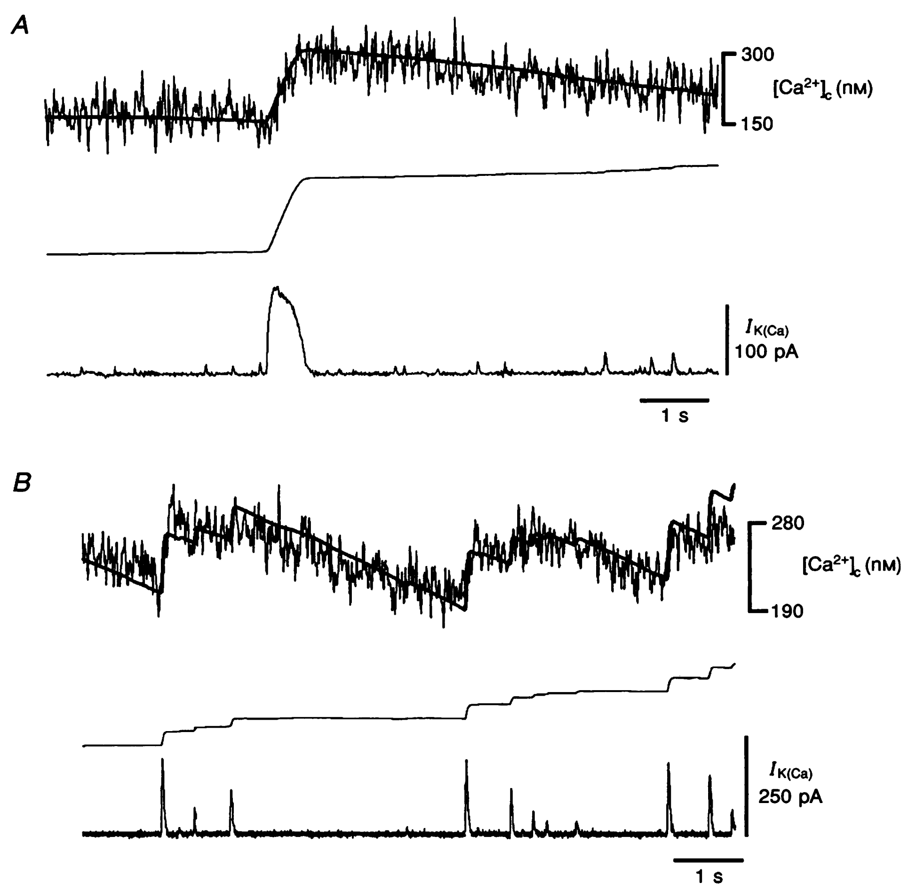

Figure 7. Time course of spontaneous $\Delta[Ca^{2+}]_c$ follows time integral of STOCs

A and *B* are computer play-backs from 2 representative cells. Traces from top to bottom in both *A* and *B* are: $\Delta[Ca^{2+}]_c$ (noisy trace); $\alpha\int\{I_{K(Ca)}(t)\}dt$ (uncalibrated); STOCs representing $I_{K(Ca)}$. The continuous line in the top fits $\alpha\int\{I_{K(Ca)}(t) - \gamma\}dt$ through $\Delta[Ca^{2+}]_c$, where α is a proportionality factor (nM pC^{-1}) and γ (pA) is a $[Ca^{2+}]_c$ independent current representing Ca^{2+} redistribution processes. *A*, holding potential, −50 mV. An unusually large spontaneous $\Delta[Ca^{2+}]_c \approx 150$ nM related to a large and long STOC. *B*, holding potential, 0 mV. The time integral of multiple STOCs followed the time course of spontaneous $\Delta[Ca^{2+}]_c$.

Modulation of STOCs during La^{3+} block of sarcolemmal Ca^{2+} fluxes

The decay of spontaneous $\Delta[Ca^{2+}]_{sl}$ could be due to Ca^{2+} diffusion from peripheral SR into the deeper cytosol, or to Ca^{2+} extrusion through the sarcolemma. To identify which process was rate limiting, it would be ideal to block Ca^{2+} extrusion, but there is no known specific blocker. Instead, all sarcolemmal Ca^{2+} fluxes can be blocked with 1 mM lanthanum (Bond, Kitazawa, Somlyo & Somlyo, 1984). The rapid change from 2·5 mM external Ca^{2+} to 0 mM external Ca^{2+} plus 1 mM external La^{3+} reduced the amplitude of STOCs immediately (Fig. 8*A*), presumably by a fast-flickering block (in analogy of Ba^{2+} block of K^+ channels, Yellen, 1984). La^{3+} moderately reduced the frequency of STOCs ($2 \pm 0{\cdot}7$ Hz before and $1{\cdot}4 \pm 1$ Hz after La^{3+}). Most importantly, however, the individual STOC decayed along a time course that was not markedly retarded by La^{3+} (Fig. 8*B* and four other cells). Assuming that the decay of a STOC reflects the decay of local subsarcolemmal $[Ca^{2+}]$, the ineffectiveness of La^{3+} suggests that Ca^{2+} extrusion via PMCA is not the rate-limiting process for the decay of $[Ca^{2+}]_{sl}$. Instead, this rate may be determined by Ca^{2+} diffusion from the subsarcolemmal gap space into the cytosol and/or by Ca^{2+} reuptake by SERCA from the gap space into the peripheral SR.

The above interpretation seems to conflict with the effect of La^{3+} on the spontaneous $\Delta[Ca^{2+}]_c$ (Fig. 8*B*, 90 nM). In the presence of 1 mM La^{3+}, $[Ca^{2+}]_c$ no longer decayed, but instead increased to a level of 280 nM (on average, 310 ± 60 nM, $n = 5$). Upon washout of La^{3+}, $[Ca^{2+}]_c$ rapidly returned to the resting level, and the spontaneous $\Delta[Ca^{2+}]_c$ reappeared. In terms of eqn (1), the effect of La^{3+} on spontaneous $\Delta[Ca^{2+}]_c$ would suggest that La^{3+}-sensitive Ca^{2+} extrusion does contribute to the redistribution

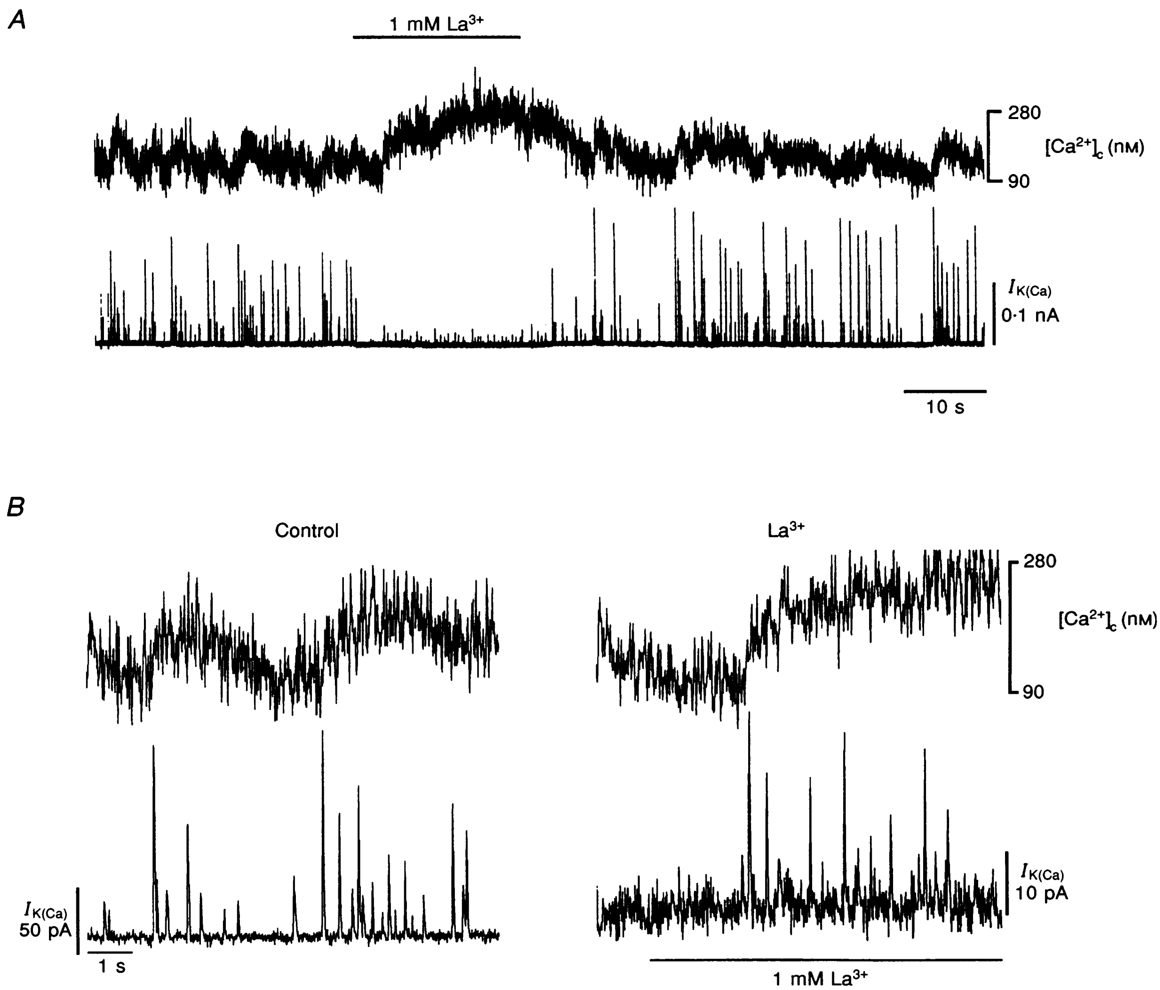

Figure 8. Block of sarcolemmal Ca^{2+} fluxes by 1 mM La^{3+} does not considerably retard the rate at which STOCs decay

Holding potential, −50 mV. *A*, on-line pen recording. *B*, computer play-backs during control (left traces) and during La^{3+} application (right traces). Note the different calibration of current and the reversible increase in $[Ca^{2+}]_c$ induced by La^{3+}.

constant γ of global $[Ca^{2+}]_c$. The discrepancy between decay of STOCs (unmodified by La^{3+}) and the decay of $\Delta[Ca^{2+}]_c$ (inhibited by La^{3+}) points out once more the discrepancy between local (STOCs) and global ($\Delta[Ca^{2+}]_c$) concentrations. On the other hand, La^{3+} has been reported as entering the chromaffin cells via the Na^+–Ca^{2+} exchanger, where it triggers catecholamine release (Powis, Clark & O'Brien, 1994); if this property could be extrapolated to the coronary smooth muscle cell, La^{3+} may have induced the increase in $[Ca^{2+}]_c$ due to a sustained Ca^{2+} release from peripheral SR. Future studies with more specific PMCA inhibitors may clarify this point.

DISCUSSION

This study presents evidence for the dissociation of the concentrations of subsarcolemmal Ca^{2+}_{sl}, indicated by $I_{K(Ca)}$ or by STOCs, and of global cytosolic Ca^{2+}, indicated by indo-1 fluorescence. For methodological reasons explained in the introduction, we did not evaluate absolute Ca^{2+} concentrations and their differences; instead, the comparison was restricted to qualitative differences in the time course of the Ca^{2+} signals due to SR Ca^{2+} release.

In vascular smooth muscle, electron probe microanalysis has suggested that some peripheral SR vesicles contain calcium in much higher concentrations than others ('calcium hot spots': Bond *et al.* 1984). Benham & Bolton (1986) speculated that a STOC is started by Ca^{2+} leaking from a highly Ca^{2+}-filled peripheral SR vesicle, the Ca^{2+} leakage increasing local $[Ca^{2+}]$ above the threshold for Ca^{2+} activation of the neighbouring ryanodine receptors, which finally leads to a regenerative Ca^{2+}-induced Ca^{2+} release (CICR) from this SR vesicle. Presumably, this local CICR does not propagate to neighbouring peripheral SR vesicles in smooth muscle cells (Kargacin & Fay, 1991; Kargacin, 1994; Ganitkevich & Isenberg, 1995).

To our knowledge, this is the first study in vascular myocytes where STOCs could be correlated with spontaneous $[Ca^{2+}]_c$ fluctuations measured fluometrically. After rupture of the patch, the spontaneous $\Delta[Ca^{2+}]_c$ could be measured only for a limited period of *ca* 3 min while the STOC activity continued. The dissociation of the two phenomena may be attributed to cell dialysis of the exogenous Ca^{2+} buffer indo-1. Inside the 20–50 nm narrow gap between the peripheral SR and the sarcolemma, indo-1 is unlikely to significantly buffer $\Delta[Ca^{2+}]_{sl}$ (cf. Stern, 1992). Outside the gap, however, cytosolic diffusion of Ca^{2+}-indo-1 can easily replace free indo-1 and vice versa (cf. the model of Nowycky & Pinter, 1993). That is, in the absence of membranes hindering diffusion, 100 μM indo-1 may buffer the local $[Ca^{2+}]$ to such an extent that it cannot reach the threshold concentration necessary for induction of Ca^{2+} release from the neighbouring ryanodine receptors. However, the washout of an unknown cytosolic constituent cannot be excluded as an alternative explanation.

The $\Delta[Ca^{2+}]_c$ fluctuations followed a time course that related to the time course of the STOCs. Equation (1) interprets the STOC as being caused by the Ca^{2+} release flux I_{rel} from peripheral SR. The fit of the results with eqn (1) suggests that $\Delta[Ca^{2+}]_c$ follows the onset of I_{rel} without measurable time delay, as the decay of $[Ca^{2+}]_c$ started immediately after the end of the STOC (I_{rel}). The absence of a delay between I_{rel} (STOC) and the change in $[Ca^{2+}]_c$ suggests that Ca^{2+} release from the peripheral SR is directed towards both sarcolemma and central cytosol. That is, this result does not support the hypothesis that Ca^{2+} is released vectorially from the peripheral SR selectively into the subsarcolemmal gap space. If this were the case, the hindered diffusion from the gap space into the cytosol would have introduced a time delay between I_{rel} and the start of $\Delta[Ca^{2+}]_c$.

The hypothesis of 'vectorial Ca^{2+} release from peripheral SR' was published with the idea that the subsarcolemmal gap space forms a functional Ca^{2+} extrusion compartment (Chen, Cannell & van Breemen, 1992); the high $[Ca^{2+}]_{sl}$ was thought to stimulate Ca^{2+} extrusion via PMCA and Na^+–Ca^{2+} exchange. The present results do not exclude such a possibility, but they suggest that Ca^{2+} extrusion is not restricted to subsarcolemmal areas with high $[Ca^{2+}]_{sl}$. The hypothesis of the 'Ca^{2+} extrusion compartment' predicts that block of Ca^{2+} efflux by La^{3+} should retard the rate of decay of $[Ca^{2+}]_{sl}$. Experimentally, such an effect was not recorded, i.e. La^{3+} did not modify the decay of the STOCs. Hence, one can conclude that the decay of $[Ca^{2+}]_{sl}$ is governed not only by Ca^{2+} extrusion but by Ca^{2+} diffusion from the gap space into the central cytosol, or by Ca^{2+} reuptake into the peripheral SR.

The large amplitude of caffeine-induced $\Delta[Ca^{2+}]_c$ suggests that caffeine synchronously activates Ca^{2+} release from peripheral and central SR in all parts of the cell. During fast application of 10 mM caffeine, caffeine rapidly equilibrates in less than 0·5 s with the cytosol (O'Neill *et al.* 1990). Correspondingly, the caffeine-induced $\Delta[Ca^{2+}]_c$ was a synchronized response, characterized not by STOCs but by a large synchronized $I_{K(Ca)}$. A similar synchronized response may hold true for inositol 1,4,5-trisphosphate (IP_3) and IP_3-induced Ca^{2+} release. Presumably, both caffeine- and IP_3-induced Ca^{2+} release can be synchronized much more easily than Ca^{2+}-induced Ca^{2+} release where the trigger effect on the release channels is diminished when a small amount of Ca^{2+} ions diffuses through the cytosol with Ca^{2+} binding and Ca^{2+} sequestration (cf. Ganitkevich & Isenberg, 1995).

In this study, caffeine increased $\Delta[Ca^{2+}]_c$ and $I_{K(Ca)}$ without a measurable delay (< 20 ms), and the signals peaked within 1·5 and 0·3 s, respectively. This result contrasts with previous reports where the caffeine-induced $\Delta[Ca^{2+}]_c$ delayed the onset of $I_{K(Ca)}$ by several seconds and peaked within 30 s (Stehno-Bittel & Sturek, 1992). Most likely, the difference results from the method of caffeine application. When we applied caffeine with the bath rather than the picospritzer, $[Ca^{2+}]_c$ increased with multiple waves (Fig. 4). If the time resolution of the recording system does not follow the time course of the individual components, the superimposition of unsynchronized Ca^{2+} release with the simultaneous Ca^{2+} redistribution via SERCA and PMCA will delay the onset of the 'main wave' of $\Delta[Ca^{2+}]_c$ and reduce its amplitude. Despite the different time course, the present results confirm the basic feature of Ca^{2+} release in coronary myocytes: during caffeine-induced Ca^{2+} release, $[Ca^{2+}]_{sl}$ precedes $[Ca^{2+}]_c$ (Stehno-Bittel & Sturek, 1992).

During caffeine-induced Ca^{2+} release, $[Ca^{2+}]_{sl}$ increased and decayed faster than global $[Ca^{2+}]_c$. This result confirms the more direct measurement of $[Ca^{2+}]_{sl}$ with the membrane-associated Ca^{2+} indicator C_{18}-fura-2 (Etter, Kuhn & Fay, 1994*a*): $[Ca^{2+}]_{sl}$ peaked 3 times earlier than $[Ca^{2+}]_c$ measured globally by fura-2. Unfortunately, only 60% of C_{18}-fura-2 is distributed to the plasma membrane, 40% to the cytosol (Etter, Poenie, Minta & Fay, 1994*b*), and this distribution hampers the quantitative evaluation of $[Ca^{2+}]_{sl}$ with a photomultiplier system. Qualitatively, the results of Etter *et al.* (1994*a*) are in line with the idea of a radial Ca^{2+} concentration gradient directed from the sarcolemma into the cell during the period of Ca^{2+} influx (Etter *et al.* 1994*a*), and with the idea of SR Ca^{2+} release (present paper and Stehno-Bittel & Sturek, 1992). Later, the direction of the Ca^{2+} concentration gradient may reverse and point from the centre towards the sarcolemma. A similar Ca^{2+} gradient may also exist during IP_3-induced Ca^{2+} release, as suggested in the literature (the time course of $I_{K(Ca)}$ is faster than that of $\Delta[Ca^{2+}]_c$ in Fig. 5 of Komori & Bolton, 1991 and in Fig. 2*B* of Loirand, Grégoire & Pacaud, 1994). Hence, cytosolic Ca^{2+} concentration gradients seem to be a general phenomenon.

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