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Regenerative caffeine-induced responses in native rabbit aortic endothelial cells

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1 Single native aortic endothelial cells obtained by enzymatic dispersion of the rabbit aortic endothelium were held under voltage clamp using patch pipette and whole-cell membrane currents were measured. In parallel experiments performed on cells from the same batches, the free internal calcium concentration, $[Ca^{2+}]_i$, in the cell was estimated by use of the Ca^{2+}-sensitive fluorescent dye, fura-2.

2 Caffeine (20 mM) applied to the cell evoked an outward current and an initial peak in $[Ca^{2+}]_i$ followed by a lower sustained rise (plateau). Ca^{2+}-free, EGTA-containing solution applied outside the cells did not reduce these responses.

3 Following caffeine stimulation there was a biphasic rising phase of outward current both in the presence and absence of extracellular Ca^{2+}.

4 Application of graded doses of caffeine revealed all-or-none type responses of both the outward current and the rise in $[Ca^{2+}]_i$.

5 Preincubation with lower doses of caffeine reduced the magnitude of both the outward current and the $[Ca^{2+}]_i$ transient evoked by 20 mM caffeine.

6 Tetraethylammonium (3 mM) applied to the bathing solution blocked unitary and spontaneous transient outward currents (STOCs) stimulated by Ca^{2+}-free solution, but only reduced the outward current evoked by caffeine (20 mM).

7 In conclusion, our results reveal the all-or-none nature of Ca^{2+} release from the endoplasmic reticulum (ER) in native aortic endothelial cells. Lower concentrations of caffeine (0.4-0.5 mM) may deplete intracellular Ca^{2+} stores. Extracellular Ca^{2+} is not necessary for maintaining the activity of spontaneous and caffeine-induced outward currents in native aortic endothelial cells. Spontaneous outward currents are believed to represent the sporadic release of calcium from store sites independent of both extracellular Ca^{2+} and the caffeine-sensitive Ca^{2+} stores which stimulate the outward current.

Keywords: Aortic endothelial cells; caffeine; Ca^{2+}-induced Ca^{2+} release; all-or-none response

Introduction

It is widely recognized that most cells employ both intracellular and extracellular sources of Ca^{2+} in cellular signaling. In native rabbit aortic endothelial cells, the dependence of both unitary and spontaneous transient outward currents (STOCs, characterized by a large amplitude $\leqslant 50$ pA) on the internal concentration of Ca^{2+} and EGTA shows that internal Ca^{2+} concentration is important for regulating this activity (Rusko *et al.*, 1992). The currents probably represent the release of stored calcium close to the internal surface of the plasma membrane, causing a temporary and localized rise in $[Ca^{2+}]_i$ which activates potassium channels similar to those described in smooth muscle cells (Benham & Bolton, 1986; Ohya *et al.*, 1987).

The studies of endothelial cells that employed fluorescent Ca^{2+} indicators revealed that endothelium-dependent vasodilators (e.g. bradykinin) activate the cells by eliciting a large transient increase in cytosolic calcium derived from intracellular stores, followed by a smaller sustained increment caused by influx of Ca^{2+} (Johns *et al.*, 1987; Schilling, 1989; Sage *et al.*, 1989; Luckhoff & Busse, 1990; Laskey *et al.*, 1990; Campbell *et al.*, 1991; Buchan & Martin, 1991). This process is probably responsible for subsequent opening of Ca^{2+}-activated K^+ channels stimulated by bradykinin and other agonists (e.g. ATP, acetylcholine) in native aortic endothelial cells (Rusko *et al.*, 1992).

It has been established that the release of Ca^{2+} from internal stores is related to an increase in inositol-1,4,5-trisphosphate (Freay *et al.*, 1989; Schilling & Elliott, 1992). The contribution of the second known Ca^{2+} release mechanism; namely, Ca^{2+}-induced Ca^{2+} release (CICR) in endothelial cells is controversial. While in the presence of extracellular Ca^{2+}, caffeine (5 mM) induced a small elevation of $[Ca^{2+}]_i$ (Buchan & Martin, 1991) this increase in $[Ca^{2+}]_i$was not observed in another study using a higher concentration of caffeine (10 mM) (Schilling & Elliott, 1992). It was suggested that Ca^{2+}-induced Ca^{2+} release either does not contribute to agonist-induced changes in $[Ca^{2+}]_i$ in endothelial cells or has a different pharmacological sensitivity compared with that in skeletal or cardiac muscle.

However, recently it has been shown that human aortic endothelial cell lines responded to caffeine (10 mM) by elevation of $[Ca^{2+}]_i$, but only when placed in low $[Mg^{2+}]_o$ (0.3 mM) (Zhang *et al.*, 1993). These results indicate the need for studying the effect of caffeine in the regulation of $[Ca^{2+}]_i$ to elucidate the mechanism(s) of intracellular Ca^{2+} release and subsequent activation of potassium channels in native aortic endothelial cells.

Recent studies have demonstrated that the membrane hyperpolarization of coronary endothelial cells induced by caffeine is concentration-dependent and is sensitive to the removal of Ca^{2+} from the perfusing solution (Chen & Cheung, 1992). Although the physiological function of the Ca^{2+}-dependent K^+ channels is unknown, channel activation may hyperpolarize the endothelial cell and increase the

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driving force for Ca^{2+} entry during stimulation by agonists (Busse *et al.*, 1988; Schilling, 1989; Luckhoff & Busse, 1990).

The purpose of the present study was to describe evidence for the relationship between intracellular stores and the plasmalemma in native aortic endothelial cells. We have examined the effects of the Ca^{2+}-release activator, caffeine, on correlation between membrane currents and $[Ca^{2+}]_i$ in the presence and absence of extracellular Ca^{2+} using whole-cell membrane current recordings under voltage clamp and parallel cytosolic $[Ca^{2+}]_i$ measurements using imaging fluorescence microscopy.

Methods

Preparation of cells

Rabbits of either sex weighing (1.5–2.2 kg) were anaesthetized and killed by carbon dioxide. Freshly dissociated endothelial cells were obtained from the aorta by procedures previously described (Rusko *et al.*, 1992). Briefly, several pieces of endothelium were incubated at 37°C for 35 min in dispersal solution containing 0.9 mg ml^{-1} papain and 0.8 mg ml^{-1} dithiothreitol. After the enzyme digestions, tissue fragments were washed with enzyme-free, Dulbecco's phosphate-buffered

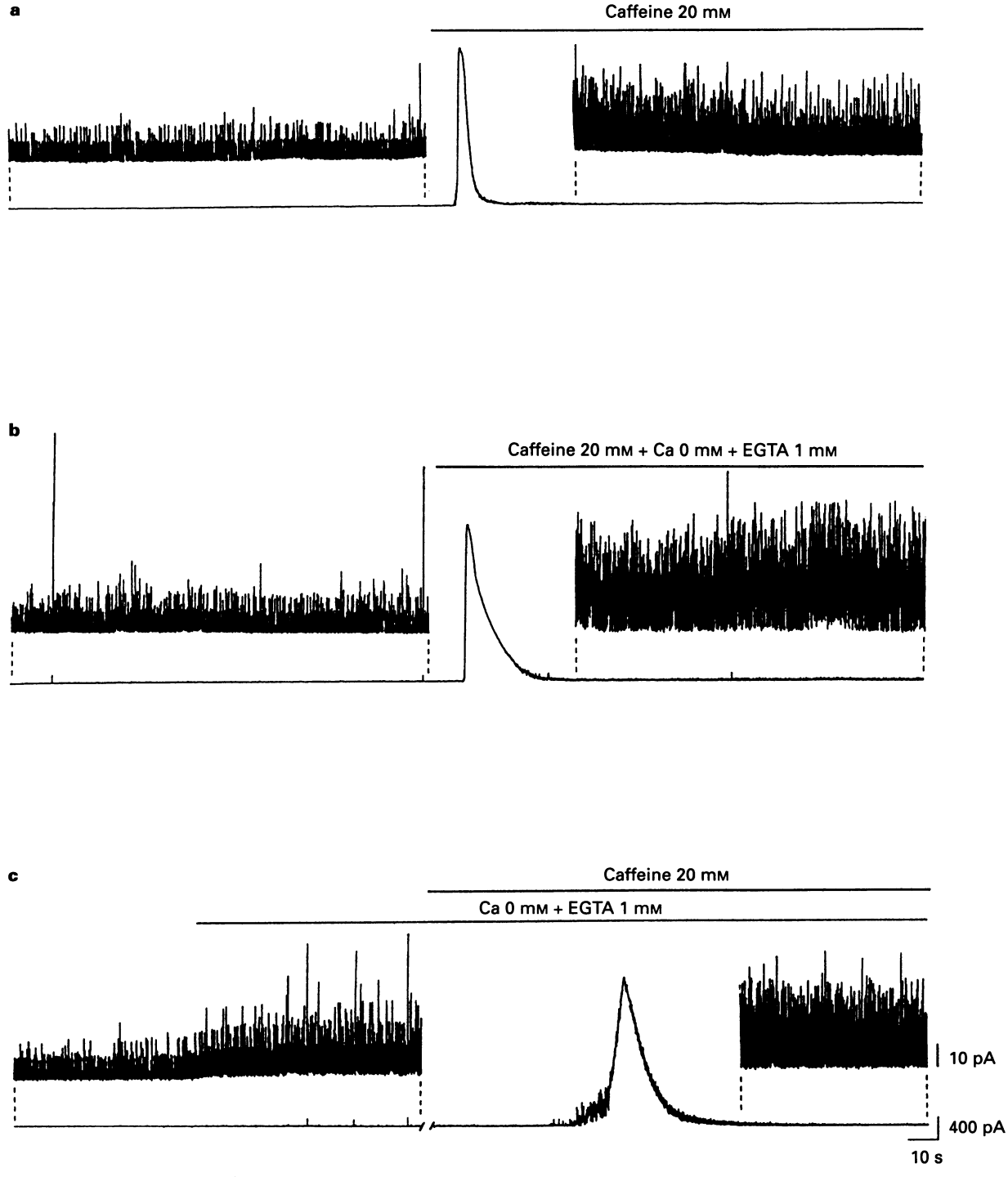

Figure 1 Responses of endothelial cells to caffeine application. Continuous records of whole cell currents in the presence (a) and in absence of extracellular Ca^{2+} (c). (b) Extracellular Ca^{2+} was omitted at the same time as caffeine was applied. The upper traces of each pair show current activity on expanded current scale. Horizontal bars indicate period during which cells were bathed in extracellular solution containing caffeine (20 mM) and no added Ca^{2+}; (a) and (b), the same scale as (c). Outward currents are upward in these and in subsequent records. Holding potential +20 mV.

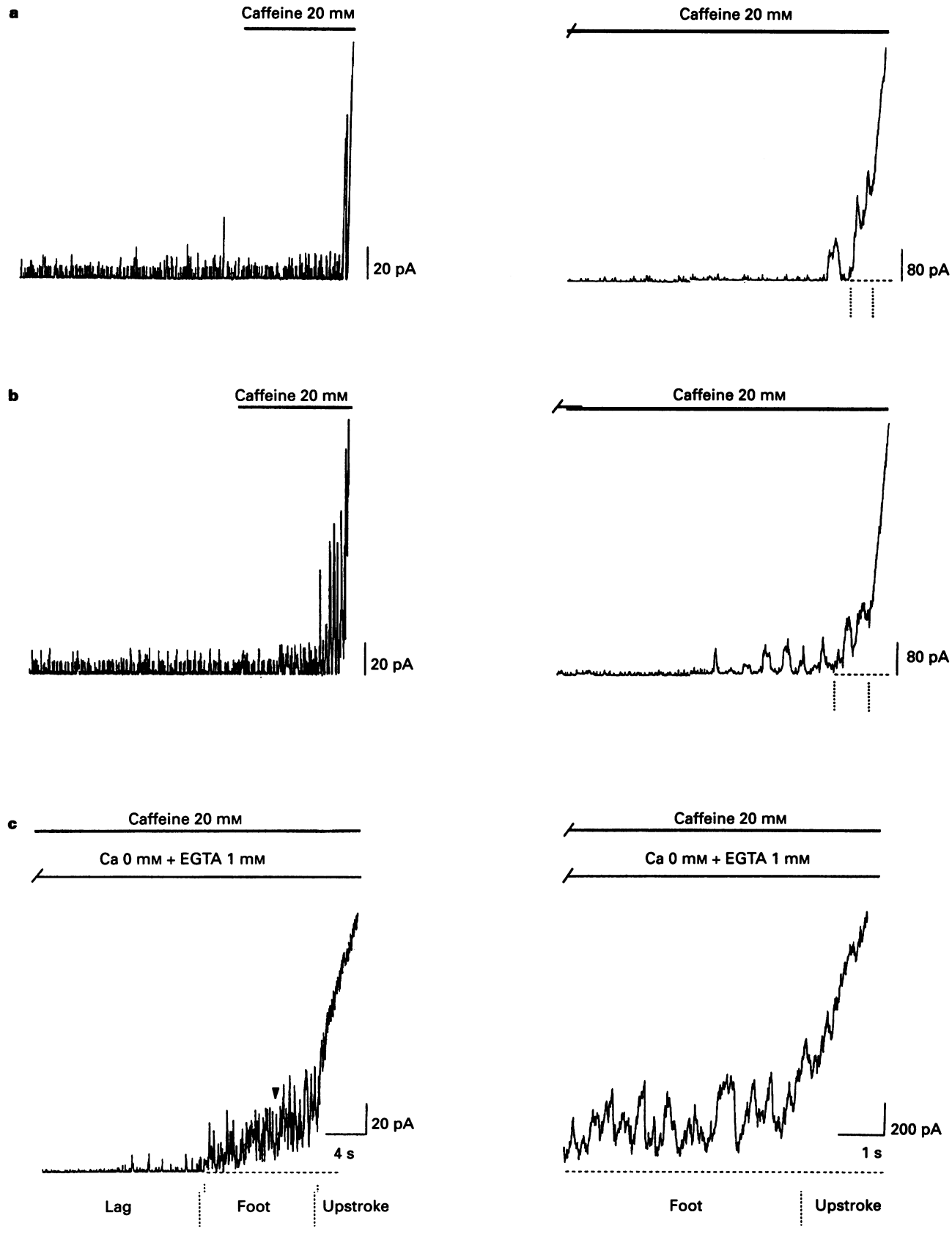

Figure 2 Sections of the rising portions of outward current responses to 20 mM caffeine. (a,b) In the presence of extracellular Ca; (c) response of the cell after 6 min bathing in a solution with no added Ca. Exposure times to caffeine and Ca^{2+}-free, EGTA containing solution are indicated by the horizontal bars. Note the different current scale for the recordings in (a) and (b) compared to (c). The time scales apply to all records in the same column. Right hand records in (a), (b) and (c) are shown at expanded time scale. The transitions to foot and upstroke phases are indicated by the dotted lines. In (c), (▼) at left side indicates the time from which the record at the expanded time scale was made (c, right side). Cells held at +20 mV.

saline, then filtrated and centrifuged. Supernatant containing the cells were resuspended in 0.8 mM-Ca^{2+} containing solution and placed on glass cover-slips. The cells were kept at 4°C until use on the same day.

Electrophysiological recording

Voltage clamp recordings were made from single endothelial cells using standard patch-clamp techniques (Hamill *et al.*, 1981). Whole-cell membrane currents were recorded with borosilicate patch pipettes of 2–5 Mohms. The currents were recorded with a List LM/EPC-7 patch-clamp amplifier (List Electronics), filtered at 2.5 kHz (–3 dB, 4-pole Bessel filter; Ithaco 4302) and stored for later analysis on videotape using a digital VCR recorder adaptor (PCM-1; Medical systems Corp., NY, U.S.A.). Membrane currents were continuously monitored and the amplitude was analyzed by direct measurement on a digital oscilloscope (Tektronix 5223). The currents were displayed on a chart recorder (Gould 220) and their outward records are shown in the upward direction.

Fura-2 digital fluorescence imagine microscopy

The endothelial cells on the coverslip were loaded with 0.75 μM fura-2/AM (acetoxymethylester) in normal PSS (1 mM stock in dimethylsulphoxide (DMSO)) for 30 min at room temperature. The coverslip chamber was mounted on an inverted microscope (Nikon, Diaphot). A Nikon 20 × phase/fluor objective was used to visualize the cells. A glass tube was used to infuse (by gravity) the chamber with fresh experimental solution (total volume of the chamber is 0.5 ml which is continually maintained using vacuum suction at the surface of the fluid). A total of 3 ml solution was used to change the solution. The endothelial cells were exposed to alternating 340 and 380 nm (bandwidth 10 nm) wavelengths of u.v. light and emission light was passed through a 510 nm (bandwidth 40 nm) filter prior to acquisition by an ICCD camera (Intensified Charge-Coupled Device; Cohu, 4810 Series). A Sun Sparc workstation and the Inovision Image Software (Inovision Corp. Research Triangle Park, NC, U.S.A.) were used to record and analyze the fluorescence ratio (340/380). Before measurement, background fluorescence at 340 nm and 380 nm was measured by bringing the cells out of the focus. Ratio images were collected every 10 s. All results given are reported as changes in the 340/380 signal ratio. Averaged numerical data obtained from dose responses of caffeine by both electrophysiological and fura-2 fluorescence methods are represented as the mean ± one standard error of the mean (s.e.mean).

Solutions

The physiological salt solution (PSS) used in the experiments had the following ionic composition (in mM): NaCl 125.4, KCl 5.9, $CaCl_2$ 1.5, $MgCl_2$ 1.2, glucose 11.5, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10, adjusted to pH 7.35 with NaOH. The pipette (intracellular solution) contained (in mM): NaCl 5, KCl 126, $MgCl_2$ 1.2, glucose 11, EGTA 0.8, HEPES 10, adjusted to pH 7.2 with KOH. The osmotic activity (280–290 mmol kg^{-1}) of the solution was monitored with a vapor pressure osmometer (Wescor 5500). Experiments were carried out at a bath temperature of 28 ± 2°C.

Drugs and chemicals

The following drugs and chemicals were used: papain, DL-dithiothreitol, caffeine all of which were purchased from Sigma Chemicals Co., St Louis, MO, U.S.A., tetraethylammonium chloride (TEA) (Eastman Kodak Co., Rochester, NY, U.S.A.), ethylene *bis* (oxyethylenenitrilo) tetra acetic acid (EGTA) (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The fluoresence indicator fura-2/AM was purchased from molecular Probes, Inc. (Eugene, OR, U.S.A.).

Results

Caffeine

The activator of calcium release from agonist-releasable stores, caffeine, was applied to the bathing solution in the presence and absence of Ca^{2+} in the extracellular solution. In the cells which were voltage clamped at potential +20 mV and bathed in PSS, 20 mM caffeine elicited an outward current up to 2400 pA (1771 ± 205 pA, $n = 7$) (Figure 1a) after an initial burst of unitary currents and STOCs (Figure 2a,b). The increased spontaneous current activity started after ≈5 s pre-

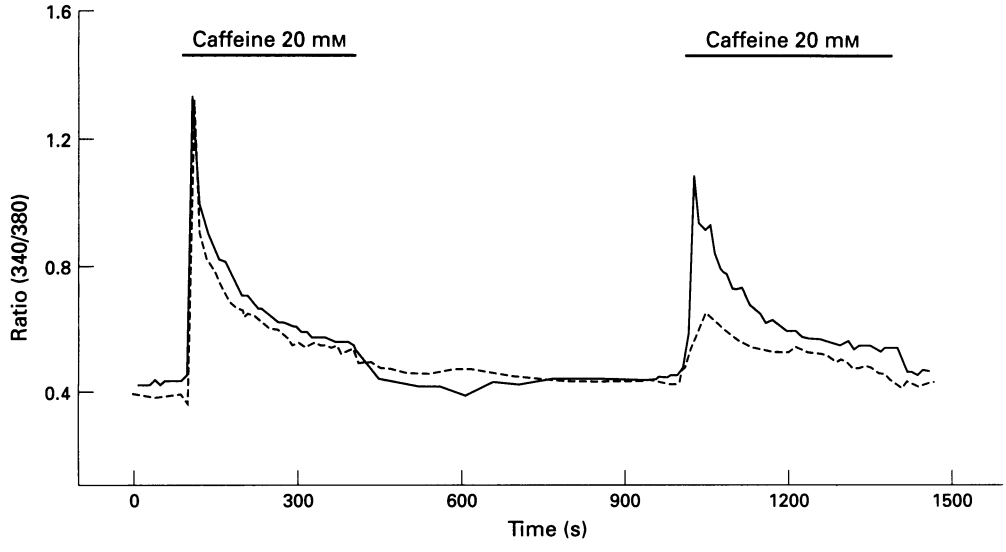

Figure 3 Tracing to show that bath application of caffeine (20 mM) elevates intracellular calcium concentration in isolated, fura-2 loaded cells. The solid line represents the average signal of 10 cells in normal PSS (1.5 mM $CaCl_2$) and the dashed line represents the average signal in Ca^{2+}-free, EGTA containing solution ($n = 10$). After the first caffeine response the cells were allowed to recover for 10 min before a second dose of caffeine was applied. In normal PSS the second application of caffeine induced a slightly lower but similar transient while the second response to caffeine in Ca^{2+}-free, 1 mM EGTA containing solution was considerably reduced.

sence of caffeine. Although in control conditions (prior to application of caffeine) STOCs were observed in approximately one third of all voltage-clamped cells, extracellularly applied caffeine (20 mM) stimulated in all cells a rapid discharge of STOCs to peaks of 50–160 pA which started from and later decayed back to the basal level (lag phase). Some cells showed only one STOC during the lag phase (Figure 2a), while others produced repetitive discharges of STOCs (2–9 events) within ≈3 s (Figure 2b) before the rising phase of the outward current developed ($n=7$).

The outward current evoked by caffeine in the absence of Ca^{2+} had a similar magnitude (1698 ± 312 pA, $n=6$) as in the

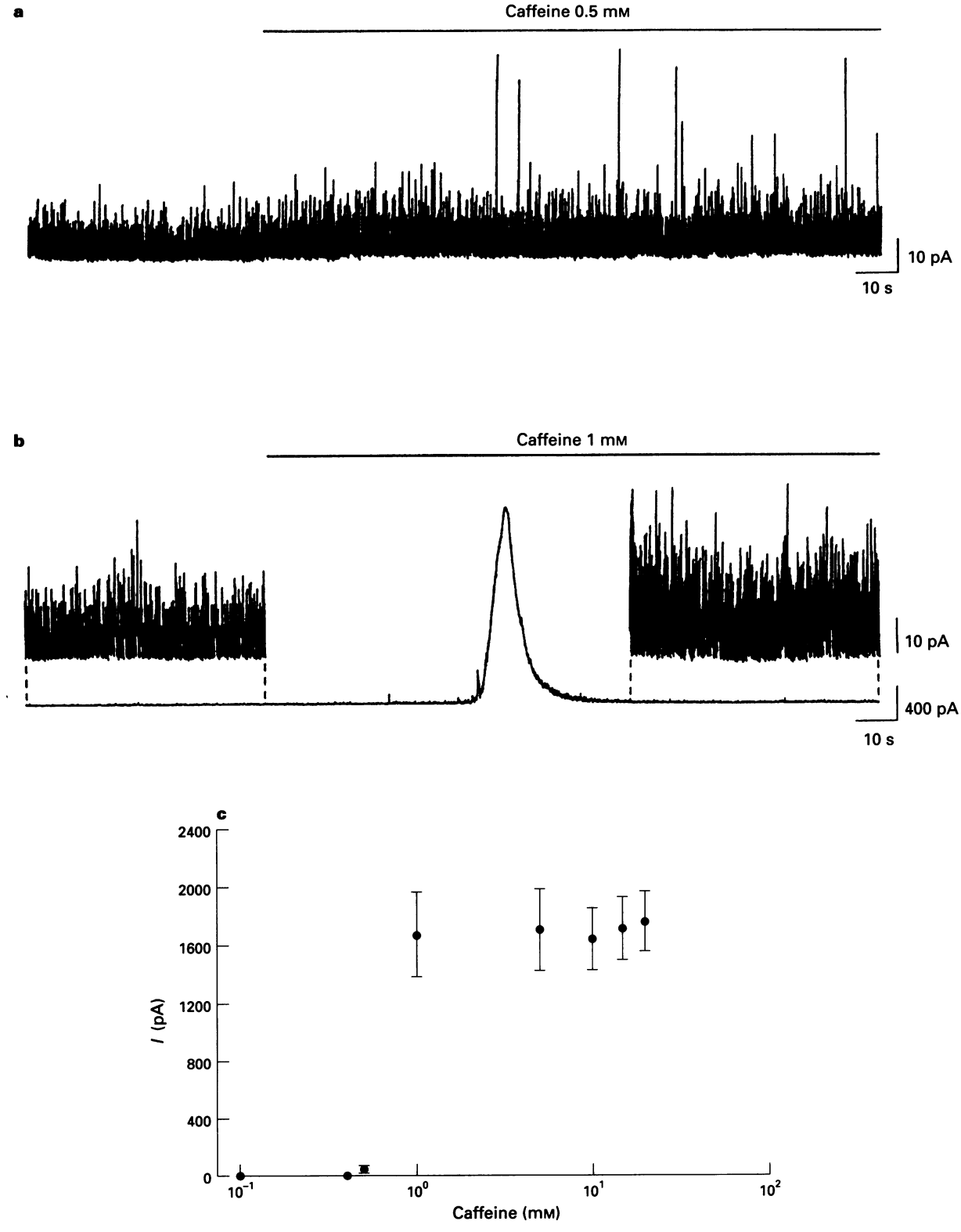

Figure 4 Dose-dependent responses of outward currents evoked by caffeine. (a) Caffeine, 0.5 mM, produced only stimulation of unitary currents and STOCs. (b) Caffeine 1 mM, elicited the outward current. (c) Dose-response relation for activation of outward currents by different doses of caffeine. Each point represents the mean from at least 5 experiments. Holding potential +20 mV.

presence of Ca^{2+}; however, it was delayed and of longer duration (Figure 1c). Moreover, while in normal Ca^{2+}-containing solution, caffeine produced an outward current which started steeply from basal current level and decayed rapidly within 15 s (Figure 1a), the shape of this caffeine-evoked transient outward current in the absence of Ca^{2+} was clearly biphasic (Figure 1c, 2c) and it decayed more slowly ($\approx$45 s) (Figure 1c). Detailed examination of the rising phase of the long lasting transient outward currents on an expanded current and time scales revealed that also in the presence of Ca^{2+} the initial rising phase is complex (Figure 2a,b). Thus, both, in the presence and absence of Ca^{2+} it was possible to determine by visual inspection a clear transition from the foot to the rapid upstroke phase. The rising portion of the outward current evoked by caffeine consists of an initial slowly rising transient component characterised by discharges of STOCs (foot phase) with amplitude of up to 250 pA in the presence of Ca^{2+} ($n=7$) and up to 400 pA in the absence of Ca^{2+} ($n=6$)

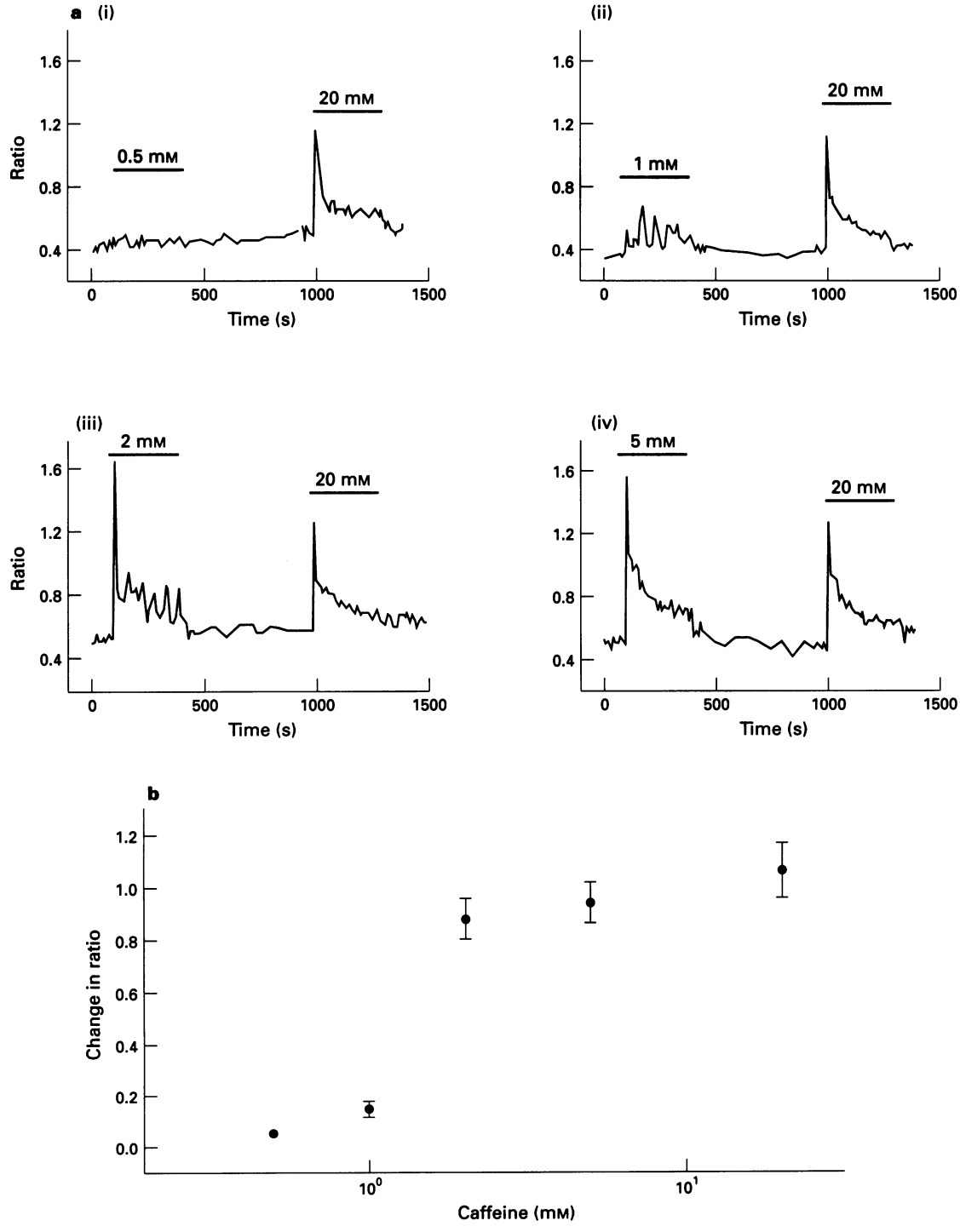

Figure 5 (a) The intracellular Ca^{2+} responses of isolated, fura-2 loaded cells obtained in normal PSS containing different concentrations of caffeine. The cells were first exposed to different caffeine concentrations: 0.5 mM (i), 1 mM (ii), 2 mM (iii) and 5 mM (iv). The caffeine was washed out after 5 min and the cells were allowed to recover in normal PSS for 10 min. A second addition of caffeine (20 mM) was made after recovery in each cell. (b) Dose-response relation for caffeine-induced transient elevations of $[Ca^{2+}]_i$ in fura-2-loaded cells bathed in normal PSS. The peak-values of the transients were averaged at different doses. Each point is the average of the peak where the number of experiments is at least 10.

and a subsequent more steeply increasing component (upstroke phase). The foot phase in the presence of Ca^{2+} was only a fraction of the length ($\approx 10\%$) of the one observed in the absence of Ca^{2+}.

When caffeine (20 mM) was applied simultaneously with Ca^{2+}-free solution, the cells responded with a steep rise in the outward current, similar to the response observed in the presence of Ca^{2+}. However, the former was followed by a slower fall to a maintained base level, similar to the action of caffeine in Ca^{2+}-free solution (Figure 1b, $n=4$).

In Ca^{2+}-free solution an outward current in response to a second application of caffeine was not observed (not shown).

A prolonged increase in frequency and amplitude of spontaneous unitary currents was observed following the outward currents in both the presence and absence of extracellular Ca^{2+} (Figure 1a,c). These effects were most pronounced when both Ca^{2+}-free solution and caffeine were applied to bathing solution at the same time (Figure 1b).

The next set of experiments was performed to investigate the effect of caffeine on intracellular Ca^{2+} release using the fluorescent Ca^{2+} indicator fura-2. Figure 3 shows that 20 mM caffeine evoked similar transient responses in both Ca^{2+}-containing and Ca^{2+}-free, 1 mM EGTA containing solution. The peak of $[Ca^{2+}]_i$ and the maintained plateau in the presence of Ca^{2+} (0.62 ± 0.04, $n=10$) were similar to that seen in Ca^{2+}-free solution (0.55 ± 0.03, $n=10$). After 5 min, caffeine was washed out from the bathing solution and the cells were allowed to recover for 10 min. It is apparent that in the presence of external Ca^{2+}, the caffeine-sensitive store was refilled. In contrast, the $[Ca^{2+}]_i$ response to the second caffeine exposure in Ca^{2+}-free solution was greatly reduced.

Concentration-dependence of the outward currents activated by caffeine

Lower concentrations of caffeine (0.1 and 0.5 M) increased both frequency and amplitude of unitary currents and evoked STOCs but did not initiate outward currents (Figure 4a). As the concentration of caffeine was increased to 1 mM, the response abruptly increased with an outward current of up to a maximum of 2400 pA (Figure 4b; $n=4$). At concentrations higher than 1 mM, the responses to caffeine were not concentration-dependent. The relationship between the outward current and the concentration of caffeine is presented in Figure 4c, where the average maximal amplitude of outward current is plotted as a function of caffeine concentration.

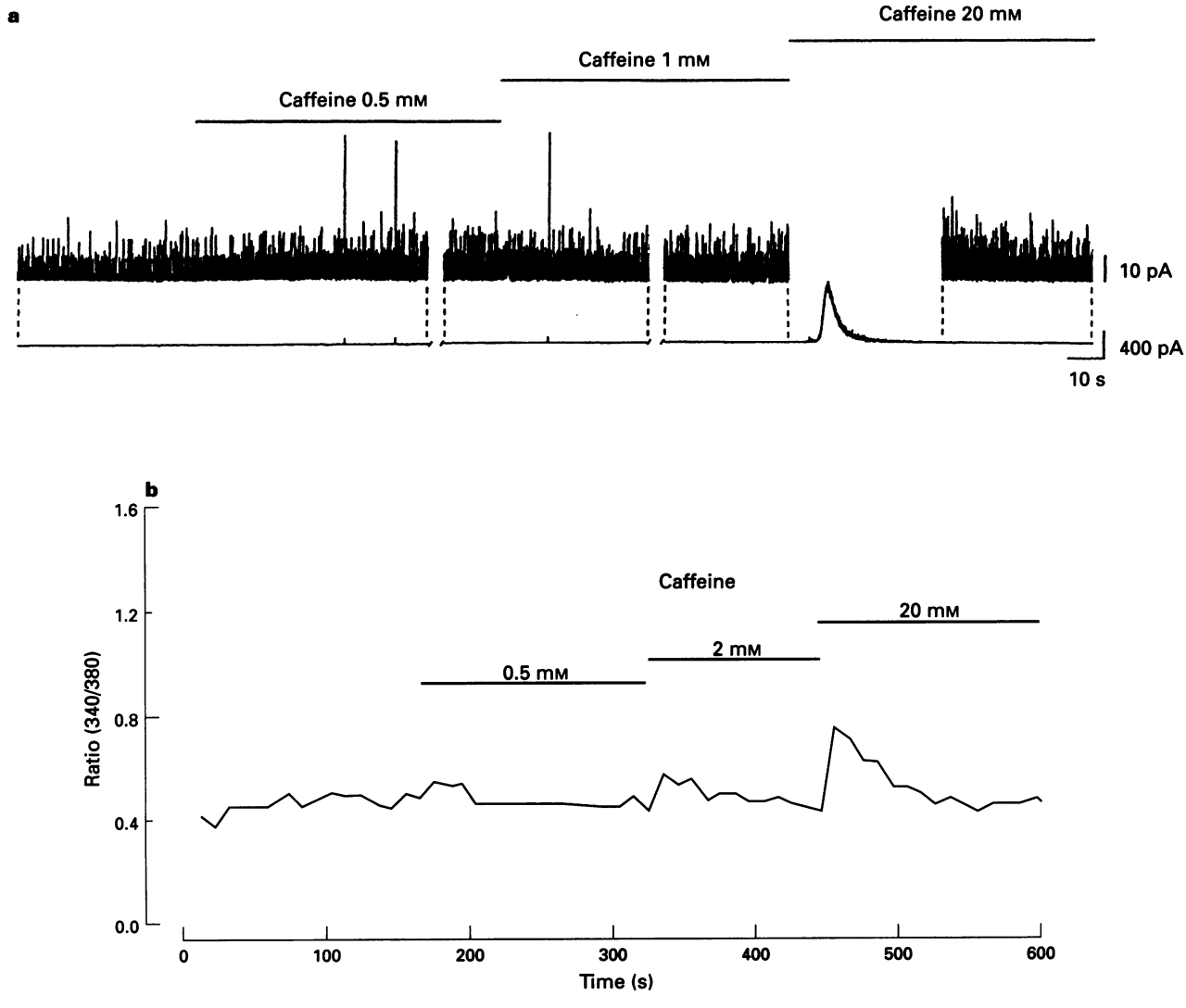

Figure 6 Sequential applications of increasing doses of caffeine. (a) Outward currents recorded at a holding potential of +20 mV during 2 min exposure to normal PSS containing 0.5, 1 and 20 mM caffeine. (b) The average of the intracellular Ca^{2+} responses of isolated, fura-2 loaded cells during exposure to normal PSS containing consecutively 0.5, 2 and 20 mM caffeine ($n=10$). Horizontal bars indicate periods during which the cell was bathed in an extracellular solution containing caffeine.

In Figure 5a the $[Ca^{2+}]_i$ responses from different cells at different concentrations of caffeine were measured as the 340/380 ratio of fura-2 fluorescence. All four panels illustrate the same protocol. The various doses of caffeine at 0.5, 1, 2 and 5 mM were applied for 5 min. After 10 min recovery, a second caffeine dose of 20 mM was applied to each preparation. All the second applications of caffeine evoked similar rises in $[Ca^{2+}]_i$. Lower doses of caffeine (0.5 mM) had no apparent effect on $[Ca^{2+}]_i$ (Figure 5a(i)) however, 1 mM caffeine induced oscillation in the $[Ca^{2+}]_i$ but no major transient peak was evoked (Figure 5a(ii)). Figure 5a(iii) shows that at 2 mM caffeine, the cells responded with a transient $[Ca^{2+}]_i$ of the same magnitude as that evoked by 5 mM and 20 mM. At this intermediate caffeine concentration, oscillations in $[Ca^{2+}]_i$ were observed. Figure 5b shows the concentration-response curve for the caffeine induced $[Ca^{2+}]_i$ responses.

When the cells were pretreated with lower doses of caffeine (0.4 or 0.5 mM) 1 mM caffeine did not evoke an outward current (Figure 6a, $n=4$) upon subsequent caffeine addition, even though this concentration would elicit a response under control conditions (see Figure 4b). Moreover, preincubation of endothelial cells with these lower concentrations of caffeine (0.4–0.5 mM) attenuated the peak of the outward current evoked by 20 mM caffeine by 60% (Figure 6a, $n=4$). Figure 6b shows the average traces of $[Ca^{2+}]_i$ ($n=10$) recorded from different doses of caffeine applied successively. The lower dose of caffeine (0.5 mM) abolished the action of subsequently added 2 mM caffeine, which in control conditions evoked a rise in $[Ca^{2+}]_i$ (Figure 5a(iii)). In addition, the response to the subsequent addition of 20 mM caffeine was reduced by 55% compared to its control (Figure 3).

Effects of TEA and reduction in $[Ca^{2+}]_o$ on spontaneous current activity

Experiments were performed to test whether the increases in both frequency and amplitude of unitary currents and STOCs evoked by Ca^{2+}-free solution reflected stimulation of Ca^{2+}-activated K^+ currents. Endothelial cells to which the K-channel blocker, TEA, was applied showed neither unitary currents nor STOCs, regardless of whether Ca^{2+} was present in the perfusing solution (Figure 7, $n=5$). In the continual presence of TEA, there was no spontaneous activity but the basal current was noisier than that in the control condition prior to TEA application. Thus, application of 3 mM TEA abolished the increase in unitary currents and STOCs seen upon removal of extracellular Ca^{2+} as would be expected if they resulted from Ca^{2+}-activated K^+ currents. However, the sustained outward current (2–12 pA, $n=9$) seen in Ca^{2+}-free solution was not affected.

The effect of TEA on the caffeine-induced outward currents in the absence of Ca^{2+}

The presence of TEA (3 mM) did not prevent the ability of caffeine to evoke outward currents in Ca^{2+}-free solution; however, their amplitude was attenuated by about 50% (Figure 8b, $n=4$). Moreover, TEA (3 mM) blocked the discharges of unitary currents and STOCs accompanying the trace of caffeine-induced outward currents.

Following wash-out of the TEA, recovery of spontaneous current activity in Ca^{2+}-free solution was allowed for 10 min before again applying 20 mM caffeine. Under these conditions, the second application of caffeine failed to evoke an outward current (not shown). Although the endothelial cells lose the ability to produce outward currents upon repetitive caffeine stimulation in the absence of extracellular Ca^{2+}, they continue to generate spontaneous outward currents.

Discussion

Caffeine induced a $[Ca^{2+}]_i$ transient and an outward current which were of the same magnitude in the presence and absence of extracellular Ca^{2+}. This indicates that both responses are the consequence of Ca^{2+} release from intracellular stores. The mechanism of caffeine-induced Ca^{2+} release involves sensitization of the ryanodine receptor-Ca^{2+} release channels in the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) to the activating action of Ca^{2+} (Endo, 1977; Martonosi, 1984). Ca^{2+}-induced Ca^{2+} release (CICR) has been reported for skeletal (Endo *et al.*, 1970; Ford & Podolsky, 1970), cardiac

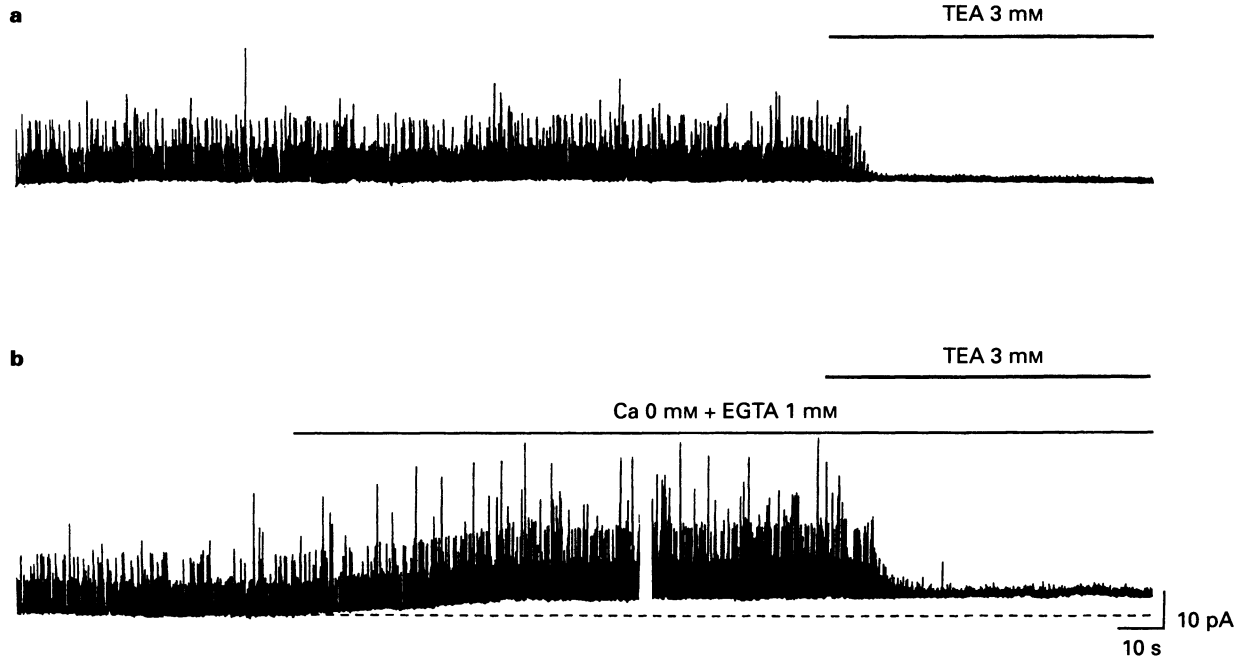

Figure 7 Membrane current records in a freshly dissociated rabbit aortic endothelial cell. Effect of applying tetraethylammonium (TEA, 3 mM) in the presence of extracellular Ca^{2+} (a) and in Ca^{2+}-free, EGTA-containing solution (b) for the period indicated by the horizontal bars. Same scale as (a). Holding potential +20 mV.

(Fabiato & Fabiato, 1972; Fabiato, 1983) and vascular smooth muscle SR (Itoh *et al.*, 1982; Leijten & van Breemen, 1984; Van Breemen & Saida, 1989) and as presented in the results section is also present in native rabbit aortic endothelial cells.

The main finding of the present study is the all-or-none nature of the caffeine-induced responses. At increasing doses of caffeine the outward current suddenly appeared at a maximum amplitude when the caffeine concentration reached 1 mM, while the $[Ca^{2+}]_i$ transient abruptly peaked at 2 mM caffeine after $[Ca^{2+}]_i$ oscillations at 1 mM. Both phenomena are undoubtedly linked, since the small difference in threshold can be readily attributed to the difference in experimental conditions (e.g. whole cell clamp recordings were made at +20 mV while fura-2 fluorescence was measured at resting membrane potentials). Another possible explanation for the difference in caffeine sensitivity is that the $[Ca^{2+}]_i$ oscillations observed at 1 mM caffeine are sufficient to achieve the threshold for activation of the outward current. The latter explanation is supported by the finding that the outward current elicited by 1 mM caffeine had a longer delay and a slower upstroke than this current elicited by 20 mM caffeine. We postulate that the regenerative CICR requires the achievement of a critical local $[Ca^{2+}]_i$ or threshold $[Ca^{2+}]_i$.

The marked reduction in the amplitude of both the outward current and $[Ca^{2+}]_i$ transients evoked by 20 mM caffeine after prior exposure to lower caffeine concentrations indicates that a subthreshold caffeine concentration activates some Ca^{2+} channels causing depletion of intracellular Ca^{2+} stores. When the ER is partially depleted, a higher concentration of caffeine is required to open more Ca^{2+} channels to achieve a sufficient Ca^{2+} release to reach the local threshold concentration for CICR. However, in spite of the fact that 20 mM caffeine was still able to induce regenerative Ca^{2+} release, the prior partial depletion of the ER caused the final response to be markedly smaller than that seen under control conditions.

Several other observations allude to the concept of threshold $[Ca^{2+}]_i$ for CICR. Both, in the presence and absence of Ca^{2+} the rising part of the outward current was biphasic. When the foot phase characterized by discharges of STOCs reached a critical level, the slope abruptly became much steeper. The threshold probably represents the critical level of $[Ca^{2+}]_i$ which trigger massive quasi-simultaneous opening of Ca^{2+}-activated K channels (upstroke) in response to sudden release of Ca^{2+} from the internal stores. The foot phase however, was much shorter in the presence of external Ca^{2+}, perhaps because continuous Ca^{2+} influx allowed the released Ca^{2+} to build up a local threshold Ca^{2+} concentration much faster. The Ca^{2+} supplied by influx would also assure that once the threshold for CICR was reached at any location in the cytoplasm, the process would propagate over all regions of the ER. Different lengths of the foot phase in the presence and absence of Ca^{2+} also indicate the supporting role of extra-

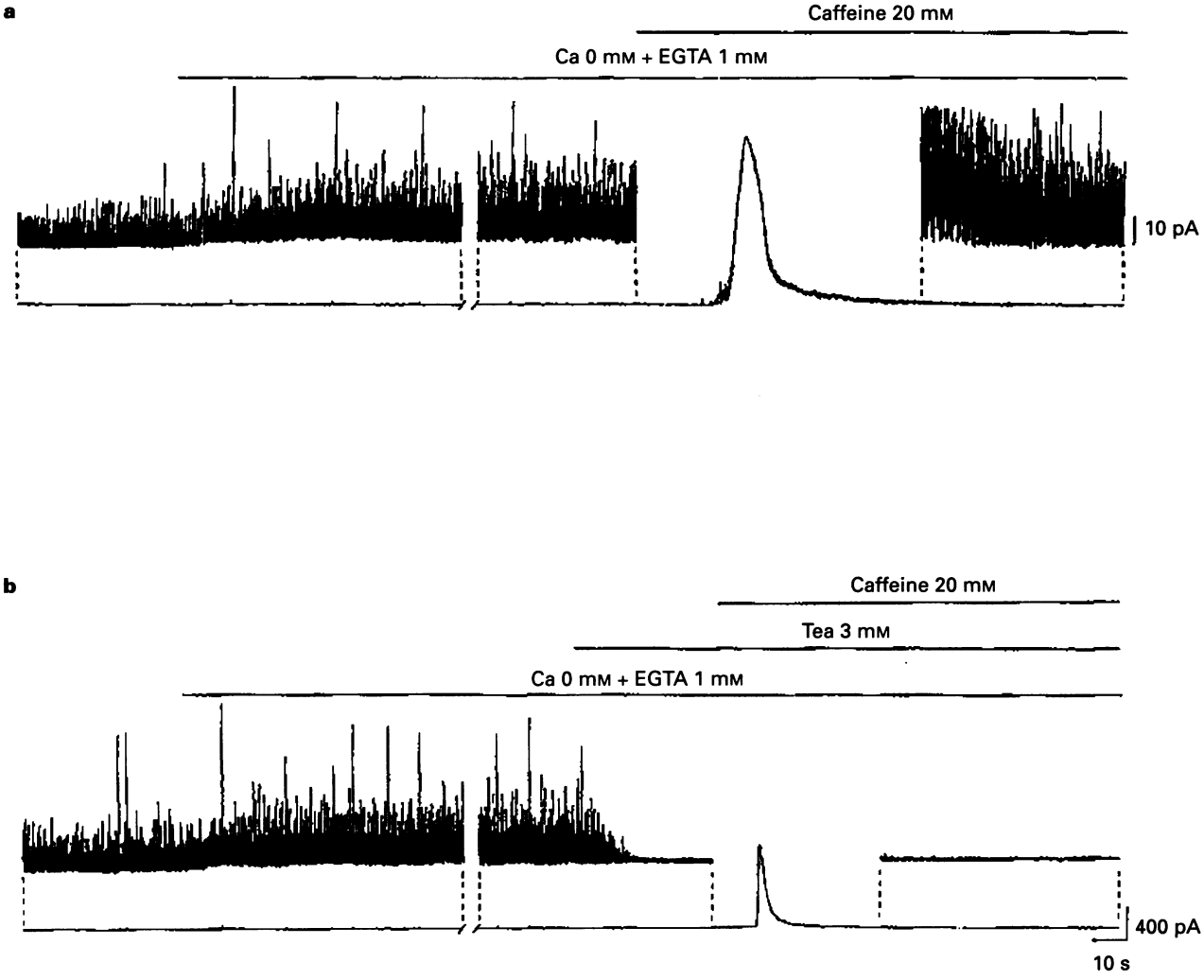

Figure 8 Effects of 3 mM tetraethylammonium (TEA) and of Ca^{2+}-free, EGTA-containing solution on the action of 20 mM caffeine. (a) Caffeine-evoked outward current was recorded after 6 min exposure to Ca^{2+}-free, EGTA-containing solution. (b) The effect of caffeine in the presence of TEA and after 6 min in Ca^{2+}-free, EGTA-containing solution. Horizontal bars indicate periods during which cells were superfused with extracellular solution containing drugs and no added Ca^{2+}. Holding potential +20 mV.

cellular Ca^{2+} in the generation of outward currents. However, it cannot be ruled out that some of the differences related to the presence and absence of extracellular Ca^{2+} are due to effects of Ca^{2+} on the outer surface of the plasmalemma.

It is also possible that local threshold values may be reached under conditions where global $[Ca^{2+}]_i$ is not high enough to sustain propagation of regenerative Ca^{2+} release throughout the cell. This situation would result in localized CICR occurring at different sites at different times leading to the oscillations of variable amplitude measured by the fura-2 fluorescence at 1 mM caffeine.

A curious observation is that persistent spontaneous current activity was increased by caffeine after the development of the outward current. This is clearly different from behaviour of the outward current in that the latter current required a latent period of ER refilling before it could be repeated. In the absence of extracellular Ca^{2+} only one outward current could be obtained because refilling of the ER from the extracellular space was prevented. In contrast, removal of external Ca^{2+} enhanced the spontaneous current activity and did not prevent its persistance after caffeine exposure. After the caffeine-sensitive stores were depleted in the absence of external Ca^{2+} a second exposure to caffeine was still able to induce a slight elevation of the steady state $[Ca^{2+}]_i$. Although we are unable at this time to provide a fully consistent explanation for the above observation, it is possible that either some refilling occurred from Ca^{2+} released into the cytoplasm and/or another non caffeine-sensitive intracellular Ca^{2+} source exists, such as mitochondria, which is normally buffered by Ca^{2+} uptake into the ER.

The caffeine-induced outward current fits the pattern of being dependent on a depletable and refillable Ca^{2+} store such as observed in smooth muscle (Leijten & van Breemen, 1984). The conductances activated by the released Ca^{2+} include both TEA-sensitive potassium channels and TEA-insensitive and at present unidentified channels. The refilling of caffeine-sensitive intracellular Ca^{2+} stores in native aortic endothelial cells however, is a relatively slow process in that at least 10 min incubation in normal physiological solution is required to elicit a second caffeine-induced outward current. The presence of a second transient $[Ca^{2+}]_i$ peak, although considerably reduced, in response to the second application of caffeine in Ca^{2+}-free solution suggests that not all of Ca^{2+} released within the cells is lost from the cell but that some released Ca^{2+} is recycled into the ER (see also Bond *et al.*, 1984; Benham & Bolton, 1986). In this case, however, the amount of $[Ca^{2+}]_i$ is probably insufficient to reach the threshold for activation of the outward current, as confirmed by our electrophysiological experiments (not shown).

Spontaneous current activity, a phenomenon found in the freshly isolated aortic endothelial cells, has been shown to be generated by a population of TEA-sensitive and highly K^+-selective channels sensitive to the cytoplasmic Ca^{2+} concentration (Rusko *et al.*, 1992). Although removal of external Ca^{2+} enhanced spontaneous current activity there is no convincing explanation for this effect. It may be related to a change in the behaviour of the BK K_{Ca} channels. This is supported to some extent by the finding that the caffeine effect and the Ca^{2+}-free effect are additive. Perhaps not only changes in $[Ca^{2+}]_i$ (Rusko *et al.*, 1992) but also changes in $[Ca^{2+}]_o$ may modulate the activity of Ca^{2+}-activated K channels. This suggestion is supported by our finding that increasing the $[Ca^{2+}]_o$ reduced the spontaneous current activity (not shown).

In conclusion, we have presented evidence for regenerative Ca^{2+} release mediated by ryanodine receptors in native aortic endothelial cells. Caffeine induced Ca^{2+} release from the ER in an all-or-none manner. Once a local threshold $[Ca^{2+}]_i$ is reached, ryanodine receptors are activated regeneratively by increasing Ca^{2+} flux from the ER lumen to the cytoplasm. At a critical caffeine concentration near 1 mM, the regenerative release may fail to propagate throughout the cell and consequently leads to irregular $[Ca^{2+}]_i$ oscillations. Ryanodine receptor-mediated Ca^{2+} release may signal NO and other relaxant factors release directly by activating constitutive NO-synthase or indirectly by activating Ca^{2+}-sensitive K channels and increasing Ca^{2+} influx through hyperpolarization.

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