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Caffeine-evoked, calcium-sensitive membrane currents in rabbit aortic endothelial cells

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1 Single cell photometry and whole-cell patch clamp recording were used to study caffeine-induced intracellular Ca^{2+} signals and membrane currents, respectively, in endothelial cells freshly dissociated from rabbit aorta.

2 Caffeine (5 mM) evoked a transient increase in $[Ca^{2+}]_i$ in fura-2-loaded endothelial cells. Pretreatment of cells with 10 μM ryanodine did not alter resting $[Ca^{2+}]_i$ but irreversibly inhibited the caffeine-induced rise in $[Ca^{2+}]_i$. The caffeine-induced increase in $[Ca^{2+}]_i$ was not attenuated by the removal of extracellular Ca^{2+} and did not stimulate the rate of Mn^{2+} quench of fura-2 fluorescence.

3 Bath application of caffeine evoked a dose- and voltage-dependent outward current. The rate of onset and amplitude of the caffeine-evoked outward current increased with higher caffeine concentrations and membrane depolarization. The relationship between caffeine-evoked current amplitude and membrane potential was non linear, suggesting that the channels underlying the current are voltage-sensitive.

4 In the absence of extracellular Ca^{2+}, the amplitude of the caffeine-evoked outward current was reduced by approximately 50% but the duration of the current was prolonged compared to that observed in the presence of external Ca^{2+}. Ca^{2+}-free external solutions produced an unexpected increase in both the frequency and amplitude of spontaneous transient outward currents (STOCs).

5 Inclusion of heparin (10 μg ml^{-1}) in the patch pipette abolished the acetylcholine (ACh)-induced outward current but failed to inhibit either STOCs or the caffeine-evoked outward current in native endothelial cells. In the absence of extracellular Ca^{2+}, heparin did not affect either STOCs or the caffeine-induced outward current.

6 Externally applied tetraethylammonium ions (TEA, 3–10 mM) reversibly inhibited unitary Ca^{2+}-activated K^+ currents and STOCs in endothelial cells but failed to inhibit completely the outward current evoked by 20 mM caffeine.

7 Bath application of 0.1 mM zinc ion (Zn^{2+}), a chloride channel blocker, did not affect unitary currents or STOCs but reduced the amplitude of the caffeine-evoked current by $>75\%$ compared to control. Replacement of extracellular NaCl with Na gluconate also reduced the amplitude of the caffeine-induced outward current. Bath application of 0.1 mM Zn^{2+} and 10 mM TEA completely blocked the caffeine-evoked outward current in endothelial cells.

8 Caffeine-induced Ca^{2+} release from intracellular stores evokes a transient rise in $[Ca^{2+}]_i$ which is correlated with a large, transient outward current. The ionic dependence and inhibition of the caffeine-sensitive current by TEA and Zn^{2+} suggests that Ca^{2+}-activated K^+ and Cl^- conductances contribute to the caffeine response in rabbit aortic endothelial cells.

Keywords: Caffeine; ryanodine; endothelium; internal calcium stores; tetraethylammonium; heparin; calcium-activated K^+ currents; calcium-activated Cl^- currents

Introduction

The secretion of endothelium-derived factors such as prostacyclin (PGI_2), nitric oxide and von Willebrand factor (vWf), is closely associated with the intracellular free calcium ion concentration ($[Ca^{2+}]_i$). Electrophysiological studies of endothelial cells freshly dissociated from rabbit aorta have shown that intracellular Ca^{2+} levels also regulate single channel activity and spontaneous transient outward currents (STOCs; Rusko *et al.*, 1992). These currents were identified as K^+ currents by their ionic dependence and sensitivity to block by the K channel blockers, tetraethylammonium ions (TEA) and charybdotoxin. The dependence of outward K^+ current activity on $[Ca^{2+}]_i$ suggests that the opening of plasmalemmal Ca^{2+}-dependent K channels is triggered by the cyclical release of Ca^{2+} from internal stores. The activation of Ca^{2+}-dependent K channels has been used to monitor the functional status of the intracellular Ca^{2+} stores in smooth muscle cells (Benham & Bolton, 1986).

The activation of cell-surface receptors by vasoactive agents (e.g. bradykinin, acetylcholine and ATP) in native aortic endothelial cells produces a biphasic increase in both the open probability of unitary currents and the amplitude of STOCs (Sauvé *et al.*, 1988; Rusko *et al.*, 1992). This current activation is correlated with a biphasic elevation in $[Ca^{2+}]_i$: an initial discharge of Ca^{2+} from intracellular stores, and a subsequent prolonged entry of Ca^{2+} from the extracellular space (see reviews by Adams *et al.*, 1993; Himmel *et al.*, 1993). In the absence of extracellular Ca^{2+}, a decrease in the magnitude of the initial agonist-evoked transient increase of $[Ca^{2+}]_i$ was observed in bovine aortic endothelial cells (Schilling *et al.*, 1988; Lückhoff *et al.*, 1988). Furthermore, depletion of the intracellular Ca^{2+} stores has been reported to be linked to the agonist-induced Ca^{2+} influx from the extracellular space (Hallam *et al.*, 1989; Jacob, 1990; Schilling *et al.*, 1992). The precise mechanism(s) which brings about these changes in cytoplasmic $[Ca^{2+}]$ in native endothelial cells is ill-defined.

The receptor-stimulated turnover of inositol phosphate has

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been shown to trigger directly the initial, transient release of intracellular Ca^{2+} from endoplasmic reticulum of cultured endothelial cells (Jaffe *et al.*, 1987; Pollock *et al.*, 1988; Freay *et al.*, 1989). There is also evidence for the release of Ca^{2+} from intracellular stores by actions of methylxanthines, such as caffeine, which are known to translocate Ca^{2+} from intracellular Ca^{2+} stores into the cytosol of skeletal, cardiac and vascular smooth muscle cells by enhancing Ca^{2+}-induced Ca^{2+}-release (see Endo, 1985). Caffeine is also able to release Ca^{2+} from the agonist-releasable intracellular Ca^{2+} stores (Benham & Bolton, 1986; Bolton & Lim, 1989). This Ca^{2+}-induced Ca^{2+} release mechanism has been shown to be present in addition to the inositol-1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+}-release mechanism in a variety of excitable and non-excitable cells (see Tsien & Tsien, 1990; Pozzan *et al.*, 1994). Evidence for the coexistence of $InsP_3$-sensitive and caffeine-sensitive Ca^{2+} stores has also recently been shown in cultured endothelial cells from bovine aorta (Thuringer & Sauvé, 1992).

This study examines caffeine-induced Ca^{2+} release from $InsP_3$-insensitive intracellular stores in endothelial cells freshly dissociated from rabbit aorta as shown by the activation of Ca^{2+}-sensitive membrane conductances. Caffeine activates an outward current correlated with the rise in $[Ca^{2+}]_i$ which is not completely blocked by bath-applied TEA and is sensitive to the external Cl^- concentration and zinc ions. Preliminary reports of some of these results has been presented previously (Adams *et al.*, 1993; Rusko *et al.*, 1993).

Methods

Experiments were carried out on freshly dissociated endothelial cells obtained from rabbit aorta. Procedures for the preparation of endothelial cells and electrophysiological recordings were as previously described (Rusko *et al.*, 1992). Briefly, pieces of endothelium were peeled from the thoracic aorta of a rabbit killed by CO_2 asphyxiation and incubated for 35 min in a solution containing 0.9 mg ml^{-1} papain and 0.8 mg ml^{-1} dithiothreitol. The tissue was washed and gently triturated in Dulbecco's phosphate-buffered saline (DPBS, pH 7.35; GIBCO Laboratories, NY, U.S.A.) containing 20% foetal calf serum. Following centrifugation, the cells were plated on glass coverslips and stored at 4°C for at least 4 h prior to use that day. Positive identification of cells as endothelial was based on morphological characteristics and specific uptake of rhodamine-fluorescent acetylated low density lipoprotein (1,1,-dioctadecyl-3,3,3,3′-tetramethyl-indocarbocyanine (DiI-Ac-LDL), Biomedical Technologies, Stoughton MA, U.S.A.) as previously described (Rusko *et al.*, 1992).

The physiological saline solution (PSS) used as the external bathing media had the following composition (mM): NaCl 125.4, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 11.5, N-2-hydroxy-ethylpiperazine-*N*′-2-ethansulphonic acid (HEPES) 10, titrated to pH 7.35 with NaOH. Whole-cell currents were recorded using a patch pipette filled with an intracellular solution with the following composition (mM): KCl 126, NaCl 5, $MgCl_2$ 1.2, glucose 11, ethyleneglycol-bis-(β-aminoethylether) *N,N,N′,N′*-tetraacetic acid (EGTA) 0.8, HEPES 10, titrated to pH 7.2 with KOH. The experiments were carried out at room temperature (23 ± 2°C).

Microfluorometric measurements

Fluorescence measurements of cytoplasmic free Ca^{2+} concentration in single, nonconfluent endothelial cells were carried out with the fluorescent Ca^{2+} indicator dye, fura-2. Freshly dissociated endothelial cells were incubated in PSS containing 1 μM of the acetoxymethyl ester of fura-2 (fura-2/AM dissolved in dimethylsulphoxide, DMSO) at room temperature (23°C) for 30 min, and excess ester was rinsed away with buffered PPS. Endothelial cells on glass coverslips were mounted in an open perfusion microincubator (PDMI-2, Medical Systems Corp., N.Y., U.S.A.) on the stage of an inverted phase contrast microscope (Nikon Diaphot) and continuously superfused at 2 ml min^{-1} with PSS. The temperature of the microincubator and bathing solution was maintained at 37°C by a bipolar temperature controller (TC-202, Medical Systems Corp., N.Y., U.S.A.). Cells were alternatively excited (60 Hz) at 340 nm and 380 nm wavelengths with an optical chopper (OC-4000, PTI) in the path of the u.v. light provided by a 75 W xenon arc lamp. Fura-2 fluorescence viewed with a × 100 Fluor objective (Nikon, 1.3 numerical aperture) was measured at 510 nm wavelength with a photon counter (Hamamatsu R928). Signals were digitized by an A-D converter and analysed with Deltascan software (PTI) on a PC 80486/50 MHz computer. Only single cells with an even dye distribution within the cytoplasm were used. The ratio of signals at the two excitation wavelengths (F_{340}/F_{380}) was determined after subtraction of autofluorescence. $[Ca^{2+}]_i$ was calculated following the method of Grynkiewicz *et al.* (1985).

In a series of experiments, quenching of intracellular fura-2 fluorescence by external Mn^{2+} was carried out to evaluate unidirectional Ca^{2+} movement into endothelial cells (Jacob, 1990). Endothelial cells were bathed in Ca^{2+}-free PSS containing 0.5 mM $MnCl_2$ and the rate of Mn^{2+} influx was determined from the rate of decrease of fura-2 fluorescence from cells excited at 360 nm wavelength. The slope of the fura-2 fluorescence, under control conditions, remained linear for ⩾ 300 s during which time either caffeine or ionomycin were bath applied. Simultaneous monitoring of the emission due to 380 nm excitation and ratioing with the 360 nm signal was used to correlate $[Ca^{2+}]_i$ changes to Mn^{2+} influx.

Electrical recordings

The membrane currents were measured by the whole-cell recording configuration of the patch clamp technique (Hamill *et al.*, 1981) and a List L/M-EPC7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). Voltage ramps (−150 to +100 mV, 4 s duration) were applied using pCLAMP programmes (Axon Instruments Inc, CA, U.S.A.) generated protocols and a PC 80386/33 MHz computer equipped with an A-D/D-A converter (12 bit resolution; Tecmar Labmaster DMA TM-40). Membrane currents were filtered at 2.5 kHz (−3dB, 4-pole Bessel Filter; Ithaco 4302), digitized using a digital VCR recorder adaptor (PCM-1; Medical Systems Corp., N.Y., U.S.A.) and stored for later analysis on videotape. Membrane currents were continuously monitored and the current amplitude was analysed by direct measurement on a digital oscilloscope (Tektronix 5223). Whole-cell and unitary currents were displayed on a chart recorder (Gould 2200S) and outward currents are shown as upward deflections. Numerical data are represented as the mean ± one standard error of the mean (s.e.mean).

Reagents

All chemical reagents used were of analytical grade. The following drugs were used: acetylcholine chloride, caffeine, papain, theophylline, ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N,N*,-tetraacetic acid (EGTA), heparin, sodium salt (mol. wt. 4,000–6,000), dimethyl sulphoxide (DMSO) (obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.), tetraethylammonium chloride (TEA; Eastman Kodak Co., Rochester, N.Y., U.S.A.), D-gluconic acid, sodium salt (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) and ryanodine, ionomycin (Calbiochem Corp., La Jolla, CA, U.S.A.).

Results

Caffeine-induced Ca^{2+} release from ryanodine-sensitive intracellular stores

Fura-2-loaded endothelial cells freshly dissociated from rabbit aorta exhibit a resting F_{340}/F_{380} ratio of 0.56 ± 0.01 ($n = 64$) which corresponds to a $[Ca^{2+}]_i$ of 82 ± 4 nM. Bath application of 5 mM caffeine evokes a transient increase in $[Ca^{2+}]_i$. Superfusion of endothelial cells with PSS containing 5 mM caffeine produced a rise in $[Ca^{2+}]_i$ to a peak F_{340}/F_{380} ratio of 2.11 ± 0.08 (866 ± 83 nM, $n = 64$) which corresponds to an approximately ten fold increase in $[Ca^{2+}]_i$ (Figure 1a). Another methylxanthine, theophylline (5 mM), also evoked a transient increase in $[Ca^{2+}]_i$ of similar magnitude and time course to that observed in response to caffeine (not shown). Pretreatment of the cells with 10 μM ryanodine did not alter resting $[Ca^{2+}]_i$; however, following >20 min exposure to ryanodine, the caffeine-induced rise in $[Ca^{2+}]_i$ was completely blocked (Figure 1b). This block was irreversible even after >30 min washout ($n = 6$).

Figure 2 shows that a higher caffeine concentration (20 mM) evokes a biphasic increase in $[Ca^{2+}]_i$: an initial transient increase, of similar magnitude to that produced by 5 mM caffeine, which decreases to a plateau phase of variable amplitude ($n = 11$). In a Ca^{2+}-free (1 mM EGTA) external solution the plateau phase following the caffeine-induced transient rise in $[Ca^{2+}]_i$ was abolished (Figure 2b). Analogous to the agonist-induced $[Ca^{2+}]_i$ response observed in endothelial cells, the transient $[Ca^{2+}]_i$ peak most probably reflects Ca^{2+} release from intracellular stores whereas the external Ca^{2+}-dependent plateau phase of the caffeine-induced $[Ca^{2+}]_i$ response may be due to an increased plasmalemmal Ca^{2+} influx.

To determine if Ca^{2+} influx is stimulated by bath application of caffeine, the fluorescence of fura-2-loaded cells was measured at excitation wavelengths of 360 and 380 nm with 0.5 mM $MnCl_2$ added to Ca^{2+}-free PSS. Assuming Mn^{2+} is a suitable marker for Ca^{2+} entry in endothelial cells (Jacob, 1990), Ca^{2+} influx can be inferred from the decrease of the fluorescence signal obtained at 360 nm excitation. The slope of the 360 nm fluorescence signal, reflecting the rate of Mn^{2+}

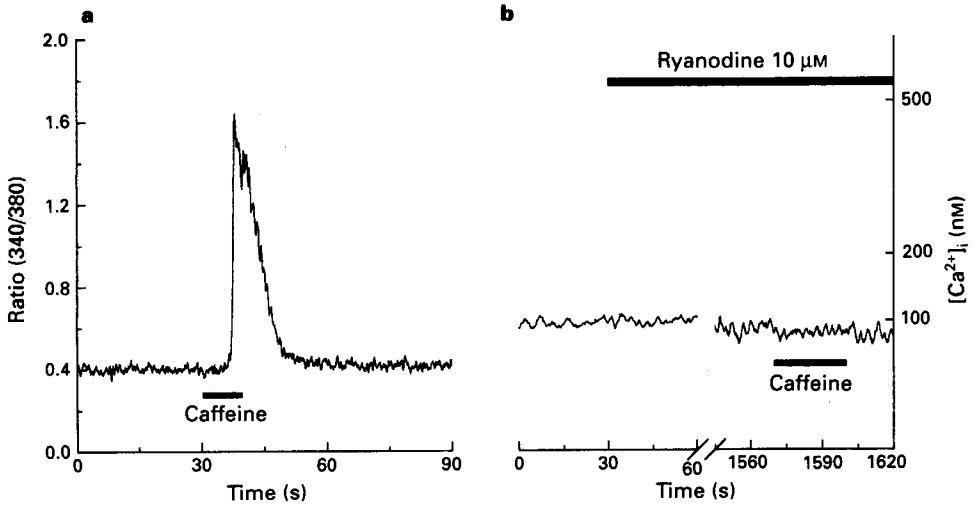

Figure 1 Caffeine- and ryanodine-sensitive $[Ca^{2+}]_i$ responses in rabbit aortic endothelial cells. (a) The intracellular Ca^{2+} response of an isolated, fura-2-loaded rabbit aortic endothelial cell to bath (PSS)-application of 5 mM caffeine. (b) Blockade of the caffeine-induced transient rise in $[Ca^{2+}]_i$ following treatment of an endothelial cell with 10 μM ryanodine for 25 min. This trace is representative of six experiments.

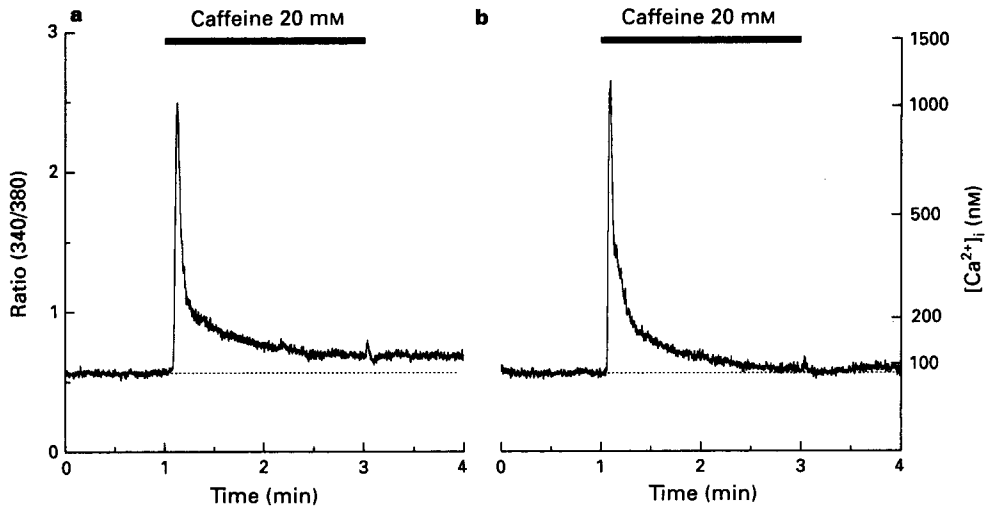

Figure 2 Caffeine-induced increase in $[Ca^{2+}]_i$ in the presence (a) and absence (b) of extracellular Ca^{2+}. Caffeine (20 mM)-induced increase in $[Ca^{2+}]_i$ in an isolated, fura-2-loaded rabbit aortic endothelial cell obtained in normal PSS containing 1.5 mM Ca^{2+} (a) and in a Ca^{2+}-free external solution containing 1 mM EGTA (b). These traces are representative of 11 experiments for each condition.

quench of fura-2 fluorescence, remained unchanged following the addition of 5–20 mM caffeine ($n = 8$). The F_{360}/F_{380} ratio exhibited a transient increase in response to caffeine due to Ca^{2+} release from intracellular stores (not shown). As a positive control, superfusion of endothelial cells with Ca^{2+}-free PSS containing 1 μM ionomycin, a divalent cation ionophore, produced a 11.9 ± 1.9-fold ($n = 3$) increase in the rate of Mn^{2+} quench of fura-2 fluorescence.

Caffeine-induced outward currents in native endothelial cells

Unitary currents and STOCs due to the activation of Ca^{2+}-dependent K^+ channels were observed in freshly dissociated endothelial cells from rabbit aorta at membrane potentials positive to −40 mV (Rusko *et al.*, 1992). Bath application of caffeine (5 and 20 mM) evoked a dose- and voltage-dependent

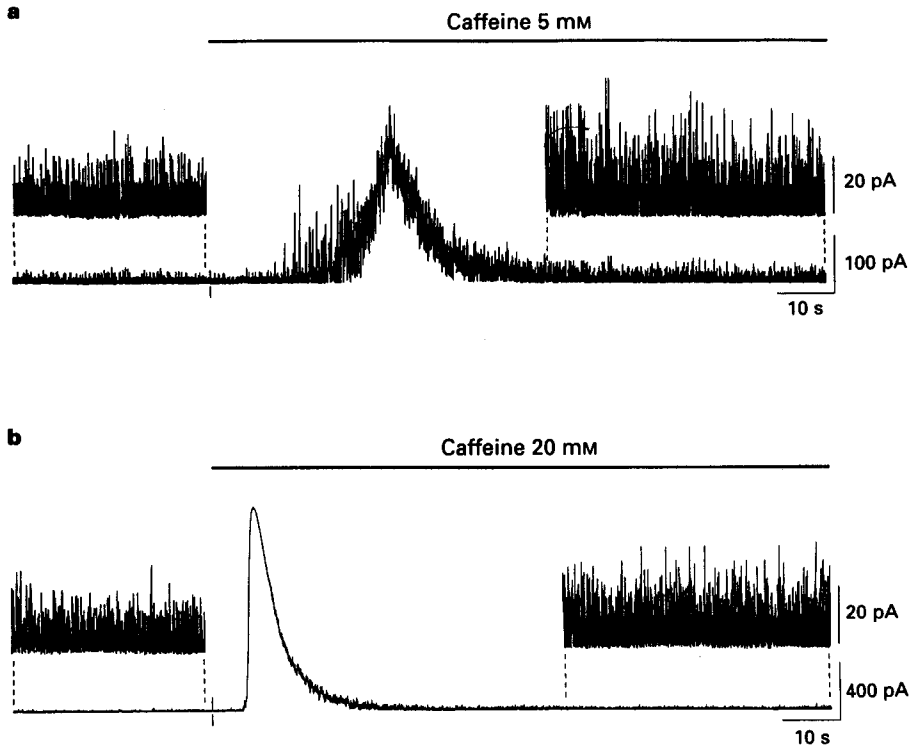

Figure 3 Caffeine-evoked outward currents in rabbit aortic endothelial cells. Continuous traces of unitary currents and STOCs recorded prior to and during exposure to 5 mM caffeine (a) and in the presence of 20 mM caffeine (b). Bath-applications of PSS containing caffeine are indicated by the horizontal bars. Upper traces show current activities at expanded current scales. Holding potential, +20 mV. Traces shown are representative of 3 experiments.

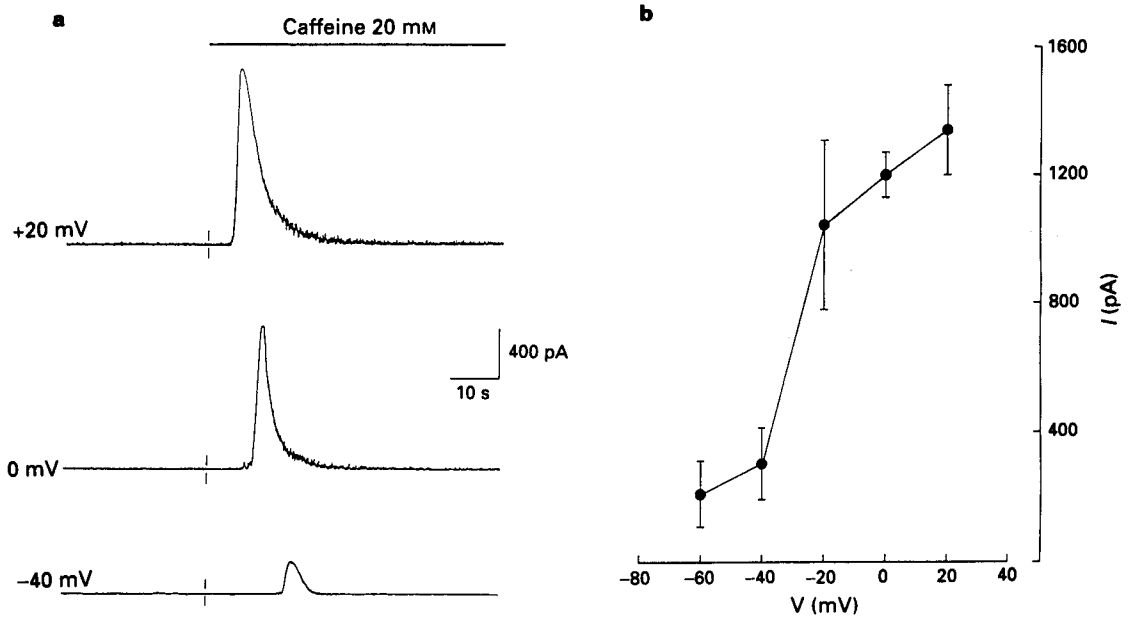

Figure 4 Voltage-dependence of transient whole-cell outward currents evoked by caffeine in rabbit aortic endothelial cells. (a) Traces of the outward currents evoked by PSS containing 20 mM caffeine at holding potentials of +20, 0, and −40 mV. (b) The current-voltage relationship obtained for the peak amplitude of the caffeine-induced outward current determined in six cells.

outward current in these endothelial cells. Figure 3a shows a typical whole-cell current response to bath application of 5 mM caffeine, producing an increase in the frequency and amplitude of unitary currents and STOCs. The frequency of STOCs increased 304 ± 18% ($n = 3$) following 3 min bath perfusion of PSS containing 5 mM caffeine ($n = 3$). The initial burst of these currents is superimposed on a slowly developing outward current which peaks at approximately 375 pA amplitude (Figure 3a). At a higher caffeine concentration (20 mM), the transient outward current amplitude increased to >1 nA (1.3 ± 0.14 nA, $n = 5$) and had durations of 7–15 s in endothelial cells held at +20 mV (Figure 3b). In the continued presence of caffeine, the frequency and amplitude of unitary currents and STOCs were increased compared to control conditions prior to caffeine application. The rapid onset of the outward current evoked by caffeine suggests a close spatial relationship between the intracellular Ca^{2+} stores and plasmalemmal Ca^{2+}-dependent K^+ channels. A long-lasting outward current in response to a second application of caffeine was not observed after >5 min washout (not shown).

Figure 4 describes the voltage-dependence of the caffeine-induced outward current in voltage-clamped endothelial cells. Bath application of 20 mM caffeine evoked an outward current that increased in amplitude with membrane depolarization, saturating at approximately +20 mV. Representative whole-cell outward currents evoked by 20 mM caffeine at three different membrane potentials are shown in Figure 4a.

The relationship between caffeine-induced current amplitude and membrane potential averaged from six cells is shown in Figure 4b. The activation of the outward current is non-linear suggesting that the ionic channels underlying the caffeine-induced current are voltage-sensitive. The membrane potential at which half-maximal activation of the caffeine-induced outward current is observed is shifted by approximately −40 mV compared to that obtained for activation of the Ca^{2+}-dependent K^+ current in rabbit aortic endothelial cells (Demirel *et al.*, 1994) suggesting that another ionic conductance may contribute to the caffeine-evoked outward current.

Dependence of outward currents on extracellular Ca^{2+}

The open-state probability of the unitary K^+ currents in rabbit aortic endothelial cells is sensitive to $[Ca^{2+}]_i$ (Rusko *et al.*, 1992; Demirel *et al.*, 1994). The unitary current activity was also affected by the extracellular $[Ca^{2+}]$. A decrease in the extracellular $[Ca^{2+}]$ may be expected to affect $[Ca^{2+}]_i$ by increasing Ca^{2+} efflux and reducing Ca^{2+} influx across the cell membrane. The dependence of unitary currents, STOCs and the caffeine-induced outward current on the extracellular $[Ca^{2+}]$ was examined in endothelial cells bathed in Ca^{2+}-free

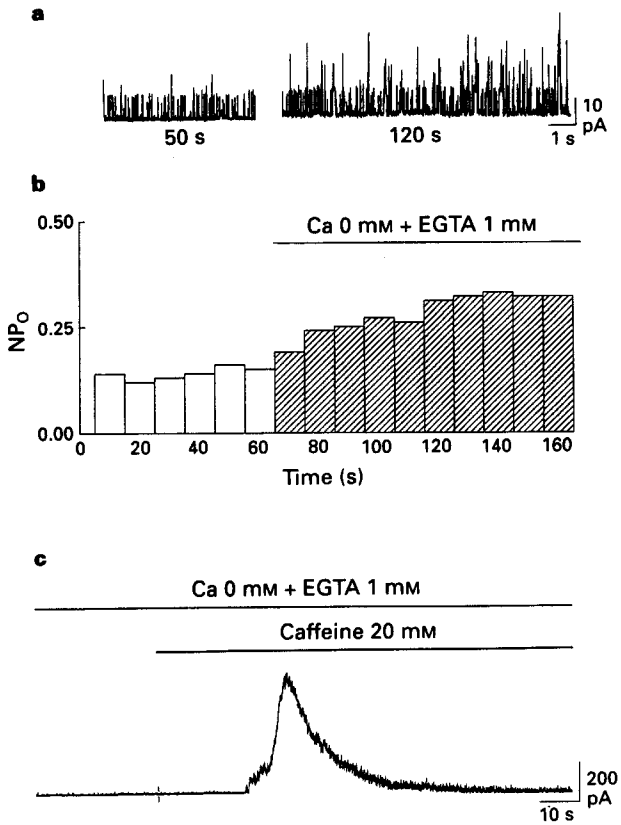

Figure 5 Effect of Ca^{2+}-free external solution on unitary currents and STOCs in aortic endothelial cells. (a) Unitary currents and STOCs recorded at holding potential +20 mV before (control, $t = 50$ s) and during exposure to Ca^{2+}-free (0 mM Ca^{2+}, $t = 120$ s) external solution. Traces shown are representative of 9 experiments. (b) Time course of change in NP_0 (10 s bin width) of unitary and STOCs before and during exposure to a Ca^{2+}-free external solution (horizontal bar). (c) Continuous record of membrane current observed following exposure to 20 mM caffeine in the presence of Ca^{2+}-free, EGTA containing solution for the periods indicated by the horizontal bars. Holding potential, +20 mV. Trace shown is representative of 4 experiments.

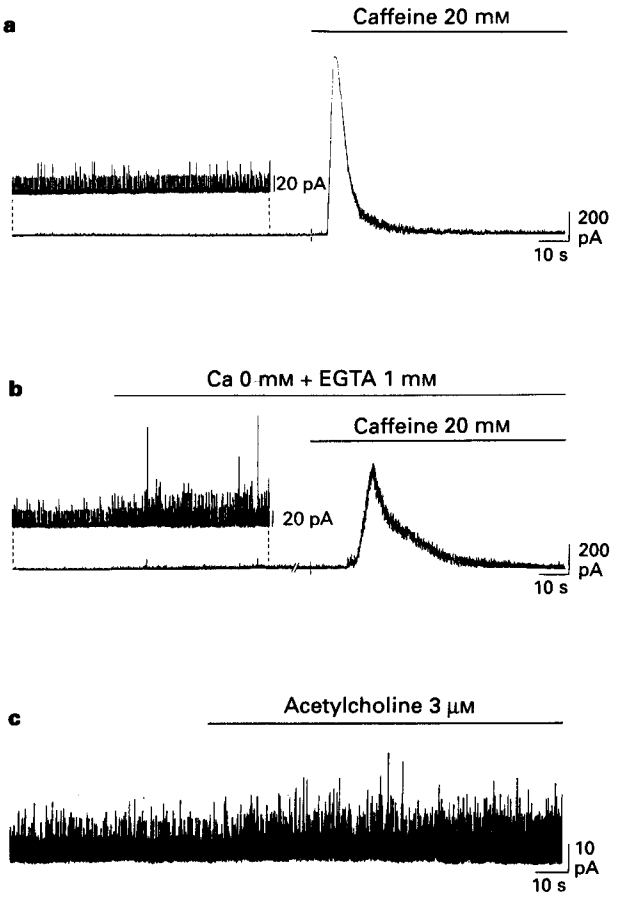

Figure 6 Effect of intracellular heparin on caffeine- and acetylcholine (ACh)-evoked outward currents in endothelial cells. (a). Caffeine-evoked outward current recorded at a holding potential of +20 mV from a cell bathed in PSS containing 1.5 mM $CaCl_2$. (b) Caffeine-evoked outward current obtained in the absence of extracellular Ca^{2+} (1 mM EGTA). (c) Unitary outward currents recorded in the absence and presence of bath applied 3 μM acetylcholine. Whole-cell currents recorded from endothelial cells dialyzed with pipette solution containing 10 μg ml^{-1} heparin. Traces shown are representative of at least 5 experiments for each condition. Horizontal bars indicate period during which the cell was bathed in an extracellular solution containing caffeine (20 mM), no added Ca^{2+}(0 mM Ca^{2+} + 1 mM EGTA) or acetylcholine (3 μM). Holding potential +20 mV.

medium containing 1 mM EGTA. Figure 5a shows an unexpected increase in both the frequency and the amplitude of STOCs produced by exposure to a Ca^{2+}-free external solution ($n = 9$). A plot of the open-channel probability (NP_0) of the unitary Ca^{2+}-dependent K^+ currents as a function of time is shown in Figure 5b.

In the absence of extracellular Ca^{2+}, caffeine (20 mM) continued to evoke a transient, outward current but the amplitude (572 ± 138 pA, $n = 4$) was reduced by approximately 50% compared to that observed in the presence of extracellular Ca^{2+} (Figure 5c). However, the caffeine-induced outward current was significantly prolonged lasting 35–45 s, a >30% increase. Moreover, the transient outward current evoked by caffeine in Ca^{2+}-free solution appears to be composed of two superimposed currents: the initial increase in current appears as a shoulder preceding a second current of larger amplitude. In the continued presence of caffeine in Ca^{2+}-free solution, unitary currents and STOCs occurred at both a higher frequency and amplitude than that observed under control conditions prior to caffeine application.

The effects of heparin on Ca^{2+} release from intracellular stores

Heparin, a specific competitive antagonist of the $InsP_3$ receptor, has been shown to inhibit $InsP_3$-mediated Ca^{2+} release from intracellular stores in a variety of cell types (Ghosh *et al.*, 1988). Inclusion of heparin (10 μg ml^{-1}) in the patch pipette had no noticeable influence on either unitary currents or STOCs in native aortic endothelial cells under resting conditions. Heparin failed to prevent the increase in both frequency and amplitude of unitary currents and STOCs observed following the removal of extracellular Ca^{2+}.

The effects of intracellular heparin on the caffeine-induced current activation were examined in voltage-clamped endothelial cells. Figure 6a shows membrane currents obtained in response to bath application of 20 mM caffeine in cells held at +20 mV in the presence of 10 μg ml^{-1} heparin in the patch pipette solution. The large, long-lasting outward current evoked by caffeine in normal PSS (1108 ± 363 pA, $n = 5$) appears similar to that observed in the absence of intracellular heparin (Figure 3b). Similarly the presence of heparin in the pipette (Figure 6b) failed to abolish the caffeine-evoked outward current in the absence of extracellular Ca^{2+}. The amplitude of the caffeine-evoked current was approximately half (557 ± 132 pA, $n = 6$) of that observed in the presence of 1.5 mM extracellular Ca^{2+}. Figure 6c shows the result of a control experiment indicating that heparin had diffused into the cell. The addition of 10 μg ml^{-1} heparin to the pipette inhibited the ACh (3 μM)-induced outward K^+ current ($n = 5$) observed in the absence of intracellular heparin (*cf.* Figure 6, Rusko *et al.*, 1992).

Pharmacological block of caffeine-induced outward currents

Evidence that Ca^{2+}-dependent K channels underlie the unitary and spontaneous transient outward currents observed in rabbit aortic endothelial cells is provided, in part, by the inhibition of these currents by Ca^{2+}-dependent K channel blockers, tetraethylammonium ions (TEA) and charybdotoxin (Rusko *et al.*, 1992). Externally applied TEA

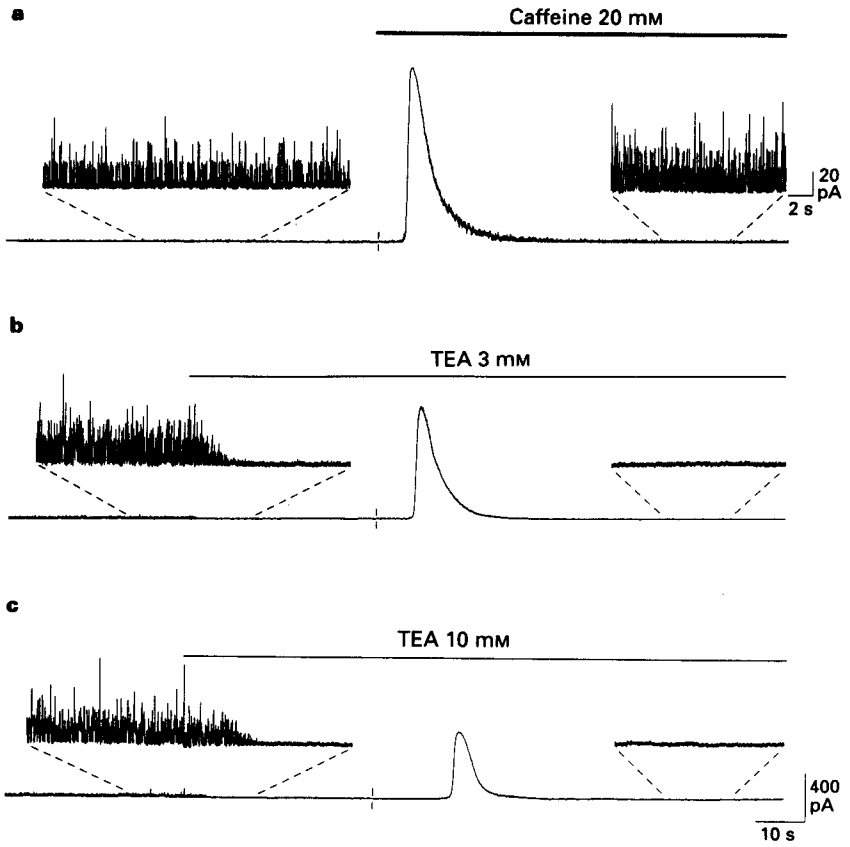

Figure 7 Caffeine-induced outward current in rabbit aortic endothelial cells obtained in the absence and presence of bath applied tetraethylammonium ion (TEA). (a) Outward current evoked in response to 20 mM caffeine in normal PSS. Caffeine-induced outward currents obtained in the presence of either 3 mM (b) or 10 mM (c) TEA. The top horizontal bar indicates the times at which caffeine was bath applied for all three traces. The horizontal bars shown in (b) and (c) indicate times at which TEA was bath-applied. Holding potential, +20 mV. Lower scale bars indicate the time and current magnitude for traces in (a), (b) and (c). Upper scale bars indicate time and current magnitudes for all expanded inserts. Traces shown are representative of at least 3 experiments.

(3–10 mM) reversibly inhibited unitary currents and STOCs observed in endothelial cells clamped at +20 mV but failed to inhibit completely the large, prolonged outward current evoked by caffeine (Figure 7b,c). Figure 7 shows that the prolonged outward current evoked by 20 mM caffeine was reduced in a concentration-dependent manner by extracellular TEA. The amplitude of the caffeine-induced outward current was reduced by 16% (1095 ± 209 pA, $n = 4$) and by 63% (480 ± 35 pA, $n = 3$) in the presence of 3 mM and 10 mM TEA, respectively. In the maintained presence of TEA, unitary currents and STOCs were not observed following caffeine stimulation. Although TEA partially inhibited the caffeine-evoked outward current, the inability of TEA to block completely the prolonged outward current evoked by caffeine suggests that another ionic conductance may be activated in the presence of caffeine.

In order to examine the contribution of a chloride conductance to the caffeine-induced outward current, zinc ion (Zn^{2+}), a chloride channel blocker, was bath-applied to voltage-clamped endothelial cells. Bath-application of 0.1 mM Zn^{2+} produced a small, sustained inward current of <5 pA amplitude but did not affect unitary currents or STOCs. The peak amplitude of the caffeine (20 mM)-induced outward current, however, was reduced by $>75\%$ (246 ± 35 pA, $n = 4$) in the presence of 0.1 mM Zn^{2+} (Figure 8a) compared to that obtained in normal PSS (Figure 3a). The caffeine-induced outward current was examined in the presence of both Zn^{2+} and TEA. Bath application of 0.1 mM Zn^{2+} and 10 mM TEA abolished unitary currents and STOCs and inhibited the caffeine (20 mM)-evoked outward current by $>95\%$ (<50 pA) after 5 min perfusion (Figure 8b). In 19 of 21 cells, bath application of both Zn^{2+} and TEA completely blocked the caffeine-induced outward current within 5 min, and longer exposure abolished the response in all 21 cells. Replacement of extracellular NaCl with Na gluconate reduced the amplitude of the caffeine (20 mM)-evoked outward current by approximately 30% (930 ± 144 pA, $n = 3$) compared to that obtained in normal PSS (not shown). Taken together, these results indicate that caffeine-induced Ca^{2+} release activates a Cl^--conductance in addition to a K^+ conductance in arterial endothelial cells.

Discussion

Results from this study demonstrate that caffeine stimulates Ca^{2+} release from intracellular stores, elevating cytoplasmic $[Ca^{2+}]$ which activates membrane currents in native endothelial cells from rabbit aorta. Ca^{2+}-dependent K^+ currents have been shown to be regulated by the $[Ca^{2+}]_i$ which is influenced by plasmalemmal Ca^{2+} influx and Ca^{2+} release from internal stores (Rusko *et al.*, 1992; Adams *et al.*, 1993). Bath application of caffeine, which stimulates Ca^{2+} release from internal stores, evoked a transient increase in $[Ca^{2+}]_i$ and an outward current both of which were sustained in the absence of extracellular Ca^{2+}. Caffeine-induced Ca^{2+} release from intracellular stores was dose-dependent and inhibited by prolonged exposure to ryanodine (10^{-5} M), an inhibitor of the Ca^{2+} release channel of sarcoplasmic reticulum (Nagasaki & Fleischer, 1988). These data indicate the presence of functional caffeine- and ryanodine-sensitive intracellular Ca^{2+} stores in freshly dissociated endothelial cells from rabbit aorta.

The inability of Ca^{2+}-free external solutions to inhibit unitary currents and STOCs suggests that Ca^{2+} entry from the extracellular space is not necessary for maintaining the activity of these currents and that the release of intracellular stores is a sufficient source of Ca^{2+} for activation of Ca^{2+}-dependent K channels in native endothelial cells. The increase in both the frequency and amplitude of unitary currents and STOCs observed in the presence of Ca^{2+}-free external solution contrasts with the inhibition of STOCs observed in jejunal and arterial smooth muscle cells (Benham & Bolton, 1986). Differences in the Ca^{2+} sensitivity of the Ca^{2+}-dependent K channels may be related to the different functions of these three cell types. Our data suggest that in the absence of extracellular Ca^{2+}, stored Ca^{2+} may be released into the cytoplasm and subsequently resequestered into intra-

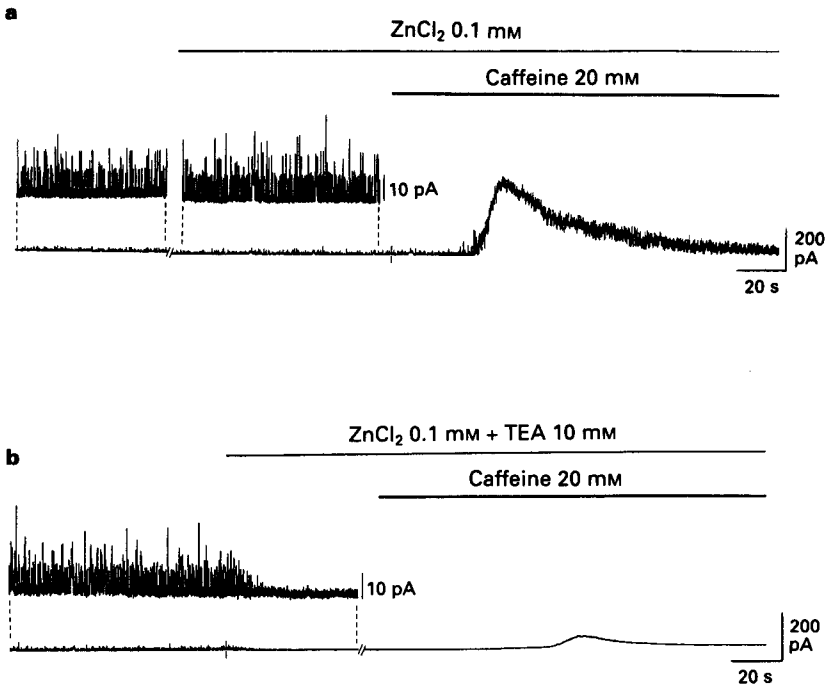

Figure 8 Caffeine-induced membrane currents in the presence of tetraethylammonium (TEA) and zinc ions. (a) Outward currents recorded in response to 20 mM caffeine in the presence of 0.1 mM $ZnCl_2$. Traces shown are representative of 4 experiments. (b) Response of a cell to 20 mM caffeine following 5 min incubation in PSS containing 0.1 mM $ZnCl_2$ and 10 mM TEA. Horizontal bars indicate times at which $ZnCl_2$, TEA and caffeine were applied. Holding potential, +20 mV.

cellular stores rather than lost from the endothelial cell. Transient local fluctuations in $[Ca^{2+}]_i$ from superficial Ca^{2+} stores adjacent to the plasmalemma may contribute to the rapid repetitive discharges of unitary currents and STOCs even in the prolonged absence (>30 min) of extracellular Ca^{2+}. The mechanisms by which the amplitude and frequency of unitary currents and STOCs are modulated by external Ca^{2+} are not yet understood.

The ability of caffeine to evoke a long-lasting outward current in Ca^{2+}-free external solution, albeit of smaller amplitude than that obtained in the presence of extracellular Ca^{2+}, also suggests that the release of intracellular Ca^{2+} stores may be an important source of Ca^{2+} for activation of Ca^{2+}-sensitive ion channels in native endothelial cells. However, it is not possible to determine whether the reduced amplitude of the caffeine-induced outward current observed in Ca^{2+}-free solution is due to a partial depletion of intracellular Ca^{2+} stores or to an impaired ability to release the complete amount of intracellular stored Ca^{2+}. Microfluorometric measurements of fura-2-loaded endothelial cells suggest that the increased unitary current activity induced by prolonged application of caffeine is probably due to sustained elevation of $[Ca^{2+}]_i$. The lack of effect of caffeine on the rate of Mn^{2+} quench of fura-2 fluorescence suggests that the sustained elevation of $[Ca^{2+}]_i$ induced by caffeine is primarily due to release of Ca^{2+} from intracellular stores rather than due to Ca^{2+} influx. In bovine cultured aortic endothelial cells, caffeine has been shown to release only a small fraction of Ca^{2+} from the intracellular stores in the absence of extracellular Ca^{2+} (Buchan & Martin, 1991). By contrast, our results obtained in freshly dissociated rabbit aortic endothelial cells indicate that a significant amount of Ca^{2+} is mobilized by caffeine. The preservation of approximately one half (~52%) of the caffeine-induced outward current following prolonged superfusion with Ca^{2+}-free solution, suggests that the release of Ca^{2+} from intracellular stores plays a significant role in caffeine-induced Ca^{2+} stimulation of vascular endothelial cells.

There is considerable evidence demonstrating that $InsP_3$ triggers the initial phase of the agonist-induced Ca^{2+} mobilization from endoplasmic reticulum in endothelial cells (Pollock *et al.*, 1988; Freay *et al.*, 1989). However, the possibility that outward currents in native endothelial cells are stimulated by cyclical release of Ca^{2+} from $InsP_3$-sensitive Ca^{2+} stores can be ruled out from the results of experiments using heparin as an inhibitor of the $InsP_3$-induced Ca^{2+} release mechanism. ACh-evoked outward K^+ currents were inhibited by the inclusion of 10 $\mu g\ ml^{-1}$ heparin in the patch pipette solution (see Figure 5c; Sakai, 1990). Heparin, however, had no effect on either the caffeine-induced long-lasting outward currents or the increased frequency and amplitude of unitary currents and STOCs evoked by Ca^{2+}-free external solutions. These results suggest the presence of $InsP_3$-insensitive Ca^{2+} stores in freshly dissociated endothelial stores which may be activated by a Ca^{2+}-induced Ca^{2+} release mechanism.

The presence of anti-ryanodine receptor antibody binding sites has recently been demonstrated in vascular and endocardial endothelium (Lesh *et al.*, 1993). Furthermore, the light microscopic distribution of endothelial immunofluorescence indicates that the binding sites are localized on the endoplasmic reticulum (ER). The presence of functional ryanodine-sensitive Ca^{2+} stores has recently been shown by both a direct effect of ryanodine on resting $[Ca^{2+}]_i$ in human cultured aortic, umbilical vein and bovine pulmonary artery endothelial cells, and by attenuation of the agonist-induced $[Ca^{2+}]_i$ transient increase in rat aortic endothelial cells by ryanodine (Ziegelstein *et al.*, 1994). These results suggest that ryanodine-sensitive Ca^{2+} stores in cultured endothelial cells can effectively deplete the $InsP_3$-sensitive intracellular Ca^{2+} pool. In contrast, our data obtained in the presence of intracellular heparin suggests that in rabbit aortic endothelial cells the caffeine- and ryanodine-sensitive Ca^{2+} stores may be functionally distinct from the agonist-sensitive Ca^{2+} stores (see Figure 6). However, ryanodine (10^{-5} M) has been shown to produce a time-dependent decrease in ACh-induced outward current amplitude in rabbit aortic endothelial cells (Sakai, 1990). A recent study of endothelial cells freshly dispersed from porcine coronary arteries suggests a heterogeneity in functional organization of endothelial Ca^{2+} stores, whereby, in a population of cells (quiet-responders), caffeine translocates Ca^{2+} towards the bradykinin ($InsP_3$)-sensitive store, while in another population (overt-responders), caffeine empties the $InsP_3$-sensitive store (Graier *et al.*, 1994).

TEA, an inhibitor of Ca^{2+}-dependent K^+ currents and STOCs in rabbit aortic endothelial cells, failed to inhibit completely the caffeine-induced outward current suggesting the presence of a TEA-insensitive component of the outward current. Evidence that Cl^- may contribute to the caffeine-evoked outward current was obtained from results of measurements made in the presence of external Zn^{2+} and when external Cl^- was replaced with gluconate. Extracellular Zn^{2+} has been shown to block a Cl^- conductance in skeletal muscle (Hutter & Warner, 1967) and inhibit unitary Cl^- currents in excised muscle membrane patches (Woll *et al.*, 1987). In the present study, external Zn^{2+} reduced the amplitude but did not completely block the caffeine-evoked outward current. However, bath-application of both Zn^{2+} and TEA completely blocked this outward current in most endothelial cells. These results suggest that both K^+ and Cl^- contribute as charge carriers to the caffeine-evoked outward current in endothelial cells. Whole-cell and single channel Cl^- currents which are blocked by Zn^{2+}, have been described in bovine pulmonary artery endothelial cells (Shapiro & DeCoursey, 1991). Ca^{2+}-activated Cl^- currents have also been recently reported in arterial endothelial cells, suggesting that the elevation of $[Ca^{2+}]_i$ may activate these Cl^- channels and produce an outward current depending on the Cl^- equilibrium potential (Groschner & Kukovetz, 1992; Vaca & Kunze, 1993).

Caffeine not only releases Ca^{2+} from intracellular stores but also affects a number of other cellular functions including membrane electrical properties, Ca^{2+} influx, Ca^{2+} extrusion and modulation of phosphodiesterases which regulate cyclic AMP levels. Caffeine has also been shown to inhibit voltage-dependent calcium channels in smooth muscle cells (Hughes *et al.*, 1990; Zholos *et al.*, 1991). Additionally, we found that prolonged exposure of endothelial cells to caffeine induces cell shrinkage. Although the mechanism underlying the caffeine-induced shrinkage is unknown, it has been shown that hypotonic volume increases activate a Cl^- current in human endothelial cells (Nilius *et al.*, 1994) suggesting that caffeine-sensitive, Ca^{2+}-activated Cl^- currents may regulate cell volume.

In conclusion, these findings suggest that Ca^{2+}-induced Ca^{2+} release may modulate ER Ca^{2+} release in arterial endothelial cells. The activation of plasmalemmal ion channels by an elevation of $[Ca^{2+}]_i$ due to caffeine-induced Ca^{2+} release from intracellular stores is characterized by a large, transient outward current. The ionic dependence and pharmacological profile of the caffeine-evoked current suggests that Ca^{2+}-sensitive K^+ and Cl^- conductances contribute to the caffeine response in rabbit aortic endothelial cells.

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