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Am J Physiol Heart Circ Physiol 275:1748-1758, 1998.

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ACh- and caffeine-induced Ca^{2+} mobilization and current activation in rabbit arterial endothelial cells

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Fransen, P., C. Katnik, and D. J. Adams. ACh- and caffeine-induced Ca^{2+} mobilization and current activation in rabbit arterial endothelial cells. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H1748–H1758, 1998.—Fura 2 microfluorometry and perforated-patch whole cell recording were carried out simultaneously to investigate the relationship between intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and membrane current activation in response to ACh and caffeine in freshly dissociated arterial endothelial cells. ACh and caffeine evoked transient increases in $[\text{Ca}^{2+}]_i$. The initial increase in $[\text{Ca}^{2+}]_i$ was accompanied by a transient outward current, which caused membrane hyperpolarization. The amplitudes of the $[\text{Ca}^{2+}]_i$ transient and outward current were dependent on caffeine concentration ($\text{EC}_{50} \sim 1 \text{ mM}$). Cyclopiazonic acid raised resting $[\text{Ca}^{2+}]_i$ levels by $\geq 50 \text{ nM}$ and failed to completely block caffeine- or ACh-induced $[\text{Ca}^{2+}]_i$ transients but slowed $[\text{Ca}^{2+}]_i$ recovery fourfold. The reversal potential of caffeine-induced currents was dependent on external K^+ and Cl^- concentrations. Caffeine-induced current amplitudes, but not $[\text{Ca}^{2+}]_i$ responses, were attenuated by external tetraethylammonium, Zn^{2+} , and La^{3+} . A consistent temporal relationship between agonist-activated membrane current and $[\text{Ca}^{2+}]_i$ increases was not observed, and, in some cases, time differences were greater than expected for simple diffusion of Ca^{2+} throughout the cell. These results suggest that Ca^{2+} -dependent current activation monitors local $[\text{Ca}^{2+}]_i$ changes adjacent to the plasmalemma, whereas single-cell photometry provides a measure of global changes in $[\text{Ca}^{2+}]_i$.

endothelium; intracellular calcium; ionic conductances; endoplasmic reticulum

THE SYNTHESIS and/or release of endothelium-derived relaxing and contracting factors in response to stimulation by vasoactive agents is triggered by an increase in the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (12). The agonist-induced increase in $[\text{Ca}^{2+}]_i$ is biphasic: a rapid, transient Ca^{2+} increase due to Ca^{2+} release from intracellular stores and a plateau phase due to a slow sustained Ca^{2+} influx across the plasma membrane (for reviews see Refs. 1 and 19). The increases in $[\text{Ca}^{2+}]_i$ are generally accompanied by changes in membrane potential due to activation of K^+ conductances (23, 32).

Caffeine, a methylxanthine, stimulates the release of Ca^{2+} from ryanodine-sensitive intracellular stores by enhancing Ca^{2+} -induced Ca^{2+} release (CICR) from the endoplasmic reticulum (ER) (8, 41). Vascular endothelial cells have been shown to possess inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores as well as

functional ryanodine-sensitive Ca^{2+} stores (4, 33, 39, 46). Caffeine has also been shown to inhibit cAMP phosphodiesterase activity, increasing intracellular cAMP concentrations, which subsequently increases Ca^{2+} reuptake by the stores (18). Furthermore, caffeine has been reported to inhibit plasma membrane Ca^{2+} channels and to suppress transient outward Ca^{2+} -independent K^+ currents in smooth muscle cells (29).

The aim of the present study was to investigate the effects of caffeine on intracellular Ca^{2+} mobilization in vascular endothelial cells and determine the temporal relationship between global changes in $[\text{Ca}^{2+}]_i$ measured fluorometrically and the activation of ionic currents and changes in membrane potential. Recent studies suggest that measurements of $[\text{Ca}^{2+}]_i$ using Ca^{2+} indicator dyes such as fura 2 are insensitive to changes in $[\text{Ca}^{2+}]_i$ adjacent to the plasma membrane (14, 21, 38). These changes in local Ca^{2+} concentration have been proposed to underlie the occurrence of spontaneous increases in $[\text{Ca}^{2+}]_i$ (" Ca^{2+} sparks") in myocytes (for review see Ref. 31) and the occurrence of spontaneous transient outward and inward currents (STOCs and STICs) in smooth muscle cells (44). These events are attributed to the spontaneous release of Ca^{2+} from caffeine-sensitive CICR channels in the ER. The mobilization of intracellular Ca^{2+} in freshly isolated endothelial cells was studied by simultaneous measurement of caffeine-induced cytosolic Ca^{2+} transients and membrane currents. The physiological relevance of these changes was assessed by comparing results from similar measurements made during agonist stimulation of cells using the vasoactive substance ACh.

MATERIALS AND METHODS

Endothelial cell preparation. Endothelial cells from rabbit arterial vessels were isolated using an enzymatic dissociation procedure described previously (22, 32). Briefly, 40 male New Zealand White rabbits (2–3 kg) were killed by cervical dislocation, and the thoracic aorta and pulmonary arteries were dissected out and placed in physiological salt solution (Na^+ -saline) with the following composition (mM): 125.4 NaCl, 5.9 KCl, 1.5 CaCl_2 , 1.2 MgCl_2 , 11.5 glucose, and 10 HEPES, adjusted to pH 7.35 with NaOH. Connective and adipose tissue from the arteries were removed, the vessels were cut longitudinally, and thin sheets of endothelium were carefully peeled off. The endothelium sheets were transferred to a high- K^+ solution (K^+ -saline) containing (mM) 126 KCl, 5.3 NaCl, 1.5 CaCl_2 , 1.2 MgCl_2 , 11.5 glucose, and 10 HEPES, adjusted to pH 7.35 with KOH, which also contained 0.45 mg/ml papain (7 U/mg) and 0.4 mg/ml dithiothreitol, and were incubated at 37°C for ~ 40 min. The tissue was then washed with K^+ -saline containing 0.5% BSA and gently triturated. The resulting suspension was filtered (62- μm nylon mesh filter) and centrifuged at 2,500 rpm for 5 min. The

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pellet of endothelial cells was resuspended in K⁺-saline, plated on glass coverslips, and maintained at 4°C for ≥1 h, allowing the cells to reequilibrate and adhere to the glass. The coverslips were placed in a recording chamber (0.5 ml volume), mounted on the stage of a Nikon Diaphot inverted microscope equipped with ultraviolet transparent optics, and visualized at ×400 magnification with phase-contrast optics. The chamber was continuously perfused at a rate of ~10 ml/min. Experiments were performed at room temperature (21 ± 2°C). Freshly dissociated cells were positively identified as endothelial cells, as described previously (22, 32).

Microfluorometric measurements. After adhesion to the coverslips, the cells were incubated for 1 h at room temperature in ~0.3 ml of Na⁺-saline solution containing 5 μM fura 2-AM (1 mM fura 2-AM in DMSO stock solution), 0.02% Pluronic F-127, and 0.5% BSA. After incubation with the dye, the cells were washed in Na⁺-saline and allowed 30 min to recover before experiments were begun. A 75-W xenon arc lamp supplied alternating (model OC-4000 Optical Chopper, Photon Technology International, South Brunswick, NJ) 340- and 380-nm illumination via a fiber-optic cable, a 450-nm dichroic mirror (model DM 400, Nikon), and a ×40 oil immersion objective (Fluor 40/1.3 NA, Nikon). Emission fluorescence (510-nm band-pass filter) was collected by a photomultiplier tube (model R928, Hamamatsu) through a variable aperture set around the cell image. The output of the photomultiplier tube was digitized using a Photon Technology International interface and sampled at 5 Hz using Felix 1.1 software (Photon Technology International) run on a Pentium 133-MHz personal computer.

Changes in [Ca²⁺]_i are measured as the ratio of the intensity of the emitted 510-nm fluorescence when the cell was illuminated with 340-nm light to the intensity when it was illuminated with 380-nm light [R_(340/380)]. This ratio was converted to approximate Ca²⁺ concentrations as follows

$$[\text{Ca}^{2+}] = K_D \times \frac{(R - R_{\min})}{(R_{\max} - R)} \times \frac{S_{f2}}{S_{b2}}$$

where the dissociation constant (K_D) is 157 nM, the minimum ratio (R_{\min}) is 0.17, the maximum ratio (R_{\max}) is 2.9, and the ratio of the fluorescence intensity of the Ca²⁺-free fura 2 at 380 nm to the fluorescence intensity of the Ca²⁺-bound fura 2 at 380 nm (S_{f2}/S_{b2}) is 4.6, as determined by a calibration procedure (17) using fura 2 pentapotassium salt and standard Ca²⁺-EGTA solutions. The times at which [Ca²⁺]_i increases were initiated, the times at which peak [Ca²⁺]_i values were attained, the integrals of the [Ca²⁺]_i response, and the rates of [Ca²⁺]_i recovery to basal levels after agonist stimulation were determined from records of [Ca²⁺]_i vs. time. The temporal relationships between changes in [Ca²⁺]_i and membrane current activation were determined using the 95% confidence limits of linear fits to the data before agonist application as threshold levels. Total [Ca²⁺]_i was calculated as the area under a [Ca²⁺]_i transient vs. time by integration, subtracting resting levels, and was used as an estimate of the total amount of Ca²⁺ mobilized during a response. Fits of the data to single-exponential functions were used to determine time constants of decay of [Ca²⁺]_i after agonist stimulation.

Electrical recording. Membrane currents and potentials were recorded using the perforated-patch whole cell recording configuration (30). Patch electrodes (1–4 MΩ) were fabricated from borosilicate glass (model GC150TF, Clark Electromedical Instruments, Reading, UK). The tip of the pipette was dipped in standard pipette solution and backfilled with the same solution containing amphotericin B. Filled pipettes were mounted on the head stage of a patch-clamp amplifier

(model EPC-7, List-Medical, Darmstadt, Germany). Pipette resistance and gigaseal formation were determined by the current responses to brief 1-mV voltage steps (pCLAMP 6, Axon Instruments, Foster City, CA). Capacitance and series resistance (R_S) compensation were applied to determine cell capacitance and access resistance. The reduction in R_S of cell-attached patches induced by amphotericin B insertion into the membrane began within seconds of seal formation, and experiments were initiated when R_S became <30 MΩ, which generally occurred within 10 min. R_S was monitored and adjusted throughout the course of the experiment. Membrane potentials were corrected for liquid junction potentials, which, by theoretical calculations (2), had values of 8.1 mV (Na⁺-saline and K⁺-saline) and 16.5 mV (SO₄²⁻-saline) at 22°C.

Current-voltage (I - V) relationships were determined by applying voltage-ramp protocols generated by pCLAMP 6 programs that consisted of holding the cell at 0 mV and applying voltage ramps from -120 to +60 mV (90 mV/s) or from -80 to +40 mV (300 mV/s). Reversal (zero-current) potentials were estimated from the intercepts of linear fits of the data about the x -axis from I - V relationships obtained in response to voltage ramps. Voltage ramps were used to measure I - V relationships during the transient currents evoked by caffeine or ACh. Membrane current and voltage signals were filtered at 10 kHz (-3 dB, 4-pole Bessel filter) and were simultaneously recorded on DAT cassettes using a digital tape recorder (model DTR-1204, BioLogic Science Instruments, Claix, France). Membrane currents and potentials were transferred to a personal computer hard disk using an analog-to-digital converter (model TL-1 DMA interface, Axon Instruments) and Axotape software. Current and voltage were continuously monitored on a digital oscilloscope and on a chart recorder.

Numerical data are presented as means ± SE.

Solutions and reagents. Freshly isolated endothelial cells were perfused with normal Na⁺-saline. In a series of experiments the external solution was changed to a high-K⁺ solution (K⁺-saline), with Na⁺ isosmotically replaced by K⁺, or to a low-Cl⁻ solution (SO₄²⁻-saline) composed of (mM) 75 Na₂SO₄, 55 NaCl, 5.9 KCl, 1.5 CaCl₂, 1.2 MgCl₂, 11.5 glucose, and 10 HEPES, adjusted to pH 7.35 with NaOH.

Perforated-patch whole cell recordings were performed using patch pipettes backfilled with a solution containing (mM) 75 K₂SO₄, 55 KCl, 5 MgSO₄, and 10 HEPES, adjusted to pH 7.2 with KOH, and 225 μg/ml amphotericin B (150 mg/ml stock solution in DMSO). Amphotericin-containing solutions were prepared daily and kept on ice and light protected. The osmotic activity of intracellular and external solutions (290–310 mmol/kg) was measured with a vapor pressure osmometer (model 5500, Wescor, Logan, UT).

All chemical reagents used were of analytic grade. The following drugs were used: ACh chloride, amphotericin B, caffeine, DMSO, dithiothreitol, lanthanum chloride, papain, and zinc chloride (Sigma Chemical, St. Louis, MO); cyclopiazonic acid (CPA) and thapsigargin (Calbiochem, La Jolla, CA); tetraethylammonium chloride (TEA; Eastman Kodak, Rochester, NY); and fura 2-AM, fura 2 pentapotassium salt, and Pluronic F-127 (Molecular Probes, Eugene, OR).

RESULTS

Fluorometric and electrophysiological experiments were performed on freshly isolated endothelial cells enzymatically dissociated from rabbit aorta ($n > 270$) and pulmonary arteries ($n = 7$). Fura 2-loaded cells

were simultaneously patch clamped using the perforated-patch whole cell recording configuration to correlate changes in [Ca²⁺]_i to membrane currents and voltages. The mean resting potential for cells studied was -35 ± 3 mV ($n = 15$), ranging from -58 to -25 mV, and the mean resting [Ca²⁺]_i was 65 ± 10 nM ($n = 22$).

Temporal relationship between [Ca²⁺]_i and membrane response when endothelial cells are activated by ACh or caffeine. Intracellular Ca²⁺ transients and whole cell currents or membrane potential changes, elicited by bath application of caffeine or ACh, were highly variable between individual cells. In 44 of 60 (73%) cells responding to 5 mM caffeine, 27 responded with measurable changes in [Ca²⁺]_i and membrane current or potential, whereas 3 exhibited a change in membrane potential or current without a corresponding change in [Ca²⁺]_i and 14 cells responded with an increase in [Ca²⁺]_i without measurable changes in membrane current or potential. Similarly, in 14 of 30 (42%) cells that responded to 100 μM ACh, 6 responded with measurable changes in [Ca²⁺]_i and membrane current or potential, whereas 3 exhibited a change in membrane potential or current with no change in [Ca²⁺]_i and 5 responded with an increase in [Ca²⁺]_i without an apparent change in membrane current or potential. Despite this heterogeneity, it was possible to characterize cellular responses in freshly dissociated endothelial cells to ACh and caffeine application.

Typical [Ca²⁺]_i responses, induced by ACh or caffeine, were biphasic with an initial rapid, transient rise, followed by a sustained, slowly decaying phase. The initial [Ca²⁺]_i transient was associated with a transient outward current or membrane hyperpolarization. No apparent change in holding current or membrane potential was associated with the sustained phase of the Ca²⁺ response. Figure 1A shows changes in [Ca²⁺]_i and temporally aligned whole cell currents from fura 2-loaded cells held at 0 mV during bath application of 5 mM caffeine and 100 μM ACh. In Fig. 1A the initial increase in the caffeine-induced [Ca²⁺]_i response led current activation, whereas the ACh-induced [Ca²⁺]_i response lagged current activation. The caffeine-induced peak [Ca²⁺]_i preceded the peak outward current, but in the presence of ACh the peak [Ca²⁺]_i occurred after maximal outward current activation. Bath application of 5 mM caffeine produced a mean increase in [Ca²⁺]_i of 279 ± 53 nM from a resting [Ca²⁺]_i of 67 ± 12 nM, which led a peak outward current amplitude at 0 mV of 317 ± 119 pA by 1.7 ± 1.0 s ($n = 11$). ACh (100 μM) evoked a mean transient increase in [Ca²⁺]_i of 143 ± 81 nM ($n = 4$).

In ~30% of the endothelial cells studied, STOCs ranging from 20 to 400 pA at 0 mV or spontaneous transient hyperpolarizations ranging from 5 to 30 mV were observed. Neither the STOCs nor the spontaneous transient hyperpolarizations were associated with measurable changes in fura 2 fluorescence. The presence of STOCs in an isolated endothelial cell voltage clamped at 0 mV is shown in Fig. 1B. The frequency of STOCs increased ~10-fold during application of 1 mM caffeine and preceded the increase in [Ca²⁺]_i. The caffeine-

induced increase in [Ca²⁺]_i was associated with a transient outward current, and the frequency of STOCs remained elevated until [Ca²⁺]_i returned to resting levels.

Voltage recordings from current-clamped, fura 2-loaded cells perfused with Na⁺-saline solutions containing caffeine or ACh are shown in Fig. 1C. In six cells, 5 mM caffeine produced maximal increases in [Ca²⁺]_i of 581 ± 237 nM at 1.6 ± 1.4 s after a hyperpolarization of 37 ± 8 mV. The initial increase in the [Ca²⁺]_i response, however, lagged the onset of membrane hyperpolarization by 0.1 ± 0.4 s. In seven cells, 100 μM ACh evoked maximal increases in [Ca²⁺]_i of 287 ± 55 nM at 1.8 ± 0.8 s after a 27 ± 8 mV peak hyperpolarization. The initial increase in the [Ca²⁺]_i response, however, led the onset of membrane hyperpolarization by 0.6 ± 0.4 s.

Overlap of ACh- and caffeine-sensitive intracellular Ca²⁺ stores. Although caffeine and ACh elicited similar changes in [Ca²⁺]_i and current or membrane potential, caffeine and ACh have been shown to affect distinct ER Ca²⁺ release channels (41). Figure 2A shows the [Ca²⁺]_i response and the corresponding changes in membrane potential in a fura 2-loaded cell successively exposed to 100 μM ACh and 5 mM caffeine. Independent of the order of agonist application, the cell responded to ACh and caffeine. The magnitudes and time courses of the [Ca²⁺]_i responses, however, depended on which agonist was applied first. When ACh was applied before caffeine, the total [Ca²⁺]_i increased 2.1-fold and the peak membrane hyperpolarization was 1.6-fold larger than when ACh was applied after caffeine. Similarly, application of caffeine before ACh caused a biphasic [Ca²⁺]_i increase, which was 1.7-fold greater than that elicited when caffeine was applied after ACh. In the latter condition, caffeine evoked only an increase in voltage noise, whereas caffeine application before ACh evoked a 10-mV hyperpolarization (Fig. 2B). Figure 2C shows [Ca²⁺]_i responses to 1 mM ACh in the absence and presence of a maximally effective concentration of caffeine. The mean peak [Ca²⁺]_i response to ACh in the presence of 20 mM caffeine was 233 ± 53 nM ($n = 5$), which was not statistically different from the maximal [Ca²⁺]_i response to ACh alone, 254 ± 97 nM ($P = 0.85$), measured in the same cells.

Concentration dependence of caffeine-induced [Ca²⁺]_i response and current activation. The [Ca²⁺]_i transient and membrane current induced by agonists such as ACh, bradykinin, and histamine have been shown to be concentration dependent in vascular endothelial cells (5, 24, 27). Caffeine-induced Ca²⁺ release from intracellular stores and activation of outward currents in endothelial cells have been described as a regenerative process with all-or-nothing activation (34) or as a dose-dependent process, with the magnitudes of the responses increasing with increasing caffeine concentration (4). Figure 3 shows a dose-dependent increase in [Ca²⁺]_i and outward current by caffeine. Changes in [Ca²⁺]_i obtained in response to increasing concentrations (0.5–5 mM) of caffeine are shown in Fig. 3A, *top traces*. The kinetics of the [Ca²⁺]_i response were also

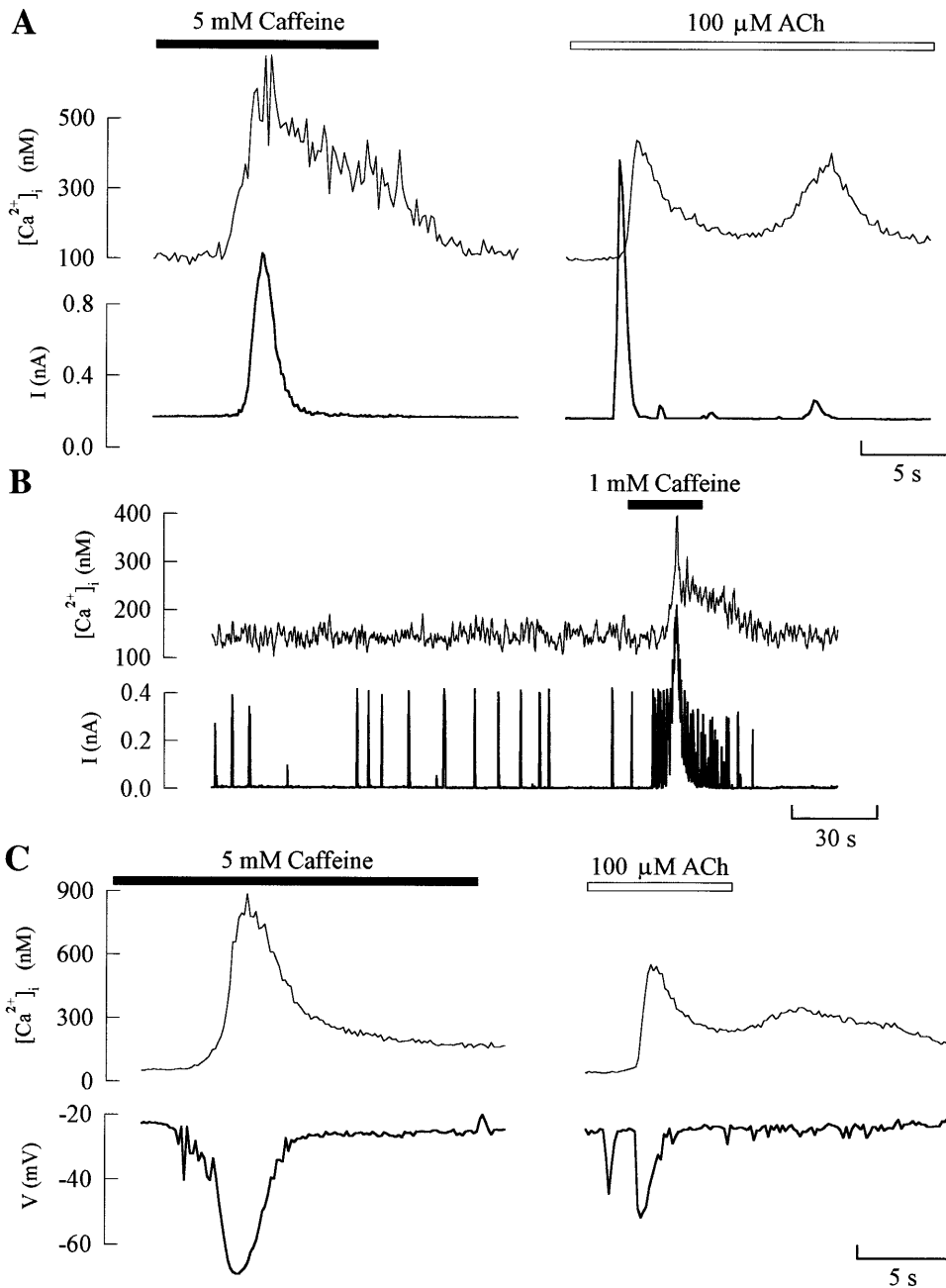


Fig. 1. Temporal relationship between intracellular Ca²⁺ concentration ([Ca²⁺]_i) and membrane current (*I*) or membrane potential (*V*) in response to stimulation by caffeine or ACh. Fura 2-loaded cells were voltage clamped at 0 mV (*A* and *B*) or current clamped (*C*) using perforated-patch whole cell recording configuration. Cells were perfused with Na⁺-saline containing caffeine or 100 μM ACh at indicated times. *Top traces* of [Ca²⁺]_i responses are temporally aligned to *bottom traces* of membrane current (*A* and *B*) or membrane potential (*C*), which were recorded simultaneously with fura 2 fluorescence.

dependent on the caffeine concentration, whereby the rate of initial rise in [Ca²⁺]_i increased with increasing caffeine concentration (from 17 nM/s at 0.5 mM caffeine to 580 nM/s at 5 mM caffeine). The caffeine-induced membrane outward currents obtained from the same cell held at 0 mV are shown in Fig. 3*A*, *bottom traces*. The peak current amplitude and rate of activation exhibit a dose dependence on caffeine similar to that observed for changes in [Ca²⁺]_i (Fig. 3*A*, *top traces*). Repeated applications of caffeine at >2 mM attenuated subsequent [Ca²⁺]_i responses. Although desensitization of caffeine responses may underestimate the magnitude of the saturating [Ca²⁺]_i increase or current with increasing caffeine concentration, the half-maximal increase in [Ca²⁺]_i was obtained with 1.2 mM caffeine

(*n* = 7–18) and 1.1 mM caffeine (*n* = 4–8) for half-maximal current activation (Fig. 3*B*). These data indicate that near-maximal [Ca²⁺]_i and current activation can be obtained with 5 mM caffeine.

Effect of inhibition of reuptake of Ca²⁺ by internal stores on responses to caffeine. The IP₃-sensitive intracellular Ca²⁺ store in endothelial cells has been reported to be rapidly refilled after Ca²⁺ influx from the extracellular space during the plateau phase of the [Ca²⁺]_i response (6, 36). The refilling of the intracellular Ca²⁺ store can be blocked by the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor CPA (20, 45). The caffeine-induced [Ca²⁺]_i transient has been shown to be independent of external Ca²⁺, suggesting that emptying of the caffeine-sensitive Ca²⁺ stores does not acti-

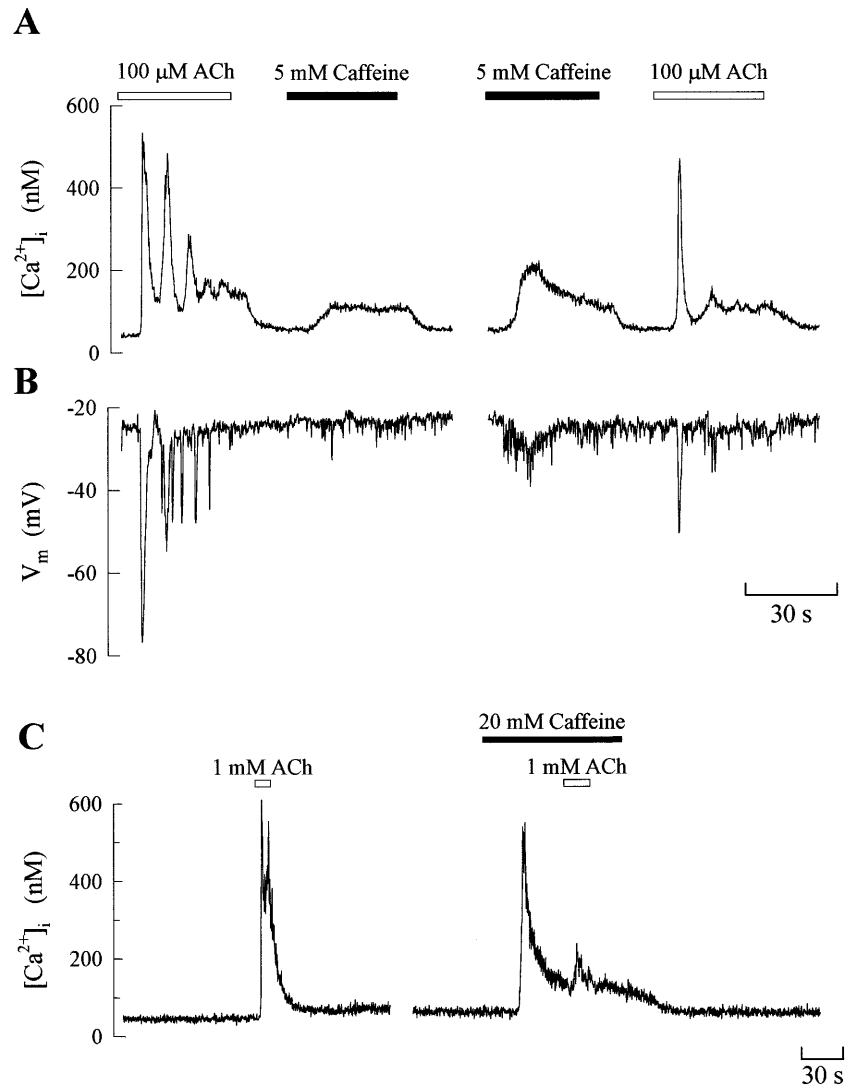


Fig. 2. Overlap between ACh- and caffeine-induced changes in $[Ca^{2+}]_i$ and membrane potential (V_m). Fura 2-loaded cells were perfused with Na⁺-saline containing ACh and/or caffeine at indicated times and concentrations. $[Ca^{2+}]_i$ (A) and membrane potential (V_m) (B) were recorded as functions of time. C: ACh-induced $[Ca^{2+}]_i$ responses obtained in absence and presence of 20 mM caffeine. ACh (1 mM) alone increased $[Ca^{2+}]_i$ to 610 nM, whereas in presence of caffeine, ACh increased peak $[Ca^{2+}]_i$ to 236 nM. Breaks in fluorescence and voltage records are 5-min intervals to allow cell to recover from prior ACh/caffeine stimulation. Membrane potential traces are temporally aligned to fluorescence traces.

vate influx of Ca²⁺ from the extracellular space (33, 34). The mobilization of intracellular Ca²⁺ and hyperpolarization of vascular endothelial cells by caffeine and the subsequent extrusion of cytoplasmic Ca²⁺ were examined after SERCA inhibition by CPA or thapsigargin.

Figure 4 shows the $[Ca^{2+}]_i$ responses to caffeine before and during prolonged exposure to 30 μ M CPA in the presence (A, top trace) or absence (B) of external Ca²⁺. Bath application of CPA (30 μ M) raised $[Ca^{2+}]_i$ from a mean resting level of 53 ± 6 nM to 111 ± 10 nM ($n = 12$, $P < 0.005$). In the presence of external Ca²⁺ and CPA, the amplitude of the caffeine-induced $[Ca^{2+}]_i$ response was $48 \pm 7\%$ ($n = 4$) of the $[Ca^{2+}]_i$ response in the absence of CPA. In the maintained presence of CPA, the mean caffeine-induced $[Ca^{2+}]_i$ increase evoked by a second application of caffeine was reduced by 74% ($n = 3$, data not shown) compared with the caffeine-induced response obtained in the absence of CPA. In the absence of external Ca²⁺ and presence of CPA, caffeine evoked an increase in $[Ca^{2+}]_i$; however, the amplitude of the response was only $16 \pm 1\%$ ($n = 4$) of control. The rate of decline of the caffeine-induced $[Ca^{2+}]_i$ increase was slowed in the maintained presence of caffeine. On

washout of caffeine, the rate of recovery of $[Ca^{2+}]_i$ to resting levels increased 2.7-fold ($n = 7$). The time constant of decay of elevated $[Ca^{2+}]_i$ after washout of caffeine was increased from 7.1 ± 0.9 to 28.3 ± 5.9 s ($n = 11$) by 30 μ M CPA. The fourfold increase in the time constant of decay was statistically significant ($P = 0.003$) compared with that observed in the absence of CPA.

The effects of ER Ca²⁺-ATPase inhibition on caffeine-induced changes in membrane potential and current were examined simultaneously with measurements of $[Ca^{2+}]_i$ in fura 2-loaded cells. Figure 4A shows membrane potential monitored simultaneously with $[Ca^{2+}]_i$ in a cell stimulated with 5 mM caffeine in the absence and presence of 30 μ M CPA. In the presence of CPA, the reduction of the caffeine-induced $[Ca^{2+}]_i$ response was accompanied by a similar decrease (~50%) in the amplitude of the caffeine-induced hyperpolarization. In seven experiments the magnitude of $[Ca^{2+}]_i$ transients and concomitant changes in current or voltage evoked by caffeine or ACh in the presence of CPA ($n = 5$) or thapsigargin ($n = 2$) were similarly reduced compared with control (absence of CPA).

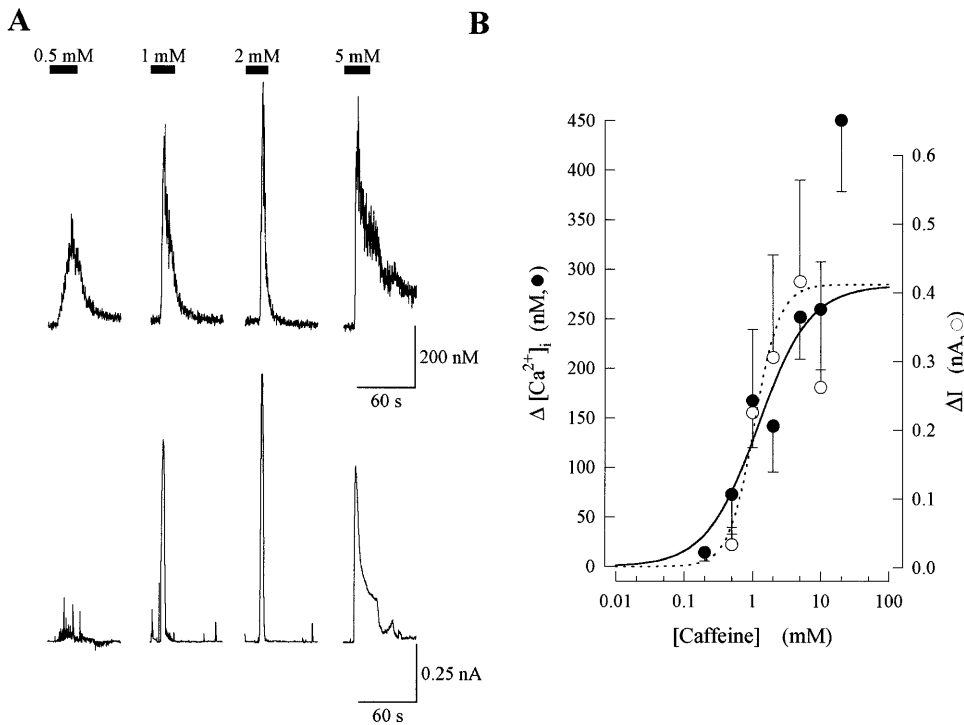


Fig. 3. Concentration dependence of caffeine-induced changes in $[Ca^{2+}]_i$ and membrane current amplitude. *A*: fura 2-loaded cell voltage clamped at 0 mV and perfused with Na⁺-saline containing 0.5, 1.0, 2.0, and 5.0 mM caffeine. $[Ca^{2+}]_i$ (top traces) and membrane current (bottom traces) were recorded simultaneously and are plotted as functions of time. Breaks in traces are 5-min intervals to allow cell to recover from previous caffeine stimulation. Current traces are temporally aligned to fluorescence traces above. *B*: dose-response curves for caffeine-induced $[Ca^{2+}]_i$ increases ($\Delta [Ca^{2+}]_i$, $n > 7$) and current activation (ΔI , $n > 4$). Lines drawn are best fits to Hill equation with calculated EC₅₀ of 1.2 and 1.1 mM and slopes of 1.1 and 2.3 for $\Delta [Ca^{2+}]_i$ and ΔI , respectively. Data points obtained for $\Delta [Ca^{2+}]_i$ at 20 mM caffeine and ΔI at 10 mM caffeine were not used in fits.

I-V relationship and ionic dependence of caffeine-induced currents. When caffeine-induced currents were recorded at hyperpolarized membrane potentials, caffeine-induced $[Ca^{2+}]_i$ transients were accompanied by small inward currents of -17 ± 5 ($n = 9$) and -13 ± 7 pA ($n = 12$) at -80 and -60 mV, respectively. Given that the K⁺ equilibrium potential (E_K) is -78 mV, these data suggest that a charge carrier other than K⁺ contributes to the caffeine-activated current. The ionic dependence of caffeine-induced currents was investigated by ion substitution. $[Ca^{2+}]_i$ transients and membrane currents were recorded simultaneously from a fura 2-loaded endothelial cell held at 0 mV in response to 5 mM caffeine in Na⁺-saline, K⁺-saline, and SO₄²⁻-saline (Fig. 5A). Replacement of external Na⁺ with K⁺ did not affect the caffeine-induced $[Ca^{2+}]_i$ response but reversed the direction of the caffeine-induced membrane current from outward to inward ($n = 3$). There was, however, no change in the caffeine-induced current amplitude when external Na⁺ was replaced with Li⁺ or *N*-methyl-D-glucamine ($n = 3$, data not shown). Similarly, replacement of external Cl⁻ with SO₄²⁻ did not markedly change the caffeine-induced $[Ca^{2+}]_i$ transient but reduced the amplitude of the caffeine-activated outward current by $\sim 50\%$ (Fig. 5A).

I-V relationships were obtained in Na⁺-saline, K⁺-saline, and SO₄²⁻-saline to identify the charge carriers and their contribution to caffeine-induced membrane currents. Net caffeine-induced currents obtained in response to voltage ramps are shown in Fig. 5B. In Na⁺-saline the reversal potential of the caffeine-activated current was -48 mV compared with E_K of -78 mV. In K⁺-saline the reversal potential shifted from -48 to 0 mV and the outward rectification of the caffeine-induced current was reduced. When external

Cl⁻ was reduced (SO₄²⁻-saline), the caffeine-induced current amplitude at all potentials was reduced and the reversal potential shifted by $+25.5$ mV ($n = 2$).

Sensitivity of caffeine-induced changes in $[Ca^{2+}]_i$ and membrane potential to external La³⁺, Zn²⁺ and TEA. The actions of the putative ion channel blockers TEA, La³⁺, and Zn²⁺ on the caffeine-evoked currents were used to further characterize the ionic dependence of the caffeine response. In the absence of caffeine, 5 mM TEA reduced the outward current in response to voltage ramps from -120 to $+60$ mV ($n = 3$), whereas 1 mM La³⁺ ($n = 2$) and 0.1–0.2 mM Zn²⁺ ($n = 5$) reduced outward and inward currents (data not shown).

Figure 6 shows the response of a cell to 5 mM caffeine obtained in the absence and presence of TEA and La³⁺. Under control conditions, caffeine evoked a typical transient increase in $[Ca^{2+}]_i$ (Fig. 6A), which was temporally related to a transient membrane hyperpolarization followed by a brief depolarization (Fig. 6B). The transient hyperpolarization was reversibly inhibited by 5 mM TEA (Fig. 6B), reducing the maximum change in membrane potential from -46 ± 7 to -4 ± 6 mV ($n = 5$). In the presence of TEA, a transient depolarization associated with the caffeine-induced $[Ca^{2+}]_i$ increase was observed (Fig. 6). Bath-applied La³⁺ (1 mM) slightly depolarized (<10 mV) the cell and abolished the transient depolarizations observed in response to caffeine (Fig. 6B). Neither TEA nor La³⁺ appreciably altered the peak $[Ca^{2+}]_i$ response to caffeine in freshly dissociated endothelial cells.

La³⁺ (1 mM) and Zn²⁺ (100 μ M) also attenuated caffeine-induced inward currents in endothelial cells. In a voltage-clamped cell held at -60 mV, caffeine evoked an inward current of -30 pA, which was inhibited by 1 mM La³⁺ or 0.2 mM Zn²⁺. After washout

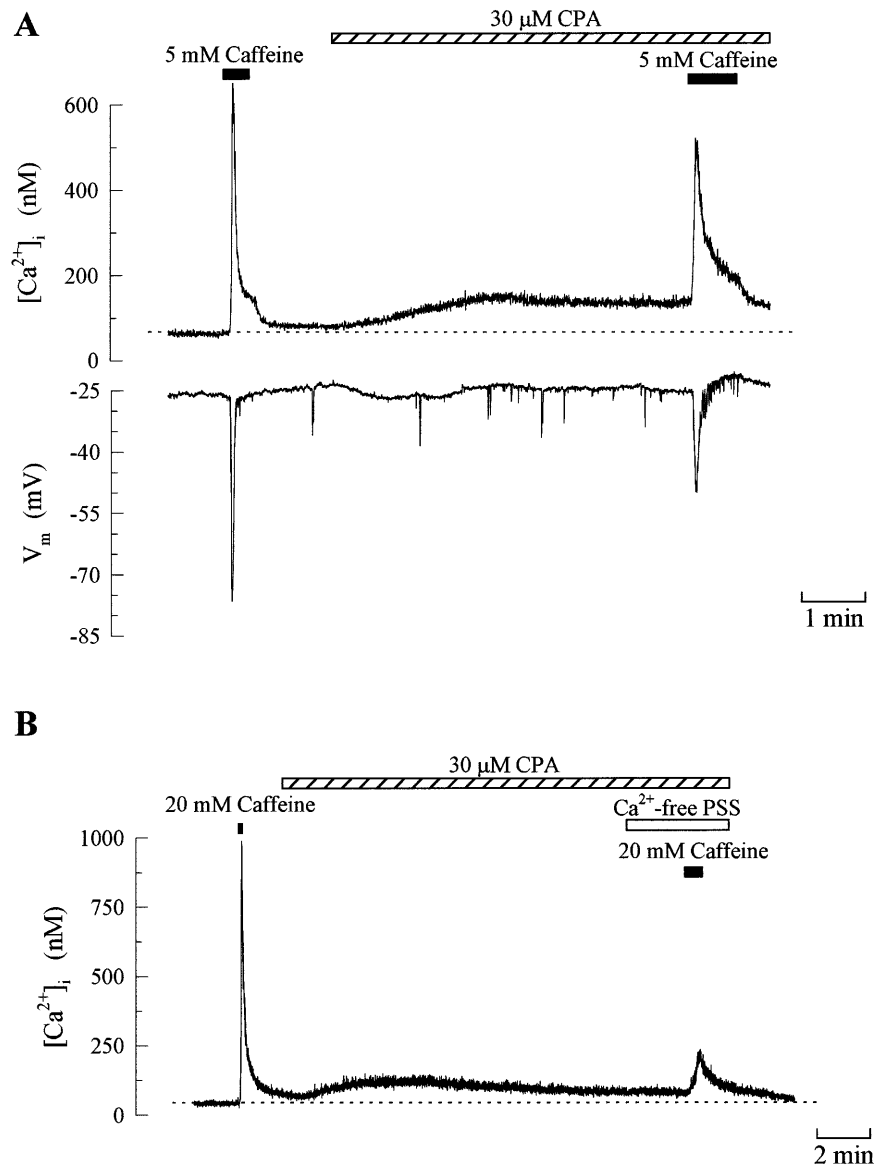


Fig. 4. Cyclopiazonic acid (CPA) inhibition of Ca²⁺ reuptake by caffeine depleted intracellular stores. Fura 2-loaded cells were perfused with Na⁺-saline containing caffeine and 30 μ M CPA at indicated times and concentrations. [Ca²⁺]_i and membrane potential are plotted as functions of time. Dashed lines, resting [Ca²⁺]_i. **A**: fura 2-loaded cell perfused with Na⁺-saline containing 5 mM caffeine in absence and presence of 30 μ M CPA. Membrane potential is temporally aligned with [Ca²⁺]_i. **B**: cell perfused with Na⁺-saline containing 20 mM caffeine in absence and presence of 30 μ M CPA in a Ca²⁺-free solution with 1 mM EGTA (physiological salt solution, PSS).

of La³⁺, the caffeine-induced inward current recovered, although it was reduced in amplitude (−18 pA, data not shown). The holding current at −60 mV was also reduced in the presence of La³⁺ or Zn²⁺, suggesting the presence of resting nonselective cation and Cl[−] conductances in vascular endothelial cells (11, 33).

DISCUSSION

These results describe quantitatively the relationship between the increase in [Ca²⁺]_i and the activation of ionic currents in arterial endothelial cells in response to ACh and caffeine. In 7% of the cells that exhibited caffeine-activated currents, no apparent changes in [Ca²⁺]_i were observed, and 32% of cells that responded to caffeine with increases in [Ca²⁺]_i had no corresponding changes in membrane current or voltage. These observations are most likely due to the fact that single-cell photometry of fura 2 fluorescence measures the average change in [Ca²⁺]_i throughout the bulk cytoplasm, whereas Ca²⁺-activated currents respond to

[Ca²⁺]_i changes adjacent to the plasma membrane. Fura 2 measurements are unlikely to resolve localized [Ca²⁺]_i changes in small, restricted volumes of the cell as postulated to exist between the ER and the plasma membrane (9, 14). The occurrence of STOCs and STICs in vascular smooth muscle cells have been proposed to be due to local release of Ca²⁺ and the concomitant activation of Ca²⁺-activated K⁺ and Cl[−] currents, respectively (43, 44). Similarly, freshly dissociated endothelial cells exhibit STOCs that have been suggested to be due to the release of Ca²⁺ from CICR channels in the ER adjacent to the plasma membrane and activation of K⁺ channels sensitive to TEA and charybdotoxin (32). The occurrence of STOCs or spontaneous transient hyperpolarizations in rabbit aortic endothelial cells was not accompanied by measurable changes in fura 2 fluorescence (Figs. 1 and 4). Agonist-induced currents without global changes in [Ca²⁺]_i suggest that cells can respond to increases in [Ca²⁺]_i in the restricted space adjacent to the plasma membrane (3). Similarly, changes in [Ca²⁺]_i

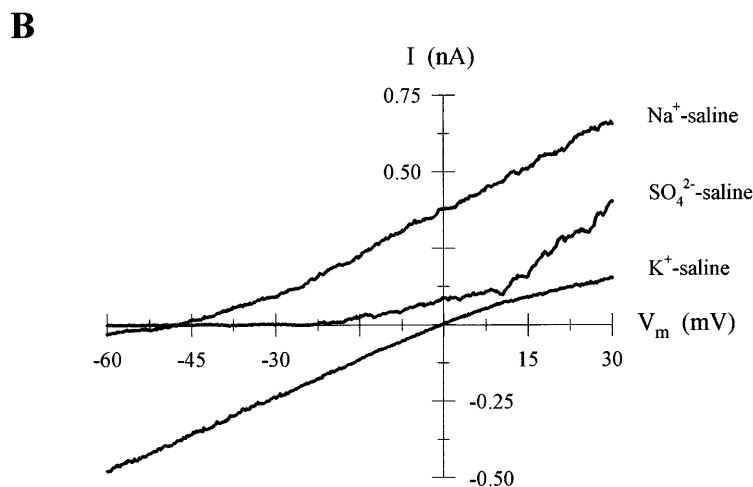
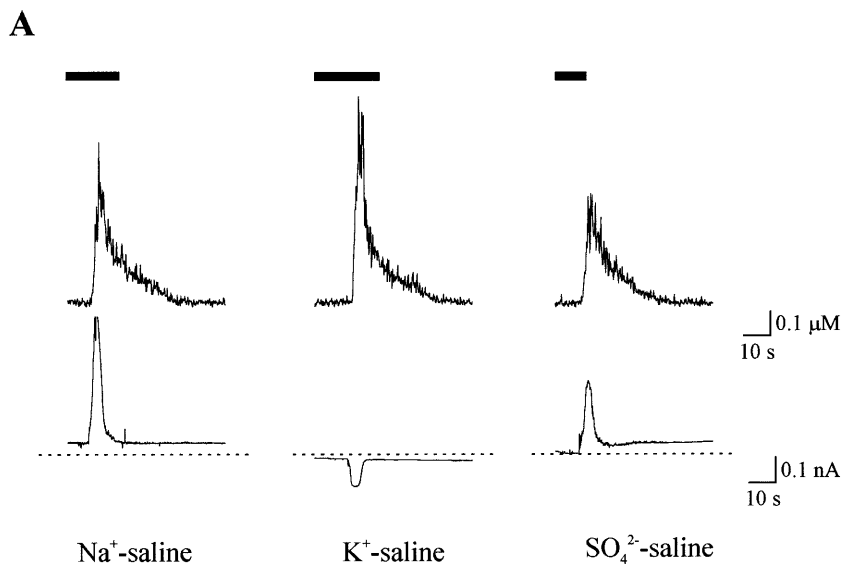


Fig. 5. Ionic and voltage dependence of caffeine-evoked membrane currents. *A*: fura 2-loaded cell voltage clamped at 0 mV and perfused with Na⁺-saline, K⁺-saline, and SO₄²⁻-saline. Caffeine (5 mM) (solid bars) was applied as indicated. [Ca²⁺]_i (top traces) and membrane current (bottom traces) in response to caffeine are shown as functions of time. Dashed line, zero-current level. Current traces are temporally aligned with fluorescence traces above. *B*: membrane currents measured in response to voltage ramps from a different cell perfused with Na⁺-saline, K⁺-saline, and SO₄²⁻-saline containing 5 mM caffeine. Voltage ramps (300 mV/s) were applied before and during peak of caffeine-induced response. Current-voltage (*I-V*) relationships represent net caffeine-induced currents obtained by subtracting currents recorded in absence of caffeine from those recorded in presence of caffeine. Membrane potentials are corrected for liquid junction potentials. Reversal potentials of caffeine-induced currents are -47.9, -0.3, and -22.3 mV in presence of Na⁺-saline [E_K equilibrium potential (E_K) = -78 mV; Cl⁻ equilibrium potential (E_{Cl}) = -25 mV], K⁺-saline (E_K = 0 mV; E_{Cl} = -25 mV), and SO₄²⁻-saline (E_K = -78 mV; E_{Cl} = 0 mV), respectively.

that occurred ~1–2 s after current activation may be due to local changes in [Ca²⁺]_i, which activated the current preceding or even stimulating the increase in global [Ca²⁺]_i.

Although ACh and caffeine elicit similar increases in [Ca²⁺]_i and changes in membrane current and potential, our results suggest that the intracellular Ca²⁺ stores sensitive to ACh and caffeine may be distinct. Fura 2 fluorescence measurements indicate that, in endothelial cells, application of caffeine does not completely empty ACh-sensitive Ca²⁺ stores (Fig. 2), suggesting that the IP₃- and ryanodine-sensitive Ca²⁺ stores do not overlap entirely. The degree of store overlap is difficult to quantitate because of desensitization of responses; however, Fig. 2 indicates that only a fraction of the Ca²⁺ stores overlap or interact. Similar observations have been reported for the caffeine- and ACh-induced changes in membrane potential, which were associated with [Ca²⁺]_i transients, and suggest the existence of partially overlapping IP₃- and ryanodine-sensitive Ca²⁺ stores (40, 45). Our results would also be consistent with a CICR mechanism, wherein

Ca²⁺ released from the IP₃-sensitive store could impact on the ryanodine-sensitive Ca²⁺ store and vice versa. A potential concern is that caffeine may attenuate the ACh-induced [Ca²⁺]_i response, given that caffeine (10 mM) has been shown to inhibit IP₃-gated channel activity in lipid bilayers (7).

Mobilization of intracellular Ca²⁺ and membrane current activation by caffeine was dose dependent, desensitized with repeated applications of high concentrations of caffeine, and highly variable from cell to cell, consistent with previous studies in vascular endothelial cells (4, 15, 39). The magnitude of the increase in [Ca²⁺]_i, as well as the kinetics of the response, was dependent on the caffeine concentration, with the initial rate of rise in [Ca²⁺]_i and the peak [Ca²⁺]_i increasing with caffeine concentration. Half-maximal caffeine concentrations for increasing [Ca²⁺]_i and current activation were similar, ~1.1 mM. Similar dose-response relations obtained for caffeine-induced [Ca²⁺]_i increases and outward current activation suggest that the events are unlikely to be independent and that caffeine-induced Ca²⁺ mobilization activates Ca²⁺-

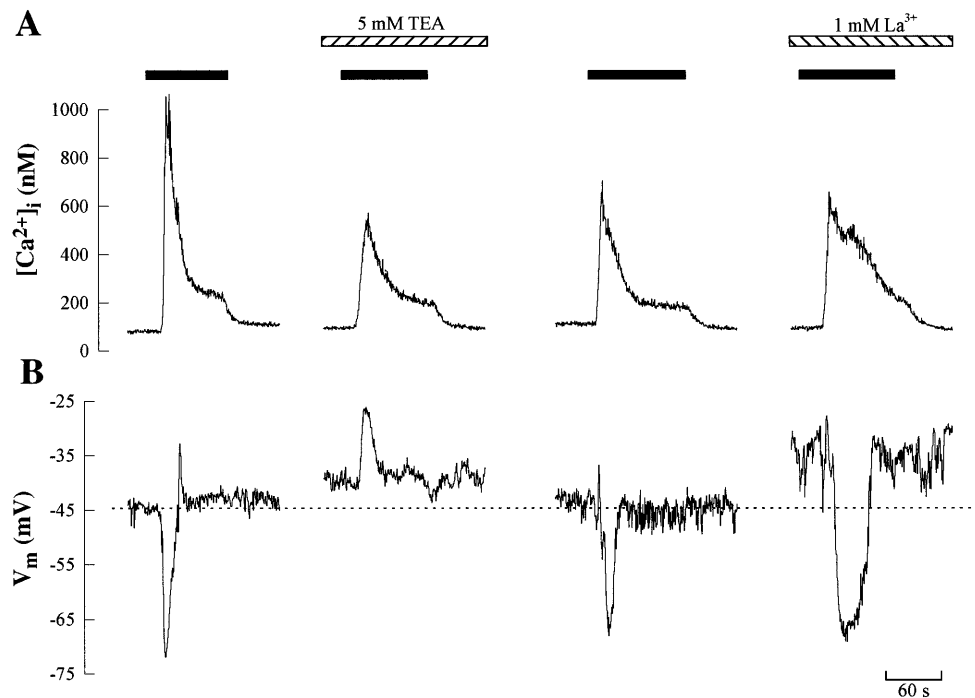


Fig. 6. Effects of tetraethylammonium (TEA) and La^{3+} on caffeine-induced changes in $[Ca^{2+}]_i$ and membrane potential. A fura 2-loaded cell was perfused with Na^+ -saline containing 5 mM caffeine (solid bars) in absence or presence of 5 mM TEA or 1 mM La^{3+} . $[Ca^{2+}]_i$ (A) and membrane potential (B) were recorded simultaneously and plotted as functions of time. Dashed line, initial resting membrane potential. Membrane potential traces are temporally aligned to fluorescence traces above and corrected for liquid junction potentials. Breaks in traces represent 5-min recovery periods after caffeine stimulation.

sensitive membrane currents. Previous studies on rabbit aortic endothelial cells, however, have suggested that $[Ca^{2+}]_i$ mobilization and current responses to caffeine, measured independently, were regenerative (34).

The initial phase of the agonist-induced $[Ca^{2+}]_i$ transient in endothelial cells has been shown to be independent of external Ca^{2+} and is due to the release of Ca^{2+} from internal Ca^{2+} stores (for reviews see Refs. 1 and 19). This initial rapid rise in $[Ca^{2+}]_i$ is assumed to be closely related to the membrane hyperpolarization due to the activation of Ca^{2+} -dependent K^+ current by Ca^{2+} released from the IP_3 -sensitive Ca^{2+} stores (27, 32, 35, 37). The sustained phase of the agonist (ACh)-induced $[Ca^{2+}]_i$ increase is dependent on external Ca^{2+} (for reviews see Refs. 1 and 19), and the influx of divalent cations from the extracellular space has been directly demonstrated by Mn^{2+} quench of the fura 2 signal (6). Activation of Ca^{2+} -sensitive cation currents (16) and associated membrane depolarizations observed in the presence of external TEA are consistent with cation influxes during the plateau phase of the $[Ca^{2+}]_i$ increase. In contrast, the $[Ca^{2+}]_i$ transient induced by caffeine is not dependent on external Ca^{2+} , and it is not accompanied by Mn^{2+} quench of the fura 2 signal (33, 34). Thus the caffeine-induced transient depolarization blocked by La^{3+} (Fig. 6) appears to differ from the sustained depolarization observed with bradykinin or thapsigargin activation of cultured bovine aortic endothelial cells (40, 42) or ACh-stimulated endothelium of intact rat aorta (26). Furthermore, La^{3+} -induced depolarization of a resting cell (Fig. 6B) suggests that La^{3+} may block an ionic current other than the Ca^{2+} release-activated Ca^{2+} current. Substitution of external Na^+ by K^+ , reduction of external Cl^- , or change in the membrane potential had no appreciable effect on caffeine-

induced $[Ca^{2+}]_i$ responses, also suggesting that the $[Ca^{2+}]_i$ increase was due to release of intracellular Ca^{2+} and not Ca^{2+} influx across the plasma membrane (15, 42).

Incubation of cells with CPA or thapsigargin caused relatively small increases in basal $[Ca^{2+}]_i$ levels (≥ 50 nM) and did not appreciably change the membrane potential or the holding current (cf. Ref. 13). In the presence of CPA, however, the magnitude of the caffeine-induced $[Ca^{2+}]_i$ response was decreased by $\sim 50\%$. The caffeine-induced $[Ca^{2+}]_i$ response was further reduced in Ca^{2+} -free external solutions containing CPA, suggesting that Ca^{2+} influx contributes to the response or that removal of external Ca^{2+} affects the state of the internal Ca^{2+} stores and thereby reduces the amount of Ca^{2+} available for release by caffeine. The $[Ca^{2+}]_i$ response to caffeine in the absence of CPA and external Ca^{2+} was not significantly different from that in the presence of external Ca^{2+} . Taken together, these results suggest that loss of Ca^{2+} from internal stores in the absence of external Ca^{2+} is balanced, in part, by Ca^{2+} reuptake, which can be blocked by CPA (Fig. 4B).

In the majority of caffeine-induced Ca^{2+} responses (84%), the rate of recovery to initial $[Ca^{2+}]_i$ levels was sensitive to the presence of caffeine, whereby washout of caffeine increased the rate of $[Ca^{2+}]_i$ decline 2.7-fold. This effect was independent of the presence of CPA, suggesting that the increased rate of $[Ca^{2+}]_i$ decline is not due to an inhibitory effect of caffeine on reuptake of Ca^{2+} into internal stores. Elevated $[Ca^{2+}]_i$ or the state of the internal stores has been proposed to increase the rate of Ca^{2+} -ATPase activity (25); however, this mechanism cannot explain the increased rate of $[Ca^{2+}]_i$ decline on washout of caffeine. If caffeine maintains an elevated $[Ca^{2+}]_i$ by preventing reuptake of Ca^{2+} into the internal stores, then, in the presence of SERCA inhibi-

tors, washout of caffeine would have no effect on the rate at which [Ca²⁺]_i declines. Reuptake, however, is an important mechanism for lowering [Ca²⁺]_i to initial levels after agonist stimulation. In the presence of caffeine or after its washout, CPA increased the time constant of decay of the caffeine-induced [Ca²⁺]_i response four- to fivefold.

The shift of reversal potential of the caffeine-evoked current after replacement of external Na⁺ with K⁺ and the inhibition of caffeine-induced hyperpolarizations by TEA, an inhibitor of K⁺ channels (32), suggest that K⁺ contributes to the caffeine-induced outward current. However, the reversal potential (−48 mV) of caffeine-induced currents is positive to E_K, and at holding potentials of no more than −60 mV caffeine evoked an inward current, indicating the activation of an ionic current with a charge carrier other than K⁺. In lowered external Cl[−] concentration, caffeine-induced outward current amplitudes were reduced and the reversal potential was shifted to more positive potentials, consistent with a decrease in the driving force for inward Cl[−] movement. Caffeine-activated currents were also partially inhibited by external Zn²⁺, an inhibitor of Cl[−] channels (11, 33). Taken together, these results indicate that Ca²⁺-activated Cl[−] and K⁺ conductance increases underlie caffeine-induced currents, consistent with previous studies suggesting that agonist- or caffeine-induced release of intracellular Ca²⁺ activates Ca²⁺-dependent K⁺ currents (16, 33), Ca²⁺-dependent Cl[−] currents (16, 28, 33), and/or nonselective cation currents (42).

In conclusion, in freshly dissociated endothelial cells, ACh and caffeine activate increases in [Ca²⁺]_i from intracellular Ca²⁺ stores, which, if distinct, must be interactive. Also in these cells, ACh and caffeine activate outward currents, which transiently cause membrane hyperpolarization. The caffeine-induced hyperpolarization is due to a dose-dependent increase in [Ca²⁺]_i and the activation of Ca²⁺-sensitive K⁺ and Cl[−] conductances; however, the peak current was not always observed to occur after the peak increase in [Ca²⁺]_i measured by fura 2 fluorescence. The lack of a clear temporal relationship between agonist-induced changes in membrane current and increases in [Ca²⁺]_i reflects the inability of single-cell photometry to resolve local agonist-induced [Ca²⁺]_i increases measured electrophysiologically. This conclusion, from results obtained in rabbit arterial endothelial cells, is consistent with recently reported results in bovine coronary arterial smooth muscle (38) and porcine coronary endothelial cells (14).

This research was supported by National Health and Medical Research Council of Australia Project Grant 961135 to D. J. Adams and National Fund for Scientific Research (NFWO) Belgium Grant to P. Fransen.

A preliminary report of some of these results has been published as an abstract (10).

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Received 6 March 1998; accepted in final form 21 July 1998.

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