J. Membrane Biol. 130, 125–137 (1992)

The Journal of
Membrane Biology

A Patch-Clamp Study of the Ca^{2+} Mobilization from Internal Stores in Bovine Aortic Endothelial Cells. I. Effects of Caffeine on Intracellular Ca^{2+} Stores

Dominique Thuringer and Rémy Sauvé
Groupe de Recherche en Transport Membranaire Département de Physiologie, Université de Montréal, Québec, Canada H3C 3J7

Summary. The effects of agents known to interfere with Ca^{2+} release processes of endoplasmic reticulum were investigated in bradykinin (BK)-stimulated bovine aortic endothelial cells (BAE cells), via the activation of Ca^{2+}-activated potassium channels [$K(Ca^{2+})$ channels]. In cell-attached patch experiments, the external application of caffeine (1 mM) caused a brief activation of $K(Ca^{2+})$ channels in Ca^{2+}-free and Ca^{2+}-containing external solutions. The application of BK (10 nM) during cell stimulation by caffeine (1–20 mM) invariably led to a drastic channel activation which was maintained during a recording period longer than that observed in caffeine-free conditions. In addition, the cell exposure to caffeine (20 mM) during the BK stimulation enhanced systematically the channel activation process. Since a rapid inhibition of BK-evoked channel activity was also produced by removing caffeine from the bath medium, it is proposed that the sustained single-channel response recorded in the concomittant presence of both agents was due to their synergic action on internal stores and/or the external Ca^{2+} entry pathway resulting in an increased $[Ca^{2+}]_i$. In addition, the local anesthetic, procaine, depressed the initial BK-induced $K(Ca^{2+})$ channel activity and completely blocked the secondary phase of the channel activation process related to the external Ca^{2+} influx into stimulated cells. In contrast, this blocking effect of procaine was not observed on the initial caffeine-elicited channel activity and could not suppress the external Ca^{2+}-dependent phase of this channel activation process. Our results confirm the existence of at least two pharmacologically distinct types of Ca^{2+}-release from internal stores in BAE cells: an inositol 1,4,5-triphosphate ($InsP_3$)-dependent and a caffeine-induced Ca^{2+}-release process.

Key Words Ca^{2+}-activated K^+ channels · caffeine · procaine · bradykinin

Introduction

The stimulation by bradykinin (BK) of B2 membrane receptors in endothelial cells is correlated with a biphasic elevation in cytosolic-free calcium concentration ($[Ca^{2+}]i$), characterized by an initial discharge of sequestered Ca^{2+} from intracellular stores, and a subsequent long-lasting entry of Ca^{2+} from the extracellular medium (Colden-Stanfield et al., 1987; Schilling et al., 1988; Sage, Adams & Van Breemen, 1989; Freay et al., 1989; Hallam et al., 1989; Thuringer, Diarra & Sauvé, 1991). The sustained phase of $[Ca^{2+}]i$ elevation is often associated with a more complex oscillatory behavior depending on both agonist and endothelial cell types (Jacob, 1991). Although the mechanism underlying $[Ca^{2+}]i$ oscillations remains undefined, it is generally admitted that the receptor-stimulated breakdown of phosphoinositides generating inositol 1,4,5-trisphosphate ($InsP_3$) triggers the initial phase of the intracellular Ca^{2+} mobilization from the endoplasmic reticulum (ER) (Freay et al., 1989; Myers & Larkins, 1989). However, evidence that the $InsP_3$-sensitive store includes all the sequestered Ca^{2+} in endothelial ER, has not been yet demonstrated. This uncertainty leaves open the possibility that there may be more than one ER compartment involved in the receptor-stimulated Ca^{2+} signal as proposed in pancreatic acinar cells and oocytes to explain calcium oscillations (Thévenod et al., 1989; Peres, 1990; Wakui, Osipchuk & Petersen, 1990; Berridge, 1991; Dehlinger-Kremer, Zeuzem & Schulz, 1991). The tendency for propagating $[Ca^{2+}]i$ waves to be superimposed on a slow rise in $[Ca^{2+}]i$ (Jacob et al., 1988) and to disappear when external Ca^{2+} is substituted by Mn^{2+} (Jacob, 1990) in agonist-simulated human endothelial cells, would allow for a portion of ER to be $InsP_3$-insensitive and capable of Ca^{2+}-induced Ca^{2+}-release. In addition to their role in the initial phase of Ca^{2+} mobilization, intracellular Ca^{2+} stores could be also involved in the agonist-induced Ca^{2+} influx from the extracellular space (Hallam et al., 1989; Jacob, 1990). According to Putney's capacitative model (1986), the external Ca^{2+} entry may be intimately regulated by emptying internal Ca^{2+} stores, although the exact nature of this regulatory mechanism remains unresolved.

The purpose of this study was to examine the possible coexistence of distinct types of intracellular Ca^{2+} stores in BAE cells of confluent monolayers, using agents with known effects on Ca^{2+}-release mechanisms. One of the basic actions of methylxanthines, such as caffeine, is to translocate Ca^{2+} from intracellular Ca^{2+} storage sites into the cytosol by an enhanced Ca^{2+}-induced Ca^{2+}-release mechanism. This process has been more especially studied in the sarcoplasmic reticulum (SR) of excitable cells (Endo, 1977). Beside its activating effect on the Ca^{2+} release channel of SR (Rousseau et al., 1988; Sitsapesan & Williams, 1990), caffeine also inhibits the cyclic adenosine 3′:5′-monophosphate (cAMP) phosphodiesterase and consequently increases cAMP content in various cell types causing an enhanced rate of Ca^{2+} resequestration into SR stores (Fabiato & Fabiato, 1975; Fredholm, Brodin & Strandberg, 1979; Saida & Van Breemen, 1984). In contrast to caffeine, local anesthetics, such as procaine, depress both Ca^{2+}-induced and $InsP_3$-induced Ca^{2+}-release mechanisms from SR (Ahn & Karaki, 1988; Kobayashi et al., 1988) and ER. For instance, Freay and co-workers (1989) have shown that the Ca^{2+}-release stimulated by $InsP_3$ in permeabilized cells or by BK in intact BAE cells was partially inhibited by procaine. This agent was also found to have additional effects on voltage-dependent Ca^{2+} channels (Spedding & Berg, 1985) and K^+ channels (Ohya, Kitamura & Kuriyama, 1987) in plasmic membranes of smooth muscle cells. We studied the effects of caffeine and procaine on BK-induced fluctuations of $[Ca^{2+}]i$ by recording the activity of $K(Ca^{2+})$ channels in BAE cells of confluent monolayers, using the cell-attached patch-clamp technique (Sauvé et al., 1990*a*; Thuringer et al., 1991). Our results argue strongly for the coexistence of an $InsP_3$-sensitive and a caffeine-sensitive Ca^{2+} store. The latter store could contribute to the BK-evoked $[Ca^{2+}]i$ increase by a Ca^{2+}-induced Ca^{2+}-release mechanism initiated by an initial Ca^{2+} discharge from the $InsP_3$-sensitive Ca^{2+} store.

Abbreviations

BAE cell: bovine aortic endothelial cell; BK: bradykinin; $InsP_3$: inositol 1,4,5-trisphosphate; EGTA: ethylene glycol-bis(*b*-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; BCECF: 2′,7′-bis(carboxyethyl)-5(6′)-carboxyfluorescein; $[Ca^{2+}]i$: cytosolic-free calcium ion concentration; $K(Ca^{2+})$ channel: calcium-activated potassium channel; iK1 channel: inward-rectifying potassium channel; Vp: potential applied in the patch pipette; cAMP: cyclic adenosine 3′:5′-monophosphate; ER: endoplasmic reticulum; SR: sarcoplasmic reticulum; *N*: number of channels; *Po*: open channel probability.

Materials and Methods

All chemical compounds were purchased from Sigma (St. Louis, MO). BAE cells of confluent monolayers were continuously superfused with Earle's medium having the following standard composition (in mM): 121.3 NaCl, 6.0 $NaHCO_3$, 1.0 NaH_2PO_4, 5.4 KCl, 0.8 $MgSO_4$, 1.8 $CaCl_2$ and 5.5 glucose; and buffered with 25 mM HEPES adequately adjusted by addition of 10 mM NaOH to pH 7.3. In a few experiments, $NaHCO_3$ was replaced by an equimolar amount of NaCl to obtain the bicarbonate-free Earle solution. The Ca^{2+}-free Earle solution was prepared by adding 2 mM EGTA with no $CaCl_2$. BK was dissolved as a stock solution at a concentration of 10 μM in H_2O. Caffeine and procaine were added to the external medium at the final concentration desired, and the pH of all solutions was checked just before use. The pipette solution contained (in mM): 200 KCl, 0.5 $MgSO_4$, 0.91 $CaCl_2$, 1 EGTA for a free calcium concentration of 1 μM. The pH was adjusted at 7.3 with 25 mM HEPES plus 10 mM KOH for a total K^+ concentration of 210 mM.

Preparation of Cell Culture

BAE cells were cultured in a Dulbecco's modified Earle's medium (GIBCO) supplemented with 10% newborn calf serum, 3.7 g/liter $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin as previously described (Sauvé et al., 1988). For experiments, cells from serial passages 21–26 were reseeded at low density onto microscope slides coated with collagen and kept in culture for five to ten days before use.

Patch-Clamp Recordings

Membrane current recordings were performed in the cell-attached patch-clamp configuration (Hamill et al., 1981) using a List EPC7 amplifier. Patch pipettes were pulled from Pyrex capillaries (Corning 7040) and had resistances ranging between 4 and 10 MΩ. The potential values reported in patch-clamp experiments are expressed as Vp. Current traces were recorded directly on FM tapes (HP 3964A) at a bandwith of 1.25 kHz, and subsequently filtered at 0.8–1.0 kHz using two low-pass four-pole Bessel filters (VVS 300B) before being digitalized at a minimum sampling rate of 2–10 kHz. Current leaks were corrected by using a successive linear interpolation procedure. The electrical recordings were performed in a continuous perfusion mode as described elsewhere (Sauvé et al., 1988). The time constant of solution change at the onset of a drug application was estimated to be 5 sec. All experiments were carried out at room temperature (usually 20°–23°C).

Signal Analysis

In order to determine the time course of the probability changes in current records containing one $K(Ca^{2+})$ channel, we used a procedure in which the values of *Po* were obtained by computing the mean open and closed time of the channel for a given set of K successive open and closed time intervals (K = 100). The time intervals were determined from current transitions detected on the basis of a two-reference levels procedure (Sauvé et al., 1986). Within this framework, the j^{th} value of *Po*, namely *P*(*j*), was given by:

$$P(j) = \sum_{i=[j-1]K+1}^{jK} \frac{t_i^o}{T(j)} \qquad 1 \leq j \leq \frac{n}{K}$$

$$T(j) = \sum_{i=[j-1]K+1}^{jK} t_i^o + \sum_{i=[j-1]K+1}^{jK} t_i^c$$

with, t_i^o and t_i^c representing the i^{th} open and closed time interval measured from the original current record, and n the total number of intervals detected over the entire recording period. Such a method was chosen to prevent statistical errors related to a variation in the number of open and closed time intervals for a fixed period of time. However, this procedure cannot be applied when several channels are active in the membrane patch. In this case, the product NPo, where N is the total number of channels, is computed successively using a selected constant period of time as shown in Fig. 5*B*.

Assessment of Changes in $[Ca^{2+}]i$ by Recording the $K(Ca^{2+})$ Channel Activity

In BAE cells, $K(Ca^{2+})$ channels are characterized by an elementary conductance of 40 pS in symmetrical 150 mM KCl, a flickering behavior showing rapid fluctuations between open and closed states, and a $[Ca^{2+}]i$ threshold level for activation estimated at 0.4 μM in inside-out patches (Sauvé et al., 1988; 1990*a*; Colden-Stanfield et al., 1990). Since the open probability of $K(Ca^{2+})$ channel was found to be voltage insensitive within the range -100 to 0 mV (Sauvé et al., 1988), changes in single-channel activity are likely to reflect changes in $[Ca^{2+}]i$. To confirm that $K(Ca^{2+})$ channels can be used as local probe for monitoring submembrane changes in free calcium, cell-attached current records and fluorescence measurements of $[Ca^{2+}]i$ were performed simultaneously in Fura-2 loaded cells (Fig. 1). Following a 45–60 min period of cell incubation with the acetoxy methyl ester form of the Ca^{2+} indicator dye Fura-2 (2 μM) at room temperature, BAE cells of confluent monolayers were continuously superfused with the recording Earle solution. The light collection was usually limited to an area including about 80–100% of the recorded cell. $[Ca^{2+}]i$ was calculated from the ratio of fluorescence measured at 350 nm and 380 nm, and the ratio of fluorescence at 380 nm in low calcium to that in high calcium (Sauvé et al., 1990*b*). Figure 1 illustrates a cell response evoked by the external addition of 10 nM BK. The cell exposure to BK caused a transient $K(Ca^{2+})$ channel activity which was intimately correlated with a transient increase in $[Ca^{2+}]i$ from a basal value (127 nM) to a peak (700 nM) then declining to a steady-state level. Both types of measurement do not lead, however, to identical conclusions concerning $[Ca^{2+}]i$ changes. For instance, a transient increase in single-channel activity was recorded during the secondary plateau phase of the Ca^{2+} signal detected by Fura-2. These results confirm that patch-clamp and Fura-2 measurements may be complementary without being actually identical. Thus, changes in $K(Ca^{2+})$ channel activity clearly reflect changes in $[Ca^{2+}]i$ evoked by BK.

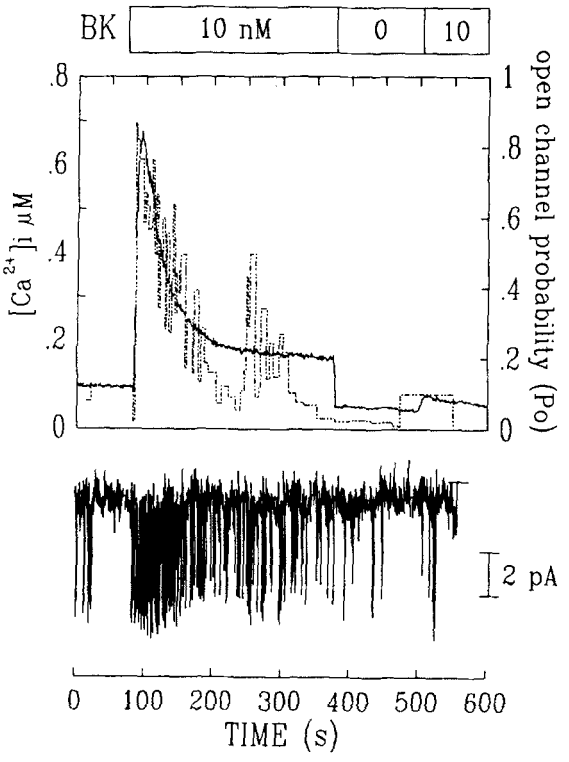


Fig. 1. Simultaneous measurements of the $[Ca^{2+}]i$ and $K(Ca^{2+})$ channel activity upon the cell exposure to BK (10 nM) (*delay not shown*). The lower trace shows cell-attached current record at a constant pipette potential (Vp) of +30 mV, and the upper trace shows the $[Ca^{2+}]i$ measured simultaneously in the same Fura2-loaded BAE cell in confluent monolayer. Variations of the open probability (*Po*) of the channel as a function of time computed from the current record are superimposed on the Fura-2 measurements. *Po* was computed with K = 50 transitions as described in Materials and Methods. Recordings were performed in the continuous perfusion mode of the Earle solution (bath exchange within 5 sec). BK (10 nM) was added to the Earle solution as indicated. Downward deflections in the current trace represent inwardly directed unitary K^+ currents flowing through $K(Ca^{2+})$ channels (pipette filled with a high K^+ solution). Fluorescence signal was calibrated according to Sauvé et al. (1990*b*) and the current trace represents 2000 points of the original record (room temperature).

Results

Single-channel K^+ currents were recorded with a high K^+ pipette solution in cell-attached patch experiments (n = 40) from BAE cells of confluent monolayers continuously superfused with an Earle solution with or without bicarbonate. As previously reported (Sauvé et al., 1990*a*; Thuringer et al., 1991), patches without channel activity were frequently observed in resting cells. Occasionally, two different elementary events could be distinguished. The unitary currents of large amplitude (-2.4 to -3.6 pA range depending on the resting potential of cells) were identified as the inward-rectifying K^+ current from iK1 channel (Colden-Stanfield et al., 1987; Sauvé et al., 1990*a*). The other unitary currents of smaller amplitude (of about -0.4 pA) were recorded in a few patches but their nature remains unknown. In contrast to iK1 channel which showed long-lasting openings (i.e., *see* Fig. 7), the second channel

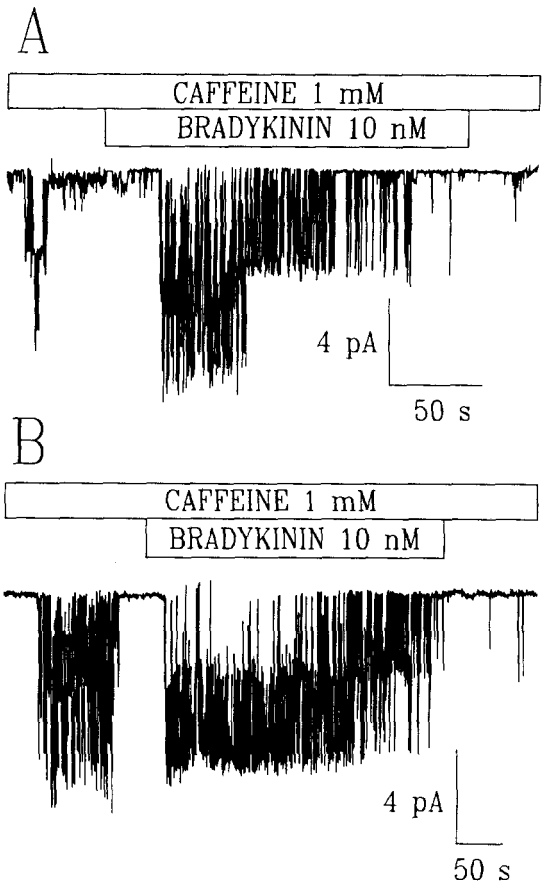


Fig. 2. Effects of caffeine on single-channel recordings from two different BAE cells in confluent monolayers exposed to a bicarbonate-free (*A*) or standard (*B*) Earle solution. In the bicarbonate-free solution, $NaHCO_3$ was replaced with equimolar NaCl from the standard Earle solution. Cell-attached experiments were carried out in the continuous perfusion mode at Vp of +30 mV applied to the pipette containing a high K^+ solution. Perfusion of solutions containing caffeine (1 mM) was started at the beginning of the current traces. BK (10 nM) was subsequently added and removed as indicated. The initial segments for the control experiment were omitted for clarity. In *A* and *B*, the transient activation of at least two $K(Ca^{2+})$ channels appears as fast single-channel openings, which can be superimposed on smaller current fluctuations coming from the unknown channel as seen in *A*. Note that the single-channel response to caffeine alone is stronger in the presence of bicarbonate, especially in the cell shown in *B*. Each trace contains 1500 points of the original records.

exhibited bursts of openings characterized by rapid fluctuations between open and closed states, as seen at the beginning of the current trace in Fig. 2*A*. Although the ionic selectivity of this channel remains to be elucidated, our results do not support in this case a Ca^{2+}-dependent activation since channel openings were observed in non-stimulated cells with a pipette solution containing mainly K^+ and Cl^- ions. Thus, the Ca^{2+}-independent cationic channel with a small conductance of 12 pS in symmetrical 140 mM KCl previously described in aortic endothelial cells (Fichtner et al., 1987) may be present in our cell preparation.

Effects of Caffeine on the Single-Channel Activity of BAE Cells

Figure 2 illustrates single-channel responses of BAE cells evoked by the addition of 1 mM caffeine to a bicarbonate-free (Fig. 2*A*) or standard Earle solution (Fig. 2*B*). The application of caffeine caused a transient $K(Ca^{2+})$ channel activity which lasted 5 to 80 sec depending on both the cells and presence of external bicarbonate (n = 20 cells). Despite cell-to-cell variations, shorter periods of channel activity (6 ± 2 sec; n = 8) were always recorded in bicarbonate-free solution. It must be noticed that the single-channel response to caffeine of the cell shown in Fig. 2*B* was more sustained than average (19 ± 10 sec; n = 8). When 10 nM BK was added to the bath medium containing 1 mM caffeine, a drastic reactivation of $K(Ca^{2+})$ channels was systematically observed in all the cells tested (n = 20). In most cases, channel openings were recorded after a lag of about 30 sec following the onset of BK application and lasted over a longer time period in standard Earle compared to bicarbonate-free solutions. This effect has been previously reported for the agonist-evoked $K(Ca^{2+})$ channel activity in BAE cells (Thuringer et al., 1991) and in Hela cells (Sauvé et al., 1990*b*). Furthermore, it is important to note that, despite cell-to-cell variations, caffeine prolonged the BK-evoked channel response, especially in bicarbonate-free conditions where the time duration of channel activity was substantially enhanced from 92.5 ± 20 sec (n = 10; Thuringer et al., 1991) to 150 ± 10 sec (n = 5). The transient behavior of the single-channel response evoked by caffeine alone is likely, therefore, to reflect a brief increase in $[Ca^{2+}]i$ rather than its direct action of $K(Ca^{2+})$ channels.

Effects of Various Caffeine Concentrations

The dose dependency of the channel activation process induced by caffeine was investigated in subsequent experiments where confluent cells were successively superfused with Earle solutions containing increasing amounts of caffeine (n = 5). An example of single-channel currents recorded in the same cell-attached patch is shown in Fig. 3. The cell exposure to 5 mM caffeine resulted in a transient activation process of repetitive single-channel bursts, which could be prolonged by increasing the dose of caffeine

to 10 mM or higher. It must be noticed that a sustained channel activity could be recorded in this particular case because of the large number of channels in the membrane patch which makes likely the recording of single-channel events even at low $[Ca^{2+}]i$. The subsequent application of 10 nM BK in the continuous presence of 20 mM caffeine invariably led to a drastic channel activation which tended to last as long as both agents were present in the bath. This result provides direct evidence that, even at high concentrations, caffeine alone was unable to increase $[Ca^{2+}]i$ to a level sufficient to activate all of the channels present in the patch area as observed with the agonist. It is, therefore, unlikely that caffeine and BK act on the same Ca^{2+} store to increase $[Ca^{2+}]i$. The removal of caffeine was followed by a rapid loss of activity within the time delay necessary to fully exchange the perfusion chamber despite the continuous presence of the agonist, and no channel opening was detected in the following period (Fig. 3, lower trace). This observation argues for a synergic action of both agonist and caffeine to maintain an elevated free Ca^{2+} level into the cytosol and, thus, a sustained channel activity as noticed before. Figure 4 illustrates the effects of sucessive caffeine applications on the cell tested in Fig. 3. Despite the expected decrease in number of reactivable channels for a second cell stimulation by BK, high caffeine concentrations could still induce repetitive single-channel bursts and BK was still able to produce an additional increase in channel activity. However, the removal of BK from the Earle solution containing caffeine did not immediately inhibit the oscillatory activation process, in contrast to the result shown in Fig. 3 where a drastic inhibition of the channel activity was observed following the removal of caffeine. The combined action of BK and caffeine is also illustrated in Fig. 5, where caffeine (20 mM) was externally applied during the BK-evoked Ca^{2+} response (n = 5). The single-channel record (Fig. 5*A*) and associated *NPo* analysis (Fig. 5*B*) provide clear evidence that caffeine not only sustains the BK-dependent $[Ca^{2+}]i$ increase, but also reactivates $K(Ca^{2+})$ channels following a caffeine-free period of cell superfusion.

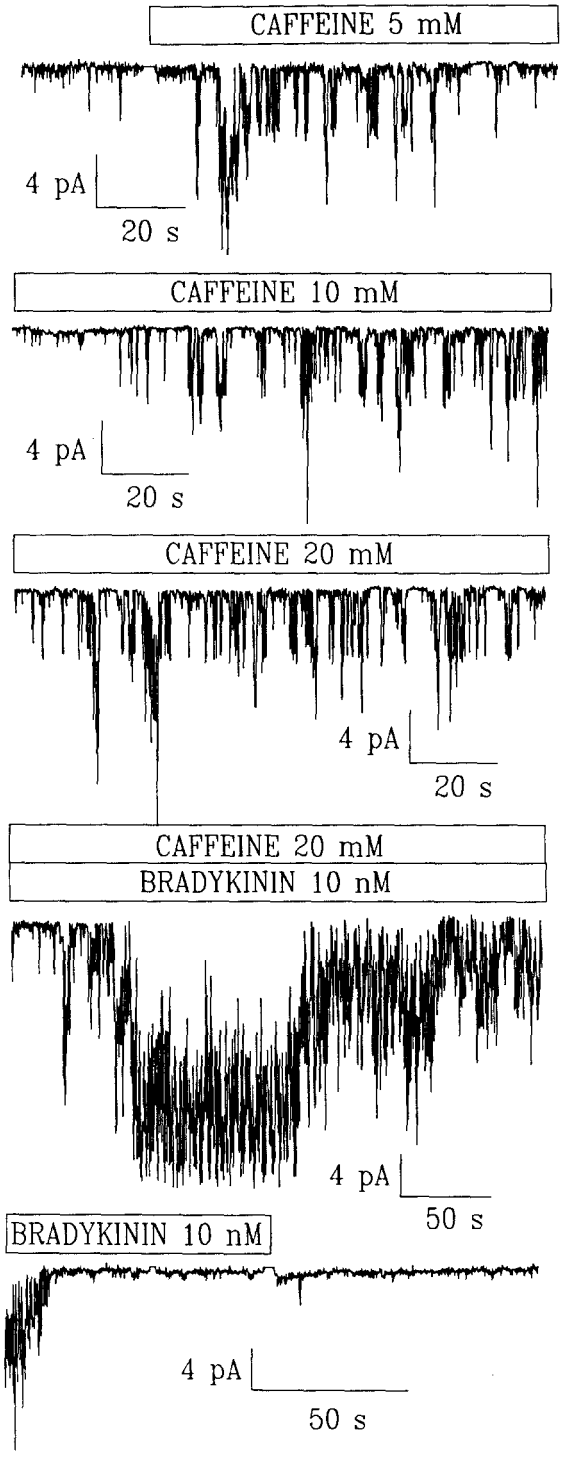


Fig. 3. Effects of various caffeine concentrations on the $K(Ca^{2+})$ channel activity. The panels show effects of increasing the dose of caffeine (5, 10 to 20 mM) on the continuous current record from the same cell-attached patch on confluent cell monolayer (Vp of +30 mV and pipette filled with a high K^+ solution). Earle solutions containing caffeine were successively perfused and 10 nM BK was subsequently added to the Earle solution containing 20 mM caffeine. Caffeine was then removed and cells were exposed for 80 sec to BK alone before returning to control conditions. Each trace contains 1500 points of the original record.

Intracellular Ca^{2+}-Release Induced by Caffeine

To assess the contribution of internally sequestered Ca^{2+} to the transient $K(Ca^{2+})$ channel activity in-

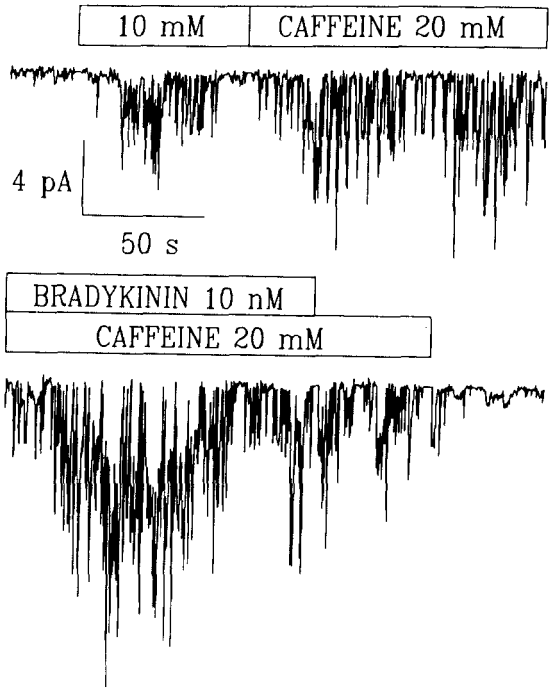


Fig. 4. Restoration of the $K(Ca^{2+})$ channel activation process evoked by caffeine. Continuous current record obtained from the same cell as in Fig. 3 after a 200-sec period in the standard Earle solution to restore control conditions. Each trace contains 1000 points of the original record.

duced by caffeine, the effects of this Ca^{2+}-releasing agent were tested on confluent BAE cells superfused with a Ca^{2+}-free Earle solution (2 mM EGTA and no $CaCl_2$ added) as shown in Fig. 6 (n = 8). After several minutes in the Ca^{2+}-free solution during which no $K(Ca^{2+})$ channel activity was observed (*not shown* in Fig. 6), a transient activation of $K(Ca^{2+})$ channel bursts was elicited by the external addition of 1 mM caffeine (Fig. 6*A*). Clearly, caffeine initiated Ca^{2+} release in BAE cells despite the decrease in the resting $[Ca^{2+}]i$ produced by removing external calcium (Thuringer et al., 1991). In the continuous presence of caffeine in the Ca^{2+}-free solution, the subsequent application of 10 nM BK still evoked a substantial channel response that could not be reproduced by a second BK application. As expected for a BK-stimulated Ca^{2+} influx (Thuringer et al., 1991), the single channel activity transiently reappeared following the readmission of calcium into the bath medium. Two obvious features appear from these results: firstly, caffeine did not prevent the Ca^{2+}-release induced by the agonist, and secondly, the released Ca^{2+} was not resequestered into the stores involved in the agonist-induced Ca^{2+} signal. Because of cell-to-cell variabilities, we cannot determine whether the duration of this BK-evoked

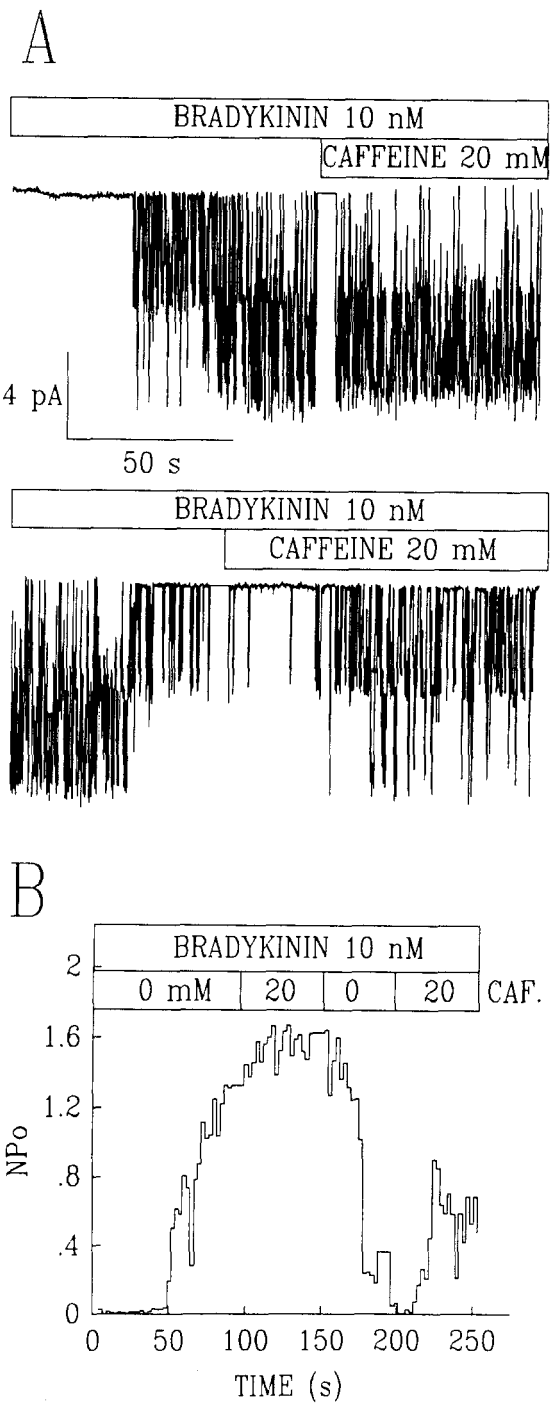


Fig. 5. Effect of 20 mM caffeine on the $K(Ca^{2+})$ channel activity induced by BK. (*A*) Continuous record of single-channel currents obtained from a cell-attached-patch at Vp of +30 mV and with a high K^+ pipette solution. During cell stimulation by 10 nM BK, caffeine 20 mM was successively added and removed from the bath medium. Each trace contains 1500 points of the original record. (*B*) Variations of the average number of open channels (*NPo*) as a function of time computed from the current record shown in *A*. *NPo* was obtained by dividing the time average of the total current amplitude, measured over successive 2.5-sec periods, by the unitary amplitude of $K(Ca^{2+})$ channel current.

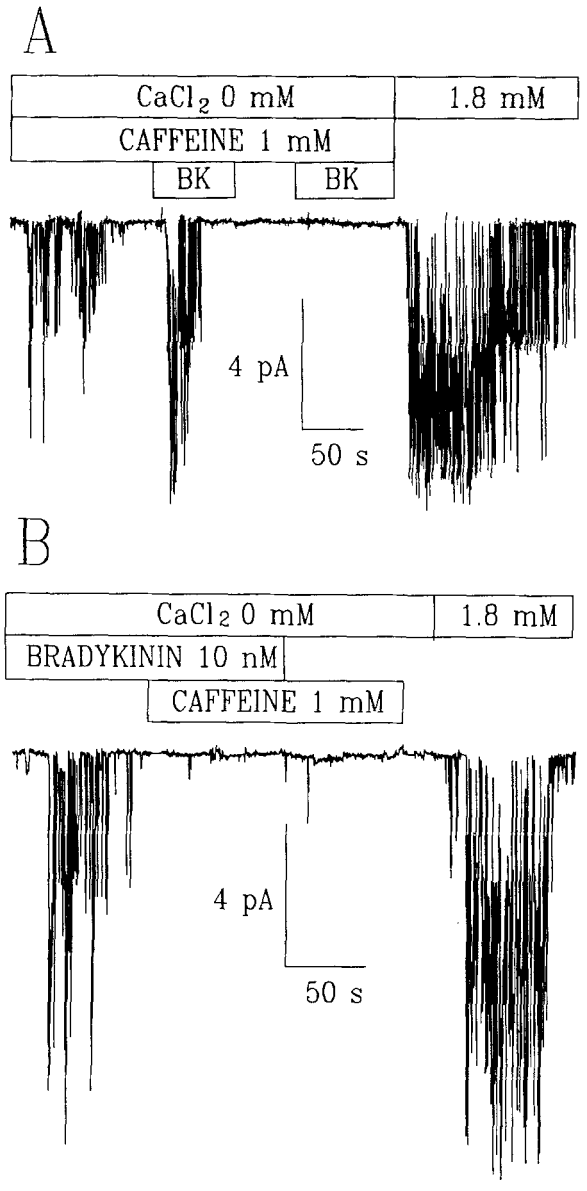


Fig. 6. Effect of 1 mM caffeine on intracellular Ca^{2+} stores. After a 2-min exposure to the Ca^{2+}-free Earle solution (2 mM EGTA and no $CaCl_2$), 1 mM caffeine (*A*) or 10 nM BK (*B*) was applied. The contribution of released Ca^{2+} from the caffeine-sensitive store to the agonist-evoked Ca^{2+} response was tested by applying BK after (*A*) or before (*B*) the caffeine exposure. Following the Ca^{2+}-free period, cells were superfused with the standard Earle solution (1.8 mM $CaCl_2$). A Vp of +30 mV was applied to the pipette containing a high K^+ solution. The current trace contains 2000 points of the original record.

channel activity became briefer than that normally produced in absence of caffeine. A reduction of duration would have shown that the $InsP_3$-sensitive store has lost a part of its Ca^{2+} content due to caffeine. However, the observation that the caffeine-induced Ca^{2+} release did not occur when agonist was applied prior to the Ca^{2+}-free solution (Fig. 6*B*), argues strongly for the contribution of the caffeine-sensitive store to the agonist-evoked Ca^{2+} mobilization. It can finally be concluded from these results, especially with regard to the ineffectiveness of caffeine to prevent the channel activation process evoked by the agonist (Fig. 3), that caffeine induces Ca^{2+}-release from an $InsP_3$-insensitive store, perhaps by acting on a Ca^{2+}-induced Ca^{2+}-release mechanism.

Inhibiting Effects of Procaine on the Ca^{2+} Signal Induced by Bradykinin

In bovine pulmonary artery endothelial cells, Freay and co-workers (1989) have shown that procaine (10 mM), a reversible blocker of Ca^{2+}-induced Ca^{2+} release in muscle, partially inhibits calcium release from ER whether it is initiated by bradykinin receptor activation or directly by $InsP_3$. Although its mechanism of action has not been investigated by these authors, the apparent specificity of procaine effects was tested on the $K(Ca^{2+})$ channel activity evoked either by BK or by caffeine, in order to confirm the coexistence of two distinct Ca^{2+} releasing stores in confluent BAE cells.

In a first series of cell-attached experiments, the single-channel activity triggered by 10 nM BK was recorded from a same membrane patch in the presence and absence of procaine (10 mM) in the Earle solution ($n = 6$). As observed in Fig. 7, the external presence of procaine depressed the $K(Ca^{2+})$ channel activity normally produced by the cell exposure to BK. However, a transient increase in the unitary current amplitude of iK1 channel related to the $K(Ca^{2+})$ channel effect on the cell membrane potential was still observed (Sauvé et al., 1990*a*). Because the additional application of the calcium-ionophore A23187 (4 μM) fully reactivated all of $K(Ca^{2+})$ channels present in other membrane patches tested (*data not shown*), procaine likely acts on Ca^{2+} mobilizing processes rather than directly on the $K(Ca^{2+})$ channel. This decrease in the BK-evoked single-channel activity appeared fully reversible following the removal of procaine from the bath. The typical fast openings of $K(Ca^{2+})$ channels were then superimposed on the transient increase in iK1 unitary amplitude, indicating that the plasma membrane was transiently hyperpolarized. The amplitude of the iK1 channel current was maintained to a lower value of -1.8 pA after the washout of the agonist. As previously reported (Sauvé et al., 1990; Thuringer et al., 1991), the activation of $K(Ca^{2+})$ channels is a biphasic phenomenon with an initial release of Ca^{2+} from internal stores followed by a secondary Ca^{2+} influx from the extracellular space. The results in

Fig. 7 provide evidence that procaine inhibits partially the initial Ca^{2+} release induced by BK in BAE cells, as previously observed in endothelial cells (Freay et al., 1989). However, within the framework of Putney's capacitative Ca^{2+} entry model (1986), the shortening of the secondary phase of channel activation process in the presence of procaine could be a consequence of a partially impaired release of Ca^{2+} from internal stores rather than a direct blockage by procaine of the Ca^{2+} entry pathway. In order to investigate further this issue, 10 mM procaine was added to the Earle solution during the BK-induced activation of the $K(Ca^{2+})$ channels as presented in Fig. 8 (n = 10). The external addition of procaine at this stage in the channel activation process resulted in a drastic loss of channel activity which could be totally recovered upon the removal of procaine from the Earle solution containing BK.

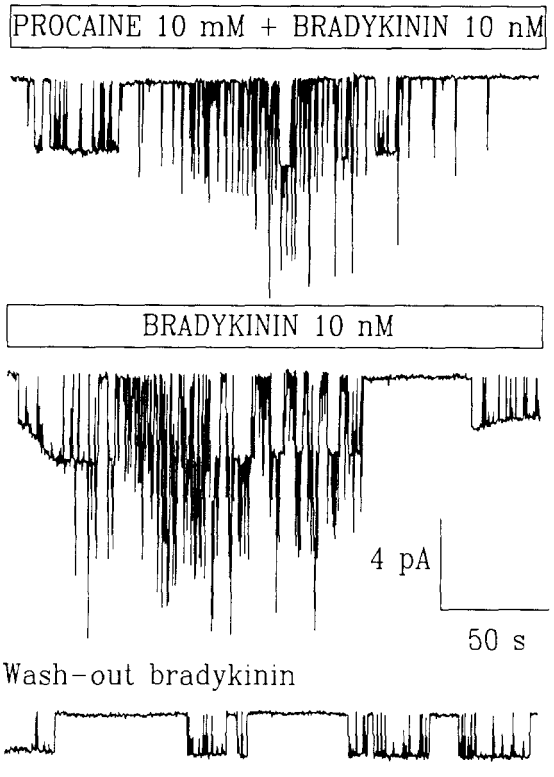


Fig. 7. Effects of procaine on the $K(Ca^{2+})$ channel activity induced by BK. Continuous record of single-channel currents obtained from the same cell-attached-patch at Vp of +30 mV and with a high K^+ pipette solution. Both 10 mM procaine and 10 nM BK were simultaneously added to the Earle solution superfusing the cells at the beginning of the upper trace, and then, were successively removed as indicated in the bottom traces. The $K(Ca^{2+})$ channel activation appears as fast single-channel openings superimposed on the slow current fluctuations coming from the iK1 channel. Each trace contains 1500 points of the original record.

Clearly, procaine also inhibits the agonist-evoked secondary Ca^{2+} influx. In addition, an increase in intracellular pH (pHi) of 0.1 U was measured fluorimetrically in cells loaded with the H^+ indicator dye BCECF, following the procaine application (*data not shown*). Although this additional effect of procaine is not documented, the consecutive cytosolic alkalinization could be responsible for a part of the inhibition of the BK-evoked $K(Ca^{2+})$ channel activity as previously shown in confluent BAE cells (Thuringer et al., 1991).

In a second series of cell-attached experiments, the possible inhibitory effects of procaine were investigated on the $K(Ca^{2+})$ channel activity evoked by 20 mM caffeine alone (n = 10). A high caffeine concentration (20 mM) was chosen because the caffeine-evoked channel activity could be reinitiated and maintained during a longer recording period in the same cell-attached patch, as described previously (Figs. 3 and 4). Figure 9 illustrates the result of such experiments in which the effects of 10 mM

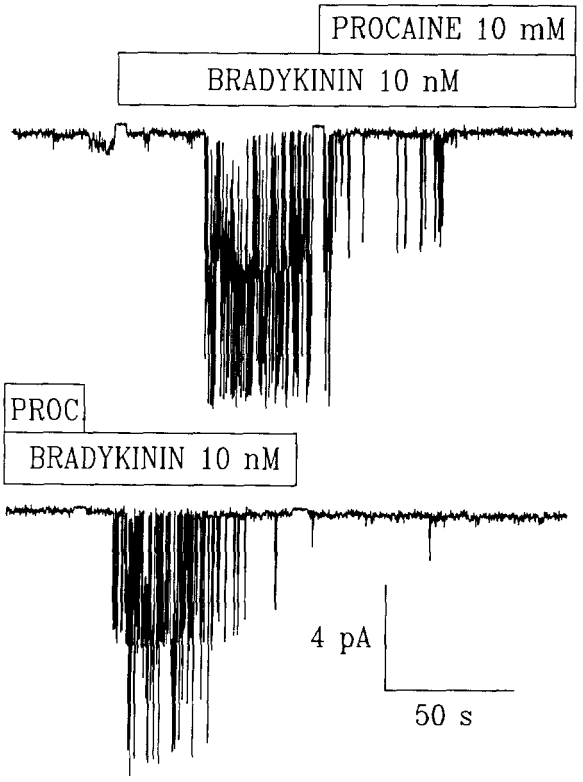
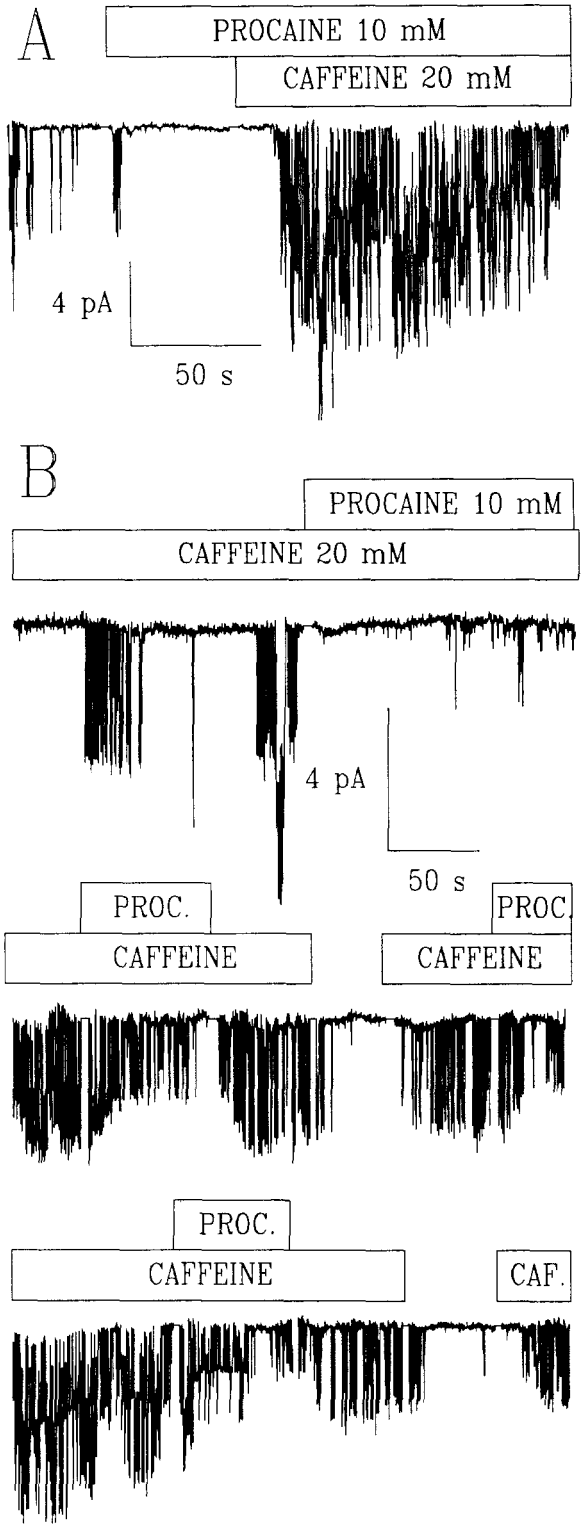


Fig. 8. Inhibition by procaine of the BK-evoked Ca^{2+} influx. Continuous perfusion experiment carried out on the same cell in the cell-attached configuration (Vp of +30 mV and pipette filled with a high K^+ solution). Cells of confluent monolayers were stimulated by the addition of 10 nM BK to Earle solution. Procaine (10 mM) was tested during the channel-activation process induced by BK. Each trace contains 1500 points of the original record.

procaine were tested before (Fig. 9*A*) and during (Fig. 9*B*) caffeine applications in two different cell-attached patches. As seen in Fig. 9*A*, the cell pretreatment with procaine succeeded to block the basal single-channel activity observed at the beginning of the experiment, but did not prevent the strong channel activation induced by 20 mM caffeine (n = 4). This result supports previous conclusion that caffeine induces Ca^{2+} release from an $InsP_3$-insensitive store probably involved in the BK-induced single-channel response. It must be noticed that the ineffectiveness of procaine to block the caffeine-induced channel activity was also observed at lower doses of caffeine (*data not shown*). In addition, we observed that the caffeine-evoked $K(Ca^{2+})$ channel response was systematically enhanced in procaine-treated (Fig. 9*A*) compared to non-treated BAE cells (*see* Figs. 3 and 9*B*). When procaine (10 mM) was applied during caffeine application (Fig. 9*B*), an inhibition of the bursts of channel openings seemed to be produced although some single-channel currents of smaller unitary amplitude and brief duration could be detected still (n = 5). Following the removal of procaine, a strong reactivation associated with a rapid increase in the unitary current amplitude of the $K(Ca^{2+})$ channel present in the patch area was observed as long as 20 mM caffeine was present in the external medium. However, in contrast to the blocking effect of procaine shown in Fig. 8, the repetitive applications of 10 mM procaine failed to decrease rapidly the sustained channel activity induced by caffeine. Returning to control conditions reversibly caused a drastic inhibition of the $K(Ca^{2+})$ channel activity as expected for a caffeine-evoked channel activation process.



Discussion

The aim of the experiments reported in this work was to examine the possible coexistence of pharmacologically distinct types of intracellular Ca^{2+} stores in endothelial cells, by using agents known to inter-

Fig. 9. Loss of inhibitory effects of 10 mM procaine on the single-channel activity induced by 20 mM caffeine. Continuous records of $K(Ca^{2+})$ channel currents obtained from two different cell-attached-patches at Vp of +30 mV and with a high K^+ pipette solution. (*A*) Following cell exposure to 10 mM procaine containing standard Earle solution, 20 mM caffeine was added to the external medium as indicated. The current trace contains 1500 points of the original record. (*B*) Repetitive applications of 10 mM procaine were performed during the channel activation process induced by caffeine 20 mM, onto another confluent cell monolayer. Note the slow decrease in amplitude of the single-channel currents in presence of procaine and the rapid inhibition of the $K(Ca^{2+})$ channel activity consecutive to the removal of caffeine. Traces contain 2000 points of the original record.

fere with the liberation of internally sequestered Ca^{2+} from endoplasmic reticulum (ER). The most striking features shown in this study are: firstly, caffeine induces Ca^{2+} release from an intracellular store in resting BAE cells and facilitates the Ca^{2+} response to BK, and secondly, the sensitivity to procaine of both the Ca^{2+} release and external Ca^{2+} entry is different in caffeine-treated, compared to BK-stimulated, cells.

Evidence of a Caffeine-Induced Ca^{2+}-Release Process in BAE Cells

Our results demonstrate that the external application of caffeine (1–5 mM) elicited a transient activation of $K(Ca^{2+})$ channels which became more sustained at higher concentrations. Because single-channel activation was also observed in the absence of external Ca^{2+} (see Fig. 6*A*), the $K(Ca^{2+})$ channel activity evoked by caffeine is likely to arise from a caffeine-induced Ca^{2+}-release process. In addition, caffeine did not substantially affect the initial phase of the channel activation process evoked by the subsequent addition of BK, even in the absence of external Ca^{2+} (*see* Figs. 2, 3, 6). Because it is generally agreed that $InsP_3$ triggers the initial phase of the agonist-induced Ca^{2+} mobilization from ER in endothelial cells (Freay et al., 1989; Myers & Larkins, 1989), it is therefore unlikely that caffeine reduces the $InsP_3$-induced Ca^{2+} discharge from internal stores by increasing the threshold concentration of $InsP_3$ required to evoke Ca^{2+} signal as reported in *Xenopus* oocytes (Parker & Ivorra, 1991). Clearly, the Ca^{2+} released by caffeine accounts for a part of the total Ca^{2+} released by the agonist since, following the cell stimulation by BK in Ca^{2+}-free external conditions, the subsequent addition of caffeine was unable to promote a transient channel activity related to an additional liberation of internally sequestered Ca^{2+} (*see* Fig. 6*B*). It might be possible that caffeine liberates Ca^{2+} from the $InsP_3$-sensitive store, and thus, reduces the amount of releasable Ca^{2+} by $InsP_3$. If the single-channel response induced by 1 mM caffeine is due to a liberation of Ca^{2+} from the $InsP_3$-sensitive store, higher doses may be expected to provoke a larger depletion of Ca^{2+} and, consequently, to prevent the subsequent release of Ca^{2+} by the agonist. However, as shown in Fig. 3, an increase in the caffeine dose up to 20 mM maintained a basal level of channel activity which was further increased by BK. Thus, the mechanisms of Ca^{2+} release induced either by caffeine or by $InsP_3$ generation appear to be distinct but become complementary upon agonist stimulation of BAE cells. It can be assumed finally from these results that caffeine induces Ca^{2+} release from an $InsP_3$-insensitive store in BAE cells as reported in pancreatic acinar cells (Delhinger-Kremer et al., 1991) and in *Xenopus* oocytes (Berridge, 1991).

Characteristics of the Caffeine-Induced Ca^{2+} Release

The well-known action of caffeine in excitable cells is to promote the release of Ca^{2+} from the sarcoplasmic reticulum (SR) by increasing the open probability of the Ca^{2+}-activated Ca^{2+}-release channel without alteration of the single-channel conductance (Endo, 1977; Konishi & Kurihara, 1987; Rousseau et al., 1988; Sitsapesan & Williams, 1990). Because no evidence of such a release process has been yet reported in endothelial cells (Jacob, 1991), it was not clear that a caffeine-induced release also occurs in BAE cells by an effect on Ca^{2+}-induced Ca^{2+} release from ER. The following observations that caffeine released Ca^{2+} in external Ca^{2+}-free conditions (Fig. 6*A*) and potentiated the effects of BK (*see* Figs. 3, 4, 5), argue for the existence of a Ca^{2+}-induced Ca^{2+}-release process, whose sensitivity to cytosolic Ca^{2+} could be increased by caffeine, as described in cardiac SR (O'Neill & Eisner, 1990; Sitsapesan & Williams, 1990). However, the sustained channel activity recorded at high doses of caffeine from BK-stimulated cells, contrasts with the inhibitory effect of this drug on the agonist-evoked Ca^{2+} signal reported in the other nonexcitable cell preparations (Wakui et al., 1990; Berridge, 1991). The possible existence of different caffeine-sensitive Ca^{2+}-release mechanisms and/or channels can be considered in endothelial cells. In this regard, evidence has been already provided for a ryanodine-insensitive but caffeine-sensitive Ca^{2+} channel in ER membranes of pancreatic acinar cells (Delhinger-Kremer et al., 1991).

Another action of caffeine is to inhibit cAMP-phosphodiesterase leading to an increase in cytosolic cAMP content. It is generally assumed in excitable cells that cAMP enhances both the capacity and the rate of Ca^{2+} sequestration into SR, and thus, permits the release of a larger amount of Ca^{2+} from more fully loaded Ca^{2+} stores (Fabiato & Fabiato, 1975; Fredholm et al., 1979; Saida & Van Breemen, 1984). In human T lymphocytes (Kelley et al., 1990), it has been recently shown that a rise in the cAMP production by agents, such as prostaglandins E, phosphodiesterase inhibitors or adenylate cyclase activators, causes a transient increase in $[Ca^{2+}]i$ related to a Ca^{2+} release from intracellular stores by a mechanism independent of the $InsP_3$ formation. The striking analogies between the cAMP effects in

T lymphocytes (Kelley et al., 1990) and the caffeine effects in BAE cells reported in this work, namely: (i) the initial cAMP-induced Ca^{2+}-release does not prevent the subsequent $InsP_3$-induced Ca^{2+}-release, (ii) the cAMP-releasable Ca^{2+} store is emptied following release of the $InsP_3$-sensitive Ca^{2+} store in stimulated cells, and (iii) cAMP-dependent Ca^{2+} release occurs in the absence of Ca^{2+} influx, may suggest that the increase in cAMP content could account for the caffeine-induced Ca^{2+}-release in our cell preparation. However, the effects of cAMP on the Ca^{2+}-response to BK and/or ATP remain still controversial in BAE cells. An increase in $[Ca^{2+}]i$ was observed by Buchan and Martin (1991) following the addition of forskolin during the external Ca^{2+}-dependent phase of BK stimulation, whereas a decrease in ATP-induced $[Ca^{2+}]i$ rise was reported by Lückhoff and co-workers (1990) in cells preincubated with isoproterenol. In our cell preparation, no noticeable effect of forskolin (1 μM) was observed on the $K(Ca^{2+})$ channel activity recorded from nonstimulated or ATP-stimulated cells (*data not shown*). Despite these differences, these results suggest strongly that cAMP does not promote a release of Ca^{2+} in resting BAE cells (Lückhoff et al., 1990; Buchan & Martin, 1991), arguing against a model where the Ca^{2+} release, induced by caffeine in unstimulated cells, would be the resulting action of an elevated production of cAMP. However, an increase in $[Ca^{2+}]i$ caused by cAMP following the external addition of 20 mM caffeine in BK-stimulated cells can partly explain the results illustrated in Figs. 3 to 5.

Action of Procaine on the Ca^{2+} Mobilization

The Ca^{2+}-induced Ca^{2+} release from SR has been reported to be blocked effectively by local anesthetics in muscle cells (Endo, 1977; Stephenson & Wendt, 1986). It has to be mentioned that the effect observed with procaine is not only inhibitory, but includes also an enhanced capacity of Ca^{2+} sequestration in SR resulting, in some conditions, in a potentiation of the caffeine-induced contraction in cardiac muscle (Stephenson & Wendt, 1986). In the present work, we found that the initial phase of the $K(Ca^{2+})$ channel activation process evoked by BK was strongly depressed by procaine (see Fig. 7), in agreement with the partial inhibition of the $InsP_3$-induced Ca^{2+}-release previously observed in bovine pulmonary artery endothelial cells (Freay et al., 1989). Furthermore, we have demonstrated that procaine inhibited entirely and reversibly the external Ca^{2+}-dependent phase of the channel activation process corresponding to the agonist-evoked secondary Ca^{2+} influx (*see* Fig. 8). In contrast, a cell pretreatment with procaine was unable to antagonize the caffeine-induced Ca^{2+}-release (*see* Fig. 9*A*). This result would be consistent with the ability of procaine to enhance the Ca^{2+} content of ER in endothelial cells as in cardiac SR (Stephenson & Wendt, 1986). The cell exposure to procaine during the $K(Ca^{2+})$ channel response evoked by caffeine seemed to block the repetitive bursts of channel openings initially induced by caffeine (*see* Fig. 9*B*). This result indicates that the site and the possible mechanism of Ca^{2+}-release by caffeine is comparable to that shown in muscle (Endo, 1977; Stephenson & Wendt, 1986). Therefore, the caffeine-evoked Ca^{2+} oscillations in BAE cells could be explained by a classical Ca^{2+}-induced Ca^{2+}-release mechanism which is normally blocked by procaine. However, subsequent applications of procaine to the same cell failed to inhibit effectively the caffeine-evoked channel response (Fig. 9*B*). The lack of effect of procaine, in this case, may be regarded as an indication that its ability to block the caffeine-induced Ca^{2+} release is a function of the amount of Ca^{2+} in the caffeine-sensitive Ca^{2+} store. In this regard, Stephenson and Wendt (1986) have shown that procaine was no longer able to significantly antagonize the caffeine-induced release of Ca^{2+} when the amount of Ca^{2+} in the SR was progressively enhanced.

An interesting observation reported in this study is the effect of bicarbonate-free external medium on the $K(Ca^{2+})$ channel activity evoked by both caffeine and bradykinin (*see* Fig. 2). Longer periods of channel activation were always recorded in standard external solution containing bicarbonate. In many cell types, the removal of bicarbonate from the external medium affects the cytosolic pH (pHi) regulation, leading to a cell alkalinization. Recent study on BAE cells has provided strong evidence that the pHi modulates directly the mechanisms responsible for the refilling of intracellular Ca^{2+} stores, in such a way that increased pHi impairs the reuptake of Ca^{2+} into the stores involved in the cell response to agonist (Danthuluri et al., 1990). In agreement with this regulatory process, we have previously shown that a rise in pHi imposed by external addition of NH_4Cl inhibits the sustained $K(Ca^{2+})$ channel activity related to the BK-induced Ca^{2+} influx in BAE cells (Thuringer et al., 1991). Because no direct action on the channels was involved in this process, the effect of alkaline pHi was interpreted in terms of an inhibition of the Ca^{2+} uptake system into the internal stores coupled to a stimulation of the Ca^{2+} extrusion to the external medium. A similar inhibitory effect of high pHi was reported on the agonist-evoked channel activation in Hela cells (Sauvé et al., 1990*b*).

An increase in pHi was observed in BCECF-loaded cells following procaine application (*data not shown*) and, consequently, the inhibiting effects of procaine on the BK-evoked $K(Ca^{2+})$ channel activity could also result from an intracellular alkalinization. In fact, high pHi has been reported to induce a rapid Ca^{2+} release from agonist-sensitive stores and to inhibit repetitive Ca^{2+} spiking in BAE cells (Danthuluri et al., 1990). However, this interpretation does not explain the different sensitivity to procaine between the $InsP_3$- and caffeine-sensitive Ca^{2+} releases.

In conclusion, our findings confirm that caffeine acts on an intracellular Ca^{2+} store which is pharmacologically distinct from that sensitive to $InsP_3$ in vascular endothelial cells. Although the mode of action of caffeine has to be explored, this caffeine-sensitive Ca^{2+} store contributes to the internal Ca^{2+} signal evoked by bradykinin, but does not appear to be involved directly in the mechanism of Ca^{2+} influx regulation in agonist-stimulated cells. Indeed, a transient channel activation was elicited upon cell exposure to 1–5 mM caffeine even though calcium was present at 1.8 mM in the external medium (*see* Fig. 2), and higher doses of caffeine did not induce a channel activity comparable to that produced by BK (*see* Fig. 3). Moreover, the external Ca^{2+}-dependent phase of the caffeine-induced channel response is less sensitive to procaine, in contrast to the secondary phase of the BK-evoked channel activation process. It can be expected from our observations that caffeine activates an external Ca^{2+} entry pathway which is different to that mainly triggered by the agonist. In addition, the origin of $[Ca^{2+}]i$ spiking in stimulated endothelial cells could be independent on the presence of the agonist on its membrane receptor.

The authors would like to thank Dr. A. Diarra for his contribution to the fluorescence measurements and Diane Vallerand for preparing cell cultures. These data were presented in part at the 14th Scientific Meeting of the International Society of Hypertension (Madrid, Spain, June 14–18, 1992), and have been published in abstract form in the Journal of Hypertension (1992). Dominique Thuringer is a fellow of the Heart and Stroke Foundation of Canada. Rémy Sauvé is a senior fellow from the Fonds de la Recherche en Santé du Québec. This work was supported by a grant from the Medical Research Council of Canada.

References

Ahn, H.Y., Karaki, H. 1988. Inhibitory effects of procaine on contraction and calcium mouvement in vascular and intestinal smooth muscles. *Br. J. Pharmacol.* **94:**789–796

Berridge, M.J. 1991. Caffeine inhibits inositol-trisphosphate-induced membrane potential oscillations in *Xenopus* oocytes. *Proc. R. Soc. Lond. B.* **244:**57–62

Buchan, K.W., Martin, W. 1991. Modulation of agonist-induced calcium mobilisation in bovine aortic endothelial cells by phorbol myristate acetate and cyclic AMP but not cyclic GMP. *Br. J. Pharmacol.* **104:**361–366

Colden-Stanfield, M., Schilling, W.P., Possani, L.D., Kunze, D.L. 1990. Bradykinin-induced potassium current in cultured bovine aortic endothelial cells. *J. Membrane Biol.* **116:**227–238

Colden-Stanfield, M., Schilling, W.P., Ritchie, A.K., Eskin, S.G., Navarro, L.T., Kunze, D.L. 1987. Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. *Circ. Res.* **61:**632–640

Danthuluri, N.R., Kim, D., Brock, T.A. 1990. Intracellular alkalinization leads to Ca^{2+} mobilization from agonist-sensitive pools in bovine aortic endothelial cells. *J. Biol. Chem.* **265:**19071–19076

Dehlinger-Kremer, M., Zeuzem, S., Schulz, I. 1991. Interaction of caffeine-, IP_3, and vanadate-sensitive Ca^{2+} pools in acinar cells of the exocrine pancreas. *J. Membrane Biol.* **119:**85–100

Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57:**71–108

Fabiato, A., Fabiato, F. 1975. Relaxing and inotropic effects of cyclic AMP on skinned cardiac cells. *Nature* **253:**556–558

Fichtner, H., Fröbe, U., Busse, R., Kohlhardt, M. 1987. Single nonselective cation channels and Ca^{2+}-activated K^+ channels in aortic endothelial cells. *J. Membrane Biol.* **98:**125–133

Freay, A., Johns, A., Adams, D.J., Ryan, U.S., Van Breemen, C. 1989. Bradykinin and inositol-1,4,5-triphosphate stimulated calcium release from intracellular stores in culture bovine endothelial cells. *Pfluegers Arch.* **414:**377–384

Fredholm, B.B., Brodin, K., Strandberg, K. 1979. On the mechanism of relaxation of tracheal muscle by theophyline and other cyclic nucleotide phosphodiesterase inhibitors. *Acta Pharmacol. Toxicol.* **45:**336–376

Hallam, T.J., Jacob, R., Merritt, J.E. 1989. Influx of bivalent cations can be independent of receptor stimulation in human endothelial cells. *Biochem. J.* **259:**125–129

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution recording from cell and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Jacob, R. 1990. Agonist-stimulated divalent cation entry into single cultured human umbilical vein endothelial cells. *J. Physiol.* **421:**55–57

Jacob, R. 1991. Calcium oscillations in endothelial cells. *Cell Calcium* **12:**127–134

Jacob, R., Merritt, J.E., Hallam, T.J., Rink, T.J. 1988. Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells. *Nature* **335:**40–45

Kelley, L.L., Blackmore, P.F., Graber, S.E., Stewart, S.J. 1990. Agents that raise cAMP in human lymphocytes release an intracellular pool of calcium in the absence of inositol phosphate production. *J. Biol. Chem.* **265:**17657–17664

Kobayashi, S., Somlyo, A.P., Somlyo, A.V. 1988. Guanine nucleotide- and inositol 1, 4, 5-trisphosphate-induced calcium release in rabbit main pulmonary artery. *J. Physiol.* **403:**601–619

Konishi, M., Kurihara, S. 1987. Effects of caffeine on intracellular calcium concentration in frog skeletal muscle fibres. *J. Physiol.* **383:**269–283

Lückhoff, A., Mülsch, A., Busse, R. 1990. cAMP attenuates autacoid release from endothelial cells: relation to internal calcium. *Am. J. Physiol.* **258:**H960–H966

Myers, D.E., Larkins, R.G. 1989. Bradykinin-induced changes

in phosphoinositides, inositol phosphate production and intracellular free calcium in cultured bovine aortic endothelial cells. *Cellular Signalling* **1**:335–343

Ohya, Y., Kitamura, K., Kuriyama, H. 1987. Cellular calcium regulates outward currents in rabbit intestinal smooth muscle cell. *Am. J. Physiol.* **252**:C401–C410

O'Neill, S.C., Eisner, D.A. 1990. A mechanism for the effects of caffeine on Ca^{2+} release during diastole and systole in isolated rat ventricular myocytes. *J. Physiol.* **430**:519–536

Parker, I., Ivorra, I. 1991. Caffeine inhibits inositol trisphosphate-mediated liberation of intracellular calcium in *Xenopus* oocytes. *J. Physiol.* **433**:229–240

Peres, A. 1990. $InsP_3$- and Ca^{2+}-induced Ca^{2+}-release in single mouse oocytes. *FEBS Lett.* **1, 2**:213–216

Putney, J.W. 1986. A model for receptor-regulated calcium entry. *Cell Calcium* **7**:1–12

Rousseau, E., LaDine, J., Liu, Q.-Y., Meissner, G. 1988. Activation of the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. *Arch. of Biochem. and Biophys.* **267**:75–86

Sage, S.O., Adams, D.J., Van Breemen, C. 1989. Synchronized oscillations in cytoplasmic free calcium in confluent bradykinin-stimulated bovine pulmonary artery endothelial cell monolayers. *J. Biol. Chem.* **264**:6–9

Saida, K., Van Breemen, C. 1984. Characteristics of the norepinephrine-sensitive calcium store in rabbit aorta. *Blood Vessels* **21**:43–52

Sauvé, R., Chahine, M., Tremblay, J., Hamet, P. 1990*a*. Single-channel analysis of the electrical response of bovine aortic endothelial cells to bradykinin stimulation: contribution of a Ca^{2+}-dependent K^+ channel. *J. of Hypertension* **8**:S193–S201

Sauvé, R., Diarra, A., Chahine, M., Simoneau, C., Garneau, L., Roy, G. 1990*b*. Single-channel and Fura-2 analysis of internal Ca^{2+} oscillations in Hela cells: contribution of the receptor-evoked Ca^{2+} influx and effect of internal pH. *Pfluegers Arch.* **416**:43–52

Sauvé, R., Parent, L., Simoneau, C., Roy, G. 1988. External ATP triggers a biphasic activation process of a calcium-dependent K^+ channel in cultured bovine aortic endothelial cells. *Pfluegers Arch.* **412**:469–481

Sauvé, R., Simoneau, C., Monette, R., Roy, G. 1986. Single-channel analysis of the potassium permeability in HeLa cancer cells. Evidence for a calcium-activated potassium channel of small unitary conductance. *J. Membrane Biol.* **92**:269–282

Schilling, W.P., Ritchie, A.K., Navarro, L.T., Eskin, S.G. 1988. Bradykinin-stimulated calcium influx in cultured bovine aortic endothelial cells. *Am. J. Physiol.* **255**:H219–H227

Sitsapesan, R., Williams, A.J. 1990. Mechanisms of caffeine activation of single calcium-release channels of sheep cardiac sarcoplasmic reticulum. *J. Physiol. Lond.* **423**:425–439

Spedding, M., Berg, C. 1985. Antagonism of Ca^{2+}-induced contraction of K^+-depolarized smooth muscle by local anaesthetics. *Eur. J. Pharmacol.* **108**:143–150

Stephenson, D.G., Wendt, I.R., 1986. Effects of procaine on calcium accumulation by the sarcoplasmic reticulum of mechanically disrupted rat cardiac muscle. *J. Physiol.* **373**:195–207

Thévenod, F., Delhinger-Kremer, M., Kemmer, T.P., Christian, A.-L., Potter, B.V.L., Schulz, I. 1989. Characterization of inositol 1,4,5-trisphosphate-sensitive (IsCaP) and -insensitive (IisCaP) nonmitochondrial Ca^{2+} pools in rat pancreatic acinar cells. *J. Membrane Biol.* **109**:173–186

Thuringer, D., Diarra, A., Sauvé, R. 1991. Modulation by extracellular pH of bradykinin-evoked activation of Ca^{2+}-activated K^+ channels in endothelial cells. *Am. J. Physiol.* **261**:H656–H666

Wakui, M., Osipchuk, Y.V., Petersen, O.H. 1990. Receptor-activated cytoplasmic Ca^{2+} spiking mediated by inositol trisphosphate is due to Ca^{2+}-induced Ca^{2+}-release. *Cell* **63**:1025–1032

Received 4 December 1991; revised 24 April 1992