RESEARCH COMMUNICATION

Heterogeneity of caffeine- and bradykinin-sensitive Ca^{2+} stores in vascular endothelial cells

Wolfgang F. GRAIER, Sabine SIMECEK, Douglas K. BOWLES and Michael STUREK*

Vascular Cell Biophysics Laboratory, Dalton Cardiovascular Research Center, and Department of Physiology, School of Medicine, University of Missouri, Columbia, MO 65211, U.S.A.

The filling state of Ca^{2+} stores in endothelial cells regulates Ca^{2+} entry. The functional relationship between the major Ca^{2+} stores [i.e. Ins(1,4,5)P_3-sensitive (= bradykinin-sensitive stores, 'BsS') and caffeine-sensitive stores] is unknown. In pig right-coronary-artery endothelial cells, caffeine failed to release Ca^{2+} in 68 % of the cells (quiet-responders), but increased bradykinin (Bk)-induced Ca^{2+} release 2.5-fold. In Bk-pre-stimulated cells, caffeine increased Ca^{2+} release upon a second stimulation with Bk 3.2-fold. In quiet-responders caffeine alone did not affect net Ca^{2+} storage, whereas Bk or caffeine followed by Bk decreased the intracellular Ca^{2+} pool to 45 % and 15 %, respectively. Blockade of the endoplasmic-reticulum Ca^{2+} pump by thapsigargin unmasked the effect of caffeine in quiet-responders, resulting in a transient increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). In 37 % of the cells caffeine alone transiently increased $[Ca^{2+}]_i$ and depleted BsS. This study suggests a heterogeneity in functional organization of endothelial Ca^{2+} stores. In quiet-responders, caffeine translocates Ca^{2+} towards the BsS, whereas in overt-responders caffeine empties the BsS.

INTRODUCTION

Vascular endothelial cells are the target of much research focused on their crucial role in regulation of blood flow (for review see [1]). Increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) are a key step in endothelial activation. These increases in endothelial $[Ca^{2+}]_i$ can occur by an increase in plasmalemmal Ca^{2+} permeability via so-called 'ligand-gated', non-selective, ion channels and by Ca^{2+} release from intracellular organelles (for review see [1]). Very recently, depletion of endothelial Ca^{2+} stores was described to stimulate Ca^{2+}-permeable channels in the plasma membrane [2–4]. Such a capacitative Ca^{2+}-influx pathway [5] is thought to regulate Ca^{2+}-permeable plasmalemmal channels by the filling state of intracellular Ca^{2+} pool(s). The second-messenger molecules that activate Ca^{2+} entry are still unknown, but the involvement of cytochrome *P*-450-related enzyme(s) [4,6] and/or phosphorylation [7] has been described.

Releasable Ca^{2+} stores in endothelial cells are described to be Ins(1,4,5)P_3-sensitive and/or caffeine-sensitive (CsS). In contrast with the Ins(1,4,5)P_3-sensitive Ca^{2+} stores, which are reported to be responsible for agonist-induced Ca^{2+} release, Ca^{2+}-induced Ca^{2+} release (i.e. the caffeine-sensitive store) has been poorly investigated to date (for review see [1]). There are some controversial reports about its existence and importance in vascular endothelium. Schilling et al. [8] described the failure of caffeine to increase basal $[Ca^{2+}]_i$ and Chu et al. [9] failed to find any ryanodine-binding sites in endothelial membranes. On the other hand, Buchan and Martin [10] found that caffeine slightly increased $[Ca^{2+}]_i$ in cultured endothelial cells, and Lesh et al. [11] reported immunolocalization of ryanodine receptors in endothelium of native aorta. Indirect evidence for caffeine-induced increases in cytosolic free Ca^{2+} was provided by the activation of Ca^{2+}-activated K^+ currents [12]. These findings were confirmed by Adams et al. [13], who clearly showed caffeine-induced increases in $[Ca^{2+}]_i$ and Ca^{2+}-activated K^+ current. Ryanodine, an antagonist of Ca^{2+}-induced Ca^{2+} release, prevented hyperpolarization upon caffeine exposure, indicating that caffeine-induced hyperpolarization is due to a Ca^{2+}-induced Ca^{2+}-release mechanism, resulting in an activation of Ca^{2+}-activated K^+ channels via an increase in endothelial $[Ca^{2+}]_i$. The aim of the present study was to determine whether the inability of caffeine to increase $[Ca^{2+}]_i$ is due to the absence of caffeine-sensitive Ca^{2+} pools in endothelial cells or whether caffeine induces Ca^{2+} translocation between Ca^{2+} pools without affecting the average $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Materials

Fura-2/AM (acetoxymethyl ester) was obtained from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell isolation

Endothelial cells were freshly prepared from pig right-coronary arteries as described previously [14–16]. Briefly, vessels were removed from the heart and stored in the refrigerator for up to 2 days in solution containing (in mM) 135 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 KCl, 0.44 KH_2PO_4, 0.34 NaH_2PO_4, 2.6 $NaHCO_3$, 20 Hepes, 10 D-glucose, plus dilutions (v/v) 0.02 amino acids, 0.01 vitamins, 0.002 Phenol Red, 0.01 penicillin/streptomycin, 0.2 % horse serum, and adjusted with NaOH to pH 7.4. Endothelial cells were dispersed by 90 min treatment with 294 units/ml collagenase (type IV), plus (in mg/ml): 2 BSA, 1 trypsin inhibitor and 0.4 DNAase I in the solution described above (0.5 mM $CaCl_2$) and loaded with 2.5 μM fura-2/AM for 30 min at 37 °C. After an equilibration period of 30 min, cells were centrifuged

Abbreviations used: AUC, area under the curve; Bk, bradykinin; BsS, bradykinin-sensitive stores; CsS, caffeine-sensitive stores; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; TG, thapsigargin.

* To whom correspondence should be addressed.

and resuspended in physiological salt solution (in mM: 135 NaCl, 1 $MgCl_2$, 5 KCl, 2 $CaCl_2$, 10 Hepes, 10 D-glucose, adjusted with NaOH at pH 7.4) containing 0.2% BSA.

Measurements of $[Ca^{2+}]_i$

One drop of the cell suspension was placed in a microfluorimetric system described by Sturek et al. [14,17]. Solutions were exchanged easily by a constant flow rate of 1 ml/min. $[Ca^{2+}]_i$ was measured in single endothelial cells or cell clusters of up to 6 cells as R, the 510 nm emission ratio from 360/380 nm excitation. Background subtraction was performed in each experiment. Because of the uncertainties of the calibration when using the equation of Grykniewicz et al. [18] reported by several authors [14,19], $[Ca^{2+}]_i$ is expressed as ratio units.

Statistics

All experiments were performed with at least three different dispersions of endothelial cells from different pigs. Data shown are expressed as means ± S.E.M. Statistical significance was evaluated by an analysis of variance and Scheffe's *post hoc* test. Significance was defined as $P < 0.05$ in all experiments (n.s., not significant).

RESULTS AND DISCUSSION

After a large number of experiments, the $[Ca^{2+}]_i$ response to caffeine was separated in two models. Each model represents one kind of endothelial response to Ca^{2+}-induced Ca^{2+} release by caffeine. Cells were differentiated according their response to 10 mM caffeine and by the ability to potentiate the $[Ca^{2+}]_i$ response to bradykinin (Bk) after 2 min exposure to caffeine: 'quiet-responders' did not show any increase in average $[Ca^{2+}]_i$ to 2 min of exposure to caffeine (10 mM), but showed an increased Ca^{2+} release to a subsequent stimulation with 100 nM Bk after caffeine treatment (63% of the experiments); 'overt-responsers' transiently increased average $[Ca^{2+}]_i$ after 2 min of stimulation with 10 mM caffeine between 0.10 to 0.30 ratio unit (37%). Differences in the response to caffeine were not due to the cold-storage time, as both types of responders were observed throughout the 2 days used.

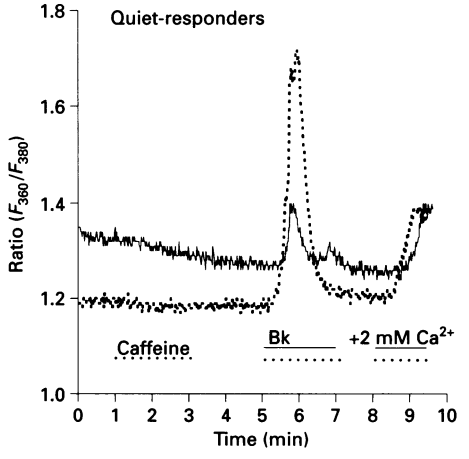

Figure 1 Representative records of the stimulatory effect of Bk on $[Ca^{2+}]_i$ in quiet-responders with or without pre-treatment with caffeine in the absence of extracellular Ca^{2+}

In Ca^{2+}-free solution, cells were pre-treated for 2 min with 10 mM caffeine (dotted line). Control cells (continuous line) were superfused for the same time in Ca^{2+}-free solution without caffeine. After 4 min in Ca^{2+}-free solution, cells were stimulated, as indicated by horizontal lines, with 100 nM Bk for 2 min. As the further horizontal lines indicate, extracellular Ca^{2+} was then increased to 2 mM.

Quiet-responders

In 31 of 49 experiments, cells did not respond with increases in cell-average $[Ca^{2+}]_i$ to 2 min of caffeine exposure. In these so-called 'quiet-responders' the effect of 100 nM Bk on $[Ca^{2+}]_i$ was significantly increased after exposure to 10 mM caffeine. As shown in Figure 1, Bk (100 nM) transiently increased endothelial $[Ca^{2+}]_i$ in Ca^{2+}-free solution from 1.26 ± 0.03 to 1.40 ± 0.05 ratio units ($n = 12$, $P < 0.05$ versus basal). In quiet-responders which were pre-treated with 10 mM caffeine for 2 min, the effect of Bk was significantly increased 2.4-fold (from 1.22 ± 0.02 to 1.56 ± 0.006 ratio units; $n = 21$, $P < 0.05$ versus basal and $P < 0.05$ versus control response to Bk; Figure 1). Addition of 2 mM extracellular Ca^{2+} at 2 min after removal of Bk yielded identical increases in $[Ca^{2+}]_i$ in caffeine-pre-treated cells and cells which were stimulated with Bk alone (Figure 1).

To compare the amount of Bk-released Ca^{2+} more accurately, the area under the curve (AUC) was calculated. In cells which were pretreated with 10 mM caffeine for 2 min, addition of 100 nM Bk yielded about 3.0-fold higher net Ca^{2+} release than under control conditions (caffeine-pre-stimulated cells 0.36 ± 0.08 $R \cdot$min, $n = 21$; control 0.13 ± 0.05 $R \cdot$min, $n = 12$; $P < 0.05$), suggesting that in 'quiet responders' caffeine translocated Ca^{2+} towards the Bk-sensitive stores (BsS).

After 3 min preincubation with 10 μM ryanodine, 10 mM caffeine failed to amplify Bk-induced Ca^{2+} release (from 1.24 ± 0.02 to 1.43 ± 0.05 ratio units, $n = 3$; $P < 0.05$ versus basal, n.s. versus control response to Bk). Incubation with 10 μM ryanodine (3 min) after caffeine did not attenuate caffeine-induced amplification of Bk-induced Ca^{2+} release (results not shown). This may exclude incomplete wash-out of caffeine, which might result in an amplification of Bk-stimulated Ca^{2+} release by activation of Ca^{2+}-induced Ca^{2+} release.

Pre-stimulation with a low dose of Bk (30 nM) in Ca^{2+}-free solution resulted in a transient increase in $[Ca^{2+}]_i$ from 1.19 ± 0.01 to 1.41 ± 0.03 ratio units ($n = 16$; $P < 0.05$ versus basal) and partial emptying of the BsS. After 6 min a second stimulation with a supramaximal concentration of Bk (1 μM to overcome receptor desensitization [20]) resulted in a transient increase in $[Ca^{2+}]_i$ from 1.17 ± 0.02 to 1.26 ± 0.04 ratio units ($n = 6$; $P < 0.05$ versus basal; Figure 2). Addition of 10 mM caffeine for 2 min between Bk stimulations did not alter basal $[Ca^{2+}]_i$, but significantly enhanced the increase in $[Ca^{2+}]_i$ to the second Bk stimulation (from 1.15 ± 0.01 to 1.37 ± 0.04 ratio units; $n = 10$; $P < 0.05$ versus basal, $P < 0.05$ versus control; Figure 2). The ratio of the Bk-induced AUC in response to the second dose to the AUC measured upon the first Bk stimulation (AUC_{Bk2}/AUC_{Bk1}) was also calculated. Caffeine (10 mM) treatment between Bk stimulations increased the AUC_{Bk2}/AUC_{Bk1} 3.0-fold, from 0.62 ± 0.27 ($n = 6$) to 2.04 ± 0.30 ($n = 10$; $P < 0.05$). The finding that caffeine amplified Bk-induced Ca^{2+} release even in cells pre-stimulated with Bk suggests that Bk [$Ins(1,4,5)P_3$] does not modulate the Ca^{2+} content of the CsS in quiet-responders.

To investigate whether caffeine translocates Ca^{2+} to the $Ins(1,4,5)P_3$-sensitive stores in quiet responders, we determined the Ca^{2+}-storage capacity after treatment with Bk, caffeine, and caffeine plus Bk. Ionomycin (1 μM) was used to deplete all Ca^{2+}

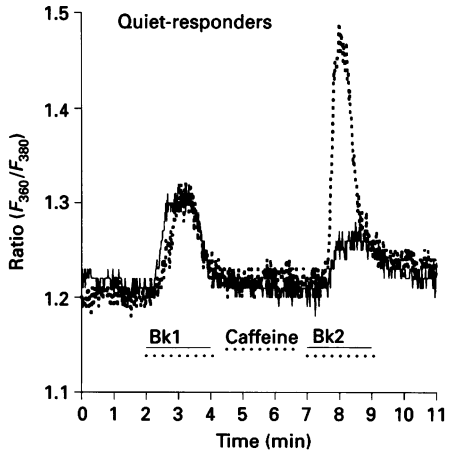

Figure 2 Representative tracings in quiet-responders of the influence of caffeine on the Bk-releasable store after pre-emptying with a small concentration of Bk

In Ca^{2+}-free solution cells were stimulated for 2 min with 30 nM Bk (Bk1). During 3 min recovery, cells were exposed for 2 min to 10 mM caffeine (broken line) or Ca^{2+}-free solution without caffeine (continuous line). A second stimulation with 1 μM Bk for 2 min (Bk2) was performed at the time indicated by the horizontal lines.

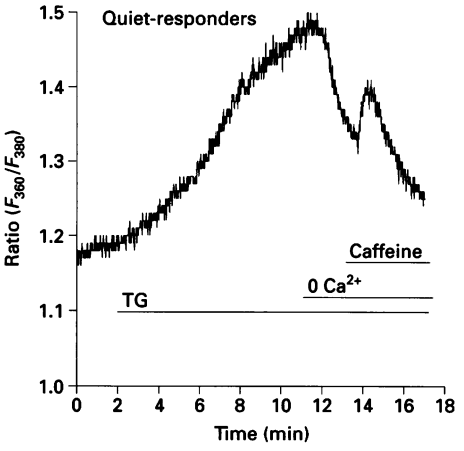

Figure 3 Representative records of the effect of inhibition of endoplasmic-reticulum Ca^{2+} pumps by TG on the response to caffeine in quiet-responders

Cells were stimulated in the presence of extracellular Ca^{2+} with 1 μM TG. After 9 min stimulation, extracellular Ca^{2+} was removed. Caffeine (10 mM) was added in the presence of TG and in the absence of extracellular Ca^{2+}.

stores in Ca^{2+}-free solution. Bk (100 nM) decreased Ca^{2+}-storage capacity to 45% of the control ($n = 5$; $P < 0.05$ versus control). Caffeine (10 mM) failed to change net Ca^{2+} storage ($n = 6$; 91% of control; n.s. versus control), whereas 100 nM Bk after 2 min exposure to 10 mM caffeine strongly diminished the Ca^{2+}-storage capacity to 15% of control ($n = 8$; $P < 0.05$ versus control and $P < 0.05$ versus Bk alone). These data suggest that, after caffeine-induced Ca^{2+} translocation towards the BsS, Bk decreases net Ca^{2+} storage much more than does Bk alone.

In quiet-responders the effect of caffeine was unmasked in the presence of 1 μM thapsigargin (TG). Figure 3 shows the effect of 1 μM TG in the presence of 2 mM Ca^{2+}. Removal of external

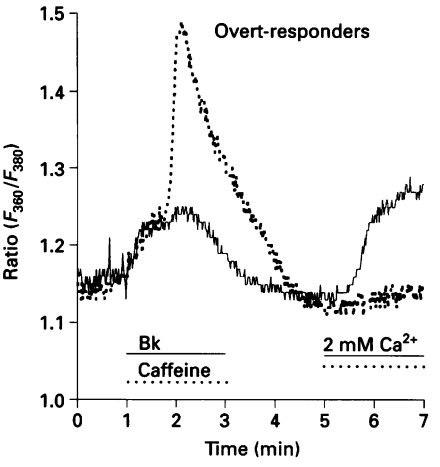

Figure 4 Effect of 10 mM caffeine (dotted line) or 100 nM Bk (continuous line) on overt-responders in the absence of extracellular Ca^{2+}

Cells were stimulated for 2 min with the compound as the lines indicate. After 2 min wash-out, store-depletion-activated Ca^{2+} entry was measured by increasing the extracellular Ca^{2+} to 2 mM as the lines indicate.

Ca^{2+} after 9 min of TG stimulation diminished the TG-induced plateau. Addition of 10 mM caffeine at this time transiently increased $[Ca^{2+}]_i$ from 1.33 ± 0.01 to 1.46 ± 0.02 ratio units ($n = 3$; $P < 0.05$ versus without caffeine). This finding may suggest that, even in quiet responders, caffeine actually releases Ca^{2+} into the cytoplasm. Inhibition of microsomal Ca^{2+}-ATPase prevents Ca^{2+} uptake into the BsS, resulting in an increase in average $[Ca^{2+}]_i$. Thus, caffeine may release Ca^{2+} from CsS into a distinct cytoplasmic area between CsS and BsS, from which Ca^{2+} is rapidly pumped into the BsS. Despite increases in local $[Ca^{2+}]_i$ in such restricted areas, no significant increase in average $[Ca^{2+}]_i$ is detectable in the absence of TG. In contrast with the Ca^{2+} translocation reported by Mullaney et al. [21] involving a GTP-controlled translocation via a direct junction between Ca^{2+} stores, our data suggest a Ca^{2+} translocation from the CsS to the BsS via some specific area of the cytoplasm. However, our data cannot rule out the involvement of some nucleotide-controlled mechanism in the event of Ca^{2+} release and re-sequestration. Since a pre-stimulation with 10 mM caffeine for 2 min directly before TG (without refilling time for CsS) prevented the caffeine-induced $[Ca^{2+}]_i$ transient in the presence of TG (results not shown), TG-induced Ca^{2+} translocation to any CsS or major changes in functional store organization by TG (except store depletion) seems unlikely. Moreover, CsS seemed to be depleted after a longer treatment TG, indicated by failure of caffeine to increase $[Ca^{2+}]_i$ after 16 min stimulation with TG (results not shown).

Overt-responders

Besides a caffeine-induced Ca^{2+} translocation towards the BsS as described above, stimulation with 10 mM caffeine for 2 min in Ca^{2+}-free solution resulted in a transient $[Ca^{2+}]_i$ increase in 37% (18 of 49) of the experiments, from 1.20 ± 0.01 to 1.43 ± 0.03 ratio units ($n = 18$; $P < 0.05$ versus basal; overt-responders; Figure 4). In these cells, the capacity of the CsS seems to be higher compared with the BsS, as indicated by a significantly smaller effect of 1 μM ionomycin in Ca^{2+}-free solution in caffeine-pre-stimulated overt responders (27%, $n = 3$; $P < 0.05$ versus con-

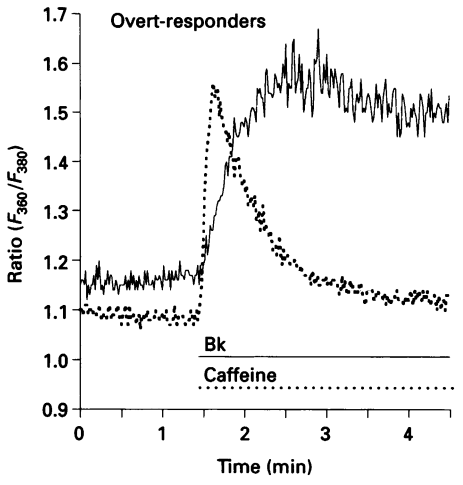

Figure 5 Representative tracings of the effects of caffeine and Bk in the presence of 2 mM extracellular Ca^{2+}

As the horizontal lines indicate, cells were stimulated with 10 mM caffeine or 100 nM Bk.

trol) than of Bk (45%, $n = 5$; $P < 0.05$ versus control). Interestingly, short pretreatment with caffeine completely prevented the effect of Bk in the absence of extracellular Ca^{2+} ($n = 7$), whereas Bk alone increased $[Ca^{2+}]_i$ in > 96% of the experiments. Preincubation with 10 μM ryanodine for 3 min prevented caffeine-induced increases in $[Ca^{2+}]_i$ and normalized the effect of Bk (results not shown). These data may suggest the existence of functional ryanodine (caffeine) receptors on BsS, or that there is a Ca^{2+} translocation from BsS towards the CsS during caffeine stimulation. Similar results were reported by Bolton and Lim [22], who demonstrated the inability of carbachol to release Ca^{2+} after caffeine exposure in smooth muscle of rabbit small intestine. Wagner-Mann and Sturek [23] showed similar effects of caffeine on endothelin-induced Ca^{2+} release in pig coronary smooth-muscle cells. Caffeine antagonism of the Ins(1,4,5)P_3 binding to its intracellular receptor, as shown by Parker and Ivorra [24], is very unlikely, since cells were stimulated with Bk in the absence of caffeine after a wash-out period of at least 4 min. As shown above in Figure 2, Bk is able to deplete BsS also under these conditions. The finding that, after a short pretreatment with ryanodine, caffeine failed to increase average $[Ca^{2+}]_i$ also in overt-responders provides further evidence that caffeine truly acts via the Ca^{2+}-induced Ca^{2+}-release mechanism, and makes any other actions of caffeine very unlikely.

It has been shown that the amount of Ca^{2+}-store depletion controls the plasmalemmal Ca^{2+} permeability in endothelial cells [3,4,25]. Since combined stimulation with caffeine and Bk results in an increased depletion of Ca^{2+} stores in quiet-responders, one might expect an amplification of Bk-induced Ca^{2+} entry in caffeine-pretreated cells. However, as shown above in Figure 1, Ca^{2+} entry induced by Bk in caffeine-pre-stimulated cells was identical with that observed with Bk alone, although pre-stimulation with caffeine enhanced store depletion even in quiet-responders.

In agreement, caffeine failed to stimulate strong long-lasting Ca^{2+} entry in overt-responders. In particular, in overt responders which were preincubated for 2 min with 10 mM caffeine, re-addition of 2 mM $CaCl_2$ to the superfusion slightly increased $[Ca^{2+}]_i$ from 1.17 ± 0.01 to 1.20 ± 0.01 ratio units ($n = 5$; Figure 4), which was not significantly different from the effect of Ca^{2+} re-addition without prior caffeine stimulation. In contrast, re-addition of 2 mM Ca^{2+} to the superfusion solution of Bk-pre-stimulated cells resulted in a large increase in endothelial Ca^{2+}, from 1.16 ± 0.02 to 1.46 ± 0.02 ratio units ($n = 7$; $P < 0.05$ versus control; Figure 4), typical of Ca^{2+}-store-depletion-activated Ca^{2+} influx.

Convincingly, addition of 10 mM caffeine in the presence of extracellular Ca^{2+} biphasically increased $[Ca^{2+}]_i$ in only 5 of 17 experiments (Figure 5). There was an initial transient increase from 1.12 ± 0.01 to 1.45 ± 0.08 ratio units ($n = 5$; $P < 0.05$ versus basal), which was very similar to that obtained with 100 nM Bk (from 1.16 ± 0.01 to 1.56 ± 0.04 ratio units; $n = 6$, $P < 0.05$ versus basal). In contrast with Bk, which yielded a high sustained plateau phase at 1.54 ± 0.03 ratio units ($n = 6$; $P < 0.05$ versus basal, n.s. versus peak) measured 6 min after stimulation, only a slight plateau phase at 1.16 ± 0.02 ratio units (i.e. 11% of the Bk effect; $n = 5$, n.s. versus basal, $P < 0.05$ versus peak) was observed in caffeine-stimulated cells (Figure 5).

Similar results were described by Adams et al. [13], who reported that caffeine activation of Ca^{2+}-activated K^+ outward current was not prolonged in the presence or the absence of extracellular Ca^{2+}, suggesting a lack of long-lasting Ca^{2+} entry in response to caffeine exposure, as shown for Bk. However, Adams et al. [13] found that the initial activation was enhanced in the presence of extracellular Ca^{2+}, indicating a transient Ca^{2+} influx in response to caffeine exposure or a partial depletion of intracellular stores. In contrast with the Bk-induced plateau phase, the very small sustained increase in $[Ca^{2+}]_i$ by caffeine was unaffected by econazole (results not shown), an inhibitor of agonist-induced Ca^{2+} entry in vascular endothelial cells [4]. Due to the unknown link between store depletion and activation of Ca^{2+}-permeable plasmalemmal channels, we may speculate that in caffeine-induced Ca^{2+}-store depletion the second messenger for channel activation, as hypothesized by several authors [6,7], is not synthesized or liberated in sufficient concentration. Since inward Ca^{2+} currents can also be stimulated by Ins(1,4,5)P_3-independent store depletion by 2,5-di-(t-butyl)-hydroquinone in endothelial cells [3,4], the absence of Ins(1,4,5)P_3 in the caffeine-induced store depletion might be excluded as being responsible for the missing Ca^{2+}-entry activation in the case of caffeine-induced store depletion. A different possibility for Ca^{2+}-entry activation, between depletion of Ins(1,4,5)P_3-sensitive and -insensitive stores, has also been discussed by Schilling et al. [25]. On the other hand, our data suggest that Ca^{2+}-store depletion alone is not responsible for activation of Ca^{2+} entry or that activation of Ca^{2+} entry does not correlate with depletion of internal Ca^{2+} pools, as recently suggested by Gosnik and Forsberg [26]. The uncoupling of Ca^{2+}-store depletion and Ca^{2+} influx is further strengthened by the finding that depletion of the BsS by caffeine failed to trigger Ca^{2+} influx.

Our results excluded direct regulation of Ca^{2+} entry by the Ca^{2+}-induced Ca^{2+}-release mechanism, as suggested by Grinstein et al. [27]. However, our results indicate that Ca^{2+}-induced Ca^{2+} release might be involved in endothelial Ca^{2+} regulation in overt-responders as an additional source for increases in average $[Ca^{2+}]_i$, and in quiet-responders as a reserve for BsS Ca^{2+} release. These data further support the increasing number of reports on the functional heterogeneity of endothelial cells [28]. Although this is the first report on the heterogeneous effect of caffeine, convincing data were also reported by Ziegelstein et al. [29], who described heterogeneous responses to ryanodine in a given endothelial cell type.

In conclusion, the present data indicate two different $[Ca^{2+}]_i$ responses to Ca^{2+}-induced Ca^{2+} release (caffeine-induced), which we propose are due to different functional organizations of

intracellular Ca^{2+} stores. Despite the existence of CsS in vascular endothelial cells, these stores seems to be partially overlapped and/or coupled with the BsS or physically close to each other. Thereby, stimulation with caffeine may result in the depletion of both types of Ca^{2+} stores (overt-responders), resulting in an inability of Bk to release Ca^{2+}. Alternatively, caffeine may cause filling of the BsS via Ca^{2+} release into a restricted area (quiet-responders), followed by the uptake into the BsS and in an enhanced response to Bk.

W.F.G. is an International Scholar of the Max Kade Foundation, New York, U.S.A. D.K.B. is an American Heart Association Missouri Affiliate Postdoctoral Fellow. We thank Mr. Qicheng Hu for his great technical assistance. This work was supported by grants from the Austrian Research Fonds (P10029 to W.F.G.), the Austrian National Bank (P4715 to W.F.G.), the U.S. National Institutes of Health (Research Career Development Award HL 02872 to M.S.) and the American Diabetes Association (to M.S.).

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Received 25 February 1994/31 March 1994; accepted 7 April 1994