Br. J. Pharmacol. (1988), **95**, 189–196

Differential role of extra- and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells

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1 The effects of extracellular Ca^{2+} on the release of endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2), and on the intracellular free calcium concentration ($[Ca^{2+}]_i$), were studied in cultured bovine aortic endothelial cells.

2 Receptor-mediated stimulation of endothelial cells with bradykinin (10 nM) elicited a transient release of EDRF (assayed by its stimulant effect on purified soluble guanylate cyclase) and of PGI_2 (measured by radioimmunoassay for 6-keto prostaglandin $F_{1\alpha}$).

3 Bradykinin (10 nM) also increased $[Ca^{2+}]_i$ (measured with the fluorescent probe indo-1) from 125 ± 11 nM to 631 ± 59 nM, with the same time course as for autacoid release.

4 In Ca^{2+}-free medium, $[Ca^{2+}]_i$ was still increased by bradykinin but declined faster (within 1 min) to resting levels than in the presence of extracellular Ca^{2+}.

5 PGI_2 release was almost completely abolished in Ca^{2+}-free medium. The intracellular calcium antagonist TMB-8 evoked a similar inhibition of PGI_2 release.

6 In contrast, bradykinin-induced EDRF release was not significantly affected by TMB-8 but was completely abolished in Ca^{2+}-free medium.

7 When endothelial cells were stimulated with the receptor-independent drug thimerosal (an inhibitor of the enzyme acyl-CoA-lysolecithin-acyl-transferase; 5 μM), a long-lasting release of EDRF (>90 min) and PGI_2 (>20 min) was observed.

8 In contrast to bradykinin stimulation, thimerosal-induced autacoid release was associated with only a slight increase of $[Ca^{2+}]_i$ to 201 ± 13 nM after 40 min.

9 After removal of extracellular Ca^{2+} from thimerosal-stimulated endothelial cells, $[Ca^{2+}]_i$ was little affected during the observation time of 90 s. EDRF release was completely abolished within 90 s whereas PGI_2 release was unchanged.

10 We conclude that EDRF production is directly controlled by extracellular Ca^{2+} during both receptor-dependent and independent stimulation. This effect of extracellular Ca^{2+} is not mediated by changes in $[Ca^{2+}]_i$. In contrast, PGI_2 release is closely correlated to $[Ca^{2+}]_i$ in bradykinin-stimulated endothelial cells. However, the results obtained during thimerosal stimulation indicate that there is not necessarily a tight coupling between the absolute level of $[Ca^{2+}]_i$ and the amount of PGI_2 released.

Introduction

The vascular endothelium plays a pivotal role in the control of vascular tone as well as of blood cell activation by release of autacoids. Among these, endothelium-derived relaxing factor (EDRF)—recently identified (Palmer *et al.*, 1987) as nitric oxide (NO)—and prostacyclin (PGI_2) are considered the most important. Simultaneous release of EDRF and PGI_2 can be stimulated by some receptor-operated agonists such as adenosine 5'-triphosphate (ATP) and bradykinin (for review see Busse *et al.*, 1985) which are known to cause breakdown of phosphatidylinositol (Derian & Moskowitz, 1986; Lambert *et al.*, 1986; Forsberg *et al.*, 1987; Pirotton *et al.*, 1987) and an increase of the intracellular free calcium concentration ($[Ca^{2+}]_i$) (Lückhoff & Busse, 1986; Hallam & Pearson, 1986; Morgan-Boyd *et al.*, 1987; Peach *et al.*, 1987) in endothelial cells. Therefore, a key control function in the synthesis of EDRF and PGI_2 has been attributed to $[Ca^{2+}]_i$. On the other hand, removal of extracellular Ca^{2+} reduces or

abolishes release of the autacoids (Singer & Peach, 1982; Long & Stone, 1985; Griffith *et al.*, 1986; Lückhoff *et al.*, 1987). However, changes in extracellular Ca^{2+} affect $[Ca^{2+}]_i$ significantly in resting as well as ATP-stimulated endothelial cells (Lückhoff & Busse, 1986). Thus, it cannot be decided from the available data whether extracellular Ca^{2+} has a distinct role, independent of cytosolic Ca^{2+} concentration, in the generation of endothelial autacoids.

To differentiate between the roles of extracellular and intracellular Ca^{2+} on autacoid release, we chose a stimulus which is different from those like bradykinin, that acts on 'calcium mobilizing receptors' (Berridge, 1987). Thimerosal acts as a receptor-independent stimulus and is known to induce a long-lasting release of EDRF and PGI_2 from endothelial cells (Förstermann *et al.*, 1986; Mülsch *et al.*, 1987). This compound blocks the enzyme acyl-CoA-lysolecithin-acyl-transferase, thereby increasing the pool of free arachidonic acid available for PGI_2 synthesis. The link between arachidonic acid metabolism and thimerosal-induced EDRF production, as well as the effect of thimerosal on $[Ca^{2+}]_i$, has not yet been clarified.

The aim of this study was to define the correlation between EDRF and PGI_2 release and intra- as well as extracellular Ca^{2+}, in endothelial cells stimulated with the receptor-independent drug thimerosal, in comparison to cells stimulated with the receptor-operated oligopeptide bradykinin. We found that extracellular Ca^{2+} has a crucial role in EDRF but not PGI_2 release. This role was independent of the level of $[Ca^{2+}]_i$, as demonstrated under experimental conditions where changes in extracellular Ca^{2+} were not readily reflected by changes in intracellular Ca^{2+}.

Methods

Bovine aortic endothelial cells were isolated and cultured as previously described in detail (Lückhoff *et al.*, 1987). Briefly, the endothelium was scraped off freshly obtained aortae and grown in standard culture dishes in Dulbecco's modified Eagle's/Ham's F-12 medium (1 : 1). Confluent cells were subcultured (passage 1–2) on microcarrier beads (Biosilon, Nunc-Intermed, Wiesbaden, F.R.G.) or quartz coverslips (1.4 × 2.5 cm) until confluence was reached. For the determination of endothelial autacoid release, 250 µl of beads (corresponding to 5×10^6 cells) were filled into small columns. The endothelial cell column was kept in a water jacket (37°C) and perfused with oxygenated Tyrode solution at a rate of 40 ml h^{-1}. The effluent from the cells was assayed for PGI_2 and EDRF.

PGI_2 was determined as 6-keto prostaglandin $F_{1\alpha}$, the stable hydrolysis product of PGI_2, by a specific radioimmunoassay, as described previously (Busse *et al.*, 1984). Samples of the effluent were collected over periods of 6 min and stored frozen until assayed.

EDRF was assayed by its stimulant effect on the activity of soluble guanylate cyclase purified from bovine lung. The activity of guanylate cyclase was determined in test tubes in terms of the formation of [^{32}P]-guanosine 3′ : 5′-cyclic monophosphate ([^{32}P]-cyclic GMP) from [α-^{32}P]-GTP. The reaction was started by adding 3 µg guanylate cyclase to a reaction mixture containing [α-^{32}P]-GTP (0.1 mM; 0.2 µCi). Simultaneously, the effluent from an endothelial cell column (standard transit time 2 s) was dropped for 15 s into the test tube and the enzymatic formation of cyclic GMP proceeded for a further 45 s at 37°C in a total volume of 180 µl. The enzyme reaction was stopped by the addition of 450 µl zinc acetate (120 mM) and 500 µl sodium carbonate (120 mM), and the amount of [^{32}P]-cyclic GMP formed was determined. Details of the method have recently been described (Mülsch *et al.*, 1987).

The intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured in endothelial cells grown on quartz coverslips by means of the fluorescent $[Ca^{2+}]_i$ indicators indo-1 and fura-2 (Grynkiewicz *et al.*, 1985). The dyes were loaded into the cytosol by incubation of the cells with the penta-acetoxymethylesters (2 µM) of the dyes for 90 min at 37°C. The coverslips were put into cuvettes filled with HEPES buffer, and fluorescence was measured in a thermostatically controlled fluorometer (Schoeffel Instruments RRS 1000). Excitation and emission wavelengths were set to 330 nm and 400 nm, respectively, when indo-1 was used as the indicator. For fura-2, 340 nm and 500 nm were used. $[Ca^{2+}]_i$ was calculated from the fluorescence intensity F as

$$[Ca^{2+}]_i = K_D \cdot (F - F_{min})/(F_{max} - F)$$

F_{max} was obtained not later than 5 min after the beginning of the experiment, by addition of the calcium ionophore ionomycin (0.1 µM) and setting the extracellular Ca^{2+} concentration to 2 mM. Saturation of the indicators with Ca^{2+} under these conditions was confirmed by spectral analysis. The background signal was obtained after addition of Mn^{2+} (2 mM) to ionomycin-treated cells. F_{min} was 1/30 F_{max} for indo-1 and 1/5 F_{max} for fura-2, as defined after addition of EGTA (2 mM, pH 8.3) to the pure dyes. K_D was taken as 250 nM (indo-1) or 200 nM (fura-2), according to Grynkiewicz *et al.* (1985). For details see Lückhoff (1986).

Solutions (concentrations in mM): Tyrode solution: Na^+ 144.0, K^+ 4.0, Mg^{2+} 1.0, Cl^- 138.4,

HCO_3^- 11.9, $H_2PO_4^-$ 0.36, calcium-disodium EDTA 0.025, glucose 11.2; Po_2 140 mmHg, pH 7.4. HEPES buffer: Na^+ 132, K^+ 4, Mg^{2+} 0.5, Cl^- 138, N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES) 9.5, glucose 5; pH 7.3. Solutions were prepared with three different Ca^{2+} concentrations: (i) $CaCl_2$, 1.6 mM; (ii) $CaCl_2$ 0 mM (Ca^{2+} was estimated to be 1 to 5 μM in these 'nominally Ca^{2+}-free solutions', due to impurities in the other salts); (iii) $CaCl_2$ 0 mM, EGTA 0.1 mM ('nominally Ca^{2+}-free EGTA solution'). Materials: bradykinin, thimerosal and 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) were obtained from Sigma, Deisenhofen, F.R.G. Ionomycin and the penta-acetoxymethylesters of indo-1 and fura-2 were purchased from Calbiochem, Frankfurt, F.R.G.

Data are presented as means ± s.e.mean. Statistical evaluation was by use of Student's *t* test for paired or unpaired data. Only data obtained from cells from the same batch were considered as paired data.

Results

Effects of bradykinin on the release of endothelial autacoids

Bradykinin at a concentration of 30 nM in the perfusate of endothelial cell columns elicited an immediate and simultaneous release of EDRF and PGI_2. The kinetics of the release reactions are given in Figure 1a. It is obvious that autacoid release was only transient despite continuous stimulation with bradykinin. The duration of the release was drastically diminished when $CaCl_2$-free perfusate was

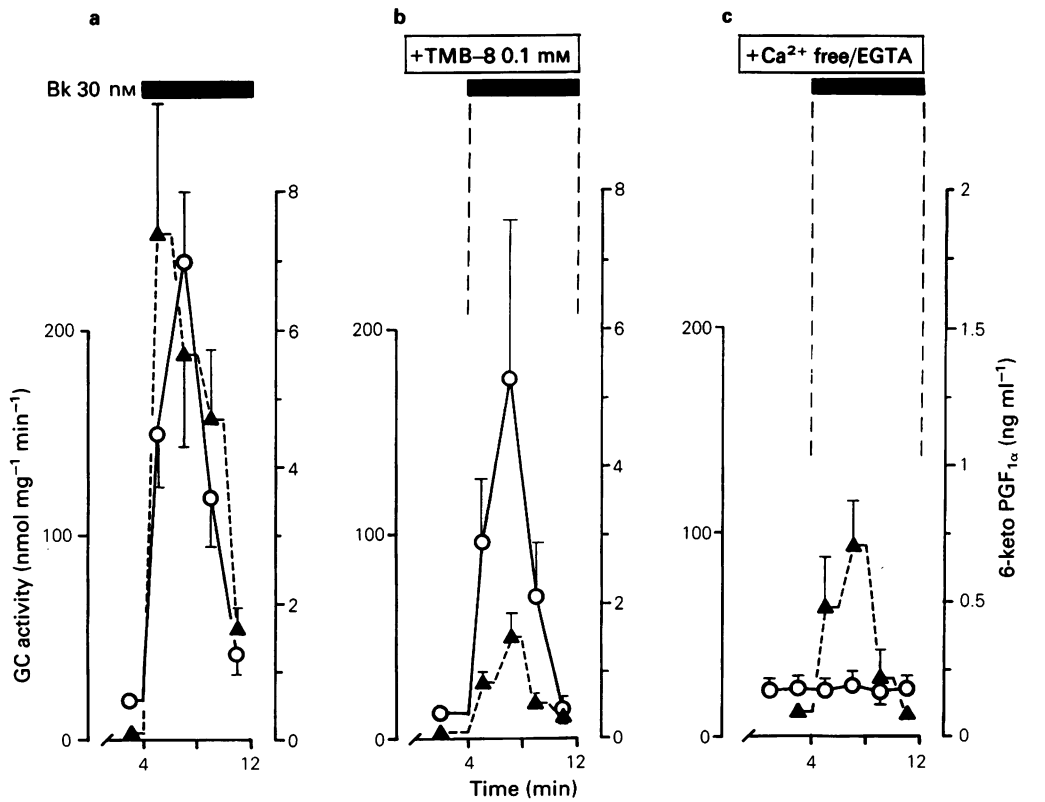

Figure 1 Simultaneous release of EDRF (○, left ordinate scale) and prostacylin (PGI_2) (6-keto prostaglandin $F_{1\alpha}$, ▲, right ordinate scale) from cultured bovine aortic endothelial cells in response to bradykinin (Bk, 30 nM). EDRF release is expressed as activity of purified soluble guanylate cyclase (GC). The duration of bradykinin stimulation is indicated by the bar in each panel. (a) Experiments performed at an extracellular Ca^{2+} concentration of 1.6 mM. Each symbol represents mean of 11 experiments; vertical lines show s.e.mean. (b) Experiments performed at an extracellular Ca^{2+} of 1.6 mM in the presence of the intracellular calcium antagonist TMB-8 (0.1 mM; $n = 3$). (c) Experiments performed in Ca^{2+}-free medium containing 0.1 mM EGTA ($n = 3$). Note that the calibration of the PGI_2 release is different in (a) and (b) from calibration in (c).

used, although the initial response was unaffected (not shown). When the cells were exposed to $CaCl_2$-free, EGTA-containing solution for 10 min, only a minimal and shortlasting PGI_2 release was observed (Figure 1c). No EDRF release could be detected under these conditions (Figure 1c). Following a 10 min pre-incubation of endothelial cell columns with Tyrode solution containing the intracellular calcium antagonist TMB-8 (0.1 mM), the bradykinin-induced PGI_2 production was almost completely abolished. By contrast, EDRF release was not significantly changed ($P > 0.05$, unpaired t test), as compared to control (Figure 1b).

Effects of bradykinin on $[Ca^{2+}]_i$

Bradykinin evoked a dose-dependent rise of $[Ca^{2+}]_i$ in indo-1-loaded endothelial cells (Figure 2). The peak occurred 5 to 20 s after the peptide was pipetted into the cuvette. After the peak, $[Ca^{2+}]_i$ declined but remained above the resting level for the next 3 min (Figure 3a). Removal of $CaCl_2$ from the bath did not affect the initial increase of $[Ca^{2+}]_i$ in response to bradykinin. However, the decline was faster than under normal Ca^{2+} (Figure 3b). Furthermore, elevation of the dose of bradykinin above 10 nM did not elicit a further increase of $[Ca^{2+}]_i$ under these conditions (not shown). It was not possible to expose endothelial cells for 10 min to $CaCl_2$-free EGTA-solutions since the cells detached from the coverslip under these conditions. The effects

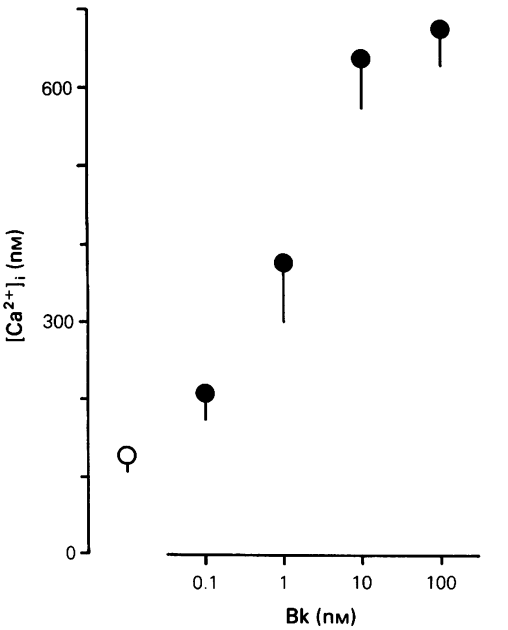

Figure 2 Dose-dependent increase of intracellular free calcium concentration ($[Ca^{2+}]_i$) in endothelial cells in response to bradykinin (Bk). Cells were stimulated with cumulative doses of bradykinin given at intervals of 15 to 30 s. Each symbol represents the mean from 11 experiments of the peak $[Ca^{2+}]_i$ elicited at each dose; vertical lines indicate s.e.mean. (○) The level of $[Ca^{2+}]_i$ in unstimulated cells.

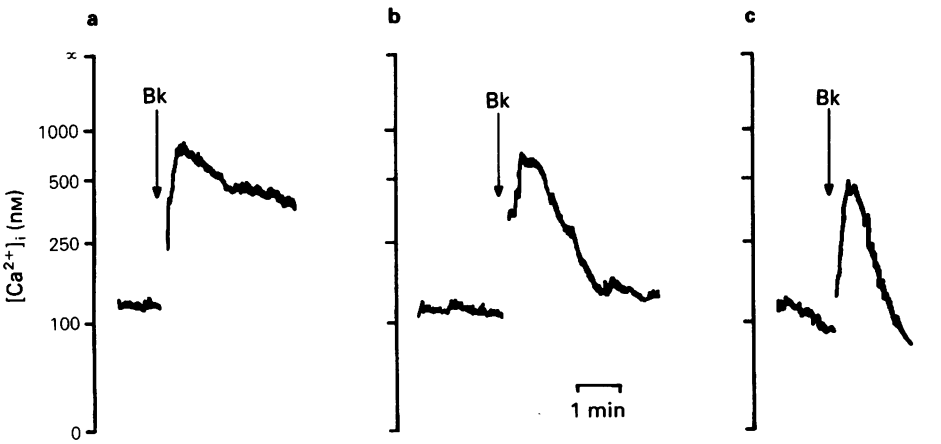

Figure 3 Effect of extracellular Ca^{2+} on the increase of intracellular free calcium concentration ($[Ca^{2+}]_i$) in response to bradykinin (Bk). The figure shows the original tracings of three experiments performed with cells from the same batch. The fluorescent signal is calibrated in terms of $[Ca^{2+}]_i$. (a) Extracellular Ca^{2+}: 1.6 mM, (b) nominally Ca^{2+}-free medium, (c) nominally Ca^{2+}-free medium with 0.1 mM EGTA. Bradykinin (10 nM) was added to the cells as indicated by the arrows.

of bradykinin (10 nM) on endothelial cells kept in $CaCl_2$-free EGTA buffer for less than 90 s could be studied ($n = 4$). The level of $[Ca^{2+}]_i$ just after stimulation was in the same range (560 ± 70 nM) as under normal Ca^{2+}, although the basal levels had declined slightly (from 121 ± 6 nM to 101 ± 16 nM) after change to nominally Ca^{2+}-free EGTA medium (Figure 3c). Sixty seconds after bradykinin stimulation, $[Ca^{2+}]_i$ had returned to values (110 ± 25 nM) not different from the basal level. In contrast, in experiments performed with normal extracellular Ca^{2+} (1.6 mM), $[Ca^{2+}]_i$ was 410 ± 60 nM (significantly different from values in Ca^{2+}-free solution, $P < 0.05$) 60 s after stimulation.

Effects of thimerosal on the release of endothelial autacoids

The time courses of the effects of thimerosal (5 μM) on the release of EDRF and PGI_2 are depicted in Figure 4. The onset of increased release of both autacoids was slow. Production of PGI_2 declined after reaching a maximum 15–20 min after the beginning of thimerosal stimulation. EDRF release continued even after thimerosal was stopped. In $CaCl_2$-free perfusate, the thimerosal-induced release of PGI_2 was only slightly affected, whereas that of EDRF was virtually abolished (Figure 5).

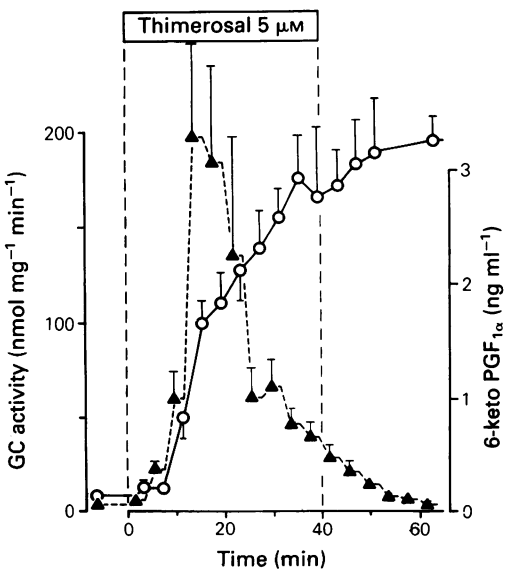

Figure 4 Simultaneous release of EDRF (○, left ordinate scale) and prostacyclin (PGI_2) (6-keto prostaglandin $F_{1\alpha}$, ▲, right ordinate scale) from cultured endothelial cells in response to thimerosal. EDRF release is expressed as activity of purified soluble guanylate cyclase (GC). The duration of the stimulation is indicated by the bar. Each symbol represents the mean of at least 5 experiments at an extracellular Ca^{2+} of 1.6 mM; vertical lines indicate s.e.mean.

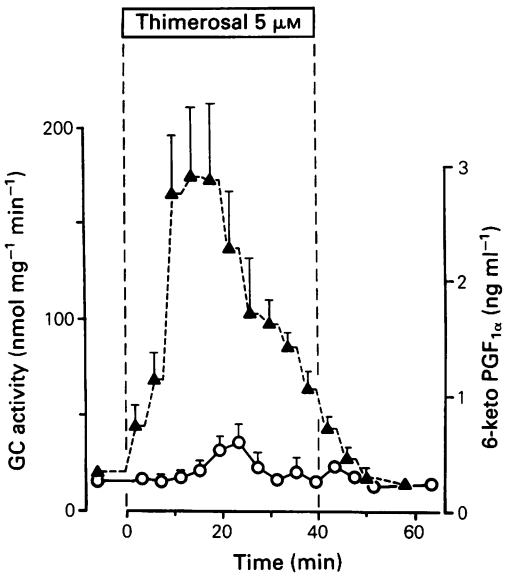

Figure 5 Thimerosal-induced release of EDRF (○, left ordinate scale) and prostacyclin (PGI_2) (6-keto prostaglandin $F_{1\alpha}$, ▲, right ordinate scale) in nominally Ca^{2+}-free medium. EDRF release is expressed as activity of purified soluble guanylate cyclase (GC). Each symbol represents the mean of 4 experiments; vertical lines indicate s.e.mean.

Figure 6 shows the results of experiments in which extracellular Ca^{2+} was removed suddenly and completely from thimerosal-stimulated endothelial cells, when the perfusate was changed to $CaCl_2$-free, EGTA-containing Tyrode solution. EDRF production ceased completely within 90 s. Restitution of extracellular Ca^{2+} restored EDRF release immediately.

In contrast, PGI_2 release was not affected when extracellular Ca^{2+} was removed from thimerosal-stimulated cells before the peak of PGI_2 release ($n = 2$; data not shown).

Effects of thimerosal on $[Ca^{2+}]_i$

Thimerosal (5 μM) was added to indo-1-loaded cells 10, 20, 40 and 60 min before $[Ca^{2+}]_i$ was measured. The drug induced significant (paired t test, $P < 0.05$) increases in $[Ca^{2+}]_i$ from 125 ± 20 nM to 190 ± 25 nM after 20 min, 201 ± 13 nM after 40 min, and 262 ± 27 nM after 60 min. After 10 min, $[Ca^{2+}]_i$ was not significantly different from control. Experiments performed with fura-2 as the $[Ca^{2+}]_i$ indica-

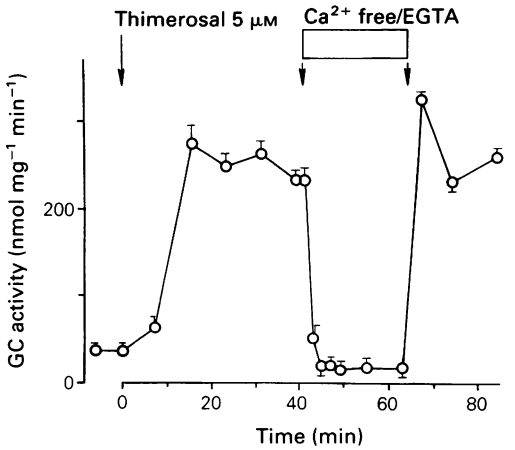

Figure 6 Effects of removal of extracellular Ca^{2+} on the thimerosal-induced release of EDRF (ordinate scale). EDRF release is expressed as activity of purified soluble guanylate cyclase (GC). Extracellular Ca^{2+} was removed when the perfusate of the endothelial cell column was switched to nominally calcium-free Tyrode solution containing EGTA (0.1 mM). The next sample for assay of EDRF was taken 90 s after Ca^{2+} removal ($n = 4$). Each symbol represents the mean and vertical lines indicate s.e.mean.

tor yielded $[Ca^{2+}]_i$ values not significantly different from those obtained with indo-1.

Even after 60 min of exposure to thimerosal, $[Ca^{2+}]_i$ was considerably and significantly ($P < 0.01$) lower than observed after stimulation with bradykinin (10 nM; Figure 3). Exposure of thimerosal-treated endothelial cells to $CaCl_2$-free medium (with or without EGTA) induced a slight decrease of $[Ca^{2+}]_i$ that did not exceed 25 nM over 90 s ($n = 7$).

Discussion

Our experiments demonstrate that the release of EDRF and PGI_2 from cultured endothelial cells differ in their sensitivity to extracellular Ca^{2+}. Moreover, similar release reactions induced by different stimuli (bradykinin or thimerosal) are accompanied by completely different levels of intracellular free Ca^{2+}.

This study reveals a close relationship between $[Ca^{2+}]_i$ and PGI_2 release in endothelial cells stimulated with bradykinin. The time courses of $[Ca^{2+}]_i$ and PGI_2 release under normal Ca^{2+} conditions were similar. Likewise, in the absence of $CaCl_2$, the durations but not the initial peaks of the bradykinin responses (increases in $[Ca^{2+}]_i$ and PGI_2 release) were equally reduced. These results are consistent with the view that bradykinin initially mobilizes calcium from intracellular stores, most likely via inositol 1,4,5-trisphosphate (Derian & Moskowitz, 1986; Lambert *et al.*, 1986). The sustained intracellular calcium response to bradykinin was abolished when extracellular Ca^{2+} was removed, indicating the possible involvement of a transmembrane influx. Recently, it has been demonstrated that the sustained $[Ca^{2+}]_i$ signal, which may be attributed to a calcium influx, does not require Ca^{2+} channels of the L-type (Colden-Stanfield *et al.*, 1987).

It is conceivable that exhaustion of intracellular calcium stores accounts for the attenuation of the endothelial response to bradykinin after a prolonged exposure to EGTA-containing solution. This can be deduced from the observation that even a short exposure to Ca^{2+}-free, EGTA-containing solution reduced the level of $[Ca^{2+}]_i$ in unstimulated cells, suggesting a transmembrane loss of calcium.

TMB-8 has been described as an intracellular Ca^{2+} antagonist that may interfere with the mobilization of Ca^{2+} from internal stores (Brand & Felber, 1984; Dougherty & Niedel, 1986). TMB-8 emits a marked fluorescence at 400 nm when excited at 330 nm. Therefore, it was not used in combination with indo-1. We observed that TMB-8 also interferes with the fluorescent signal of fura-2. In particular, the background signal obtained from endothelial cells treated with ionomycin and manganese appeared considerably changed by TMB-8. Therefore, it is difficult to calibrate, in terms of $[Ca^{2+}]_i$, the signal from endothelial cells loaded with fura-2 and TMB-8. However, since fluorescence of those cells increased little in response to bradykinin (10 nM) but virtually normally in response to ionomycin, these experiments are consistent with the notion that TMB-8 inhibits the bradykinin-induced rise of $[Ca^{2+}]_i$.

TMB-8 was more effective in reducing PGI_2 than EDRF release but a small portion of PGI_2 release was not abolished by the drug. It cannot be decided whether this residual release is really independent of $[Ca^{2+}]_i$, as the release observed in $CaCl_2$-free EGTA solution might be. A small fraction of PGI_2 synthesis may be controlled by $[Ca^{2+}]_i$-independent release of arachidonic acid from phospholipids (Martin & Wysolmerski, 1987). However, most of the PGI_2 synthesis stimulated by bradykinin is quantitatively related to $[Ca^{2+}]_i$.

Bradykinin-stimulated EDRF release, on the other hand, does not exhibit this close relationship to $[Ca^{2+}]_i$, although both variables change in parallel under most experimental conditions. A discrepancy is apparent from a comparison of Figure 1b and c. Removal of extracellular Ca^{2+} by omission of $CaCl_2$ and addition of EGTA had a far more striking effect on EDRF release than on PGI_2 release. In contrast,

the intracellular calcium antagonist TMB-8 inhibited EDRF release only marginally. This result was the first experimental indication that EDRF release is more closely related to transmembrane calcium flux than to intracellular Ca^{2+} levels. Further evidence was provided by the experiments with thimerosal as the stimulus for endothelial autacoid release.

Thimerosal was a strong stimulus for EDRF and PGI_2 release. The kinetics of the release are consistent with the known cellular action of thimerosal, the inhibition of the enzyme acyl-CoA-lysolecithin-acyltransferase which reincorporates cytoplasmic free fatty acids into lysolecithin (Goppelt-Struebe *et al.*, 1986). A gradual increase of the intracellular free arachidonic acid pool may provide an explanation for the slow onset of PGI_2 release. The mechanism whereby thimerosal causes generation of EDRF is not yet known. PGI_2 release reached a maximum 15–20 min after the beginning of the stimulation and then declined, even as EDRF release continued to rise. The decline may be due to an autoinhibition of cyclo-oxygenase (Brotherton & Hoak, 1983) or prostaglandin synthetase (Egan *et al.*, 1976) by the intermediate product prostaglandin H_2 (PGH_2).

The continuous production of EDRF from thimerosal-stimulated endothelial cells made it possible to study the contribution of extracellular Ca^{2+} to the release of the autacoid when the perfusate was suddenly switched to a $CaCl_2$-free, EGTA-containing perfusate. EDRF release stopped almost immediately (yet reversibly), much more quickly than could be explained by calcium exhaustion, even if one assumes that the slightly elevated $[Ca^{2+}]_i$ was somehow involved in EDRF synthesis.

Our experiments discriminate between the effects of intra- and extracellular Ca^{2+}. This was not possible in previous studies involving endothelial cells *in situ* stimulated with acetylcholine (Long & Stone, 1985; Griffith *et al.*, 1986). In native endothelial cells intracellular calcium measurements have not been performed. Provided that acetylcholine evoked the same change of $[Ca^{2+}]_i$ as bradykinin evokes in cultured endothelial cells, one would expect an immediate decrease of $[Ca^{2+}]_i$ after removal of extracellular Ca^{2+}.

Therefore, one could argue from these *in situ* studies (Long & Stone, 1985; Griffiths *et al.*, 1986) that removal of extracellular Ca^{2+} affected primarily $[Ca^{2+}]_i$ and only secondarily EDRF release. However, our experiments show that the presence of extracellular Ca^{2+} is a prerequisite, independent of cytosolic Ca^{2+} homeostasis, for EDRF release. This is because thimerosal-induced EDRF release was accompanied by levels of $[Ca^{2+}]_i$ far lower than those observed under bradykinin stimulation, and the decrease of $[Ca^{2+}]_i$ in Ca^{2+} free medium was moderate (less than 25 nM in 90 s).

In contrast to EDRF release, PGI_2 release was stimulated by thimerosal even in the absence of extracellular Ca^{2+}. Hence, the results of our experiments contra-indicate an involvement of extracellular or elevated intracellular Ca^{2+} in PGI_2 release from thimerosal-stimulated endothelial cells.

We conclude that the release of EDRF depends critically on the presence of extracellular Ca^{2+}, via some mechanisms other than that of raising the level of $[Ca^{2+}]_i$. Further studies will test whether stimulation of EDRF release requires an increased transmembraneous Ca^{2+}-turnover or whether Ca^{2+} may affect EDRF release by physical interaction with the cell membrane.

The expert technical assistance of Cornelia Kellner, Christine Kircher, and Isabel Winter is gratefully acknowledged. The study was supported by Stiftung Volkswagenwerk (grant I/62689).

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(Received February 27, 1988
Revised April 15, 1988
Accepted April 19, 1988)