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Bradykinin-induced release of PGI_2 from aortic endothelial cell lines: responses mediated selectively by Ca^{2+} ions or a staurosporine-sensitive kinase

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1 Bradykinin (100 nM) triggers release of nitric oxide and prostacyclin from both AG07680A and AG04762 bovine cultured aortic endothelial cells. The exposure of these cells to bradykinin is in each case associated with a striking rise in intracellular calcium ion concentration.

2 Exposure of AG07680A cells to 250 nM ionomycin was followed also by a significant release of prostacyclin, whereas 250 nM ionomycin had no capacity to stimulate release of prostacyclin from AG04762 cells.

3 There was a similar concentration-dependent increase in intracellular calcium ion concentration on exposure of AG07680A and AG04762 cells to ionomycin.

4 Exposure of AG04762 cells for 10 min to staurosporine produced a concentration-dependent inhibition (IC_{50} = 107 ± 14 nM) in bradykinin-stimulated prostacyclin release. There was no similar inhibitory effect of staurosporine in AG07680A cells.

5 Bradykinin (10 nM) triggered release of nitric oxide from both AG07680A and AG04762 cells, and the effect was not inhibited by 500 nM staurosporine. There was a similar ionomycin-dependent release of nitric oxide from both cell types.

6 These results identify a common pathway for bradykinin-dependent nitric oxide release from both AG07680A and AG04762 cells, involving increases in intracellular calcium ion concentration. In contrast, the bradykinin-dependent release of prostacyclin may involve one of two pathways (involving an increase in intracellular calcium or activation of a staurosporine-sensitive kinase), and the two pathways are selectively exploited in AG07680A and AG04762 cells, respectively.

Keywords: Endothelial cells; prostacyclin; bradykinin; calcium; protein kinase C; nitric oxide; endothelium-derived relaxing factor

Introduction

A monolayer of endothelial cells lines the luminal surface of blood vessels and provides a physical barrier between the circulating blood and underlying vascular smooth muscle. Endothelial cells also serve a role in regulating vascular smooth muscle tone by release of dilator substances such as prostacyclin (epoprostenol, PGI_2; Moncada *et al.*, 1976) and nitric oxide (endothelium-derived relaxing factor, NO; Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987; Ignarro, 1991), or constrictor substances such as endothelin (Yanagisawa *et al.*, 1988). The release of PGI_2 and NO from endothelial cells may be triggered by hormones or vasoactive mediators whose receptors are coupled to phospholipase C (PLC). Examples include bradykinin, angiotensin II and acetylcholine (reviewed in Jacob *et al.*, 1990). Receptor-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by PLC yields inositol 1,4,5-trisphosphate and diacylglycerol which are implicated in release of Ca^{2+} from endoplasmic reticulum to the cytosol, and activation of protein kinase C (PKC) respectively (Berridge, 1987). The receptor-dependent release of NO involves activation of the constitutively expressed NO synthase, an effect which is mediated by increases in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ (Moncada *et al.*, 1991). In contrast, the release of PGI_2 may involve more than one pathway of transduction, with complex patterns of interaction between these pathways (reviewed in Jacob *et al.*, 1990). PGI_2 release may be triggered by Ca^{2+}-dependent activation of PLA_2 (with subsequent release of arachidonic acid) (Hallam *et al.*, 1988). Alternatively, PGI_2 release may involve activation of PKC, with little or no significant rise in $[Ca^{2+}]_i$ (Carter *et al.*, 1989). The mechanism of PKC-dependent release of PGI_2 appears to involve altered sensitivity to $[Ca^{2+}]_i$, mediated by phosphorylation of an unidentified substrate, which (it has been proposed) might be PLA_2 itself, or perhaps a G protein that couples activated cell-surface receptors to PLC (Carter *et al.*, 1989).

In primary human endothelial cultures, the release of PGI_2 appears to be mediated by a variable contribution from both signalling pathways, although the consensus view suggests a primary role for receptor-dependent elevations in $[Ca^{2+}]_i$ (Hallam *et al.*, 1988). However, we have now identified two related bovine aortic endothelial lines, which are available from the Institute of Aging Cell Repository (U.S.A.), in which the two pathways involved in receptor-dependent release of PGI_2 segregate between the two cell lines. These cell lines provide a unique resource for further examination of the complex signalling pathways involved in release of PGI_2 from endothelium.

Methods

Cells

Bovine aortic endothelial cells were obtained from the National Institute of Aging Cell Repository (Institute for Medical Research, Copewood and Davis Streets, Camden, NJ 08103, U.S.A.). Two cell lines were obtained, namely

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AG04762 (previously listed as AG4762) and AG07680A (previously listed as AG7680). These cells were obtained originally by collagenase digestion of a bovine thoracic aortic segment (Holstein breed), and they are known to express factor VIII immunoreactivity and angiotensin converting enzyme. AG04762 is reported to be a later passage of the AG07680A cell line.

The cells were cultured in 75 cm^2 flasks or 3.5 cm (diameter) wells in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 15% (v/v) foetal calf serum (Gibco), 1.5 mM glutamine, 60 u ml^{-1} penicillin, 50 $\mu g\ ml^{-1}$ streptomycin and 25 $\mu g\ ml^{-1}$ gentamicin. The culture medium in each flask was changed twice a week.

Confluent cells were divided in a ratio 1:4. The medium was aspirated and the cells washed once with Dulbecco's phosphate-buffered saline (without Ca^{2+} or Mg^{2+} ions, Gibco). Thereafter the cells were exposed to 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in PBS (Flow Laboratories) for 1–2 min at 37°C. The detached cells were pelleted by centrifugation at 150 g for 3 min, resuspended in culture medium and plated into new flasks or dishes, or on to cover slips for fluorescence measurements.

For some experiments, cells were sub-cultured on to microcarrier beads which were then loaded on to 2 ml columns (described fully in Parsaee *et al.*, 1992). This arrangement allowed perfusion of the cells on the column with Krebs-Henseleit buffer (gassed with 5% CO_2 and 95% air) at 2 ml min^{-1}. The eluate from these columns was then analysed for its content of PGI_2 and NO. Although it was not possible to measure exactly the cell number on each column, similar numbers of confluent beads were loaded on to all columns, and the cell number approximated 2×10^7. The release of PGI_2 was also measured from endothelial cells cultured in 3.5 cm plates as described previously (Carter *et al.*, 1988; Parsaee *et al.*, 1992).

Bioassay for NO

NO was measured by a modification (described fully in Parsaee *et al.*, 1992) of the method of Furchgott & Zawadzki (1980). Briefly, eluate from the columns was allowed to superfuse a rat aortic ring, preconstricted with 1 μM phenylephrine. Measurements were then made of the relaxation of the ring. In pilot experiments, endothelial cell-dependent relaxation of the aortic ring was shown to be inhibited by prior perfusion of the column with N^{ω}-monomethyl-L-arginine (L-NMMA), or simultaneous superfusion of the aortic ring with haemoglobin. These findings confirmed the identity of NO in the column eluate.

Prostacyclin determination

Prostacyclin was measured as its stable hydrolysis product 6-oxo-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) in 50 μl fractions taken from the column eluate. The analysis was by radioimmunoassay, and the antibody was a generous gift from Dr Susan Barrow (UMDS, University of London).

Measurement of free intracellular calcium ion concentration $[Ca^{2+}]_i$

The method employed was a modification (Parsaee *et al.*, 1992) of that described by Hallam *et al.* (1988). Briefly, fluorescence intensity was measured in Fura 2-loaded endothelial cells cultured on 10 mm glass cover slips (Chance Proper). Recordings were made at 37°C in a Shimadzu RF5000 spectrophotofluorimeter, with excitation at 340 nm and 380 nm, and emission measured at 500 nm. The value of $[Ca^{2+}]_i$ was calculated from the ratio of the two fluorescence signals according to the equation of Grynkiewicz *et al.* (1985).

The results were analysed with GraphPAD InPlot computer software, and curves were fitted to a simple one-site model, assuming Michaelis-Menton kinetics. [^{3}H]-6-oxo-$PGF_{1\alpha}$ was obtained from Amersham International (UK); Fura 2-AM was obtained from Calbiochem (UK). 6-Oxo-prostaglandin $F_{1\alpha}$ (free acid), ionomycin (Ca^{2+} salt), bradykinin (triacetate salt), phenylephrine (free base) and staurosporine were obtained from Sigma (UK). General reagents were also obtained from Sigma (UK) except where indicated.

Results

The release of 6-oxo-$PGF_{1\alpha}$ from AG07680A cells was measured. Figure 1a shows that exposure of these cells to a saturating (100 nM) concentration of bradykinin was followed by a rapid release of 6-oxo-$PGF_{1\alpha}$ which was sustained for about 5–10 min. In similar experiments, AG04762 cells were also shown to release 6-oxo-$PGF_{1\alpha}$ following exposure to 100 nM bradykinin (Figure 1b). Figure 1a and b is representative of three similar experiments. Basal release from AG07680A and AG04762 cells was 6.0 ± 3.6 and 10.4 ± 3.0 pg 6-oxo-$PGF_{1\alpha}$ 50 μl^{-1}, and stimulated release reached a maximum of 1698 ± 87 and 523 ± 112 pg 6-oxo-$PGF_{1\alpha}$ 50 μl^{-1} respectively ($n = 3$).

In contrast to the effect of bradykinin on the two endothelial cell lines, there was a striking difference in their sensitivity to ionomycin. In separate experiments ($n = 3$ for each cell line) AG07680A or AG04762 cells were exposed to 250 nM ionomycin, and measurements made of the release of

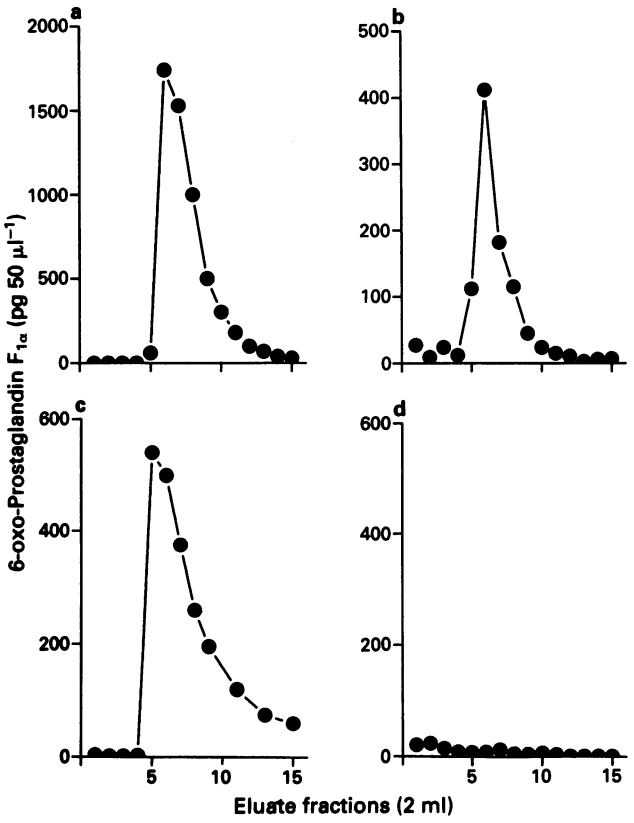

Figure 1 Release of prostacyclin (PGI_2) (measured as 6-oxo-$PGF_{1\alpha}$) from cultured endothelial cells. AG07680A cells (a and c) or AG04762 cells (b and d) were subcultured on to microcarrier beads and perfused on columns with Krebs-Henseleit buffer. Prior to any measurements or stimulation of the cells, the system was allowed to settle at a perfusion rate of 2 ml min^{-1} for at least 30 min.The cells were then exposed to 100 nM bradykinin (a and b) or 250 nM ionomycin (c and d) in the perfusate during the interval of collection of fractions 2–4 (3 min). Eluate factions (2 ml) from the columns were collected and analysed for their content of 6-oxo-$PGF_{1\alpha}$/50 μl. The results are representative of 3 similar experiments.

6-oxo-$PGF_{1\alpha}$. Figure 1c and d shows representative examples of the responsiveness of AG07680A cells to ionomycin, with absolutely no response seen in AG04762 cells.

These results prompted further experiments in which the capacity of bradykinin or ionomycin to trigger rises in $[Ca^{2+}]_i$ was measured. The resting $[Ca^{2+}]_i$ levels in AG07680A and AG04762 cells were 141 ± 12 nM ($n = 11$) and 172 ± 26 nM ($n = 6$), and increased on exposure to 500 nM bradykinin to 517 ± 56 nM ($n = 9$) and 548 ± 41 nM ($n = 6$) respectively. The response to bradykinin is concentration-dependent in AG07680A cells, and the bradykinin concentration (EC_{50}) required for the half-maximum increase in $[Ca^{2+}]_i$ has been reported previously by us as 4.8 nM (Parsaee *et al.*, 1992). A similar result was obtained with AG04762 (data not presented).

In later experiments, cells of both endothelial cell lines were exposed to ionomycin at concentrations between 10 nM and 500 nM, and the results are shown in Figure 2. There was no difference between the two cell lines in the rise in $[Ca^{2+}]_i$ with increasing ionomycin concentrations, notwithstanding their striking difference in sensitivity to ionomycin in terms of 6-oxo-$PGF_{1\alpha}$ release.

These findings prompted us to compare the effect of staurosporine on bradykinin-dependent release of 6-oxo-$PGF_{1\alpha}$ in the two cell lines. Exposure of AG04762 cells for 10 min to 500 nM staurosporine attenuated by about 55% the increase in 6-oxo-$PGF_{1\alpha}$ triggered by 100 nM bradykinin (Figure 3b). In contrast, the same concentration of staurosporine had no capacity to reduce bradykinin-dependent release of 6-oxo-$PGF_{1\alpha}$ from AG07680A cells (Figure 3a). Exposure of AG04762 cells to selected concentrations of staurosporine revealed a concentration-dependent inhibition of 6-oxo-$PGF_{1\alpha}$ release triggered by bradykinin, and the IC_{50} value for this effect was 107 ± 14 nM (Figure 4). Analysis of the data in Figure 4 revealed that the bradykinin-dependent effect could only be inhibited by staurosporine under these experimental conditions by 65.2%.

Finally, measurements were made of the capacity of bradykinin or ionomycin to release NO. In these experiments no differences were observed between NO release from AG07680 or AG04762 cells. The bradykinin-dependent release of NO is shown in Figure 5, and the effect was unaltered by the prior exposure of these cells to 500 nM staurosporine. Exposure of AG07680A cells to selected concentrations of bradykinin revealed a concentration-dependent response, with an EC_{50} value reported previously by us (Parsaee *et al.*, 1992) of 0.70 ± 0.14 nM ($n = 3$). Similar results were obtained with AG04762 cells (data not presented). Ionomycin has been reported previously by us to trigger release of NO from AG07680A cells (Parsaee *et al.*, 1992), and once again similar results were obtained with AG04762 cells (data not presented).

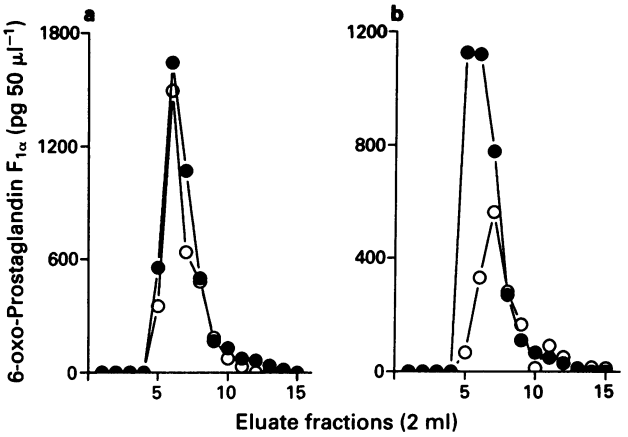

Figure 3 Bradykinin-dependent release of prostacyclin (measured as 6-oxo-$PGF_{1\alpha}$) from endothelial cells. AG07680A cells (a) or AG04762 cells (b) were perfused on columns as described in the legend to Figure 1. Prior to exposure of the cells to 100 nM bradykinin (during collection of fractions 2–4), the column was perfused for 10 min with Krebs-Henseleit buffer containing 500 nM staurosporine (○), or Krebs-Henseleit buffer alone (●). Individual 2 ml fractions from the columns were analysed for their content of 6-oxo-$PGF_{1\alpha}$. The results are typical of 3 similar experiments.

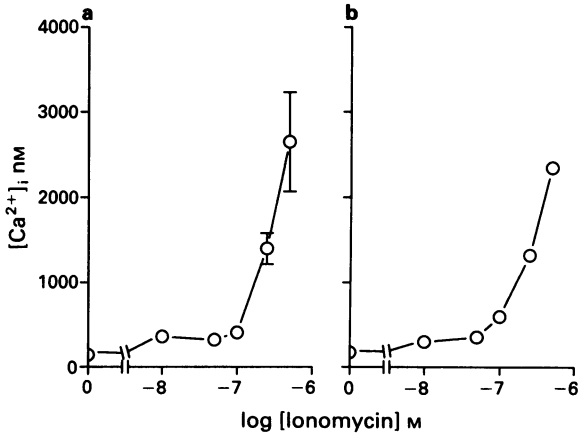

Figure 2 Concentration-response relationships of ionomycin-dependent increases in $[Ca^{2+}]_i$ in cultured endothelial cells. AG07680A cells (a) or AG04762 cells (b) were subcultured on to glass cover slips and loaded with Fura-2. The cells were then exposed to selected concentrations of ionomycin, and fluorescence intensity measured in a spectrophotofluorimeter. The results show mean values for increases in $[Ca^{2+}]_i$ ($n = 3$–6, ± s.e.mean. When omitted, the error bars are incorporated into the symbol).

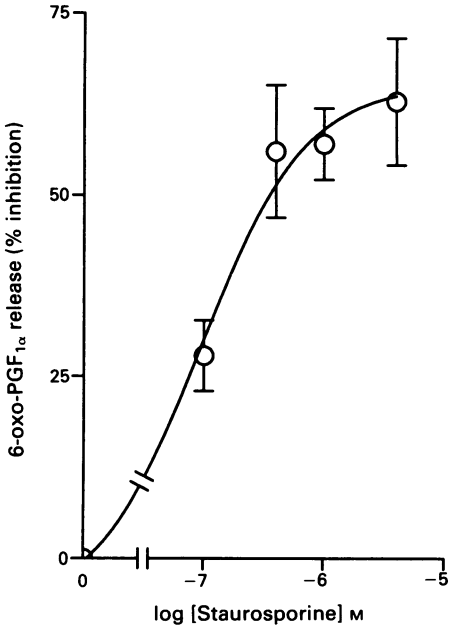

Figure 4 Concentration-response relationship for staurosporine-dependent inhibition of 6-oxo-$PGF_{1\alpha}$ release from AG04762 cells following exposure to bradykinin. Cells were cultured in 3.5 cm (diam.) dishes, and then exposed to selected concentrations of staurosporine (or culture medium control) for 10 min. Thereafter, the medium was changed, the cells washed, and the cells then exposed to 100 nM bradykinin for 5 min. The culture medium was then analysed for its content of 6-oxo-$PGF_{1\alpha}$, and the results are expressed as the mean staurosporine-dependent inhibition of the bradykinin-dependent increase in 6-oxo-$PGF_{1\alpha}$ release ($n = 4$; values given ± s.e.mean).

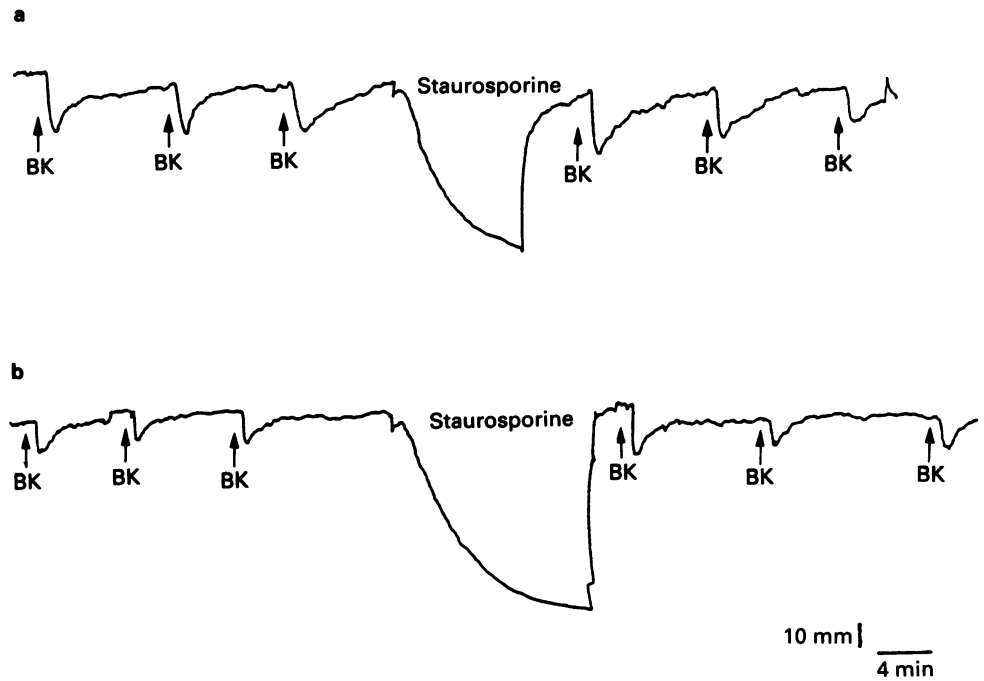

Figure 5 The release of nitric oxide from cultured endothelial cells. AG04762 cells (a) or AG07680A cells (b) were perfused on columns as described in the legend to Figure 1. The column eluates were superfused on to rat aortic rings, which had been pre-constricted with 1 μM phenylephrine. The results show the relaxation mediated by repeated exposure to 10 nM bradykinin for 45 s. At a point shown on the curve, the eluate from the column was temporarily diverted, and the ring allowed to relax. The endothelial cells were then exposed to 500 nM staurosporine for 10 min. The ring then contracted again with 1 μM phenylephrine, and the eluate from the column used to superfuse the ring. The results show the nitric oxide-dependent relaxations of the rings mediated by subsequent exposure of the endothelial cells to 10 nM bradykinin. The relaxations produced by 10 nM bradykinin were 19.2 ± 1.7 before, and 18.3 ± 3.0 mm ($n = 3$) after exposure of AG04762 cells to staurosporine. The bradykinin-dependent relaxations were 12.8 ± 0.6 before, and 13.7 ± 2.0 mm ($n = 3$) after exposure of AG07680A cells to staurosporine (results are means ± s.e.mean).

Discussion

Results published previously have suggested that the release of PGI_2 from endothelial cells may be triggered either by increases in $[Ca^{2+}]_i$ (Hallam *et al.*, 1988), or by a complex pathway involving reduced sensitivity to $[Ca^{2+}]_i$ mediated by activation of PKC (Carter *et al.*, 1989). Either (or both) pathways might be activated by receptor-dependent activation of PLC (reviewed in Jacob *et al.*, 1990), and results are now presented which show that two closely related endothelial cell lines exploit selectively a rise in $[Ca^{2+}]_i$ or activation of a staurosporine-sensitive kinase in bradykinin-dependent release of PGI_2. In contrast, we confirm earlier reports that the release of NO from endothelial cells appears to be coupled in all cases to rises in $[Ca^{2+}]_i$, most probably by direct activation of NO synthase (Moncada *et al.*, 1991). Intriguingly, the threshold of $[Ca^{2+}]_i$ required for activation of NO synthase is substantially lower than that involved in activation of PLA_2 and release of PGI_2 (Parsaee *et al.*, 1992).

Bradykinin triggers a rapid and significant rise in $[Ca^{2+}]_i$ in both AG07680A and AG04762 cells, and these changes in $[Ca^{2+}]_i$ are accompanied by a very striking increase in the release PGI_2. Ionomycin also increased $[Ca^{2+}]_i$, and to very high levels when the cells were exposed to ionomycin at concentrations above 100nM. However, despite $[Ca^{2+}]_i$ at concentrations above 2 μM in both cell types, ionomycin triggered release of PGI_2 from only AG07680A cells and not from AG04762 cells.

It followed that bradykinin-dependent release of 6-oxo-$PGF_{1\alpha}$ from AG04762 cells was not simply dependent on increases in $[Ca^{2+}]_i$. Further experiments revealed that the bradykinin-dependent release of 6-oxo-$PGF_{1\alpha}$ from these cells was inhibited by staurosporine, which suggests the involvement of a staurosporine-sensitive kinase. In view of the previous work on endothelial cell signalling, this kinase is identified most probably as one of the many PKC isoforms. The finding that staurosporine inhibited bradykinin-dependent release of PGI_2 only partially (about 60%) also suggests the possibility that other signalling pathways may be involved (perhaps in a cascade of protein phosphorylation). Further extensive studies will be required to identify with confidence the individual kinase(s), and these are not included in the present report. There are now upwards of 11 PKC isoenzymes that have been identified or sequenced, some of which are insensitive to phorbol ester, and for many of which no specific inhibitors are currently available. The published evidence to date suggests, at least in the rat, that the most abundant PKC isoenzyme in endothelial cells is PKC_α (Mattila, 1991).

Details of the pathways implicated in receptor-dependent release of PGI_2 have proved very difficult to examine experimentally in endothelial cells, most particularly because of the variable involvement of $[Ca^{2+}]_i$. Exploitation of these two cell lines in further studies may permit more precise assignment of the biochemical pathways involved.

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References

BERRIDGE, M.J. (1987). Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.*, **56**, 159–193.

CARTER, T.D., HALLAM, T.J., CUSACK, N.J. & PEARSON, J.D. (1988). Regulation of P_{2y} purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br. J. Pharmacol.*, **95**, 1181–1190.

CARTER, T.D., HALLAM, T.J. & PEARSON, J.D. (1989). Protein kinase C activation alters the sensitivity of agonist-stimulated endothelial-cell prostacyclin production to intracellular Ca^{2+}. *Biochem. J.*, **262**, 431–437.

FURCHGOTT, R.F. & ZADAWZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HALLAM, T.J., PEARSON, J.D. & NEEDHAM, L.A. (1988). Thrombin-stimulated elevation of human endothelial-cell cytoplasmic free calcium concentration causes prostacyclin production. *Biochem. J.*, **251**, 243–249.

IGNARRO, L.J. (1991). Signal transduction mechanisms involving nitric oxide. *Biochem. Pharmacol.*, **41**, 485–490.

JACOB, R., SAGE, S.O. & RINK, T.J. (1990). Aspects of calcium signalling in endothelium. In *Endothelium: an Introduction to Current Research*. ed. Warren, J.B. pp. 33–44. New York: Wiley-Liss.

MATTILA, P. (1991). Protein kinase C subtypes in endothelial cells. *FEBS Lett.*, **289**, 86–90.

MONCADA, S., GRYGLEWSKI, R.J., BUNTING, S. & VANE, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxide to an unstable substance that inhibits platelet aggregation. *Nature*, **263**, 663–665.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PARSAEE, H., MCEWAN, J.R. & MACDERMOT, J. (1992). Differential sensitivities of the prostacyclin and nitric oxide biosynthetic pathways to cytosolic calcium in bovine aortic endothelial cells. *Br. J. Pharmacol.*, **107**, 1013–1019.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

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