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Inhibition of inositol 1,4,5-trisphosphate formation by cyclic GMP in cultured aortic endothelial cells of the pig

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1 In cultured endothelial cells of the pig the endothelium-derived relaxing factor (EDRF) releasing agent thrombin ($2\,u\,ml^{-1}$) caused a significant increase in basal levels of both guanosine 3':5'-cyclic monophosphate (cyclic GMP) and inositol 1,4,5-trisphosphate (IP_3). This increase was time dependent, with peak levels occurring at 2 min and returning towards basal values after 5 min.

2 Pretreatment of the cells with the EDRF inhibitors haemoglobin (1 μM) or L-N^G-nitro arginine (50 μM) significantly reduced the cyclic GMP response to thrombin. Both agents also resulted in significant elevations in basal levels of IP_3. The IP_3 response to thrombin was significantly enhanced at all time points by haemoglobin and at 5 min for L-N^G-nitro arginine, when compared with the response to thrombin alone.

3 Pretreatment of the cells with either sodium nitroprusside (10 μM) or atrial natriuretic peptide (1 μM) caused a significant elevation of basal cyclic GMP levels. Although subsequent exposure to thrombin caused a further increase in cyclic GMP, which together with the rise induced by the previous two agents was significantly greater than the increase caused by thrombin alone, the incremental increase induced by thrombin was markedly less in the presence of nitroprusside or atrial natriuretic peptide. Both these agents, as well as 8-bromo cyclic GMP, resulted in a significant suppression of the IP_3 response to thrombin.

4 These findings show that one mechanism for the inhibitory effect of cyclic GMP on EDRF release from endothelium may be through the inhibition of IP_3 formation in response to EDRF releasing agents.

Introduction

Inositol 1,4,5-trisphosphate (IP_3) is the second messenger which mobilizes intracellular stores of calcium in a number of cell types (Berridge & Irvine, 1984; Hashimoto *et al.*, 1986; Berridge, 1987). It is produced when phosphatidyl-inositol 4,5-bisphosphate (PIP_2) is hydrolysed by phospholipase C in response to cell-surface receptor activation (Berridge & Irvine, 1984; Berridge, 1984; Hokin, 1985; Downes & Michell, 1985). The other product of this hydrolysis is sn 1,2-diacylglycerol which activates protein kinase C (Nishizuka, 1984). In many cell types a guanyl nucleotide transducing-protein (G protein) couples the receptor to phospholipase C and in some cases this step is sensitive to inhibition by pertussis toxin (Berridge, 1987).

A rapid formation of IP_3 in endothelial cells occurs in response to several agonists including thrombin (Moscat *et al.*, 1987; Pollock *et al.*, 1988), bradykinin (Derian & Moskowitz, 1986; Lambert *et al.*, 1986), ADP and ATP (Forsberg *et al.*, 1987; Pirotton *et al.*, 1987) and mellitin, a direct activator of phospholipase C (Loeb *et al.*, 1988). These agonists also stimulate the release of endothelium-derived relaxing factor (EDRF) (for review see Angus & Cocks, 1989), the critical signal for EDRF release being elevation of intracellular Ca^{2+} levels (for review see Newby & Henderson, 1990).

EDRF, recently discovered to be nitric oxide (Palmer *et al.*, 1987) acts like the nitrovasodilator drugs through stimulation of soluble guanylate cyclase and the elevation of intracellular levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Katsuki *et al.*, 1977; Ignarro *et al.*, 1981; Rapoport *et al.*, 1983a,b; Griffith *et al.*, 1985; Forstermann *et al.*, 1986). Atrial natriuretic peptide (ANP) similarly causes intracellular cyclic GMP levels to be elevated in vascular smooth muscle (Winquist *et al.*, 1989), doing so by specific activation of particulate guanylate cyclase (Waldman *et al.*, 1984).

The stimulated rise in cyclic GMP produced by EDRF and other cyclic GMP-elevating agents which results in vascular smooth muscle relaxation is associated with both the inhibition of influx of extracellular calcium as well as a decrease in the release of intracellular calcium (Karaki *et al.*, 1984; Collins *et al.*, 1986). The cause of the inhibition of contraction and of intracellular Ca^{2+} release, was thought to result from the inhibitory effect of cyclic GMP on phosphatidylinositol hydrolysis (Rapoport, 1986) in rat aorta, and inhibition of IP_3 formation by cyclic GMP has now been shown also in rabbit aorta (Lang & Lewis, 1989).

Stimulation of EDRF release in cultured endothelium results in elevation of cyclic GMP levels in the endothelium itself (Martin *et al.*, 1988; Smith & Lang, 1990). Furthermore, elevation of endothelial cell levels of cyclic GMP with either 8-bromo cyclic GMP (Evans *et al.*, 1988) or ANP (Hogan *et al.*, 1989) inhibits EDRF release. The mechanism of this effect remains unknown however, but might be through inhibition of the rise in IP_3 levels in endothelium induced by EDRF releasing agents. To investigate this possibility in the present study, we have examined the effects of alteration of endothelial cell levels of cyclic GMP on IP_3 formation in cultured cells stimulated with thrombin.

Methods

Preparation of cultured endothelial cells

Aortae, from approximately 16 week old pigs, were removed immediately after slaughter at the local abattoir, and flushed with 0.9% (w/v) sterile NaCl containing benzylpenicillin $200\,u\,ml^{-1}$ with streptomycin $200\,\mu g\,ml^{-1}$. The proximal end of the vessel was tied off and the distal end cannulated with a 50 ml syringe containing the same saline. The lumen of the vessel was then filled with the saline for transportation back to the laboratory.

Endothelial cells were isolated essentially as described by Gordon & Martin (1983). Briefly, the intercostal arteries were ligated, the lumen emptied of the saline and filled with 0.2% collagenase (type II, Sigma) in Medium E199 and incubated at 37°C for 20 min. The cells were then harvested into 40 ml of Medium E199 supplemented with 10% foetal calf serum, 10%

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newborn serum, glutamine 6 mM, benzylpenicillin 200 u ml^{-1}, streptomycin 200 $\mu g\,ml^{-1}$ and kanamycin 100 $\mu g\,ml^{-1}$. The cells were subsequently seeded into three six-well plates (well area = 9.62 cm^2) The culture medium was replaced the next day and then every other day until the cells became confluent, usually within 5–7 days.

Experimental protocol

The culture medium was removed and the cells washed with 2 × 2 ml of Krebs-Ringer bicarbonate (KRB) solution of the following composition (mM): NaCl 95.5, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. The cells were then incubated in 2 ml of KRB containing lithium chloride 10 mM at 37°C under an atmosphere of 5% CO_2 in air for at least 90 min. Drugs were added at the concentrations and times indicated in the Results.

At the appropriate time the KRB was rapidly removed and the reaction terminated by the addition of 0.5 ml of ice cold 5% (v/v) perchloric acid (PCA). The cells were scraped from the well and together with a further 0.5 ml of PCA were placed in plastic tubes. This combined 1 ml volume of PCA was then centrifuged at 13000*g* for 2 min. The resulting supernatant was aspirated into separate plastic tubes, previously cooled on dry ice and, along with the cell debris pellet stored at −20°C until assay within 1 month. Supernatants were frozen immediately in this way to prevent breakdown of the inositol phosphates.

Measurement of IP_3 cyclic GMP and DNA

The supernatants were thawed and 400 μl of each transferred to separate tubes containing 100 μl of 10 mM EDTA (pH 7.0). Samples were then neutralized by adding 300 μl of 1:1 (v/v) mixture of 1,1,2-trichloro-trifluoroethane and tri-n-octylamine followed by vigorous vortexing for 90 s. The IP_3 and cyclic GMP content of the aqueous upper layer was measured with commercially available kits (Amersham International, U.K. and New England Nuclear Research Products, F.R.G., respectively).

The DNA content of the pellet was measured by the fluorimetric method of Kissane & Robins (1958). The IP_3 content of each well was expressed as pmol μg^{-1} DNA and the cyclic GMP as fmol μg^{-1} DNA.

Drugs

Atrial natriuretic peptide (human sequence), sodium nitroprusside, thrombin (human), L-N^G-nitro arginine and haemoglobin (bovine) were obtained from Sigma Chemical Company, UK. All were dissolved in distilled water immediately prior to use except in the case of L-N^G-nitro arginine where the water was acidified with HCl.

Haemoglobin solutions were reduced to the ferrous form with dithionite as described by Martin *et al.* (1986). Sera, culture medium, glutamine and kanamycin were obtained from Flow Laboratories, U.K., benzyl penicillin (crystopen) from Glaxo, U.K., and streptomycin sulphate from Evans Medicals Limited, U.K.

Statistics

Both IP_3 and cyclic GMP values are expressed as the means ± standard error of the mean (s.e.mean). For analysis of within-group data, a one-way analysis of variance was used followed by Dunnett's multiple range test to identify significant differences at the 0.05 level. For between-group data Tukey's test was used; comparisons were considered significantly different when $P < 0.05$.

Results

Thrombin

Figure 1 shows the levels of IP_3 and cyclic GMP following incubation of the cultured endothelial cells with thrombin

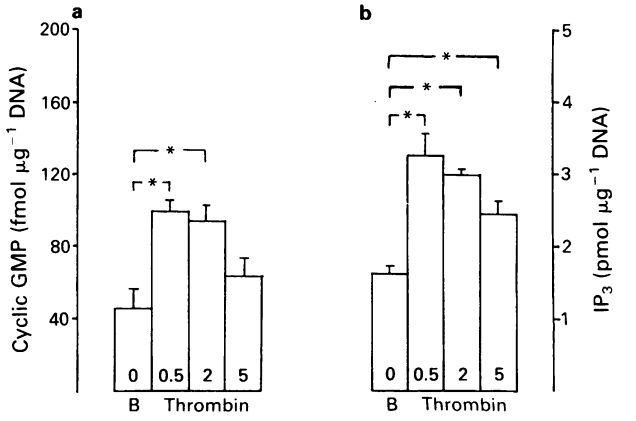

Figure 1 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of guanosine 3′: 5′-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and following stimulation with thrombin (2 u ml^{-1}) for 0.5, 2 and 5 min. (* $P < 0.05$ cf basal values; $n \geqslant 5$).

(2 u ml^{-1}) for 30 s, 2 min and 5 min. The data show a significant rise in IP_3 levels after 30 s declining thereafter towards basal values but remaining significantly elevated at 5 min. The pattern of change in cyclic GMP levels is similar to that of IP_3 with a significant increase at 30 s remaining elevated at 2 min and declining back to basal levels at 5 min.

Haemoglobin

Figure 2 shows the results in thrombin-stimulated cells but following preincubation of the cells for 15 min with haemoglobin (1 μM). The data again shows a significant rise in IP_3 levels at each time point following thrombin stimulation when compared with basal levels in the absence of haemoglobin ($P < 0.05$ at all time points). The rise in IP_3 observed at 30 s, 2 min and 5 min after thrombin addition was significantly greater than that observed in the absence of haemoglobin ($P < 0.05$ at all time points). Haemoglobin alone also caused a significant increase in IP_3 compared with basal levels ($P < 0.05$). There were no significant changes in cyclic GMP levels either in the presence of haemoglobin alone or after the addition of thrombin.

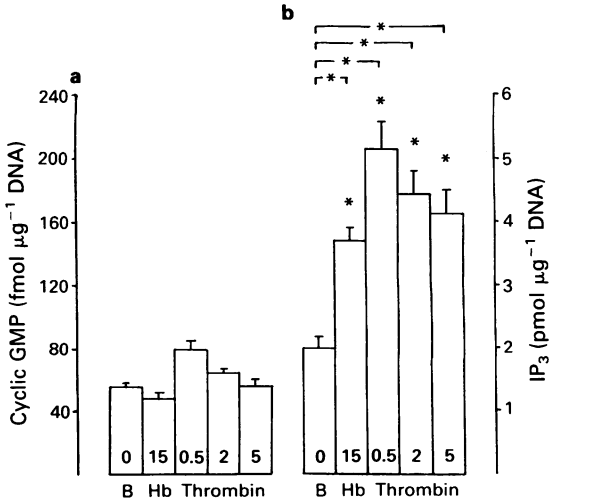

Figure 2 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bar) of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and after incubation with haemoglobin (Hb; 1 μM) for 15 min followed by stimulation with thrombin as for Figure 1. (*(in brackets) $P < 0.05$ cf. basal values; * $P < 0.05$ cf. values for thrombin in the absence of haemoglobin; $n \geqslant 5$).

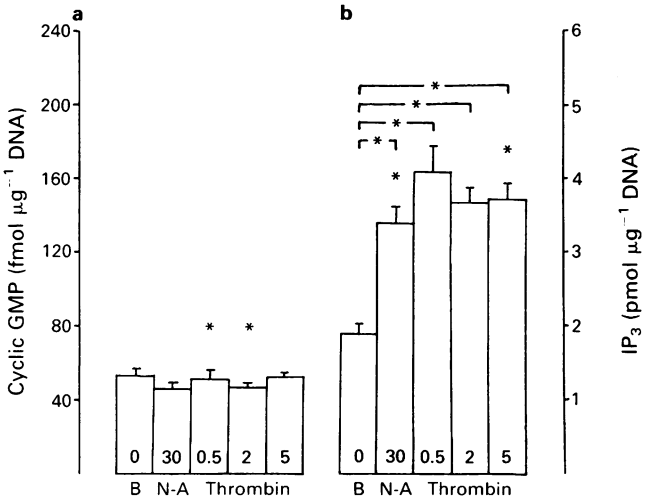

Figure 3 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and after incubation with L-N^G-nitro arginine (N-A; 50 μM) for 30 min followed by incubation with thrombin as for Figure 1. (*(in brackets) $P < 0.05$ cf. basal values; * $P < 0.05$ cf. values for thrombin in the absence of L-N^G-nitro arginine; $n \geqslant 5$).

L-N^G-nitro arginine

Figure 3 shows the changes in IP_3 and cyclic GMP levels following pre-incubation of the cells for 30 min with L-N^G-nitro arginine (50 μM). Like haemoglobin, this inhibitor of EDRF production resulted in a significant increase in IP_3 levels, compared with basal values in the absence of thrombin ($P < 0.05$). In the presence of thrombin, IP_3 increased significantly at each time point ($P < 0.05$) and remained significantly higher at 5 min when compared with the cells stimulated with thrombin alone ($P < 0.05$). Cyclic GMP levels did not alter following thrombin stimulation in the presence of this agent.

Sodium nitroprusside

Figure 4 shows the effect of pre-incubation of the cells for 30 s with sodium nitroprusside (10 μM). The data show no significant rise in IP_3 levels above basal levels at any of the time points studied, following thrombin stimulation. However, incubation with nitroprusside alone caused a significant increase in cyclic GMP over basal levels ($P < 0.05$), which

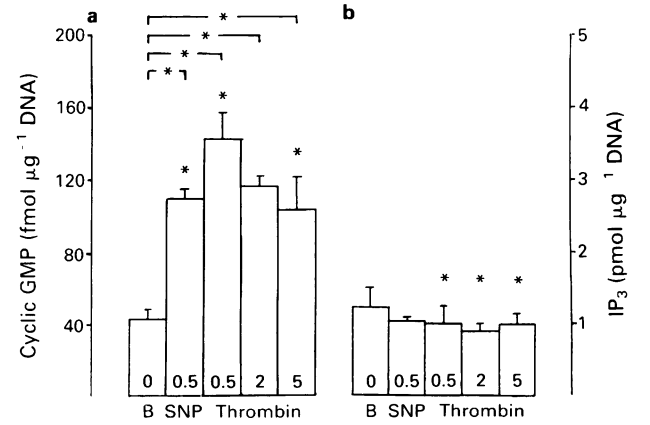

Figure 4 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and after incubation with sodium nitroprusside (SNP; 10 μM) and for 0.5 min followed by incubation with thrombin as for Figure 1. (*(in brackets) $P < 0.05$ cf. basal values; * $P < 0.05$ cf. values for thrombin in the absence of sodium nitroprusside; $n \geqslant 5$).

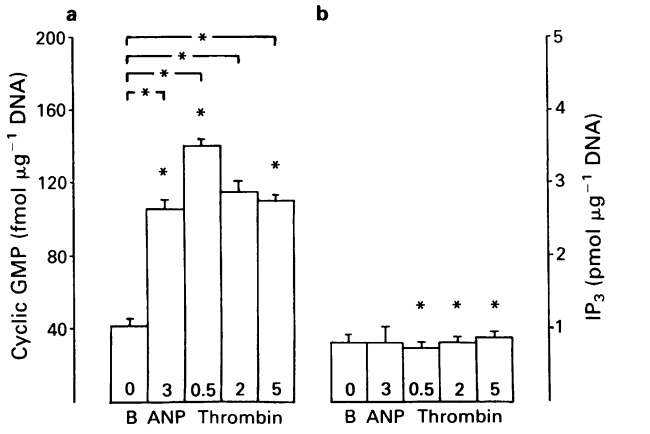

Figure 5 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (a) and inositol 1,4,5-trisphosphate (IP_3) (b) in cultured aortic endothelial cells of the pig and after incubation with atrial natriuretic peptide (ANP; 1 μM) for 3 min followed by incubation with thrombin as for Figure 1. (*(in brackets) $P < 0.05$ cf basal values; * $P < 0.05$ cf. values for thrombin in the absence of atrial natriuretic peptide; $n \geqslant 5$).

were significantly enhanced by subsequent incubation with thrombin when compared with basal values ($P < 0.05$ at all time points) or when compared to levels obtained following incubation with thrombin alone ($P < 0.05$ at 30 s and 5 min).

Atrial natriuretic peptide

Figure 5 again shows a thrombin time course but following preincubation of the cells for 3 min with ANP (1 μM). As for nitroprusside, the presence of ANP resulted in complete inhibition of the IP_3 response to thrombin. ANP alone significantly elevated cyclic GMP levels above basal values ($P < 0.05$) and significantly enhanced the thrombin-stimulated rise when compared to basal ($P < 0.05$ at all time points) or when compared with values in the absence of ANP ($P < 0.05$ at 30 s and 5 min).

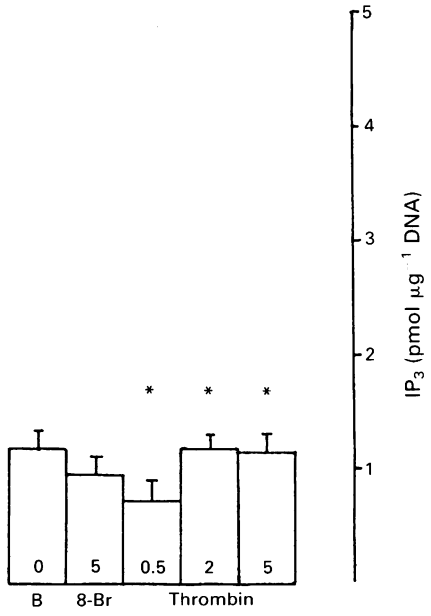

Figure 6 Histogram showing basal (B) concentrations (mean with s.e.mean shown by vertical bars) of inositol 1,4,5-trisphosphate (IP_3) in cultured aortic endothelial cells of the pig and after incubation with 8-bromo cyclic GMP (8-Br; 100 μM) for 5 min followed by incubation with thrombin as for Figure 1. (* $P < 0.05$ cf. values for thrombin in the absence of 8-bromo cyclic GMP; $n \geqslant 5$).

8-bromo cyclic GMP

In the experiments with this lipid-soluble analogue of cyclic GMP, IP_3 levels only were measured since this agent directly elevates cyclic GMP levels in cells. As shown in Figure 6 incubation with 100 μM for 5 min produced complete inhibition of IP_3 formation in response to thrombin.

Discussion

The data show that there is a rapid rise in both IP_3 and cyclic GMP levels in cultured endothelial cells of the pig following exposure to the EDRF-releasing agent thrombin. When the cells were preincubated with the two cyclic GMP elevating agents sodium nitroprusside and ANP, not only were basal cyclic GMP levels increased but, in the presence of thrombin the level remaining elevated for longer. Furthermore, in the presence of elevated basal cyclic GMP levels, the subsequent response of the cells to thrombin was blunted i.e. the incremental increase in cyclic GMP was markedly reduced. Under these conditions, the IP_3 response to thrombin was completely inhibited. Conversely when the cells were preincubated with the EDRF inhibitor haemoglobin (Martin *et al.*, 1985) or the inhibitor of EDRF formation L-N^G-nitro arginine (Moore *et al.*, 1990), the increase in cyclic GMP following thrombin, was significantly less when compared to thrombin alone, and the IP_3 response was significantly enhanced.

These findings therefore confirm earlier work which showed that elevated levels of cyclic GMP inhibit phosphatidylinositol turnover in platelets (Takai *et al.*, 1981) and also in vascular smooth muscle (Rapoport, 1986). They also confirm our own studies showing an inhibitory effect of cyclic GMP on stimulated IP_3 levels in vascular smooth muscle (Lang & Lewis, 1989). The observation that the thrombin-induced incremental increase in cyclic GMP in the presence of nitroprusside or atrial natriuretic factor was reduced, also suggests that EDRF release from the cells was inhibited by these agents. A finding which again confirms our earlier studies that elevation of endothelial cell levels of cyclic GMP inhibits EDRF release (Evans *et al.*, 1988; Hogan *et al.*, 1989).

The mechanism responsible for the cyclic GMP-induced inhibition in endothelial cells is unknown at present. It is possible that, as suggested for the action of cyclic GMP in vascular smooth muscle and platelets (Takai *et al.*, 1981; Rapoport, 1986; Lang & Lewis, 1989), there is inhibition of the transduction mechanisms between the cell surface receptors and IP_3 formation. This is likely to be at the level of either a G protein or possibly phospholipase C. Evidence in favour of these sites of action of cyclic GMP in vascular smooth muscle has recently been provided by Hirata and colleagues (1990). These workers showed that the inhibitory effect of cyclic GMP on phosphoinositide hydrolysis and GTPase activity in homogenates and membrane preparations of cultured bovine aortic smooth muscle cells, resulted from an inhibition of guanine nucleotide regulatory protein activation and the interaction between guanine nucleotide regulatory protein and phospholipase C. Thrombin-induced EDRF release is thought to involve a G protein in its transduction mechanism since pertussis toxin blocks EDRF release by this agent (Flavahan *et al.*, 1989).

Although we have previously shown an inhibitory effect of cyclic GMP on acetylcholine- and substance P-induced EDRF release from rabbit blood vessels (Evans *et al.*, 1988; Hogan *et al.*, 1989), it is not known whether cyclic GMP will inhibit EDRF release, and possibly IP_3 formation, in endothelial cells from all species, with all agonists. If the mechanism of action of cyclic GMP is by inhibition of the transduction mechanisms between receptor and IP_3 information, it is possible that only those agonists coupled to a specific G protein would be inhibited by cyclic GMP. We have previously shown that cyclic GMP has no effect on EDRF release induced by ATP (Evans *et al.*, 1988). By use of intracellular calcium measurements as an indication of endothelial cell activation, it has also been demonstrated that 8-bromo cyclic GMP did not inhibit increases in intracellular calcium induced by thrombin in human umbilical vein endothelial cells (Jaffe *et al.*, 1987), or by histamine in human aortic endothelial cells (Ryan *et al.*, 1988). It has been shown that ADP utilizes a different G protein from thrombin in the transduction mechanism for EDRF release (Flavahan *et al.*, 1989). The nature of the G proteins involved in the activation of human endothelium by thrombin and histamine is not known but if they resemble those for ADP, and maybe the related purine ATP, this could explain the previous negative findings of the effect of 8-bromo cyclic GMP on EDRF release induced by these agents in human cells.

It is likely therefore that the inhibitory effects of cyclic GMP and of EDRF itself on its own release, is important only for those agonists utilizing a specific G protein coupled to IP_3 formation.

In conclusion, the present study provides a possible explanation for the inhibitory effects of cyclic GMP on EDRF release observed by us previously (Evans *et al.*, 1988; Hogan *et al.*, 1989). However, it is unlikely that endothelial cell activation and EDRF release induced by all agonists from endothelial cells of all species will be affected in this way.

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References

ANGUS, J.A. & COCKS, T.M. (1989). Endothelium-derived relaxing factor. *Pharmacol. Ther.*, **41**, 303–352.

BERRIDGE, M.J. (1984). Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.*, **220**, 345–360.

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

BERRIDGE, M.J. & IRVINE, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*, **312**, 315–321.

COLLINS, P., GRIFFITH, T.M., HENDERSON, A.H. & LEWIS, M.J. (1986). Endothelium-derived relaxing factor alters calcium fluxes in rabbit aorta: a cyclic guanosine monophosphate mediated effect. *J. Physiol.*, **381**, 427–437.

DERIAN, C.K. & MOSKOWITZ, M.A. (1986). Polyphosphoinositide hydrolysis in endothelial cells and carotid artery segments. *J. Biol. Chem.*, **261**, 3831–3837.

DOWNES, C.P. & MICHELL, R.H. (1985). Inositol phospholipid breakdown as a receptor-controlled generator of second messengers. In *Molecular Mechanisms of Transmembrane Signalling*. ed. Cohen, P. & Houslay, M. pp. 3–56. Amsterdam: Elsevier Science Publishers.

EVANS, H.G., SMITH, J.A. & LEWIS, M.J. (1988). Release of endothelium-derived relaxing factor is inhibited by 8-bromo-cyclic GMP. *J. Cardiovasc. Pharmacol.*, **12**, 672–677.

FLAVAHAN, N.A., SHIMOKAWA, H. & VANHOUTTE, P.M. (1989). Pertussis toxin inhibits endothelium-dependent relaxations to certain agonists in porcine coronary arteries. *J. Physiol.*, **408**, 549–560.

FORSBERG, E.J., FEUERSTEIN, G., SHOHAMI, E. & POLLARD, H.B. (1987). Adenosine triphosphate stimulates inositol phospholipid metabolism and prostacyclin formation in adrenal medullary endothelial cells by means of P_2-purinergic receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 5630–5634.

FORSTERMAN, U., MÜLSCH, A., BÖHME, E. & BUSSE, R. (1986). Stimulation of soluble guanylate cyclase by an acetylcholine-induced endothelium-derived factor from rabbit and canine arteries. *Circ. Res.*, **58**, 531–538.

GORDON, J.L. & MARTIN, W. (1983). Endothelium-dependent relaxation of the pig aorta: relationship to stimulation of ^{86}Rb efflux from isolated endothelial cells. *Br. J. Pharmacol.*, **79**, 531–542.

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J. & HENDERSON, A.H. (1985). Evidence that cyclic guanosine monophosphate (cGMP)-

mediates endothelium-dependent relaxation. *Eur. J. Pharmacol.*, **112**, 195–202.

HASHIMOTO, T., HIRATA, M., ITOH, T., KANMURA, Y. & KURIYAMA, H. (1986). Inositol 1,4,5 trisphosphate activates pharmacomechanical coupling in smooth muscle of the rabbit mesenteric artery. *J. Physiol.*, **370**, 605–618.

HIRATA, M., KOHSE, K.P., CHANG, C.-H., IKEBE, T. & MURAD, F. (1990). Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells. *J. Biol. Chem.*, **265**, 1268–1273.

HOGAN, J.C., SMITH, J.A., RICHARDS, A.C. & LEWIS, M.J. (1989). Atrial natriuretic peptide inhibits EDRF release from blood vessels of the rabbit. *Eur. J. Pharmacol.*, **165**, 129–134.

HOKIN, L.E. (1985). Receptors and phosphoinositide-generated second messengers. *Annu. Rev. Biochem.*, **54**, 205–255.

IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.L. & GREUTTER, C.A. (1981). Mechanism of vascular smooth muscle relaxation of organic nitrates, nitroprusside and nitric oxide. Evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.*, **218**, 739–749.

JAFFE, E.A., GRULICH, J., WEKSLER, B.B., HAMPEL, G. & WATANABE, K. (1987). Correlation between thrombin-induced prostacyclin production and inositol trisphosphate and cytosolic free calcium levels in cultured human endothelial cells. *J. Biol. Chem.*, **262**, 8557–8565.

KARAKI, H., NAKAZAWA, H. & URAKAWA, N. (1984). Comparative effects of verapamil and sodium nitroprusside on contraction and ^{45}Ca uptake in the smooth muscle of rabbit aorta and guinea-pig taenia coli. *Br. J. Pharmacol.*, **81**, 393–400.

KATSUKI, S., ARNOLD, N., MITTAL, C. & MURAD, F. (1977). Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J. Cyclic Nucleotide Res.*, **3**, 23–25.

KISSANE, J.M. & ROBINS, E. (1958). The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.*, **233**, 184–188.

LAMBERT, T.L., KENT, R.S. & WHORTON, A.R. (1986). Bradykinin stimulation of inositol polyphosphate production in porcine aortic endothelial cells. *J. Biol. Chem.*, **261**, 15288–15293.

LANG, D. & LEWIS, M.J. (1989). Endothelium-derived relaxing factor inhibits the formation of inositol trisphosphate by rabbit aorta. *J. Physiol.*, **411**, 45–52.

LOEB, A.L., IZZO, N.J., JOHNSON, R.M., GARRISON, J.C. & PEACH, M.J. (1988). Endothelium-derived relaxing factor release associated with increased endothelial cell inositol trisphosphate and intracellular calcium. *Am. J. Cardiol.*, **62**, 36G–40G.

MARTIN, W., SMITH, J.A. & WHITE, D.G. (1986). The mechanisms by which haemoglobin inhibits relaxation of rabbit aorta induced by nitrovasodilators, nitric oxide and bovine retractor penis inhibitory factor. *Br. J. Pharmacol.*, **89**, 563–572.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation of rabbit aorta by certain ferrous haemoproteins. *J. Pharmacol. Exp. Ther.*, **233**, 679–685.

MARTIN, W., WHITE, D.G. & HENDERSON, A.H. (1988). Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. *Br. J. Pharmacol.*, **93**, 229–239.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

MOSCAT, J., MORENO, F. & GARCIA-BARRENO, P. (1987). Mitogenic activity and inositol metabolism in thrombin-stimulated pig aorta endothelial cells. *Biochem. Biophys. Res. Commun.*, **145**, 1302–1309.

NEWBY, A.C. & HENDERSON, A.H. (1990). Stimulus-secretion coupling in vascular endothelial cells. *Annu. Rev. Physiol.*, **52**, 661–664.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature*, **308**, 693–697.

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

PIROTTON, S., RASPE, E., DEMOLLE, D., ERNEUX, C. & BOEYNAEMS, J.M. (1987). Involvement of inositol 1,4,5-tris-phosphate and calcium in the action of adenine nucleotides on aortic endothelial cells. *J. Biol. Chem.*, **262**, 17461–17466.

POLLOCK, W.K., WREGGET, K.A. & IRVINE, R.F. (1988). Inositol phosphate production and Ca^{2+} mobilization in human umbilical vein endothelial cells stimulated by thrombin and histamine. *Biochem. J.*, **256**, 371–376.

RAPOPORT, R.M. (1986). Cyclic guanosine monophosphate inhibition of contraction may be mediated through inhibition of phosphatidylinositol hydrolysis in rat aorta. *Circ. Res.*, **18**, 407–410.

RAPOPORT, R.M., DRAZNIN, M.B. & MURAD, F. (1983a). Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature*, **306**, 174–176.

RAPOPORT, R.M., DRAZNIN, M.B. & MURAD, F. (1983b). Endothelium-dependent vasodilator- and nitrovasodilator-induced relaxation may be mediated through cyclic GMP formation and cyclic GMP-dependent protein phosphorylation. *Trans. Ass. Am. Phys.*, **96**, 19–30.

RYAN, U.S., AVDONIN, P.V., POSIN, E.Y., POPOV, E.G., DANILOV, S.M. & TKACHUK, V.A. (1988). Influence of vasoactive agents on cytoplasmic free calcium in vascular endothelial cells. *J. Appl. Physiol.*, **65**, 2221–2227.

SMITH, J.A. & LANG, D. (1990). Release of endothelium-derived relaxing factor from pig cultured endothelial cells, as assessed by changes in endothelial cell cyclic GMP content, is inhibited by a phorbol ester. *Br. J. Pharmacol.*, **99**, 565–571.

TAKAI, Y., KAIBUCHI, K., MATSUBARA, T. & NISHIZUKA, Y. (1981). Inhibitory action of guanosine 3′:5′-monophosphate on thrombin induced phosphatidylinositol turnover and protein phosphorylation in human platelets. *Biochem. Biophys. Res. Commun.*, **101**, 61–67.

WALDMAN, S.A., RAPOPORT, R.M. & MURAD, F. (1984). Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. *J. Biol. Chem.*, **259**, 14332–14334.

WINQUIST, R.J., FAISON, E.P., WALDMAN, S.A., SCHWARTZ, R., MURAD, F. & RAPOPORT, R.M. (1989). Atrial natriuretic factor illicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 7661–7664.

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