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Stimulation of endothelial prostacyclin production plays no role in endothelium-dependent relaxation of the pig aorta

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1 Stimulation of prostacyclin production by pig aortic endothelial cells adhering to microcarrier beads superfused in columns, or ^{3}H release from cells prelabelled with [^{3}H]-arachidonate, was studied in response to a range of agents that induce endothelium-dependent vascular relaxation.

2 Bradykinin, adenosine triphosphate (ATP) and ionophore A23187 each stimulated release of prostacyclin from unlabelled cells and of ^{3}H from prelabelled cells but acetylcholine did not.

3 Bradykinin induced a parallel, dose-dependent increase in ^{3}H release and ^{86}Rb efflux, measured simultaneously from columns of aortic endothelial cells preloaded with ^{86}Rb and [^{3}H]-arachidonate.

4 The rank-order of effectiveness at inducing both ^{3}H and ^{86}Rb release, measured simultaneously from columns of aortic endothelial cells prelabelled with ^{86}Rb and [^{3}H]-arachidonate and challenged with maximal doses of each agonist, was: A23187 > bradykinin > ATP.

5 The similarity between agonist-induced ^{3}H release (from cells prelabelled with [^{3}H]-arachidonate) and ^{86}Rb efflux indicates that a common mechanism may be responsible, and the effectiveness of ionophore A23187 suggests that a rise in the intracellular level of calcium may be involved.

6 The lack of effect of acetylcholine on release of prostacyclin from unlabelled cells or of ^{3}H from cells prelabelled with [^{3}H]-arachidonate provides further evidence that acetylcholine acts on endothelial cells by a mechanism that does not involve calcium mobilisation.

7 Although bradykinin, ATP and ionophore A23187 each induced release of prostacyclin from aortic endothelial cells, prostacyclin did not relax the pig aorta. Furthermore, endothelium-dependent relaxation was unaffected by pretreating aortic strips with aspirin. It therefore appears that neither prostacyclin nor any other cyclo-oxygenase product mediates endothelium-dependent relaxation of the pig aorta.

Introduction

Bradykinin, adenosine triphosphate (ATP), acetylcholine and the calcium ionophore A23187 each relaxes a wide variety of isolated arterial and venous preparations by an unknown mechanism initiated in the endothelium (Furchgott & Zawadzki, 1980; Altura & Chand, 1981; De Mey & Vanhoutte, 1981; 1982; Cherry, Furchgott, Zawadzki & Jothianandan, 1982; Gordon & Martin, 1982; 1983). Studies of endothelium-dependent relaxation have provided valuable information about the range of substances that act on the vascular endothelium, but these experiments do not allow direct measurements of endothelial responses *per se*. We have recently developed a technique for assessing the responsiveness of vascular cells directly (Gordon & Martin, 1982; 1983; Martin & Gordon, 1983a,b), by measuring agonist-induced potassium efflux (using ^{86}Rb) from preloaded cells. Although this technique has been used successfully to examine the effects of bradykinin, ATP and A23187 on isolated endothelial cells, acetylcholine, a powerful endothelium-dependent relaxant, has no effect on ^{86}Rb efflux. It has been previously shown that vasoactive stimuli, including bradykinin and A23187, stimulate the release of

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prostaglandins from cultured vascular endothelial cells (Gimbrone & Alexander, 1975; Weksler, Ley & Jaffe, 1978; Hong, 1980), although the time courses of these responses have not been analysed. We therefore measured prostacyclin production by unlabelled endothelial cells and ^3H release from cells prelabelled with [^3H]-arachidonate, stimulated by vasodilator agents (with particular interest in acetylcholine), to determine whether release of prostacyclin or any other metabolite of arachidonic acid was involved in endothelium-dependent relaxation of the pig aorta. The time courses of the responses were analysed by monitoring the eluate from columns packed with microcarrier beads covered with pig aortic endothelial cells (Ryan, Mortara & Whitaker, 1980; Davies, 1981; Busch, Cancilla, DeBault, Goldsmith & Owen, 1982; Pearson, Carleton & Hutchings, 1983a; Pearson, Slakey & Gordon, 1983b).

Methods

Isolation of aortic endothelial cells

Pig aortic endothelial cells were isolated as previously described (Gordon & Martin, 1983). The small sheets of endothelial cells were dispersed by resuspension in a solution of disodium edetate (EDTA, 0.7 mM) in phosphate-buffered saline (PBS), followed by vortexing. Cells ($1-5 \times 10^6$) were then resuspended in DMEM containing 20% foetal calf serum (FCS, Sera-Lab, U.K.), added to a sterile, siliconised flask containing 2 ml of Biosilon plastic microcarrier beads (~200 μm diameter, NUNC, obtained from Gibco, U.K.), and incubated at 37°C under an atmosphere of 5% CO_2 in air. Incubation overnight was sufficient time for cells to adhere to the microcarrier beads. Cells on beads were visualised by light microscopy after fixation in absolute alcohol and staining with a 0.1% solution of methyl violet.

Preparation of columns

All experiments were performed with cells one day after isolation. Cells on beads were separated from non-adherent endothelial cells and contaminating erythrocytes by filtration through a nylon mesh. Columns were packed by pouring a suspension of beads into the barrel of a 1 ml syringe with a glass-fibre plug at the tip. The packed volume of each column was 0.3–0.5 ml. Care was taken to ensure that the columns contained no air bubbles and never became dry. For prelabelling experiments, 4 μCi of [5,6,8,9,-11,12,14,15-^3H]-arachidonic acid (final loading conc. 360 nM; Amersham International) was evaporated to dryness under oxygen-free nitrogen, reconstituted in 100 μl of either Krebs solution alone or Krebs containing 50 μCi ml^{-1} ^{86}RbCl (0.1–0.5 mM; Amersham International), placed on top of the packed cell column and allowed to run over the beads. Columns were then incubated for 1 h at 37°C. After replacing the syringe plunger, with a PP_{30} polythene delivery tube inserted through the rubber seal, columns were washed for 30 min by perfusion, from the bottom upwards, at a rate of 0.35 ml min^{-1}, with a Krebs solution of the following composition (mM): NaCl 119, KCl 3.1, $MgSO_4$ 0.6, $NaHCO_3$ 25, KH_2PO_4 1, $CaCl_2$ 1.3 and glucose 11.1, gassed with 5% CO_2 in O_2. Thereafter, 1 min fractions of perfusate were collected and the effects of vasodilator drugs were determined by replacing the normal Krebs solution with one containing the test compound (Pearson *et al.*, 1983a,b). ^3H and ^{86}Rb in each fraction were determined by dual-channel liquid-scintillation counting, after correcting for channel overlap. The amount of radioactivity in the cells at the end of each experiment was determined after solubilisation in a 0.5% solution of Triton X-100 in distilled water. ^3H release from cells prelabelled with [^3H]-arachidonate was expressed as c.p.m.; ^{86}Rb efflux was calculated as the first-order rate constant and expressed in units of min^{-1} (Gordon & Martin, 1983).

Measurement of prostacyclin release

Prostacyclin released into the perfusate, from columns of endothelial cells which had not been isotopically labelled, was measured by radioimmunoassay of its stable product, 6-keto-prostaglandin $F_{1\alpha}$, as previously described (Pearson, Ager, Trevethick & Gordon, 1979; Ager, Gordon, Moncada, Pearson, Salmon & Trevethick, 1982). The perfusate (0.1 ml) was added to the assay tubes without any solvent extraction. The detection limit of the assay was <3 pg/sample, and cross-reactivities (at 50% displacement) were: PGE_2 3%; $PGF_{2\alpha}$ 0.5%; thromboxane $B_2 < 0.05\%$; $PGB_2 < 0.01\%$. The antiserum was a generous gift from Dr B.A. Peskar.

Aortic strips

Babraham pigs, 10–14 days old, were killed by stunning and exsanguination. A section of aorta was removed between the renal and iliac arteries and cut into spiral strips (2 cm long by 2 mm wide). In some experiments, the endothelium was removed from aortic strips by scraping the luminal surface with a scalpel blade. Strips were mounted under 1 g resting tension on glass hooks in 4 ml organ baths and bathed at 37°C in Krebs solution gassed with 5% CO_2 in O_2. Tension was measured using Gould Statham UC3 isometric transducers coupled to a Lectromed MX 212 pen recorder.

Drugs

Acetylcholine chloride, adenosine diphosphate, adenosine triphosphate, aspirin (acetylsalicyclic acid), bradykinin triacetate and histamine dihydrochloride were purchased from Sigma; ionophore A23187 was purchased from Calbiochem-Behring, U.K.; prostacyclin was a gift from Dr P.L. Walton, I.C.I., Macclesfield. The A23187 stock solution (10^{-2} M) was made up in dimethylsulphoxide and dilutions were made using distilled water. The prostacyclin stock solution (10^{-3} M) was made up in glycine/NaOH buffer (pH 10.7) and diluted in PBS immediately before testing. The biological activity of prostacyclin was checked by monitoring its effect on platelet aggregation induced by ADP, using the photometric technique of Born (1962) as modified by Gordon & Drummond (1974).

Results

Production of prostacyclin by pig aortic endothelial cells and release of ^3H from cells prelabelled with [^3H]-arachidonate

In the absence of stimuli, the basal rate of prostacyclin release from superfused pig aortic endothelial cells in microcarrier bead columns was ~70 pg 10^{-6} cells min^{-1}, as measured by radioimmunoassay of its stable product 6-keto-prostaglandin $F_{1\alpha}$.

Acetylcholine (10^{-6} M) had no effect on prostacyclin production, but ATP (10^{-4} M), bradykinin (10^{-7} M) and ionophore A23187 (10^{-5} M) induced maximal increases of 8.5 ± 2.5, 28 ± 5.2 and 52 ± 3.2 times the basal rate of release respectively (mean values ± s.e.; $n = 4$) (Figure 1a). Previous experiments (Gordon & Martin, 1983) had established that

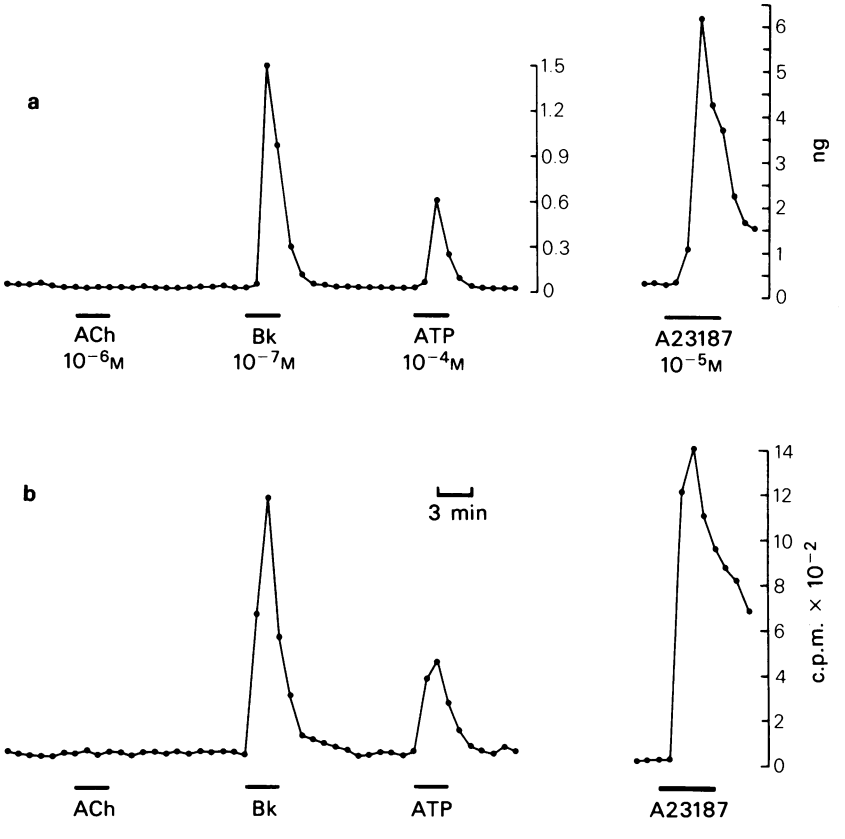

Figure 1 (a) The effects of acetylcholine (ACh), bradykinin (Bk), ATP and ionophore A23187 on prostacyclin production by superfused columns of pig aortic endothelial cells on microcarrier beads, measured (in ng) by radioimmunoassay of 6-keto-prostaglandin $F_{1\alpha}$ in 1 min fractions of superfusate. (b) The effects of acetylcholine, bradykinin, ATP and A23187 on ^3H release (expressed in c.p.m.) from pig aortic endothelial cells prelabelled with [^3H]-arachidonate in microcarrier bead columns.

these concentrations induced maximal endothelium-dependent relaxation of pig aortic strips. Responses were not sustained and began to fall before infusions of the agents were terminated.

A similar pattern was observed when the release of ^{3}H from columns of endothelial cells prelabelled with [^{3}H]-arachidonic acid was monitored (Figure 1b). After a 30 min washout period the basal release of ^{3}H (in c.p.m.) was stable for at least 90 min, which was the maximum duration of most experiments. Acetylcholine (10^{-6} M) had no effect on ^{3}H release, but ATP (10^{-4} M), bradykinin (10^{-7} M) and ionophore A23187 (10^{-5} M) stimulated release transiently (Figure 1b; Figure 3).

The ability of ATP, bradykinin and ionophore A23187, but not acetylcholine, to evoke prostacyclin production, and ^{3}H release from cells prelabelled with [^{3}H]-arachidonate, parallels the ability of these agents to elicit potassium efflux from isolated endothelial cells (Gordon & Martin, 1983), and suggests that agonist-induced arachidonate and potassium release may arise by a common mechanism. If so, for any single agent the dose-response relationships for ^{3}H release from cells prelabelled with [^{3}H]-arachidonate and ^{86}Rb efflux should be parallel, and for a range of agents the rank-order of effectiveness at inducing ^{3}H and ^{86}Rb release should be the same. Subsequent experiments were designed to investigate these possibilities.

Comparison of ^{3}H release and ^{86}Rb efflux from cells prelabelled with [^{3}H]-arachidonate and ^{86}Rb

Bradykinin induced a parallel, dose-dependent increase in both ^{3}H release and ^{86}Rb efflux, measured simultaneously from columns of aortic endothelial cells prelabelled with [^{3}H]-arachidonate and ^{86}Rb (Figure 2a). Figure 2b shows dose-response relationships for the effects of bradykinin on the release of both isotopes, obtained from five experiments; the threshold concentration was 10^{-10} M, and maximal responses were obtained at 10^{-7} M.

The maximum release of both ^{3}H and ^{86}Rb was measured simultaneously from columns of aortic endothelial cells in response to acetylcholine (10^{-6} M), ATP (10^{-4} M), bradykinin (10^{-7} M) and ionophore A23187 (10^{-5} M) (Table 1). Individual responses to acetylcholine, ATP and ionophore A23187, taken from separate experiments, are shown in Figure 3.

The rank-order of effectiveness was A23187 > bradykinin > ATP > acetylcholine for both ^{3}H and ^{86}Rb release.

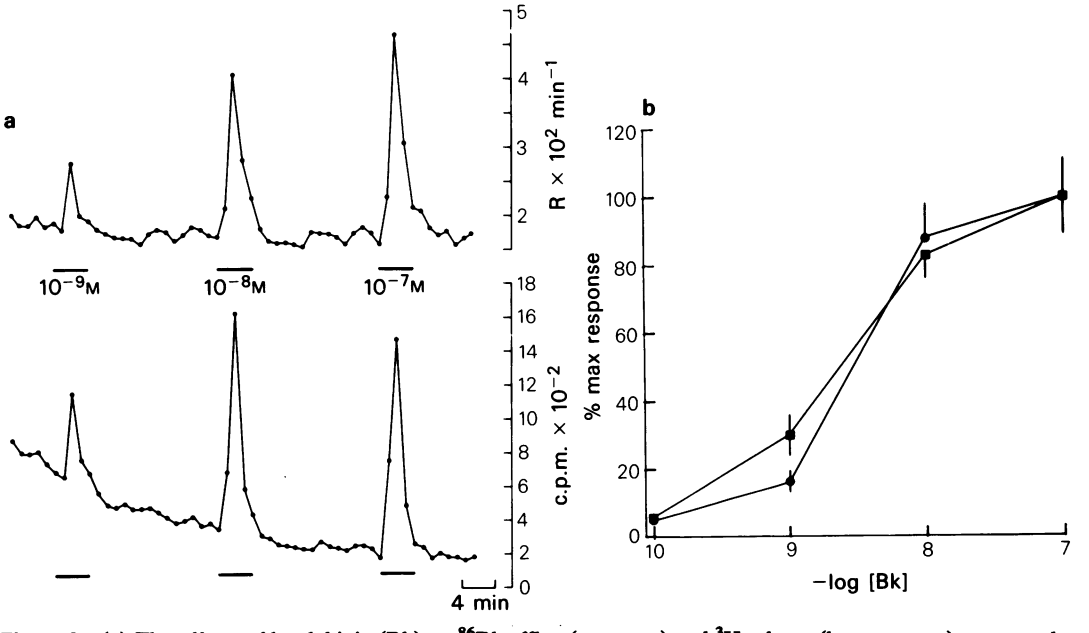

Figure 2 (a) The effects of bradykinin (Bk) on ^{86}Rb efflux (top trace) and ^{3}H release (bottom trace), measured simultaneously from a superfused column of pig aortic endothelial cells prelabelled with ^{86}Rb and [^{3}H]-arachidonate. ^{86}Rb efflux is expressed as the first-order rate constant; ^{3}H release is expressed in c.p.m. (b) Dose-response relationships showing the effects of bradykinin on ^{86}Rb efflux (●) and ^{3}H release (■), expressed as percentages (mean of $n = 5$; vertical lines indicate s.e.) of the maximum responses.

Table 1 Maximal ^{86}Rb efflux and ^{3}H release induced by acetylcholine (ACh), ATP, bradykinin and ionophore A23187, measured simultaneously from columns of pig aortic endothelial cells (pre-labelled with ^{86}Rb and [^{3}H]-arachidonate) adhering to microcarrier beads

	ACh (10^{-6} M)	*ATP* (10^{-4} M)	*Bradykinin* (10^{-7} M)	*A23187* (10^{-5} M)
^{86}Rb efflux	108±6	170±6	380±41	820±40
^{3}H Release	100±2	240±10	470±50	780±45

Responses are expressed as percentages (mean ± s.e., $n = 4-6$) of the basal efflux of each isotope.

Role of prostacyclin in endothelium-dependent relaxation

As bradykinin, ATP and ionophore A23187 each induces the release of prostacyclin, a powerful dilator of some vascular beds (Bunting, Gryglewski, Moncada & Vane, 1976), from aortic endothelial cells, we investigated the effects of prostacyclin on pig aortic strips and its possible role in endothelium-dependent relaxation.

Prostacyclin, in concentrations up to 10^{-5} M, failed to relax histamine-contracted pig aortic strips, both with and without endothelium present (Figure 4b). Bradykinin relaxed the strips with endothelium present, demonstrating that the ineffectiveness of prostacyclin was not a consequence of unresponsive tissues. In a photometric assay the same sample of prostacyclin was found to be active in inhibiting platelet aggregation induced by adenosine disphosphate (2.5×10^{-6} M), with an IC_{50} value of ~1.0 nM (data not shown).

Pretreatment of pig aortic strips for 60 min with aspirin (3×10^{-4} M), previously shown to inhibit porcine aortic endothelial prostacyclin production by

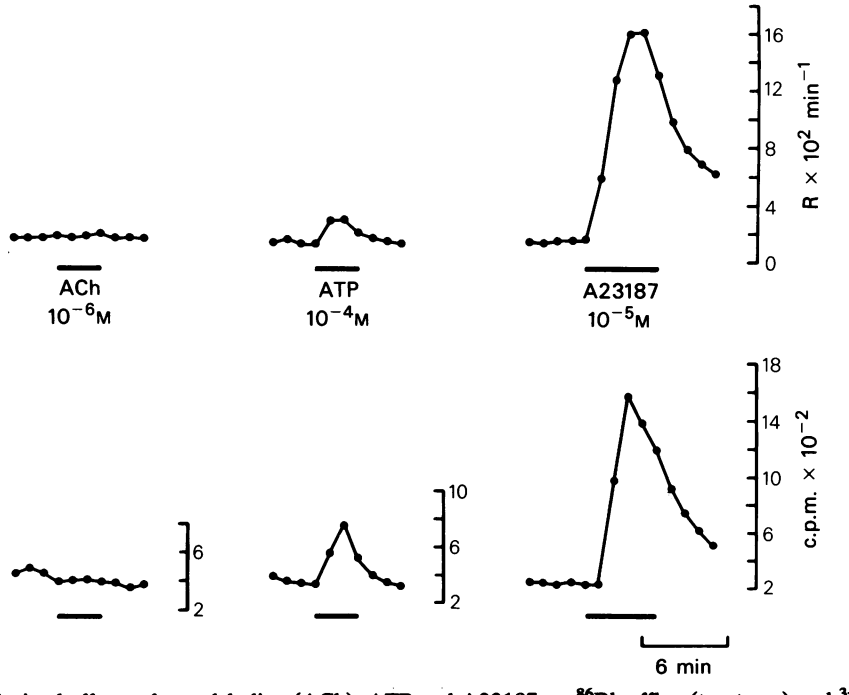

Figure 3 Maximal effects of acetylcholine (ACh), ATP and A23187 on ^{86}Rb efflux (top trace) and ^{3}H release (bottom trace), measured simultaneously from superfused microcarrier bead columns of pig aortic endothelial cells prelabelled with ^{86}Rb and [^{3}H]-arachidonate.

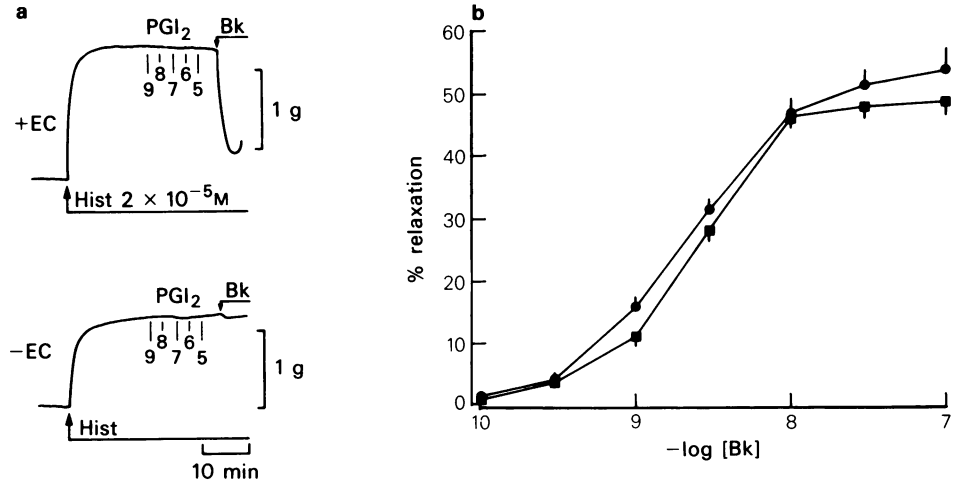

Figure 4 (a) The failure of prostacyclin (PGI_2) to relax histamine-contracted pig aortic strips, either with (+EC) or without (−EC) the endothelium present. When tone had stabilized, prostacyclin was added cumulatively in 10 fold increments begining at 10^{-9} M. Bradykinin (Bk, 10^{-7} M) relaxed the aortic strip with endothelium present, indicating that the lack of effect of prostacyclin was not because the tissues were unresponsive to vasodilator stimuli. (b) Dose-response curves for the endothelium-dependent relaxant effect of bradykinin on strips of pig aorta with (■) or without (●) aspirin pretreatment (3×10^{-4} M for 1 h). Each point is the mean of $n = 6$; vertical lines show s.e.mean.

virtually 100% (Gordon & Pearson, 1978), had no effect on the endothelium-dependent relaxation induced by bradykinin (Figure 4b), ATP or ionophore A23187 (data not shown).

Discussion

Using superfused columns of endothelial cells on microcarrier beads we were able to measure the kinetics of prostacyclin production in a way that is not possible with conventional static cultures. In columns with endothelial cells attached to beads and tested 24 h after isolation, basal production (i.e. in the absence of added stimuli) was high enough that 6-keto-prostaglandin $F_{1\alpha}$ could be measured in fractions collected every 5 s, if required. It is worth noting that basal release from these cells is greater than from columns of subcultured cells (Pearson *et al.*, 1983a,b), possibly because cells tested after 24 h have still not recovered from the trauma of isolation, which itself stimulates prostacyclin release (Ager *et al.*, 1982).

Previous studies have shown that bradykinin and ionophore A23187 stimulate prostacyclin release into the medium bathing conventional cultures of vascular endothelial cells (Weksler *et al.*, 1978; Hong, 1980). Of the endothelium-dependent relaxants we tested, bradykinin, ATP and ionophore A23187 stimulated release of prostacyclin from superfused columns of pig aortic endothelial cells, but acetylcholine did not. Stimulation of prostacyclin release was transient and began to decline even in the continued presence of the stimulus. Similarly, bradykinin, ATP and ionophore A23187, but not acetylcholine, induced a transient release of ^3H from endothelial cells prelabelled with [^3H]-arachidonate. This pattern reflects the ability of these agents to evoke ^{86}Rb efflux from aortic endothelial cells (Gordon & Martin, 1982; 1983) and suggests that these two processes may arise by a common mechanism. This concept is supported by the findings that for bradykinin the dose-response relationships for stimulation of ^3H release and ^{86}Rb efflux from cells prelabelled with [^3H]-arachidonate and ^{86}Rb are similar, and that for all the agents tested the rank-order of effectiveness at inducing ^3H release (A23187 > bradykinin > ATP > acetylcholine) is the same as that for ^{86}Rb efflux. The effectiveness of ionophore A23187 strongly suggests that a rise in the intracellular concentration of calcium is involved in both ^{86}Rb efflux and ^3H release; this is in agreement with previous findings indicating that stimulated release of potassium and activation of phospholipases which liberate arachidonic acid from endothelial cells are calcium-dependent processes (Gordon & Martin, 1982; 1983; Hong & Deykin, 1982). The inability of acetylcholine to evoke ^3H release from cells prelabelled with [^3H]-arachidonate provides additional evidence that this agent acts on endothelial cells to

induce endothelium-dependent relaxation by a mechanism that does not involve calcium mobilisation (Gordon & Martin, 1982, 1983).

Our present results show that measuring prostacyclin production, or ^3H release from cells prelabelled with [^3H]-arachidonate, can be used to assess directly the responsiveness of vascular endothelial cells to some agents that induce endothelium-dependent relaxation, but apparently only to those agents that also induce ^{86}Rb efflux. Responses of endothelial cells to acetylcholine have still to be demonstrated directly.

Bradykinin, ATP, ionophore A23187 and acetylcholine all induce endothelium-dependent relaxation of the pig aorta, but only the first three stimulate prostacyclin release. If all four agents induce relaxation by stimulating release of a single vasodilator substance from endothelial cells, then this agent cannot be prostacyclin. This argument is supported by the finding that prostacyclin, although a potent dilator of some blood vessels (Bunting *et al.*, 1976), does not relax the pig aorta. It is also unlikely that any other cyclo-oxygenase product of arachidonic acid is responsible for endothelium-dependent relaxation of the pig aorta since pre-treatment with aspirin has no effect on relaxation induced by any of the agents tested in this study. Experiments from other laboratories suggest that a lipoxygenase product of arachidonic acid may mediate endothelium-dependent relaxation (Furchgott & Zawadzki, 1980; Chand & Altura, 1981; De Mey & Vanhoutte, 1982). However, acetylcholine failed to evoke release of ^3H from pig aortic endothelial cells prelabelled with [^3H]-arachidonate, suggesting that the involvement of any metabolic product of arachidonic acid in acetylcholine-induced relaxation of the pig aorta is unlikely. It is possible that acetylcholine could release arachidonic acid from a phospholipid pool not labelled with [^3H]-arachidonate under the conditions of our experiments; there is evidence for compartmentalisation of arachidonate from experiments with kidneys perfused with bradykinin and ATP (Schwartzman & Raz, 1982).

In conclusion, by using superfused columns of pig aortic endothelial cells adhering to microcarrier beads we have been able to monitor the magnitude and time course of prostacyclin production and arachidonate release stimulated by some (but not all) agents that induce endothelium-dependent relaxation. Stimulation of prostacyclin production by such agents appears to play no role in endothelium-dependent relaxation of the pig aorta.

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