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Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells

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1 A cascade superfusion technique has been developed for the differential bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) released from porcine aortic endothelial cells cultured on microcarriers, packed into a column and perfused.

2 Bradykinin (Bk; 20–100 nM) released prostacyclin (9.6 ± 1.5 nM per 10^6 cells; mean ± s.e.mean, $n = 9$) and prostaglandin E_2 (PGE_2; 2.1 ± 0.6 nM per 10^6 cells) from the column measured by relaxation of strips of bovine coronary artery (BCA) and rabbit mesenteric or coeliac artery, respectively. The presence of these prostanoids in the effluent was confirmed by specific radioimmunoassays.

3 A23187 (500–2000 nM) also released both prostacyclin and PGE_2 from the cells. This release was long-lasting and not reproducible.

4 Bk (20–100 nM) and A23187 (30–300 nM) released EDRF from the column. This was detected in a cascade of four rabbit aortic strips (RbA), denuded of endothelium and contracted with U46619 or phenylephrine. The relaxation of the RbA strips caused by EDRF was progressively attenuated down the cascade (half-life < 7 s) and was not affected by indomethacin.

5 EDRF and prostacyclin could be differentially bioassayed in a cascade of alternating RbAs and BCAs as prostacyclin did not relax RbAs and the time delay to the BCAs destroys EDRF. EDRF could be bioassayed on its own when the endothelial cells were treated with indomethacin.

6 5-Hydroxytryptamine 0.2, noradrenaline 1.0, platelet-activating factor (Paf-acether) 1.0, formyl-methionyl-leucyl-phenylalanine 1.0, acetylcholine 0.5, bethanecol 0.5, adenosine diphosphate 0.25 and angiotensin II 0.1 μM did not release either prostanoids or EDRF from the column.

Introduction

Prostacyclin (Moncada *et al.*, 1976; Gryglewski *et al.*, 1976) and endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) are both products of vascular endothelium which play a role in vasodilatation. Cultures of vascular endothelial or smooth muscle cells release prostacyclin and prostaglandin E_2 (PGE_2) either spontaneously (Ager *et al.*, 1982) or in response to mechanical (Johnson, 1980) or chemical stimulation (Gimbrone & Alexander, 1975; Weksler *et al.*, 1978; Hong, 1980). The development of a technique in which endothelial cells are cultured on microcarrier beads, packed into columns and perfused (Ryan *et al.*, 1980; Davies, 1981) has allowed the study of the metabolism of arachidonic acid (AA; Pearson *et al.*, 1983a, b; Gordon & Martin, 1983). More recently, Cocks *et al.* (1985) have used a similar approach to generate EDRF and detect it using a ring of canine coronary artery denuded of endothelium. We have now combined the technique of perfused cultured endothelial cells with the superfusion technique for the detection of biologically active substances (Vane, 1964) and developed a method for studying the simultaneous release and actions of both prostacyclin and EDRF by differential bioassay.

Methods

(a) Isolation and culture of porcine aortic endothelial cells

Fresh porcine thoracic aortae were obtained from an abattoir in phosphate buffered saline (PBS; pH 7.2) containing antibacterial-antimycotic mixture, and

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washed externally with 70% ethanol and rinsed in PBS. The lumen was then washed with PBS, the connective tissue and fat removed and the intercostal arteries ligated. The lumen was washed again and the lower end clamped prior to filling with PBS containing 1 mg ml^{-1} collagenase. After 15 min incubation at 37°C the solution was removed and the cells obtained washed twice by centrifugation at 180 *g* for 10 min in culture medium (Hepes buffered Dulbecco's modification of Eagle's medium containing 20% foetal calf serum and antibacterial-antimycotic mixture).

The endothelial cells from each aorta were suspended in culture medium and incubated separately overnight in 25 cm^2 culture flasks to allow small patches of endothelial cells to adhere. The non-adherent cells were then removed by washing three times with medium and culture continued for a further 24 h to allow the cell clusters to spread.

The cells from 6–8 aortae were then removed from the culture flasks with 0.125% trypsin-versene at room temperature and washed twice in culture medium before being seeded onto 1 g hydrated Cytodex 3 microcarrier beads. The cells and beads were incubated at 37°C for 8 cycles, each consisting of 5 min stirring at 40 r.p.m. and 30 min at rest, before being continuously stirred at 40 r.p.m. in 500 ml culture medium on a microcarrier stirrer system (Techne). After 3 to 4 days in culture the cells reached confluence on the beads. This was confirmed by phase contrast microscopy. Before packing the column for perfusion experiments, the cells on 0.1 ml of beads were counted by incubation for 1 h in 1 ml of 0.1% crystal violet in 0.1 M citric acid and the nuclei released counted in a haemocytometer (Sanford *et al.*, 1951). The volume of the microcarriers in the column was determined at the end of the experiment.

(b) Electron microscopy

Microcarriers coated with cells were fixed for light and electron microscopy in 0.1 M cacodylate buffered 3% glutaraldehyde (pH 7.2) for at least 1 h. The microcarriers were prepared for scanning electron microscopy (Malick & Wilson, 1975) and examined on a Philips 500 SEM. The cells exhibited the 'cobblestone' morphology and numerous microvilli characteristic of endothelial cells (Figure 1).

(c) Preparation of the cell column

Between 1 and 3 ml of microcarrier beads containing $1-9 \times 10^7$ endothelial cells (5–14 day old cultures) were packed into a K16 chromatographic column, cut down to 10 cm length and maintained at 37°C (Pharmacia). The ends of the column were closed with modified flow adaptors (Pharmacia) which prevented microcarrier beads from escaping from the column

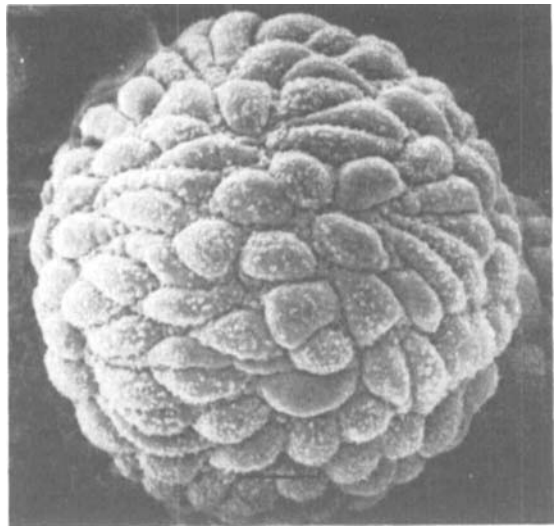

Figure 1 Electronmicrograph of a microcarrier bead covered with endothelial cells. Bar = 10 μ.

(Figure 2). After packing, the column was continuously perfused with Krebs buffer gassed with a mixture of 95% O_2 and 5% CO_2 containing (mM): Na^+ 144.0, K^+ 5.9; Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25.0, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.0. The Krebs solution was pumped through the column at 5 ml min^{-1} via a heated (37°C) glass coil which also served as a gas trap and as an injection port for the test substances (Figure 2). The void volume between the upper layer of the cells and the outlet of the capillary tubing leading to the superfusion cascade was 80 μl. The time taken for the superfusion fluid to reach the first bioassay tissue was 1 s. After 15 min perfusion to waste, the column effluent was used to superfuse the bioassay tissues.

(d) Bioassay

The bioassay was carried out using the cascade superfusion technique (Vane, 1964). Spirally cut strips of bovine coronary artery (BCA; Dusting *et al.*, 1977), rabbit mesenteric or coeliac arteries (RbMA, RbCA; Bunting *et al.*, 1976), rabbit thoracic aorta (RbA; Furchgott, 1955) and occasionally rat stomach strip (RSS; Vane, 1957) were superfused with either warmed (37°C) Krebs solution or with the effluent from the column. The delay between each tissue in the cascade was 3 s. During the experiment, the column could be moved and mounted at any height of the cascade such that its outflow could superfuse any of the assay tissues first. Occasionally a second cascade was superfused in series, with a 4 min delay interposed

between them, to study the disappearance of prostacyclin.

(e) Preparation and superfusion of the arterial strips

Fresh bovine hearts were obtained from an abattoir in ice-cold saline. The circumflex and left coronary arteries were dissected 3–4 h after the animals were killed and cut spirally into 4–5 cm long strips which were used immediately for bioassay or stored in ice-cold Krebs buffer for 1–2 days before use. New Zealand White rabbits (2–3 kg) of either sex were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.v.) and RbA, RbMA and RbCA were removed and cut into 3–4 cm long spiral strips.

The arterial strips were mounted in heated (37°C) glass chambers and superfused (5 ml min^{-1}) in cascade with Krebs buffer for 2–3 h before superfusion with

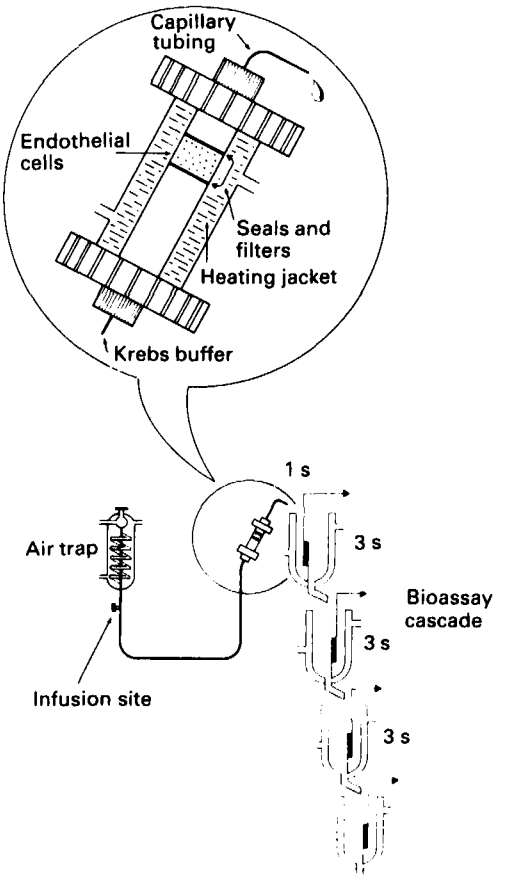

Figure 2 Diagrammatic representation of the bioassay cascade and the column containing the endothelial cells.

effluent from the column. Changes in the length of the tissues were detected by auxotonic levers attached to Harvard 386 heart/smooth muscle transducers, the display of which was recorded by a 6-channel Watanabe recorder.

For assay of EDRF the detector arteries were threaded onto a cotton pipe-cleaner, immersed in Krebs buffer and the endothelium removed by gentle circular and longitudinal movements. The arteries were then cut into spiral strips which were considered to be completely denuded of endothelium when they did not relax to an infusion of the muscarinic agonists 5-methyl-furmethide (1 μM) and acetylcholine bromide (1 μM) or the calcium ionophore A23187 (1 μM). For assay of EDRF the endothelium-denuded arterial strips were contracted with a continuous infusion over the tissues (O.T.) of the 11,9-epoxymethano analogue of prostaglandin H_2–U46619 (15–30 nM), or with phenylephrine hydrochloride (50–200 nM). Stable stimulators of smooth muscle guanylate cyclase such as sodium nitroprusside (NP) or glyceryl trinitrate (GTN) were infused (10–200 nM O.T.) to calibrate the relative sensitivity of the RbAs to guanylate cyclase-mediated relaxation.

Stimuli for the release of prostanoids or EDRF from the column were given as one minute infusions. Test substances were infused either O.T. or through the column (T.C.) using a peristaltic minipump (Pierce) or a syringe pump (Braun Perfusor VI). Short infusions (up to 60 s) were given manually using 1 ml polypropylene syringes. Indomethacin (5 μM) was always infused O.T. to inhibit the synthesis of prostanoids by the assay tissues. When inhibition of prostanoid synthesis by the cells in the column was required, the infusion of indomethacin was moved from O.T. to T.C.

When the release of prostanoids or EDRF was attempted with acetylcholine, 5-hydroxytryptamine, noradrenaline or angiotensin II, the direct musculotropic effects of these compounds were blocked by infusing specific antagonists such as atropine sulphate (1.0 μM), phenoxybenzamine (2.0 μM), methysergide (0.3 μM) and saralasine (0.2 μM) O.T.

(f) Radioimmunoassay of prostanoids

The superfusate from some experiments in which prostacyclin and PGE_2 were bioassayed was collected, after superfusing the assay tissues, in consecutive fractions of 2.5 ml for radioimmunoassay (RIA) of 6-keto-$PGF_{1\alpha}$ and PGE_2 as previously described (Salmon, 1978).

(g) Chemicals

The following chemicals were used: prostacyclin, glyceryl trinitrate, 5-methyl-furmethide, trypsin-ver-

sene (Wellcome); sodium nitroprusside, angiotensin II, saralasine, arachidonic acid, atropine sulphate, 5-hydroxytryptamine creatinine sulphate, noradrenaline hydrochloride, formyl-methionyl-leucyl-phenylalanine (FMLP) platelet-activating factor (Paf)-acether, adenosine diphosphate, acetylcholine bromide, Type II collagenase and phenylephrine hydrochloride (Sigma); bradykinin (Sandoz or Sigma); A23187 (Calbiochem); indomethacin (Merck, Sharp and Dohme); methysergide (Sandoz); phenoxybenzamine (Smith, Kline and French); prostaglandin E_2 and U46619 (Upjohn Co.); HEPES buffered Dulbecco's modification of Eagle's medium and antibacterial-antimycotic solution (Gibco); foetal calf serum (Flow Laboratories) and Cytodex 3 (Pharmacia).

Results

Release of prostanoids

For bioassay of prostanoids, the cascade comprised BCA, RbMA or RbCA and RSS. These tissues responded to 1 min infusions (O.T.) of prostacyclin and PGE_2 in a characteristic fashion (Figure 3). PGE_2 was 10 to 20 times more potent than prostacyclin in inducing relaxation of the RbMA and RbCA. The BCA was contracted by PGE_2 and relaxed by prostacyclin. The RSS contracted to PGE_2 but not to prostacyclin at concentrations up to 300 nM. In the presence of indomethacin (5 μM, O.T.) the detection limits for both prostanoids was between 10 and 30 nM.

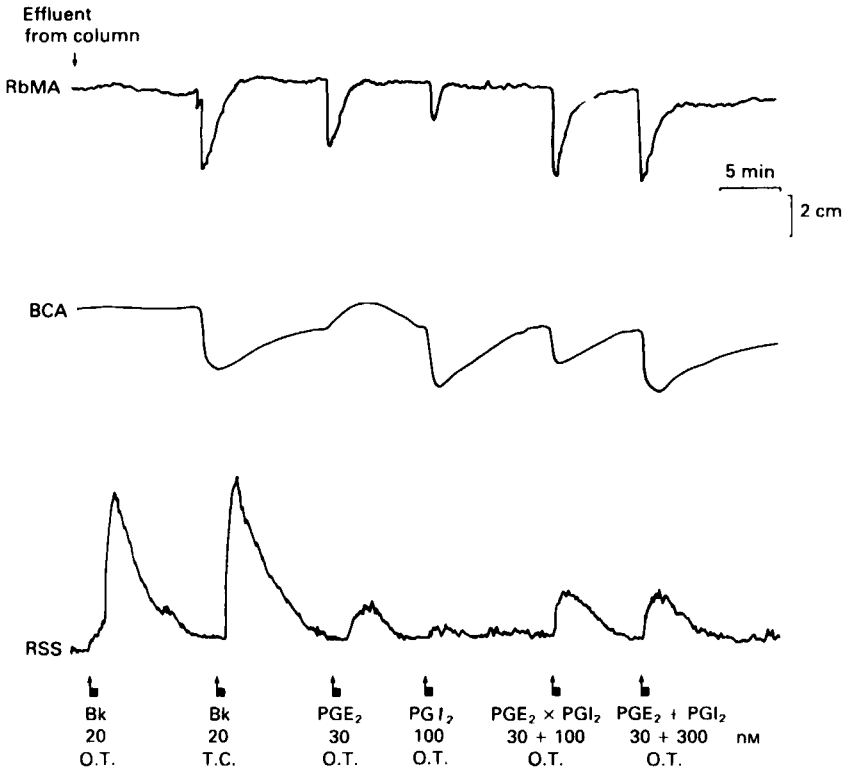

Figure 3 Effects of bradykinin (Bk, infused over the tissue (O.T.) or through the column (T.C.)), prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2, O.T.) on rabbit mesenteric artery (RbMA) bovine coronary artery (BCA) and rat stomach strip (RSS). Bk (20 nM, O.T.) induces a contraction of the RSS and has no effect on RbMA and BCA. The same concentration of Bk given T.C. induces the release of a substance(s) which relax(es) the RbMA and the BCA. PGE_2 (30 nM, O.T.) induces a relaxation of the RbMA and a contraction of the BCA and RSS while PGI_2 (100 nM, O.T.) relaxes RbMA and BCA without much effect on the RSS. The effect of mixtures of PGE_2 and PGI_2 at different concentrations O.T. are also shown.

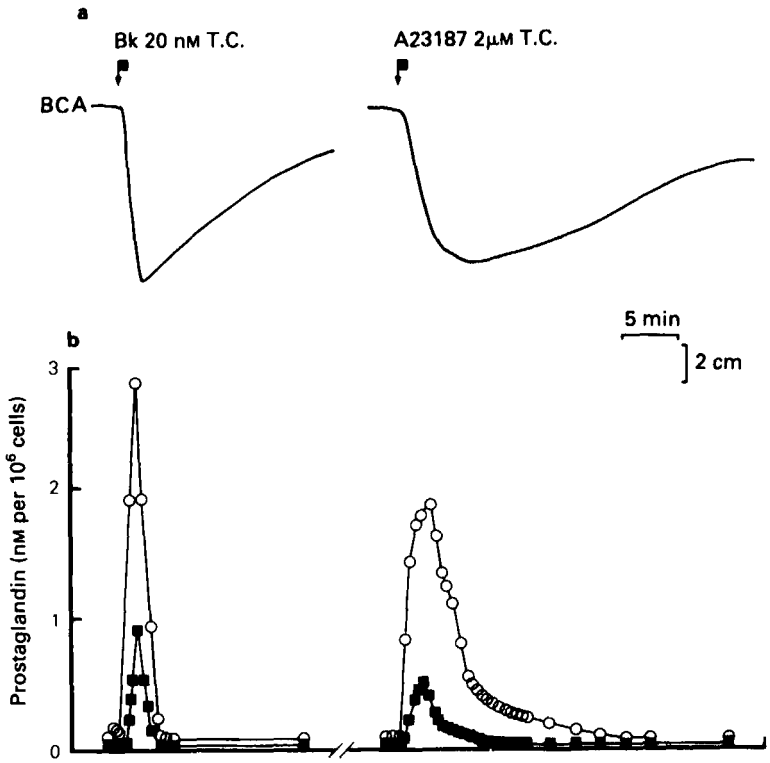

Figure 4 (a) Bioassay of prostacyclin (relaxations of the bovine coronary artery) and (b) radioimmunoassay of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$ ○) and PGE^2 (■) released by bradykinin (Bk, 20nM), and A23187 (2 μM) infused through the column (T.C.).

Bradykinin (Bk; 10–50 nM, T.C.) or A23187 (0.5–2 μM, T.C.) induced, in a concentration-dependent manner, the release of a mixture of prostacyclin and PGE_2 as shown by the presence of an unstable BCA relaxing substance (half-life≙4 min) and a stable RbMA/RbCA relaxing material which also contracted the RSS. Calculation of the absolute amounts of prostacyclin and PGE_2 released by the column was difficult since both prostanoids had synergistic (RbMA/RbCA) or opposing activities (BCA) on the assay tissues (Figure 3). Nonetheless, bioassay of mixtures of known amounts of prostacyclin and PGE_2 enabled the amount of each prostanoid released to be estimated. In nine experiments in which 6 to 11 day old cultures containing $4.4–8.6 \times 10^7$ cells per column were used, Bk (20 nM) T.C. released 9.6 ± 1.5 nM per 10^6 cells of prostacyclin and 2.1 ± 0.6 nM per 10^6 cells of PGE_2 (mean ± s.e.mean). The ratio of the released prostacyclin/PGE_2 was 4.6. Because of the difficulties in the bioassay, the amounts of prostanoids released were also measured by specific RIA (Figure 4). Using this method, the amounts of 6-keto-$PGF_{1\alpha}$ and PGE_2 released were lower than those detected by bioassay; i.e. 2.9 ± 0.3 nM per 10^6 cells for 6-keto-$PGF_{1\alpha}$ and 1.1 ± 0.2 nM per 10^6 cells for PGE_2 (mean ± s.e.mean, $n = 7$). The ratio of 6-keto-$PGF_{1\alpha}$/PGE_2 was 2.6

In contrast to Bk, A23187 induced a long lasting and non-reproducible release of prostanoids (Figure 4). When calculated on a molar basis A23187 was approximately 100 times less potent than Bk in releasing peak concentrations of prostanoids (Figure 4). AA at concentrations of 10–30 μM T.C. produced a moderate release of a BCA-relaxing material that decomposed by approximately 50% after passing through a 4 min delay coil, indicating prostacyclin generation (data not shown).

The following test substances failed to release prostacyclin from the column when administered T.C. at the concentrations given (in μM); acetylcholine bromide 0.5; bethanechol 0.5; noradrenaline hydro-

chloride 1.0; 5-hydroxytryptamine creatinine sulphate 0.2; adenosine diphosphate 0.25; angiotensin II 0.1; Paf-acether 1.0 and FMLP 1.0. The above concentrations of these substances were the highest that could be used without affecting the tone of the assay tissues when administered O.T.

Release of endothelium-derived relaxing factor.

For bioassay of EDRF, the cascade consisted of three or four RbAs denuded of endothelium and submaximally contracted by U46619 (15–30 nM) or phenylephrine (50–200 nM). Some of the spiral strips did not respond to U46619 and therefore were contracted with phenylephrine.

In the experiments designed for the simultaneous detection of EDRF and prostacyclin, BCAs denuded of endothelium were included in the cascade (Figure 5). These tissues develop spontaneous tone in the cascade and therefore their response to U46619 or to phenylephrine was smaller than that of the RbAs.

EDRF relaxed RbAs and BCAs, whereas prostacyclin relaxed BCAs and had no effect on the tone of RbAs (Figure 5). EDRF was characterized in our bioassay system as a RbA-relaxing substance that could be released from the column by chemical (Bk and A23187; Figure 6) or mechanical stimulation (gentle compression of the cells in the column during

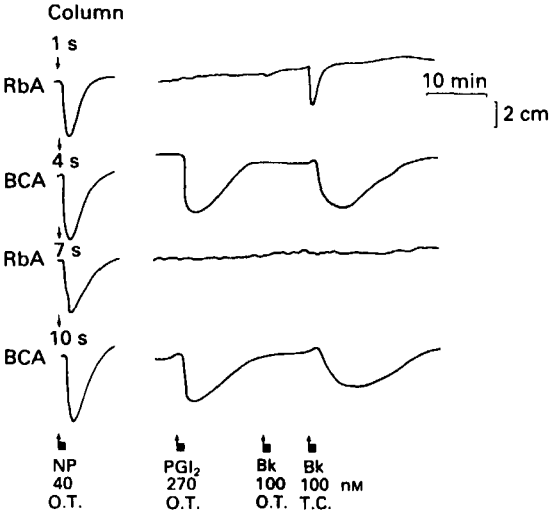

Figure 5 Relaxations of contracted rabbit aorta strips (RbAs) and bovine coronary arteries (BCAs) induced by sodium nitroprusside (NP, 40 nM, O.T.), prostacyclin (PGI_2, 270 nM, O.T.) and bradykinin (Bk, 100 nM, T.C.). Bk (100 nM, O.T.) has no effect on the bioassay tissues. The tissues were contracted by U46619 (30 nM). Abbreviations as in Figure 3 legend.

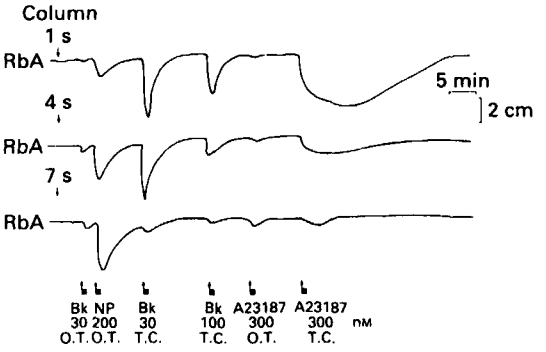

Figure 6 Relaxations of contracted rabbit aortic strips (RbAs) induced by bradykinin (Bk, 30 nM, O.T.), sodium nitroprusside (NP, 200 nM, O.T.) and Bk (30 and 100 nM, T.C.). A23187 (300 nM, O.T.) has very little effect on the assay tissues but induces the release of a labile relaxing substance when given T.C. (300 nM). The tissues were contracted by U46619 (15 nM). Abbreviations as in Figure 3 legend.

the packing of the column or during the experiment, Figure 7). EDRF was very unstable, as shown by the progressive attenuation of the relaxation of the RbAs which were separated from the upper layer of the endothelial cells in the column by delays of 1, 4, 7 and 10 s, respectively. By moving the column down the cascade, it was possible to reduce the delay to each RbA to 1 s (Figure 8). This allowed measurement of the sensitivity of each RbA to EDRF and a clear demonstration of its instability. In most of the experiments the half-life of EDRF appeared to vary between 4 and 7 s, however, detailed calculation of half life was not performed since the rate of disappearance of EDRF seemed to be influenced by the amount detected by the first tissue in the cascade.

The release of EDRF induced by Bk (10–100 nM) or A23187 (30–300 nM) was concentration-dependent and was not inhibited by indomethacin. Bk induced a short-lasting release which tended to disappear during the 1 min infusion whereas the release by A23187 was longer lasting (Figure 6). The release of EDRF was optimal with columns of microcarriers of about 1 ml containing $1-2 \times 10^7$ cells, unlike the release of prostanoids which increased in proportion to the number of cells packed in the column. The release of EDRF by Bk was detectable with concentrations between 1 and 3 nM and was maximal at concentrations between 20 and 100 nM. The release induced by the first challenge was always greater than that induced by subsequent challenges (Figure 6) which were then reproducible for one or two hours, after which a decline in the release was observed. In contrast, A23187 induced a sustained release which was difficult to reproduce and therefore

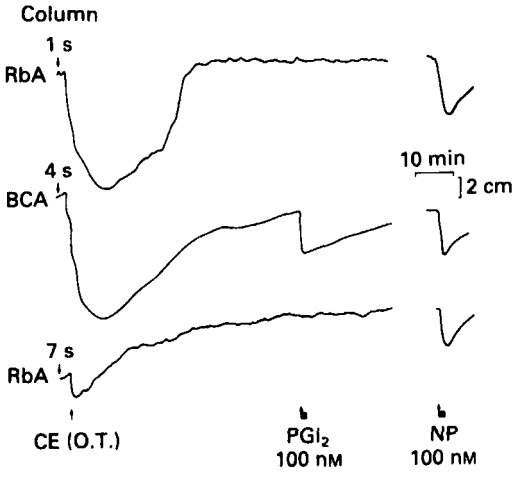

Figure 7 Relaxation of contracted rabbit aortic strips (RbAs) and bovine coronary artery (BCA) induced by the effluent of a column packed with cells (CE). The effects of prostacyclin (PGI_2, 100 nM) and sodium nitroprusside (NP, 100 nM) are also shown. The tissues were contracted by phenylephrine (50 nM). Abbreviations as in Figure 3 legend.

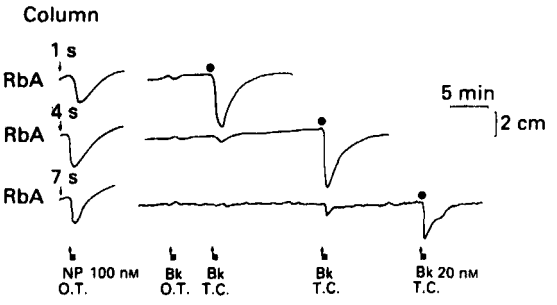

Figure 8 Relaxation of contracted rabbit aortic strips (RbAs) induced by sodium nitroprusside (NP, 100 nM, O.T.). Bradykinin (Bk, 20 nM) has no effect when given O.T., however, when given T.C. it induces the release of a labile relaxing substance. The second and third Bk infusions T.C. are given when the effluent from the column directly superfuses the second and the third tissues in the cascade. The tissues were precontracted by U46619 (15 nM). Abbreviations as in Figure 3 legend.

was not used regularly. EDRF was not released from the column by 5-hydroxytryptamine, noradrenaline, Paf-acether, FMLP, acetylcholine or bethanechol at the concentrations described above.

AA at concentrations up to 100 μM had no effect on the bioassay tissues either given O.T. or T.C. At higher concentrations (300 μM O.T.) it relaxed the assay tissues. When administered T.C. it produced an additional increase in relaxation in the first RbA of the cascade.

Discussion

Vascular endothelium generates two unstable vasodilators, prostacyclin (Moncada *et al.*, 1976; Gryglewski *et al.*, 1976) and EDRF (Furchgott & Zawadzki, 1980). Because of the lack of stability of prostacyclin and of EDRF in their physiological environment (half-lives of about 3 min and 6 s respectively) they can only be detected by biological methods.

The elucidation of the structure of prostacyclin and its chemical degradation product, 6-keto-$PGF_{1\alpha}$, has permitted the use of other methods like RIA, high pressure liquid chromatography and gas chromatography/mass spectrometry for the detection and quantitation of prostacyclin. At present, however, our knowledge of EDRF is very limited and bioassay is the only technique by which it may be studied. Until now, the evidence for the release of EDRF and its action on tissues away from the site of release comes from experiments in which the products of arteries with intact endothelium are detected on arteries denuded of endothelium (Furchgott, 1984; Griffith *et al.*, 1984). More recently Cocks *et al.* (1985) have described experiments similar to our own in which the effluent of a volume of endothelialised beads packed in the barrel of a syringe was used to superfuse a single arterial ring.

We have now adapted the bioassay superfusion cascade of Vane (1964) to the assay of EDRF. This method has been successfully used to study the release of many vasoactive substances from a variety of perfused organs (Vane, 1969; Moncada *et al.*, 1978). In the present study we have replaced the heterogeneous population of cells which are present in any perfused organ with a homogeneous population of cultured vascular endothelial cells on microcarrier beads, packed in a chromatographic column and perfused in the same way as an isolated organ. The effluent from this column superfused in a cascade a series of vascular smooth muscle strips which detect prostacyclin and PGE_2 such as BCA (Dusting *et al.*, 1977), RbMA/RbCA (Bunting *et al.*, 1976) and RSS (Vane, 1957). Inclusion in the cascade of RbA strips (Furchgott, 1955) contracted and denuded of endothelium (Furchgott & Zawadski, 1980) permitted the simultaneous bioassay of EDRF (see Figure 5).

Cultures of porcine aortic endothelial cells (6–14

days old) do not release prostanoids or EDRF in response to stimulation by some receptor agonists at the concentrations used in this study. Other authors have, however, demonstrated release of prostacyclin with higher concentrations of adenosine diphosphate and angiotensin II under similar experimental conditions (Pearson *et al.*, 1983b). Our results indicate that these cells lose their responsiveness to these agonists after a few days in culture, confirming a previous finding (Cocks *et al.*, 1985). However, these agonists do release prostacyclin and/or EDRF from intact vascular tissue *in vitro* and *in vivo* (Furchgott & Zawadski, 1980; Beetens *et al.*, 1982; Gryglewski, 1982; Cocks & Angus, 1983; Forstermann & Neufang, 1984). Paf-acether did not release prostacyclin or EDRF from the column.

Bk and A23187 were the most potent releasers of prostacyclin and EDRF, therefore Bk (20–100 nM) was used as the standard stimulus in most of our studies. The release of EDRF induced by both Bk and A23187 is temporally similar to the release of prostanoids induced by infusions of these agents into guinea-pig isolated lungs. Release induced by Bk is rapid, short-lasting and disappears during the infusion (Palmer *et al.*, 1973; Bakhle *et al.*, 1985) while that induced by A23187 is slow, long-lasting and reaches a peak several minutes after the end of the infusion (Bakhle *et al.*, 1985). A23187 is generally considered to be the most potent releaser of prostacyclin and EDRF from vascular endothelium (Peach *et al.*, 1985). However, in our system Bk is considerably more potent than A23187 in releasing prostacyclin and marginally more potent in releasing EDRF, confirming the findings of Cocks *et al.* (1985).

It is interesting that unlike prostacyclin the release of EDRF does not increase with the number of cells in the column. It is likely that this is due to inactivation of EDRF as it passes from the lower to the upper layers of endothelium inside the column.

AA (10–30 μM) released prostacyclin but not EDRF. Ten fold higher concentrations were required to generate a trace of EDRF. Unlike cyclo-oxygenase, simple additions of substrate are not enough for the lipoxygenase to generate its products (Borgeat & Samuelsson, 1979). Therefore the small and erratic release of EDRF following stimulation with high concentrations of AA neither confirmed nor excluded the possibility that EDRF might be a metabolite of this fatty acid (Furchgott & Zawadzki, 1980; Forstermann & Neufang, 1984; Peach *et al.*, 1985).

Both prostacyclin and EDRF were also released from the column by mechanical stimulation (Figure 7). Indeed, this may be a good method of producing large amounts of EDRF or its degradation products.

Bk and A23187-induced release of prostacyclin was recorded as the relaxation of BCA and RbMA/RbCA. However, when compared with the standard prostacyclin response, the Bk-induced release of prostacyclin from endothelial cells was obviously influenced by the presence of another substance which contracted BCA and relaxed RbMA/RbCA more profoundly than prostacyclin itself (Figure 3). This substance was identified as PGE_2 from its profile of biological activity (Figure 3) and RIA data (Figure 4). Concomitant release of prostacyclin and PGE_2 occurred at a ratio 2.6–4.6 compromising the precise quantification of those two prostanoids by bioassay. The bioassay of prostacyclin, therefore, poses more problems than that of EDRF. The interference of EDRF with the bioassay of prostacyclin is easily eliminated by moving BCAs down the cascade to create a delay of at least 10 s from the upper layer of endothelial cells in the column.

The bioassay of EDRF is not influenced by the presence of prostacyclin or PGE_2 since neither of these two prostanoids affected the tone of the RbAs. We have shown that inhibition of prostanoid generation by indomethacin does not influence the generation of EDRF by endothelial cells as previously reported (Furchgott *et al.*, 1984; Peach *et al.*, 1985), thus EDRF can be assayed on its own if the cells in the column are treated with indomethacin.

Bioassay of EDRF alone requires 3–4 RbAs mounted in a cascade and separated by a 3 s delay between each. The attenuation of RbA relaxations down the cascade is characteristic of EDRF. In most of the experiments EDRF did not survive for 7 s; indeed, in some experiments a 4 s delay was sufficient for EDRF to disappear (Figure 7) indicating that the half-life of EDRF was, on average, less than 7 s. This disappearance of EDRF down the cascade did not seem to be linear but rather depended on the amount of EDRF being released, as recorded by the relaxation of the uppermost tissue in the cascade (Figure 6). Detailed analysis of this phenomenon is difficult without a knowledge of the dose-response curve of EDRF on the bioassay tissues.

In conclusion, we have described a method for the bioassay of prostacyclin and EDRF resulting from the combination of several established techniques. The disadvantages of this method include the following: firstly, the generation of the endothelium-derived vasodilators occurs in cultured endothelial cells which have probably lost their receptors for several physiological agonists. Secondly the cells are separated from the underlying smooth muscle so a biochemical collaboration between those two vascular layers cannot be studied. Finally, the bioassay of prostacyclin is influenced by the presence of PGE_2 in the effluent. On the other hand, the advantage of this technique include the simultaneous detection of prostacyclin and EDRF released from endothelial cells; the lack of interference of these vasodilators on bioassay

tissues and the potential for studying the effects of chemical and physical factors on the generation of the endothelium-derived vasodilators.

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