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Different patterns of release of endothelium-derived relaxing factor and prostacyclin

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1 Release of endothelium derived relaxing factor (EDRF) and prostacyclin (PGI_2) from endothelial cells (EC) cultured from bovine aortae was measured by bioassay and radioimmunoassay, respectively, during infusions (10 min) of bradykinin (BK), adenosine diphosphate (ADP), arachidonic acid (AA), alkaline buffers and the free-bases (FB) of L-arginine or D-arginine. Release of EDRF from the luminally perfused rabbit aorta was also measured during infusions (10 min) of acetylcholine (ACh), substance P and ADP.

2 Bradykinin (10 or 30 nM) infused through the column of EC induced release of both EDRF and PGI_2, neither of which was maintained for the duration of the infusion.

3 ADP (1.6 or 4 μM) infused through the column of EC induced release of a EDRF which was maintained for the duration of the infusion and a release of PGI_2 which lasted for a much shorter period.

4 Arachdonic acid (30 or 90 μM) infused through the column of EC caused a sustained release of EDRF and PGI_2, both of which outlasted the infusion of AA.

5 L-Arginine FB, D-arginine FB or alkaline buffer infused through the column of EC released EDRF, but only small amounts of PGI_2. The release of EDRF outlasted the period of infusion and was due to an increase in the pH of the Krebs solution perfusing the EC.

6 Infusions of ACh (0.25–1 μM) or ADP (4–16 μM) caused a sustained release of EDRF from the luminally-perfused rabbit aorta, whereas infusion of substance P (3.3–10 μM) caused only a transient release of EDRF.

7 These results show that distinct patterns of EDRF release exist to different agonists in both cultured and *in situ* EC, and that EDRF and PGI_2 do not necessarily follow the same time course of release. Furthermore, sustained release of EDRF does not require the constant infusion of the precursor, L-arginine, whereas sustained release of PGI_2 only occurs when AA, the precursor of PGI_2, is present in the extracellular medium.

Keywords: Bradykinin; adenosine diphosphate; arachidonic acid; L-arginine; D-arginine; alkaline buffer; acetylcholine; substance P

Introduction

Prostacyclin (PGI_2; Moncada *et al.*, 1976) and endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) are both released from endothelial cells (EC) by a variety of agonists including acetylcholine (ACh), adenosine diphosphate (ADP), bradykinin (BK) and substance P (see Furchgott *et al.*, 1984; Gryglewski *et al.*, 1988). This receptor-mediated release of EDRF and PGI_2 is coupled most probably at the level of phospholipase C (de Nucci *et al.*, 1988a). In contrast the free base (FB) of arginine can change the pH of Krebs buffer by about 1.0 pH unit and thereby selectively release EDRF with very little accompanying PGI_2, through a mechanism which is receptor-independent (Mitchell *et al.*, 1991a). Thus, alkaline buffers appear to induce the release of EDRF by a mechanism that bypasses the coupled release stage, thereby provoking a release of EDRF independent from that of PGI_2.

Although EDRF and PGI_2 are co-released by several agonists their chemical nature, biosynthetic pathway and mode of action differ. PGI_2 is a metabolite derived from the 20 carbon fatty acid, arachidonic acid (AA; Moncada *et al.*, 1976) whereas EDRF is NO or a related molecule (Palmer *et al.*, 1987; Myers *et al.*, 1990) formed from L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988). The biosynthetic pathways of EDRF and PGI_2 also differ with regard to substrate availability. The level of free AA available for PGI_2 synthesis is tightly controlled (see Irvine, 1982) whereas intracellular levels of L-arginine are relatively high (approximately 0.1–1 mM), and remain stable during prolonged EDRF release (Mitchell *et al.*, 1990a) due to constant generation from intracellular stores (Mitchell *et al.*, 1990b). On the other hand there are similarities between the two pathways for both EDRF (Förstermann *et al.*, 1991; Mitchell *et al.*, 1991b) and PGI_2 (Moncada *et al.*, 1976) are synthesized by particulate enzymes (EDRF synthase and cyclo-oxygenase/prostacyclin synthase respectively). In addition, both cyclo-oxygenase and the EDRF synthase-related enzyme, NO synthase in macrophages, utilize molecular oxygen (Ryhage & Samuelsson, 1965; Stuehr *et al.*, 1991).

Although the release of EDRF induced by several agonists has been extensively studied, the kinetics of release has not been fully explored. Moreover, the temporal relationship between EDRF and PGI_2 release from cultured EC has been investigated primarily with receptor-activating agents (White & Martin, 1989). The objective of this study was to determine the duration of release from cultured EC of EDRF and PGI_2 following infusions of their respective precursors, or stimulation of the EC by receptor-dependent or -independent agonists.

Some of these results were presented to the British Pharmacological Society (de Nucci *et al.*, 1988b).

Methods

Endothelial cell culture

Endothelial cells were isolated by treatment of bovine aortae with 0.02% (w/v) collagenase. Cells were grown to confluence

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in plastic vessels, then removed by treatment with 0.05% (w/v) trypsin, and seeded onto Cytodex 3 microcarrier beads (Pharmacia), as previously described (de Nucci *et al.*, 1988a). The beads were packed into a jacketed column and perfused with Krebs solution (5 ml min^{-1} at 37°C) containing superoxide dismutase (SOD, 10 units ml^{-1}; Gryglewski *et al.*, 1986).

Preparation of the rabbit aorta

New Zealand White rabbits were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.v.) and exsanguinated via the common carotid artery. The thorax and abdomen were opened and the aorta exposed and carefully cleared of connective tissue *in situ*. The mesenteric and coeliac arteries were ligated close to their origins from the aorta and a segment of the aorta from the aortic arch to the left renal artery was carefully removed. A cannula was inserted into the cardiac end of the aorta, secured with thread, mounted in a heated tissue bath (37°C) and the aorta was perfused through the lumen at 5 ml min^{-1} with warmed (37°C) gassed (95% O_2:5% CO_2) Krebs solution containing SOD (10 units ml^{-1}) and indomethacin (5 μM) (Warner *et al.*, 1989).

EDRF detection by bioassay

The effluent from either the column or from the luminally-perfused rabbit aorta superfused in a cascade (Vane, 1964) four-spirally cut rabbit aortic strips (RbAs) that were denuded of endothelium. Effluent from the column or donor aorta reached the consecutive RbAs after 1, 4, 7, and 10 s. Drugs were infused either over the assay tissues (o.t.) as a control, or through the EC column (t.c.) or donor aorta (t.a.). The assay tissues were superfused first with Krebs solution containing U46619 (30 nM) until a stable contraction was obtained. Aortic strips prepared from 5–10% of rabbits were insensitive to U46619 (30–300 nM) and were consequently contracted with noradrenaline (300 nM). The assay tissues were then calibrated by the relaxant effects of glyceryl trinitrate (GTN), and the sensitivities on the recordings of the RbAs adjusted electronically to be roughly equal. The sensitivity of batches of EC to EDRF/PGI_2 releasing agonists varied; it was therefore necessary to test each batch with doses of a given agonist in order to choose an optimum concentration (causing EDRF release which relaxed the first RbA $\geqslant$ 20 pmol GTN and PGI_2 release $\geqslant$ 0.5 ng ml^{-1}). The relaxations of the assay tissues were recorded with auxotonic levers (Paton, 1957), attached to Harvard isotonic transducers and displayed on a six channel Watanabe Recorder (type WR3101). Indomethacin (5 μM) was infused o.t. throughout the experiments to prevent synthesis of cyclo-oxygenase products by the assay tissues. When AA was infused, the dual cyclo-oxygenase and lipoxygenase inhibitor BW755C (30 μM; Higgs *et al.*, 1979) was infused o.t. to prevent the formation of eicosanoids in the tissues in the cascade. In experiments where arginine FB or alkaline buffers were used the column of EC was perfused with Krebs solution without KH_2PO_4 to avoid precipitation of calcium. An appropriate infusion o.t. of KH_2PO_4 restored the phosphate level in the cascade. In experiments using the luminally-perfused rabbit aorta homatropine (1.5 μM), phenoxybenzamine (0.3 μM), mepyramine (1 μM) and methysergide (0.3 μM) were infused o.t. The time course of release of EDRF, as measured by activation of isolated soluble guanylate cyclase, correlates with relaxation of rabbit aortic strips used in bioassay (Kondo *et al.*, 1989). Thus bioassay tissues give a reliable assessment of the duration of EDRF release.

Measurement of PGI_2 by radioimmunoassay

The effluent from the column was collected after passing over the bioassay cascade tissues and analyzed by radioimmunoassay (RIA) for 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$; Salmon, 1978) as a measure of PGI_2 release from the EC. The detection limit on the RIA was approximately 0.5 ng ml^{-1}.

Measurement of pH

The pH was measured with a Corning pH 105 meter.

Materials

The Krebs solution (pH; 7.5–8.0) had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.17, $CaCl_2 \cdot 6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Bradykinin acetate (BK), adenosine diphosphate di (monocyclohexylammonium) salt (ADP), arachidonic acid sodium salt (AA), superoxide dismutase (SOD) from bovine erythrocytes, free base (FB) of L-arginine or D-arginine, indomethacin, substance P acetate, acetylcholine chloride (ACh), homatropine hydrobromide, trizma and glycine buffers were supplied by Sigma (Poole, U.K.). Sodium carbonate buffer (Na_2CO_3; anhydrous, Analar) was freshly prepared each day (100 mg ml^{-1}) in distilled water. This was then diluted to produce a solution of the same buffering capacity as infusions of L- or D-arginine FB (1–3 mM). The Krebs solution salts, glucose and Na_2CO_3 were obtained from B.D.H. (Dagenham, U.K.). Methysergide bimaleate was obtained from Sandoz Prod. Ltd. (Leeds, U.K.), mepyramine maleate from May & Baker (Dagenham, U.K.), and phenoxybenzamine from S.K. & F. (Stevenage, U.K.). Collagenase (Cooper Biomedical) was obtained from Lorne Diagnostics (Suffolk, U.K.) and trypsin and Dulbecco's modified Eagle's medium were obtained from Flow Laboratories. [^{3}H]-6oxo-$PGF_{1\alpha}$ was purchased from New England Nuclear. The antiserum and 6-oxo-$PGF_{1\alpha}$ for the RIA and BW755C 3-amino-1-[*m*-(trifluoromethyl)-phenyl] -2 pyrazoline) were gifts from J.A. Salmon and G. Higgs (Wellcome Research Laboratories, Beckenham, U.K.) respectively. U46619 (9,11-dideoxy-9α-methano epoxyprostaglandin $F_{2\alpha}$) was a gift from J. Pike (Upjohn, Kalamazoo, U.S.A.).

Statistics

Results are shown as mean $\pm$ s.e.mean for n experiments. Student's unpaired t test was used to assess the difference between means and a P value of <0.05 was taken as significant.

Results

Cultured endothelial cells

Release of EDRF and PGI_2 by bradykinin or ADP Neither BK (10 or 30 nM) nor ADP (1.6 or 4 μM) affected the strips of RbAs when infused o.t. Infusions of BK t.c. provoked transient release of EDRF (declining after 2.5 $\pm$ 0.3 min, $n = 6$). However, the release of EDRF induced by ADP (1–4 μM) was sustained throughout the 10 min infusion period (Figure 1a, $n = 6$).

The release of PGI_2 induced by infusions of BK or ADP reached a maximum in the 2nd–4th minute and then returned rapidly to basal levels (Figure 1b).

The transient release of EDRF induced by BK and the sustained release induced by ADP occurred at all concentrations used.

Release of EDRF and PGI_2 by arachidonic acid The release of EDRF induced by AA (30 or 90 μM) was sustained ($n = 4$) and in 2 experiments outlasted the infusion period (Figure 2a). The release of PGI_2 from EC induced by infusions of AA (30 or 90 μM) was not dose-dependent and always outlasted the infusion period (Figure 2b, $n = 4$).

Release of EDRF and PGI_2 by the free bases (FB) of L-arginine or D-arginine The free bases (FB) of L- or D-arginine (0.5–3 mM) or Na_2CO_3, at concentrations that increased the pH of the Krebs solution by about 1 pH unit to 8.6–9.0, infused o.t.

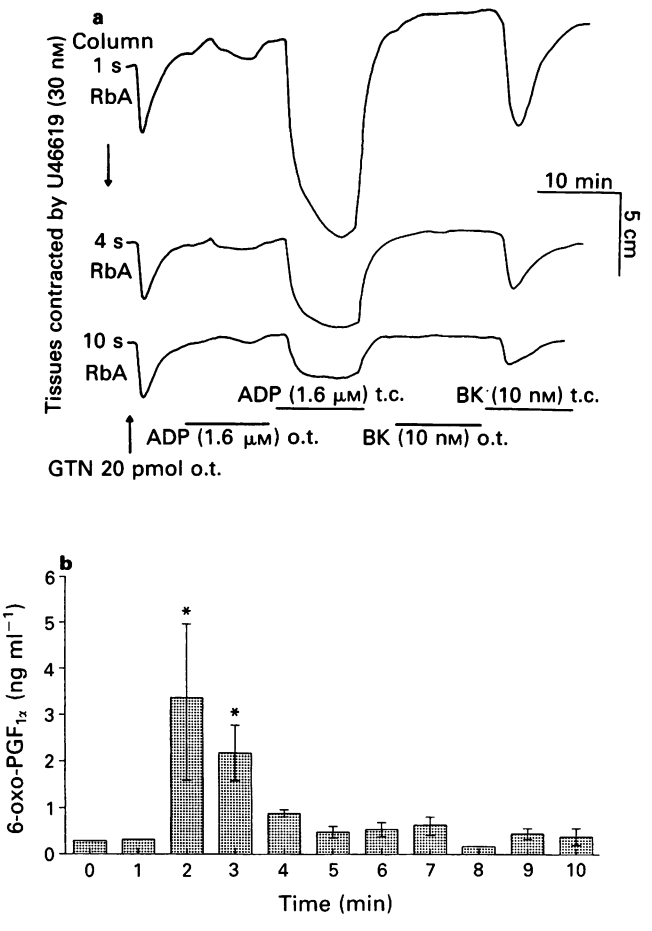

Figure 1 (a) Release of EDRF induced by 10 min infusions of ADP and bradykinin (BK) from bovine aortic cultured endothelial cells treated with superoxide dismutase (10 units ml^{-1}). The perfusate from the column of endothelial cells superfused a cascade of four rabbit aortic strips which were denuded of endothelium (RbAs; the first, second and fourth are shown). The RbAs were contracted by infusion of U46619 (10 nM). Control infusions of ADP (1.6 μM) or BK (10 nM) were given over the assay tissues (o.t.). Injections of glyceryl trinitrate (GTN) o.t. (40 pmol) caused relaxation of the RbAs. Infusions of ADP and BK through the column of endothelial cells (t.c.) caused release of EDRF. The release of EDRF induced by ADP was sustained throughout the infusion period and stopped abruptly on termination of the infusion. However the release of EDRF induced by BK was transient, the RbAs returning to their original baseline during the course of the infusion. This is a bioassay trace from a typical experiment. (b) Release of prostacyclin (PGI_2) induced by infusions of ADP. Effluent from a column of endothelial cells was collected at 1 min intervals and PGI_2 release measured by RIA for 6-oxo-$PGF_{1\alpha}$. Infusions of ADP (1.6 or 4 μM) through the column of endothelial cells (t.c.) for 10 min periods induced a transient release of PGI_2, that was maximal during the second and third minute, and subsequently returned to basal levels. Similar results were obtained with infusions of BK (10 or 30 nM). Data with different agonist concentrations were pooled due to variability of the endothelial cells (see methods). $^*P < 0.05$.

had small contractile effects on the RbAs. However, when infused t.c. they provoked a sustained release of EDRF similar to that seen with AA (Figure 3, $n = 4$). The accompanying release of PGI_2 was small and variable. There was no significant increase above basal in the case of L- or D-arginine free base ($n = 4$). However, there was a 3.5 ± 0.75 fold increase in PGI_2 during the 4th and 5th minutes of a 10 min infusion of Na_2CO_3 ($n = 4$). Infusions of L- or D-arginine FB increased the pH of the Krebs solution by 0.6–1.2 units when infused o.t. but the increase was less pronounced when the buffers were infused t.c. (Figure 3 lower panel). Trizma base or glycine buffer which changed the pH of the Krebs solution to a similar extent also resulted in the selective release of EDRF (data not shown).

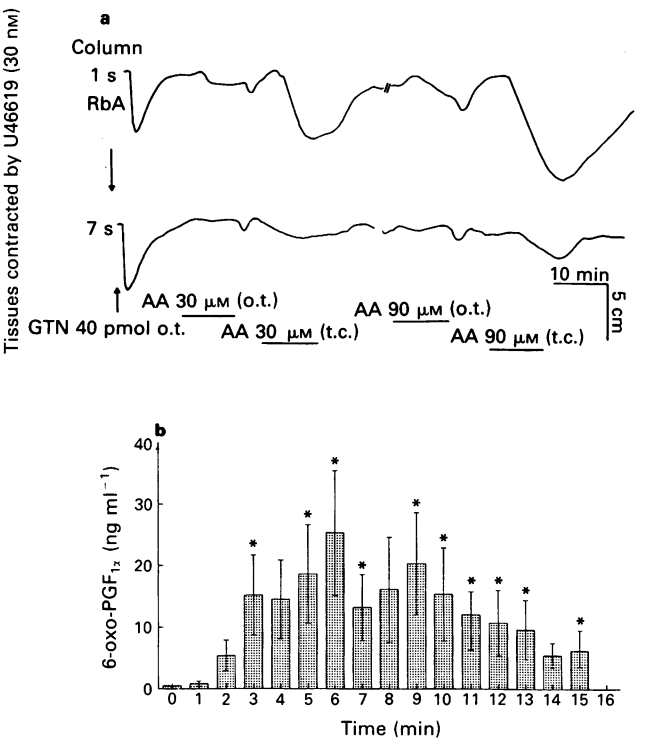

Figure 2 (a) Release of EDRF induced by infusions of arachidonic acid (AA) from columns of bovine aortic cultured endothelial cells. Experimental details are the same as for Figure 1. Infusions of AA (30 and 90 μM) over the RbAs (o.t.) induced small relaxations. When AA was infused for a period of 10 min through the column of endothelial cells (t.c.) it provoked a sustained and prolonged release of EDRF. Note that when the infusion of AA was stopped, the release of EDRF only gradually decreased. (b) Release of prostacyclin (PGI_2) induced by infusions of arachidonic acid (AA). The experimental procedures were the same as in Figure 1b. When AA (30–90 μM) was infused through the column of endothelial cells (t.c.) the level of PGI_2 released increased reaching a maximum by the third minute and remaining at this level for the duration of the infusion (10 min). When the infusion was removed the release of PGI_2 did not return immediately to basal but remained at an elevated level ($\geq$5 min, $^*P < 0.05$ are shown for values as compared to the basal level).

Luminally perfused rabbit aorta

Release of EDRF by ACh or ADP Acetylcholine (250 nM) had no effect on the assay tissues when given o.t. However, when infused for 10 min through the aorta (t.a.) of ACh provoked a sustained release of EDRF (Figure 4, $n = 4$). With the luminally perfused rabbit aorta higher concentrations of ADP (4 or 16 μM) were needed than with the EC columns to provoke the release of EDRF. ADP (4–16 μM) caused a sustained relaxation of the assay tissues when infused o.t. When ADP was infused t.a. the relaxation was greater ($n = 3$ out of 4; Figure 4) and this release of EDRF was sustained throughout the infusion period.

Release of EDRF by substance P Substance P (3.3 μM) had no effect when infused o.t. When infused t.a., substance P provoked a transient release of EDRF (Figure 4, $n = 4$).

Discussion

Here we have shown that different patterns of EDRF release occur from EC both in culture and *in situ* and the duration of PGI_2 release does not necessarily correlate with the duration of EDRF release induced by a given agonist.

Bradykinin infusions did not cause sustained release of either PGI_2 or EDRF. This desensitization to BK has been previously noted in guinea-pig isolated lungs, where infusions

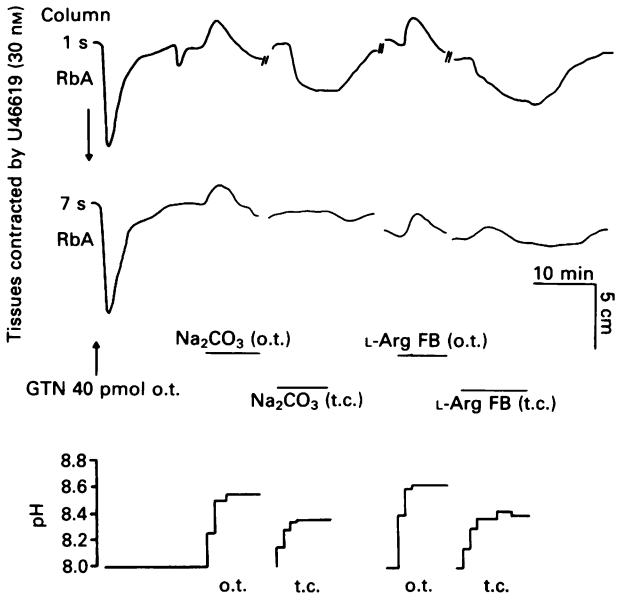

Figure 3 Release of EDRF induced by infusions of L-arginine free base (L-Arg, 3 mM) and Na_2CO_3 from bovine aortic cultured endothelial cells. Experimental details are the same as in Figure 1a. The first and second of 4 RbAs are shown. L-Arg and Na_2CO_3 infused over the RbAs (o.t.) had a contractile effect. However, when either agent was infused through the column of endothelial cells (t.c.) it induced a sustained and prolonged release of EDRF. Similar results were seen using the free base of D-arginine (3 mM; $n = 4$). The histogram at the bottom represents the change in the pH of the Krebs solution induced by L-Arg and Na_2CO_3 respectively.

of BK neither caused sustained release of thromboxane A_2 (Piper & Vane, 1969) nor of PGI_2 (Bakhle *et al.*, 1985), and in cultured EC where the release of PGI_2 induced by infusions of BK was transient (White & Martin, 1989). In our cells this short lived action of BK is unlikely to be due to inactivation of BK by angiotensin converting enzyme (ACE) for they have little or no ACE activity (de Nucci *et al.*, 1988c).

Substance P also induced a transient release of EDRF from the whole rabbit aorta, confirming previous observations on rabbit aortic strips with intact endothelium (Beny *et al.*, 1987). Thus this pattern of transient EDRF release is not peculiar to EC in culture. Whether this desensitization of the endothelium

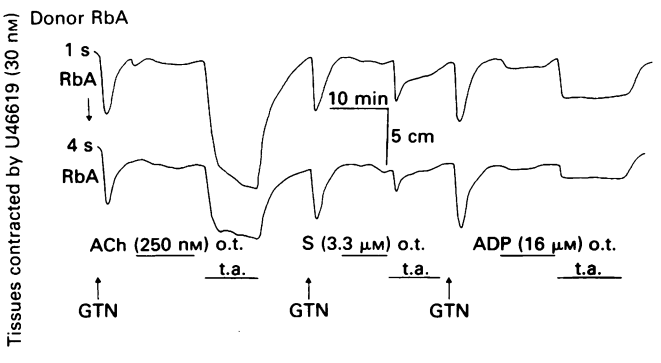

Figure 4 Release of EDRF induced by infusions of acetylcholine (ACh), adenosine diphosphate (ADP) and substance P (SP) from a luminally perfused rabbit aorta. Experimental details are the same as in Figure 1a with the exception of the source of EDRF generation. Infusions of ACh (250 nM; 10 min) or ADP (16 μM) through the rabbit aorta donor (t.a.) induced the release of EDRF which was sustained. The tissues returned to their original baseline immediately on removal of the infusion of ACh or ADP. In contrast, SP (3.3 μM; 10 min) infused t.a. induced a transient release of EDRF, the RbAs returning to their original baseline during the course of the infusion. o.t., over the RbAs.

to BK or substance P is due to rapid phosphorylation of G-proteins by protein kinase C and consequent uncoupling of the receptor complex remains to be clarified.

It is interesting that agents which increase the pH of the Krebs solution can cause a sustained release of EDRF. These observations support our previous findings that the free bases of L or D-arginine release EDRF to similar extents due to elevated pH and not by supplying substrate to NO synthase (Mitchell *et al.*, 1991a). Release by alkalinisation is mainly dependent on the presence of extracellular calcium, as opposed to receptor-mediated release where intracellular calcium mobilization may be important (Mitchell *et al.*, 1991a). This suggests that elevation of extracellular pH causes the entry of extracellular calcium, which would directly stimulate EDRF synthesis, for the enzyme responsible for EDRF formation (EDRF synthase; Förstermann *et al.*, 1991; Mitchell *et al.*, 1991b) by EC is calcium-dependent. However, an increase in the extracellular pH to 8.6 is unlikely to be a physiological stimulus for EDRF. Although EC respond to, and attempt to buffer, an elevated pH (possibly by exporting H^+ into the extracellular medium in exchange for Na^+), local changes in the blood will not produce this degree of alkalinisation.

The finding that ACh induces a sustained release of EDRF from the luminally perfused rabbit aorta, as suggested from experiments on isolated strips (Beny *et al.*, 1987) highlights the paradox in that ACh is a potent and prolonged releaser of EDRF, but is an unlikely endogenous agonist (see Kalsner, 1988). It has not been demonstrated that ACh diffuses from parasympathetic nerve terminals to activate the EC.

The sustained release of EDRF induced by ADP is common to columns of EC and to luminally perfused aortae and could indicate that ADP is an important physiological or pathophysiological agonist. One of the more likely pathophysiological sources of ADP may be activated platelets (Haslam, 1964), in which case it would be desirable for areas with an intact endothelium to release EDRF to decrease platelet adhesion, and possibly inhibit further platelet aggregation. As ADP increases intracellular calcium in EC initially by releasing calcium from intracellular stores, and subsequently by stimulating the entry of extracellular calcium (Pritton *et al.*, 1987; Luckhoff & Busse, 1986) the transient release of PGI_2 induced by ADP, which we describe here, is most probably correlated with the transient release of calcium from intracellular stores, as has been shown to be the case for porcine EC (Pearson *et al.*, 1983).

The rapid decrease in PGI_2 release following stimulation of the EC by agonists other than AA is unlikely to be due to diminished activity of cyclo-oxygenase, for infusions of exogenous AA produced a sustained release of PGI_2. However, it may possibly be explained by an increase in the reacylation of the released AA or the depletion of AA from the available pool of phospholipids. In contrast to PGI_2 the release of EDRF does not appear to be suppressed by the effective removal of L-arginine, the putative precursor. Indeed, as we show here, EC release EDRF for a prolonged period of time in the absence of extracellular L-arginine. This is explained by a high intracellular level of L-arginine (100 μM; Mitchell *et al.*, 1990a), some thirty times higher than the apparent K_m for purified endothelial NO synthase (Pollock *et al.*, 1991) and by the ability of EC to maintain intracellular arginine, despite continuous EDRF release, by generation from intracellular stores (Mitchell *et al.*, 1990b). Thus, it seems that the availability of substrate for EDRF and PGI_2 synthesis regulates the duration of release of these autocoids following stimulation of the endothelium.

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