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Alkaline buffers release EDRF from bovine cultured aortic endothelial cells

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1 Release of endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2) from bovine cultured aortic endothelial cells (EC) was measured by bioassay and radioimmunoassay, respectively.

2 Bradykinin (BK, 3–30 pmol), adenosine diphosphate (ADP, 2–6 nmol) or the sodium ionophore monensin (40–100 nmol) injected through a column of EC released EDRF. L-Arginine free base (FB; 10–20 μmol) or D-arginine FB (10–20 μmol) injected through the column of EC released similar amounts of EDRF and also caused an increase in pH of the Krebs solution perfusing the EC from 7.5–8.0 to 8.6–9.5. Sodium carbonate (Na_2CO_3) an alkaline buffer which caused the same changes in the pH of the Krebs solution also induced the same release of EDRF. The hydrochloride salts of L- or D-arginine did not cause either release of EDRF when injected through the column of EC or increases in the pH of the Krebs solution.

3 Inhibitors of either diacylglycerol lipase (RHC 80267) or kinase (R59022) inhibited the release of EDRF induced by BK or ADP but potentiated the release induced by L-arginine FB, monensin (40–100 nmol) or alkaline buffer (Na_2CO_3). R59022 and RHC 80267 infused through the EC increased the basal release of EDRF.

4 When calcium chloride was omitted from the Krebs solution the release of EDRF induced by alkaline buffer (Na_2CO_3; pH 8.6–9.5) or L-arginine FB (10–20 μmol) was selectively inhibited when compared to that induced by BK (3–30 pmol) or ADP (2–6 nmol). This inhibition was reversed when calcium (2.5 mM) was restored.

5 N^G-monomethyl-L-arginine (NMMA; 30 μM) inhibited release of EDRF induced by BK (10–30 pmol) or alkaline buffers (Na_2CO_3 or D-arginine FB; pH 8.6–9.5). This inhibition was partially reversed by L- but not D-arginine FB or HCl (30–100 μM).

6 Prostacyclin was released when BK (10 pmol), ADP (2 nmol) or arachidonic acid (30 nmol) were injected through the column of EC. However, monensin (40 nmol) or alkaline buffers (pH 8.6–9.5) did not release detectable amounts of PGI_2 as measured by radioimmunoassay for 6-oxo-prostaglandin $F_{1\alpha}$.

7 Thus alkalinisation of the external bathing solution can release EDRF from cultured EC by a mechanism which does not involve receptor activation and which depends on the presence of extracellular calcium.

Keywords: Prostacyclin; N^G-monomethyl-L-arginine; monensin; calcium; diacylglycerol kinase inhibitor; diacylglycerol lipase inhibitor; bradykinin; adenosine diphosphate; pH

Introduction

Macrophages can synthesize nitrates and nitrites from the guanidino group of L-arginine (Iyengar *et al.*, 1987; Hibbs *et al.*, 1987), a process which is inhibited by N^G-monomethyl-L-arginine (L-NMMA; Hibbs *et al.*, 1987). After the demonstration that nitric oxide (NO) accounted for the biological activity of endothelium-derived relaxing factor (EDRF, Palmer *et al.*, 1987), several groups showed that NO was formed in macrophages and that this pathway was dependent on the metabolism of L-arginine (Marletta *et al.*, 1988; Hibbs *et al.*, 1988; Stuehr *et al.*, 1989). L-Arginine is also a precursor of EDRF in the endothelial cell (EC; Palmer *et al.*, 1988a) and EDRF release is reversibly inhibited by L-NMMA (Palmer *et al.*, 1988b). Although L-arginine does not cause or augment the release of EDRF from porcine (Palmer *et al.*, 1988a) or bovine (this paper) cultured EC, the inhibition by L-NMMA of EDRF release is reversed by L-arginine (Palmer *et al.*, 1988b).

We have shown that the receptor-mediated release of EDRF and prostacyclin (PGI_2) is coupled (de Nucci *et al.*, 1988a) although other agonists which do not act on receptors such as the calcium ionophore A23187 (Gryglewski *et al.*, 1986a) or thimerosal (Förstermann *et al.*, 1986; Mülsch *et al.*, 1987) can also release EDRF and prostacyclin (PGI_2) from EC. Here we demonstrate that L-arginine or D-arginine free base (FB) but not their hydrochloride (HCl) salts induce release of EDRF from EC without coupled release of PGI_2 and that this EDRF release is not due to receptor activation. This novel mechanism of EDRF release is based on alkalinization of the Krebs solution and depends on the presence of extracellular calcium. Some of this work has been 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% (wt/vol) trypsin, and seeded onto Cytodex 3 microcarrier beads (Pharmacia). The beads were stirred for 3–4 days until they were covered with a confluent layer of EC, as previously described (de Nucci *et al.*, 1988a).

Detection of EDRF by bioassay

The EC on beads (2–3 ml carrying 1–2 × 10^7 cells) were packed into a jacketed column and perfused (5 ml min^{-1} at

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37°C) with gassed (95% O_2:5% CO_2) Krebs solution, which contained superoxide dismutase (SOD, 10 u ml^{-1}; Gryglewski *et al.*, 1986b) and indomethacin (5 μM). The effluent from the column superfused a cascade (Vane, 1964) of four spirally cut strips of rabbit aortae (RbA) that were denuded of endothelium. The effluent from the column reached the consecutive RbA after 1, 4, 7 and 10 s. Drugs were given over the assay tissues (OT) as a control or through the column of endothelial cells (TC).

The assay tissues were superfused with Krebs solution containing either U46619 (30 nM) or noradrenaline (100–300 nM) until a stable contraction was achieved. Glyceryl trinitrate (GTN) was then used to calibrate the relaxations of the RbA. The recording of the RbA was electrically adjusted so that they showed a similar relaxation with a given dose of GTN.

Measurement of prostacyclin release

In experiments to measure PGI_2 release, indomethacin was omitted from the Krebs solution perfusing the EC but added as an infusion over the bioassay tissues. The column effluent was collected and analysed by radioimmunoassay (RIA) for 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) as a measure of PGI_2 release by the EC (Salmon, 1978).

Removal of phosphate from the Krebs solution

When L- or D-arginine FB or sodium carbonate (Na_2CO_3) buffer were used, the column of EC was perfused with Krebs solution without KH_2PO_4 to avoid precipitation of phosphates. An appropriate solution of KH_2PO_4 was infused over the RbA in order to complete the salt requirement of the Krebs solution.

Measurement of pH

The pH was measured with a Corning pH 105 meter.

Statistics

During OT infusions of the test drugs (R59022, RHC 80267, calcium chloride or L-NMMA) control injections of EDRF-releasing agents were made through the column of EC. The resulting relaxations of the 4 RbAs were each measured in mm and added together to express the control release. Test drugs were then infused TC and the EDRF-releasing agents were retested. The relaxations of the 4 RbAs were again measured and added together and the result expressed as a % of the control release. Any changes in the sensitivity of the RbAs occurring during the course of the experiment were assessed by changes in relaxations induced by a given dose of GTN. Thus, if the GTN response was increased or decreased by x fold with respect to control it was assumed that this represented a similar change in the sensitivities of the tissues to EDRF. The measured responses to EDRF at the same time point were therefore multiplied by the factor x before being compared with control. Unpaired Student's t test was used and a P value of less than 0.05 taken to be significant.

Materials

The Krebs solution (pH 7.5–8) 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), L-arginine free base (FB), L-arginine hydrochloride (HCl), D-arginine FB, D-arginine HCl, monensin sodium salt and indomethacin were supplied by Sigma (Poole, Dorset). Glyceryl trinitrate (GTN) was obtained from Lipha (Middlesex). Trypsin was obtained from Flow Laboratories (Hertfordshire). Superoxide dismutase from bovine erythrocytes was a gift from Grunenthal (Stolberg, W. Germany). Sodium carbonate (Na_2CO_3; anhydrous, Analar, BDH Chemicals Ltd, Poole, Dorset) buffer was freshly prepared each day (100 mg ml^{-1}) in distilled water. This was then diluted with distilled water to produce a solution of the same buffering capacity in Krebs solution as the equivalent volume of L- or D-arginine FB (1 M stock) such that 10–20 μl of either injected into 5 ml of Krebs solution increased the pH from 7.5–8.0 to 8.6–9.5.

[^{3}H]-6-oxo-$PGF_{1\alpha}$ was purchased from New England Nuclear; 6-oxo-$PGF_{1\alpha}$ and the antiserum for the RIA were gifts from Dr J.A. Salmon (Wellcome Research Laboratories, Beckenham). U46619, the code name for 9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$ was a gift from Dr J. Pike (Upjohn Co., Kalamazoo, U.S.A.). R59022, the code name for 6-(2)-4((4-fluorophenyl)phenylmethylene)-1-piperidinyl)-7-methyl-5H-thiazolo-(3,2-a)pyrimidin-5-one, was supplied by Janssen Pharmaceutical (Oxon). RHC 80267 (1,6-di(o-(carbamoyl) cyclohexanone oxime)hexane) was a gift from Dr J. Sutherland (Revlon Health Care Group, U.S.A.). N^G-monomethyl-L-arginine citrate (L-NMMA) was obtained from Ultrafine Chemicals (Manchester).

Results

Release of EDRF by extracellular alkalinization

L- or D-arginine FB (10 or 20 μmol) injected TC released equal amounts of EDRF with a concomitant increase in the pH of the Krebs solution from 7.5–8.0 to 8.6–9.5 (D-arginine FB caused the release of EDRF which was 101 $\pm$ 4% of that induced by the same concentration of L-arginine FB; $n = 4$). L- or D-arginine HCl (10 or 20 μmol) did not release EDRF or increase the pH of the Krebs solution. When Na_2CO_3 solution was added to L- or D-arginine HCl in order to increase the pH, injections TC resulted in EDRF release which was of a similar magnitude to that induced by Na_2CO_3 solution given alone ($n = 4$). Injections of Na_2CO_3 which caused the same increase in the pH of the Krebs solution as L-arginine FB resulted in the release of EDRF which was 99 $\pm$ 16% ($n = 10$) of that induced by L-arginine FB. Figure 1 shows the pH-dependent release of EDRF induced by L-arginine FB (20 μmol) or L arginine HCl (20 μmol; with the addition of Na_2CO_3).

The effect of RHC 80267 on the release of EDRF

Bradykinin (3–30 pmol), ADP (2–6 nmol), L-arginine FB (10–20 μmol) or monensin (40–100 nmol) injected TC induced release of EDRF in the presence of the diacylglycerol lipase inhibitor RHC 80267 (10 μM; Sutherland & Amin, 1982) infused OT. When RHC 80267 was infused TC there was a relaxation of the RbAs which diminished down the cascade, indicating an increase in the basal release of EDRF (45 $\pm$ 12%, $n = 4$). The stimulated release of EDRF induced by BK or ADP was inhibited in the presence of RHC 80267 ($n = 5$) whereas that induced by L-arginine FB ($n = 5$) or monensin ($n = 3$) was potentiated when compared to the control release of EDRF ($P < 0.05$). Figure 2a shows the effects of RHC 80267 (30 μM) on EDRF release induced by BK (10 pmol), ADP (2 nmol), L-arginine FB (20 μmol) and monensin (40 pmol). Figure 2b shows the release of EDRF as the % $\pm$ the s.e.mean of control for 3–5 experiments.

The effect of R59022 on the release of EDRF

Similar effects to those obtained with RHC 80267 were observed when the diacylglycerol kinase inhibitor R59022 (10–30 μM; de Chaffoy de Courcelles *et al.*, 1985) was infused through the column of EC. The maintained basal release of EDRF was increased (37 $\pm$ 9%) in 5 out of 8 experiments and

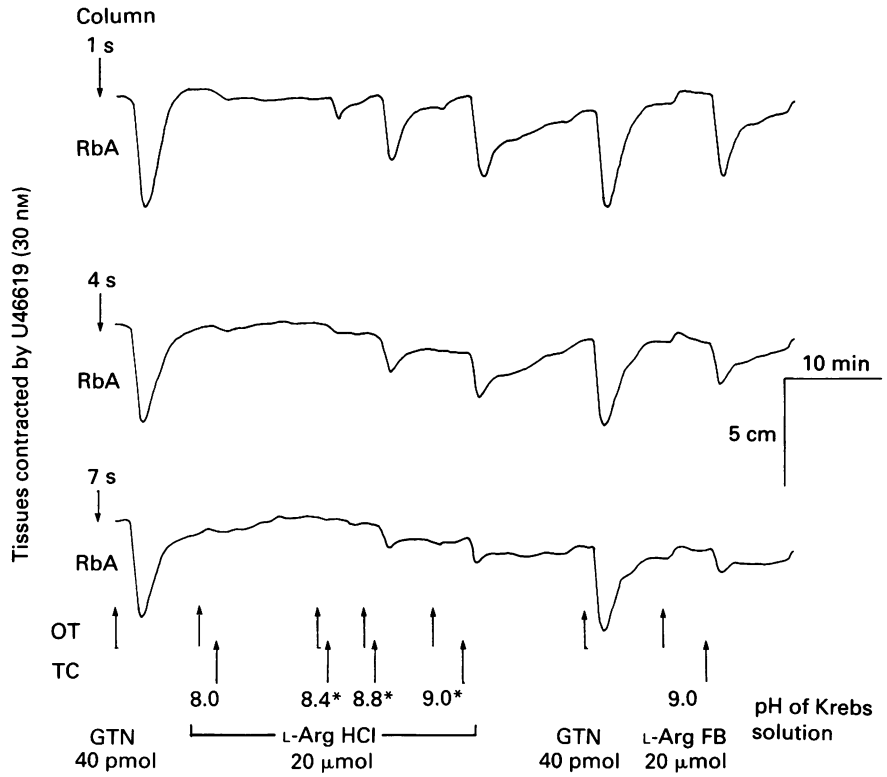

Figure 1 The effect of increasing the extracellular pH of endothelial cells on endothelium-derived relaxing factor (EDRF) release. Effluent from a column of endothelial cells superfused a cascade of four rabbit aortic strips (RbA) precontracted by an infusion of U46619 (30 μM), three of which are shown. Glyceryl trinitrate (GTN; 40 pmol) injected over the tissues (OT) caused a similar relaxation of the three RbA. L-Arginine free base (L-Arg FB; 20 μmol) injected OT caused an increase in the pH of the Krebs solution to pH 9 with no effect on the assay tissues. When injected through the column of endothelial cells (TC) it caused the release of EDRF characterized by relaxation of the RbAs which diminished down the cascade. L-Arginine HCl (L-Arg HCl; 20 μmol) neither increased the pH of the Krebs solution nor released EDRF when given TC. Increasing amounts of a solution of Na_2CO_3, denoted in the figure by *, which increased the pH of the Krebs solution to pH 8.4, 8.8 or 9.0 induced pH-dependent release of EDRF when added to the solution of L-Arg HCl. Similar results were obtained in three other experiments.

transiently increased in the other 3; the EDRF release induced by BK (10–30 pmol; $n = 6$) or ADP (2–6 nmol; $n = 8$) was inhibited; and the release induced by L-arginine FB (10–20 μmol; $n = 6$) or alkaline buffer (Na_2CO_3; pH 8.5–9.0; $n = 3$) was potentiated when compared to the control release of EDRF ($P < 0.05$). Figure 3a shows the inhibition of EDRF release induced by ADP (2 nmol) in the presence of R59022 (30 μM) TC and potentiation of release induced by L-arginine FB (20 μmol). Figure 3b shows the release of EDRF as the % of control release for 3–6 experiments.

The removal of extracellular calcium on the release of EDRF

Endothelial cells were perfused either with normal Krebs solution containing 2.5 mM Ca^{2+} or with a low Ca^{2+} buffer which consisted of Krebs solution without $CaCl_2$, thus leaving a small concentration of Ca^{2+} due to contamination from the other salts. At all times the RbA were perfused with Krebs solution containing the full complement of Ca^{2+}. In conditions of normal Ca^{2+} EDRF was released from the EC by BK (3–30 pmol), ADP (2–6 nmol), L-arginine FB (10–20 μmol) or alkaline buffer (Na_2CO_3 buffer; pH 8.6–9.5). In low Ca^{2+} Krebs solution the release induced by L-arginine FB or alkaline buffer was inhibited to a greater extent than the release induced by BK or ADP. When normal Ca^{2+} was restored to the buffer the inhibition of EDRF release was reversed ($n = 3$–4). Figure 4a shows the selective inhibition of EDRF release induced by L-arginine FB (20 μmol) when extracellular Ca^{2+} (2.5 mM) was removed TC. Figure 4b shows the release of EDRF as the % of control release for 3–4 experiments.

L-Arginine (at concentrations which do not alter pH) on the release of EDRF

L-Arginine FB or HCl (100–300 μM), infused through the column of EC, did not affect the pH of the Krebs solution, the basal release of EDRF or potentiate the release of EDRF induced by other agonists (results not shown; $n > 10$).

N^G-monomethyl-L-arginine (L-NMMA) on the release of EDRF

N^G-monomethyl-L-arginine (10–30 μM) had no direct effect on the RbAs or on the relaxant effects of GTN but when infused TC caused an increase in tone of the RbAs which diminished down the cascade, indicating a decrease of the basal release of EDRF. L-NMMA, dose-dependently inhibited the release of EDRF induced by BK (10–30 pmol; $n = 3$) or by alkaline buffer (D-arginine FB; 10–20 μmol; $n = 4$). This inhibition and increase in tone of the RbA was lessened by L-arginine FB or HCl (30–100 μM; $n = 4$) but not by D-arginine FB or HCl (30–100 μM; $n = 4$; data not shown). Figure 5a shows the inhibition of EDRF release induced by D-arginine FB (20 μmol) with L-NMMA (30 μM) infused TC and the partial reversal in the presence of L-arginine HCl (100 μM) TC. Figure 5b shows the release of EDRF as the % of control release for 3–5 experiments.

Release of prostacyclin from endothelial cells

BK (30 pmol; $n = 26$), ADP (2 nmol; $n = 11$) or arachidonic acid (30 nmol; $n = 7$) given TC induced the release of pros-

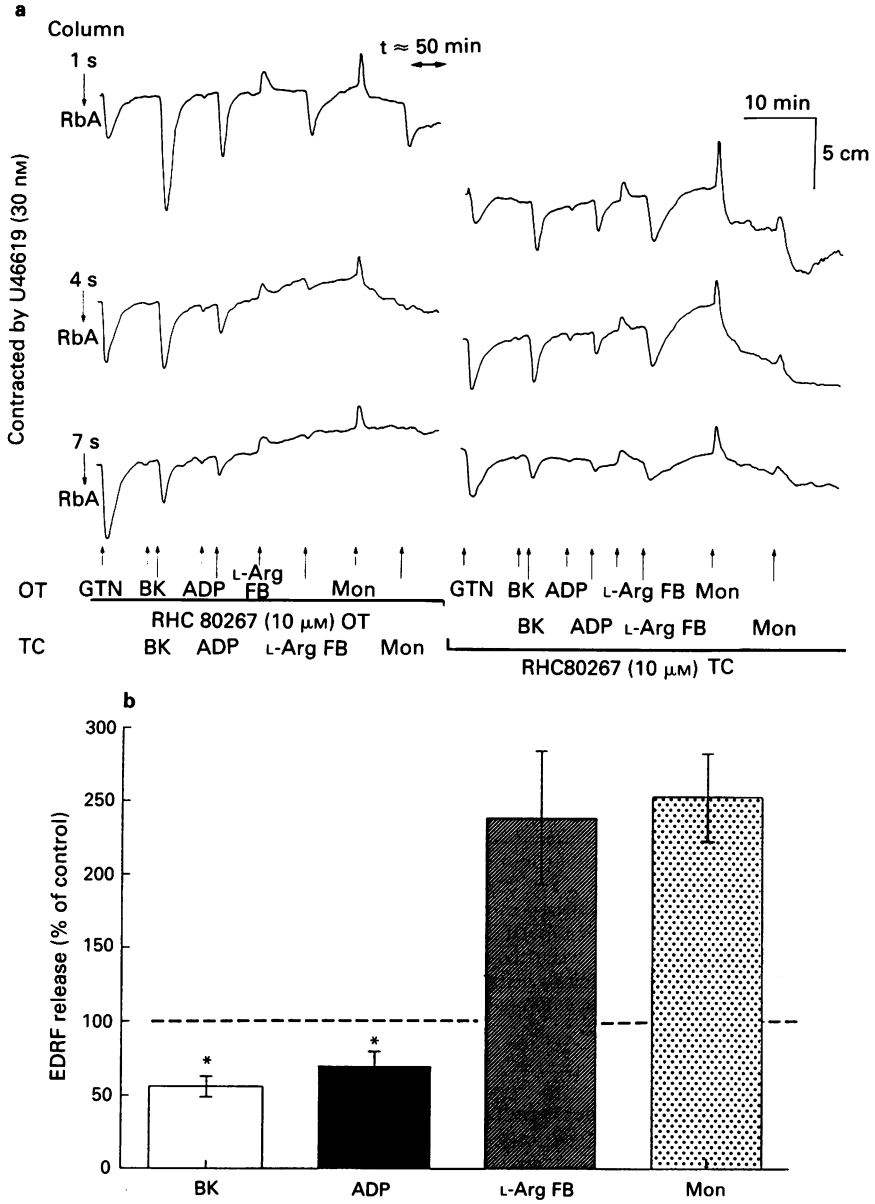

Figure 2 (a) Effect of diacylglycerol lipase inhibitor (RHC 80267) on endothelium-derived relaxing factor (EDRF) release. Experimental details as in Figure 1. When RHC 80267 (10 μM) was infused over the assay tissues (OT) glyceryl trinitrate (GTN; 40 pmol, OT) induced similar relaxations of all three RbA. Bradykinin (BK; 10 pmol) or ADP (2 nmol) given through the column of endothelial cells (TC) released more EDRF than L-arginine free base (L-Arg FB; 20 μmol) TC as shown by the larger relaxations of the assay tissues. Monensin (Mon; 40 nmol) TC also caused a release of EDRF. When RHC 80267 was infused TC there was a clear increase in the basal release of EDRF seen as a graded loss of tone of the assay tissues. However, the stimulated release of EDRF induced by BK or ADP was smaller while that induced by L-Arg FB or Mon was larger, such that they released more EDRF than when RHC 80267 was infused OT. Similar results were obtained in three other experiments. (b) The effect of RHC 80267 (10 μM) on the release of EDRF induced by BK (3–30 pmol; $n = 5$; open column), ADP (2–6 nmol; $n = 5$; solid column), L-Arg FB (10–20 μmol; $n = 5$; hatched column) or Mon (40–100 nmol; $n = 3$; stippled column) as % of control (dotted line); s.e.mean shown by vertical bars. RHC 80267 decreased the release of EDRF in response to BK or ADP, but increased that in response to L-Arg FB or Mon. Thus, in the presence of RHC 80267 the release of EDRF induced by either BK or ADP was significantly different from that induced by either L-Arg FB or Mon (indicated by * $P < 0.05$ for both comparisons).

tacyclin into the Krebs solution perfusing the column of EC as measured by RIA for 6-oxo-$PGF_{1\alpha}$. However, L-arginine FB (20 μmol; $n = 10$), alkaline buffer (Na_2CO_3; pH 8.6–9.5; $n = 4$) or monensin (40 nmol; $n = 3$) given TC did not induce the release of prostacyclin from EC above the limit of detection of the assay (0.5 ng ml^{-1}) (Table 1).

Discussion

Our results demonstrate that L-arginine FB and D-arginine FB, but not the hydrochloride salts can release EDRF from bovine cultured aortic EC. The recent proposal that L-arginine is the precursor of EDRF (Schmidt *et al.*, 1988; Palmer *et al.*, 1988a) could indicate that the selective release of EDRF by L-arginine FB occurs because of an increased synthesis of EDRF. However the findings that: (i) L-arginine HCl neither released EDRF nor potentiated EDRF release when given to the EC at the same concentrations; (ii) D-arginine FB released similar amounts of EDRF as L-arginine FB with similar increases in pH; (iii) a buffer, such as Na_2CO_3, which caused a similar increase in pH of the Krebs solution as L- or D-arginine FB, also released similar amounts of EDRF, all point to the conclusion that an increase in extracellular pH leads to the release of EDRF. The failure of R59022 to inhibit the release of EDRF induced by L- or D-arginine FB, Na_2CO_3

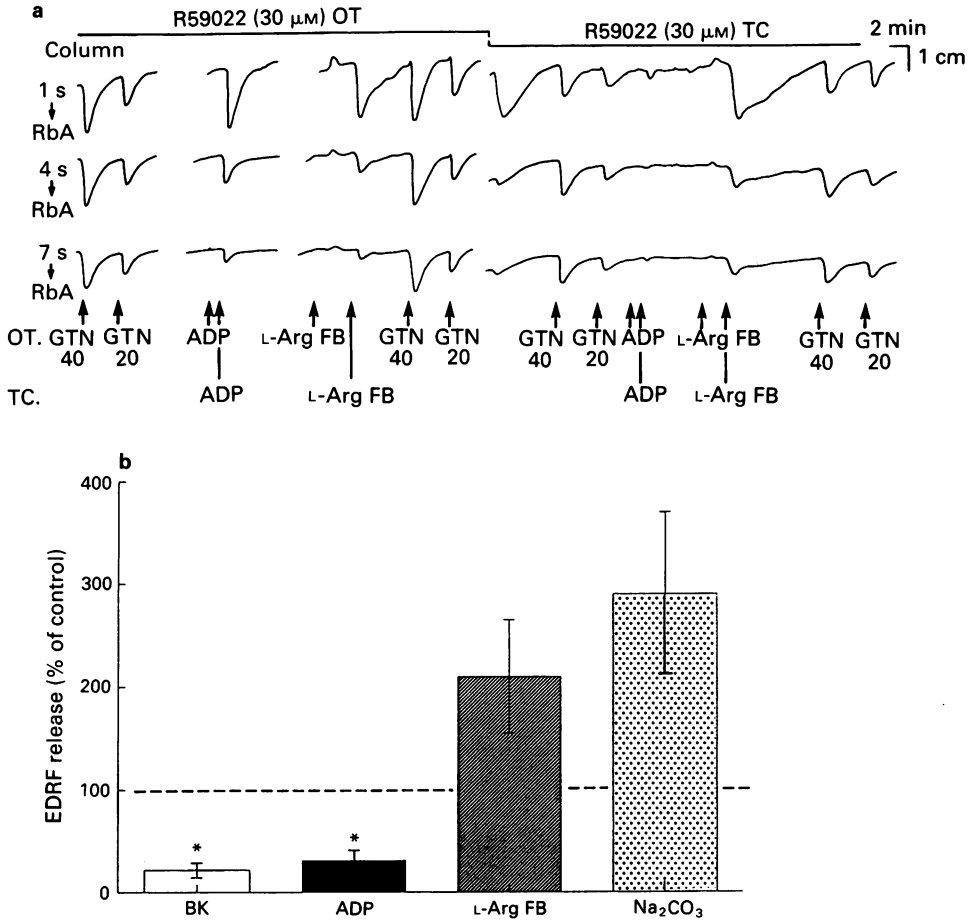

Figure 3 (a) Effect of diacylglycerol kinase inhibitor (R59022) on endothelium-derived relaxing factor (EDRF) release. Experimental details as in Figure 1. When R59022 (30 μM) was infused over the assay tissues (OT) glyceryl trinitrate, 20 or 40 pmol (GTN; 20 or 40) OT induced similar relaxations of all three rabbit aortic strips (RbA). ADP (2 nmol) or L-arginine free base (L-Arg FB; 20 μmol) injected through the column (TC) induced release of EDRF that caused relaxations of the top RbA equivalent to that induced by GTN 40. Infusions of R59022 TC caused a transient release of EDRF and inhibited the release of EDRF induced by ADP (smaller on the top RbA than GTN, 40). However, the EDRF release induced by L-Arg FB was potentiated (larger on the top RbA than GTN 40). Similar results were obtained in three other experiments. (b) The effect of R59022 (10–30 μM) on the release of EDRF induced by BK (10–30 pmol; $n = 6$; open column), ADP (2–6 nmol; $n = 8$; solid column), L-Arg FB (10–20 μmol; $n = 6$; hatched column) or alkaline buffer (Na_2CO_3; pH 8.6–9.5; $n = 3$; stippled column) as % of control release (dotted line); s.e.mean shown by vertical bars. R59022 decreased the release of EDRF in response to BK or ADP, but increased that in response to L-Arg FB or Na_2CO_3. Thus, in the presence of R59022 the release of EDRF induced by either BK or ADP was significantly different from that induced by either L-Arg FB or Na_2CO_3 (indicated by * $P < 0.05$ for both comparisons).

or monensin contrasts with its inhibitory action on the release of EDRF induced by other agonists (de Nucci *et al.*, 1988a). These results provide evidence that the release induced by L- or D-arginine FB, monensin or Na_2CO_3 is not receptor-mediated and shares a common mechanism for the activation of endothelial cells.

The novel finding that the diacylglycerol lipase inhibitor RHC 80267 (Sutherland & Amin, 1982) inhibits the release of EDRF induced by BK or ADP confirms our previous observation that the mechanism of EDRF release by these agonists involves activation of a phospholipase C (de Nucci *et al.*, 1988a). RHC 80267 should increase the concentrations of diacylglycerol in the cells leading to activation of protein kinase C and phosphorylation of G-proteins (Yamanishi *et al.*, 1983). This would act as a negative feedback system as previously reported for neutrophils (Smith *et al.*, 1987) and endothelial cells (de Nucci *et al.*, 1988a). The finding that the release of EDRF induced by L- or D-arginine FB, Na_2CO_3 or monensin

Table 1 The release of prostacyclin induced by different agonists

	6-oxo-$PGF_{1\alpha}$ (ng ml^{-1})				
Time (min)	0	1	2	3	*n*
BK (10 pmol)	<0.5	1.3 ± 0.3	2.5 ± 0.4	1.7 ± 0.3	26
ADP (2 nmol)	<0.5	1.2 ± 0.4	1.3 ± 0.3	<0.5	11
AA (30 nmol)	<0.5	4.8 ± 1.0	6.4 ± 1.2	4.0 ± 1.5	7
L-Arg FB (20 μmol)	<0.5	<0.5	<0.5	<0.5	10
Na_2CO_3 (pH 8.6–9.5)	<0.5	<0.5	<0.5	<0.5	4
Mon (40 nmol)	<0.5	<0.5	<0.5	<0.5	3

Prostacyclin release induced by bradykinin (BK), ADP, arachidonic acid (AA), L-arginine free base (L-Arg FB), alkaline buffer (Na_2CO_3) and monensin (Mon) was estimated by radioimmunoassay of 6-oxo-$PGF_{1\alpha}$ at times 0, 1, 2 and 3 min after doses of the above agents. The concentrations of 6-oxo-$PGF_{1\alpha}$ are given as the mean ± s.e.mean of *n* experiments.

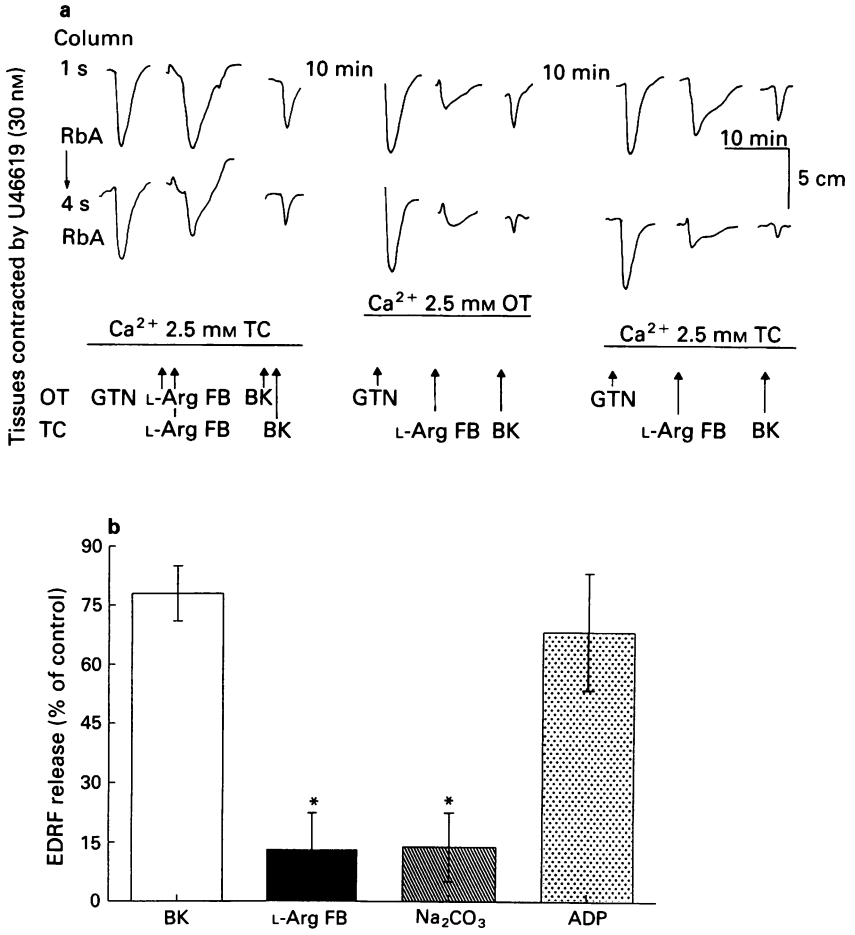

Figure 4 (a) Effect of lowering the extracellular calcium on endothelium-derived relaxing factor (EDRF) release. Experimental details as in Figure 1 but only two of four RbA are shown. Glyceryl trinitrate (GTN; 40 pmol) given over the assay tissues (OT) caused relaxations which were similar down the cascade. When calcium (Ca^{2+}; 2.5 mM) was infused through the column of endothelial cells (TC) EDRF release induced by L-arginine free base (L-Arg FB; 20 μmol) was greater than that induced by bradykinin (BK; 3 pmol). When the infusion of Ca^{2+} was changed from TC to OT and the endothelial cells were perfused with low Ca^{2+} Krebs solution, the release of EDRF induced by L-Arg FB was similar in magnitude to that induced by BK. When Ca^{2+} was returned TC, the release of EDRF induced by L-Arg FB was again greater than that induced by BK. Similar results were obtained in three other experiments. (b) The effect of removal of extracellular calcium on the release of EDRF induced by BK (3–30 pmol; $n = 4$; open column), L-Arg FB (10–20 μmol; $n = 4$; solid column), alkaline buffer (Na_2CO_3; pH 8.6–9.5; $n = 3$; hatched column) or ADP (2–6 nmol; $n = 3$; stippled column) as % of control; s.e.mean shown by vertical bars. Removal of the extracellular calcium significantly decreased the release of EDRF induced by L-Arg FB or Na_2CO_3 with respect to control and with respect to BK or ADP under the same conditions (indicated by * $P < 0.05$ all ways).

is potentiated by RHC 80267 reinforces the view that these agents are releasing EDRF by a mechanism different from that of BK or ADP.

It is interesting that the sodium ionophore, monensin causes a similar pattern of release of EDRF and PGI_2 to alkaline pH in that it releases EDRF with no simultaneous release of PGI_2. This could indicate that the release of EDRF in response to an increased extracellular pH is mediated by a similar mechanism to that of monensin. Most eukaryotic cells have several pH regulating mechanisms and the interaction between them is complicated. There is general agreement that all cells possess an electro-neutral Na^+/H^+ antiporter and that this antiporter has a pH regulating function after acid loads (Deitmer & Ellis, 1980; Krulwich, 1983). In addition most cells have a pH regulating anion antiporter, through which they can normalize pH after acid or alkali loads (Russel & Boron, 1976; Madshus, 1988). Adams *et al.* (1989) recently showed that monensin could elevate intracellular calcium in bovine pulmonary arterial cells and associated this action with the presence of a $Na^+:Ca^{2+}$ exchanger. Whether an increase in extracellular pH can activate a similar ion exchanger in the EC which leads to EDRF release remains to be investigated. A possible mechanism could be that after an increase in the Na^+ content of the EC, due to an alkali load, the EC exchanges Na^+ for Ca^{2+} and the resulting increase in intracellular Ca^{2+} causes EDRF release. This hypothesis is supported by the finding that extracellular Ca^{2+} has a more important function in the release of EDRF induced by alkaline pH than for that induced by BK or ADP where mobilisation of intracellular Ca^{2+} is probably involved (Lambert *et al.*, 1986; Derian & Moskowitz, 1986). The release of EDRF with little or no PGI_2 release by alkaline buffers and its dependence on extracellular Ca^{2+} fits well with the suggestion that a mobilisation of intracellular Ca^{2+} is vital for PGI_2 release (Luckoff, 1988).

The finding that L-NMMA inhibits the release of EDRF induced by an increase in pH as well as by receptor activation and that L-arginine lessens this inhibition suggests that L-NMMA acts at a fundamental point in the EDRF-generating system. The finding that L-arginine does not increase basal or stimulated release of EDRF in our system confirms previous observations using porcine aortic cultured EC (Palmer *et al.*, 1988a) or fresh vascular preparations (Thomas *et al.*, 1989). The intracellular concentration of L-arginine in cultured EC is approximately 100 μM and EC have the ability to maintain this arginine concentration during pro-

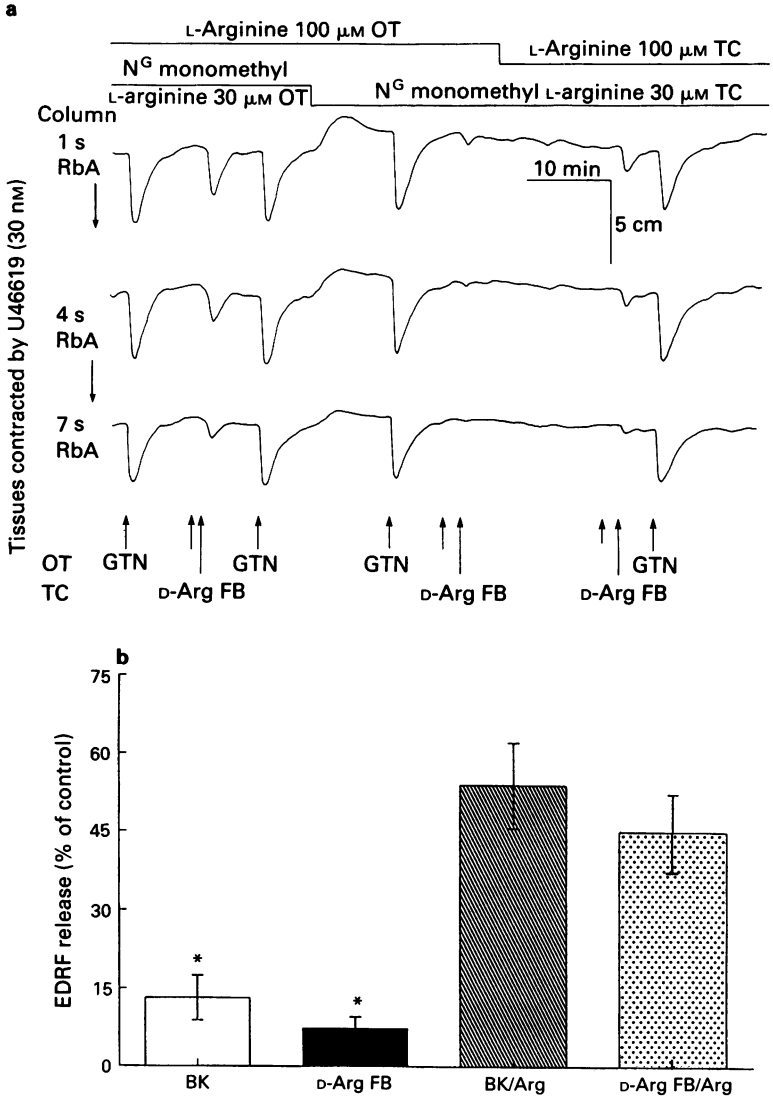

Figure 5 (a) The effect of N^G-monomethyl-L-arginine (L-NMMA) on endothelium-derived relaxing factor (EDRF) release induced by D-arginine free base (D-Arg FB). Experimental details as in Figure 1. Glyceryl trinitrate (GTN; 40 pmol) given over the RbA (OT) induced relaxations which were similar on all the RbAs. Infusion of L-NMMA (30 μM) OT had no direct effect on the tone of the RbA nor on the detection of EDRF release induced by D-Arg FB (20 μmol). Infusion of L-NMMA through the column of endothelial cells (TC) caused contraction of the RbAs and an inhibition of the release of EDRF induced by D-Arg FB. Additional infusions of L-Arg HCl (100 μM) partially reversed the inhibition, but did not fully restore the release of EDRF induced by D-Arg FB. Similar results were obtained in three other experiments. (b) The effect of L-NMMA (30 μM) on EDRF release induced by BK (10–30 pmol; $n = 4$; open column) or alkaline buffer (D-Arg FB; 10–20 μmol; $n = 4$; solid column) and the effect of the addition of L-Arg (100 μM) for BK ($n = 4$; hatched column) or D-Arg FB ($n = 4$; dotted column) as % of control release; s.e.mean shown by vertical bars. In the presence of L-NMMA the release of EDRF induced by BK or D-Arg FB was significantly different from control and from the release in the presence of a co-infusion of L-Arg (both indicated by, * $P < 0.05$).

longed EDRF release (Mitchell *et al.*, 1990a) by generating L-arginine from an intracellular store (Mitchell *et al.*, 1990b). This facility of EC could explain why the application of L-arginine does not affect the synthesis of EDRF.

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