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ENDOTHELIUM-DERIVED RELAXING FACTOR INHIBITS THE FORMATION OF INOSITOL TRISPHOSPHATE BY RABBIT AORTA

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SUMMARY

1. The effects of endothelium-derived relaxing factor (EDRF), sodium nitroprusside, 8-bromo-cyclic GMP and atrial natriuretic factor (ANF) on inositol trisphosphate (IP_3) levels were studied in isolated rabbit aortic preparations stimulated with noradrenaline.

2. In endothelium-containing preparations, acetylcholine, which stimulated EDRF release, inhibited noradrenaline-stimulated IP_3 formation. The EDRF inhibitor haemoglobin reversed this effect.

3. In endothelium-denuded preparations, sodium nitroprusside, 8-bromo-cyclic GMP and ANF each similarly inhibited the rise in IP_3 levels stimulated by noradrenaline.

4. These findings show that in rabbit aorta, agents which increase cyclic GMP inhibit the noradrenaline-induced rise in IP_3 levels and may provide an explanation for the previously reported observations that cyclic GMP inhibits the noradrenaline-stimulated increase in calcium influx and release of intracellular calcium in vascular smooth muscle.

INTRODUCTION

Endothelium-derived relaxing factor (EDRF) is the humoral agent responsible for endothelium-dependent vascular smooth muscle relaxation (Furchgott & Zawadzki, 1980; Griffith, Edwards, Lewis, Newby & Henderson, 1984). Endothelium-derived relaxing factor has recently been identified as a nitric oxide-containing moiety (Palmer, Ferrige & Moncada, 1987). Like nitrovasodilators, the action of EDRF is mediated through stimulation of soluble guanylate cyclase and elevation of intracellular levels of cyclic guanosine monophosphate (cyclic GMP) (Katsuki, Arnold, Mittal & Murad, 1977; Ignarro, Lippton, Edwards, Baricos, Hyman, Kadowitz & Greutter, 1981; Rapoport, Draznin & Murad, 1983*a*,*b*; Rapoport & Murad, 1983; Griffith, Edwards, Lewis & Henderson, 1985; Forstermann, Mülach, Böhme & Busse, 1986). Atrial natriuretic factor (ANF) similarly causes elevation of intracellular levels of cyclic GMP in vascular smooth muscle (Winquist, Faison, Waldman, Schwartz, Murad & Rapoport, 1984). Unlike EDRF, ANF acts via specific activation of the particulate isoenzyme form of guanylate cyclase (Waldman, Rapoport & Murad, 1984).

It is generally accepted that hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C, in response to agonist stimulation, produces *sn*-1,2-diacylglycerol (DAG) (Nishizuka, 1984) and inositol 1,4,5-trisphosphate (IP_3) (Berridge, 1984; Berridge & Irvine, 1984; Hokin, 1985; Downes & Michell, 1985). Both these products have important intracellular secondary messenger functions. DAG activates protein kinase C (Nishizuka, 1984) and IP_3 causes release of calcium from the sarcoplasmic reticulum (Berridge, 1984; Berridge & Irvine, 1984; Hashimoto, Hirata, Itoh, Kanmura & Kuriyama, 1986).

Increased levels of cyclic GMP in response to EDRF, nitrovasodilators and ANF, cause vascular smooth muscle relaxation (Griffith *et al.* 1985; Rapoport, Schwartz & Murad, 1985). This increase in cyclic GMP is accompanied by a decrease in calcium influx and the intracellular release of calcium (Collins, Griffith, Henderson & Lewis, 1986). In addition, it has been suggested recently that cyclic GMP may inhibit contraction of vascular smooth muscle through inhibition of phosphoinositol hydrolysis (Rapoport, 1986). Since changes in calcium fluxes may be secondary to changes in the intracellular levels of inositol phosphates, in the present study we have investigated the effects of agents which increase cyclic GMP levels on IP_3 production in isolated aortic preparations of the rabbit.

METHODS

Tissue preparation

Male New Zealand White rabbits (approximately 2–2·5 kg) were killed by a blow to the neck and exsanguination. Their aortae were removed and placed in Krebs–Ringer–bicarbonate (KRB) solution of the following composition (mM): NaCl, 118·5; KCl, 4·74; $MgSO_4$, 1·18; KH_2PO_4, 1·18; $CaCl_2$, 2·5; $NaHCO_3$, 24·9; and glucose, 10, gassed with 95% O_2/5% CO_2 at room temperature. After removal of all fat and connective tissue each aorta was cut into 4 cm lengths. The endothelium was removed from some preparations by gently rubbing the intimal surface with a wooden stick.

The 4 cm lengths were then incubated with 25 μCi/ml of *myo*[2-^{3}H(*N*)]inositol (specific activity: 12·8–19·0 Ci/mmol) in KRB for 5 h at 37 °C to label endogenous phospholipid stores in smooth muscle (which comprises > 90% of rabbit aorta; unpublished observations). The tissues were placed in fresh KRB containing 10 mM-lithium chloride (LiCl) and incubated for 20 min at 37 °C to inhibit inositol phosphate break-down. After the various interventions were carried out (see figure legends), the tissues were immersed in liquid nitrogen to inhibit further reactions. The tissues were then homogenized in 1·5 ml of ice-cold 3·6% (w/v) perchloric acid (PCA) and centrifuged at 2500 r.p.m. for 15 min at 5%. The supernatant was added to an equal volume of 0·5 M-KOH/9 mM-sodium tetraborate/1·9 mM-EDTA/3·8 mM-sodium hydroxide to remove the PCA, bringing the pH of the supernatant to 8–9. The samples were then frozen and stored at −20 °C until required for inositol phosphate analysis.

Measurement of [^{3}H]*inositol phosphates*

The same procedure as described by Berridge and co-workers was used (Berridge, Dawson, Downes, Heslop & Irvine, 1983). Briefly, previously frozen samples were thawed and applied to 1 ml volumes of AGIx-8 exchange resin (formate form) and eluted sequentially with (a) 20 ml of distilled water; (b) 20 ml of 5 mM-disodium tetraborate/60 mM-sodium formate; (c) 10 ml of 0·2 M-ammonium formate in 0·1 M-formic acid; (d) 20 ml of 0·4 M-ammonium formate in 0·1 M-formic acid; (e) 10 ml of 0·8 M-ammonium formate in 0·1 M-formic acid; (f) 10 ml of 1 M-ammonium formate in 0·1 M-formic acid. Two millilitre fractions were collected, 1 ml of which was used for measurement of ^{3}H by standard liquid-scintillation counting techniques.

The products eluted with the distilled water and the 5 mM-disodium tetraborate–60 mM-sodium formate solution were free inositol and phosphoglycerol inositol respectively (Berridge *et al.* 1983)

which were discarded. The subsequent fractions (numbered 1–25) contained the various inositol phosphates.

To test the elution position of the [^{3}H]IP_3 fraction from the anion exchange column, 1 nCi purified [^{3}H]IP_3 (specific activity 1 Ci/mmol) was added to 1·5 ml ice-cold 3·6% (w/v) PCA containing a 4 cm length of rabbit aorta. Following homogenization and preparation as described earlier, the sample was chromatographed as described and the fractions measured for ^{3}H content.

Statistical analysis

Results are expressed as mean values. For clarity standard errors of the mean are given for IP_3 levels only. Results were compared using Student's t test for unpaired data and considered significantly different when $P < 0{\cdot}05$. Only IP_3 levels have been statistically compared.

Materials and drugs

Acetylcholine chloride, sodium nitroprusside, (−)-noradrenaline bitartrate, human atrial natriuretic factor and haemoglobin were obtained from Sigma Chemical Co., UK. 8-bromo-cyclic GMP from Boehringer Corporation Ltd, London, UK. *myo*[2-^{3}H(*N*)]inositol from New England Nuclear Research Products, FRG and D-*myo*-[2-^{3}H]inositol 1,4,5-trisphosphate from Amersham International Ltd, UK. Haemoglobin, as obtained from Sigma Chemical Co., UK, is a mixture of reduced and oxidized forms. Since it is only the ferrous form which acts as an EDRF inhibitor, this was prepared as described by Martin, Villani, Jothianandan & Furchgott, 1985.

RESULTS

The purified [^{3}H]IP_3 sample eluted from the columns between fractions 16 and 20 inclusive. The efficiency of extraction was $68{\cdot}5 \pm 2{\cdot}3$% ($n = 6$).

Noradrenaline increased the levels of all inositol phosphates above basal values. Neither the basal IP_3 levels nor the noradrenaline-stimulated IP_3 levels in the endothelium-intact preparations were significantly different from the corresponding levels in the endothelium-denuded preparations. The noradrenaline-stimulated increase in IP_3 levels was significantly inhibited by acetylcholine and this inhibition was significantly reduced by haemoglobin (Fig. 1).

Sodium nitroprusside, 8-bromo-cyclic GMP and ANF each significantly inhibited the noradrenaline-stimulated increases in IP_3 levels (Figs 2*A*, *B* and *C*).

DISCUSSION

The data show that there is a rapid increase in IP_3 levels in the rabbit aorta following exposure to noradrenaline. The exposure time of 30 s was chosen from preliminary experiments which showed that the peak IP_3 response occurred at this time (data not shown). These changes in IP_3 levels in rabbit aorta are similar to those previously described for the mesenteric artery of the rabbit (Hashimoto *et al.* 1986).

Endothelium-derived relaxing factor and each of the other interventions which increase intracellular cyclic GMP levels resulted in a significant reduction in the noradrenaline-stimulated levels of IP_3. The present findings thus confirm earlier reports showing a reduced phosphatidylinositol turnover by agents which increase cyclic GMP in both platelets (Takai, Kaibuchi, Matsubara & Nishizuka, 1981) and vascular smooth muscle (Rapoport, 1986). In these earlier studies, however, IP_3 levels were not measured and in the study by Rapoport, long agonist incubation times were used (30 min) during which time the intracellular IP_3 levels were likely to have returned to baseline values.

The mechanism of the cyclic GMP-induced inhibition of IP_3 levels is unknown at present. One possible site of action of cyclic GMP may be at the transduction point between cell surface receptors and phospholipase C. Increased levels of cyclic GMP may prevent the GTP binding protein from coupling to phospholipase C and so inhibit phosphatidylinositol hydrolysis and hence IP_3 production. A phospholipase

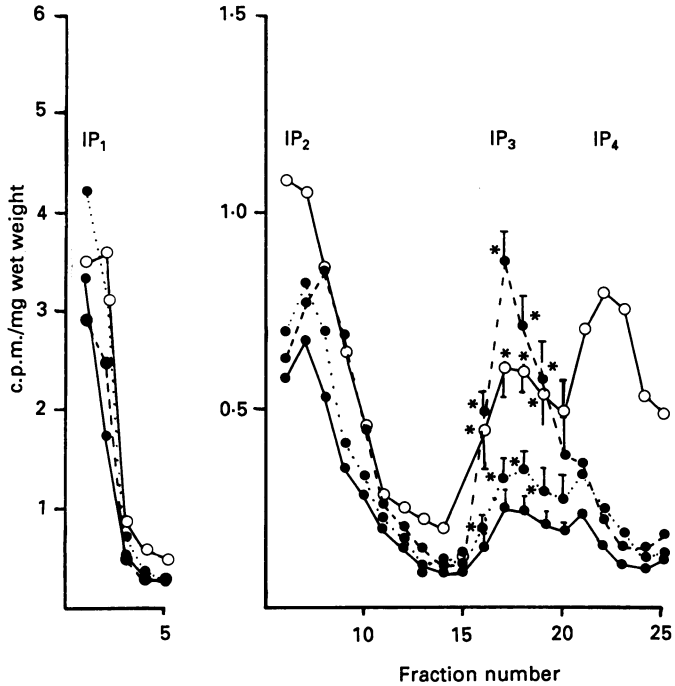

Fig. 1. Levels of [^{3}H]inositol 4-phosphate (IP_1), inositol 4,5-bisphosphate (IP_2), inositol 1,4,5-trisphosphate (IP_3) and inositol 1,3,4,5-tetrakisphosphate (IP_4) as counts per minute (c.p.m.) per milligram tissue in column fractions 1–25 from endothelium-intact aortae in the absence of agonist stimulation (●——●); following 30 s stimulation with noradrenaline (10^{-5} M) (●– –●); following 1 min pre-incubation with acetylcholine (10^{-6} M) before 30 s stimulation with noradrenaline (10^{-5} M) (●·····●); and following 2 min pre-incubation with haemoglobin (10^{-6} M) before 1 min pre-incubation with acetylcholine (10^{-6} M) prior to 30 s stimulation with noradrenaline (○——○). $n \geqslant 6$; $^*P < 0{\cdot}05$.

C inhibitory action of cyclic GMP in platelets has already been suggested by other workers (Nakashima, Tohmatsu, Hattori, Okano & Nozawa, 1986), though no evidence exists for this mechanism in vascular smooth muscle.

Previously we have shown that EDRF, acting through cyclic GMP, inhibits calcium influx and intracellular calcium release in rabbit aorta (Collins *et al.* 1986). The cyclic GMP-induced inhibition of IP_3 formation shown by the present study provides an explanation for the inhibition of intracellular calcium release by cyclic GMP since it is known that the intracellular release of calcium in vascular smooth muscle is mediated by IP_3 (Yamomoto & van Breemen, 1985) though this is not directly affected by cyclic GMP (Twort & van Breemen, 1988).

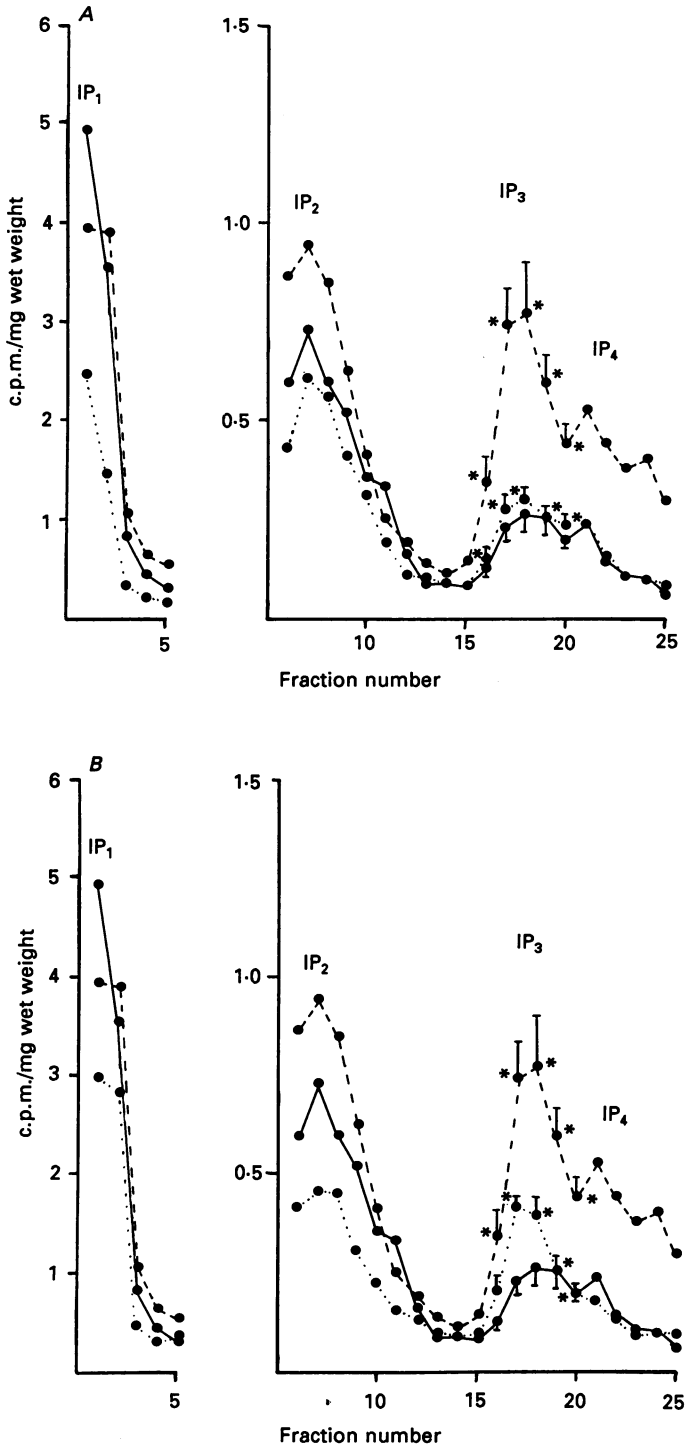

Fig. 2*A* and *B*. For legend see page 50.

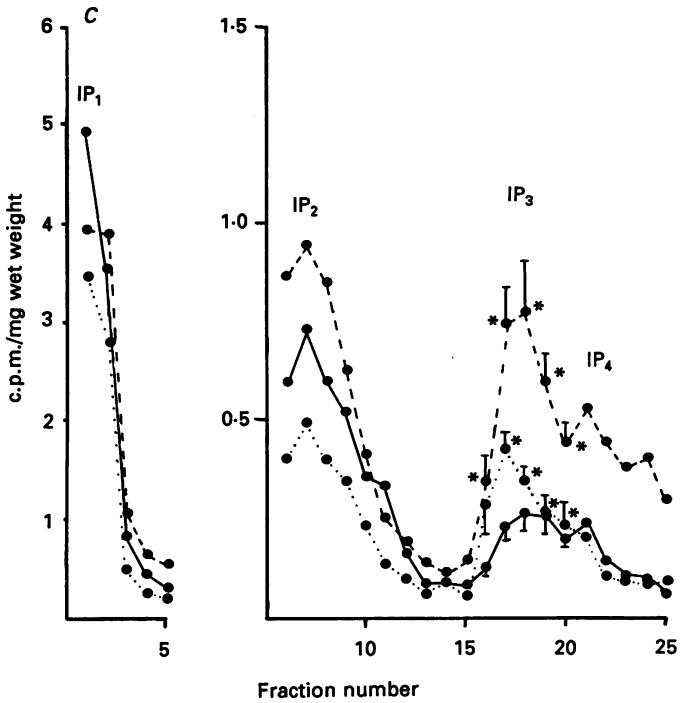

Fig. 2. Levels of [^{3}H]IP_1, IP_2, IP_3 and IP_4 from endothelium-denuded aortae in the absence of agonist stimulation (●——●); following 30 s stimulation with noradrenaline (10^{-5} M) (●– –●); and following 30 s stimulation with noradrenaline (10^{-5} M) preceded by 1 min pre-incubation with sodium nitroprusside (10^{-5} M) (*A*) or 15 min pre-incubation with 8-bromo-cyclic GMP (10^{-4} M) (*B*) or 5 min pre-incubation with ANF (10^{-6} M; ●·····●) (*C*). $n \geqslant 6$; $^*P < 0{\cdot}05$. Conventions as for Fig. 1.

The effect on calcium influx is less easily explained by the present findings since a direct association with inositol phosphate formation and calcium influx has not been demonstrated in vascular smooth muscle.

Although the present data may explain the previously observed cyclic GMP-mediated inhibition of calcium fluxes in vascular smooth muscle, they do not provide an explanation for cyclic GMP-mediated inhibition of vascular smooth muscle tone during the tonic phase of contraction when intracellular levels of IP_3 and calcium have returned close to baseline values (Hashimoto *et al.* 1986; Bradley & Morgan, 1987). During tonic contraction, diacylglycerol formation is likely to be more important than inositol phosphate formation since diacylglycerol levels remain elevated at this time (Griendling, Rittenhouse, Brock, Ekstein, Gimbrone & Alexander, 1986), with resulting stimulation of protein kinase C and maintenance of tone (Forder, Scriabine & Rasmussen, 1985). It is possible therefore that inhibition of phosphatidylinositol hydrolysis by cyclic GMP would also reduce diacylglycerol formation, protein kinase C activation and hence inhibition of contraction. Further studies will be necessary to answer this question, however.

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