Br. J. Pharmacol. (1994), **112**, 311–315 © Macmillan Press Ltd, 1994

Regulation by hypoxia of endothelin-1-stimulated phospholipase D activity in sheep pulmonary artery cultured smooth muscle cells

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1 The aim of the study was to characterize the effects of hypoxia on agonist-stimulated phospholipase D (PLD) and phospholipase C activity of sheep pulmonary artery cultured smooth muscle cells.

2 Endothelin-1 (ET-1), 5-hydroxytryptamine (5-HT) and the protein kinase C (PKC) activator tetradecanoylphorbol acetate (TPA), stimulated a time- and concentration-dependent increase in [^{3}H]-phosphatidylbutanol accumulation. This was abolished by pretreatment of the cells with the PKC inhibitor, Ro-318220, suggesting that agonist-stimulated phospholipase D activity is dependent upon the activation of PKC.

3 Hypoxia (Po_2 20 mmHg for 30 min) stimulated basal [^{3}H]-phosphatidylbutanol accumulation by approximately 2 fold and this activity was abolished by preincubation of the cells with 10 μM Ro-318220.

4 In cells preincubated in low O_2 containing medium for 30 min, the subsequent agonist-stimulated accumulation of [^{3}H]-phosphatidylbutanol was reduced. However, the decrease in stimulation was greater for ET-1 and 5-HT than for TPA.

5 ET-1 and TPA stimulated a time-dependent increase in protein kinase C- mediated psuedosubstrate phosphorylation. Following preincubation for 30 min in low O_2 containing media, basal pseudosubstrate phosphorylation increased whilst the fold stimulation by TPA and ET-1 decreased.

6 In cells preincubated in low O_2 containing medium, ET-1-stimulated [^{3}H]-inositol phosphate accumulation was reduced by approximately 30–40%. This reduction was reversed by preincubation of the cells with Ro-318220.

7 These results suggest a role for PKC in the effects of hypoxia on PLD in pulmonary artery smooth muscle cells.

Keywords: Pulmonary artery smooth muscle cells; phospholipase D; endothelin-1; hypoxia; protein kinase C

Introduction

Acute and chronic hypoxia have been shown to affect agonist-stimulated contraction and growth of pulmonary artery smooth muscle. This is due not only to modulation of the release of vasoactive substances from endothelial cells and fibroblasts (Rakugi *et al.*, 1990), but also through a direct action upon the underlying smooth muscle cells (Murray *et al.*, 1990; Dempsey *et al.*, 1991; Butler *et al.*, 1991). The molecular events underlying such an effect are unclear although the hydrolysis of phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P_2], the mobilisation of both intra- and extracellular calcium and the activation of protein kinase C (PKC) have been implicated (Dempsey *et al.*, 1991; Maclean & Nally 1992; Jin *et al.*, 1992).

An intracellular signalling pathway which may also play a role in the regulation of smooth muscle cell growth and contraction involves the hydrolysis of phosphatidylcholine by phospholipase D (PLD) (Cook & Wakelam 1989; Billah & Anthes 1990; MacNulty *et al.*, 1990; Plevin *et al.*, 1992). The product from this reaction, phosphatidic acid, may have second messenger roles in agonist-stimulated smooth muscle contraction (Ohanian *et al.*, 1990). In addition, phosphatidic acid may be converted by phosphatidic acid phosphohydrolase to sn-1,2 diacylglycerol, the physiological activator of PKC (Nishizuka, 1984).

The aims of the present experiments were: (1) to characterize the effect of hypoxia upon agonist-stimulated PLD activity in primary cultures of pulmonary artery smooth muscle cells under oxygenated and hypoxic conditions and (2) to determine the role of PKC in modulating these effects. A preliminary account of these findings has been presented to the British Pharmacological Society (Plevin *et al.*, 1993).

Methods

Sheep pulmonary artery smooth muscle cells were obtained by collagenase digestion and cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 15% foetal calf serum, glutamine 2 mM, fungizone 25 μg ml^{-1}, and penicillin 250 i.u. ml^{-1}, streptomycin 250 μg ml^{-1}, at 37°C in an atmosphere of 95/5% air/CO_2. Passages 3–9 were used routinely for the experiments described below. The identity of the cells was confirmed by immunoblotting for smooth muscle α-actin (results not shown).

Phospholipase D (PLD) activity was measured as the accumulation of [^{3}H]-phosphatidylbutanol. Sheep pulmonary artery smooth muscle cells were prelabelled for 40 h in serum-free DMEM containing [^{3}H]-palmitic acid (specific activity 12–20 kBq mmol^{-1}) at a concentration of 2 μCi ml^{-1}. On the day of the experiment, the cells were preincubated in gassed Krebs solution composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.7, pH 7.4 for 30 min at 37°C then incubated in Krebs solution containing 30 mM butanol for a further 5 min. The reaction was initiated by the addition of agonist in Krebs solution containing 30 mM butanol. However, during the low oxygen experiments, agonist and butanol were added simultaneously. The reaction then was terminated by rapid aspiration followed by the addition of

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0.5 ml ice cold methanol. After 10 min on ice, the cell wells were scraped and washed and the extracts transferred into glass vials. Labelled phospholipid products were extracted at room temperature for 60 min by the addition of 0.35 ml chloroform and aqueous and organic phases split by the further addition of chloroform (0.35 ml) and water (0.5 ml) followed by centrifugation. The aqueous phase was removed and the lower chloroform layer dried down under vacuum. The extract was redissolved in 50 µl chloroform and 25 µl applied to Whatman t.l.c. plates. The resolving solvent was the upper phase of ethylacetate/2,2 trimethylpentane/water and acetic acid (50/110/100/20 v/v). The peak corresponding to labelled phosphatidylbutanol ([^{3}H]-PtdBuOH) was excised and counted by liquid scintillation.

For measurement of [^{3}H]-inositol phosphate accumulation ([^{3}H]-IP), sheep pulmonary artery smooth muscle cells were prelabelled in serum-free media as outlined above but in the presence of [^{3}H]-inositol (specific activity 20.0 kBq mmol^{-1}). On the day of the experiments the cells were preincubated with Krebs for 20 min and then for a further 10 min in Krebs solution containing 10 mM lithium chloride before stimulation by the addition of agonist in a final volume of 0.5 ml. The reaction was terminated by aspiration and the addition of ice cold methanol (0.5 ml). Following scraping and transfer to plastic vials, the inositol polyphosphates were extracted by the addition of chloroform (0.5 ml) and methanol (0.5 ml) to give a final ratio of 2:1. The phases were then split by the addition of chlorofom/methanol/water to a final ratio of 1:1:0.8 and a sample of the aqueous phase was assayed for inositol phosphates by ion anion exchange chromatography on Dowex formate columns as previously described (Plevin *et al.*, 1992).

For the measurement of agonist-stimulated PKC activity, a modified version of the method used by Alexander *et al.* (1990) was employed. Sheep pulmonary artery smooth muscle cells, grown to confluency and quiescence on 6 cm^2 plates, were preincubated with Krebs solution at 37°C for 30 min. The cells were washed twice in a buffer containing (mM): KCl 150, $MgCl_2$ 5.16, PIPES 12.5, EGTA 12.5 and $CaCl_2$ 8.17, pH 7.4 and the reaction initiated in the same buffer containing agonist and 0.5 units ml^{-1} of streptolysin-O, 200 µM ATP [γ-^{32}P]ATP, (300–450 c.p.m. pmol^{-1}, specific activity = 2200 Ci mmol^{-1}) and 200 µM of a PKC peptide pseudosubstrate in a final volume of 400 µl. Following a 10 min incubation period the reaction was terminated by the addition of 100 µl of 25% (w/v) trichloroacetic acid in 2 M acetic acid. After 10 min on ice, the cells were scraped and the extracts transferred to microfuge tubes and spun for 5 min for 14,000 *g* at 4°C. Aliquots of the supernatants were spotted onto 3 cm squares of P81 Whatman ion exchange chromatography paper. The squares were washed (× 3) in 100 ml of 75 mM phosphoric acid (10 min each wash) and then once in ethanol. The squares were dried and measured by scintillation counting. Non-specific phosphorylation (3–5% of total phosphorylation) in the absence of peptide was measured in each assay and subtracted from all samples. Two different PKC substrates were used in the experiments which yielded identical results (PKC 19-31ser^{25} and GS-peptide).

For low oxygen experiments, sheep pulmonary artery smooth muscle cells were preincubated in Krebs solution degassed with nitrogen/CO_2 95/5%. During preincubations and cell stimulations, a stream of nitrogen/CO_2 was passed over the cells. Under these conditions a P_{O_2} value of approximately 20 mmHg was achieved in the Krebs solution bathing the cells.

All dose-response data were analysed by an iterative curve fitting procedure (Delean *et al.*, 1980). Statistical comparisons were made with Student's unpaired *t* test.

The peptide pseudosubstrate, GS-peptide (Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys) was synthesized and purified by Dr E.G. Rowan of the Strathclyde Institute for Drug Research and Ro-31-8220 (3-{1-[3-(amidinothio)propyl]-3-indoyl}-4-(-methyl-3-indoyl)-1H-pyrrole 2,5-dione) was a kind gift from Dr G. Lawton of Roche Products Ltd., Welwyn Garden City, Herts. All radiochemicals were purchased from Amersham International. PKC 19-31ser^{25} was purchased from Calbiochem. All other chemicals were of the highest quality commercially available.

Results

Figure 1 shows the time course of [^{3}H]-PtdBuOH accumulation in sheep pulmonary artery smooth muscle cells in culture in response to endothelin-1 (ET-1), 5-hydroxytryptamine (5-HT) and the protein kinase C activator, tetradecanoylphorbol acetate (TPA). Endothelin-1 (100 nM) and 5-HT (30 µM) stimulated a rapid accumulation of [^{3}H]-PtdBuOH in pulmonary artery smooth muscle cells which reached a peak between 1–2 min, after which no further increase in accumulation was observed (Figure 1a). The maximum stimulation was different for the two agonists being 4.0 ± 1.5 fold in response to ET-1 (n = 6), and 2.8 ± 0.7 fold in response to 5-HT (n = 4). Following a lag time of approximately 30 s, TPA also stimulated the accumulation of [^{3}H]-

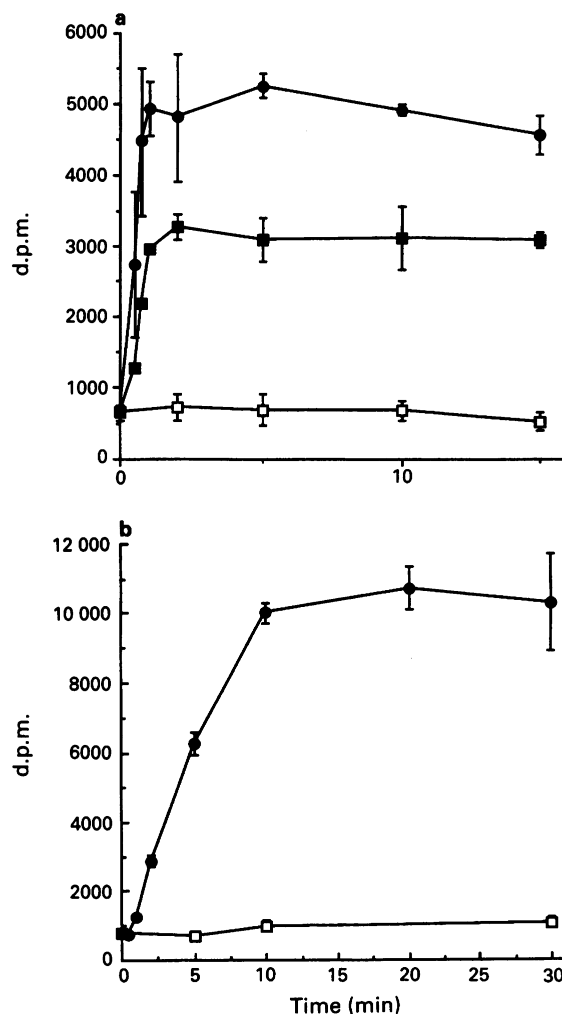

Figure 1 Time course of endothelin-1 (ET-1)-, 5-hydroxytryptamine (5-HT)- and tetradecanoyl phorbol acetate (TPA)-stimulated [^{3}H]-phosphatidylbutanol accumulation in sheep pulmonary smooth muscle cells in culture. Cells prelabelled with [^{3}H]-palmitate, were incubated with maximum concentrations of ET-1 (100 nM) and 5-HT (30 µM) (a), and TPA (100 nM) (b), in the presence of 30 mM butanol. [^{3}H]-phosphatidylbutanol was assayed as outlined in the Methods section. Each point represents that mean ± s.d. of triplicate determinations from a single experiment representative of at least 3 others. (□) Control; (a) (■) 5-HT; (●) ET-1; (b) (●) TPA.

PtdBuOH in pulmonary artery smooth muscle cells (Figure 1b). However, in response to TPA, accumulation was sustained for a longer period reaching a peak at 10 min at approximately 8-10 fold the basal response (9.3 ± 2.0, $n = 7$).

ET-1, 5-HT and TPA stimulated [^{3}H]-PtdBuOH accumulation in a concentration-dependent manner. The ET-1 and TPA response was observed over the low nanomolar concentration range (EC_{50} values: ET-1 = 1.9 ± 1.4 nM, TPA = 12.5 ± 4.9 nM, $n = 3$). 5-HT was less potent with an EC_{50} value in the low micromolar range (IC_{50} = 10.8 ± 3.7 μM, $n = 3$).

Figure 2 shows the effect of the specific PKC inhibitor Ro-318220 (Davis *et al.*, 1989) on both ET-1 and TPA stimulated PLD activity in pulmonary artery smooth muscle cells. Ro-318220 at concentrations of 10 μM or above virtually abolished the response to both compounds. However, there was a significant difference in the IC_{50} value obtained for this effect against either the TPA or the ET-1 response (IC_{50} for Ro-318220: TPA = 0.59 ± 0.09 μM, ET-1 7.3 ± 2.3 μM, $n = 4$; $P < 0.05$). Ro-318220 at 10 μM also abolished 5-HT-stimulated PLD activity (results not shown, $n = 1$).

Following 30 min incubation in low oxygen containing Krebs solution (20 mmHg), basal accumulation of [^{3}H]-phosphatidylbutanol increased by approximately 2 fold (2.01 ± 0.15, $n = 5$). The increase in basal accumulation observed in low oxygen containing buffer was abolished in the presence of the PKC inhibitor, Ro-318220 (Figure 3). In cells preincubated in low oxygen containing medium for 30 min the subsequent stimulation by TPA, ET-1 and 5-HT was reduced (Table 1). However, the decrease in stimulation in fold terms was greater for ET-1 and 5-HT than for TPA (Table 1). In contrast, when hypoxia was initiated simultaneously with agonist addition there was no change in the maximum accumulation of [^{3}H]-phosphatidylbutanol accumulation in response to ET-1 or TPA (results not shown).

TPA and ET-1 also stimulated PKC-mediated peptide pseudosubstrate phosphorylation in pulmonary artery smooth muscle cells (Figure 4). ET-1 (100 nM)-stimulated peptide pseudosubstrate phosphorylation reached a peak by 10 min (2.8 ± 0.5 fold, $n = 3$) and did not increase during the remainder of the time course. TPA (100 nM) also stimulated PKC activity which was sustained for up to 30 min, the longest time point studied (4.4 ± 1.2 fold, $n = 4$). In cells preincubated in low O_2 conditions for 30 min, basal PKC activity also increased by approximately 2 fold (Table 2). The maximum PKC activity stimulated by TPA remained the same resulting in an apparent reduction in the fold stimulation in response to the phorbol ester. However, the response to ET-1 was reduced to a greater extent than that observed for TPA (71 ± 12% vs 48 ± 8%, $n = 4$).

The effects of hypoxia upon ET-1-stimulated [^{3}H]-IP accumulation are shown in Figure 5 and Table 3. Preliminary experiments showed that ET-1 stimulated a linear increase in [^{3}H]-IP accumulation for up to 30 min. Following pretreatment in low oxygen containing medium, ET-1-stimulated accumulation of [^{3}H]-IP was reduced by 34 ± 9% ($n = 4$) whilst basal values were not affected. In control cells, pretreatment with 10 μM Ro-318220 enhanced ET-1 stimulated accumulation of [^{3}H]-IP by approximately 50% (52 ± 22% $n = 4$). Pretreatment of the cells with 10 μM Ro-318220 also prevented the hypoxia-induced reduction in ET-1-stimulated [^{3}H]-IP accumulation (Table 3).

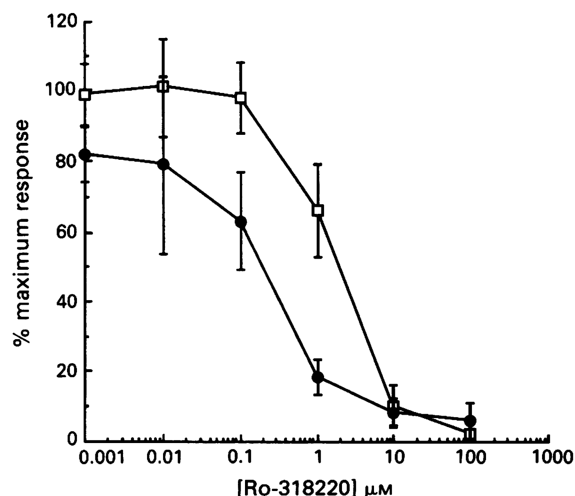

Figure 2 The effect of Ro-318220 upon tetradecanoyl phorbol acetate (TPA)- and endothelin-1 (ET-1)-stimulated [^{3}H]-phosphatidylbutanol accumulation in sheep pulmonary smooth muscle cells in culture. Cells prelabelled with [^{3}H]-palmitate were preincubated with increasing concentrations of Ro-318220 for 20 min prior to stimulation with either ET-1 (100 nM) or TPA (100 nM) and assayed for [^{3}H]-phosphatidylbutanol accumulation as outlined in the Methods section. Each point represents the mean ± s.d. of triplicate determinations from a single experiment representative of at least 3 others. (●) TPA; (□) ET-1.

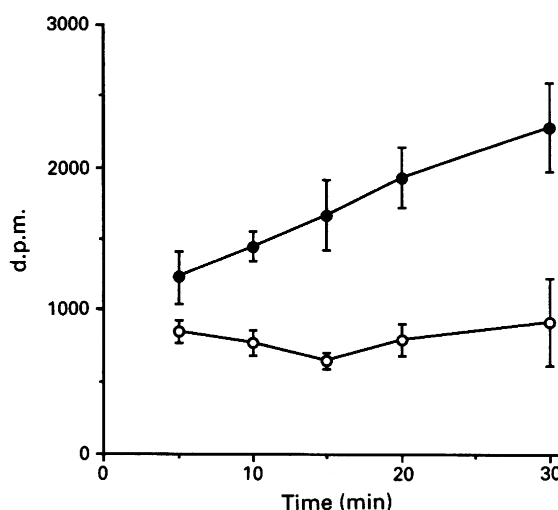

Figure 3 The effect of Ro-318220 on hypoxia-induced [^{3}H]-phosphatidylbutanol accumulation in sheep pulmonary artery smooth muscle cells in culture. Cells prelabelled with [^{3}H]-palmitate were preincubated with 0.05% DMSO (●) or 10 μM Ro-318220 (○) for 20 min before incubation in low oxygen containing Krebs for the times indicated. [^{3}H]-phosphatidylbutanol accumulation was assayed as outlined in the Methods section. Each point represents the mean ± s.d. of triplicate determinations from a single experiment performed at least three times.

Table 1 The effect of hypoxia upon agonist-stimulated [^{3}H]-phosphatidylbutanol accumulation in sheep pulmonary artery smooth muscle cells

	[^{3}H]-phosphatidylbutanol accumulation (% basal)	
	Control	*Hypoxia* (PO_2 20 mmHg)
TPA	802 ± 196	347 ± 119
ET-1	486 ± 134	157 ± 68
5-HT	313 ± 87	108 ± 19

Cells prelabelled with [^{3}H]-palmitate were incubated in normal or low oxygen containing Krebs for 30 min then stimulated with either TPA (100 nM, 10 min) ET-1 (100 nM, 5 min) or 5-HT (30 μM, 5 min) in the presence of 30 mM butanol. Cell extracts were assayed for [^{3}H]-phosphatidylbutanol as outlined in the Methods section. Each value represents the mean ± s.d. from three experiments performed in triplicate. TPA = tetradecanoyl phorbol acetate; ET-1 = endothelin-1; 5-HT = 5-hydroxytryptamine.

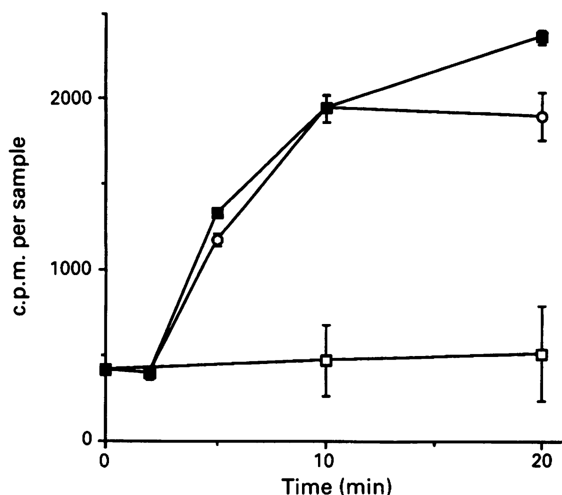

Figure 4 Time course of tetradecanoyl phorbol acetate (TPA) and endothelin-1 (ET-1)-stimulated protein kinase C (PKC)-mediated peptide pseudosubstrate phosphorylation in sheep pulmonary artery smooth muscle cells in culture. Unlabelled cells were stimulated with either 0.05% DMSO (□), 100 nM TPA (■) or 100 nM ET-1 (○), for the times indicated then assayed for PKC activity as outlined in the Methods section. Each point represents the mean ± s.d. of triplicate determinations from a single experiment representative of three others.

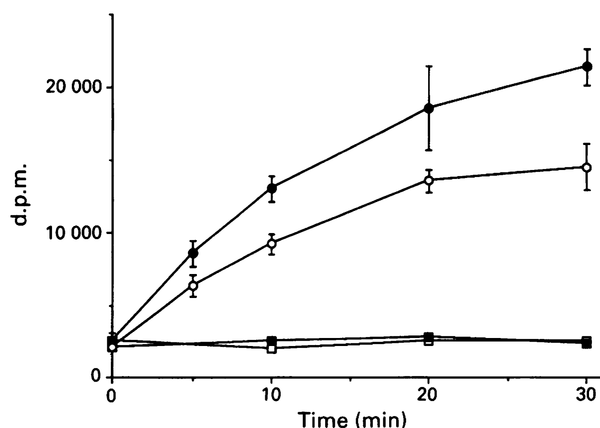

Figure 5 The effect of hypoxia upon endothelin-1 (ET-1)-stimulated [^{3}H]-inositol phosphate accumulation in sheep pulmonary artery smooth muscle cells in culture. Cells prelabelled with [^{3}H]-inositol were preincubated in normal or low oxygen-containing Krebs for 30 min then stimulated with vehicle or 100 nM ET-1 for the times indicated. Cell extracts were assayed for total [^{3}H]-inositol phosphates as outlined in the Methods section. Each point represents the mean ± s.d. of triplicate determinations from a single representative experiment ($n = 4$). Control basal (□); low oxygen basal (■); control ET-1 (●); low oxygen ET-1 (○).

Table 2 The effect of hypoxia upon tetradecanoyl phorbol acetate (TPA) and endothelin-1 (ET-1) stimulated protein kinase C (PKC)-mediated GS-peptide pseudosubstrate phosphorylation in sheep pulmonary artery smooth muscle cells in culture

	GS-peptide phosphorylation (c.p.m.)	
	Control	*Low* O_2 (20 mmHg)
Basal	478 ± 33	907 ± 99
TPA	1916 ± 89	2016 ± 127
ET-1	1389 ± 79	1080 ± 78

Cells were incubated in normal or low oxygen containing Krebs for 30 min and then stimulated with vehicle, 100 nM TPA or 100 nM ET-1 for a further 10 min. GS-peptide phosphorylation was measured as outlined in the Methods section. Each value is the mean ± s.d. from a single experiment performed in triplicate which is representative of at least four independent experiments.

Table 3 The effect of Ro-318220 on hypoxia-induced reduction of endothelin-1 (ET-1) stimulated [^{3}H]-IP accumulation in sheep pulmonary artery smooth muscle cells in culture

	Inositol phosphates (d.p.m.)	
	Control	*Low* O_2 (20 mmHg)
Basal	2627 ± 415	2727 ± 239
Basal + Ro	2887 ± 432	2562 ± 122
ET-1	12320 ± 732	8512 ± 458
ET-1 + Ro	16555 ± 1257	15936 ± 963

Cells prelabelled with [^{3}H]-inositol were preincubated with 10 μM Ro-318220 or vehicle (0.05% DMSO) for 15 min before further incubation in normal or low oxygen containing Krebs for 30 min. Cells were incubated for a further 10 min with 100 nM ET-1. Each value represents the mean ± s.d. of results from a single experiment performed in triplicate and representative of 2 others.

Discussion

In sheep pulmonary artery cultured smooth muscle cells, both ET-1 and 5-HT produce a rapid and transient stimulation of PLD activity. This finding is consistent with results obtained in vasopressin-stimulated A10 smooth muscle cells and in bombesin-stimulated Swiss 3T3 fibroblasts (Plevin *et al.*, 1992; Plevin & Wakelam 1992; Briscoe *et al.*, 1993). We also observed that the response was dependent upon prior PKC activation since incubation with the PKC inhibitor, Ro-318220, abolished both TPA and agonist-stimulated PLD activity. However, it must be noted that the IC_{50} values for Ro-318220 inhibition were different for TPA and ET-1 responses. We have previously suggested this difference to reflect a divergent effect of PKC isoforms upon the activation of PLD and initial receptor /G-protein/PLC coupling (Plevin *et al.*, 1992). These results are consistent with the presence of a sequential pathway in the agonist activation of PLD, involving initial hydrolysis of PtdIns(4,5)P_2 and the intermediate activation of PKC by sn-1,2 DAG (Cook & Wakelam, 1989; Cook *et al.*, 1990).

Hypoxia (Po_2 20 mmHg for 30 min) introduced simultaneously with agonist did not affect the absolute maximum level of accumulation in response to ET-1. This is probably due to the fact that the agonist activation of PLD activity is rapidly desensitized and that changes in oxygen tension were not rapid enough to effect PLD activity. However, over a period of 30 min, [^{3}H]-phosphatidylbutanol accumulation increased to approximately twice the basal value. This increase was abolished by Ro-318220 suggesting that the effect was PKC mediated. In addition, 30 min by hypoxia also reduced the subsequent agonist activation of [^{3}H]-phosphatidylbutanol accumulation. For TPA this reduction reflected the increase in basal PLD activity but for ET-1 and 5-HT an additional decrease was observed. This suggested the involvement of another mechanism in the effect of hypoxia as well as an action upon basal PLD activity.

We further addressed the effect of hypoxia upon PKC activity by measuring PKC-mediated phosphorylation of a defined PKC pseudosubstrate in cells permeabilized with streptolysin-O. The onset of PKC pseudosubstrate phosphorylation was delayed in response to both ET-1 and TPA reflecting the time for permeabilisation to occur. In other systems agonist-stimulated pseudosubstrate phosphorylation has been shown to be abolished by either Ro-31822 or prolonged TPA pretreatment, suggesting that this assay is a

suitable marker for PKC activity (Saville *et al.*, unpublished observations). In cells preincubated in low O_2 containing media for 30 min we also observed that the maximum pseudosubstrate phosphorylation stimulated by TPA was not reduced suggesting that the level of maximum activity of PKC was not compromised. However, the basal PKC activity was increased by 2 fold and ET-1-stimulated PKC activity was reduced. The relative differences in the decreases in fold stimulation by TPA and ET-1 again suggested an additional effect of hypoxia upon receptor-mediated events upstream of PKC.

Further experiments suggested that the effect of hypoxia upon agonist-stimulated PLD and PKC activity may involve the additional regulation of the receptor/G-protein/PLC interaction since pretreatment of the cells in low oxygen containing Krebs reduced the subsequent ET-1-stimulated $[^3H]$-IP accumulation. This hypoxia-mediated effect may also involve the activation of PKC. Protein kinase C-mediated-negative feedback inhibition of agonist-stimulated inositol phosphate formation has been observed in a number of systems including bombesin-stimulated Swiss 3T3 fibroblasts and bradykinin-stimulated chromaffin cells in culture (Plevin *et al.*, 1990; Boarder & Challis 1992). We found that ET-1 stimulated $[^3H]$-inositol accumulation was reduced by pretreatment with the cells in low oxygen containing media and that this reduction was reversed by Ro-318220, results consistent with this proposal. Hypoxia-induced activation of PKC may therefore uncouple the receptor from the G-protein, reducing the subsequent stimulation of inositol phosphate accumulation and other second messenger formation. However, we cannot discount a PKC-mediated decrease in the number of ET-1 receptors, as observed in other vascular smooth muscle cells (Resink *et al.*, 1990), nor the possible inhibition of receptor-operated calcium channel activity. Since PLD activity in vascular smooth muscle cells is dependent on both PKC and external calcium (Lassegue *et al.*, 1991) one or both mechanisms could account for the subsequent reduction in agonist-activation of PLD and PKC.

In the context of these findings, it is clear that hypoxia-induced activation of PKC isoforms may have an important regulatory role on the subsequent activation of second messenger pathways such as PtdIns(4,5)P_2 hydrolysis in smooth muscle cells. This effect, whilst stimulating the cells to grow may not allow activation of pathways detrimental to the long term viability of the cell, for example sustained calcium mobilisation. Of additional importance in delineating the effects of hypoxia on pulmonary artery smooth muscle cell growth, may be a consideration of the temporal relationship between the exposure of the smooth muscle cell to vasoconstrictors and growth factors and the development of hypoxia.

This study was funded by the Wellcome Trust (R.P.). N.K. was a recipient of a Nuffield Foundation Undergraduate Bursary Award (1992). The authors wish to thank Dr G. Lawton of Roche Products Ltd. (Welwyn Garden City, Herts) for the kind gift of Ro-318220.

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(Received December 2, 1993
Revised January 26, 1994
Accepted January 27, 1994)