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# Stimulation of the hydrolysis of phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine by endothelin, a complete mitogen for Rat-1 fibroblasts

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The mitogenic activity of endothelin and its ability to stimulate PtdIns(4,5)$P_2$ and phosphatidylcholine turnover in Rat-1 fibroblasts was studied. Stimulated incorporation of [$^3$H]thymidine occurred in the absence of any other added growth factors. The endothelins stimulated rapid generation of both Ins(1,4,5)$P_3$ and choline. Endothelin-1 and endothelin-2 were equipotent in stimulating both responses, but endothelin-3 was less potent. Endothelin-1-stimulated Ins(1,4,5)$P_3$ generation reached a maximum at 5 s and then declined; however, the response was long-lived, with a 4.5-fold elevation over basal still observed after 15 min. Endothelin-stimulated choline generation was observed with no increase in choline phosphate; indeed, the apparent level of this metabolite fell after 30 min of stimulation, presumably due to the observed stimulation of phosphatidylcholine synthesis. The endothelin-stimulated increase in choline generation was abolished in cells where protein kinase C was down-regulated. However, endothelin-stimulated choline generation was greater than that observed in response to a protein kinase C-activating phorbol ester, raising the possibility that the peptide activates phospholipase D by both protein kinase C-dependent and -independent mechanisms.

## INTRODUCTION

The endothelins are a family of 21-amino-acid potent vasoactive peptides. Endothelin-1 (ET-1) was originally isolated by Yanagisawa *et al.* [1] from medium conditioned by pig endothelial cells. Cloning and sequence analysis have now shown that there are in fact three ET-related genes in human, pig and rat genomes which encode very similar peptides known as ET-1, ET-2 and ET-3 [2]. Most recently, the existence of another member of this family, the vasoactive intestinal contractor (VIC) peptide, was reported [3]. ET-1 also shows remarkable sequence similarity to a family of 21-residue toxins from the venom of the Israeli burrowing asp, *Atractaspis engaddensis*, known as the sarafotoxins S6 (SS6) [4].

ET-1 is the most potent mammalian vasoconstrictor peptide known and has a very strong and sustained constrictor activity *in vitro* and pressor activity *in vivo* [5]. Constriction of isolated arterial strips is slow in onset, long lasting and extremely difficult to wash out, and is dependent on extracellular $Ca^{2+}$ [1]. However, ET-1 has now been shown to have pharmacological effects in tissues other than blood vessels [5], including contraction of non-vascular smooth muscles, stimulation of atrial natriuretic peptide secretion, inhibition of renin release and positive inotropic and chronotropic effects on myocardium, and has been found to act as a potent mitogen in both smooth muscle cells [6,7] and Swiss 3T3 fibroblasts [8,9]. In agreement with these observations, specific high-affinity binding sites for ET-1 have been demonstrated in tissues other than the vascular system [10].

The mechanism of action of the ETs is unknown. ET-1-induced increases in intracellular free $Ca^{2+}$ have been demonstrated in many systems. In some cases this has been attributed to the mobilization of $Ca^{2+}$ from intracellular stores by Ins(1,4,5)$P_3$ generated by phospholipase C-catalysed hydrolysis of PtdIns(4,5)$P_2$ [6,8,11,12]. However, activation of plasma membrane $Ca^{2+}$ channels has also been reported and in some systems the increases in intracellular $Ca^{2+}$ have been shown to be partly dependent on the influx of extracellular $Ca^{2+}$ [6,8,13,14]. The other product of PtdIns(4,5)$P_2$ breakdown is *sn*-1,2-diacylglycerol (DAG) which can activate protein kinase C (PKC). Elevated cellular DAG levels in response to ET-1 stimulation have been reported in several systems [8,12,15,16].

Both increases in intracellular free $Ca^{2+}$ concentration and elevated DAG levels are thought to be early signals in the stimulation of DNA synthesis, and the inositol phospholipid second-messenger-generating pathway is generally considered to be a major signalling pathway in mitogenesis [17]. There is also, however, increasing evidence for stimulated phosphatidylcholine (PtdCho) breakdown to provide an additional source of DAG in cells in the absence of elevated Ins(1,4,5)$P_3$ [18,19]. Using Rat-1 fibroblasts as a model system, we have found ET-1 to be a complete mitogen for these cells and capable of stimulating both PtdIns(4,5)$P_2$ and PtdCho hydrolysis. Comparison of second messenger generation in response to ET-1 with that produced by ET-2, ET-3 and SS6 suggests that both pathways are controlled by the same receptor. We have also investigated further the mechanism of ET-1-stimulated PtdCho hydrolysis, which our results indicate to be predominantly PKC-dependent.

## MATERIALS AND METHODS

Rat-1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum at 37 °C in a humidified atmosphere of air/$CO_2$ (19:1). For experiments, cells were seeded in 24-well plates and labelled to isotopic equilibrium with either [$^3$H]choline chloride (5 $\mu$Ci/ml) in DMEM containing 1% newborn calf serum or [$^3$H]inositol (2 $\mu$Ci/ml) in inositol-free DMEM containing 1% dialysed newborn calf serum for 48 h. The cells were used when confluent and quiescent.

On the day of experiments, the labelling medium was removed and the cells were washed twice with 0.5 ml of Hanks' buffered

Abbreviations used: ET, endothelin; PKC, protein kinase C; DAG, *sn*-1,2 diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; HBG, Hanks' buffered saline containing 1% (w/v) BSA and 10 mM-glucose; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; PLD, phospholipase D; SS6, sarafotoxins S6; PtdCho, phosphatidylcholine; $EC_{50}$, concentration causing 50% of maximum effect.

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saline, pH 7.4, containing 1% (w/v) BSA and 10 mM-glucose (HBG). For choline experiments the cells were preincubated with 0.5 ml of fresh HBG for a further 30 min and stimulation was carried out with agonists in HBG. For inositol phosphate experiments the cells were incubated for 10 min with HBG containing 10 mM-LiCl (HBG/LiCl) and stimulation was carried out with agonists in HBG/LiCl for 20 min. All incubations were performed at 37 °C. Reactions were terminated by the addition of 0.5 ml of ice-cold methanol. For experiments in which intracellular choline levels were to be measured, the incubation medium was aspirated before the addition of methanol. The cells in each well were then scraped and transferred to vials. Chloroform was added to give a chloroform/methanol ratio of 1:2 (v/v) and the samples were extracted overnight. The phases were split by the addition of chloroform and water to give final proportions of chloroform/methanol/water of 1:1:0.9 (by vol.) and the upper phase was taken for analysis of either water-soluble choline metabolites or total inositol phosphates.

Total inositol phosphates were analysed by batch chromatography on Dowex-1 formate as previously described [20]. Water-soluble choline metabolites were analysed by cation-exchange chromatography on Dowex-50-W-$H^+$ columns as described by Cook & Wakelam [18]. Changes in PtdCho were determined by dissolving the dried lipid phase in chloroform/methanol (19:1, v/v) and separating the lipids by t.l.c. on silica-gel G plates in a chloroform/methanol/acetic acid/water (75:45:3:1, by vol.) solvent system [21].

For Ins(1,4,5)$P_3$ mass measurements, cells were grown to confluency in 75 $cm^2$ flasks and mechanically harvested into their culture medium. After a 45 min incubation time at 37 °C the cells were spun down and washed twice in HBG. Samples (50 $\mu$l) containing approx. $10^6$ cells were then stimulated with 25 $\mu$l of agonist and the reactions were terminated by the addition of 25 $\mu$l of 10% (v/v) ice-cold $HClO_4$. The samples were extracted on ice and neutralized with 1.5 M-KOH/60 mM-Hepes. Ins(1,4,5)$P_3$ levels were then determined by the mass assay of Palmer *et al.* [22]. Briefly, Ins(1,4,5)$P_3$ in the cell extract was competed with [$^3$H]Ins(1,4,5)$P_3$ for the Ins(1,4,5)$P_3$-specific binding site of bovine adrenocortical microsomes. The quantity of Ins(1,4,5)$P_3$ in the cell extract was then determined from a standard curve based on pure Ins(1,4,5)$P_3$. Quantitatively and qualitatively similar results were obtained when Ins(1,4,5)$P_3$ generation was determined in cells attached to plastic.

Phorbol 12,13-dibutyrate (PDBu) binding was determined on confluent and quiescent cells in 24-well plates. The monolayers were washed twice with 0.5 ml of HBG at 37 °C and binding was then carried out by incubating the cells at 37 °C with 150 $\mu$l of 100 nM-[$^3$H]PDBu (specific radioactivity 25 Ci/mmol) in HBG for 20 min. Specific binding was defined by the inclusion of 20 $\mu$M unlabelled PDBu. The incubations were terminated by washing three times with 0.5 ml of ice-cold HBG. The cells were solubilized with 0.5 ml of 0.5 M-NaOH/0.1% (v/v) Triton X-100 and bound radioactivity was determined by scintillation counting.

For measurement of DNA synthesis, cells were grown to confluence and quiescence in DMEM containing 10% newborn calf serum. The medium was then changed to serum-free DMEM for 24 h. Additions of mitogens were made at the concentrations indicated in serum-free DMEM containing 1 $\mu$Ci of [$^3$H]thymidine/ml and the cells were incubated for a further 24 h. The medium was then aspirated and the monolayers were washed twice with 0.5 ml of HBG, three times with 0.5 ml of ice-cold 5% (v/v) trichloroacetic acid and twice with 0.5 ml of ice-cold ethanol. The radioactivity associated with the acid-insoluble material was determined by scintillation counting after solubilizing the cells in 0.3 M-NaOH.

Tissue culture materials were from Gibco, Paisley, Scotland, U.K.; radiochemicals were from Amersham International, Amersham, Bucks., U.K.; ET-1 was from BioMac, University of Glasgow, Glasgow, Scotland, U.K.; ET-2 was from Scientific Marketing Associates, London, U.K.; ET-3 was from Novabiochem, Nottingham, U.K.; and Dowex 50 × 8-400 $H^+$ cation-exchange resin, Dowex 1 × 8-200 $Cl^-$ anion-exchange resin and phorbol esters were from Sigma, Poole, Dorset, U.K. All other reagents were of the highest grade available.

## RESULTS

ET-1 is a complete mitogen for Rat-1 cells. Although not as potent as 10% serum or EGF (10 nM), a maximal dose of ET-1 (100 nM) stimulated [$^3$H]thymidine incorporation in quiescent cells in serum-free medium in the absence of any other added growth factors. Bombesin, on the other hand, did not significantly stimulate mitogenesis in the absence of other growth factors [results from a typical experiment: $(1.20 \pm 0.14) \times 10^5$ d.p.m. in control; $(8.0 \pm 0.2) \times 10^5$ d.p.m. with 10% serum; $(4.0 \pm 0.84) \times 10^5$ d.p.m. with 100 nM-ET-1; $(1.93 \pm 0.43) \times 10^5$ d.p.m. with 617 nM-bombesin; 24 h incubation; $n = 3$].

ET-1 (100 nM)-stimulated PtdIns(4,5)$P_2$ hydrolysis was measured by a rapid increase in the mass of Ins(1,4,5)$P_3$ which peaked at 5–10 s with about a 10-fold increase over controls (results from one typical experiment out of a total of three, $n = 3$: $0.8 \pm 0.4$ pmol in control; $7.9 \pm 0.9$ pmol with ET-1) and then declined towards basal levels (Fig. 1). However, levels remained stimulated above controls at 15 min (results from a typical experiment: $0.8 \pm 0.3$ pmol in control; $3.7 \pm 0.3$ pmol with ET-1; $n = 3$). The generation of [$^3$H]inositol phosphates in response to ET-1 was dose-dependent, with a maximal response at approx. 10 nM and an $EC_{50}$ value (concentration causing 50% of the maximum effect) of $1.9 \pm 1.0$ nM ($n = 5$) (Fig. 2*a*). Pharmacological characterization of inositol phosphate generation using ET-2, ET-3 and SS6 yielded $EC_{50}$ values of $1.2 \pm 0.3$ nM, $54 \pm 18$ nM and $1.7 \pm 0$ nM respectively ($n = 3$), thus giving a rank order of potency of ET-1 = ET-2 = SS6 > ET-3.

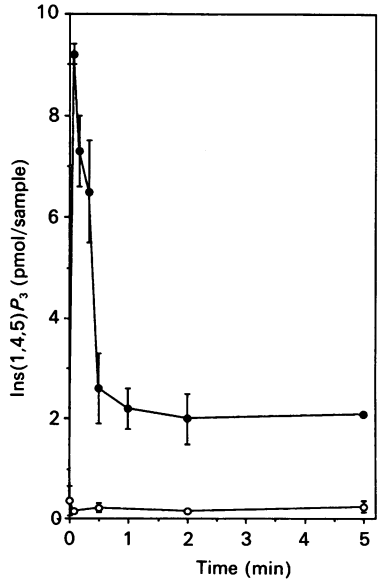


**Fig. 1. Time course of ET-1-stimulated Ins(1,4,5)$P_3$ generation**

Rat-1 cells were grown to confluency and harvested as described in the Materials and methods section. Stimulation was carried out with 100 nM-ET-1 for the stated times and Ins(1,4,5)$P_3$ levels were measured by the mass assay of Palmer *et al.* [22]. The results are expressed as means ± S.D. from a typical experiment where $n = 3$. ○, Control cells; ●, ET-1 stimulated cells.

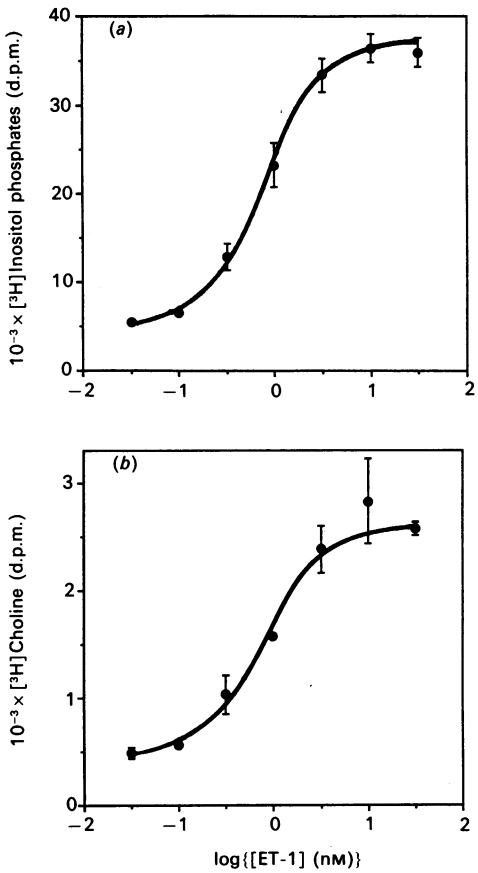


**Fig. 2. Dose-dependence of ET-1-stimulated (*a*) inositol phosphate and (*b*) choline generation**

[$^3$H]Inositol- or [$^3$H]choline-labelled Rat-1 cells were stimulated with increasing concentrations of ET-1 and the radioactivity associated with (*a*) total inositol phosphates and (*b*) the choline fraction was determined as described. The results are expressed as means ± S.D. from single typical experiments where $n = 5$.

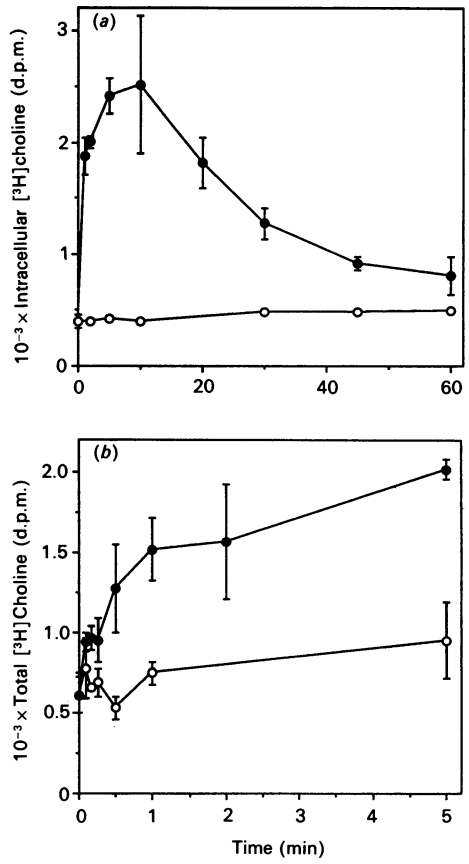


**Fig. 3. Time course of ET-1-stimulated changes in (*a*) intracellular and (*b*) total choline**

Rat-1 cells were labelled with [$^3$H]choline for 48 h and then stimulated with 100 nM-ET-1 for the stated times. The [$^3$H]choline-containing fractions were separated as described in the text. The results are expressed as means ± S.D. from single typical experiments where $n = 3$–5. ○, Control cells; ●, ET-1 stimulated cells.

The generation of [$^3$H]choline in response to a maximal dose of ET-1 was also very rapid. There was a significant increase in both intracellular and total choline (intracellular + extracellular) after 10 s which had at least doubled by 1 min (Figs. 3*a* and 3*b*). Thereafter, the rate of production of total [$^3$H]choline decreased, and by 5 min it paralleled the basal increase. Both stimulated and basal levels of total [$^3$H]choline reached steady state after about 30 min. The kinetics of intracellular choline changes were somewhat different, peaking at about 5 min and remaining elevated for a further 10–15 min before decreasing and returning to near-basal levels at 60 min. The maximum obtainable increase in both intracellular and total choline levels in the presence of ET-1 over controls varied between 2- and 5-fold. The production of [$^3$H]choline in response to ET-1 was also dose-dependent, with an $EC_{50}$ value of 0.8 ± 0.4 nM ($n = 5$), which is close to that for PtdIns(4,5)$P_2$ hydrolysis (Fig. 2*b*). The pharmacology of choline and inositol phosphate generation was very similar. $EC_{50}$ values for choline generation for ET-2, ET-3 and SS6 of 0.7 ± 0.2 nM, 56 ± 18 nM and 1.2 ± 0.2 nM ($n = 3$) respectively were obtained, yielding the same rank order of potency (ET-1 = ET-2 = SS6 > ET-3).

There was no significant change in [$^3$H]choline phosphate content until approx. 30 min after stimulation with ET-1, when a decrease of 15–30% below controls was observed (Fig. 4*a*). In order to determine whether this might be due to stimulated incorporation into the parent lipid, PtdCho synthesis in response to ET-1 stimulation was investigated. The stimulated [$^3$H]choline incorporation into PtdCho was evident as early as 5 min after stimulation and increased up to 20 min, when it was paralleled by the basal increase (Fig. 4*b*). The maximum response was 150–180% of the basal (results from a typical experiment: 5097 ± 861 d.p.m. in control; 8357 ± 453 d.p.m. with ET-1; 20 min stimulation, $n = 3$). Glycerophosphocholine generation did not change in response to ET-1 stimulation (results not shown).

The generation of [$^3$H]choline in response to the PKC-activating phorbol ester phorbol 12-myristate 13-acetate (PMA) was also time- and dose-dependent. PMA stimulated choline generation after a lag time of 1–2 min (Fig. 5) with an $EC_{50}$ value of 27 ± 14 nM ($n = 2$). The inactive $\beta$-phorbol was without effect (results from a typical experiment: 708 ± 35 d.p.m. in control; 750 ± 51 d.p.m. with 300 nM-$\beta$-phorbol; 2954 ± 257 d.p.m. with 300 nM-PMA; 10 min stimulation). The magnitude of the PMA response was, however, generally lower than that obtained with ET-1 (results from a typical experiment: 1106 ± 306 d.p.m. in control; 2130 ± 38 d.p.m. with 300 nM-PMA; 3045 ± 248 d.p.m. with 100 nM-ET-1; 10 min stimulation, $n = 4$).

Long-term pretreatment with high concentrations of PMA has frequently been used to down-regulate PKC activity (e.g. [23]). In Rat-1 cells in which PKC had been down-regulated by 48 h of treatment with 400 nM-PMA, choline generation in response to

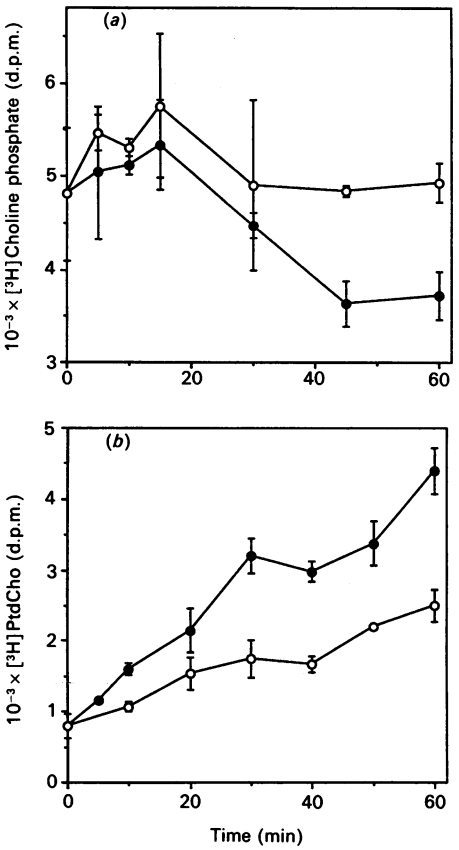


**Fig. 4. Time course of ET-1-stimulated changes in (*a*) choline phosphate and (*b*) PtdCho**

(*a*) Rat-1 cells were labelled for 48 h with [$^3$H]choline and then stimulated with 100 nM-ET-1 for the stated times. [$^3$H]Choline phosphate-containing fractions were separated as described in the text. (*b*) Rat-1 cells were grown to confluency and quiescence and then preincubated with 28 $\mu$M-choline chloride and 5 $\mu$Ci of [$^3$H]choline/ml for 30 min. Stimulation was carried out with 100 nM-ET-1 for the stated times and the samples were extracted as described in the text. The lipid phases were dried down, redissolved in chloroform/methanol (19:1, v/v) and separated by t.l.c. as described in the Materials and methods section. Results are expressed as means ± S.D. from single typical experiments where $n = 3$. ○, Control cells; ●, ET-1 stimulated cells.

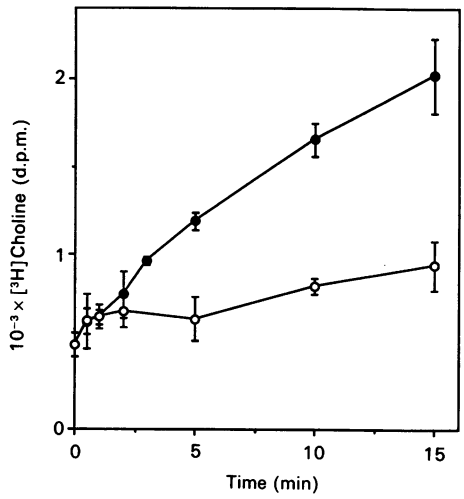


**Fig. 5. PMA-stimulated choline generation**

[$^3$H]Choline-labelled Rat-1 cells were stimulated with 300 nM-PMA for the indicated times. The [$^3$H]choline-containing fractions were separated as described. The results are expressed as means ± S.D. from a single typical experiment where $n = 3$. ○, Control cells; ●, PMA-stimulated cells.

**Table 1. ET-1- and PMA-stimulated choline generation in control and PKC-down-regulated cells**

Rat-1 cells were labelled with [$^3$H]choline for 48 h in the presence of PMA (400 nM) or the inactive $\beta$-phorbol (400 nM). Stimulation was carried out with 100 nM-ET-1 or 300 nM-PMA and the radioactivity associated with the choline fraction was measured. Results are expressed as means ± S.D. from a single typical experiment where $n = 3$.

| Treatment | Choline generation (d.p.m.) | |
|---|---|---|
| | +PMA | +$\beta$-Phorbol |
| Control (0 min) | 324±108 | 287±34 |
| Control (2 min) | 354±118 | 438±158 |
| ET-1 (2 min) | 497±221 | 1248±162 |
| Control (10 min) | 488±133 | 521±58 |
| ET-1 (10 min) | 433±19 | 1667±158 |
| PMA (10 min) | 477±67 | 1242±154 |

both ET-1 (100 nM) and PMA (300 nM) was completely abolished. In control cells which were treated with the inactive $\beta$-phorbol for an equivalent length of time the stimulated generation of choline was as observed in normal cells (Table 1). [$^3$H]PDBu binding was utilized as a measure of PKC activity, and suggested that PKC had indeed been down-regulated, although a residual activity still remained (results from a typical experiment: 5054±114 d.p.m. in control; 5356±113 d.p.m. in $\beta$-phorbol-treated cells; 1748±12 d.p.m. in PMA-treated cells; 20 min incubation, $n = 2$). Prolonged PMA treatment had little effect on ET-1-stimulated inositol phosphate generation [results from a typical experiment: 11352±4035 d.p.m. (basal) and 37812±5679 d.p.m. (stimulated) in control cells; 15716±2033 d.p.m. (basal) and 49151±5687 d.p.m. (stimulated) in PMA-treated cells; 20 min of ET-1 stimulation], indicating that coupling of ET to the inositol phosphate system was still intact.

## DISCUSSION

Since the discovery of ET-1 by Yanagisawa *et al.* [1], many investigators have sought to determine the second-messenger-generating pathways which may mediate its mechanism of action. So far most attention has focused on the hydrolysis of PtdIns(4,5)$P_2$ as a means of generating intracellular signals. However, there is accumulating evidence from other systems for agonist-induced PtdCho hydrolysis, and many hormones and growth factors have now been shown to stimulate PtdCho hydrolysis in addition to PtdIns(4,5)$P_2$ breakdown (see [24] for a review). This generates an alternative source of DAG in the absence of any further increase in intracellular $Ca^{2+}$ due to Ins(1,4,5)$P_3$ formation, and could therefore cause a more sustained activation of PKC after the PtdIns(4,5)$P_2$ response has been desensitized [19]. This may be important in long-term receptor-mediated events such as proliferation and differentiation. PtdCho turnover is of interest when investigating the mechanism of action of ET, since this vasoconstrictor peptide has been reported to cause extremely long-lasting pressor responses *in vivo* as well as sustained constriction in isolated smooth muscle preparations [5], and also to possess mitogenic activity [6–9].

We have shown that in Rat-1 fibroblasts ET-1 (100 nM) is a

complete mitogen. This is therefore a relevant model system in which to investigate further the signalling pathways stimulated by ET-1. A previous report [12] demonstrated PtdIns(4,5)$P_2$ hydrolysis stimulated by ET-1 in Rat-1 cells and we confirm this (Fig. 1). However, our results are the first mass measurements of Ins(1,4,5)$P_3$ generation in response to ET-1 stimulation in any system. Previous studies have employed [$^3$H]inositol-labelled cells and have generally measured Ins$P_3$ generation in the presence of LiCl, often with no attempt to separate the different isomers. The magnitude of the response that we have observed is much greater than that reported previously, being approx. 10-fold at 5 s, probably due to the greater sensitivity of the Ins(1,4,5)$P_3$ mass assay. The Ins(1,4,5)$P_3$ response was very rapid, peaking at 5–10 s and returned towards basal levels in 30 s. It was, however, still elevated above control levels at 15 min. This contrasts with other work from this laboratory using bombesin-stimulated Swiss 3T3 cells, in which the Ins(1,4,5)$P_3$ signal was completely desensitized by 30 s, possibly reflecting differences in the mechanisms of action of the two peptides. The sustained phase of the ET-1 response, which suggests sustained PtdIns(4,5)$P_2$ hydrolysis, obviously has important implications for both the intracellular free $Ca^{2+}$ concentration and DAG levels which may be important in the mitogenic activity of ET-1 in Rat-1 cells. We have also shown for the first time that ET-1 stimulates PtdCho breakdown in addition to PtdIns(4,5)$P_2$ hydrolysis. The response was rapid and dose-dependent, with an $EC_{50}$ comparable with that obtained for PtdIns(4,5)$P_2$ hydrolysis [$1.9 \pm 1.0$ nM for PtdIns(4,5)$P_2$ hydrolysis; $0.8 \pm 0.4$ nM for PtdCho hydrolysis]. This value is similar to the $K_d$ reported for ET-1 binding to rat vascular smooth muscle cells [13] and the $EC_{50}$ values for ET-1-stimulated DNA synthesis [7], inositol phosphate generation and intracellular $Ca^{2+}$ transients [25] and arachidonate release [26] in vascular smooth muscle cells.

Pharmacological characterization of PtdIns(4,5)$P_2$ and PtdCho hydrolysis using ET-1 and the analogous peptides ET-2, ET-3 and SS6 yielded the same rank order of potency, i.e. ET-1 = ET-2 = SS6 > ET-3, for both responses, with each peptide having a similar $EC_{50}$ value for both responses ( $\sim$ 1–2 nM for ET-1, ET-2 and SS6; $\sim$ 50 nM for ET-3). These results therefore suggest that the two second-messenger-generating pathways are controlled via the same receptor. There is, however, the possibility of the existence of multiple ET receptor subtypes. Several recent reports have proposed two or more ET receptors. Watanabe *et al.* [27] have suggested the presence of two distinct types of ET receptors of chick cardiac membranes, one having higher affinity for ET-1 and ET-2 and the other with higher affinity for ET-3, whereas Kloog *et al.* [28] have proposed the presence of three apparent receptor subtypes in rat tissues based on their relative affinities for ET-1, ET-3, $SS6_b$ and $SS6_c$.

The hydrolysis of PtdCho was observed as an early 10 s increase in [$^3$H]choline, with no significant change in [$^3$H]choline phosphate by 30 min. These results suggest that ET-1-stimulated hydrolysis of PtdCho in Rat-1 cells proceeds via a phospholipase D (PLD)-catalysed mechanism. PLD-mediated hydrolysis of PtdCho has now been proposed in a number of systems, including vasopressin-stimulated hepatocytes [29] and REF52 cells [30], ATP-stimulated BPAEC cells [31], fMet-Leu-Phe-stimulated HL-60 granulocytes [32] and bombesin-stimulated Swiss 3T3 fibroblasts [18]. The lipid product of a PLD hydrolysis would be phosphatidic acid, which can be further metabolized by phosphatidic acid phosphohydrolase to generate DAG. There have been several reports of increased DAG levels in response to ET-1 stimulation. Griendling *et al.* [15] observed a biphasic accumulation of DAG in response to 100 nM-ET-1 in vascular smooth muscle cells which was sustained above the baseline for at least 20 min, and they raised the possibility of a role for PtdCho hydrolysis, a proposal confirmed by us. Ins$P_3$ production in the same cell line was transient and had returned to baseline by 5 min [33], which supports a role for an alternate DAG source.

There was no change in [$^3$H]choline phosphate levels until approx. 30 min after stimulation with ET-1, when about a 30% decrease below controls was observed. This may be the result of incorporation into lipid due to the activation of the PtdCho synthetic pathway, since we have detected increased PtdCho synthesis as early as 5 min after agonist stimulation. In fact, there may be generation of choline phosphate at these earlier time points due to choline kinase or phospholipase C activity, but this may not be detected due to rapid reincorporation into the parent lipid. Price *et al.* [34] have also reported stimulated PtdCho synthesis in Swiss 3T3 cells in response to a number of growth factors, but their observations were made only after a 30 min stimulation.

PtdCho hydrolysis in Rat-1 cells could also be stimulated by the PKC-activating phorbol ester PMA, suggesting that activation of PKC in these cells can lead to breakdown of PtdCho. This is supported by the inability of the inactive $\beta$-phorbol to elicit a response. The similarity between the ET-1 and PMA-stimulated responses suggests that they have a common mechanism of action. In order to investigate further the role of PKC in stimulating PLD activity, cells were treated with 400 nM-PMA for 48 h, since this treatment has been reported to down-regulate PKC [23]. The PMA- and ET-1-stimulated generation of choline was abolished in the down-regulated cells, but not in cells which had been treated with the inactive control, $\beta$-phorbol. The loss in PKC activity was confirmed by PDBu binding; specific binding of [$^3$H]PDBu was decreased in PMA-treated cells but remained the same as controls in $\beta$-phorbol-treated cells. Long-term PMA pretreatment did not affect the ability of ET-1 to stimulate inositol phosphate generation, suggesting that it had not affected events upstream from PKC such as receptor function or coupling to the inositol phosphate pathway. This contrasts with the effect of phorbol ester treatment in human vascular smooth muscle cells, where pretreatment of the cells for 24 h with PMA resulted in decreased ET-1 binding and inhibited both the inositol phosphate and DAG responses to ET-1 [35]. Our results favour a role for PKC in stimulating PLD activity. This also appears to be the case in other systems where phorbol ester stimulation resulted in PtdCho turnover via a PLD activity. Work from this laboratory [18] has shown PMA- and bombesin-stimulated choline generation in Swiss 3T3 cells, both of which were abolished by PMA-stimulated down-regulation of PKC, while in REF52 cells [36] pretreatment with phorbol diester or staurosporin, a PKC-kinase inhibitor, inhibited subsequent phorbol diester-stimulated PtdCho hydrolysis. Results such as these imply that stimulation of PLD activity may be secondary to the activation of PKC, for example as a result of PtdIns(4,5)$P_2$ breakdown. This is supported by comparing the kinetics of Ins(1,4,5)$P_3$ generation and choline generation, as the former appears to precede the latter. However, it is also noteworthy that in our Rat-1 system the magnitude of the choline response stimulated by PMA was usually smaller than that obtained with ET-1. Also, although PMA pretreatment totally abolished the ET-1-stimulated choline response, not all of the PKC activity as defined by PDBu binding was down-regulated, though it may be that the residual activity is incapable of stimulating PLD. Therefore there are two possible mechanisms of ET-1-stimulated PtdCho hydrolysis: it may be occurring downstream from PtdIns(4,5)$P_2$ hydrolysis via PKC-activated PLD as suggested above or, alternatively, there may be direct receptor-mediated activation of PLD. The smaller choline response obtained with PMA raises the possibility of both PKC-dependent and

-independent mechanisms of activation. The loss of stimulated choline generation of the PKC down-regulated cells may be due to the loss of other components of the PLD-activating pathway.

Mitogenesis is clearly a complex event, probably involving the interaction of many different signalling pathways. Our results show ET-1 to be a complete mitogen in Rat-1 cells and active in stimulating the PtdIns(4,5)$P_2$ and PtdCho second-messenger-generating pathways. Pharmacological characterization of both responses using ET-1 and the analogous peptides ET-2, ET-3 and SS6 suggests that both pathways are controlled by the same receptor. The choline-generating pathway appears to take place kinetically downstream from Ins(1,4,5)$P_3$ generation via a PLD-catalysed mechanism involving a predominantly PKC-dependent means of activation. The lipid product of such an hydrolysis would be phosphatidic acid. The further metabolism of phosphatidic acid to DAG by phosphatidic acid phosphohydrolase and the sustained hydrolysis of PtdIns(4,5)$P_2$ may be important for prolonged activation of PKC. This may be relevant in the mechanism of action of ET-1 due to its long-lasting physiological effects and mitogenic activity.

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