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# Endothelin-1 stimulated phospholipase D in A10 vascular smooth muscle derived cells is dependent on tyrosine kinase

## Evidence for involvement in stimulation of mitogenesis

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The mechanism whereby endothelin stimulates motogenesis of vascular smooth muscle cells is not understood. Here we show that endothelin-1 stimulates phospholipase D by a protein kinase C and tyrosine kinase dependent mechanism, and present evidence that implicate the phosphatidic acid formed by phospholipase D in the mitogenic response.

Endothelin; Phospholipase D; Tyrosine kinase; Protein kinase C; Mitogenesis; Smooth muscle cell

## 1. INTRODUCTION

Endothelin-1 (ET-1) is known to be mitogenic for vascular smooth muscle cells [1,2] and fibroblasts [3–5] maintained in culture. This mitogenic activity has been associated with the stimulated expression of the proto-oncogenes c-*fos* and c-*myc* in both smooth muscle cells [2] and fibroblasts [4]. ET-1 acting at $ET_A$ receptors is known to stimulate both phospholipase C (PLC) [3,4] and phospholipase D (PLD) [5] in vascular smooth muscle cells. Phosphatidic acid is the primary lipid product of PLD, and it (or its *lyso* derivative) may be mitogenic in its own right [6,7]. Phospholipase D has been reported as being activated in several ways, including stimulation by protein kinase C (PKC) (e.g. [8–13]) and elevation of intracellular free $Ca^{2+}$ (e.g. [10,14]).

Two recent reports suggest the possibility that tyrosine kinases may be involved in the mitogenic response. Firstly, it was demonstrated that ET-1, along with vasopressin and angiotensin II, enhance tyrosine phosphorylation in glomerular mesangial cells [15]. These agonists all share the PLC receptor–effector mechanism. It was also shown that fMet-Leu-Phe acting on neutrophils activates PLD by a mechanism involving enhanced protein tyrosine phosphorylation [16]. This agonist, which stimulates PLC, has previously been reported to activate tyrosine phosphorylation by an unresolved mechanism. Following from this, it has recently been shown that pervanadate induced enhancement of tyrosine phosphorylation leads to PLD activation in neutrophils [17].

In view of these various reports, we set out to study the mechanism by which ET-1 regulates PLD in cultured vascular smooth muscle cells. We provide evidence that both PKC and tyrosine kinases are involved and ask whether this activation of PLD could be instrumental in mediating endothelin stimulated mitogenesis.

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*Abbreviations:* ET-1, endothelin-1; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; PA, phosphatidic acid; PBut, phosphatidylbutanol; PMA, phorbol myristate acetate; $IC_{50}$, concentration giving 50% of maximal inhibition.

## 2. EXPERIMENTAL

A10 cells were maintained as previously described [3], were seeded into 24-well multiwell plates and grown to confluence. Experiments were performed in a balanced salt solution consisting of (mM) NaCl 120, KCl 5.4, $NaHCO_3$ 16.2, $MgSO_4$ 0.8, $NaH_2PO_4$ 1, $CaCl_2$ 1.8, D-glucose 5.5, 4-(2-hydroxethyl)-1-piperazine ethane sulphonic acid 30, at pH 7.4.

The procedure for labelling of A10 cells with $^{32}P$ and analysis of phospholipids was as described previously for endothelial cells [16]. Briefly, monolayer cultures of A10 cells were loaded with $^{32}P_i$ at a concentration of 0.25 MBq/ml in a 0.4 ml phosphate-free balanced salt solution for 24 h. In order to study PLD the generation of [$^{32}$P]PBut (phosphatidylbutanol) was measured: this is generated in the presence of butanol by the transphosphatidylation reaction unique to PLD. Cells were exposed to 50 mM butanol for 10 min prior to, and during, experimentation. We have fully characterised this procedure elsewhere [13], and shown that 50 mM butanol converts all the PLD product to PBut, so that residual agonist stimulated [$^{32}$P]PA is a product of the PLC/diacylglycerol kinase route. Where PKC activating phorbol myristate acetate (PMA), PKC inhibitor Ro 31-8220 (a kind gift of Roche Products Ltd., Welwyn, UK)/or tyrosine kinase inhibitor ST 271 [16] (a kind gift of Wellcome Foundation Ltd., Beckenham, UK)

were used they were included for 10 or 20 min prior to and during the 3 min incubation. The reaction was terminated with methanol, lipids were extracted with chloroform and separated on oxalate-coated Whatman silica gel 60A thin layer chromatography plates in ethylacetate/acetic acid/trimethylpentane (9:2:5).

Assay of inositol phospholipid PLC in [$^3$H]inositol loaded A10 cells by formation of total [$^3$H]inositol (poly)phosphates in the presence of 10 mM lithium was as described previously [3].

For determination of [$^3$H]thymidine incorporation into nucleic acid, cells were maintained serum-free for 24 h, followed by stimulation with 1 $\mu$M insulin ± ET-1 for 24 h. During the last 4 h, [$^3$H]thymidine was added to 1 $\mu$Ci/ml, following which the cells were washed sequentially in 5% trichloroacetic acid, ethanol, and solubilised and counted in 0.1 M NaOH. Statistical analysis was on log transformed data to correct for unequal variance: one way analysis of variance was followed by Duncan's multiple range test at significances of 0.05 and 0.01. The values for $IC_{50}$ were determined by GraphPad.

## 3. RESULTS

### 3.1. *Stimulation of PLD*

Table I shows the accumulation of [$^{32}$P]PA and [$^{32}$P]PBut in response to 100 nM ET-1 in the presence of 50 mM butanol. These results show the stimulated accumulation of [$^{32}$P]PBut, clearly demonstrating stimulation of PLD by ET-1 in these cells. Further studies on stimulation by ET-1 of [$^{32}$P]PBut showed a response reaching a maximum at 3 min and with an $EC_{50}$ of about 3 nM (data not shown). PMA also elevated [$^{32}$P]PBut accumulation in these cells, with little or no elevation of [$^{32}$P]PA (Tables I and II). PKC inhibitor Ro 31-8220 (compound 3 in [18]) inhibited agonist stimulated [$^{32}$P]PBut (Table I) with no effect on unstimulated cells.

To investigate a possible role for tyrosine kinase in the ET-1 stimulated events we used a potent and selective tyrosine kinase inhibitor ST 271 [16]. Table II shows that 100 $\mu$M ST271 had no effect on [$^{32}$P]PA accumulation in control and stimulated (ET-1 or PMA) cells. However, ST 271 (100 $\mu$M) caused a substantial reduction in the ET-1 stimulated [$^{32}$P]PBut (over four separate, experiments the reduction by ST271 was to 22%, $P < 0.05$ by paired Student's $t$-test) with only a minor reduction in the PMA stimulated [$^{32}$P]PBut. It

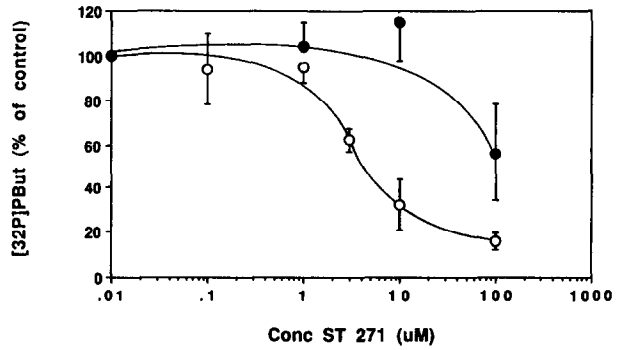


Fig. 1. Effect of ST 271 on stimulation of [$^{32}$P]PBut accumulation in A10 cells by 50 nM ET-1 (open circles) and 100 nM PMA (closed circles). Stimulation was for 3 min in the presence of 50 mM butanol. Data are normalised to 100% of response (stimulated minus basal) to either ET-1 or PMA in the absence of ST 271 and pooled across four separate experiments each in triplicate.

can be seen from Fig. 1 that ST 271 had an $IC_{50}$ of about 3 $\mu$M when stimulation was with ET-1, while the $IC_{50}$ was greater than 100 $\mu$M when stimulation was with PMA. At 10 $\mu$M, ST 271 inhibited the response to ET-1 by about 70% while the response to PMA was unaffected.

Further experiments were designed to assess the effect of ST 271 on related activities; e.g. in the range 0.1–100 $\mu$M there was no effect on stimulation of total [$^3$H]inositol (poly)phosphates in [$^3$H]inositol labelled cells [3] by 100 nM ET-1. Furthermore 100 $\mu$M ST 271 had no effect on the augmentation by Ro 31-8220 of this ET-1 stimulated PLC response.

### 3.2. *Stimulation of [$^3$H]thymidine incorporation*

Using [$^3$H]thymidine incorporation as an index of mitogenesis in cultured A10 cells maintained serum-free for 24 h prior to stimulation, we found only an unreliable stimulation with 24 h ET-1 alone (up to 100 nM) but a reproducible stimulation in the presence of insulin. In a typical experiment [$^3$H]thymidine incorporation per well (dpm × $10^{-3}$, $\bar{x}$ ± S.E.M., $n = 3$) was: control,

Table I

Accumulation of [$^{32}$P]PA and [$^{32}$P]PBut: effect of PKC stimulation and inhibition

| | [$^{32}$P]PA | [$^{32}$P]PBut |
|---|---|---|
| Control | 189 ± 21 | 315 ± 63 |
| ET-1 | 726 ± 242 | 1791 ± 63 |
| ET-1 + Ro 31-8220 | 1243 ± 120* | 769 ± 9* |
| PMA | 334 ± 47 | 2143 ± 351 |
| PMA + Ro 31-8220 | 378 ± 34 | 443 ± 82 |

ET-1 was at 100 nM, Ro 31-8220 at 10 $\mu$M and PMA at 100 nM. Data are cpm, mean ± S.E.M. ($n = 9$) from 3 separate experiments each in triplicate. Significantly different from ET-1 alone, *$P < 0.01$.

Table II

Effect of ST 271 on stimulated [$^{32}$P]PA and [$^{32}$P]PBut accumulation

| | [$^{32}$P]PA | | [$^{32}$P]PBut | |
|---|---|---|---|---|
| | Control | ST 271 | Control | ST 271 |
| Control | 460 ± 41 | 423 ± 54 | 568 ± 32 | 579 ± 3 |
| ET-1 | 732 ± 165 | 770 ± 205 | 1413 ± 249 | 768 ± 136* |
| PMA | 359 ± 79 | 351 ± 60 | 1859 ± 349 | 1204 ± 93 |

ET-1 was at 100 nM, ST 271 at 100 $\mu$M and PMA at 100 nM. Data are cpm, mean ± S.E.M. ($n = 3$) from a single experiment representative of 3 separate experiments. Significantly different from ET-1 alone, *$P < 0.01$.

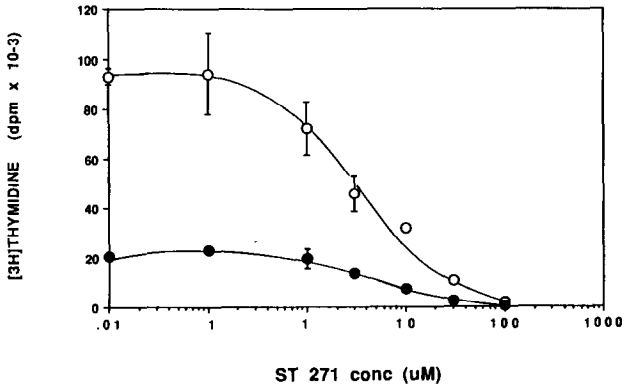


Fig. 2. Effect of ST 271 on [$^3$H]thymidine incorporation in response to 24 h 1 $\mu$M insulin (open circles) or unstimulated controls (closed circles). Data are mean ± S.E.M. ($n$ = 3) from a single experiment representative of 3.

22.5 ± 1.1; insulin (1 $\mu$M), 31.8 ± 2.3; insulin (1 $\mu$M) and ET-1 (10 nM), 72.6 ± 3.5.

To study the possible involvement of tyrosine kinases and PLD generated PA in this system we incubated cells with various conditions for 24 h. ST 271 (100 $\mu$M) and butanol (50 mM) for 24 h left cells with unchanged morphology and similar protein levels (e.g. control, 22.4 ± 2.4; butanol (50 mM), 21.4 ± 2.2; ST 271 (100 $\mu$M), 23.5 ± 1.8; figures are $\mu$g protein per well, mean ± S.E.M., $n$ = 4). Since the effects of insulin are assumed to be mediated by tyrosine kinase activity then ST 271 will not enable separate investigation of the mechanism of ET-1 stimulated mitogenesis. Fig. 2 shows that insulin stimulated [$^3$H]thymidine incorporation was inhibited by ST 271. The $IC_{50}$ over three separate experiments, each in triplicate, was 3.35 ± 0.74 $\mu$M. In separate experiments we showed that ST 271 has similar effects on the [$^3$H]thymidine incorporation stimulated by combined insulin and ET-1 (data not shown). Preliminary experiments showed that insulin did not stimulate PLD in these cells, so butanol might be expected to be selective for events downstream of the ET-1 stimulation of PLD. To exclude a possible effect of butanol on related signal transduction mechanisms we preincubated [$^3$H]inositol labelled A10 cells with 50 mM butanol (24 h) and showed no effect on ET-1 stimulation of PLC or its augmentation by Ro 31-8220 (data not shown).

Experiments were undertaken in which the ET-1 stimulation of [$^3$H]thymidine incorporation in the presence of insulin was compared with and without 50 mM butanol. The stimulation of incorporation by insulin alone, expressed as -fold over basal (no insulin or ET-1) was still apparent although in most cases (3 experiments out of 4) it was reduced (2.29 ± 0.44-fold in the absence of butanol, and 1.96 ± 0.31-fold in the presence of butanol over the 4 experiments). However, the response to ET-1 in the presence of insulin was lost when butanol was also present (Fig. 3). The results from the three individual experiments, expressed as a ratio of stimulation by ET-1 compared to stimulation by 100 nM insulin alone, were 5.4, 2.1 and 1.49 with no butanol, and 1.42, 0.96 and 0.52, respectively, with 50 mM butanol.

## 4. DISCUSSION

We show here that ET-1 stimulates PLD in vascular smooth muscle A10 cells; that this activation of PLD involves PKC is suggested by inhibition by Ro 31-8220, and supported by stimulation of PLD by PMA.

A role for tyrosine kinase is suggested by the inhibitory effects of ST 271. ET-1 has been shown to stimulate tyrosine kinase by acting at $ET_A$ receptors on glomerular mesangial cells [15], and ST 271 has been shown to potently attenuate agonist stimulated PLD, but not PMA stimulated PLD, by inhibition of tyrosine kinase activity [16]. We show here that stimulation by ET-1, but not PMA, in A10 cells is potently inhibited by ST 271 with a similar $IC_{50}$ to the inhibition of insulin stimulated [$^3$H]thymidine incorporation, a tyrosine kinase dependent process. We report that ST 271 does not affect the inositol (poly)phosphate response to ET-1; therefore it presumably does not affect ET-1 binding to its receptor. Nor does it act as an inhibitor of PLC, PLD or PKC. It seems likely therefore that the attenuation of the PLD response to ET-1 by ST 271 is due to its effectiveness as a tyrosine kinase inhibitor.

In subsequent experiments butanol (50 mM) was shown to leave cells intact and with functional signal transduction pathways even after 24 h. Butanol will have a selective effect on PLD derived PA, diverting it to PBut. Since ET-1 but not insulin (unpublished data) stimulates PLD in these cells, butanol treatment provides a strategy for selectively investigating the role of ET-1 stimulated PLD in the mitogenic response. Here

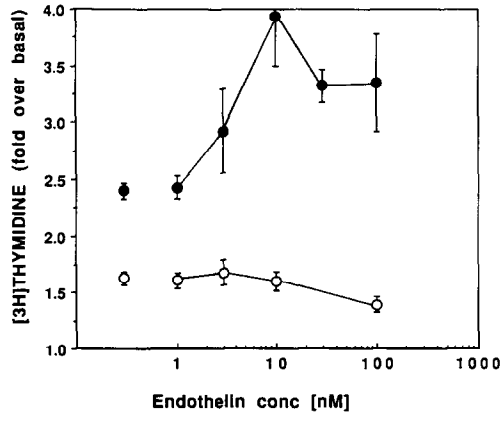


Fig. 3. Stimulation by ET-1 (100 nM) in the presence of insulin (1 $\mu$M) of mitogenesis measured by [$^3$H]thymidine incorporation in the presence (open circles) and absence (closed circles) of 50 mM butanol during the 24 h incubation. Data are mean ± S.E.M. ($n$ = 3) from a single experiment representative of three, expressed as -fold over basal (no insulin or ET-1).

we show that butanol does abolish the ET-1 augmentation of [$^3$H]thymidine incorporation, while leaving a response to insulin intact. These results are consistent with the hypothesis that ET-1 stimulated mitogenesis is mediated via PLD derived PA.

In summary, these studies point to the conclusion that ET-1 stimulation of PLD is dependent on both PKC and tyrosine kinases, and the incorporation of [$^3$H]thymidine is dependent on PLD derived PA.

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