CELL REGULATION, Vol. 2, 897–903, November 1991

Endothelin-1 activates phospholipase D and thymidine incorporation in fibroblasts overexpressing protein kinase $C_{\beta 1}$

Jin-Keon Pai, Emily A. Dobek, and W. Robert Bishop
Schering-Plough Research
Bloomfield, New Jersey 07003

Endothelins (ETs) are a family of extremely potent vasoconstrictor peptides. In addition, ET-1 acts as a potent mitogen and activates phospholipase C in smooth muscle cells and fibroblasts. We examined the effects of ET-1 on phosphatidylcholine (PC) metabolism and thymidine incorporation in control Rat-6 fibroblasts and in cells that overexpress protein kinase $C_{\beta 1}$ (PKC). PC pools were labeled with [^{3}H]myristic acid, and formation of phosphatidylethanol (PEt), an unambiguous marker of phospholipase D (PLD) activation, was monitored. ET-1 stimulated much greater PEt formation in the PKC overexpressing cells. ET-1 action was dose-dependent with a half-maximal effect at 1.0×10^{-9} M. With increasing ethanol concentrations, [^{3}H]PEt formation increased at the expense of [^{3}H]phosphatidic acid (PA). Propranolol, an inhibitor of PA phosphohydrolase, increased [^{3}H]PA accumulation and decreased [^{3}H]diacylglycerol (DAG) formation. These data are consistent with the formation of [^{3}H]DAG from PC by the sequential action of PLD and PA phosphohydrolase. Phorbol esters are known to stimulate thymidine incorporation and PLD activity to a greater extent in PKC overexpressing cells than in control cells. ET-1 also stimulates thymidine incorporation to a greater extent in the PKC overexpressing cells. The effect of ET-1 on thymidine incorporation into DNA in the overexpressing cells was also dose-dependent with a half-maximal effect at 0.3×10^{-9} M. Enhanced PLD activity induced by ET-1 in the overexpressing cells may contribute to the mitogenic response, especially in light of a possible role of the PLD product, PA, in regulation of cell growth.

Introduction

Endothelins (ETs)[1] are a family of extremely potent vasoconstrictor peptides that have recently been described. Three genes encoding homologous endothelin peptides—ET-1, ET-2, and ET-3—have been discovered in the human, rat, and porcine genomes (Inoue *et al.,* 1989). ET-1, the first endothelin identified, is a 21-residue peptide produced by endothelial cells and is one of the most potent vasoconstrictors and bronchoconstrictors known (Yanagisawa *et al.,* 1988). ET-1 is synthesized from proendothelin ("big" ET) by the action of ET-converting enzyme (Sawamura *et al.,* 1990).

ET has slowly developing and sustained pressor and vasoconstrictive activity *in vivo* (Yanagisawa and Masaki, 1989). Infused ET causes transient vasorelaxation mediated by release of endothelium-derived relaxing factor (and possibly eicosanoids) and followed by vasoconstriction and a prolonged increase in blood pressure (Yanagisawa and Masaki, 1989). ET-1 also elevates intracellular Ca^{2+} levels and stimulates proliferation of vascular smooth muscle cells in culture (Nakaki *et al.,* 1989). Recently, ET-1 was demonstrated to stimulate mitogenesis in Swiss 3T3 (Takuwa *et al.,* 1989) and Rat-1 fibroblasts (Muldoon *et al.,* 1989).

ET-1 potently stimulates phosphoinositide-specific phospholipase C in both fibroblasts and smooth muscle cells (Muldoon *et al.,* 1989; Takuwa *et al.,* 1989). This enzyme catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate to generate two second messengers: inositol-1,4,5-triphosphate, which mobilizes intracellular calcium, and 1,2-diacylglycerol (DAG), which activates protein kinase C (PKC) (Kikawa *et al.,* 1989). ET-1-induced formation of DAG is biphasic (Sunako *et al.,* 1990). The diverse functions of ETs are thought to be mediated by G protein-coupled receptors (Arai *et al.,* 1991; Sakurai *et al.,* 1991) linked to the phosphoinositide hydrolysis pathway.

[1] Abbreviations used: DAG, 1,2-*sn*-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ET, endothelin; PA, phosphatidic acid; PC, phosphatidylcholine; PEt, phosphatidylethanol; PLD, phospholipase D; PMA, phorbol-12-myristate-13-acetate; PKC, protein kinase C; R6-C1, control Rat-6 fibroblasts; R6-PKC3, PKC overexpressing cells; TCA, trichloroacetic acid.

Recent evidence indicates that many receptors coupled to inositol phospholipid hydrolysis are also coupled (directly or indirectly) to a distinct phospholipase, phospholipase D (PLD) (Billah and Anthes, 1990). PLD catalyzes hydrolysis of the terminal diester bond of phosphatidylcholine (PC) (and possibly other glycerophosphatides) with the formation of phosphatidic acid (PA) and choline. PA can serve as a substrate for DAG biosynthesis by the action of PA phosphohydrolase. In addition to hydrolysis, PLD also catalyzes a transphosphatidylation reaction in which the phosphatidyl moiety of phospholipid is transferred to a nucleophilic alcohol such as ethanol producing phosphatidylethanol (PEt) (Kanfer, 1980). This unique transphosphatidylation reaction has been employed as an unambiguous marker of PLD. Recent evidence indicates an important role for PLD in receptor-mediated signal transduction (Bocckino *et al.*, 1987; Pai *et al.*, 1988a,b; Kiss and Anderson, 1989; Liscovitch and Amsterdam, 1989).

We recently reported that overexpression of $PKC_{\beta 1}$ in Rat-6 fibroblasts enhances PLD activation in response to phorbol-12-myristate-13-acetate (PMA) (Pai *et al.*, 1991). These PKC overexpressing cells exhibit multiple growth abnormalities (Housey *et al.*, 1989), display an enhanced mitogenic response to growth factors (Hoshina *et al.*, 1990), and are more susceptible to transformation by activated Ha-*ras* oncogenes (Hsiao *et al.*, 1989). Because this cell line provides a useful and sensitive system for exploring PLD activation and its regulation by PKC, we have used it to examine the effects of ET-1 on PEt and PA formation and [^{3}H]thymidine incorporation.

In this report we demonstrate that PKC overexpressing cells have an enhanced PLD response (PEt, PA, and DAG formation) to ET-1. This may account for the biphasic nature of DAG formation in ET-1-stimulated cells (Sunako *et al.*, 1990). In addition, we show that ET-1 exerts a mitogenic effect in PKC overexpressing cells but not in control cells. While this work was in progress, it was reported that ET-1 induces release of [^{3}H]choline from PC in Rat-1 fibroblasts (MacNulty *et al.*, 1990), suggesting that ET-1 activates the hydrolysis of PC in these cells. Our demonstration that ET-1 also induces formation of PLD-generated lipid metabolites and formation of PEt provides definitive evidence that ET-1 activates the PLD pathway and further suggests a link between activation of PLD and the mitogenic response.

Results

Activation of PLD by ET

To determine whether ET-1 stimulates PLD, PC pools were labeled with [^{3}H]myristic acid and cells were stimulated with ET-1 in the presence of ethanol (0.5%). In a number of experiments, synthesis of [^{3}H]PEt was maximal between 30 and 60 min in the PKC overexpressing cells (R6-PKC3) (Figure 1). PEt formation in the presence of ethanol is an unambiguous marker of PLD activation (Pai *et al.*, 1988a,b; Billah and Anthes, 1990; Kanfer, 1980). Induction of [^{3}H]PEt formation by ET-1 was dose-dependent (Figure 2). Formation of [^{3}H]PEt was maximal at 5 nM ET-1 and half-maximal at 1.0 nM, indicating that it is one of the most potent agonists known for PLD activation (Figure 2). The biologically inactive precursor of ET-1, proendothelin ("big" ET), did not activate PLD (data not shown). When R6-PKC3 cells were labeled with [^{3}H] or [^{32}P]alkyl-lysoPC, formation of [^{3}H]/[^{32}P] alkyl-PEt was also observed in response to ET-1 (data not shown).

ET-1 was much more effective in stimulating [^{3}H]PEt formation in cells overexpressing PKC than in control Rat-6 fibroblasts (R6-C1) (Figures

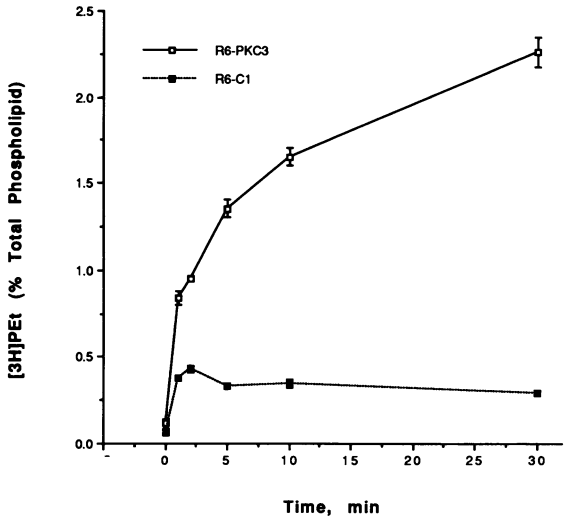

Figure 1. **Formation of [^{3}H]PEt in ET-1-stimulated fibroblasts.** Control (R6-C1) or PKC-overexpressing (R6-PKC3) fibroblasts were grown to confluency in 60-mm dishes and were serum-deprived for 18 h. Cells were then labeled with [^{3}H]myristic acid and stimulated with 20 nM ET-1 in the presence of 0.5% ethanol for various times. Lipids were extracted, and [^{3}H]PEt was isolated as described in Materials and methods. The radioactivity was quantified by liquid scintillation counting. Data presented are means ± range of duplicate determinations. Similar results were obtained in at least five independent experiments.

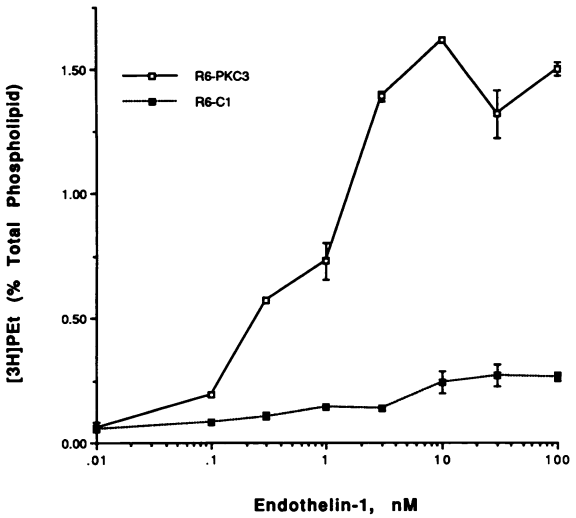

Figure 2. **Effect of ET-1 on the formation of [^{3}H]PEt.** This experiment was performed as described in Figure 1 except that cells were stimulated with the indicated concentration of ET-1 for 30 min. Data shown are representative of three independent experiments.

1 and 2). Although PEt formation was maximal in R6-C1 cells at ~2 min after stimulation, this response was sustained in the PKC overexpressers (Figure 1). Formation of PEt unequivocally proves that ET-1 induces activation of PLD in fibroblasts. These results also indicate that, similar to our observations in phorbol ester-stimulated cells (Pai *et al.*, 1991), PKC overexpression results in an enhanced PLD response.

PEt formation is dependent on exogeneous ethanol

In [^{3}H]myristate-labeled R6-PKC3 cells, ET-1 also induced formation of [^{3}H]PA and [^{3}H]DAG (Figure 3; ET-1 only). [^{3}H]PA formation was maximal at 5 min after ET-1 stimulation (Figure 3A), whereas [^{3}H]DAG continued to increase over the 30-min time course (Figure 3B). The transient nature of [^{3}H]PA formation is consistent with its subsequent metabolism to DAG (and other products).

Because PLD-catalyzed transphosphatidylation using ethanol as nucleophile is a competing reaction with hydrolysis, PEt formation should occur at the expense of PA formation. The amount of ET-1-stimulated PEt formation in the PKC overexpressing cells was dependent on the concentration of ethanol in the media (Figure 4A). Increasing concentrations of ethanol also led to a decrease in the amount of [^{3}H]PA (Figure 4B). Inhibition of PA formation was near maximal at about 0.5% ethanol. The loss of radioactivity from PA, however, could not be entirely accounted for by the increased labeling of PEt. For example, at 1% ethanol there is a gain in PEt of 1.5% of total phospholipid label, whereas PA shows a loss equal to ~2.5% of phospholipid label. This disparity is more pronounced at lower ethanol concentrations and has been observed in several independent experiments. Our data may be accounted for in part by a redirection of a common phosphatidyl-PLD intermediate from PA to PEt in the presence of ethanol, indicating a role for PLD in the formation of [^{3}H]PA in response to ET-1. However, ethanol may be having additional effects on PA metabolism in this system. In addition, we have observed that ethanol also inhibits [^{3}H]DAG formation in a concentration-dependent manner (data not shown), suggesting in-

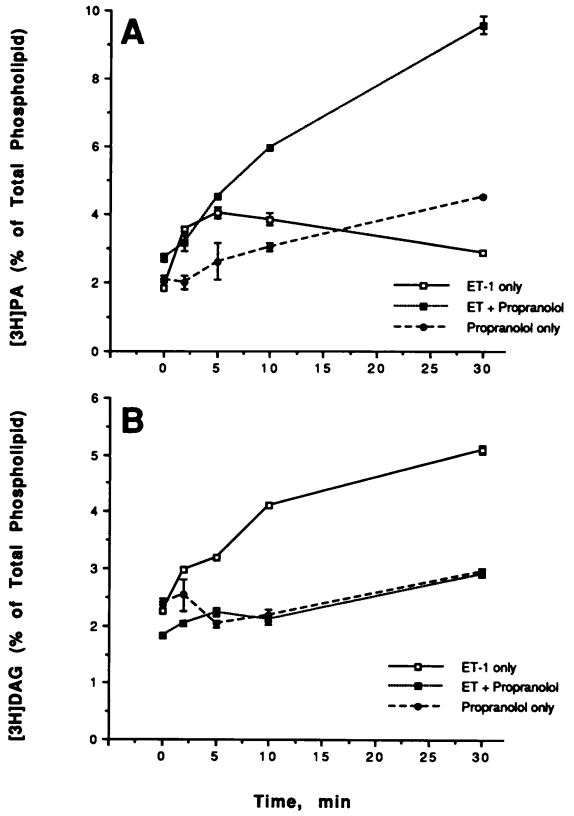

Figure 3. **Effect of propranolol on ET-1-stimulated formation of [^{3}H]PA and [^{3}H]DAG in R6-PKC3 fibroblasts.** This experiment was performed as described in Figure 1 except that cells were preincubated with 200 μM propranolol, then stimulated with 20 nM ET-1 in the presence of 0.1% ethanol for various times. After the extraction, lipids were separated by thin-layer chromatography and quantified by liquid scintillation counting. Data shown are representative of three independent experiments.

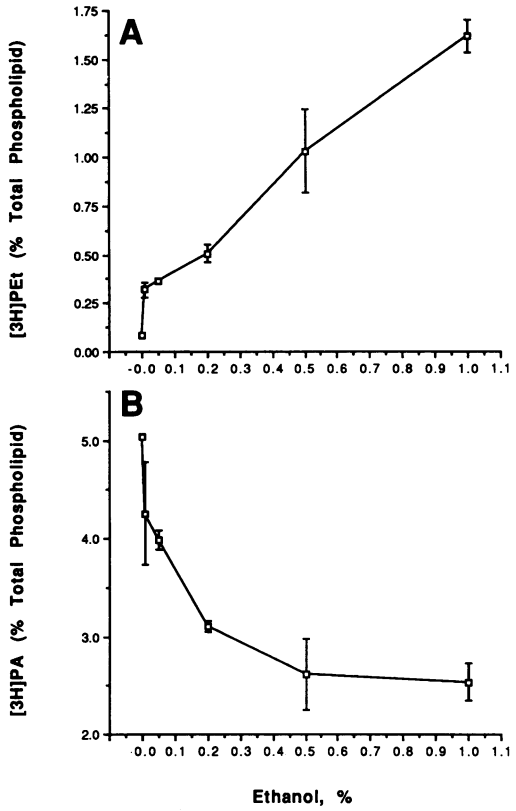

Figure 4. **Effect of ethanol on ET-1-stimulated formation of [^{3}H]PEt and [^{3}H]PA.** This experiment was performed as described in Figure 1 except that cells were stimulated with ET-1 for 5 min with the indicated concentration of ethanol in the medium. Data shown are representative of two independent experiments.

direct formation of DAG from PA by the action of PA phosphohydrolase.

PLD-derived PA is hydrolyzed by PA phosphohydrolase to form DAG

The amphiphilic cationic drug L-propranolol is an antagonist of β-adrenergic receptors and antagonizes sympathomimetic amines at cardiac β-1 receptors. Due to its amphiphilic nature, higher concentrations of D,L-propranolol can interact with anionic membrane phospholipids such as PA. Therefore, propranolol can inhibit PA phosphohydrolase and is currently the only available inhibitor of this enzyme (Witter and Kanfer, 1985). The effect of propranolol was examined to further determine the contribution of the PLD pathway to [^{3}H]DAG formation. Propranolol (200 μM) greatly inhibited [^{3}H]DAG formation in response to ET-1 (Figure 3B). Concomitantly, accumulation of [^{3}H]PA was increased (Figure 3A). Propranolol alone had only a small effect on [^{3}H]DAG formation (from 2.41% of total phospholipid at 0 min to 2.94% at 30 min). Propranolol alone increased [^{3}H]PA formation from 2.10% of total phospholipid at 0 min to 4.53% at 30 min. However, the combination of propranolol and ET-1 increased the formation of [^{3}H]PA in a more than additive manner (from 2.72% of total phospholipid at 0 min to 9.58% at 30 min). These data suggest that PA, generated by the action of PLD, is subsequently dephosphorylated to DAG by a propranolol-sensitive PA phosphohydrolase.

Effect of ET-1 on DNA synthesis in Rat-6 fibroblasts

Increased thymidine incorporation in response to ET-1 has been demonstrated in Rat-1 (Muldoon *et al.*, 1990) and Swiss 3T3 (Takuwa *et al.*, 1989) fibroblasts. The ability of ET-1 to stimulate DNA synthesis in these cells is markedly reduced by chronic phorbol ester treatment to deplete cellular PKC (Takuwa *et al.*, 1989; Muldoon *et al.*, 1990). To further examine the role of PKC in this mitogenic response, we measured the effect of ET-1 on [^{3}H]thymidine incorporation in control and PKC overexpressing cells. When quiescent cells are incubated in the presence of 10% serum, both cell lines showed similar maximal stimulation of thymidine incorporation (Figure 5). Treatment with PMA alone was sufficient to induce near-maximal thymidine incorporation in the PKC overexpressing cells, although having little effect on R6-C1 cells, in agreement with the observations of Hoshina *et al.* (1990). The ability of ET-1 to induce thymidine incorporation into DNA in the overexpressing cells was also dose-dependent with a half-maximal effect at 0.3×10^{-9} M, a concentration that induces a strong PEt response in these cells (Figure 2). At 20 nM, ET-1 induced [^{3}H]thymidine incorporation in the R6-PKC3 cells (17–27% of serum stimulation) but not in control cells (2–4% of serum stimulation) (Figure 5). This mitogenic response was less than that induced by PMA alone.

Discussion

The actions of ETs are thought to be mediated by two distinct, recently cloned receptors with predicted transmembrane topology similar to that of other G protein-coupled receptors (Arai *et al.*, 1991; Sakurai *et al.*, 1991). Receptors coupled to phosphoinositide hydrolysis frequently lead to activation of PLD. Our results clearly demonstrate that in PKC overexpressing Rat-6 fibroblasts ET-1 induces formation of PEt,

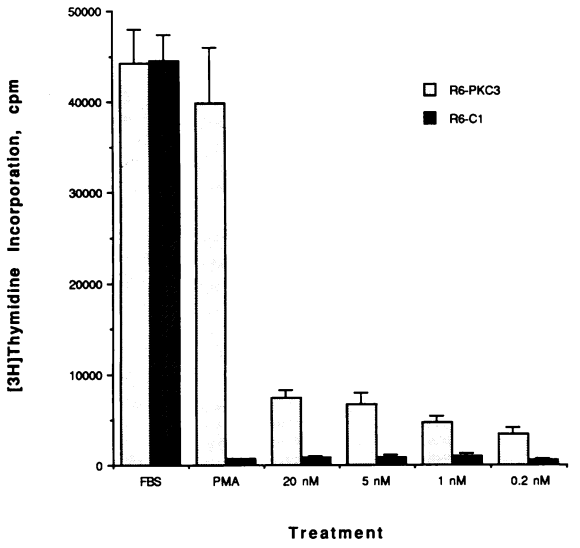

Figure 5. **Effect of serum, PMA, and ET-1 on [^{3}H]thymidine incorporation into DNA.** Serum-deprived cells were labeled with [^{3}H]thymidine (0.4 μCi/ml) in the presence of various agents for 24 h; the medium was removed and 1 ml of ice-cold 10% (vol/vol) trichloroacetic acid was added. After 30 min on ice, the cells were dissolved in 0.5 ml of 0.4 N NaOH and the radioactivity was counted. The data are the means ± SD of three determinations. The data were shown after subtracting the control of 5652 cpm from R6-PKC3 and that of 1346 cpm from R6-C1. Data shown are representative of five independent experiments.

a definitive marker for PLD activation, and exerts a mitogenic effect. This is in agreement with the recent report by MacNulty *et al.* (1990) that ET-1 stimulates [^{3}H]choline release via PLD in Rat-1 fibroblasts. The increase in [^{3}H]PA we observed on ET-1-stimulation is likely due to PLD action because it is inhibited by increasing concentrations of ethanol (Figure 4B). This is consistent with a diversion of phosphatidyl groups away from PA and into PEt. However, the loss of radioactivity from PA was not quantitatively accounted for by the increased labeling of PEt, leaving open the possibility that ethanol may have other effects in this system than simply competing with water for the PLD-catalyzed hydrolysis of PC.

Our data also suggest that ET-induced [^{3}H]DAG formation occurs, at least in part, by the PLD/PA phosphohydrolase pathway as suggested by the finding that the PA phosphohydrolase inhibitor, propranolol, inhibits [^{3}H]DAG formation (Figure 3A). In addition, we have observed that ethanol also inhibits [^{3}H]DAG formation (data not shown), presumably by decreasing the formation of PA substrate for PA phosphohydrolase (Figure 4).

The mitogenic response of fibroblasts to growth-stimulating factors is preceeded by rapid alterations in second messenger levels. As discussed by Muldoon *et al.* (1990), the involvement of these early events in the stimulation of proliferation is unclear, but because the continued presence of growth factors is required throughout the G_1 phase of the cell cycle, at least some signal must persist. Little is known about the nature of these sustained signaling events (Pardee, 1989). Activation of PLD by ET-1 in R6-PKC3 cells is persistent (Figure 1). Similarly, ET-1-dependent responses are sustained in other systems. For example, the *in vivo* pressor effects of ET are long-lasting, and ET causes a slowly developing and sustained contraction of vascular smooth muscle strips (Yanagisawa *et al.*, 1989). Phorbol esters also induce sustained activation of PLD in various cell types (Billah and Anthes, 1990; Pai *et al.*, 1991) and, similar to ET, they induce sustained contraction of vascular smooth muscle (Rasmussen *et al.*, 1984). It is possible that the mitogenic response elicited by ET and phorbol esters in R6-PKC3 cells and the persistent nature of vascular smooth muscle contraction induced by these agents can be attributed to the prolonged activation of PLD. It is interesting to note that in R6-C1 where only a modest, unsustained activation of PLD is observed, ET-1 fails to elicit a mitogenic response.

The effect of PKC overexpression on the PLD response to ET-1 may be due to a combination of factors. First, PKC may directly regulate PLD activity by phosphorylation, as suggested by enhanced phorbol ester stimulation of PLD in R6-PKC3 fibroblasts (Pai *et al.*, 1991). In support of this, MacNulty *et al.* (1990) found that ET-stimulated choline release was abolished on PKC down-regulation. Second, the R6-PKC3 cells may be altered in their level of expression of ET-1 receptors or some other component of the signal-transducing machinery in addition to PKC. These alternatives are currently under investigation.

As we proposed previously (Pai *et al.*, 1991), enhanced PLD activity can generate an enhanced DAG signal by the PLD-PA phosphohydrolase pathway, and this DAG may lead to further PKC activation, thus perpetuating the signal through a feed-forward loop of cell stimulation (Pai *et al.*, 1991). A role for sustained DAG formation in the mitogenic response of fibroblasts has previously been suggested (Muldoon *et al.*, 1990; Wright *et al.*, 1990). Recent reports contending that the sustained phase of DAG in agonist-stimulated cells does not sup-

port persistent PKC activation (Martin *et al.*, 1990; Leach *et al.*, 1991) argue against such a model. However, these studies focused on the better characterized, calcium-dependent forms of PKC and did not evaluate the activation state of the more recently discovered isoforms (Kikawa *et al.*, 1989). Although we observed that propranolol inhibits sustained [^{3}H]DAG formation in response to ET-1, we were unable to examine the effects of this drug on mitogenesis due to cytotoxicity on overnight incubation (data not shown).

The direct product of PLD activity, PA, and its lyso derivative have also been suggested to play possible second messenger roles in the regulation of cell growth (Jalink *et al.*, 1990; Tsai *et al.*, 1990). These observations along with our results emphasize the potential importance of PLD activation in the mitogenic response.

Materials and methods

Materials

ETs were obtained from Peninsula Laboratories (Belmont, CA). [9,10 (n)-^{3}H]Myristic acid and [^{3}H]alkyl-lysoPC (92 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). [methyl-^{3}H]Thymidine was purchased from New England Nuclear (Boston, MA). Other lipids were from Avanti Polar Lipids (Birmingham, AL). Precoated silica gel G plates (0.25-mm thick) were purchased from E. Merck (Darmstadt, Germany). Fatty acid-free bovine serum albumin, trypsin, and β-octylglucoside were obtained from Calbiochem (San Diego, CA). PMA and 4-α-PMA were obtained from LC Services (Boston, MA) and K252a was from Kyowa Hakko USA Inc. (New York, NY). All other reagents were purchased from Sigma (St. Louis, MO).

Maintenance of cultures

R6-C1 and R6-PKC3 were grown to confluency on 100-mm dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and G418 (50 μg/ml) as described previously (Housey *et al.*, 1989; Pai *et al.*, 1991). They were then serum-deprived for 24 h in DMEM containing 0.5% fetal bovine serum.

Labeling of cultures

To label PC pools with [^{3}H]myristic acid (5 μCi/ml), the labeled material was dried under nitrogen and dissolved in dimethylsulfoxide (DMSO). DMEM was added to yield a final DMSO concentration of 0.03%. Labeling was for 2 h with [^{3}H]myristic acid. [^{3}H]Myristic acid was readily incorporated into phospholipids; of this, 89% was incorporated into PC and the remainder in phosphoinositides (3%), phosphatidylethanolamine (5%), and lyso-PC.

Analysis of PEt formation

After labeling, cells were washed twice with phosphate-buffered saline and 5 ml of DMEM containing ET-1 (20 nM; final concentration), or PMA (100 nM) was added to each plate along with 0.5% (vol/vol) ethanol. To examine the effects of propranolol or K252a, cells were preincubated for 5 min with the indicated concentration of compound and then DMEM containing ET-1 was added to give the final concentration stated above. To stop the reaction, the incubation media was aspirated and the cells were scraped into 1 ml of methanol. The plate was washed again with 1 ml of methanol to remove any remaining cells. Phases were separated by the procedure of Bligh and Dyer (1959). Standard samples (10 μg each) of PA, PEt, and DAG were added to the lower chloroform phase. This phase was then dried under nitrogen and spotted on silica gel G thin-layer chromatography plates. The plates were developed halfway using the upper phase of a solvent mixture consisting of ethyl acetate/isooctane/acetic acid/water (110:50:20:100, vol/vol) and then developed again in hexane/diethyl ether/methanol/acetic acid (80:20:8:2, vol/vol). Lipids were located by staining with iodine vapor or by autoradiography, and the areas containing PEt, DAG, and PA were scraped and counted by liquid scintillation spectrometry. PEt, PA, and DAG were normalized to the percent of total amount of the labeled phospholipid (mainly PC)(Pai *et al.*, 1991).

Thymidine incorporation

Cells were seeded at 5×10^4 cells per well in 24-well plates and grown to confluency. They were then serum-deprived for 24 h, washed once with DMEM and 0.5 ml of DMEM containing [methyl-^{3}H]thymidine (0.2 μCi/well), and various concentrations of test substances were added. After 24 h incubation, the medium was removed and ice-cold 10% trichloroacetic acid (TCA) (1 ml/well) was added. After 30 min on ice, wells were washed with 1 ml of ice-cold 10% TCA and subsequently with ethanol/ether (2:1, vol/vol) to remove residual TCA. Cells were solubilized with 0.5 ml of 0.4 N NaOH, and aliquots were counted by liquid scintillation spectrometry (Takuwa *et al.*, 1987).

Acknowledgments

We express our thanks to Dr. I. Bernard Weinstein for providing the Rat-6 control and R6-PKC3 cell lines and to Dr. Meeta Chatterjee for her critical reading of this manuscript. We also thank Dr. Jonathan Pachter for useful discussions. We are grateful to Dr. J. Schwartz for supporting this work.

Received: May 28, 1991.
Revised and accepted: August 5, 1991.

References

Arai, H., Hori, S., Ohkubo, H., and Nakanishi, S. (1991). Cloning and expression of cDNA encoding an endothelin receptor. Nature *348,* 730–732.

Billah, M., and Anthes J. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem. J. *269,* 281–291.

Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. *37,* 911–917.

Bocckino, S.B., Blackmore, P.F., Wilson, P.B., and Exton, J.H. (1987). Phospatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. J. Biol. Chem. *262,* 15309–15315.

Hoshina, S., Ueffing, M., and Weinstein, I.B. (1990). Growth factor-induced DNA synthesis in cells that overproduce protein kinase C. J. Cell. Physiol. *145,* 262–267.

Housey, G.M., Johnson, M.D., Hsiao, W.-L. W., O'Brian, C.A., Murphy, J.P., Kirschmeier, P., and Weinstein, I.B. (1989). Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. Cell *52,* 343–354.

Hsiao, W.-L. W., Housey, G.M., Johnson, M.D., and Weinstein, I.B. (1989). Cells that overproduce protein kinase C

are more susceptible to transformation by an activated H-*ras* oncogene. Mol. Cell. Biol. *9,* 2641–2647.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., and Masaki, T. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc. Natl. Acad. Sci. USA *86,* 2863–2867.

Jalink, K., van Corven, E.J., and Moolenaar, W.H. (1990). Lysophosphatidic acid, but not phosphatidic acid, is a potent Ca^{2+}-mobilizing stimulus for fibroblasts. J. Biol. Chem. *265,* 12232–12239.

Kanfer, J.N. (1980). The base exchange enzyme and phospholipase D of mammalian tissue. Can. J. Biochem. *58,* 1370–1380.

Kikawa, U., Kishimoto, A., and Nishizuka, Y. (1989). The protein kinase C family: heterogeneity and its implications. Annu. Rev. Biochem. *58,* 31–44.

Kiss, Z., and Anderson, W.B. (1989). Phorbol ester stimulates the hydrolysis of phosphatidylethanolamine in leukemic HL-60, NIH 3T3, and baby hamster kidney cells. J. Biol. Chem. *264,* 1483–1487.

Leach, K.L., Ruff, V.A., Wright, T.M., Pessin, M.S., and Raben, D.M. (1991). Dissociation of protein kinase C activation and *sn*-1,2-diacylglycerol formation. J. Biol. Chem. *266,* 3215–3221.

Liscovitch, M., and Amsterdam, A. (1989). Gonadotropin-releasing hormone activates phospholipase D in ovarian granulosa cells. J. Biol. Chem. *264,* 11762–11767.

MacNulty, E.E., Plevin, R., and Wakelam, M.J.O. (1990). Stimulation of the hydrolysis of phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine by endothelin, a complete mitogen for Rat-1 fibroblasts. Biochem. J. *272,* 761–766.

Martin, T.F.J., Hsieh, K.-P., and Porter, B.W. (1990). The sustained second phase of hormone-stimulated diacylglycerol accumulation does not activate protein kinase C in GH_3 cells. J. Biol. Chem. *265,* 7623–7631.

Muldoon, L.L., Probnow, D., Rodland, K.D., and Magun, B.E. (1990). Endothelin-1 stimulates DNA synthesis and anchorage-independent growth of Rat-1 fibroblasts through a protein kinase C-dependent mechanism. Cell Regul. *1,* 379–390.

Muldoon, L.L., Rodland, K.D., Forsythe, M.L., and Magun, B.E. (1989). Stimulation of phosphatidylinositol hydrolysis, diacylglycerol release, and gene expression in response to endothelin, a potent new agonist for fibroblasts and smooth muscle cells. J. Biol. Chem. *264,* 8529–8536.

Nakaki, T., Nakayama, M., Yamamoto, S., and Kato, R. (1989). Endothelin-mediated stimulation of DNA synthesis in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. *158,* 880–883.

Pai, J.-K., Pachter, J.A., Weinstein, I.B., and Bishop, W.R. (1991). Overexpression of protein kinase C β1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. Proc. Natl. Acad. Sci. USA *88,* 598–602.

Pai, J.-K., Siegel, M.I., Egan, R.W., and Billah, M.M. (1988a). Activation of phospholipase D by chemotactic peptide in HL-60 granulocytes. Biochem. Biophys. Res. Commun. *150,* 355–364.

Pai, J.-K., Siegel, M.I., Egan, R.W., and Billah, M.M. (1988b). Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL-60 granulocytes. J. Biol. Chem. *263,* 12472–12477.

Pardee, A.B. (1989). G_1 events and regulation of cell proliferation. Science *246,* 603–608.

Rasmussen, H., Forder, J., Kojima, I., and Scriabine (1984). TPA-induced contraction of isolated rabbit smooth muscle. Biochem. Biophys. Res. Commun. *122,* 776–784.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., and Masaki, T. (1991). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature *348,* 732–735.

Sawamura, T., Kimura, S., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K., and Masaki, T. (1990). Purification and characterization of putative endothelin converting enzyme in bovine adrenal medula: evidence for a cathepsin D-like enzyme. Biochem. Biophys. Res. Commun. *168,* 1230–1236.

Sunako, M., Kawahara, Y., Hirata, K., Tsuda, T., Yokoyama, M., Fukuzaki, H., and Takai, Y. (1990). Mass analysis of 1,2-diacylglycerol in cultured rabbit vascular smooth muscle cells. Hypertension *15,* 84–88.

Takuwa, N., Takuwa, Y., Bollag, W.E., and Rasmussen, H. (1987). The effects of bombesin on polyphosphoinositide and calcium metabolism in Swiss 3T3 cells. J. Biol. Chem. *262,* 182–188.

Takuwa, N., Takuwa, Y., Yanagisawa, M., Kamejiro, Y., and Masaki, T. (1989). A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. J. Biol. Chem. *264,* 7856–7861.

Tsai, M.-H., Yu, C.-L., Wei, F.-S., and Stacey, D.W. (1990). The effect of GTPase activating protein upon ras is inhibited by mitogenetically responsive lipids. Science *243,* 522–527.

Witter, B., and Kanfer, J.N. (1985). Hydrolysis of endogeneous phospholipids by rat brain microsomes. J. Neurochem. *44,* 155–162.

Wright, T.M., Shin, H.S., and Raben, D.M. (1990). Sustained increase in 1,2-diacylglycerol precedes DNA synthesis in epidermal-growth-factor-stimulated fibroblasts. Biochem. J. *267,* 501–507.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature *332,* 411–415.

Yanagisawa, M., and Masaki, T. (1989). Endothelin, a novel endothelium-derived peptide. Pharmacological activities, regulation and possible roles in cardiovascular control. Biochem. Pharmacol. *38,* 1877–1883.