Biochem. J. (1991) **280**, 609–615 (Printed in Great Britain)

Differences in the regulation of endothelin-1- and lysophosphatidic-acid-stimulated Ins(1,4,5)P_3 formation in Rat-1 fibroblasts

Robin PLEVIN, Elisabeth E. MACNULTY, Susan PALMER and Michael J. O. WAKELAM*
Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland U.K.

Endothelin-1 (ET-1)- and lysophosphatidic acid (LPA)-stimulated PtdIns(4,5)P_2 hydrolysis has been studied in Rat-1 fibroblasts. Although both agonists caused the dose-dependent accumulation of inositol phosphates, a number of differences were observed. LPA induced a transient increase in Ins(1,4,5)P_3 mass which returned to basal levels within 90 s, whereas the response to ET-1 did not desensitize, with levels remaining at 3–4 times basal values for up to 15 min. Stimulated decreases in mass levels of PtdIns(4,5)P_2 mirrored Ins(1,4,5)P_3 formation for both agonists. Experiments with electropermeabilized cells demonstrated that the effects of both agonists are stimulated by a phospholipase C controlled by a guanine-nucleotide-binding regulatory protein; however, there are differences in the nature of these interactions. The inositol phosphate response to ET-1 is poorly potentiated by guanosine 5′-[γ-thio]triphosphate (GTP[S]) and markedly inhibited by guanosine 5′-[β-thio]diphosphate (GDP[S]), whereas that to LPA is potentiated by GTP[S] but is relatively insensitive to GDP[S]. In addition, LPA decreased the lag time for the onset of GTP[S]-stimulated [^{3}H]InsP_3 accumulation, whereas ET-1 was without effect. Phorbol 12-myristate 13-acetate treatment of the cells inhibited LPA-stimulated, but not ET-1-stimulated, inositol phosphate formation in both intact and permeabilized cells, suggesting that the site of protein kinase C-mediated phosphorylation may be blocked in ET-1-stimulated Rat-1 cells. The results indicate that the receptor–G-protein–phospholipase C interaction for the two agonists may not conform to the same model.

INTRODUCTION

It is recognized that the majority of agonist receptors linked to the hydrolysis of PtdIns(4,5)P_2 do so by interacting with a specific guanine-nucleotide-binding protein (G-protein) (Cockcroft & Gomperts, 1985; Gilman, 1987). Although this system is poorly defined in comparison with both agonist-stimulated adenylate cyclase or rhodopsin activation of cyclic GMP phosphodiesterase, several criteria have been established to confirm the involvement of a G-protein in receptor-activated hydrolysis by phospholipase C (PIC). Firstly, agonist-stimulated formation of the second messenger Ins(1,4,5)P_3 in permeabilized cells and membranes is enhanced specifically by non-hydrolysable analogues of GTP such as guanosine 5′-[γ-thio]triphosphate (GTP[S]) and guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) and is inhibited by the GDP analogue guanosine 5′-[β-thio]diphosphate (GDP[S]). This has been shown to be the case for hormones and neurotransmitters such as bombesin, angiotensin II, bradykinin, histamine and ATP (Pfeilshifter & Bauer, 1987; Harden *et al.*, 1988; Chiang & Hauser, 1989; Claro *et al.*, 1989; Plevin *et al.*, 1990). Secondly, as with the adenylate cyclase system, the dissociation of GDP from the G-protein is thought to be the rate-limiting step in the activation process. Agonist receptor occupation is therefore believed to accelerate GTP/GDP exchange and enhance the onset of PtdIns(4,5)P_2 hydrolysis (Boyer *et al.*, 1989*a,b*). Thirdly, agonist-stimulated inositol phosphate formation is inhibited by prior treatment with the protein kinase C-activating phorbol ester phorbol 12-myristate 13-acetate (PMA). Coupling of the receptor to the effector, PIC, is believed to be impaired through protein kinase C-mediated phosphorylation at several sites, possibly including the G-protein itself (Brown *et al.*, 1987; Orellana *et al.*, 1987; Geny *et al.*, 1989). Regulation of the system is thus also achieved by negative feedback inhibition.

Two agonists whose postulated receptors have been reported to be linked to the inositol phosphate system are lysophosphatidic acid (LPA) and endothelin (ET). Phospholipids such as LPA and phosphatidic acid have previously been shown to activate several second messenger pathways including stimulation of PtdIns(4,5)P_2 hydrolysis, cyclic GMP formation and the inhibition of adenylate cyclase (Ohsako & Deguchi, 1981; Murayama & Ui, 1987; van Corven *et al.*, 1989). One or more of these pathways may therefore mediate the mitogenic effect of these phospholipids in fibroblast cell lines (Moolenaar *et al.*, 1986; van Corven *et al.*, 1989). However, it is not completely clear if the biological action of LPA is mediated through interaction with a receptor *per se*, or is secondary to its effect on the membrane itself. ET, a novel 21-amino-acid peptide originally isolated from vascular endothelium (Yanagisawa *et al.*, 1988; see review by Yanagisawa & Masaki, 1989), is a potent vasoconstrictor and a complete mitogen for smooth muscle cells (Nakaki *et al.*, 1989) and fibroblasts, including both the Swiss 3T3 and Rat-1 cell lines (Takuwa *et al.*, 1989; Brown & Littlewood, 1989; MacNulty *et al.*, 1990*a*). ET stimulates inositol phosphate accumulation in these and in other systems, including rat glomerulosa cells, atria and brain (Resink *et al.*, 1988; Simonson *et al.*, 1989; Muldoon *et al.*, 1989; Takuwa *et al.*, 1989; Ambar *et al.*, 1989). Recently we have shown for the first time rapid and sustained formation of Ins(1,4,5)P_3 mass in response to ET-1 in Rat-1 fibroblasts which appeared to be mediated through interaction with an ET-1 receptor subtype (MacNulty *et al.*, 1990*a,b*).

Although preliminary reports have shown both LPA- and ET-stimulated inositol phosphate accumulation to be enhanced by

Abbreviations used: LPA, L-α-lysophosphatidic acid (1-oleoyl); ET, endothelin; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; HBG, Hanks buffered saline containing 1% (w/v) BSA and 10 mM-glucose; PIC, phospholipase C; G-protein, guanine-nucleotide-binding regulatory protein; GTP[S], guanosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate; EC_{50}, concn. causing 50% of maximum effect.

* To whom correspondence should be addressed.

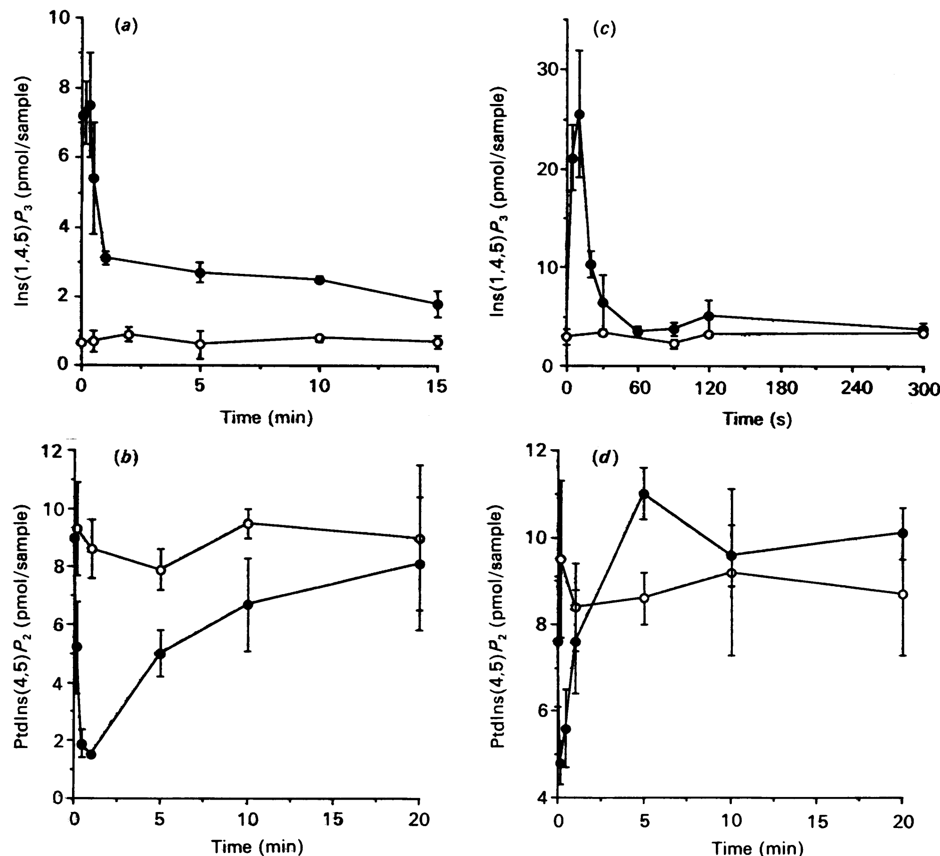

Fig. 1. Time course of LPA- and ET-1-stimulated Ins(1,4,5)P_3 mass formation (*a*, *c*) and PtdIns(4,5)P_2 breakdown (*b*, *d*) in Rat-1 fibroblasts

Cells treated as outlined in the Materials and methods section were incubated with agonist (●) (*a*, *b* 100 nM-ET-1; *c*, *d* 30 μM-LPA), or vehicle (○) for the times indicated. Each point represents the mean ± S.D. of triplicate determinations from one of at least three other experiments.

GTP[S] in permeabilized Rat-1 cells and renal glomerulosa membranes respectively (van Corven *et al.*, 1989; Takuwa *et al.*, 1990), little is known about the nature of the receptor–G-protein–effector interaction for these two important compounds. We therefore examined the characteristics of LPA- and ET-1-stimulated PtdIns(4,5)P_2 hydrolysis and Ins(1,4,5)P_3 formation in both intact and electropermeabilized Rat-1 fibroblasts. In the present paper we show that, although both agonists stimulate PIC apparently via a G-protein-linked receptor, only LPA conforms to the criteria expected in terms of the kinetics of Ins(1,4,5)P_3 formation, interaction with guanine nucleotides and inhibition by PMA, whereas significant differences are observed in the ET-1-stimulated inositol phosphate response. We conclude that the current model describing a receptor–G-protein interaction is not appropriate for the ET response in Rat-1 fibroblasts. Furthermore the data also suggest that the ET receptor–G-protein interaction in Rat-1 cells may be distinguished from other ET receptor systems previously characterized.

MATERIALS AND METHODS

Rat-1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland, U.K.) containing 20 mM-glutamine, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 10 % (v/v) newborn calf serum at 37 °C in air/CO_2 (19:1). For [^{3}H]inositol phosphate studies, cells were grown in 24-well plates and labelled with 2 μCi of [^{3}H]inositol/ml (Amersham International, Amersham, Bucks., U.K.) for 48 h in inositol-free DMEM containing penicillin, streptomycin, glutamine and 1 % (v/v) dialysed newborn calf serum. For permeabilization studies, the cells were labelled in 75 cm^3 flasks. After the labelling period, the medium was removed and the cells were washed twice in Hanks buffered saline (137 mM-NaCl, 5.4 mM-KCl, 4.0 mM-$NaHCO_3$, 1.26 mM-$CaCl_2$, 0.5 mM-$MgCl_2$, 0.4 mM-$MgSO_4$, 0.35 mM-NaH_2PO_4, pH 7.4) containing 1 % (w/v) BSA and 10 mM-glucose (HBG), then preincubated for a further 10 min at 37 °C in HBG containing 10 mM-LiCl. The reaction was initiated by the addition of agonist or vehicle in a final volume of 150 μl of HBG/LiCl, and the cells were incubated for a further 15 min at 37 °C. The reaction was terminated by the addition of 0.5 ml of ice-cold methanol, the wells were scraped and the cells were transferred to plastic vials. Chloroform was added to give a methanol/chloroform ratio of 2:1 (v/v) and, following overnight extraction at 4 °C, the phases were separated by the addition of chloroform (0.31 ml) and water (0.31 ml). Water-soluble [^{3}H]inositol phosphates were separated using Dowex formate (Sigma) anion-exchange chromatography as described previously (Wakelam *et al.*, 1986).

For the measurement of agonist-stimulated formation of Ins(1,4,5)P_3 mass, unlabelled cells were harvested from 75 cm^3 flasks using a cell scraper, preincubated for 45 min in conditioned DMEM and then washed twice in HBG. Aliquots (50 μl) of resuspended cells (approx. 10^6 cells) were then incubated with agonist or vehicle in a final volume of 75 μl for the times indicated. The reaction was terminated by the addition of 25 μl of ice-cold 10 % (v/v) $HClO_4$, and the samples were extracted on ice and then neutralized using 1.5 M-KOH/60 mM-Hepes. Ins(1,4,5)P_3 mass was assayed by competitive displacement of

[^{3}H]Ins(1,4,5)P_3 binding to bovine adrenal cortex microsomes, quantified using an Ins(1,4,5)P_3 standard curve (Palmer *et al.*, 1989).

For the measurement of agonist-stimulated hydrolysis of PtdIns(4,5)P_2 mass, unlabelled cells were stimulated with agonist in a final volume of 100 μl and the reaction was stopped by the addition of 20% (v/v) $HClO_4$. The inositol-containing phospholipids were extracted as outlined by Creba *et al.* (1983). The lipids were then deacylated and deglycerated by incubation with 1 M-NaOH for 30 min at 100 °C. A re-neutralized extract of each sample was then dried down and assayed for Ins(1,4,5)P_3 mass as outlined above.

For labelled permeabilization studies, cells were grown, labelled and harvested as described above and, after the 45 min preincubation, washed twice and resuspended in assay buffer [20 mM-Hepes (pH 7.5), containing 120 mM-KCl, 10 mM-LiCl, 6 mM-$MgCl_2$, 2 mM-KH_2PO_4, 2.5 mM-NaCl, 2.5 mM-HCl, 2 mM-Na_2ATP, 0.1 mM-EGTA and 1 mg of BSA/ml]. The final cell suspension was then exposed to six discharges of a 3 μF capacitor with a field strength of 2 kV/cm, spaced by 2 s intervals. Approx. 95% of cells were found to be permeable to ethidium bromide following this procedure. Cells [2–2.5 × 10^4 per 50 μl aliquot] were incubated with agonists or vehicle in assay buffer (250 μl final volume) at 37 °C for 5 min. The reaction was terminated by the addition of 0.94 ml of chloroform/methanol (1:2, v/v) and the inositol phosphates were extracted and the radioactivity was determined as outlined above. For kinetic analysis of [^{3}H]InsP_3 accumulation in permeabilized cells the reaction was terminated with $HClO_4$ (10%, v/v) after the stated times, and the inositol phosphates were extracted and the samples neutralized as outlined above, before analysis on Dowex columns. Dose–response curves were fitted to a logistic equation as defined by DeLean *et al.* (1980).

ET-1 was obtained from BioMac, University of Glasgow, Scotland, U.K. All other materials were from previously reported sources (Cook *et al.*, 1990; Plevin *et al.*, 1990) or were of the highest commercial grades available.

RESULTS

In Rat-1 fibroblasts both ET-1 and LPA stimulated the formation of total [^{3}H]inositol phosphates ([^{3}H]Ins$P$) in a dose-dependent manner. ET-1 gave the larger response, stimulating a 7–8-fold increase in [^{3}H]inositol phosphate accumulation over basal values after 15 min. By comparison, a smaller 2–3-fold increase was seen in response to LPA. In addition, ET-1 was also the more potent agonist, being maximally active at approx. 10 nM [concn. producing 50% of maximal effect (EC_{50}) 1.89 ± 0.8 nM, $n = 5$], whereas LPA stimulated [^{3}H]Ins$P$ accumulation over the micromolar range (EC_{50} 2.1 ± 1.2 μM, $n = 3$). These values compare favourably with those previously obtained in Rat-1 cells and in other systems (Resink *et al.*, 1988; Muldoon *et al.*, 1989; van Corven *et al.*, 1989). Fig. 1 shows the time course of Ins(1,4,5)P_3 mass formation in response to maximum concentrations of ET-1 (Fig. 1*a*) and LPA (Fig. 1*c*). ET-1 (100 nM) stimulated the rapid formation of Ins(1,4,5)P_3, with a peak response at 5–10 s of an 8–10-fold increase over control values. Levels then declined between 10 and 60 s, but did not return to basal values; instead, a new steady-state level of Ins(1,4,5)P_3 mass was obtained which was some 3–4-fold above control levels. LPA also stimulated the formation of Ins(1,4,5)P_3 mass, with a smaller peak response (5.2 ± 2.3-fold, $n = 4$) being observed between 10 and 20 s. However, in contrast to the response to ET-1, stimulated levels quickly returned to basal and by 90 s were not significantly different from the control (control, 2.26 ± 0.48 pmol; LPA, 3.77 ± 0.70 pmol).

The increases in the mass of Ins(1,4,5)P_3 in response to stimulation were paralleled by decreases in the cellular mass of PtdIns(4,5)P_2 (Figs. 1*b* and 1*d*). ET-1 stimulated a rapid decrease in the mass of this lipid with the level only returning to control values after approx. 20 min (Fig. 1*b*). Although LPA also stimulated a rapid decrease in the mass of PtdIns(4,5)P_2 the response was transient, with the concentration of the lipid returning to control levels within 2 min.

Further differences in the characteristics of LPA- and ET-1-stimulated inositol phosphate generation in intact cells are shown in Fig. 2. Following a 15 min pretreatment with 300 nM-PMA [^{3}H]Ins$P$ formation in response to LPA was decreased by some 50–60% (Fig. 2*a*) over the observed agonist concentration range. However, the response to ET-1 was unaffected by PMA pretreatment (Fig. 2*b*). Increased concentrations of PMA (up to 1 μM) or a prolonged preincubation period (up to 60 min) were also without any effect on the ET-1 response (results not shown).

In order to assess potential differences in the coupling of the LPA and ET-1 receptors to inositol phosphate formation, experiments using electropermeabilized cells were performed. Fig. 3 shows the effect of the non-hydrolysable GTP analogue GTP[S] on the dose–response curves for LPA- and ET-1-

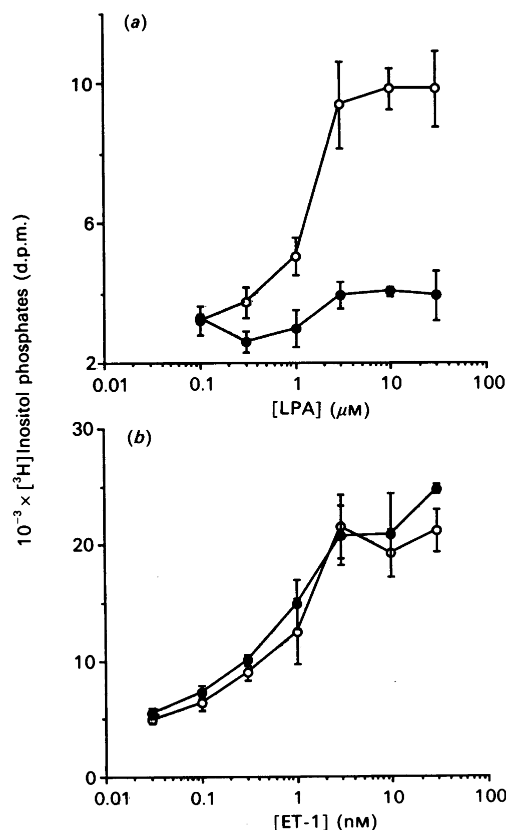

Fig. 2. Effect of PMA on LPA and ET-1 dose-dependent stimulation of [^{3}H]inositol phosphate accumulation in Rat-1 fibroblasts

Cells labelled as outlined in the Materials and methods section were preincubated with vehicle (○) or 300 nM-PMA (●) for 15 min prior to incubation for a further 20 min with increasing concentrations of either LPA (*a*) or ET-1 (*b*). Each point represents the mean ± S.D. of triplicate determinations from a single representative experiment ($n = 3$). Radioactivity in unstimulated samples was (d.p.m.): (*a*) −PMA, 2778 ± 192; +PMA, 2232 ± 315, (*b*) −PMA, 4344 ± 779; +PMA, 4781 ± 90.

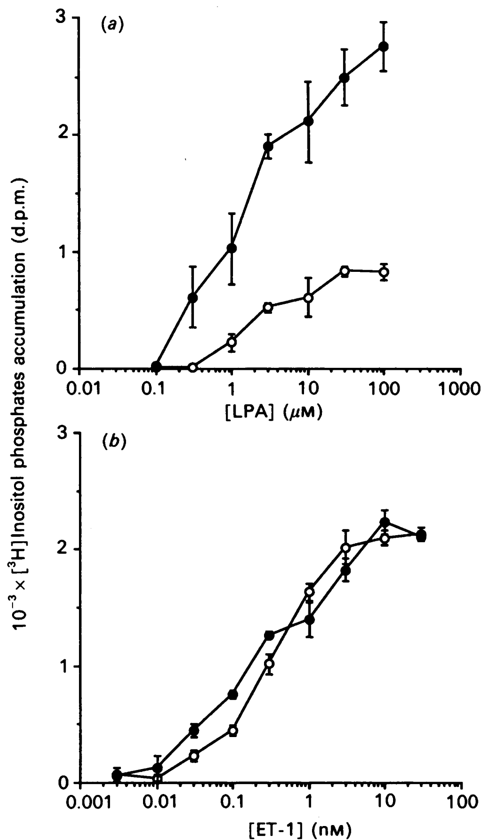

Fig. 3. Effect of GTP[S] on LPA- and ET-1-stimulated [³H]inositol phosphate accumulation in permeabilized Rat-1 fibroblasts

Labelled cells, permeabilized as outlined in the Materials and methods section, were incubated with increasing concentrations of LPA (*a*) or ET-1 (*b*) in the presence (●) or the absence (○) of 30 μM-GTP[S] for 5 min. Each point represents the mean ± S.D. of triplicate determinations from one of three to five individual experiments.

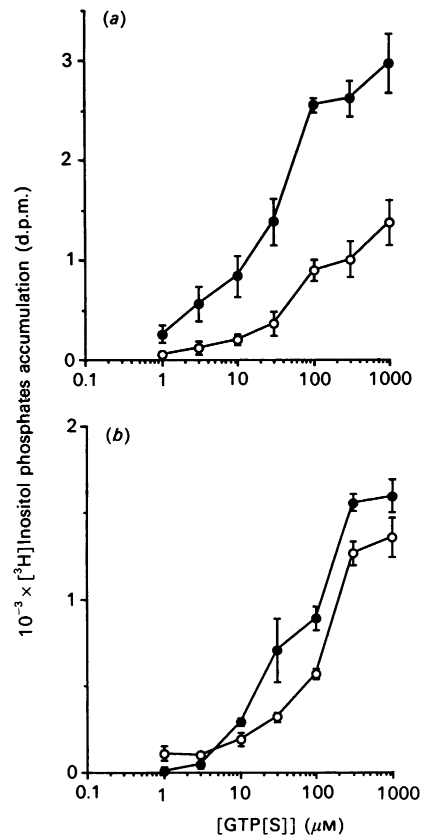

Fig. 4. Effect of LPA and ET-1 on dose-dependent GTP[S]-stimulated [³H]inositol phosphate accumulation in permeabilized Rat-1 fibroblasts

Cells treated as outlined previously were incubated with increasing concentrations of GTP[S] in the presence (●) or the absence (○) of 30 μM-LPA (*a*) or 0.3 nM-ET-1 (*b*). Each point represents the mean ± S.D. of triplicate determinations from a single experiment representative of at least two others.

stimulated [³H]inositol phosphate accumulation. LPA alone gave a modest 2–3-fold response over basal values, but there was a clear potentiation of the LPA response on the addition of 30 μM-GTP[S] at all concentrations of the agonist employed (Fig. 3*a*). Potentiation of the response was specific for non-hydrolysable analogues of GTP. p[NH]ppG was approximately half as effective in potentiating the LPA response (54 ± 3 % of the GTP[S] effect, $n = 2$) or in stimulating [³H]Ins*P* accumulation alone (24 % of GTP[S] response, $n = 2$), whereas GTP, GDP, GMP and ATP were without effect in both instances ($n = 3$). In contrast, GTP[S] had little effect upon ET-1-stimulated [³H]Ins*P* accumulation in permeabilized Rat-1 cells. (Fig. 3*b*). At 30 μM-GTP[S] there was not significant enhancement of the ET-1 response, which was not improved by varying the concentration of the nucleotide.

Fig. 4 shows the effect of LPA and ET-1 on the [³H]Ins*P* dose–response curve for GTP[S] in permeabilized Rat-1 cells. Maximum concentrations of LPA markedly enhanced the response to GTP[S] by some 200–300 % and shifted the EC_{50} value for the nucleotide to the left (−LPA, 57 ± 21 μM; +LPA, 16.5 ± 10.25 μM, $n = 3$) (Fig. 4*a*). In contrast ET-1 had no significant effect upon the GTP[S] response (Fig. 4*b*) and did not lower the EC_{50} value for the nucleotide in this instance.

The kinetics of [³H]InsP_3 formation in response to ET-1, LPA and GTP[S] are shown in Fig. 5. GTP[S] alone stimulated the formation of [³H]InsP_3, but only after a lag time of at least 30 s (Figs. 5*a* and 5*b*). However, on addition of LPA (30 μM, Fig. 5*a*), a potentiating effect of GTP[S] was observed as early as 10 s, the earliest time point recorded, indicating that the lag time for the onset of the nucleotide response was lowered by the addition of the agonist. A similar phenomenon was observed for the kinetics of [³H]InsP_2 formation (results not shown). However, ET-1 at a maximum concentration (10 nM) failed to decrease the lag time for the onset of the potentiating effect of GTP[S] (Fig. 5*b*), and in this instance ET-1 alone gave a maximum InsP_3 response. At sub-maximal concentrations (e.g. 1 nM-ET-1) a potentiating effect was observed (results not shown).

The effects of the non-hydrolysable GDP analogue GDP[S] on LPA-, ET- and GTP[S]-stimulated [³H]Ins*P* accumulation are shown in Table 1. Following a 5 min preincubation with a maximally effective GDP[S] concentration (1 mM) the response to each agonist was decreased. GDP[S] was most effective against GTP[S] and ET-1, alone or in combination, decreasing these responses by approx. 40–55 % ($n = 6$). However, although the LPA plus GTP[S] response was also lowered by some 50 %, the LPA alone response was only marginally affected (10–20 %).

Table 2 shows the effect of short-term PMA pretreatment on stimulated [³H]Ins*P* accumulation in permeabilized cells. Following preincubation with 300 nM-PMA for 15 min, the response to LPA was lowered by approx. 50 % (−PMA, 197 ± 18; +PMA,

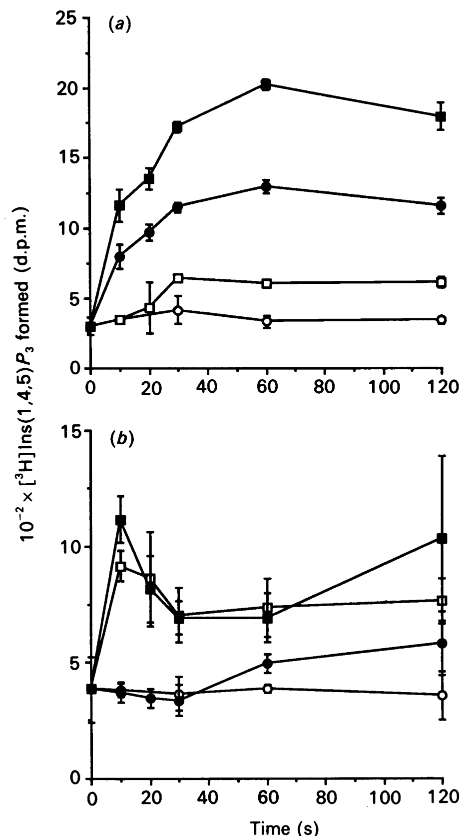

Fig. 5. Kinetics of agonist- and GTP[S]-stimulated [^{3}H]InsP_3 formation in permeabilized Rat-1 fibroblasts

Cells, harvested and electroporated as outlined in the Materials and methods section, were incubated with: (*a*) vehicle (○), 200 μM-GTP[S] (□), 30 μM-LPA (●) or 30 μM-LPA + 200 μM-GTP[S] (■); and (*b*) vehicle (○), 200 μM-GTP[S] (●), 10 nM-ET-1 (□) or 10 nM-ET-1 + 200 μM-GTP[S] (■) for the times indicated. Each point represents the mean ± S.D. of triplicate determinations from one of three individual experiments.

Table 1. Effect of GDP[S] on agonist- and nucleotide-stimulated [^{3}H]inositol phosphate accumulation in permeabilized Rat-1 fibroblasts

Cells treated as outlined in the Materials and methods section were preincubated with vehicle or 1 mM-GDP[S] for 5 min prior to stimulation for a further 5 min with LPA (30 μM), ET-1 (0.3 nM) and GTP[S] (30 μM) alone and in combination. Basal values were (d.p.m.): control, 1511 ± 93; GDP[S], 717 ± 21. Each value represents the mean ± S.D. of triplicate determinations from one of five individual experiments.

	[^{3}H]Ins$P$ accumulation (% of basal)	
	−GDP[S]	+GDP[S]
LPA	307 ± 17	258 ± 22
GTP[S]	222 ± 4	118 ± 5
LPA + GTP[S]	653 ± 26	307 ± 15
ET-1	743 ± 9.5	363 ± 25
ET-1 + GTP[S]	831 ± 4	385 ± 10

152 ± 9 % of basal values). The response to GTP[S] was only marginally affected by PMA (−PMA, 150 %; +PMA, 138 % of basal values). As with the intact cell system the response to ET-

Table 2. Effect of PMA on agonist- and nucleotide-stimulated [^{3}H]inositol phosphate accumulation in permeabilized Rat-1 fibroblasts

Cells treated as outlined in the Materials and methods section were preincubated for 15 min with vehicle or 300 nM-PMA, permeabilized and then stimulated for a further 5 min with LPA (30 μM), ET-1 (0.3 nM) or GTP[S] (30 μM) alone and in combination. Basal values were (d.p.m.): control, 629 ± 83; 300 nM-PMA, 474 ± 14. Each value is the mean ± S.D. of triplicate determinations from a single experiment representative of two others.

	[^{3}H]Ins$P$ accumulation (% of basal)	
	−PMA	+PMA
LPA	197 ± 18	152 ± 9
GTP[S]	150 ± 10	138 ± 4
LPA + GTP[S]	359 ± 15	268 ± 8
ET-1	231 ± 14	256 ± 8
ET-1 + GTP[S]	326 ± 6	367 ± 13

1 alone or together with GTP[S] was not significantly affected by PMA.

DISCUSSION

The intracellular mechanisms through which a number of different classes of agonists stimulate mitogenesis are still not fully understood. Two such agonists, the phospholipid LPA and the peptide ET-1, are complete mitogens for Rat-1 fibroblasts (van Corven *et al.*, 1989; MacNulty *et al.*, 1990*b*). However, the agonists stimulate mitogenesis through the activation of different intracellular pathways. LPA is thought to act through the inhibition of adenylate cyclase (van Corven *et al.*, 1989). We have been unable to detect any inhibitory or stimulatory effects of ET-1 upon adenylate cyclase activity (results not shown). Indeed, ET-1-stimulated DNA synthesis is dependent on protein kinase C activity and is thus consistent with the involvement of inositol phospholipid breakdown (Muldoon *et al.*, 1990). LPA also stimulates inositol phosphate formation in Rat-1 cells, but activation of this pathway is not thought to be involved in the LPA mitogenic response. By implication, this suggests that any differences in the characteristics of ET-stimulated PtdIns(4,5)P_2 hydrolysis, in comparison with those of LPA, may be of significance in relation to its potency as a mitogen. We therefore examined the receptor–G-protein–effector interaction for both ET- and LPA-stimulated inositol phosphate formation in Rat-1 fibroblasts to determine if such differences do indeed exist at this level.

The characteristics of LPA-stimulated inositol phosphate formation in both intact and permeabilized Rat-1 fibroblasts conformed closely to the criteria expected for a typical receptor–effector interaction involving a G-protein. In permeabilized cells, LPA-stimulated inositol phosphate formation was enhanced by GTP[S]. This agrees with the preliminary findings of van Corven *et al.* (1989). However, unlike this earlier study, we performed full dose–response analysis for this effect. In the presence of GTP[S] a significant decrease in the EC_{50} value for the agonist response was observed. The shift in the apparent affinity of the agonist for the receptor, rather than an enhancement of the maximum response, is a more critical test for the involvement of a receptor–G-protein interaction in agonist-stimulated second messenger formation. In addition, by studying the kinetics of LPA-stimulated InsP_3 formation in permeabilized Rat-1 cells, we also show that addition of the agonist decreased the lag time for the onset of the nucleotide response, suggesting that the agonist

accelerates GDP/GTP exchange. This is an essential criterion for defining the involvement of a G-protein in receptor coupling to a second messenger pathway (Harden *et al.*, 1988; Boyer *et al.*, 1989*a*) and thus strongly suggests that LPA indeed acts through an authentic receptor. Consistent with this finding, we also found that the EC_{50} value for GTP[S]-stimulated inositol phosphate accumulation after 5 min was significantly decreased in the presence of maximum concentrations of LPA.

Further evidence for the LPA response being mediated through a G-protein-linked receptor was derived from PMA inhibition studies. In intact cells, preincubation with PMA markedly decreased the subsequent LPA-stimulated inositol phosphate response in a manner characteristic for a receptor linked to PIC through activation of a G-protein. The site of protein kinase C-mediated inhibition seems to be upstream of the G-protein–PIC interface, since the response to GTP[S] was unaffected by PMA pretreatment. This is a commonly observed site for protein kinase C-mediated down-regulation of the inositol phosphate pathway (Pfeilschifter & Bauer, 1987; Plevin *et al.*, 1990). However, lack of a suitable labelled ligand prevented us from determining if the putative LPA receptor is modified by protein kinase C activity. Finally, by measuring changes in the mass of Ins(1,4,5)P_3 and its precursor phospholipid, PtdIns(4,5)P_2, and not merely the accumulation of total labelled inositol phosphates over long periods as previously reported (van Corven *et al.*, 1989), we observed that LPA stimulation of this second messenger pathway is both rapid and transient. These data are consistent with the interaction of LPA with a specific phospholipid receptor and strongly argue against a non-specific interaction with the plasma membrane.

Surprisingly, we observed many differences in the characteristics of the ET-1-stimulated and LPA-stimulated inositol phosphate formation in Rat-1 fibroblasts. In intact cells, ET-1 stimulated sustained PtdIns(4,5)P_2 hydrolysis, as determined by measurement of both an increase in the mass of Ins(1,4,5)P_3 and a sustained decrease in the mass level of PtdIns(4,5)P_2. These experiments demonstrate that ET-1-stimulated hydrolysis is sustained, whereas that stimulated by LPA is desensitized. Comparison between the effects of the two agonists also demonstrates that the sustained ET-1-stimulated Ins(1,4,5)P_3 response is unlikely to be due to regulation at the level of the InsP_3 kinase or phosphatase. Sustained PtdIns(4,5)P_2 hydrolysis is not a feature commonly associated with peptide receptors linked to the inositol phosphate pathway via a G-protein (Fu *et al.*, 1988; Cook *et al.*, 1990), although it may be observed for non-peptide agonists such as prostaglandin $F_{2\alpha}$, carbachol (M1 receptor) and the tyrosine kinase receptor agonists platelet-derived growth factor and epidermal growth factor (Tilly *et al.*, 1988; Blakely *et al.*, 1989; Seuwen *et al.*, 1990). These findings are also inconsistent with the kinetics of labelled InsP_3 formation observed in other ET-receptor-linked systems, such as vascular smooth muscle (van Renterghem *et al.*, 1988) and brain capillary endothelial cells (Vigne *et al.*, 1990) where apparent complete desensitization was detected. In addition, we observed that both short- and long-term PMA pretreatment was without effect upon ET-stimulated inositol phosphate formation. Again, this is in contrast with the result expected for receptors linked to PtdIns(4,5)P_2 hydrolysis via a G-protein, and is at variance with results described in other tissues for ET-1-stimulated inositol phosphate formation (Araki *et al.*, 1989; Roubert *et al.*, 1989; Resink *et al.*, 1990).

In permeabilized cells we also found that ET-1-stimulated inositol phosphate formation did not conform to all the criteria expected for a receptor linked to a G-protein, in terms of the effect of guanine nucleotides and the ability to accelerate GDP/GTP exchange. These results strongly suggest that the presently accepted model of receptor-stimulated guanine nucleotide exchange and subsequent activation of the intrinsic GTPase activity of the α subunit is inadequate to describe the receptor–G-protein interaction for this peptide.

There are several possibilities which may explain the apparent difficulties in observing a potentiation of the ET response by GTP[S]. Electropermeabilization may not allow full equilibration of nucleotide pools within the cells. However, this would still suggest differences in the G-protein input required for both receptors to illicit full inositol phosphate responses. Also, this potential artifact does not explain the lack of an effect of PMA in intact cells. Also, using cells permeabilized with streptolysin-O we have obtained essentially the same results (results not shown). Another possibility is that, in addition to a classical receptor–G-protein coupling mechanism, ET-1 may also interact with the G-protein itself, in a manner similar to that recently described for certain types of venoms, toxins and neuropeptides such as mastoparan, bombesin and bradykinin (Mousli *et al.*, 1990). This could adequately account for the absence of desensitization, the small effect of GTP[S] and the large inhibition by GDP[S]. Such an explanation could also account for the lack of potentiation by guanine nucleotides at maximal ET-1 concentrations, although there was a potentiation at sub-maximal agonist concentrations.

Our findings are also surprising in relation to the recent cloning of two subtypes of the ET receptor (Arai *et al.*, 1990; Sakurai *et al.*, 1990). Both subtypes have seven transmembrane spanning domains, which is consistent with a receptor which is coupled to a G-protein. The ET-1 receptor contains sequences which are putative sites for protein kinase C-mediated phosphorylation. These findings correlate with the biochemical characteristics observed for ET-1-stimulated inositol phosphate accumulation in smooth muscle cells. Although our initial characterization also suggested the presence of an ET-1 type of receptor in Rat-1 fibroblasts (MacNulty *et al.*, 1990*b*), none of the biochemical characteristics correspond to these structural observations. Therefore we may indeed be dealing with a subtype of the ET-1 receptor, one which displays unusual characteristics in relation to the receptor–G-protein interaction. In this regard, it is worth noting that the non-selective ET receptor contains unusual sequences within the third cytoplasmic loop, the putative site of the G-protein interaction. Therefore not all subtypes of the ET receptor may interact with the G-protein in the same manner.

Although the biochemical basis for the unusual and novel phenomena highlighted in this study remains unclear, the findings may be of significance in relation to the physiological effects of ET. It is able to maintain contractile responses for up to several hours *in vitro* and *in vivo* (Yanagisawa *et al.*, 1988; Yanagisawa & Masaki, 1989) and is a mitogen in the absence of any other cofactors (Brown & Littlewood, 1989; MacNulty *et al.*, 1990*b*). Recently it has been shown that sustained PtdIns(4,5)P_2 hydrolysis may indeed play a role in early events in mitogenesis (Seuwen *et al.*, 1990). Therefore the unusual receptor–G-protein interaction which seems to underlie the sustained PtdIns(4,5)P_2 hydrolysis in response to ET-1 in Rat-1 fibroblasts may indeed help to explain its potency as a mitogen.

This work was supported by grants from the Cancer Research Campaign, the Medical Research Council (U.K.) and the British Heart Foundation. E. E. M. is a Glasgow University postgraduate scholar.

REFERENCES

Ambar, I., Kloog, Y., Schuartz, I., Hazum, E. & Sokolovsky, M. (1989) Biochem. Biophys. Res. Commun. **158**, 195–201

Arai, H., Hori, S., Aramori, I., Ohkubo, H. & Nakanishi, S. (1990) Nature (London) **348**, 730–732

Araki, S., Kawahara, Y., Kariya, K., Sunako, M., Fukuzaki, H. & Taki, Y. (1989) Biochem. Biophys. Res. Commun. **159**, 1072–1079

Blakely, D. M., Corps, A. N. & Brown, K. D. (1989) Biochem. J. **258**, 177–185

Boyer, J. L., Downes, C. P. & Harden, T. K. (1989*a*) J. Biol. Chem. **264**, 884–890

Boyer, J. L., Helper, J. P. & Harden, T. K. (1989*b*) Trends Pharmacol. Sci. **10**, 360–365

Brown, K. D. & Littlewood, C. J. (1989) Biochem. J. **263**, 977–980

Brown, K. D., Blakely, D. M., Hamon, M. H., Laurie, M. S. & Corps, A. N. (1987) Biochem. J. **245**, 631–639

Chiang, C.-F. & Hauser, G. (1989) Biochem. Biophys. Res. Commun. **165**, 175–181

Claro, E., Garcia, A. & Picatoste, F. (1989) Biochem. J. **261**, 29–35

Cockcroft, S. & Gomperts, B. D. (1985) Nature (London) **314**, 534–536

Cook, S. J., Palmer, S., Plevin, R. & Wakelam, M. J. O. (1990) Biochem. J. **165**, 617–620

Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) Biochem. J. **212**, 733–747

DeLean, A., Munson, P. J. & Rodbard, D. (1980) Am. J. Physiol. **235**, E97–E102

Fu, T., Okano, Y. & Nozawa, Y. (1988) Biochem. Biophys. Res. Commun. **157**, 1429–1435

Geny, B., Stutchfield, J. & Cockcroft, S. (1989) Cell. Signalling **1**, 165–172

Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649

Harden, K. T., Hawkins, P. T., Stephens, L, Boyer, J. L. & Downes, C. P. (1988) Biochem. J. **252**, 583–593

MacNulty, E. E., Plevin, R. & Wakelam, M. J. O. (1990*a*) Biochem. Soc. Trans. **18**, 482–483

MacNulty, E. E., Plevin, R. & Wakelam, M. J. O. (1990*b*) Biochem. J. **272**, 761–766

Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J. & de Laat, S. W. (1986) Nature (London) **323**, 171–173

Mousli, M., Bueb, J. L., Bronner, C., Rouot, B. & Landry, Y. (1990) Trends Pharmacol. Sci. **11**, 358–362

Muldoon, L. L., Rodland, K. D., Forsythe, M. L. & Magun, B. E. (1989) J. Biol. Chem. **264**, 8529–8536

Muldoon, L. L., Pribnow, D., Rodland, K. D. & Magnum, B. E. (1990) Cell. Regul. **1**, 379–390

Murayama, T. & Ui, M. (1987) J. Biol. Chem. **262**, 5522–5529

Nakaki, T., Nakayama, M., Yamamoto, S. & Kato, R. (1989) Biochem. Biophys. Res. Commun. **158**, 880–883

Ohsako, S. & Deguchi, T. (1981) J. Biol. Chem. **256**, 10945–10948

Orellana, S., Solski, P. A. & Brown, J. H. (1987) J. Biol. Chem. **262**, 1638–1643

Palmer, S., Hughes, K. T., Lee, D. C. & Wakelam, M. J. O. (1989) Cell. Signalling **1**, 147–156

Pfeilschifter, J. & Bauer, C. (1987) Biochem. J. **248**, 209–215

Plevin, R., Palmer, S., Gardener, S. D. & Wakelam, M. J. O. (1990) Biochem. J. **268**, 605–610

Resink, T. J., Scott-Burden, T. & Buhler, F. R. (1988) Biochem. Biophys. Res. Commun. **157**, 1360–1368

Resink, T. J., Scott-Burden, T., Weber, E. & Buhler, F. R. (1990) Biochem. Biophys. Res. Commun. **166**, 1213–1219

Roubert, P., Gillard, V., Plas, P., Guillon, J. M., Chabrier, P. E. & Braquet, P. (1989) Biochem. Biophys. Res. Commun. **164**, 809–815

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. & Masaki, T. (1990) Nature (London) **348**, 732–735

Seuwen, K., Kahan, C., Hartmann, T. & Pouyssegur, J. (1990) J. Biol. Chem. **265**, 22292–22299

Simonson, M. S., Wann, S., Mene, P., Dubyak, G. R., Kester, M., Nakazato, Y., Sedor, J. R. & Dunn, M. J. (1989) J. Clin. Invest. **83**, 708–712

Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K. & Masaki, T. (1989) J. Biol. Chem. **264**, 7856–7861

Takuwa, Y., Kasuya, Y., Takuwa, N., Kudo, M., Yanagisawa, M., Goto, K., Masaki, T. & Yamashita, K. (1990) J. Clin. Invest. **85**, 653–658

Tilly, B. C., van Pardon, P. A., Verlaan, I., de Laat, S. W. & Moolenaar, W. H. (1988) Biochem. J. **252**, 857–863

van Corven, E. J., Groenink, A., Jalink, K., Eicholtz, T. & Moolenaar, W. H. (1989) Cell **59**, 45–54

van Renterghem, C. V., Vigne, P., Barhanin, J., Schmid-Alliana, J., Frelin, C. & Lazdunski, M. (1988) Biochem. Biophys. Res. Commun. **157**, 977–985

Vigne, P., Marsault, R., Breittmayer, J. P. & Frelin, C. (1990) Biochem. J. **266**, 415–420

Wakelam, M. J. O., Murphy, G. J., Hruby, V. J. & Houslay, M. D. (1986) Nature (London) **323**, 68–71

Yanagisawa, M. & Masaki, T. (1989) Biochem. Pharmacol. **38**, 1877–1883

Yanagisawa, M., Kurihara, H., Kimura, S., Tombe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. A. (1988) Nature (London) **332**, 411–415

Received 31 December 1990/2 July 1991; accepted 12 July 1991