Regulation of endothelin-1- and lysophosphatidic acid-stimulated tyrosine phosphorylation of focal adhesion kinase (pp125^{fa}) in Rat-1 fibroblasts

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The characteristics of protein tyrosine phosphorylation were examined in Rat-1 fibroblasts in response to endothelin-1 (ET-1) and 1-oleoyl-lysophosphatidic acid (LPA). Both agonists stimulated the biphasic tyrosine phosphorylation of at least three major proteins of approx. 120 kDa (pp116, pp120 and pp130) and two of 80 kDa (pp80 and pp70). Immunoprecipitation experiments indicated that the pp120 protein corresponded to the recently described focal adhesion protein kinase pp125^{fa}. Phorbol 12-myristate 13-acetate, alone or in combination with the calcium ionophore A23187, also stimulated the phosphorylation of pp125^{fa} but to a smaller extent than LPA or ET-1. Removal of both extracellular and intracellular Ca²⁺ did not significantly reduce LPA- and ET-1-stimulated tyrosine phosphorylation of pp125^{fa}. In cells where protein kinase C

activity was down-regulated or inhibited, ET-1-stimulated tyrosine phosphorylation of pp125^{fa} was reduced to a greater extent than phosphorylation in response to LPA. In addition, ET-1-stimulated tyrosine phosphorylation of pp80 was decreased by 50–70% in response to protein kinase C inhibition at both 2 and 60 min whereas LPA-stimulated tyrosine phosphorylation of this protein was only reduced at 2 min. Pretreatment with pertussis toxin reduced the tyrosine phosphorylation of pp42 and pp44 forms of mitogen-activated protein kinase in response to both ET-1 and LPA but reduced the tyrosine phosphorylation of pp125^{fa} only in response to LPA. These results indicate agonist-specific differences in the regulation of pathways mediating the tyrosine phosphorylation of pp125^{fa} and other target proteins.

INTRODUCTION

The intracellular signalling pathways involved in the regulation of cell growth and division in response to a number of mitogens with receptors coupled to G-proteins have not been clearly defined. A large body of evidence has implicated a role for protein kinase C (PKC) in this response, as it is activated via receptor-mediated PtdIns(4,5)P₂ and phosphatidylcholine hydrolysis in numerous cell types [1–4]. Recently, attention has focused on the possible involvement of intermediates of the tyrosine kinase cascade in mitogen-stimulated cell division. These include mitogen-activated protein kinase (MAP kinase), MAP kinase kinase and Raf-1 [5–7]. Activation of this pathway also involves stimulation of p21^{ras} nucleotide exchange by the Ras activator mammalian homologue of Son of sevenless (mSos) [8,9].

The G-protein-receptor-coupled agonists 1-oleoyl-lysophosphatidic acid (LPA) and endothelin-1 (ET-1) exhibit differences in their efficacy as mitogens for Rat-1 fibroblasts in culture [10–12] and this may be directly related to the characteristics of cell-signalling events initiated by each agonist. ET-1 stimulates the sustained hydrolysis of both PtdIns(4,5)P₂ and phosphatidylcholine, resulting in the formation of 1,2-diacyl-sn-glycerol and activation of PKC [11–15]. LPA also stimulates the hydrolysis of PtdIns(4,5)P₂ but only transiently; the mitogenic activity of the phospholipid is believed to be due to a receptor-mediated inhibition of adenylate cyclase involving a G_i protein [10]. As LPA-stimulated InsP₃ formation is not sensitive to

pertussis toxin, this suggests that for LPA, multiple receptor–G-protein–effector–coupling interactions may occur.

Recently it has been shown that the activation of a number of G-protein-coupled receptors linked to polyphosphoinositide hydrolysis may also result in tyrosine phosphorylation of several intracellular proteins including MAP kinase [16–18]. For a number of these agonists, including thrombin and LPA, the regulation of MAP kinase may involve the activation of p21^{ras} by a pertussis-toxin-sensitive mechanism [19]. However, the mechanisms by which ET-1 and LPA regulate the tyrosine phosphorylation of other tyrosine kinase substrates are still unclear.

In the present paper we show that both ET-1 and LPA stimulate the biphasic tyrosine phosphorylation of a number of major target proteins in Rat-1 fibroblasts. These include proteins pp130, pp120, pp116, pp80 and pp70. One of these substrates, pp120, corresponds to focal adhesion kinase, pp125^{fa} [20,21]. We have examined the dependence of phosphorylation of this and other tyrosine-phosphorylated proteins on PKC and Ca²⁺. We show that both ET-1- and LPA-stimulated tyrosine phosphorylation of pp125^{fa} is only partly dependent on PKC activation or Ca²⁺ mobilization, suggesting an additional pathway involved in the regulation of pp125^{fa} tyrosine phosphorylation in response to G-protein-coupled receptor agonists. Moreover, we show that, whereas tyrosine phosphorylation of MAP kinase in response to both ET-1 and LPA are inhibited by pertussis toxin pretreatment, only LPA-stimulated tyrosine phosphorylation of pp125^{fa} is affected. This suggests differential

Abbreviations used: LPA, 1-oleoyl-lysophosphatidic acid; ET-1; endothelin-1; PDGF, platelet-derived growth factor; HBG, Hanks buffered saline containing 2% (w/v) BSA and 10 mM glucose; MAP kinase, mitogen-activated protein kinase; HRP, horseradish peroxidase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

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involvement of a pertussis-toxin-sensitive G-protein in the activation of tyrosine kinase pathways by ET-1 and LPA.

MATERIALS AND METHODS

Materials

All materials were of the highest commercial grade available. [γ - 32 P]ATP was from Amersham International (Amersham, Bucks., U.K.). All purchased anti-phosphotyrosine and anti-(MAP kinase) antibodies were from Affiniti Research Products Ltd., Nottingham, U.K. The PKC substrate (GS-peptide; Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys-COOH) was synthesized by Dr. E. G. Rowan of the Strathclyde Institute for Drug Research, Strathclyde University, Glasgow, U.K. Antibody 2A7 was a gift from Professor T. J. Parsons, University of Virginia, Charlottesville, VI, U.S.A.

Cell culture

Rat-1 fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) newborn calf serum, 27 mg/ml glutamine and penicillin/streptomycin (250 units/ml and 250 μ g/ml respectively) at 37 °C in a humidified atmosphere of air/CO₂ (19:1, v/v). All experiments were performed in cells grown to confluency on 6 cm² plates and made quiescent by serum deprivation for 40 h.

Cell stimulation and phosphotyrosine immunoblotting

Cells were incubated in Hanks buffered saline containing 10 mM glucose and 2% (w/v) BSA (HBG) for 30 min and then stimulated with agonist or vehicle for the appropriate times. The reaction was terminated by rapid aspiration followed by washing in ice-cold 20 mM Hepes buffer, pH 7.4, containing 150 mM NaCl, 50 mM NaF, 10 mM Na₂P₂O₇, 4 mM EDTA, 2 mM EGTA and 2 mM Na₃VO₄ (HPFEV). After the final aspiration, 0.75 ml of SDS sample buffer (70 °C) was added and the sample passed repeatedly through a 21G needle. The samples were boiled for 5 min and then stored at -80 °C until analysis. Portions of each sample (30–50 μ g of protein) were subjected to SDS/PAGE (7.5%) and then transblotted on to nitrocellulose. The nitrocellulose blots were blocked for non-specific binding for 3 h in 50 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 0.2% (v/v) Tween (NaTT) and 3% (w/v) BSA, then incubated in the same buffer containing 0.2% BSA and 200 ng/ml of a rabbit anti-phosphotyrosine antibody overnight. The blots were rinsed in NaTT buffer, washed for 90 min (six changes of NaTT buffer) and then incubated in buffer containing 0.2% BSA and donkey anti-(rabbit immunoglobulin) linked to horseradish peroxidase (HRP) for 60 min. The blots were then washed for 120 min (eight changes of NaTT buffer) and developed using the ECL detection system (Amersham). The blotting procedure was assessed for specificity for phosphotyrosine, appropriate antibody dilution and linearity with protein concentration (results not shown). For analysis of the tyrosine phosphorylation of MAP kinase, the 42 and 44 kDa forms of MAP kinase were separated by SDS/PAGE (12% gels) and probed with either anti-phosphotyrosine or anti-(MAP kinase) antibodies under the conditions outlined above.

Immunoprecipitation of focal adhesion kinase pp125^{fa}

Cells, incubated as outlined above, were washed three times in HPFEV and then solubilized by the addition of HPFEV containing 1% (v/v) Triton X-100, 0.25% deoxycholate, 50 μ g/ml

leupeptin, 10 μ g/ml soya bean trypsin inhibitor, 10 μ g/ml aprotinin, 100 μ M phenylmethanesulphonyl fluoride, 1 mM benzamide and 5 μ M pepstatin A (immunoprecipitation buffer). After 30 min on ice, the samples were centrifuged at 50000 *g* for 30 min at 4 °C to remove any insoluble material. The samples were then precleared by incubation with rabbit serum precoupled to protein A-agarose for 60 min at 4 °C and then incubated with the anti-pp125^{fa} antibody 2A7 (2 μ l/250 μ g of protein) for 2 h. Samples were then immunoprecipitated by incubation with Protein A-agarose coupled to rabbit anti-(mouse IgG) for 2 h at 4 °C. The immunoprecipitates were recovered by centrifugation in a Microfuge (20 s at 14000 *g*), and washed three times with immunoprecipitation buffer and twice in immunoprecipitation buffer not containing Triton or deoxycholate. Immunoprecipitated pp125^{fa} was eluted from the Protein A-agarose by boiling in SDS sample buffer. Immunoprecipitated extracts were immunoblotted for phosphotyrosine using mouse monoclonal antibody PY54 (10 ng/ml) or for pp125^{fa} using antibody 2A7 (2 μ l/ml). Anti-phosphotyrosine blots were reprobed for pp125^{fa} to confirm the identity of the 120 kDa band. For Western-blot analysis of pp125^{fa} an anti-(mouse IgG) HRP-linked second antibody was used.

PKC assay

Assay of agonist-stimulated PKC activity was as described previously [22,23]. Cells were washed twice in HBG. The reaction was initiated by the addition of agonist for the times specified in a final volume of 0.5 ml at 37 °C. The cells were washed twice in buffer containing 150 mM KCl, 5.16 mM MgCl₂, 12.5 mM Pipes, 12.5 mM EGTA and 8.17 mM CaCl₂, pH 7.4, and then incubated in the same mixture containing 100 μ g/ml digitonin, 200 μ M ATP/[γ - 32 P]ATP (300–450 c.p.m./pmol) and 200 μ M of the GS peptide. After a 10 min incubation period, the reaction was terminated by the addition of 100 μ l of 25% (w/v) trichloroacetic acid in 2 M acetic acid. After 10 min on ice the cells were scraped and the extracts transferred to Microfuge tubes and spun for 5 min at 14000 *g* at 4 °C. Portions of the supernatants were spotted on to 3 cm squares of P81 Whatman ion-exchange chromatography paper. The squares were washed ($\times$ 3) in 30% acetic acid containing 1% phosphoric acid (10 min each wash) and then once in ethanol. They were then dried and measured by liquid-scintillation counting. Non-specific phosphorylation (5–10% of total phosphorylation in the absence of the peptide) was measured in each assay and subtracted from all values.

EC₅₀ values were estimated by computer-assisted fitting of concentration–response curves using an Allfit program. Concentrations of free Ca²⁺ and Mg²⁺ were calculated by a chelation computer program using appropriate dissociation constants for Ca²⁺, Mg²⁺ and H⁺.

RESULTS

Initial experiments revealed that ET-1 (100 nM) and LPA (30 μ M) stimulated the tyrosine phosphorylation of at least three proteins at approx. 120 kDa (pp116, pp120 and pp130) and two at 80 kDa (pp80 and pp70) (Figure 1). Phosphorylation of pp120 was also observed for other G-protein-linked agonists including thrombin (100 nM) and 5-hydroxytryptamine (30 μ M) but not angiotensin II (100 nM) or bombesin (100 nM) (results not shown). Platelet-derived growth factor (PDGF) (30 ng/ml) also stimulated tyrosine phosphorylation of a similar set of proteins but to a lesser extent than that observed for agonists activating G-protein-coupled receptors. For ET-1 and LPA the time courses of phosphorylation of pp120 were similar (Figures

Figure 1 Agonist-stimulated protein tyrosine phosphorylation in Rat-1 fibroblasts

Cells were treated as outlined in the Materials and methods section and then immunoblotted for phosphotyrosine. Lane 1, control; lane 2, PMA (100 nM, 10 min); lane 3, ET-1 (100 nM, 2 min); lane 4, LPA (30 μ M, 2 min); lane 5, PDGF-BB (30 ng/ml, 10 min); lane 6, thrombin (100 nM, 2 min); lane 7, 5-hydroxytryptamine (30 μ M, 2 min). This blot is representative of five others.

Figure 2 Time course of ET-1- and LPA-stimulated protein tyrosine phosphorylation in Rat-1 fibroblasts

Rat-1 fibroblasts were incubated with 100 nM ET-1 (a) or 10 μ M LPA (b) for the given times and then immunoblotted for phosphotyrosine levels as outlined in the Materials and methods section (see insets). Each point represents the density of staining of pp120 as a percentage of the maximum stimulation at 2 min. Error bars represent the S.E.M. from five experiments.

2a and 2b). An initial peak of phosphorylation was observed between 2 and 5 min of stimulation which fell towards basal values at 10 min before increasing once again between 15 and

Figure 3 Concentration-dependence of LPA-stimulated tyrosine phosphorylation

Rat-1 fibroblasts were incubated with increasing concentrations of LPA for 2 min and then immunoblotted for phosphotyrosine as outlined in the Materials and methods section. Each blot is a representative experiment from at least four individual experiments. Each point represents the density of staining of pp120 as a percentage of the maximum staining with 10 μ M LPA at 2 min. Error bars are S.E.M. where $n = 4$.

Figure 4 Immunoprecipitation of pp125^{lak} in ET-1-stimulated Rat-1 fibroblasts

Cells were stimulated with 100 nM ET-1 for 2 min and cell extracts incubated with increasing concentrations of antibody 2A7. Immunoprecipitates were assayed for phosphotyrosine or pp125^{lak} content as outlined in the Materials and methods section. This experiment is representative of one performed three times. (a) Phosphotyrosine blot; (b) pp125^{lak} blot. Lane 1, 0.25 μ l/250 μ g of protein; lane 2, 1 μ l/250 μ g; lane 3, 2.5 μ l/250 μ g.

60 min. The tyrosine phosphorylation of other proteins (pp130, pp116, pp80 and pp70) followed the same biphasic pattern in response to either LPA or ET-1. Both agonists stimulated tyrosine phosphorylation of pp120 and other proteins in a concentration-dependent manner (see Figure 3 for LPA; results for ET-1 not shown). Combined EC_{50} values for the stimulation of pp120 phosphorylation by ET-1 and LPA were 0.96 ± 33 and 52.37 ± 14 nM respectively (single determinations from five separate experiments). For LPA, this value is at least two orders of magnitude to the left of that observed for [3 H]inositol phosphate accumulation in the same system [10,14].

Figures 4(a) and 4(b) show analysis of the phosphotyrosine levels in extracts from cells stimulated with ET-1 and immuno-

Figure 5 ET-1- and LPA-stimulated tyrosine phosphorylation of pp125^{fak} in Rat-1 fibroblasts

Stimulated cells were immunoprecipitated for pp125^{fak} using antibody 2A7 then immunoblotted for phosphotyrosine **(a) (i)** and **(b)** or pp125^{fak} **(a) (ii)** as outlined in the Materials and methods section. Each experiment was performed at least three times. **(a)** Lane C, control (2 min); lane E 100 nM ET-1 (2 min); lane L, LPA (10 μ M, 2 min); lane T, thrombin (100 nM, 2 min); lane PF, PDGF (30 ng/ml, 15 min); lane P, PMA (100 nM, 20 min). **(b)** Lanes C represent control values at 2 and 60 min, and lanes E represent ET-1 stimulations from 2 to 60 min.

Table 1 Effect of PMA and A23187 on tyrosine phosphorylation of pp120 and pp80 in Rat-1 fibroblasts

Cells were incubated with vehicle or agonist and samples were then analysed for tyrosine phosphorylation as outlined in the Materials and methods section. A23187 and PMA incubations were for 15 min and 2 min for ET-1 and LPA. Phosphorylation was determined by densitometry and control values were subtracted. Each value is the mean $\pm$ S.D. of single determinations from at least six individual experiments.

Agent	Percentage of basal stimulation	
	pp120	pp80
PMA (100 nM)	128 $\pm$ 2.3	211 $\pm$ 14.8
A23187 (5 μ M)	124 $\pm$ 5.5	151 $\pm$ 24.1
PMA (100 nM) + A23187 (5 μ M)	139 $\pm$ 4.6	336 $\pm$ 36.2
ET-1 (100 nM)	380 $\pm$ 44.0	456 $\pm$ 49.4
LPA (10 μ M)	319 $\pm$ 50.0	357 $\pm$ 62.0

precipitated with the anti-pp125^{fak} antibody, 2A7 [20]. Incubation of cell lysates with increasing concentrations of antibody 2A7 resulted in an increased recovery of a tyrosine-phosphorylated protein which was identified as pp125^{fak} by Western blotting.

Both LPA and ET-1 stimulated a significant increase in the tyrosine phosphorylation of pp125^{fak} [Figure 5a(i)]. However, the response to the PKC activator phorbol 12-myristate 13-acetate (PMA) was small in comparison with that of ET-1, LPA or thrombin. This was not due to a reduced amount of recovery

Figure 6 Effect of removal of extracellular Ca²⁺ on ET-1- and LPA-stimulated pp120 and pp125^{fak} tyrosine phosphorylation in Rat-1 fibroblasts

Cells were incubated for 15 min in the presence of EGTA to give a free [Ca²⁺] of 200 nM before stimulation for 2 min with either ET-1 (100 nM) or LPA (10 μ M). In **(a)** cell extracts were assayed for phosphotyrosine (see the Materials and methods section). In **(b)** cell extracts were immunoprecipitated for pp125^{fak} before assay for phosphotyrosine (see the Materials and methods section). Each blot is representative of at least three experiments. Lane 1, control; lane 2, control — Ca²⁺; lane 3, ET-1; lane 4, ET-1 — Ca²⁺; lane 5, LPA; lane 6, LPA — Ca²⁺.

of pp125^{fak} from stimulated and non-stimulated samples, as recovery of pp125^{fak} was consistent throughout the precipitation procedure [Figure 5a(ii)]. The time course of ET-1-stimulated tyrosine phosphorylation of pp125^{fak} (Figure 5b) follows closely the time course observed for pp120 tyrosine phosphorylation. A similar sustained tyrosine phosphorylation of pp125^{fak} was also observed in response to LPA (results not shown).

PMA also stimulated tyrosine phosphorylation of several proteins in a time-dependent manner. However, relative to either LPA or ET-1, PMA stimulated only a very small increase in the tyrosine phosphorylation of pp120, which showed a considerable delay in onset (results not shown). The calcium ionophore A23187 (5 μ M) also stimulated a small increase in the tyrosine phosphorylation of pp120 but did not act in synergy with PMA (Table 1). PMA stimulated pp80 and pp70 tyrosine phosphorylation to a much greater extent than pp120, approx. 30–40% of that observed for LPA and ET-1 (see Table 1 for pp80; results for pp70 not shown). In addition, there was synergy between A23187 and PMA for pp80 phosphorylation. However, even together, the degree of stimulation was only 60–80% of that observed for the agonists.

Reducing the extracellular free Ca²⁺ concentration to approx. 200 nM by preincubating the cells with EGTA did not cause a significant reduction in the initial phase of tyrosine phosphorylation in response to either ET-1 or LPA (Figure 6a). This corresponded to the levels of tyrosine phosphorylation of pp125^{fak} which were only marginally reduced by external Ca²⁺ removal (Figure 6b). However, the sustained phase of tyrosine phosphorylation of pp120, corresponding to pp125^{fak}, was reduced during prolonged stimulation with either ET-1 or LPA (mean $\pm$ S.D. percentage of stimulation of pp120 at 60 min in the presence of Ca²⁺: ET-1 = 31 $\pm$ 22, LPA = 57 $\pm$ 23; n = 3). In

Figure 7 Time course of PMA-, ET-1- and LPA-induced GS-peptide phosphorylation in Rat-1 fibroblasts

Cells were incubated with vehicle or agonist for the indicated times and extracts assayed for GS-peptide phosphorylation as outlined in the Materials and methods section. Each point represents the mean $\pm$ S.D. of duplicate determinations from a single experiment performed at least three times. ■, Vehicle; ●, ET-1 (100 nM); □, LPA (10 μ M); ○, PMA (100 nM).

Table 2 Effect of Ro-318220 preincubation or PMA pretreatment on ET-1-, LPA- and PMA-stimulated GS-peptide phosphorylation in Rat-1 fibroblasts

Rat-1 fibroblasts were preincubated with vehicle (dimethyl sulphoxide) or 10 μ M Ro-318220 for 20 min or pretreated with 300 nM PMA or 4 α -phorbol for 40 h. Samples were assayed for GS-peptide phosphorylation as outlined in the Materials and methods section. Each value is expressed in c.p.m. and represents the mean $\pm$ S.D. of duplicate determinations from single experiments performed at least three times.

	GS-peptide phosphorylation			
	Basal	ET-1 (100 nM)	LPA (10 μ M)	PMA (100 nM)
Vehicle	539 $\pm$ 50	1485 $\pm$ 158	1013 $\pm$ 66	2440 $\pm$ 347
Ro-318220	400 $\pm$ 37	497 $\pm$ 88	469 $\pm$ 19	577 $\pm$ 61
4 α -Phorbol	417 $\pm$ 33	1038 $\pm$ 151	699 $\pm$ 74	1864 $\pm$ 234
PMA	377 $\pm$ 94	382 $\pm$ 83	404 $\pm$ 41	404 $\pm$ 193

Figure 8 Effect of Ro-318220 on ET-1 and LPA stimulation of tyrosine phosphorylation of pp125^{lak} in Rat-1 fibroblasts

Cells were pretreated with 10 μ M Ro-318220 for 20 min before the initiation of the reaction. Samples were immunoprecipitated for pp125^{lak} using antibody 2A7 and immunoblotted for phosphotyrosine as outlined in the Materials and methods section. (a) 2 min; (b) 60 min. Lane 1, control; lane 2, control + Ro-318220; lane 3, ET-1 (100 nM); lane 4, ET-1 + Ro-318220; lane 5, LPA (10 μ M); lane 6, LPA + Ro-318220. These experiments were performed at least three times.

Table 3 Effect of Ro-318220 preincubation or PMA pretreatment on ET-1- and LPA-stimulated tyrosine phosphorylation of pp120 and pp80 in Rat-1 fibroblasts

Cells were pretreated with Ro-318220 or vehicle (0.05% dimethyl sulphoxide) for 20 min (A) or preincubated with PMA (300 nM) or 4 α -phorbol (300 nM) for 40 h (B). Samples were immunoblotted for phosphotyrosine as outlined in the Materials and methods section. Values are expressed as a percentage of maximum ET-1 and LPA stimulations at 2 and 60 min in the presence of vehicle (0.05% dimethyl sulphoxide or 4 α -phorbol). Each value represents the mean $\pm$ S.D. of at least six experiments.

	2 min		60 min	
	pp120	pp80	pp120	pp80
A				
ET-1	70.2 $\pm$ 14	30.7 $\pm$ 5	61.0 $\pm$ 12	44 $\pm$ 10
LPA	91.7 $\pm$ 10	38.7 $\pm$ 14	80.7 $\pm$ 11	112 $\pm$ 16
B				
ET-1	71 $\pm$ 18	41.6 $\pm$ 21	63.1 $\pm$ 18	27.0 $\pm$ 11
LPA	89.3 $\pm$ 8	33.6 $\pm$ 13	77 $\pm$ 14	96.7 $\pm$ 18

Figure 9 Effect of Ro-318220 on ET-1- and LPA-stimulated tyrosine phosphorylation of pp80 in Rat-1 fibroblasts

Cells were preincubated with vehicle (0.05% dimethyl sulphoxide) or 10 μ M Ro-318220 for 20 min before initiation of the reaction. Agonist incubations were for 2 or 60 min. Cell extracts were subsequently immunoblotted for phosphotyrosine as outlined in the Materials and methods section. (a) ET-1 (100 nM); (b) LPA (10 μ M). C, control; C + R, control plus Ro-318220; E, ET-1; E + R, ET-1 plus Ro-318220; L, LPA; L + R, LPA plus Ro-318220. Each blot is a representative of at least four experiments.

cells pretreated with 10 μ M thapsigargin for 10 min in order to deplete intracellular Ca²⁺ stores, the initial activation of tyrosine phosphorylation of pp120 and other proteins by both ET-1 and LPA was not affected (mean $\pm$ S.D. percentage of maximum

Figure 10 Effect of pertussis toxin pretreatment on ET-1- and LPA-stimulated tyrosine phosphorylation of MAP kinase and pp125^{fak} in Rat-1 fibroblasts

Cells were pretreated with 50 (a and b) or 100 (c) ng/ml pertussis toxin for 18 h before stimulation of the cells with 100 nM ET-1 or 10 μ M LPA for 2 min. In (a) and (b) whole-cell extracts were immunoblotted for phosphotyrosine (a) or MAP kinase (b). In (c) cell extracts were immunoprecipitated for pp125^{fak} with antibody 2A7 then immunoblotted for phosphotyrosine content (see the Materials and Methods section). Lane 1, control; lane 2, control + pertussis toxin; lane 3, ET-1; lane 4, ET-1 + pertussis toxin; lane 5, LPA; lane 6, LPA + pertussis toxin. Each blot is representative of at least three others. The arrows in (a) indicate the position of MAP kinase isoforms.

stimulation of pp120 in the absence of thapsigargin: ET-1 = 97 ± 7.0 , LPA 91.3 ± 11.8 ; $n = 3$).

ET-1 and LPA also stimulated PKC activity in Rat-1 fibroblasts (Figure 7). A rapid initial peak in GS-peptide phosphorylation was obtained between 2 and 5 min of stimulation. PMA also stimulated GS-peptide phosphorylation, to a much greater extent than that observed with either agonist and showed a delay of 60 s before the onset of the response was observed. In cells preincubated with the PKC inhibitor Ro-318220 (10 μ M) [24] or pretreated with 300 nM PMA for 48 h in order to down-regulate PKC, LPA- and ET-1-stimulated GS-peptide phosphorylation was abolished (Table 2).

In the presence of either Ro-318220 or after PMA pretreatment, LPA- and ET-1-stimulated tyrosine phosphorylation of pp125^{fak} was reduced by approx. 20–40% at both 2 and 60 min

(Figures 8a and 8b). Over a number of experiments this reduction was found to be greater for ET-1 stimulation than for LPA ($43.2 \pm 12.0\%$ compared with $21.3 \pm 13.0\%$ at 2 min; $n = 4$). This difference in the level of inhibition was also reflected in tyrosine phosphorylation of the 120 kDa band corresponding to pp125^{fak} (Table 3). Phosphorylation of pp116, pp80 and pp70 was reduced by 50–70% under the same conditions (see Table 3 for pp80; results not shown for pp116 and pp70). PMA-stimulated tyrosine phosphorylation of both pp120 and pp80 was abolished by Ro-318220, confirming that the interventions were effective against PKC-mediated phosphorylation (results not shown). In addition, a time-dependent difference in the effect of PKC inhibition on agonist-induced phosphorylation was observed. At 2 min both ET-1- and LPA-induced phosphorylation of pp80 was significantly reduced (Figure 9 and Table 3). However, at 60 min, although the tyrosine phosphorylation of pp80 in response to ET-1 was inhibited, LPA-induced phosphorylation was unaffected (Figure 9b).

Figure 10 shows the effect of pertussis toxin pretreatment on LPA- and ET-1-stimulated tyrosine phosphorylation of pp42 and pp44 MAP kinase and pp125^{fak} in Rat-1 fibroblasts. Concentrations of 50 ng/ml of pertussis toxin were partially effective against both ET-1- and LPA-induced tyrosine phosphorylation of proteins corresponding to the pp42 and pp44 isoforms of MAP kinase (Figure 10a). The identity of these bands were confirmed by reprobing with an anti-(MAP kinase) antibody (not shown) and evaluated in separate experiments by examining the shift in migration of each isoform (Figure 10b). At 50 ng/ml, pertussis toxin was ineffective against either ET-1- or LPA-stimulated tyrosine phosphorylation of the pp120 protein which corresponds to pp125^{fak} (Figure 10a). This finding was also observed in cell lysates immunoprecipitated with antibody 2A7. However, increasing the concentration of pertussis toxin to 100 ng/ml we found that LPA- but not ET-1-induced tyrosine phosphorylation of pp125^{fak} was reduced (Figure 10c). These findings were also observed for the 60 min time point (results not shown). Finally, pretreating cells with 8-bromocyclic AMP (0.5 mM) for up to 60 min also failed to affect significantly either ET-1 or LPA-stimulated tyrosine phosphorylation of pp120 and pp80 (results not shown).

DISCUSSION

In this study we have shown that both ET-1 and LPA stimulate the tyrosine phosphorylation of a number of target proteins in Rat-1 fibroblasts. One of these proteins corresponds immunologically to focal adhesion kinase, pp125^{fak}, previously identified by Parsons and co-workers [20,25]. This kinase has been shown to be co-localized with a number of cytoskeletal proteins and is implicated in the regulation of cell structure during mitogenic activation. In addition, it is one of the major substrates for tyrosine phosphorylation in response to integrin activation and pp60^{v-src} overexpression and is believed to represent a significant site of convergence for oncogenes, integrins and neuropeptides [21,26–29].

We hypothesized that the initial phase of protein tyrosine phosphorylation of pp120 (corresponding to pp125^{fak}) would be dependent on prior PKC activation, as it has recently been shown that the initial phase of tyrosine phosphorylation of other tyrosine kinases such as MAP kinase in response to G-protein-coupled receptor agonists are dependent on PKC activation [30]. This is due to the presence of upstream intermediate kinases of the growth-factor-initiated tyrosine kinase cascade, such as Raf-1 or MAP kinase kinase, which may be substrates for PKC-mediated serine phosphorylation [31,32]. Also the initial peak of

tyrosine phosphorylation coincided with the initial peak of PKC-mediated GS-peptide phosphorylation in response to both agonists, being maximal between 2 and 5 min. However, the tyrosine phosphorylation state of pp120 corresponding to pp125^{lak} was only partially reduced after PKC down-regulation or inhibition. These results strongly suggest that a component of the LPA and ET-1 response does not involve the PKC-mediated activation of an intermediate tyrosine kinase for which pp125^{lak} is a substrate. Consistent with this finding is the observation that PMA alone or in combination with the calcium ionophore A23187 stimulated the phosphorylation of pp120 to a very small extent relative to both LPA and ET-1. These findings indicate that an additional PKC-independent regulatory pathway is involved in coupling classical G-protein-linked receptors to pp125^{lak}.

It must be noted that for the tyrosine phosphorylation of other proteins such as pp80 and pp70 stimulated by ET-1 and LPA, there is a much higher degree of dependence on prior activation of PKC. However, agonist-induced tyrosine phosphorylation of pp80 is stimulated to a much greater extent in response to LPA or ET-1 than when stimulated by PMA alone or in combination with A23187. These results again suggest the possibility of two pathways, one PKC-dependent and one PKC-independent, which may interact upstream of the target substrates, or the presence of sites on target substrates which require serine phosphorylation for full tyrosine phosphorylation. Whatever the mechanism involved, a convergence between the PKC and tyrosine kinase pathways is likely to occur at these protein targets.

The reduction of the second phase of agonist-induced tyrosine phosphorylation after extracellular Ca²⁺ removal suggests that Ca²⁺ influx may regulate the late phase of phosphorylation. Both ET-1 and LPA stimulate sustained Ca²⁺ influx in a number of cell systems [33,34] and it is possible that removal of extracellular Ca²⁺ may change the balance between sustained tyrosine kinase and phosphatase activity. This phenomenon has recently been observed for thrombin-stimulated tyrosine phosphorylation of pp72^{src} in platelets [35]. However, the inability of A23187 to elicit a substantial increase in tyrosine phosphorylation of pp120 phosphorylation and the negligible effects of Ca²⁺ removal on pp125^{lak} phosphorylation suggest that agonist-stimulated Ca²⁺ influx *per se* does not account for the activation of this tyrosine kinase pathway. In addition, pretreatment of cells with thapsigargin in order to deplete intracellular Ca²⁺ stores failed to modify the magnitude of pp120 phosphorylation in response to both ET-1 and LPA, excluding the possibility that mobilization of Ca²⁺ from intracellular stores is obligatory for the initial agonist-induced tyrosine phosphorylation event.

Recently it has been shown that LPA may activate p21^{ras} by interaction with a pertussis-toxin-sensitive G-protein, resulting in downstream activation of tyrosine kinase substrates including MAP kinase [19]. Consistent with these findings we have observed a reduction in the tyrosine phosphorylation of MAP kinase in response to both LPA and ET-1 after pertussis toxin pretreatment. However, at an equivalent concentration of pertussis toxin (50 ng/ml), we did not observe a significant reduction in the tyrosine phosphorylation of pp125^{lak}. Only by increasing the concentration of pertussis toxin did we observe a reduction in tyrosine phosphorylation and this was only in response to LPA. ET-1-induced tyrosine phosphorylation of pp125^{lak} was not affected. Although these results suggest the activation of a pertussis-toxin-sensitive G-protein as a requirement for LPA-induced activation of both MAP kinase and pp125^{lak}, it may also indicate possible stoichiometric differences in the activation of each tyrosine kinase pathway by G-protein subunits. It is also

possible that different ET receptor subtypes may be involved in regulating pertussis-toxin-dependent and -independent signalling events in the same cell [36]. Thus a considerable degree of 'plasticity' may be observed in the apparent regulation of tyrosine phosphorylation by PKC and pertussis-toxin-dependent mechanisms in ET receptor systems.

Additional differences between ET-1 and LPA-induced tyrosine phosphorylation were also observed. The EC₅₀ value for LPA-induced tyrosine phosphorylation of pp120 and other proteins was at least two orders of magnitude to the left of that obtained for [³H]inositol phosphate formation in this cell system [10,11]. In preliminary studies we have observed a similar concentration-response relationship for LPA-stimulated tyrosine phosphorylation of pp42 MAP kinase in endothelial cells (A. McLees, A. Graham, K. Malarkey and R. Plevin, unpublished work). This suggests possible differences in the coupling of the putative LPA receptor(s) to the tyrosine kinase and PKC pathways. The concentration-response relationships for a number of ET-1-receptor-coupled events are the same and could explain the relative sensitivity of ET-1- and LPA-induced tyrosine phosphorylation of pp125^{lak} and other proteins, for example pp80, to PKC inhibition (Table 3). Although the characteristics of LPA-stimulated [³H]inositol phosphate production suggests that the putative LPA receptor is linked to the G-protein α -subunit, designated Gq/G11, our results are consistent with the hypothesis that part of the receptor signal activated by LPA may also be transduced through an additional tyrosine kinase pathway, at lower levels of receptor occupancy.

Focal adhesion kinase pp125^{lak} does not contain either SH-2 or SH-3 domains in its structure, suggesting that direct activation by an activated growth-factor receptor is unlikely [29]. However, PDGF also stimulated tyrosine phosphorylation of pp125^{lak} in Rat-1 fibroblasts. Thus tyrosine phosphorylation of pp125^{lak} by the activation of intrinsic tyrosine kinase and non-tyrosine kinase receptors must require activation of other intermediates. This may include receptor-associated tyrosine kinases such as members of the pp60^{src} tyrosine kinase family [37], low-molecular-mass G-proteins [38] or an SH-2 docking protein analogous to the 185 kDa insulin receptor substrate [39,40].

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