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Regulation of lysophosphatidic acid-stimulated tyrosine phosphorylation of mitogen-activated protein kinase by protein kinase C- and pertussis toxin-dependent pathways in the endothelial cell line EAhy 926

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In the endothelial cell line EAhy 926, 1-oleoyl-lysophosphatidic acid (LPA) stimulated the tyrosine phosphorylation of the pp42 isoform of mitogen-activated protein (MAP) kinase. Maximum phosphorylation was observed within 5 min of LPA addition, but the response was sustained for up to 120 min. Re-addition of LPA after 60 min stimulated a further sustained increase in the tyrosine phosphorylation of MAP kinase. In cells pretreated with phorbol 12-myristate 13-acetate (PMA; 24 h) or preincubated with the protein kinase C inhibitor Ro-318220, LPA-induced tyrosine phosphorylation of pp42 MAP kinase was substantially reduced at 2 min but potentiated at 60 min. Ro-318220 in combination with either PMA or pertussis toxin pretreatment abolished the LPA response at all time points, suggesting an involvement of protein kinase C in the pertussis toxin-sensitive part of the pathway. Agents which raised intracellular cyclic AMP levels did not affect the initial phase of LPA-stimulated MAP kinase activation, but abolished the late phase. However, this effect was prevented by Ro-318220, implicating a greater role for protein kinase C than protein kinase A in the regulation of sustained MAP kinase responses. LPA stimulated an increase in the tyrosine phosphorylation of focal adhesion kinase pp125 ($pp125^{FAK}$) in EAhy 926 cells which was both protein kinase C- and pertussis toxin-independent. These results are discussed in terms of the pathways regulating both MAP kinase and $pp125^{FAK}$ in response to LPA in the EAhy 926 endothelial cell line.

INTRODUCTION

The phospholipid 1-oleoyl-lysophosphatidic acid (LPA) is a potent mitogen for a number of fibroblast cell lines in culture [1,2]. This effect is not believed to be due to perturbation of the plasma membrane or the formation of an active metabolite, but via an interaction with an extracellular receptor protein [3–5]. In a number of cells LPA stimulates the hydrolysis of both phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine, the mobilization of intracellular Ca^{2+} and activation of protein kinase C (PKC) [1,6–9]. However, the mitogenic efficacy of this phospholipid is believed to be at least partially dependent upon a pertussis toxin-dependent pathway involving activation of the inhibitory G-protein G_i2 and inhibition of adenylate cyclase [1,10].

A major pathway by which a number of agonists whose receptors are coupled to G-proteins stimulate cell growth and division involves the activation of intermediate kinases by phosphorylation on tyrosine residues. This includes a family of serine/threonine-specific kinases, known collectively as mitogen-activated protein (MAP) kinase, which play an intimal role in transducing the initial receptor signal to the nucleus [11,12]. For the majority of G-protein-linked receptors the activation of MAP kinase is believed to be PKC-dependent [13]. However, recently it has been shown that LPA may stimulate the tyrosine phosphorylation of MAP kinase by activating nucleotide exchange on $p21^{ras}$ [14–16]. This pathway is inhibited by pertussis toxin pretreatment, indicating the involvement of a G_i protein in the activation of $p21^{ras}$ by LPA. Thus the regulation of MAP kinase activity by both PKC- and pertussis toxin-sensitive pathways may be important in determining the mitogenic response to LPA.

Activation of other kinases by phosphorylation on tyrosine residues may also be important in the regulation of cell growth and division initiated by LPA. This includes the activation of the novel focal adhesion kinase $pp125^{FAK}$ [9,16–19]. This kinase has been proposed to be involved in changes in cell structure and adhesion following stimulation with mitogens [20].

Recently it has been shown that LPA may be synthesized by thrombin-activated platelets, suggesting a potential action for LPA at the site of vessel injury [21]. We have therefore examined the effects of LPA on the tyrosine phosphorylation of MAP kinase and $pp125^{FAK}$ in the endothelial cell line EAhy 926. In this study we show that LPA stimulates the sustained tyrosine phosphorylation and activation of the pp42 isoform of MAP kinase. Increased tyrosine phosphorylation is also observed following re-addition of LPA. Contrary to results in Rat-1 fibroblasts [16], we show that LPA stimulation of MAP kinase is wholly dependent upon the activation of PKC. However, a pertussis toxin-sensitive mechanism is also involved in the activation of MAP kinase. Our studies also implicate PKC in the termination of the MAP kinase response, possibly through the activation of a MAP kinase phosphatase. We also observed an increase in the tyrosine phosphorylation of $pp125^{FAK}$ in response to LPA which was not affected by PKC inhibition or pertussis toxin pretreatment. The activation of both MAP kinase, which is

Abbreviations used: LPA, 1-oleoyl-lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PKA, protein kinase A; $pp125^{FAK}$, focal adhesion kinase pp125; cAMP, cyclic AMP.

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involved in cellular growth, and pp125^{FAK}, which is integral to changes in cell shape and adhesion, suggests a possible role for LPA as a novel angiogenic factor for endothelial cells.

EXPERIMENTAL

Materials

Cell culture equipment was from Gibco, Paisley, Scotland, U.K. All antibodies used were from Affiniti Research Products Ltd, Nottingham, U.K. Second antibodies, ECL detection reagents and [γ-^{32}P]ATP were purchased from Amersham. The BIOTRAK MAP kinase assay kit was a gift from Dr. D. A. Cohen (Amersham). All other compounds were purchased from Sigma.

Cell culture

The human endothelial hybrid cell line EAhy 926 [22] was maintained in Dulbecco's modified Eagles medium containing 10% foetal calf serum, 27 mg/ml glutamine, hypoxanthine/aminopterin/thymidine supplement and penicillin/streptomycin (250 units/ml and 250 μg/ml) at 37 °C in a humidified atmosphere of air/CO_2 (19:1). All experiments were performed in cells grown to confluency on 6 cm^2 plates and made quiescent by serum deprivation for 40 h.

Cell stimulation and immunoblotting

Cells were incubated in Hanks' buffered saline containing 10 mM glucose and 2% (w/v) BSA for 30 min, 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 $Na_4P_2O_7$, 4 mM EDTA, 2 mM EGTA and 2 mM Na_3VO_4 (HPFEV). Following the final aspiration 0.5 ml of SDS sample buffer (70 °C) was added and the sample was passed repeatedly through a 21 G needle. The samples were boiled for 5 min and then stored at −80 °C until analysis. Aliquots of each sample (50–75 μg of protein) were run on 7.5% or 10% PAGE gels in the presence of SDS and then transblotted on to nitrocellulose. The nitrocellulose blots were blocked for non-specific binding for 3 h in 50 mM Tris buffer containing 150 mM NaCl, 0.02% (v/v) Tween, pH 7.4 (NaTT), and 3% BSA, then incubated in the same buffer containing 0.2% BSA and 200 ng/ml of a rabbit anti-phosphotyrosine antibody (Affiniti) overnight. The blots were rinsed in NaTT buffer, washed for 90 min (six changes of NaTT) and then incubated in buffer containing 0.2% BSA and a 1:6000 dilution of donkey anti-(rabbit Ig) linked to horse-radish peroxidase for 60 min. The blots were then washed for 120 min (eight changes of NaTT) 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 MAP kinase immunoblotting, conditions were employed as outlined above except that 10 ng/ml of a mouse monoclonal anti-(MAP kinase) antibody, recognizing both the 42 and 44 kDa forms of MAP kinase was used as the primary antibody and the second antibody was an anti-(mouse Ig) antibody linked to horseradish peroxidase. Re-probing of the phosphotyrosine blots with anti-(MAP kinase) antibody confirmed the identity of the pp42 isoform.

For PKC immunoblotting conditions were employed as outlined above using 1 μg/ml of mouse monoclonal antibodies raised against the α, β, δ and ϵ isoforms of PKC.

Assay of MAP kinase activity

MAP kinase activity was assayed *in vitro* following partial purification of activated MAP kinase on phenyl-Sepharose [23]. Aliquots of protein (20–30 μg) were incubated in 20 mM Tris, pH 8.0, containing 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP/[γ-^{32}P]ATP (0.5 μCi/tube) and 15 μg of myelin basic protein for 30 min at 30 °C in a final volume of 100 μl. The reaction was terminated by the addition of 5 × SDS sample buffer and the samples were run on SDS/15%-PAGE. The gels were fixed in 10% acetic acid/20% methanol, dried and then subjected to autoradiography overnight. In other experiments protein was incubated with 2 mM of the epidermal growth factor receptor peptide pseudosubstrate KRELVEPLT^{669}PAGEAPNALLR (BIOTRAK MAP kinase assay). The reaction was terminated by the addition of 2 M acetic acid. Phosphorylated peptide was separated by ion-exchange chromatography on Whatman p81 paper using 75 mM H_2PO_4. Activity was measured by liquid scintillation counting.

Immunoprecipitation of pp125^{FAK}

Cells, incubated as outlined above, were washed (× 3) in HPFEV and then solubilized by the addition of HPFEV containing 1% Triton X-100, 0.25% deoxycholate, 50 μg/ml leupeptin, 10 μg/ml soya bean trypsin inhibitor, 10 μg/ml aprotinin, 100 μM PMSF, 1 mM benzamidine and 5 μM pepstatin A (immunoprecipitation buffer). Following 30 min on ice the samples were spun at 14000 ***g*** for 5 min at 4 °C to remove any insoluble material. The samples (250 μg of protein) were then incubated with 2 μg of the anti-pp125^{FAK} antibody for 2 h. This was followed by incubation with Protein A–agarose which had been precoupled to rabbit anti-(mouse IgG) for 2 h at 4 °C. The immunoprecipitates were recovered by centrifugation in a Microfuge (20 s, 14000 ***g***), and washed twice with immunoprecipitation buffer and three times with immunoprecipitation buffer which did not contain Triton or deoxycholate. Immunoprecipitated pp125^{FAK} was eluted from the Protein A–agarose by boiling in SDS sample buffer. Trichloroacetic acid (8% final concn.) was added to the supernatants which were then spun at 14000 ***g*** for 15 min at 4 °C. Trichloroacetic acid precipitates were washed three times with 50 mM Tris/HCl, pH 7.4, then 10 μl of Tris base (1 M) was added. Samples were then boiled in SDS-sample buffer until the pellet dissolved and stored at −80 °C until immunoblotted. Immunoprecipitated extracts were immunoblotted for phosphotyrosine using mouse monoclonal antibody PY54 (0.10 μg/ml), or for pp125^{FAK} using antibody 2A7 (0.125 μg/ml). Anti-phosphotyrosine blots were re-probed for pp125^{FAK} to confirm the identity of the 120 kDa band. For the analysis of pp125^{FAK} by Western blotting, detection utilized an anti-mouse horseradish peroxidase-linked second antibody.

RESULTS

Preliminary experiments showed that LPA stimulated the tyrosine phosphorylation of a number of proteins in the EAhy 926 cell line, including a major band at approx. 40 kDa (Figure 1a). Re-probing of phosphotyrosine blots with anti-(MAP kinase) showed that this band corresponded to the 42 kDa isoform of MAP kinase (results not shown). Evaluation of MAP kinase levels in separate experiments also indicated that, following stimulation with LPA, a proportion of the pp42 isoform migrated to a different position on the gel (Figure 1b). The time course of LPA-stimulated tyrosine phosphorylation of pp42 MAP kinase is shown in Figure 1. An initial peak in phosphorylation was

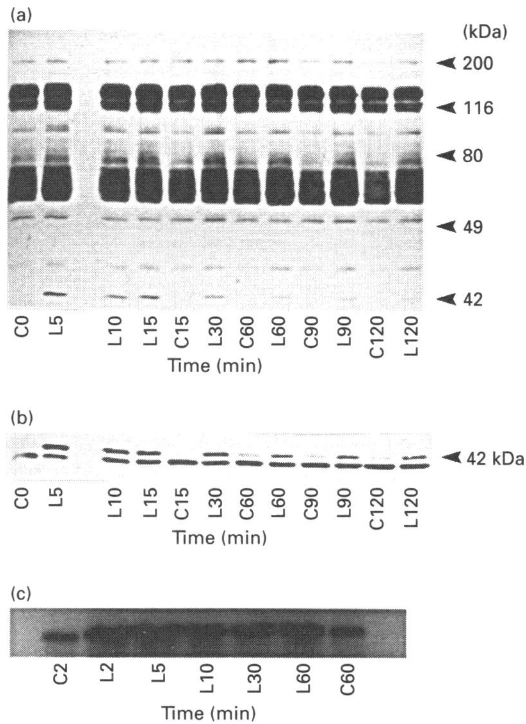

Figure 1 Time course of LPA-stimulated tyrosine phosphorylation and activation of pp42 MAP kinase in EAhy 926 cells

Cells were stimulated with 10 μM LPA (**a** and **b**) for the times indicated and then immunoblotted for phosphotyrosine (**a**) or MAP kinase (**b**) content as outlined in the Materials and methods section. In (**c**), cells were assayed for *in vitro* kinase activity. Each blot is a representative experiment of at least five others. Annotations: C, control; L, LPA.

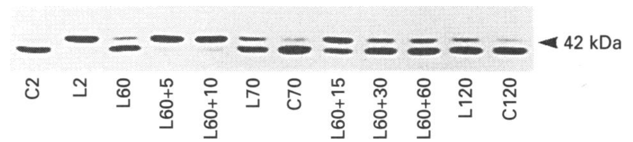

Figure 2 Re-stimulation of LPA-induced activation of pp42 MAP kinase in EAhy 926 endothelial cells

Cells were stimulated with 10 μM LPA for 60 min, re-stimulated with 10 μM LPA for the times indicated and then assayed for MAP kinase content as outlined in the Materials and methods section. Numbers represent stimulation time alone or with 60 min pre-stimulation (60+). Annotations: C, control; L, LPA. Each blot is a representative experiment of at least two others.

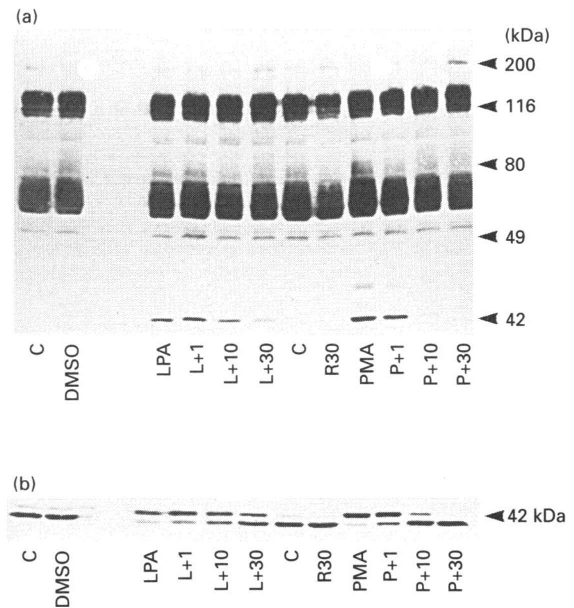

Figure 3 Effect of Ro-318220 on PMA- and LPA-stimulated tyrosine phosphorylation of pp42 MAP kinase in EAhy 926 cells

Cells were preincubated with increasing concentrations of Ro-318220 for 30 min before stimulation with 10 μM LPA for 2 min or 100 nM PMA for 10 min. Cell extracts were assayed for phosphotyrosine (**a**) and MAP kinase content (**b**) as outlined in the Materials and methods section. Each blot is a representative experiment performed at least four times with similar results. Annotations: C, control; P, PMA; L, LPA; R, Ro-318220. Numbers (1, 10 and 30) indicate the concentration of Ro (μM).

observed approx. 5 min after stimulation with maximal concentrations of LPA (10 μM). Phosphorylation levels then decreased over time, but a significant proportion of the signal remained after 2 h. The kinetics of the tyrosine phosphorylation of pp42 corresponded closely to the shift in migration of the 42 kDa form of MAP kinase (Figure 1b).

LPA also stimulated MAP kinase activity, as measured by an increase in the phosphorylation of myelin basic protein following partial purification of MAP kinase (Figure 1c). A peak in phosphorylation was observed between 2 and 5 min; however, phosphorylation was still observed after 60 min. In addition, an increase in the phosphorylation of another MAP kinase substrate, the epidermal growth factor receptor peptide, was also observed (pmol of phosphate$\cdot$min$^{-1}\cdot$mg^{-1}: control 2 min, 25.4 $\pm$ 7.0; LPA 2 min, 100.0 $\pm$ 8.2; control 60 min, 30.1 $\pm$ 1.0; LPA 60 min, 58.6 $\pm$ 1.8; $n = 2$).

Re-application of LPA to the cells 60 min after the initial stimulation resulted in a further increase in the activation of MAP kinase (Figure 2). Activation again peaked at approx. 5 min after re-addition, and was sustained for a further 60 min.

LPA-stimulated tyrosine phosphorylation of pp42 MAP kinase was observed over a concentration range of 0.01–1 μM. The EC_{50} obtained for this response (187 $\pm$ 14 nM; $n = 4$) is at least one order of magnitude to the left of that observed for inositol phosphate accumulation. There was no apparent difference in the concentration–response curves obtained for the activation of MAP kinase at 5 or 60 min.

The PKC activator phorbol 12-myristate 13-acetate (PMA) also stimulated a sustained increase in MAP kinase tyrosine phosphorylation in EAhy 926 cells (results not shown). Figure 3 shows the effect of the PKC inhibitor Ro-318220 [24] on PMA- and LPA-stimulated tyrosine phosphorylation of pp42 MAP kinase. While DMSO vehicle was without effect on agonist activation of MAP kinase, at concentrations of 10 μM or above Ro-318220 completely abolished the PMA-stimulated response. However, the response to LPA at 2 min was only partly reduced, a substantial proportion of the signal still remaining in the presence of 30 μM Ro-318220 (inhibition of 63.4 $\pm$ 12.0 %; $n = 4$).

In cells pretreated with PMA for 24 h the levels of the α and ϵ isoforms of PKC were substantially reduced (Figure 4a). Under these conditions LPA-induced MAP kinase tyrosine phosphorylation was reduced by approx. 70 % at 2 min (Figure 4b). In

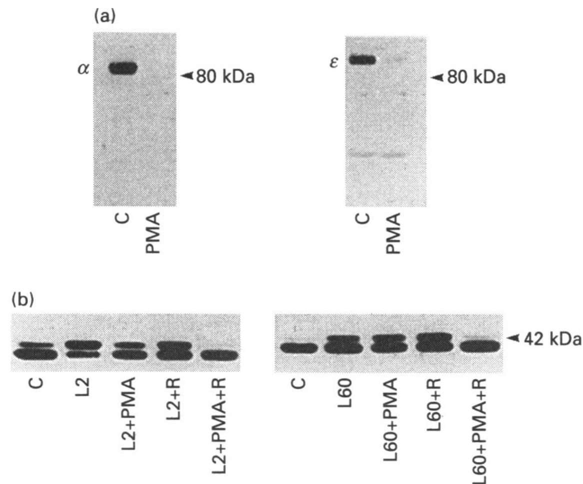

Figure 4 Effect of PMA and Ro-318220 pretreatment on LPA-stimulated activation of MAP kinase in EAhy 926 cells

(**a**) Cell extracts from cells pretreated with 100 nM PMA were assayed for α and ϵ PKC isoforms. Cells were incubated for 24 h in the presence of vehicle or PMA and for 30 min in the presence of 10 μM Ro-318220 before stimulation with LPA for 2 or 60 min, as indicated. The extracts were assayed for MAP kinase levels as outlined in the Materials and methods section. Each blot is a representative experiment of at least four others. Annotations: C, control; L, LPA; R, Ro-318220.

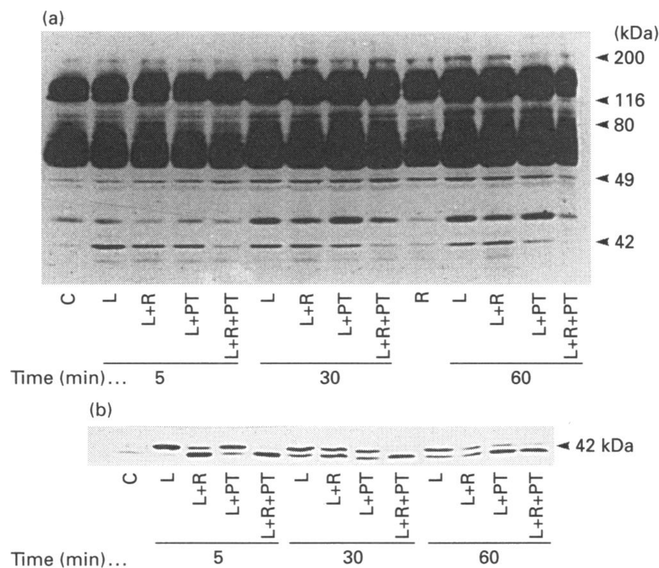

Figure 5 Effect of pertussis toxin and Ro-318220 on LPA-stimulated tyrosine phosphorylation of pp42 MAP kinase in EAhy 926 cells

Cells were pretreated for 18 h with 50 ng/ml pertussis toxin before being preincubated with 10 μM Ro-318220 for 30 min. Cells were stimulated with 10 μM LPA for the given times and extracts were assayed for phosphotyrosine (**a**) or MAP kinase content (**b**). Each blot is a representative example from at least five separate experiments. Annotations: PT, pertussis toxin; R, Ro-318220; L, LPA; C, control.

contrast, at 60 min, LPA-stimulated activation of MAP kinase was enhanced to a small extent. A larger enhancement of the late phase of the response was observed when the cells were pre-incubated with Ro-318220 (Figures 4b and 5). When both Ro-318220 and PMA were used in combination, LPA-stimulated

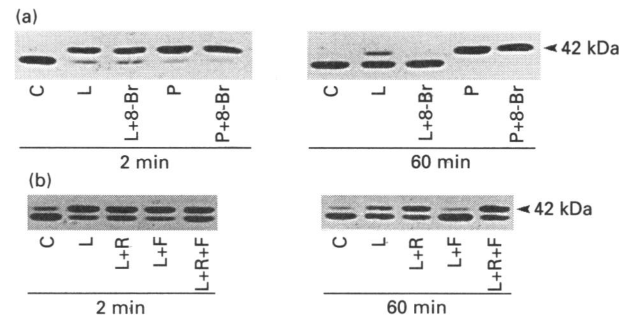

Figure 6 Effect of raising intracellular cAMP levels on LPA-induced activation of pp42 MAP kinase in EAhy 926 cells

(**a**) Cells were incubated with 0.5 mM 8-bromo-cAMP for 30 min prior to stimulation with 10 μM LPA or PMA for 2 or 60 min. (**b**) Cells were preincubated with 10 μM Ro-318220 for 30 min before addition of 10 μM forskolin + 100 μM 3-isobutyl-1-methylxanthine for 15 min. Cells were stimulated with LPA for 2 or 60 min. Samples were assayed for MAP kinase content as outlined in the Materials and methods section. Each blot is a representative of at least three others. Annotations: C, control; L, LPA, 8-Br, 8-bromo-cAMP; R, Ro-318220; F, forskolin + isobutylmethylxanthine.

MAP kinase activation was completely abolished at both 2 and 60 min.

LPA-stimulated activation of MAP kinase was also affected by pertussis toxin pretreatment in a time-dependent manner (Figure 5). However, under these conditions, the early phase of the response was reduced by approx. 25% (inhibition of 28 ± 12%; $n = 3$) following pertussis toxin pretreatment. As the stimulation period increased, the effect of pertussis toxin was greater, reducing the signal by 73 ± 21% at 60 min. Ro-318220 and pertussis toxin in combination abolished LPA-stimulated tyrosine phosphorylation of MAP kinase at all time points studied.

The effect of raising intracellular cyclic AMP (cAMP) levels on LPA-stimulated MAP kinase activation is shown in Figure 6. In cells treated for 30 min with 0.5 mM 8-bromo-cAMP, no reduction in LPA-stimulated MAP kinase activation was observed at 2 min (Figure 6a). However, at 60 min the activation of MAP kinase was abolished. No effect was observed on PMA-stimulated MAP kinase at either 2 or 60 min. This phenomenon was also observed in cells pretreated with 10 μM forskolin in the presence of 0.1 mM 3-isobutyl-1-methylxanthine (results not shown). Figure 6(b) shows the effect of preincubation of the cells with Ro-318220 in combination with forskolin on LPA-stimulated MAP kinase activation. There was little additional effect of forskolin on the inhibition of MAP kinase activation by Ro-318220 at 2 min. Ro-318220 also prevented forskolin-mediated abolition of LPA-induced MAP kinase activation at 60 min. A similar result was observed in cells where 8-bromo-cAMP was used instead of forskolin (results not shown).

LPA also stimulated the tyrosine phosphorylation of a number of other proteins in a time- and concentration-dependent manner, including two highly abundant proteins of approx. 120 and 130 kDa. Immunoprecipitation experiments indicated that the pp120 protein corresponded to $pp125^{FAK}$ (Figure 7a). LPA stimulated the tyrosine phosphorylation of $pp125^{FAK}$ over a time course of 2–60 min (Figure 7b). LPA-stimulated tyrosine phosphorylation of $pp125^{FAK}$ and pp130 was not reduced by Ro-318220 or pertussis toxin pretreatment (Figure 7c). A small decrease in the signal was observed in the LPA response when these two agents were used in combination.

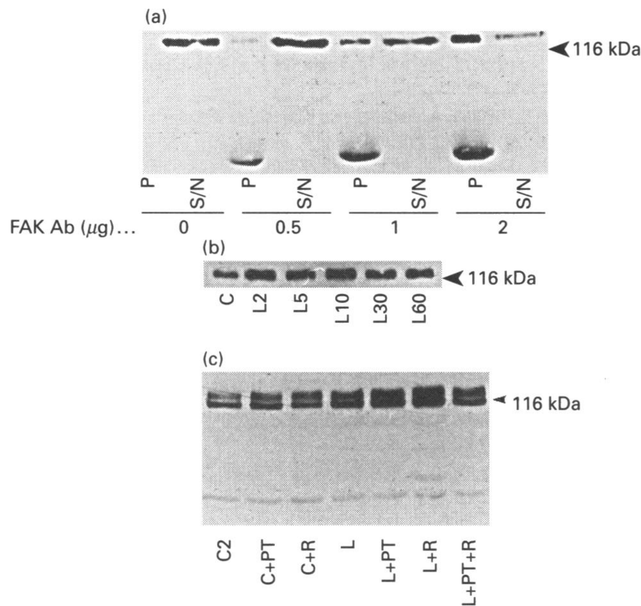

Figure 7 LPA-stimulated tyrosine phosphorylation of pp130 and pp125^{FAK} in EAhy 926 cells

(**a**) Cells were stimulated for 2 min with LPA and immunoprecipitated with increasing concentrations of anti-pp125^{FAK} antibody. Extracts were immunoblotted for pp125^{FAK} as outlined in the Materials and methods section. Numbers represent concentrations of anti-FAK antibody in μg/250 μg of protein used in the immunoprecipitation. P, pellet; S/N, supernatant. (**b**) Cells were incubated with LPA for the times indicated and then assayed for phosphotyrosine content following immunoprecipitation with 2 μg of anti-pp125^{FAK} antibody/250 μg of protein. Annotations: L, LPA; C, control. (**c**) Cells were pretreated with 50 ng/ml pertussis toxin for 18 h and preincubated for a further 30 min in the absence or presence of 10 μM Ro-318220. Cells were incubated with 10 μM LPA for 2 min. Cell extracts were assayed for phosphotyrosine content as outlined in the Materials and methods section. Annotations: R, Ro-318220; PT, pertussis toxin; C, control; L, LPA. Each blot is representative of at least three others.

DISCUSSION

In the endothelial cell line EAhy 926, LPA stimulated the tyrosine phosphorylation of the pp42 form of MAP kinase as determined by immunoblotting and immunoprecipitation experiments. This was consistent with an increase in the activity of MAP kinase as judged by increased phosphorylation of myelin basic protein and the epidermal growth factor receptor peptide. LPA also stimulated the tyrosine phosphorylation of MAP kinase in EC304 cells and in primary cultures of bovine aortic endothelial cells (A. Graham, A. McLees and R. Plevin, unpublished work), suggesting the presence of LPA receptors on endothelial cell types. In these cell types and in Rat-1 fibroblasts, LPA stimulated a sustained increase in the tyrosine phosphorylation of MAP kinase. The sustained kinetics of phosphorylation differ from those of a number of other agonists whose receptors are G-protein-linked, such as vasopressin and angiotensin II, which have been shown to activate MAP kinase transiently ([25,26]; K. Malarkey and R. Plevin, unpublished work). The prolonged activation of MAP kinase is believed to be of considerable importance in determining the dynamics of translocation of MAP kinase to the nucleus and the mitogenic efficacy of a given agonist [27]. In addition, we have also shown that MAP kinase may be re-activated by re-addition of LPA 60 min after the initial stimulation. Activation was once again sustained for at least an additional 60 min. This is a significant finding, since LPA has been proposed to be released from serum to activate early events in mitogenesis [28]. Thus, *in vivo*, the activation of MAP kinase may be sustained over a number of hours by continual re-stimulation from serum-derived LPA.

Our results implicate a role for PKC in the regulation of LPA-induced MAP kinase activation. In the initial phase the LPA response was markedly reduced by either PMA pretreatment or Ro-318220 preincubation. This observation is consistent with an inhibition of a PKC-mediated phosphorylation of Raf-1 [29]. However, we also found that by combining pretreatment protocols designed to inhibit the activation of PKC, LPA activation of MAP kinase was completely abolished. This might suggest that our individual treatments fail to inhibit sufficiently the main isoforms of PKC involved in the regulation of Raf-1. However PMA-induced activation of MAP kinase was clearly abolished following Ro-318220 preincubation or PMA pretreatment (Figure 3 and results not shown), and PKC α and ϵ expression was essentially abolished. Furthermore, during more prolonged pretreatment with PMA (40 h) in which the expression of both the β and δ isoforms was also markedly reduced, the LPA signal was still only partially inhibited (results not shown). Unfortunately, this condition was also associated with a reduction in MAP kinase expression and could not be used routinely. Despite such caveats, these results suggest the possible involvement of a number of PKC isoforms, including atypical isoforms [30], in the activation of MAP kinase in response to LPA. However, this hypothesis can only be confirmed by measurement of LPA-induced PKC activity in this cell line.

It must be noted that individually Ro-318220 and PMA, while inhibiting the initial MAP kinase signal, enhanced the late phase of the response. This was particularly a feature when using the PKC inhibitor, and it is possible that Ro-318220 was acting in a non-specific manner. However, two MAP kinase phosphatases have recently been isolated, one of which is rapidly induced by PKC activation [31,32], and it is also possible that preventing the induction of such a MAP kinase phosphatase may help to sustain the MAP kinase response stimulated by LPA. This possibility is at present being addressed in our laboratory.

The results in this study also show that the PKC-dependent pathway which regulates LPA-induced tyrosine phosphorylation of MAP kinase involves the activation of a pertussis toxin-sensitive G-protein. Consistent with this observation is the recent finding that, in Rat-1 fibroblasts, LPA stimulates the activation of p21^{ras} in a pertussis toxin-sensitive manner [14,16]. The identity of the G-protein involved in coupling the activated LPA receptor to p21^{ras} is unclear, but it has been shown recently by Carr and co-workers that the LPA receptor can directly interact with the G-protein α-subunit of G_i2 [33]. It has also been suggested that the activation of an intermediate tyrosine kinase may also be involved in the activation of p21^{ras} induced by LPA [14,16]. Our studies implicate an additional isoform of PKC as a possible candidate for the link between G_i2 and p21^{ras}. However, we cannot discount an input from a third pathway involving, for example, the recently identified MEK kinase [34].

LPA may also inhibit adenylate cyclase activity, and it is possible that the uncoupling of G_i from the LPA receptor may result in a compensatory activation of cAMP in EAhy 926 cells. Recently it has been shown that an increase in intracellular cAMP concentration results in a decrease in agonist activation of MAP kinase in Rat-1 fibroblasts through protein kinase A (PKA)-mediated phosphorylation of Raf-1 [35]. Thus it is possible that the pertussis toxin-induced decrease in LPA-stimulated MAP kinase activation may be mediated by an indirect effect. Preliminary results have shown that cAMP-raising agents abolished LPA- and platelet-derived growth factor-induced activation of MAP kinase in Rat-1 fibroblasts, as

previously observed [35]. However, in EAhy 926 cells 8-bromo-cAMP or forskolin abolished LPA-stimulated MAP kinase activation only at 60 min, and were ineffective at 2 min. These results imply that PKA-mediated phosphorylation of Raf-1 may not be a major regulatory mechanism in EAhy 926 cells, but confirm that the effect of pertussis toxin is not due to raising cAMP levels. The finding that both 8-bromo-cAMP and forskolin abolished the late phase of LPA-induced tyrosine phosphorylation of MAP kinase suggests that a PKA-dependent MAP kinase phosphatase may be induced or activated; however, further experimentation is required to confirm this hypothesis. Ro-318220 also reversed the effect of 8-bromo-cAMP on the sustained phase of the LPA response, suggesting that PKC, acting directly, is more important than PKA in regulating the sustained MAP kinase response.

In EAhy 926 cells we found that LPA also stimulated the tyrosine phosphorylation of other proteins such as pp130 and $pp125^{FAK}$. Thus the potential angiogenic effects of LPA may involve a role in regulating changes in cytoskeletal structure as endothelial cells migrate to form new capillaries. Previously it has been shown that both pertussis toxin-sensitive and -insensitive pathways may regulate the tyrosine phosphorylation of $pp125^{FAK}$ in response to agonists [16,36]. However, in EAhy 926 cells the response was not affected by PKC inhibition or pertussis toxin pretreatment, at concentrations which affected LPA-stimulated MAP kinase activation. These findings are consistent with results previously observed for bombesin-stimulated Swiss 3T3 fibroblasts and endothelin-1 and LPA-stimulated Rat-1 fibroblasts [9,16,37], and suggest that the activation of MAP kinase and $pp125^{FAK}$ do not lie on the same intracellular signalling pathway.

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