Kinetic and biochemical correlation between sustained p44ERK1 (44 kDa extracellular signal-regulated kinase 1) activation and lysophosphatidic acid-stimulated DNA synthesis in Rat-1 cells

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Rat-1 fibroblasts were used to study the role of the sustained activation of extracellular signal-regulated kinase 1 (ERK1) in lysophosphatidic acid (LPA)-stimulated mitogenic signalling. Mitogenic doses of LPA, like serum, stimulated biphasic, sustained, ERK activation that persisted towards the G1/S boundary. The EC_{50} for LPA-stimulated ERK activation after 10 min, the time of peak response, was 2 orders of magnitude to the left of that for the sustained response after 3 h or that for DNA synthesis after 22 h, with the result that non-mitogenic doses stimulated a maximal peak response but no second phase. To complement these studies, we examined the role of different signal pathways in regulating the sustained and acute phases of ERK activation using defined biochemical inhibitors and mimetics. Activation of protein kinase C and Ca^{2+} fluxes played a minor and transient role in regulation of ERK1 activity by LPA in Rat-1 cells. Sustained ERK1 activation stimulated by

LPA was completely inhibited by pertussis toxin, whereas the early peak response was only partly affected; this is correlated with the specific inhibition of LPA-stimulated DNA synthesis by pertussis toxin. The selective tyrosine kinase inhibitor herbimycin A completely inhibited sustained ERK1 activation by LPA but, again, the early phase of the response was only partially inhibited. In addition, low doses of staurosporine inhibited ERK1 activation by LPA. The effects of herbimycin A and staurosporine were selective for the response to LPA but did not affect that to epidermal growth factor. The results suggest a strong correlation between sustained ERK1 activation and DNA synthesis in LPA-stimulated Rat-1 cells. Furthermore, the two discrete phases of ERK activation by LPA are regulated by a combination of at least two different signalling pathways; the sustained activation of ERK1 in Rat-1 cells proceeds via a G_i - or G_o -mediated pathway which may also involve a tyrosine kinase.

INTRODUCTION

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycero-3-phosphate) exhibits a variety of biological activities in higher eukaryotes, promoting cell proliferation, smooth muscle contraction, reversal of neuroblastoma differentiation and changes in cell morphology and adhesion [1,2]. LPA is a growth factor for vascular smooth muscle cells and a variety of normal and transformed cell lines [3,4], and acts as an autocrine growth factor in ovarian tumours [5]. For these reasons, there is considerable interest in the biochemical signal transduction pathways utilized by LPA to stimulate cell cycle progression. In common with thrombin [6,7], LPA activates a receptor of the 'serpentine' or G-protein-coupled receptor superfamily [8]. To date, no LPA receptor has been cloned; however, the ability of LPA to stimulate DNA synthesis is inhibited by pertussis toxin [4], indicating a role for a trimeric GTPase of the G_i or G_o family, whereas $Ins(1,4,5)P_3$ formation is insensitive to pertussis toxin but is modulated by GDP/GTP analogues in Rat-1 cells [9], indicating a role for a G_q -related GTPase in regulating phospholipase $C\beta$ (PLC β) activity.

LPA, like many serpentine receptor growth factors, stimulates $PtdIns(4,5)P_2$ hydrolysis by PLC to generate the second messengers $Ins(1,4,5)P_3$ and *sn*-1,2-diradylglycerol [10]. The

resultant increases in intracellular free Ca^{2+} concentration and protein kinase C (PKC) activity may be important signals in the early cell cycle [10], but reconstitution of this pathway in many cell types is insufficient to stimulate DNA synthesis [11] and many growth factors do not stimulate this pathway. Other phospholipid signalling pathways, including phosphatidylcholine-specific PLC and phospholipase D, are also activated by growth factors [12], but again it is unclear if these pathways correlate well with DNA synthesis [13].

LPA and α -thrombin stimulate DNA synthesis via a pertussis toxin-sensitive pathway, indicating a role for a G_i or G_o trimeric GTPase in regulating key mitogenic signalling pathways [4,6]. Both of these agonists stimulate the pertussis toxin-sensitive inhibition of adenylate cyclase [4,6], but it seems likely that the more important ' G_i pathway' for proliferation involves the activation of serine/threonine kinase cascades that are responsible for transducing signals into the nucleus. Both α -thrombin [14] and LPA [15] activate the extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinases (MAP kinases) [16]. ERK1 and ERK2 [17] are serine/threonine kinases which are activated by phosphorylation [18] catalysed by a dual-specificity MAP kinase kinase called MAP or ERK kinase (MEK) [19]. MEK is in turn regulated by two different upstream kinases: the Raf proto-oncogene product [20] and MEK kinase

Abbreviations used: BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra(acetoxymethyl) ester; DiC₈, dioctanoylglycerol; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP or ERK kinase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate-13-acetate.

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[21]. The Ras proteins play a central role in regulating the ERK cascade [22–24]. Genetic analysis has previously shown that Raf is downstream of Ras [25], and recent studies suggest that Ras binds to Raf and recruits it to the plasma membrane where it is activated [26,27], resulting in activation of MEK and ERK.

Sustained activation of ERKs leads to their accumulation in the nucleus [28,29], allowing phosphorylation of transcription factors such as p62^{TCF}/Elk-1 [30,31], thereby regulating gene expression. In PC12 cells, sustained ERK activation is required to commit cells to a defined differentiation programme [32]. For example, nerve growth factor elicits sustained ERK activation and promotes differentiation, whereas epidermal growth factor (EGF) results in transient activation of ERKs and does not promote differentiation, but rather acts as a growth factor. While this model may apply well to neuronal differentiation, the situation in classical proliferative systems is much less clear, and only in the case of α -thrombin has a link between sustained ERK activation and DNA synthesis been satisfactorily established [33,34].

Rat-1 cells have proved to be a useful system in which to study the mitogenic effects of LPA [4], which stimulates DNA synthesis and ERK1 activation by activating the Ras pathway [15,35]. However, the ability of LPA to stimulate sustained ERK activity and the biochemical pathways by which LPA regulates ERK are subject to some debate. A recent study in Rat-1 cells examined ERK activation during a 20 min time course and concluded that the response was essentially transient [36]; however, LPA is required to be present for several hours to commit cells to enter S-phase [37]. Furthermore, other serpentine receptor growth factors activate ERK in a transient manner [38,39]. In addition, PKC plays the major role in regulating ERK activation by LPA in endothelial cells [40], but appears to play little or no role in other cell types [36,40a]. Since in Rat-1 cells LPA is able to couple to at least two trimeric-GTPase-regulated pathways (G_q -PLC β and G_i -Ras), we were interested in determining the kinetics of ERK activity in Rat-1 cells and the relative contributions made by these different pathways during the response.

In this paper we show a striking temporal and pharmacological correlation between sustained ERK activation and LPA-stimulated DNA synthesis in Rat-1 cells. In addition, by several biochemical criteria the sustained phase of ERK1 activation is regulated differently to the peak response, and this is also correlated with DNA synthesis. Taken together, the results provide strong support for a role for sustained ERK1 activation in LPA-stimulated cell proliferation.

MATERIALS AND METHODS

Materials

Cell culture reagents were from Irvine Scientific. Pre-poured SDS/PAGE reagents were from Novex Gel Systems. 1-Oleoyl-LPA was obtained from Avanti Polar lipids. EGF was from Boehringer Mannheim. Pertussis toxin was from List Biologicals or Calbiochem. Herbimycin A and GF109203X were from Calbiochem. Staurosporine, BAPTA-AM [1,2-bis-(*o*-amino-phenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra(acetoxymethyl) ester] and thapsigargin were from LC Laboratories. [γ -³²P]ATP and [³H]thymidine were from NEN-DuPont. All other reagents, including phorbol esters and myelin basic protein (MBP), were from Sigma.

Cells and cell culture

The Rat-1 cells used in this and previous studies [15,41] were originally provided by Dr. Johannes L. Bos, Utrecht University,

The Netherlands. Rat-1 cells were maintained in Dulbecco's modified Eagle's medium containing penicillin (100 units/ml)/streptomycin (100 μ g/ml), glutamine (4 mM) and 10% (v/v) foetal bovine serum (FBS). Cells were washed once in serum-free medium and then placed in fresh serum-free medium for at least 24 h prior to the experiments described herein. Pretreatment with various agents was as follows: pertussis toxin, 100 ng/ml for 18 h; herbimycin A, 5 μ M for 4 h; staurosporine, 1 μ M for 20 min; GF109203X, 4 μ M for 20 min. For PKC down-regulation studies, confluent Rat-1 cells were treated with serum-free Dulbecco's modified Eagle's medium containing 1 μ M phorbol 12-myristate 13-acetate (PMA) or control (vehicle) for 48 h prior to stimulation.

Cell stimulation

For ERK1 assays, experiments were performed upon 6-well plates of confluent, quiescent, cells that had been serum-starved for 24–36 h. Growth factors were added to the final concentrations indicated and stimulation proceeded at 37 °C for the times indicated. Incubations were terminated by aspiration and addition of ice-cold TG lysis buffer [20 mM Tris/HCl (pH 8), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 50 mM NaF, 1 mM Na₃VO₄, 1 mM Pefabloc, 20 μ M leupeptin, 10 μ g/ml aprotinin]. All manipulations of cell lysates were carried out at 4 °C. Lysates, prepared by rocking for 20 min, were collected into Eppendorf tubes and cleared of nuclei and detergent-insoluble material by centrifuging for 10 min at 16000 *g*. The protein content in cell lysates was determined using the Bio-Rad 'micro' protein assay protocol, and values were routinely found to vary by no more than 10%.

Immune-complex kinase assays for ERK1

Anti-peptide antibodies directed to the extreme C-terminus of ERK1 (E1.2) were described previously [15]; this serum exclusively immune-precipitates ERK1. We have been unable to derive immune-precipitating antisera to ERK2 using C-terminal peptide conjugates. All the experiments described herein are ERK1 assays; however, we have recently constructed a Rat-1 cell line expressing physiological levels of an epitope-tagged ERK2 construct (mycERK2) and find that it behaves similarly to ERK1 in all assays tested to date (K. Cadwallader, F. McCormick and S. Cook, unpublished work).

Cell lysates, typically 100 μ g of protein, were immunoprecipitated with 3 μ l of crude antiserum and Protein A-Sepharose beads at 4 °C for 2–3 h. Immune precipitates were collected by centrifuging for 10 s at 16000 *g* and washed with 2 $\times$ 1 ml of lysis buffer. Immune complexes were washed in kinase buffer (30 mM Tris, pH 8, 20 mM MgCl₂, 2 mM MnCl₂) before being resuspended in 30 μ l of kinase assay cocktail containing kinase buffer, 6 μ g of MBP, 10 μ M unlabelled ATP and 2.5 μ Ci of [γ -³²P]ATP per sample. Incubations were for 30 min at 30 °C and were terminated by the addition of hot 4 $\times$ SDS/PAGE sample buffer, followed by boiling for 5 min at 95 °C. Samples were resolved on a 14% SDS/PAGE gel using Novex gel systems. The gel was stained with Coomassie Brilliant Blue, dried and autoradiographed. The Coomassie Blue-stainable MBP band was excised from the dried gel and the incorporated radioactivity determined by scintillation counting.

Assay of DNA synthesis by [³H]thymidine incorporation

Matched confluent cultures of Rat-1 cells were washed once in serum-free medium and then incubated in fresh serum-free medium for 24 h before stimulation with the appropriate growth

factors for a further 24 h. Reinitiation of DNA synthesis was assayed by incorporation of a pulse of [^3H]thymidine (1 $\mu\text{Ci}/\text{ml}$; 5 μM unlabelled) during the final 4 h of stimulation. At the end of the stimulation time, radioactivity incorporated into trichloroacetic acid-precipitable material was determined by liquid scintillation counting following solubilization in 0.1 M NaOH.

Reproducibility of results

Results from single experiments representative of between three and nine giving similar results are shown. In addition, in each case results from several experiments were pooled and statistical analysis of differences was performed by Student's *t* test using the StatView program for Macintosh. For ERK MBP kinase assays, results are expressed as raw c.p.m. of ^{32}P incorporated into MBP, except in cases where several data sets have been combined for comparison and are expressed as percentage of maximum response or fold increase over control. Time courses of ERK1 immune complex kinase activity were performed as single-point assays; we and others [14,15,33] have found this to be a highly sensitive and reproducible assay. In addition, some cells were stimulated and assayed in duplicate and gave identical results with errors of generally less than 10%. [^3H]Thymidine incorporation assays were performed on duplicate or triplicate cell samples.

RESULTS

Kinetic and pharmacological correlation between sustained ERK1 activation and DNA synthesis in Rat-1 cells

To test for a role for sustained ERK activity in cell proliferation, we first examined the ability of LPA to stimulate sustained ERK1 activation in Rat-1 cells. We analysed the kinase activity of p44ERK1 immunoprecipitated from Rat-1 cell lysates that had been prepared from cells stimulated with LPA or 10% (v/v) FBS for time periods ranging from 10 min to 7 h. Both treatments resulted in a biphasic, sustained, activation of ERK1 which peaked at 5 or 10 min. In the case of LPA this response declined markedly before a second phase was apparent from 30 or 60 min onward which persisted significantly above basal for up to 7 or 8 h (Figure 1A and Table 1). The second phase of the response to FBS was reproducibly larger than that with LPA, and this was correlated with the greater proliferative response and earlier kinetics of S-phase entry in the presence of FBS (Figure 1B). ERK1 activity was significantly elevated over controls throughout the time course in response to proliferative stimuli such as LPA, EGF and serum (Table 1), but not with PMA (see Figure 3). These results indicate that sustained ERK activation is a common response to mitogenic stimuli in Rat-1 cells.

Sustained activation of ERK1 by growth factors persisted until late in the G1 phase of the cell cycle in Rat-1 cells. By analysing the time course of [^3H]thymidine incorporation into trichloroacetic acid-precipitable material we observed significant DNA synthesis in response to FBS after 9 h which then increased rapidly to peak at 16–20 h (Figure 1B). With LPA, DNA synthesis was slightly delayed but rose after 10–12 h (Figure 1B); EGF gave similar results to LPA (not shown). The magnitude of maximal LPA-stimulated DNA synthesis relative to that induced by serum was $52 \pm 15\%$ ($n = 3$); we have never observed LPA to be as or more effective than serum as others have reported [4]. We conclude that ERK activation by LPA persists until very close to the G1/S boundary in Rat-1 cells.

If ERK activation is important for the proliferative response, we might expect a simple pharmacological correlation between ERK activation and DNA synthesis. This was clearly the case for

Figure 1 ERK1 activity persists until late G1 in response to LPA and FBS in Rat-1 cells

(A) Confluent, serum-starved, Rat-1 cells were stimulated with LPA (100 μM), FBS (10%, v/v) or vehicle (control) for the times indicated up to 8 h. Following lysis, ERK1 was immunoprecipitated with E1.2 antiserum and assayed for activity using MBP as substrate. (B) The kinetics of ERK1 activation are compared with those of S-phase entry stimulated by FBS, LPA or vehicle. ERK1 activity and incorporation of [^3H]thymidine into DNA were assayed as described in the text. Similar results were obtained in $n = 3$ –6 separate experiments.

Table 1 Sustained activation of ERK1 by LPA, EGF and FBS in Rat-1 cells

Serum-starved Rat-1 cells were stimulated for various time periods up to 7 h with 100 μM LPA, 10 nM EGF, 20% (v/v) FBS or vehicle (as a control). Results are fold increases over the zero-time control from pooled results of $n = 4$ –7 experiments for stimulation times at 10 min, 2 or 3 h and 6 or 7 h. Significance of differences from control (vehicle) response: * $P < 0.05$, ** $P < 0.01$.

Stimulus	Increase in ERK1 activity (fold)		
	10 min	2 or 3 h	6 or 7 h
Control	1.9 $\pm$ 1.4	1.5 $\pm$ 0.7	1.8 $\pm$ 0.4
LPA	17.1 $\pm$ 3.3**	7.4 $\pm$ 3.1*	6.5 $\pm$ 2.6*
FBS	24.3 $\pm$ 2.5**	22 $\pm$ 1.7**	9.3 $\pm$ 2.2*
EGF	27 $\pm$ 4.5**	9.1 $\pm$ 2*	6.3 $\pm$ 2.8*

EGF, where stimulated increases in ERK1 MBP kinase activity and DNA synthesis were in good agreement, with EC_{50} values of 0.3 nM and 0.6 nM respectively (Figure 2A). The EC_{50} for the

Figure 2 Non-mitogenic doses of LPA give only transient ERK1 activation in Rat-1 cells

(A) Quiescent, serum-starved, Rat-1 cells were stimulated with increasing concentrations of EGF for 10 min (ERK activity; ■) or 24 h (DNA synthesis; □). (B) Quiescent, serum-starved, Rat-1 cells were stimulated with increasing concentrations of LPA for 10 min (■) or 3 h (□) for ERK activity, or 24 h for DNA synthesis (●). Data are pooled from $n = 2$ experiments. Similar results were obtained in three experiments. Incorporation of [3 H]thymidine was assayed as described in the text. (C) Quiescent, serum-starved Rat-1 cells were stimulated with mitogenic (100 μ M; ■) or non-mitogenic (1 μ M; □) doses of LPA, or vehicle (○), for the times indicated. Following lysis and immune precipitation with E1.2 antiserum, ERK1 activity was assayed as described in the text. Results are expressed as radioactivity incorporated into MBP (ERK1) or trichloroacetic acid precipitates (DNA synthesis) (c.p.m.).

LPA-stimulated increase in ERK1 activity assayed after 10 min was 40.3 ± 45.2 nM (mean $\pm$ S.D., $n = 4$); this value was nearly 2 orders of magnitude to the left of that for DNA synthesis (12 ± 6.8 μ M; mean $\pm$ S.D., $n = 5$) assayed after 24 h (Figure 2B). The EC_{50} for sustained ERK1 activation after 3 h of LPA treatment, 20 ± 7 μ M (mean $\pm$ S.D., $n = 2$), was in much closer agreement with that for DNA synthesis. These experiments were performed with the same aliquots of LPA, and so the differences in apparent potency did not reflect differences in preparation or supplier of LPA.

By comparing the kinetics of ERK1 activation in response to 1 μ M or 100 μ M LPA (Figure 2C), we observed that both concentrations of LPA elicited the same peak response at 10 min, but the response to 1 μ M LPA was transient and monophasic, returning to basal within 30 min. In contrast, 100 μ M LPA, a maximal mitogenic dose, resulted in a biphasic, sustained, response persisting for 3 h (Figure 2C) and 7 h (Figure 1). These results indicate that the peak activation of ERK1 by LPA can be fully reconstituted by 1 μ M LPA without any stimulation of DNA synthesis, making the magnitude of this early peak response an unreliable indicator of proliferative efficacy.

Ca²⁺- and PKC-dependent pathways make a minor and transient contribution to LPA-stimulated ERK1 activation in Rat-1 cells

The preceding results provided a correlation between sustained ERK1 activation and LPA-stimulated DNA synthesis in Rat-1 cells. We wished to define the biochemical pathways by which the sustained and peak responses were regulated.

LPA stimulates a G_q -PLC β pathway in Rat-1 cells, resulting in an increase in the intracellular free Ca²⁺ concentration and activation of PKC [9,10]; EGF does not activate this pathway in Rat-1 cells [37]. We first compared ERK activation by LPA with that by PMA and the cell-permeant diacylglycerol dioctanoylglycerol (DiC₈) (Figure 3). LPA again stimulated a pronounced peak of ERK1 activity at 10 min which then declined to give a second phase which persisted above basal for up to 2 h (Figure

Figure 3 Persistent activation of PKC elicits a minor and transient activation of ERK1 in Rat-1 cells

Quiescent, serum-starved, Rat-1 cells were stimulated with 100 μ M LPA, 100 nM PMA, 50 μ M DiC₈ or vehicle (Con) for the indicated times. Detergent lysates were immune-precipitated with E1.2 antiserum, and ERK1 activity was determined by immune complex kinase assays using MBP as substrate. Results are expressed as radioactivity incorporated into MBP (c.p.m.). Similar results were obtained in four other experiments

3) and 8 h (see Figures 1 and 2). We routinely used time points of 2 or 3 h as indicators of the sustained phase of the response and 5 or 10 min as the peak response. The second phase of the response was variable in magnitude (68 ± 26 % of the peak response; mean $\pm$ S.D. of $n = 13$ determinations) but was a highly reproducible and significant component of the response to LPA (Table 1). In side-by-side analysis, the peak response seen with LPA at 10 min (13.2 ± 2 -fold, mean $\pm$ S.D. of $n = 13$ experiments) was significantly greater than that seen with DiC₈ (3.7 ± 1.7 -fold; $n = 4$, $P < 0.01$) or PMA (4.4 ± 1.4 -fold; $n = 7$,

Table 2 Effect of chronic PMA pretreatment on ERK1 activation in Rat-1 cells

Serum-starved Rat-1 cells were pretreated with control vehicle (Control) or with 1 μ M PMA for 48 h before stimulation in duplicate for 10 min with PMA (100 nM), LPA (100 μ M) or EGF (10 nM) as indicated. Alternatively, cells were pretreated with vehicle (Control) or with 2 μ M GF109203X for 30 min prior to a 10 min stimulation with PMA (100 nM) or LPA (100 μ M) as indicated. ERK assays are quantified as radioactivity incorporated into MBP (c.p.m.), and results represent means $\pm$ S.D. from duplicate cell stimulations. A single representative experiment is shown, and values in parentheses indicate responses as a percentage of the maximum control response pooled from $n = 5$ experiments. Significance of differences from control response: * $P < 0.05$; ** $P < 0.01$. nd, not determined.

Stimulus	ERK1 activity (32 P incorporated into MBP; c.p.m.)			
	Expt. 1		Expt. 2	
	Control	1 μ M PMA, 48 h	Control	+ GF109203X
Basal	2502 $\pm$ 179	3493 $\pm$ 595	8750 $\pm$ 754	nd
	(-)	(-)	(-)	(-)
PMA	14584 $\pm$ 216	3514 $\pm$ 1077	43785 $\pm$ 120	4947 $\pm$ 214
	(100 $\pm$ 2%)	(13 $\pm$ 12%)**	(100 $\pm$ 2%)	(16 $\pm$ 15%)**
LPA	40553 $\pm$ 4266	31017 $\pm$ 1613	90588 $\pm$ 12387	52587 $\pm$ 16388
	(100 $\pm$ 11%)	(70 $\pm$ 10%)*	(100 $\pm$ 10%)	(61 $\pm$ 24%)*
EGF	79844 $\pm$ 1505	87124 $\pm$ 18801	nd	nd
	(100 $\pm$ 6%)	(124 $\pm$ 23%)		

$P < 0.01$). In addition, a maximal dose of DiC₈ or PMA gave only a small and transient activation of ERK1 which declined to basal within 30–60 min and was no longer significantly elevated above basal. Thus strong and persistent activation of diacylglycerol- and PMA-sensitive isoforms of PKC is not sufficient to account for the magnitude or kinetics of LPA-stimulated ERK1 activation in Rat-1 cells.

Pretreatment of Rat-1 cells with 1 μ M PMA for 48 h to 'down-regulate' PKC levels [42] reduced the response to a subsequent PMA stimulus to 13 $\pm$ 12% of control values ($n = 8$ determinations; significant at $P < 0.01$), indicating that PMA pretreatment had removed a large proportion of the intracellular PMA-responsive PKC (Table 2). In the same experiments the response to LPA was only reduced to 70 $\pm$ 10% ($n = 7$ experiments; significant at $P < 0.05$), suggesting only a minor role for PKC. The response to EGF was not significantly affected by down-regulation of PKC.

To complement these studies we used the selective PKC inhibitor GF109203X [43]. GF109203X reduced PMA-stimulated ERK1 activation to 16 $\pm$ 15% ($n = 7$, $P < 0.01$) of control values (Table 2), and did not affect the response to EGF (results not shown). In the same experiments the drug had a minor effect on the peak response to LPA, reducing it to 61 $\pm$ 24% of control ($n = 5$ determinations, $P < 0.05$) (Table 2). In time-course experiments we examined the effect of GF109203X on both the early peak of ERK activation by LPA and the sustained phase of the response. In contrast to its partial inhibitory effect at early times, GF109203X had no significant inhibitory or stimulatory effect on the sustained phase of the response when assayed after 2 or 3 h (110 $\pm$ 15% of the control response; $n = 2$, $P > 0.1$; results not shown). This suggests that a PKC-dependent pathway for ERK activation is confined to the early part of the response to LPA.

The poor correlation between activation of PKC and ERK1 was also reflected in the involvement of PMA-sensitive forms of PKC in proliferation. In assays of [3 H]thymidine incorporation, PMA alone elicited only a weak response and down-regulation

Figure 4 A23187 stimulates a transient ERK1 activation in Rat-1 cells

Quiescent, serum-starved, Rat-1 cells were stimulated with 100 μ M LPA, 50 μ M A23187 or 300 nM thapsigargin for the indicated times. ERK1 activity was determined as described in the text, and results are expressed as radioactivity incorporated into MBP (c.p.m.). Similar results were obtained in two other experiments.

of PKC did not inhibit LPA-stimulated DNA synthesis. For example, in control cells, incorporation of [3 H]thymidine (in c.p.m.) was: basal, 1819 $\pm$ 215; PMA, 4929 $\pm$ 512; LPA, 12759 $\pm$ 912; FBS, 18172 $\pm$ 672. In cells pretreated with PMA for 48 h, values were: basal, 3162 $\pm$ 398; PMA, 3339 $\pm$ 211; LPA, 12060 $\pm$ 437; FBS, 19176 $\pm$ 1311 ($n = 3$). Thus PKC is neither sufficient nor necessary for stimulation of ERK1 or DNA synthesis [4] by LPA in Rat-1 cells.

The calcium ionophore A23187 gave a weaker activation of ERK1 compared with that by LPA (7.2 $\pm$ 1.0-fold compared with 13.2 $\pm$ 2.0-fold for LPA; $P < 0.05$) and the response was transient, declining to basal within 60–90 min (Figure 4). In addition, thapsigargin, which elicits a transient mobilization of Ins(1,4,5) P_3 -sensitive Ca^{2+} stores by inhibiting the endoplasmic reticulum Ca^{2+} -ATPase [44], was unable to significantly increase ERK1 activity (Figure 4).

Chelation of extracellular Ca^{2+} with EGTA to prevent agonist-induced Ca^{2+} entry completely inhibited ERK1 activation observed in response to A23187, but had no effect on LPA-stimulated ERK1 activation (Table 3). In addition, we used the cell-permeant Ca^{2+} -chelating agent BAPTA-AM in the presence of EGTA to buffer the increases in intracellular free Ca^{2+} concentration resulting from LPA-stimulated Ins(1,4,5) P_3 generation and Ca^{2+} entry. EGTA and BAPTA-AM partially inhibited LPA-stimulated ERK1 activation (50 $\pm$ 3.5%; Table 3), but completely inhibited the response to A23187.

Since the effects of A23187 and PMA are likely to be non-physiological overstatements of the normal agonist-stimulated responses, these results suggest that Ca^{2+} and PKC make only a minor and transient contribution to ERK activation by LPA, with little role in the sustained phase.

Pertussis toxin inhibits DNA synthesis and sustained ERK activation by LPA

The ability of LPA to reinitiate DNA synthesis in Rat-1 cells is pertussis toxin-sensitive, with half-maximal inhibition occurring at 0.1–1 ng/ml; under the same conditions the response to EGF was totally unaffected ([4]; results not shown). The ability of

Table 3 Effects of BAPTA-AM and EGTA on ERK1 activation in Rat-1 cells

Serum-starved Rat-1 cells were incubated in Dulbecco's modified Eagle's medium in the absence (Control) or in the presence of 3 mM EGTA alone or EGTA + BAPTA-AM (15 μ M) for 20 min prior to addition of LPA (50 μ M) or A23187 (50 μ M). ERK assays are quantified as radioactivity incorporated into MBP (c.p.m.) and results represent means $\pm$ S.D. from duplicate cell stimulations. A single representative experiment is shown, and values in parentheses indicate response in presence of EGTA or BAPTA-AM + EGTA as a percentage of the maximum control response from $n = 3$ experiments. Significance of differences from control response: * $P < 0.05$; ** $P < 0.01$.

Stimulus	ERK1 activity (32 P incorporated into MBP; c.p.m.)			
	Expt. 1		Expt. 2	
	Control	+ EGTA	Control	+ BAPTA/EGTA
Basal	7121 $\pm$ 443	9038 $\pm$ 1590	1179 $\pm$ 332	945 $\pm$ 223
A23187	58340 $\pm$ 1941 (100 $\pm$ 9%)	10784 $\pm$ 1021 (7 $\pm$ 6%)**	3109 $\pm$ 461 (100 $\pm$ 9%)	918 $\pm$ 51 (2 $\pm$ 1%)**
LPA	97777 $\pm$ 15179 (100 $\pm$ 13%)	73628 $\pm$ 1154 (92 $\pm$ 16%)	9236 $\pm$ 952 (100 $\pm$ 13%)	5458 $\pm$ 2 (50 $\pm$ 4%)*

Figure 5 Pertussis toxin selectively inhibits sustained ERK1 activation by LPA in Rat-1 cells

Quiescent, serum-starved, Rat-1 cells were pretreated with control vehicle (■) or 100 ng/ml pertussis toxin (□; Ptx) for 18 h before stimulation with 100 μ M LPA for 10–180 min. A vehicle time course is also shown (○). ERK1 MBP kinase activity was assayed as described in the text, and is expressed as radioactivity incorporated into MBP (c.p.m.). Similar results were obtained in four other time course experiments.

LPA to activate ERK1 was greatly inhibited by pertussis toxin treatment, but the drug had quite different effects at different times in the response (Figure 5). The initial peak response was inhibited by 72 $\pm$ 13 % (mean $\pm$ S.D. from 8 separate experiments; $P < 0.01$) in a saturable, dose-dependent manner, with an IC_{50} of 0.4 $\pm$ 0.1 ng/ml (mean $\pm$ S.D., $n = 2$). In contrast, the smaller sustained phase of LPA-stimulated ERK1 activation at 2 and 3 h was totally abolished by the same concentrations of pertussis toxin (decreased to 7 $\pm$ 6 % of the maximum response; $n = 4$, $P > 0.001$) (Figure 5). These results suggest that, at early time points, LPA uses pertussis toxin-sensitive and -insensitive pathways for ERK1 activation, whereas the sustained response proceeds exclusively via a pertussis toxin-sensitive pathway. In the same series of experiments, EGF- and PMA-stimulated ERK1 activation was not inhibited by pertussis toxin treatment

Figure 6 Herbimycin A selectively inhibits sustained ERK1 activation by LPA in Rat-1 cells

Quiescent, serum-starved, Rat-1 cells were pretreated with medium alone (■) or with 5 μ M herbimycin A (Herb' A; □) for 4 h before stimulation with LPA (100 μ M) for the times indicated; a vehicle time course is also shown (○). ERK1 MBP kinase activity was assayed as described in the text, and is expressed as radioactivity incorporated into MBP (c.p.m.). Similar results were obtained in three other experiments.

at all time points tested (144 $\pm$ 48 % and 130 $\pm$ 53 % of control response respectively).

Herbimycin A selectively inhibits sustained ERK1 activation by LPA in Rat-1 cells

Much attention has focused recently on the possibility that serpentine receptor growth factors may activate tyrosine kinases [36–40]. We examined the effects of the tyrosine kinase inhibitors herbimycin A [45] and staurosporine upon the ability of LPA to stimulate DNA synthesis and ERK1 in Rat-1 cells.

Stimulation of Rat-1 cells with EGF or LPA in the presence of increasing concentrations of herbimycin A resulted in a dose-dependent, ultimately complete, inhibition of DNA synthesis. For example, [3 H]thymidine incorporation (in c.p.m.) was 426 $\pm$ 25 for basal, 6238 $\pm$ 1121 for LPA and 2760 $\pm$ 36 for EGF. In the presence of 5 μ M herbimycin A these responses were decreased to 112 $\pm$ 49 for LPA and 138 $\pm$ 10 for EGF (means $\pm$ S.D. of duplicate determinations, representative of $n = 3$ experiments). The IC_{50} values were very similar for both growth factors, being 1.43 $\pm$ 1.0 μ M against EGF and 0.93 $\pm$ 0.9 μ M against LPA (means $\pm$ S.D., $n = 3$). These results suggested that a herbimycin A-sensitive component was absolutely required for quiescent Rat-1 cells to traverse G1 and enter S-phase, whether stimulated by EGF or LPA. This inhibitory effect was confined to G1. If herbimycin A was added to cells at $t = 0$ or at any time throughout G1 (10–12 h; see Figure 1B), the result was complete inhibition of subsequent DNA synthesis. From 10–12 h post-LPA addition onwards, herbimycin A had progressively less effect on DNA synthesis (results not shown). Thus it seems that a herbimycin A-sensitive tyrosine kinase is required throughout G1 of the cell cycle for commitment to S-phase in response to LPA. Similar results were obtained with EGF.

Since Ras is required for LPA to fully activate ERK1 [15], and a variety of tyrosine kinases converge on the Ras pathway [15,23–25,32], we investigated whether herbimycin A inhibited ERK activation. Pretreatment of Rat-1 cells with 5 μ M herbimycin A did inhibit activation of ERK1 by LPA, but had different effects at different stages in the response (Figure 6,

Table 4 Effects of herbimycin A and staurosporine on LPA- and EGF-stimulated ERK1 activation in Rat-1 cells

Quiescent, serum-starved, Rat-1 cells were pretreated with vehicle (Control) or with 5 μ M herbimycin A or 1 μ M staurosporine as indicated before stimulation in duplicate with LPA (50 μ M) or EGF (10 nM) for 10 min. ERK1 was immunoprecipitated from detergent lysates and assayed against MBP kinase as described in the text. Values in parentheses are responses as a percentage of the control response derived from data pooled from four experiments. Significance of differences from control response: * $P < 0.05$; ** $P < 0.01$. nd, not determined.

Stimulus	ERK1 activity (32 P incorporated into MBP; c.p.m.)			
	Expt. 1		Expt. 2	
	Control	+ Herbimycin A	Control	+ Staurosporine
Basal	43327 $\pm$ 8076	nd	2479 $\pm$ 386	nd
LPA	327760 $\pm$ 36927 (100 $\pm$ 10%)	141724 $\pm$ 19308 (38 $\pm$ 19%)*	14752 $\pm$ 2367 (100 $\pm$ 11%)	3392 $\pm$ 99 (17 $\pm$ 11%)**
EGF	612749 $\pm$ 114965 (100 $\pm$ 14%)	637390 $\pm$ 88241 (106 $\pm$ 31%)	12211 $\pm$ 148 (100 $\pm$ 6%)	10907 $\pm$ 281 (81 $\pm$ 23%)

Table 4). Inhibition of the first phase of the response, at 5 or 10 min, was partial (62 $\pm$ 19% inhibition; mean $\pm$ S.D. of five experiments, $P < 0.05$), but the second, sustained, phase of ERK1 activity at 60 or 120 min seemed particularly sensitive to the drug (90 $\pm$ 9% inhibition; mean $\pm$ S.D. of three experiments, $P < 0.01$). In the same series of experiments herbimycin A did not inhibit PMA-stimulated ERK activation, and the response to EGF was not significantly affected by herbimycin A (Table 4 and results not shown).

In contrast to other reports [46], to date we have been unable to demonstrate a reproducible inhibition of ERK1 activation by genistein; this drug is able to inhibit agonist-stimulated DNA synthesis in Rat-1 cells, but only at high concentrations (100 μ M), at which we observe significant toxicity. Staurosporine completely inhibited PMA-stimulated ERK activation (reduced response to 9 $\pm$ 13% of control). Despite this, staurosporine at 1 μ M did exert some selective effects, since it greatly inhibited the response to LPA (83 $\pm$ 11% inhibition; mean $\pm$ S.D. of four experiments) but only poorly inhibited the response to EGF (19 $\pm$ 23%; mean $\pm$ S.D. of three experiments). The selective effect of staurosporine on the response to LPA was dose-dependent, with an approximate IC_{50} of 300 nM (results not shown); at concentrations of 3 μ M and above we did observe some modest inhibition of the response to EGF (results not shown). Since selective PKC inhibition has only a small effect on the response to LPA (Table 2), these results suggest that, at low doses, staurosporine is inhibiting a kinase (distinct from PKC) that is selectively used by LPA to activate ERK1.

DISCUSSION

A major pathway by which LPA regulates cell proliferation is activation of Ras and the Raf-MEK-ERK cascade [15,35,47]. A working model suggests that sustained ERK activation is required for neuronal differentiation of PC12 cells [32], but it remains unclear how well this model applies to proliferative systems such as fibroblasts. We initiated the present study to examine the role of sustained ERK activation as a signal in LPA-stimulated cell proliferation and to characterize the biochemical pathway by which LPA activates ERK1 during this response.

Sustained activation of ERK1 is correlated with DNA synthesis in LPA-stimulated Rat-1 cells

In PC12 cells, sustained activation of ERKs promotes differentiation, whereas growth factors give a transient response [29,32]. In this way it is proposed that quantitative changes in ERK activation can be translated into qualitative changes in gene expression and cell fate [32]. It is unclear how well this model applies to proliferative systems, e.g. in fibroblasts, will growth factors require sustained or transient ERK activation to promote proliferation? The only major study to address this has been that of Pouyssegur and colleagues [14,33,34,48], who found that thrombin requires sustained ERK activity for several hours to stimulate DNA synthesis in CCL39 cells. In contrast, agonists such as vasopressin and angiotensin II stimulate only transient ERK activation in vascular smooth muscle cells [38,39], and the ability of LPA to stimulate sustained ERK activation is subject to debate [15,36].

To test this model, we examined the kinetics of ERK activation by LPA in Rat-1 fibroblasts. We found that agonists which can stimulate DNA synthesis (LPA, EGF and FBS in Rat-1 cells) activate ERK1 in a co-ordinated, sustained, manner that persists towards the G1/S boundary, whereas non-mitogenic stimuli, such as PMA, stimulate only transient ERK activation in Rat-1 cells. This correlation is particularly strong for LPA. In side-by-side analysis, 100 μ M LPA (a maximum dose for DNA synthesis) elicits biphasic, sustained, ERK activation, whereas a non-mitogenic dose (1 μ M) results in a transient, monophasic, response, indicating that sustained ERK activation is intimately linked to LPA-stimulated DNA synthesis.

The disparity in EC_{50} values between that for the peak activation of ERK and that for the sustained phase and DNA synthesis is consistent with previous reports for LPA (reviewed in [1,2]). To commit cells to DNA synthesis, LPA is required throughout G1, during which it may be subject to metabolic interconversion to inactive species [37,48a], thereby reducing the effective dose and limiting the magnitude and duration of cellular responses. This may explain why higher concentrations are required for long-term responses, whereas short-term responses reflect more closely the apparent K_d of the putative receptor [8], although we cannot rule out the possibility of multiple LPA receptors coupling differentially to G_q and G_i pathways, or indeed a single receptor coupling to these different pathways at different occupancies. Regardless, there remains a clear correlation between sustained activation of the ERK pathway and DNA synthesis for LPA. This has only been elucidated by thorough kinetic analysis, since examination of ERK activity only at the time of the peak response revealed no difference between a mitogenic and non-mitogenic dose of LPA.

The sustained phase of ERK activation by LPA was highly reproducible, but exhibited a 25% variation in magnitude in 13 independent determinations. The reason for this variation is unclear, but one possibility is that it represents experimental variation in the level of *de novo* expression of MAP kinase phosphatase-1, the product of the immediate-early gene that inactivates ERK during prolonged stimulation [49]. We are currently investigating a possible role for this phosphatase by using cycloheximide to block its expression and immunoblotting of samples to detect expression.

LPA activates ERK1 by two pathways in Rat-1 cells; a G_i -mediated pathway is more important for the sustained response

By several criteria, the two distinct phases of ERK activation by LPA are differentially regulated. There is evidence for at least two pathways for ERK1 activation: a minor and transient

pathway which may be regulated by Ca^{2+} and PKC, and a quantitatively greater, G_i -mediated, pathway which may involve activation of a tyrosine kinase and appears to be more important for the sustained phase of the response.

Increases in the intracellular free Ca^{2+} concentration and in PKC activity as a result of LPA-stimulated polyphosphoinositide hydrolysis seem to play a minor role in the early peak of ERK activation. Persistent activation of phorbol ester-sensitive forms of PKC by PMA mimicked at best 30% of the early peak response, but did not stimulate a sustained response, while inhibition of PKC had a small effect on the response to LPA which was confined to the early phase. Likewise, even quite stringent intervention in intracellular Ca^{2+} homeostasis had only minor effects on LPA-stimulated ERK activation. A transient input for PKC- and Ca^{2+} -stimulated pathways may be due to two reasons. First, activation of PKC and Ca^{2+} fluxes is regulated by phospholipase signal pathways which are largely desensitized within a few minutes of agonist addition [12,13]. Secondly, increases in Ca^{2+} or PKC may activate adenylate cyclase, thereby elevating cAMP levels and inhibiting ERK activation by a recently described cAMP-mediated inhibitory cross-talk pathway [41,50].

Several studies have defined a ' G_i pathway' for the stimulation of proliferation by thrombin and LPA, based on the observation that DNA synthesis is inhibited by pertussis toxin treatment [2,6]. LPA-stimulated ERK1 activation was inhibited by pertussis toxin with an IC_{50} value similar to that for inhibition of DNA synthesis. However, in kinetic terms pertussis toxin exerted a quite selective effect; at least 30% of the peak response was reproducibly insensitive to pertussis toxin, whereas sustained activation of ERK1 was completely abolished in pertussis toxin-treated cells in all experiments. This is in contrast with the results of Hordijk et al. [36], and may simply be due to the use of a more sensitive and quantitative assay which allows us to demonstrate a clear pertussis toxin-insensitive component to the response. The major role for a G_i pathway in both sustained ERK activity and DNA synthesis induced by LPA underlines the correlation between these events.

Selective inhibition of sustained LPA-stimulated ERK activity was also observed with the tyrosine kinase inhibitor herbimycin A. The effect of herbimycin A was reasonably selective, since the response to PMA was unaffected, suggesting that PKC does not utilize a tyrosine kinase to activate ERKs and that herbimycin A does not simply inhibit Raf or MEK. The lack of effect on ERK activation by EGF is more intriguing, but is supported by the selective inhibition of the response to LPA by low doses of staurosporine. While staurosporine is known to inhibit PKC, the results in Table 2 indicate by two criteria that selective PKC inhibition has little effect on the response to LPA. The results suggest that LPA uses a specific tyrosine kinase, not used by EGF, to activate the Ras pathway. Previous studies have shown that both staurosporine and genistein can inhibit signalling between LPA and Ras [35,36]. Herbimycin A is an inhibitor of the Src-family tyrosine kinases, and thrombin has recently been demonstrated to activate both Src and Fyn in CCL39 cells in a pertussis toxin-sensitive manner [51], but to date there is no evidence that this is the case in Rat-1 cells. The selective effects of pertussis toxin, staurosporine and herbimycin A suggest that they may prove to be useful tools in identifying components in the coupling of the LPA receptor to the Ras-Raf-ERK cascade. However, since the anti-proliferative effects of herbimycin A can be dissociated from the inhibition of ERK1, for example with EGF, the use of this agent in proliferative assays may be limited.

Recent studies in PC12 cells suggest that it is simply the duration of receptor signalling to the ERK pathway that

determines sustained ERK activation and resultant biological responses [29,32]. This should not be taken to mean that qualitative differences in signalling pathways do not matter. Reconstitution of the $\text{G}_q/\text{PLC}\beta$ pathway in CCL39 cells with the M1 carbachol receptor does not stimulate sustained ERK1 activation or DNA synthesis [11], and this is consistent with our inability to observe sustained ERK1 activation in response to PMA or A23187. This probably reflects the fact that PMA does not apparently use Ras-dependent pathways for ERK activation, or indeed does not activate Ras in Rat-1 cells [52]. The ability of LPA and thrombin to elicit sustained ERK activity appears to be related to their ability to couple to a G_i -Ras pathway. It is interesting to note that angiotensin II and vasopressin, which do not couple to G_i in vascular smooth muscle cells, stimulate a transient activation of ERK [38,39].

Use of Ras N17 or Rap V12 to antagonize Ras function abolishes sustained ERK1 activation by both LPA and EGF, but has less effect on the peak response at early times [15,47], suggesting that Ras-dependent and Ras-independent pathways regulate ERK at early times but that Ras may be more important for the smaller sustained responses. Such a model is consistent with the constitutive activation of ERKs observed in cell lines harbouring activated oncogenic Ras mutants [22]. These cells exhibit a modest activation of ERKs which is reminiscent of the G_i - and tyrosine kinase-dependent sustained phase described here rather than of the large peak response seen at earlier time points.

In conclusion, the results presented here provide strong support for the notion that sustained ERK1 activation is an important signal for LPA-stimulated DNA synthesis in Rat-1 cells. It appears that, just as sustained ERK activity is important for commitment to differentiation in PC12 cells, it also plays a role in DNA synthesis in fibroblasts, as originally proposed for thrombin [33,34]. This kinetic analysis is supported by biochemical studies. The activated LPA receptor couples to the G_q - $\text{PLC}\beta$ pathway, but this plays only a minor and transient role in activation of ERK1 and little role in proliferation. The more important pathway for ERK activation may involve either α_i -GTP or $\beta\gamma$ subunits [53] activating an effector system, perhaps a tyrosine kinase, which results in activation of the Ras pathway. Combination of these pathways may account for the early peak of activity, but the G_i -Ras pathway appears to be more important for sustained signalling. Experiments are under way to investigate whether this is reflected in the regulation of immediate-early gene expression.

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