Lysophosphatidic acid inhibits gap-junctional communication and stimulates phosphorylation of connexin-43 in WB cells: possible involvement of the mitogen-activated protein kinase cascade

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Lysophosphatidic acid (LPA) was shown to be a powerful inhibitor of gap-junctional communication between cultured rat liver WB cells, as determined by the transfer of Lucifer Yellow, with 50% inhibition obtained at about 0.3 μ M LPA. Inhibition of communication was rapid (5 min) and was maintained for at least 80 min. After incubation for 3 h with LPA, communication competence was partially restored and dye transfer was refractory to further addition of LPA. Communication in LPA-refractory cells retained sensitivity to inhibition by phorbol ester and by epidermal growth factor (EGF). LPA-induced inhibition was associated with phosphorylation of connexin-43 protein, as detected by slower migration of the protein detected on Western blots, which could be eliminated by incubation of samples with

alkaline phosphatase. A close correspondence was observed between the time- and dose-dependency of LPA effects on communication and the induction of mitogen-activated protein kinase (MAP kinase). Activation of both the 42 kDa and 44 kDa subspecies were confirmed by mobility shifts on Western blots using an anti-(MAP kinase R1) (erk 1-III) antibody and by fractionation on Mono Q columns. Cells pretreated with phorbol ester for 24 h were insensitive to phorbol ester inhibition of communication or activation of MAP kinase, but retained their sensitivity to LPA. The results indicate that LPA initiates the activation of protein kinase cascades in WB cells that are probably independent of protein kinase C and identifies connexin-43 as one substrate for the activated kinases.

INTRODUCTION

A number of recent studies have established that exogenously added lysophosphatidic acid (LPA) is mitogenic for some fibroblastic and epithelial cell lines [1–5]. Overall, the evidence supports the view that this simple phospholipid interacts directly with receptors on the surface of cells, rather than by acting as a general membrane perturbant, or as a precursor of intracellular metabolites. Thus photoaffinity-labelling studies with a LPA analogue have identified a binding protein of apparent molecular mass 38–40 kDa in a range of cell types, including fibroblasts [6]. Binding of LPA to this protein was inhibited by suramin, a polysulphonated naphthylurea derivative that also blocks the biological response of intact cells to LPA [7]. The initiating events in LPA-induced mitogenesis are not established, but rapid responses include inhibition of G-protein-linked adenylate cyclase [3], activation of both inositol lipid hydrolysis by phospholipase C [8] and phosphatidylcholine hydrolysis by phospholipase D [9], and activation of p21^{ras} [10]. In addition, LPA has been shown to modulate the assembly of actin stress fibres via the GTP-binding protein rho [11].

In this paper we establish that LPA activates mitogen-activated protein (MAP) kinase in WB cells, a result which has also recently been observed in Swiss 3T3 cells [12]. MAP kinase is part of a protein kinase cascade activated by many mitogens and by some non-mitogenic signals (see [13–15] for references). The enzyme is a serine/threonine kinase which phosphorylates a number of protein substrates *in vitro* and which requires phosphorylation on both threonine and tyrosine residues for activation. Recently, it has been reported that purified MAP kinase phosphorylates connexin-43 (Cx43), a major structural protein of gap junctions in heart and a number of cultured cell lines [16].

The authors concluded that epidermal-growth-factor (EGF)-induced inhibition of communication through the channels formed by transmembrane connexin proteins correlated with activation of MAP kinase, leading to phosphorylation of Cx43. There is considerable evidence that junctional permeability can be modulated by phosphorylation of the connexin proteins (see [17–19] for references). We now report that LPA is also a potent inhibitor of gap-junctional communication between WB cells and that this is associated with the phosphorylation of Cx43 and the activation of MAP kinase. Our data are consistent with an involvement of MAP kinase in regulating connexon function.

MATERIALS AND METHODS

Cell culture

WBF344, a rat epithelial cell line with the phenotypic properties of 'oval' cells, was obtained from Dr. J. W. Grisham [20] and cells were cultured as described previously [18]. In all experiments cells were used 1 day post-confluent.

Measurement of cell–cell communication

Communication was measured as described previously [21]. Briefly, cells in 35 mm-diam. dishes were rinsed with Ca²⁺/Mg²⁺-free PBS and incubated in medium without serum for 2–3 h before the addition of test substances. In all experiments, LPA was made up in PBS containing 1% fat-free BSA; controls were incubated with the vehicle alone. Stock solutions of phorbol 12-myristate 13-acetate (PMA) were in dimethyl sulphoxide (Me₂SO; final assay concn. 0.1%) and controls contained Me₂SO alone. After the appropriate incubation period, cells were

Abbreviations used: LPA, lysophosphatidic acid; Cx43, connexin-43; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; Me₂SO, dimethyl sulphoxide; MAP kinase, mitogen-activated protein kinase.

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rinsed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and incubated in the same solution containing 0.05% Lucifer Yellow CH (Sigma). Dye was loaded by making two or three scrape lines on the monolayer with a surgical blade. After 3 min, the cells were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS, fixed in 4% formaldehyde/1% glutaraldehyde in PBS and immediately examined under a Nikon epifluorescence microscope. Fluorescently labelled cells in eight fields of vision were counted (in control cultures a field of vision contained 230–250 fluorescent cells). Results were presented as a percentage of the relevant control.

Pretreatment with PMA

Cells in either 35 mm- or 100 mm-diam. dishes were incubated with PMA (100 nM) or Me_2SO for 24 h. The cells were washed in PBS and incubated for a further 2 h in serum-free medium containing either PMA or Me_2SO .

Partial purification and assay of MAP kinase

Cells (from 100 mm dishes) were rinsed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and incubated with LPA, PMA or EGF. At appropriate times the medium was removed and the cells were washed with cold PBS. Buffer A (0.9 ml; [22]) was added, and the cells were collected by scraping and disrupted by sonication. After centrifugation (100 000 g; 12 min) the supernatant was collected and MAP kinases were partially purified by using phenyl-Sepharose (Pharmacia) as described [23]. The assay mixture (50 μl) contained 25 mM Tris/HCl (pH 7.4), 50 mM glycerophosphate, 0.24 mM dithiothreitol, 0.1 mM Na_3VO_4 , 10 mM MgCl_2 , 40 μM ATP, 1.5 mM EGTA, 0.33 mg/ml myelin basic protein (Sigma), 0.4 mg/ml protein kinase A inhibitor (Sigma), 0.3–0.4 μCi of [^{32}P]ATP (4 Ci/ μmol ; Brestec, Adelaide, Australia) and MAP kinase preparation (2–4 μg of protein). After incubation for 20 min at 30 °C, 45 μl samples were spotted on P81 paper. Radioactivity incorporated into myelin basic protein was determined after extensive washes with 85 mM H_3PO_4 .

In some experiments, soluble extracts were chromatographed on Mono Q columns essentially as described [24].

Immunoblotting

To detect Cx43 protein, cells in 100 mm dishes were washed in PBS and gap-junction-enriched pellets were prepared by alkali extraction [18]. The pellet was resuspended in 1 mM NaHCO_3 containing 1 mM phenylmethanesulphonyl fluoride and solubilized in SDS sample buffer. Protein was determined by a modified Lowry procedure [25]. Samples containing 20 μg of protein were separated on 10%-acrylamide gels, transferred to nitrocellulose (100 V, 2 h), and immunoreaction and detection were carried out as described [18]. The affinity-purified antibody to amino acids 314–322 of rat heart Cx43 was a gift from Dr. D. Gros [26]. A similar procedure was used to detect MAP kinase protein by using partially purified extracts (see above) and an anti-(MAP kinase R1) (erk 1-111) antibody raised against residues 63–98 of the rat 43 kDa erk1 MAP kinase [27]. This antibody was a gift from Dr. S. L. Pelech.

Phosphatase digestion

Alkali-extracted pellets prepared as described above were divided into three equal portions and diluted to 40 μl in phosphatase reaction buffer (50 mM Tris/HCl, pH 8, 10 mM MgCl_2 , 50 mM NaCl). Two portions were incubated for 30 min at 30 °C in the presence or absence of calf intestinal alkaline

phosphatase (4 units; molecular biology grade, Boehringer Mannheim). The third portion was incubated with phosphatase in the presence of a phosphatase-inhibitor mixture (50 mM NaF, 100 mM Na_3VO_4 , 10 mM KH_2PO_4). Reactions were terminated with SDS sample buffer and the samples were analysed by immunoblotting (see above).

RESULTS AND DISCUSSION

Previous studies [18,19] have shown that PMA and EGF, which both inhibit gap-junctional communication between WB cells, stimulate the phosphorylation of Cx43 in these cells. Similar experiments were undertaken after treatment with LPA. As shown in Figure 1 (lane 4), alkali extracts of WB cells contained a number of proteins of 42–47 kDa which reacted with the anti-Cx43 antibody. Some of these proteins were constitutively phosphorylated in the WB cells, as treatment with alkaline phosphatase induced migration with a lower apparent molecular mass (lane 5). Incubation of the cells with 50 μM LPA for 10 min induced the appearance of immunoreactive bands with slower migration in the denaturing gels (Figure 1, lane 1). These bands were abolished by incubation with alkaline phosphatase (lane 2), an effect which was blocked by the inclusion of phosphatase inhibitors in the incubation (lane 3). In separate experiments using cells prelabeled with ^{32}P as described [19], it was established that LPA stimulated the serine-phosphorylation of Cx43 (results not shown).

Next, a series of experiments were carried out to determine the effect of LPA on cell–cell communication, and its interaction with EGF and PMA (Figure 2). Both of the last two compounds have been shown to inhibit communication in WB cells [18,19]. As shown in Figures 2(a) and 2(b), incubation with 25 μM LPA for 15 min caused a marked inhibition of the transfer of Lucifer Yellow between cells, which was maintained for about 80 min (results not shown), but had partially recovered after 3 h incubation. The cells were refractory to LPA at this time, as addition of further LPA did not decrease dye transfer. However, communication in the LPA-refractory cells was inhibited by either EGF (Figure 2a) or PMA (Figure 2b). Similarly, cells became refractory to the EGF-induced inhibition of communication after 4 h (Figures 2c and 2d). Cells refractory to EGF retained their sensitivity to LPA (Figure 2b) and PMA (Figure 2d). The most likely explanation of these data is that all three

Figure 1 LPA stimulation of Cx43 phosphorylation

Immunoblot analysis of confluent WB cells incubated for 10 min in the absence (lanes 4, 5 and 6) or presence (lanes 1, 2 and 3) of 50 μM LPA as described in the Materials and methods section. Samples from stimulated and unstimulated cells were also incubated with alkaline phosphatase (lanes 2 and 5) or with alkaline phosphatase plus phosphatase inhibitors (lanes 3 and 6). All tracks were loaded with 20 μg of protein. Arrows indicate the LPA-stimulated phosphorylated proteins and the fastest migrating dephosphorylated protein. The migration of molecular-mass (kDa) markers is indicated on the right of the Figure.

Figure 2 Inhibition of gap-junctional communication by LPA

Cells were incubated with LPA (25 μ M; **a** and **b**) or with EGF (10 ng/ml; **c** and **d**) for the times indicated, and dye transfer was measured as described in the Materials and methods section. At the times indicated by the arrows, EGF (■; 10 ng/ml), LPA (●; 25 μ M), PMA (▲; 100 nM), BSA (○; 0.001%, w/v) or Me₂SO (△; 0.1%, v/v) was added, incubation was continued for various times, and dye transfer was measured. Data are means $\pm$ S.E.M. of 8 determinations.

Table 1 Effect of PMA and LPA on cell-cell communication in PMA-pretreated cells

Confluent WB cells were pretreated for 26 h with 100 nM PMA or 0.5% Me₂SO and then stimulated for 10 min with 100 nM PMA or 50 μ M LPA. Cell-cell communication was determined as described in the Materials and methods section. Results are the means ($\pm$ S.E.M.) of 10 separate determinations.

Treatment	Cell-cell communication (% of control)
PMA (26 h)	100
PMA (26 h) + PMA (10 min)	99.5 $\pm$ 4.2
PMA (26 h) + LPA (10 min)	2 $\pm$ 0.4
Me ₂ SO (26 h)	100
Me ₂ SO (26 h) + PMA (10 min)	0.4 $\pm$ 0.1
Me ₂ SO (26 h) + LPA (10 min)	3.2 $\pm$ 0.4

agonists initiate their effects on communication through different receptor systems that are down-regulated by the specific ligand. In the case of PMA, the receptor is likely to be the protein kinase C family of proteins, and preincubation of WB cells with the phorbol ester for 6 h has been shown to cause a marked loss of Ca²⁺- and phospholipid-dependent kinase activity [21]. Separate experiments established that pretreatment of WB cells with PMA blocked the capacity of the phorbol ester to inhibit dye transfer, but did not decrease the inhibitory effect of LPA (Table 1). Similarly, pretreatment with PMA did not abolish the capacity of LPA to induce the mobility shift of Cx43 proteins in extracts of WB cells (Figure 3). It cannot be concluded from these data that protein kinase C is not required for the effects of LPA on Cx43 phosphorylation, as it has not been established that PMA down-regulates all sub-species of the enzyme in WB cells. It is

Figure 3 LPA stimulation of Cx43 phosphorylation in PMA-pretreated cells

Immunoblot analysis of Cx43 from WB cells pretreated with PMA (100 nM) for 24 h and then stimulated for 10 min with 100 nM PMA (lane 2) or with 25 μ M LPA (lane 3) (lane 1, untreated control). All tracks were loaded with 20 μ g of protein. The migration of molecular-mass (kDa) markers is indicated on the right of the Figure.

Figure 4 Time course of LPA-induced inhibition of dye transfer and activation of MAP kinase

Cells were incubated with 25 μ M LPA for the times indicated. Dye transfer (**a**) and MAP kinase activity in partially purified extracts (**b**; □ symbols represent cells incubated with BSA alone) were determined as described in the Materials and methods section. Data represent means $\pm$ S.E.M. of 8 determinations (**a**) or means of duplicate determinations (**b**). Similar data were obtained in two replicate experiments.

clear, however, that sub-species of protein kinase C which are sensitive to PMA are not involved.

The results shown in Figures 1, 2 and 3 suggest that LPA stimulates the phosphorylation of Cx43 by a protein kinase(s) which is probably independent of protein kinase C and that the phosphorylation is associated with decreased cell-cell communication. Although the kinases responsible for Cx43 phosphorylation are not known, previous reports have indicated that LPA activates MAP kinase [12] and that Cx43 protein is a substrate for purified MAP kinase [16]. In the present experiments, we observed a close correlation between the ability of LPA to inhibit communication and to activate MAP kinase activity (Figure 4). In this experiment, MAP kinase in soluble extracts was partially purified by phenyl-Sepharose chromatography and assayed with myelin basic protein as substrate. LPA induction of MAP kinase

Figure 5 Effect of LPA concentration on inhibition of dye transfer and activation of MAP kinase

Cells were incubated with 25 μM LPA; dye transfer was measured after 10 min (a) and MAP kinase activity after 2 min (b). Data are means ± S.E.M. of 8 determinations (a) or means of duplicate determinations (b). Similar data were obtained in a replicate experiment.

Table 2 Effects of agonist pretreatment on MAP kinase activity

Cells were pretreated with LPA (25 μM, 3 h), EGF (10 ng/ml, 4 h) or PMA (100 nM, 26 h) and were further incubated for 5 min with BSA (0.001%), LPA (25 μM), buffer, EGF (10 ng/ml), Me₂SO (0.1%) or PMA (100 nM). MAP kinase activity was assayed as described in the Materials and methods section. Results are expressed as fold stimulation over the appropriate controls. Kinase activity in the control samples after LPA, EGF or PMA pretreatment was 306, 886 or 166 fmol of P_i/μg of protein respectively. Abbreviation: ND, not determined. In a separate experiment, incubation of control cells with 100 nM PMA, 25 μM LPA or 10 ng/ml EGF for 5 min caused a 4.8-, 6.5- or 7.7-fold increase in MAP kinase activity respectively.

Treatment	MAP kinase activity (fold stimulation over control)		
	LPA-pretreated	EGF-pretreated	PMA-pretreated
LPA	0.87	2.60	3.20
EGF	5.50	1.04	9.60
PMA	ND	ND	1.10

activity was rapid and transient (Figure 4), with a clear increase after 1 min. Dye transfer was not inhibited after incubation for 1 min, but was marked after 2 min (Figure 4). Similarly, in Western-blotting experiments, mobility shift of Cx43 proteins could not be detected after 1 min, but was apparent after 2 min (results not shown). Effects on both communication and MAP kinase activation were apparent at a concentration of 0.3 μM LPA (Figure 5). In addition, cells which had been made refractory to the inhibition of communication by long-term incubation with LPA (see Fig. 2) were also refractory to the LPA-induced activation of MAP kinase, but retained sensitivity to EGF (Table 2). Similarly, cells pretreated with EGF were refractory to the further addition of EGF, but were stimulated by incubation with LPA. Preincubation of WB cells with PMA abolished the induction of MAP kinase by the phorbol ester, but did not block the activation by EGF or by LPA (Table 2).

Figure 6 Immunoblot analysis of MAP kinase incubated with EGF, LPA and PMA

Cells were incubated with buffer (lane 1), EGF (10 ng/ml; lane 2), BSA (0.001%; lane 3), LPA (25 μM; lane 4), Me₂SO (0.1%; lane 5) or PMA (100 nM; lane 6) for 5 min. MAP kinase was partially purified as described in the Materials and methods section by hydrophobic adsorption to phenyl-Sepharose CL-4B. Samples (1.7 μg of protein) were electrophoresed and subjected to Western blotting using anti-(MAP kinase) antibody (see the Materials and methods section). Arrows indicate the migration of the 42 and 44 kDa MAP kinase isoforms in control cells.

Although the mechanism for the LPA induction of MAP kinase activity is not known, it has recently been reported that LPA activates p21^{ras} in fibroblasts [10]. It is therefore possible that activation of ras is part of the LPA-induced MAP kinase cascade, as has been reported for a number of mitogens [13–15].

In separate experiments we established that proteins of molecular mass 42 and 44 kDa could be detected in the partially purified extracts by Western blotting using an antibody directed against amino acids 63–98 of rat 43 kDa erk1 MAP kinase (Figure 6, lanes 1, 3 and 5). This antibody has been reported to recognize both the 42 and 44 kDa forms of MAP kinase [27]. Incubation of WB cells with EGF (Figure 6, lane 2), LPA (Figure 6, lane 4) or PMA (Figure 6, lane 6) for 5 min resulted in the appearance of immunologically reactive protein with a mobility intermediate between those of the 42 and 44 kDa isoforms and another greater than 44 kDa (Figure 6). This result suggests that all three agonists cause the phosphorylation and activation of predominately the 42 and 44 kDa forms of MAP kinase. In the case of EGF, there was an almost complete conversion into the more slowly migrating forms. The above result was confirmed by chromatography of the soluble extracts on Mono Q columns (Figure 7). It is clear that incubation of WB cells with EGF, LPA or PMA results in two major peaks of MAP kinase activity. Immunoblotting of peak fractions confirmed that the early-eluted peak contained predominantly the 42 kDa form and the later-eluted peak the 44 kDa form of MAP kinase (results not shown).

This is the first report of a specific phosphorylation event triggered by LPA. This simple phospholipid has all the hallmarks of a new class of signalling molecule, and it will be of great interest to determine whether LPA or PA generated metabolically have similar signalling functions. However the observations that serum contains LPA [28], and that activated platelets secrete the phospholipid, suggest that a model involving interaction with a surface receptor is physiologically feasible. It is also of considerable interest that LPA activates the MAP kinase cascade (see also [12]), raising the possibility that a kinase associated with this cascade is responsible for Cx43 phosphorylation. It is noteworthy that all the agonists used in this study (LPA, EGF and PMA) which inhibit cell–cell communication in WB cells also activate the two major isoforms of MAP kinase. It therefore appears that divergent signalling pathways associated with different receptor systems converge at some point to activate MAP kinase. It is therefore possible that MAP kinase may be responsible for phosphorylation of Cx43 by all three agonists. However, we stress that we currently have no direct evidence that a component of the MAP kinase cascade is responsible for the phosphorylation of Cx43 protein and decrease in cell–cell communication in WB cells. No attempt has been made in the present studies to compare quantitatively the extent of MAP kinase

Figure 7 Resolution of MAP kinase isoforms by Mono Q chromatography

Cells were incubated with BSA (0.001 %; ●), EGF (10 ng/ml) plus BSA (○), or LPA (25 μ M; ■) (a) or with Me₂SO (0.1 %; ●) or PMA (100 nM; □) (b) for 5 min. Cells were disrupted in buffer A, centrifuged, and the supernatants were chromatographed on Mono Q columns as described in the Materials and methods section. MAP kinase activity in fractions was assayed as in the Materials and methods section.

induction, Cx43 phosphorylation and inhibition of communication by EGF, LPA and PMA. Definitive establishment of a relationship between these events will require identification of the sites on Cx43 which are phosphorylated *in vitro* and *in vivo*, and site-directed-mutagenesis studies to establish whether phosphorylation of these sites is involved in regulating gap-junction permeability. However, in a separate report it has been shown that recombinant Cx43 is a substrate for MAP kinase *in vitro* [16]. We have also shown that a synthetic peptide corresponding to amino acids 247–260 of rat heart Cx43 [26] is a substrate for partially purified MAP kinase from LPA- or EGF-stimulated WB cells (results not shown). This peptide contains the sequence proline–leucine–serine–proline, which is the optimal primary sequence for phosphorylation by MAP kinase [15]. In current experiments we are attempting to identify the protein kinase responsible for phosphorylating Cx43 and to characterize the phosphorylation sites on the protein. However, although phosphorylation of recombinant Cx43 by MAP kinase has been reported [16], in preliminary experiments we have been unable to phosphorylate CX43 protein present in the alkali-enriched gap-junctional preparations used in the present studies (C. S. T. Hii, unpublished work). Other reports have indicated that Cx32 protein in rat liver gap junctions is a poor substrate for

cyclic-AMP-dependent protein kinase, protein kinase C or Ca²⁺/calmodulin-dependent protein kinase [29]. The latter authors concluded that the alkaline extraction caused denaturation of protein, which limited access of the kinases to the connexin protein. We are currently exploring alternative ways of isolating gap junctions (e.g. detergent extraction) which may yield protein which is an improved substrate for added kinases.

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