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Identification of a putative membrane receptor for the bioactive phospholipid, lysophosphatidic acid

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Lysophosphatidic acid (LPA) is a naturally occurring phospholipid with hormone- and growth factor-like activities. Exogenous LPA stimulates GTP-dependent phosphoinositide hydrolysis and inhibits adenylate cyclase in its target cells, but the site of action of LPA is unknown. We now report the identification by photoaffinity labeling of a putative LPA membrane receptor in various LPA-responsive cell types. A ^{32}P-labeled LPA analogue containing a photoreactive fatty acid, [^{32}P]diazirine-LPA, labels a membrane protein of apparent molecular mass of 38–40 kDa in various cell types, including neuronal cells, brain homogenates, carcinoma cells, leukemic cells and normal fibroblasts. Labeling of the 38–40 kDa protein is competitively inhibited by unlabeled 1-oleoyl-LPA (IC_{50} ~10 nM), but not by other phospholipids. Specific labeling is not detected in rat liver membranes or in human neutrophils, which are physiologically unresponsive to LPA. Suramin, an inhibitor of both early and late events in the action of LPA, completely inhibits the binding of photoreactive LPA. We suggest that the 38–40 kDa protein represents a specific LPA cell surface receptor mediating at least part of the multiple cellular responses to LPA.

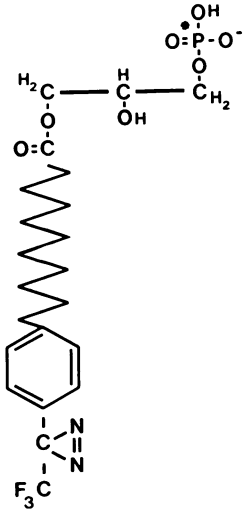
Key words:lysophosphatidic acid/photoaffinity labeling/receptor

Introduction

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycerol-3-phosphate) is an intriguing phospholipid with a broad spectrum of biological activities, including platelet activation (Benton *et al.*, 1982), smooth muscle contraction (Tokumura *et al.*, 1980), induction of neuronal shape changes (K.Jalink, T.Eichholtz and W.H.Moolenaar, submitted for publication) and, in fibroblasts, cell proliferation (van Corven *et al.*, 1989, 1992). LPA is rapidly produced in activated platelets (Watson *et al.*, 1985; Gerrard and Robinson, 1989) and could possibly be secreted following its formation, in common with other bioactive lipids (e.g. platelet activating factor; Prescott *et al.*, 1990). Although exogenous LPA is partially metabolized by cells, current evidence suggests that the biological activity of LPA is not attributable to one of its metabolites (van der Bend *et al.*, 1992a)

LPA initiates its action at the level of the plasma membrane by stimulating phospholipases C and D and inhibiting adenylate cyclase in a G protein-dependent manner (van Corven *et al.*, 1989; Jalink *et al.*, 1990; Plevin *et al.*, 1991; van der Bend *et al.*, 1992b). A key question concerns the mechanism by which extracellular LPA activates G protein-mediated signaling pathways at the inner side of the plasma membrane. An attractive and plausible hypothesis is that LPA interacts directly with its own G protein-coupled receptor. The finding that LPA action is cell type-specific and that the cellular responses to LPA are subject to homologous desensitization (Jalink *et al.*, 1990), provide support for the receptor hypothesis. On the other hand, it is conceivable that LPA acts by inducing specific structural changes in the lipid bilayer and thereby activates signal transduction cascades in a receptor-independent fashion; however, there is currently no precedent, to our knowledge, for such a 'bilayer-perturbation' mechanism of cell activation.

In the present study, we have used a newly designed photoaffinity analogue of LPA containing 11-{4-[3-(trifluoromethyl)-diazirinyl]phenyl}-undecanoic acid (Harter *et al.*, 1988; Niggli *et al.*, 1990) as a photoreactive fatty acid residue ([^{32}P]diazirine-LPA; see Figure 1) in an attempt to identify by photo-crosslinking membrane proteins that interact with LPA in a specific manner. Upon photolysis, the diazirine group forms a highly reactive carbene, which



[^{32}P]–diazirine–LPA

Fig. 1. Structure of photoreactive LPA used in the present study. The photoreactive fatty acyl chain is 11-{4-[3-(trifluoromethyl)-diazirinyl]phenyl}-undecanoic acid; the ^{32}P label is in the polar headgroup as indicated (*). See Materals and methods for chemical synthesis and further details.

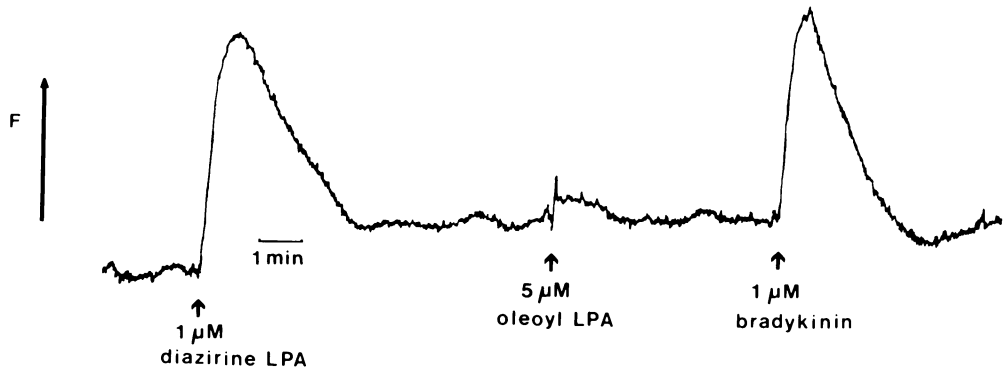


Fig. 2. Ca^{2+} signals in LPA-responsive A431 cells. Typical time course of changes in cytoplasmic free Ca^{2+} in human A431 cells following stimulation with diazirine-LPA, 1-oleoyl-LPA and bradykinin as indicated. Arrows indicate time of addition of agonist. Ca^{2+} transients were recorded as changes in indo-1 fluorescence (F). Resting $[Ca^{2+}]_i$ in A431 cells is in the 170–190 nM range (Moolenaar *et al.*, 1986), while peak values are estimated to be close to 500 nM.

binds covalently to acyl chains of neighboring lipids and amino acid side chains of integral membrane proteins (Brunner, 1989). Here we show that diazirine-LPA can be cross-linked in a specific manner to a membrane protein with apparent molecular mass 38–40 kDa in various LPA-responsive cell types and in rat brain homogenates. We conclude that the photolabeled protein represents a specific LPA cell-surface receptor mediating at least part of the multiple biochemical and biological activities of LPA.

Results

Binding studies using ^{32}P-labeled LPA

In initial experiments we sought to detect specific and saturable binding of 1-oleoyl-[^{32}P]LPA to various LPA-responsive cell types. Although time-dependent association of [^{32}P]LPA with either Rat-1 fibroblasts, human A431 carcinoma cells or mouse N1E-115 neuroblastoma cells was readily detectable at 0°C, our efforts to measure significant displacement of the label in the presence of excess unlabeled LPA were unsuccessful. Thus, [^{32}P]LPA binding appeared to be largely non-specific, perhaps not surprising in view of the high lipophilicity of the ligand, resulting in extensive intercalation into the lipid bilayer. We therefore turned to a different strategy that involves photoaffinity labeling of LPA binding proteins using newly designed [^{32}P]diazirine-LPA as a probe (Figure 1).

Biological activity of diazirine-LPA

We first examined whether diazirine-LPA (non-radiolabeled) shows biological activity in LPA-responsive cells. When tested over the concentration range 0.1–1 μM, diazirine-LPA turned out to be about as potent as natural 1-oleoyl- or 1-palmitoyl-LPA at inducing Ca^{2+} mobilization in human A431 cells (Figure 2) and evoking rapid shape changes in mouse N1E-115 neuroblastoma cells (results not shown). Moreover, the Ca^{2+} response to 1-oleoyl-LPA, but not that to bradykinin, was fully desensitized by pretreatment of the cells with 1 μM diazirine-LPA (Figure 2), strongly suggesting that diazirine-LPA acts at the same cellular site(s) as natural LPA.

Photoaffinity labeling of an LPA binding protein in intact N1E-115 cells

Photoreactive [^{32}P]diazirine-LPA, used at a concentration of ~1 nM, was cross-linked at 0°C to mouse N1E-115 neuroblastoma cells, a cell type showing various rapid responses to LPA including phosphoinositide hydrolysis and cytoskeletal reorganization (K.Jalink, T.Eichholtz and W.H.Moolenaar, manuscript submitted). Following cell lysis, SDS gel electrophoresis reveals the presence of one major labeled polypeptide of 38 kDa (Figure 3). The labeled material migrating with the dye front presumably represents free [^{32}P]diazirine-LPA and photolabeled lipids. Labeling of the 38 kDa protein was already detectable after 2 min of UV irradiation (at 0°C), while optimal labeling was observed at 5–15 min (Figure 4). A similar labeling pattern was observed when cells were preincubated for 1 h at 0°C

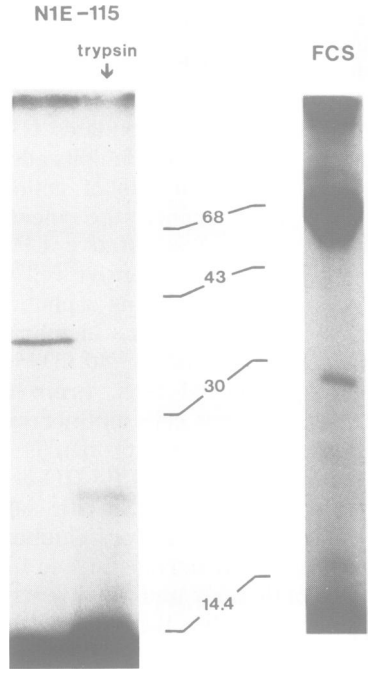


Fig. 3. Photoaffinity cross-linking of [^{32}P]diazirine-LPA to N1E-115 cells and serum proteins. N1E-115 cells (left two lanes) and serum proteins (1% FCS in PBS; right lane) were incubated with [^{32}P]diazirine-LPA (~1 nM), irradiated for 10 min on ice and prepared for SDS–PAGE and autoradiography as described in Materials and methods. Trypsin sensitivity was determined by treating labeled cells (2×10^6/ml) with 1 mg/ml trypsin (in PBS) for 15 min on ice, followed by centrifugation and lysis in sample buffer. 100 μg of FCS-protein was loaded on the gel. The molecular mass markers in the middle are in kDa.

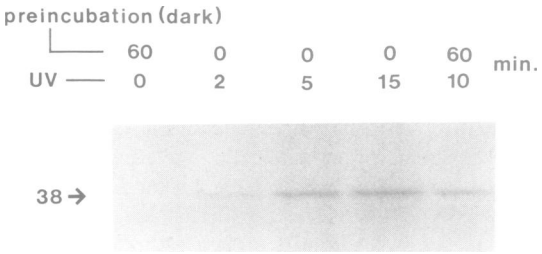


Fig. 4. Time dependency of photoaffinity labeling of the 38 kDa protein. N1E-115 cells were preincubated with photoreactive LPA (0.5 nM) for the indicated periods of time (0 or 60 min) followed by UV irradiation (0–15 min) as described in Materials and methods. No labeling is detectable in non-irradiated cells.

with photoreactive LPA followed by irradiation for 10 min (Figure 4). Running the gel under non-reducing conditions did not affect the overall labeling pattern. After treatment of labeled cells with trypsin (1 mg/ml, 15 min on ice) prior to cell lysis, the 38 kDa polypeptide is completely digested resulting in the formation of a single labeled fragment of ~23 kDa (Figure 3).

Although cells were extensively washed prior to photoaffinity labeling, we wished to eliminate the possibility that the 38 kDa protein represents a residual serum component adsorbed onto the cell surface to which diazirine-LPA is artifactually cross-linked. When diazirine-LPA was photolysed in a fetal calf serum (FCS)-containing buffer, we observed binding of the probe to serum albumin (68 kDa) and to 28 and 15 kDa serum proteins, with no detectable labeling at the 38 kDa region of the autoradiograph (Figure 3). Furthermore, the intensity of the 38 kDa band was not decreased after long-term serum deprivation of the cells (24–48 h). From these observations we conclude that the 38 kDa LPA binding protein is of cellular origin.

Labeling specificity and inhibition by suramin

Photolabeling of the 38 kDa polypeptide in N1E-115 cells is competitively inhibited by unlabeled LPA in a concentration-dependent manner (Figure 5A). The concentration of unlabeled LPA required for 50% inhibition of diazirine-LPA cross-linking (IC_{50}) is close to 10 nM, as estimated by quantitative densitometric analysis; this value falls within the dose–response relationship for LPA-induced early events (Jalink *et al.*, 1990). The cross-linking was not inhibited by other phospholipids, including lysophospholipids, phosphatidic acid (Figures 5B and C) and the lipid mediator 1-*O*-hexadecyl-2-acetylglycero-3-*sn*-phosphocholine (platelet activating factor; PAF) (result not shown), even when added at >1000-fold molar excess (1 μM), although lysophosphatidylglycerol at this high concentration reduced labeling to some extent (Figure 5B). When the photoaffinity experiments were carried out with [^{32}P]diazirine-phosphatidic acid as a probe, no protein band was labeled (not shown).

Specific antagonists of LPA have not been described until now; however, the polyanionic compound suramin, a broad-specificity inhibitor of ligand–receptor interactions, was recently reported to reversibly inhibit LPA-induced phosphoinositide hydrolysis and DNA synthesis (van Corven *et al.*, 1992 and references therein). Suramin can form complexes with proteins, thereby modifying the protein tertiary structure, which might explain its ligand-displacing properties (Müller and Wolpert, 1976). Figure 5D illustrates

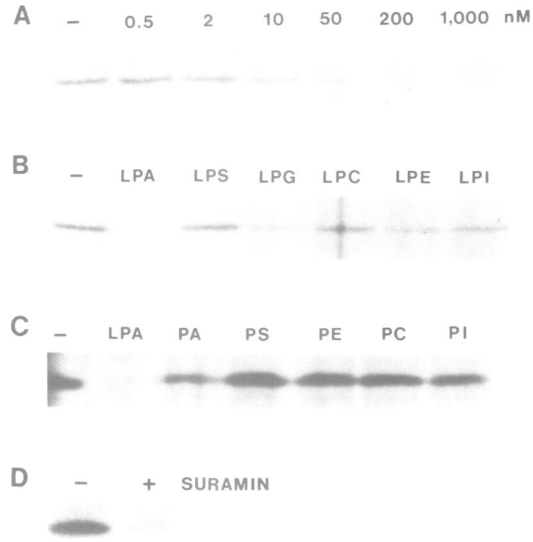


Fig. 5. Competition for 38 kDa protein labeling by unlabeled LPA, other phospholipids and suramin. N1E-115 cells (2×10^6/ml) were reacted with 0.3 nM [^{32}P]diazirine-LPA in the presence of varying concentrations of oleoyl-LPA (**A**) or other lysophospholipids (1 μM; **B**). A preincubation period of 1 h in the dark was followed by UV irradiation for 5 min. Similar results were obtained when the period of preincubation in the dark was omitted. Other phospholipids (1 μM) (**C**) do not inhibit photolabeling (10 min) with [^{32}P]diazirine-LPA (0.5 nM). (**D**) photolabeling (10 min) with 0.2 nM [^{32}P]diazirine-LPA in the absence (–) or presence (+) of suramin (70 μM), added 10 min prior to photolysis. Samples were analyzed as described in Materials and methods.

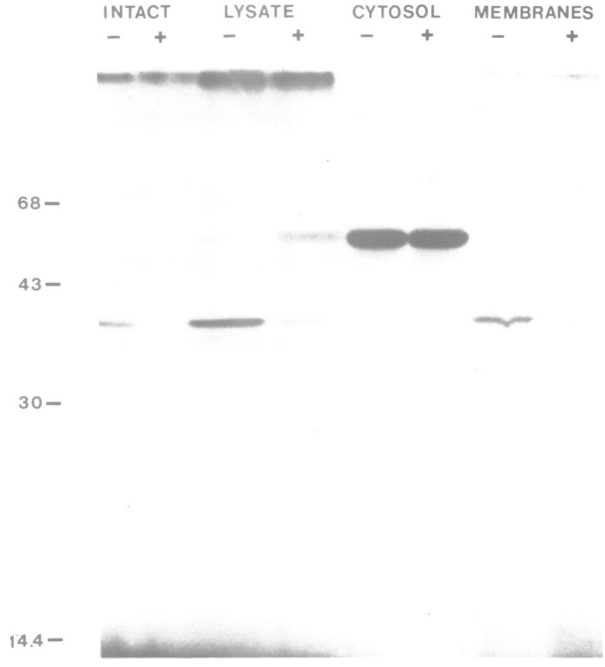


Fig. 6. Photoaffinity labeling of membranes and cytosol prepared from N1E-115 cells. Intact cells (2×10^6 cells/ml), cell lysate, cytosolic and membrane fractions, prepared as described in Materials and methods, were photolabeled with [^{32}P]diazirine-LPA (0.8 nM) in the absence (–) or presence (+) of 1 μM oleoyl-LPA. Protein concentrations of cell fractions were ~1 mg/ml. 150 μg of protein was loaded per slot.

that suramin strongly inhibits labeling of the 38 kDa protein in N1E-115 cells, when used at a concentration that has been shown to block LPA biological activity in fibroblasts (70 μM; van Corven *et al.*, 1992).

Further characterization of the LPA binding protein

Specific cross-linking of diazirine-LPA to the 38 kDa protein is equally effective in the membrane fraction of N1E-115 cells as in intact cells, whereas the 38 kDa protein is not detectable in the cytosolic fraction (Figure 6). In contrast, cross-linking to the cytosolic fraction reveals a dominant LPA binding protein of ~55 kDa. However, labeling of this cytosolic protein is not inhibited by excess unlabeled LPA, indicative of 'non-specific' binding. Figure 6 also illustrates that the binding of LPA to the 55 kDa cytosolic protein is significantly reduced in the presence of membranes ('whole cell lysates'), presumably due to partitioning of LPA into lipid bilayers. The labeled 38 kDa protein could be completely solubilized in 0.5% (v/v) Triton X-100, suggesting that the protein is not tightly associated with the cytoskeleton.

We also examined the effects of several glycosylation inhibitors on the specific labeling pattern. Neither tunicamycin (20 μg/ml; 48 h), nor 1-deoxymannojirimycin (0.5 mM; 48 h), neuraminidase (5 U/ml), or endoglycosidase F/N-glycanase F (1.3 U; incubation with a NP-40 cell lysate overnight) were able to affect the electrophoretic mobility of the cross-linked protein (not shown). This suggests that the LPA binding protein contains little or no N-linked oligosaccharides.

LPA binding proteins in other cell types

Specific LPA cross-linking to a 38 kDa polypeptide is also observed in membranes isolated from rat brain homogenates; labeling is undetectable in rat liver membranes, whereas membranes from lung tissue show a very faint band at 38 kDa, which is absent in the presence of 1 μM unlabeled oleoyl-LPA (Figure 7). NG108-15 neuroblastoma×glioma hybrids show a similar labeling pattern as N1E-115 cells (Figure 8). In non-neuronal cells, including human A431 carcinoma cells, K562, HL60 and Jurkat T leukemic cells, a similar polypeptide is specifically labeled, but its apparent molecular mass is slightly higher (40 kDa) than in the neuronal cells tested (Figure 8). In marked contrast, no specific protein labeling is detected in human neutrophils (Figure 8), also at higher (14 nM) concentrations of [^{32}P]diazirine-LPA (results not shown). These cells are known to be unresponsive to LPA with respect to Ca^{2+} mobilization, chemotaxis and oxygen radical production (Jalink *et al.*, 1990; A.J.Verhoeven, unpublished observations; we note that, contrary to previous findings, LPA does evoke calcium mobilization in Jurkat and K562 cells; Jalink *et al.*, 1990 and unpublished observations).

LPA-responsive Rat-1 cells (van Corven *et al.*, 1989; van der Bend *et al.*, 1992b) also show specific binding of diazirine-LPA to a 40 kDa protein, although elevated concentrations (8 nM) of photoreactive ligand were required for optimal visualization of the photolabeled band in these cells (Figure 9). A similar observation was made for human fibroblasts (results not shown).

Discussion

Apart from its critical precursor role in *de novo* lipid biosynthesis, the simple phospholipid LPA has remarkable hormone- and growth factor-like activities when added exogenously to cells (reviewed by Moolenaar, 1991). Although the intracellular signaling pathways activated by exogenous LPA have been elucidated in some detail (van

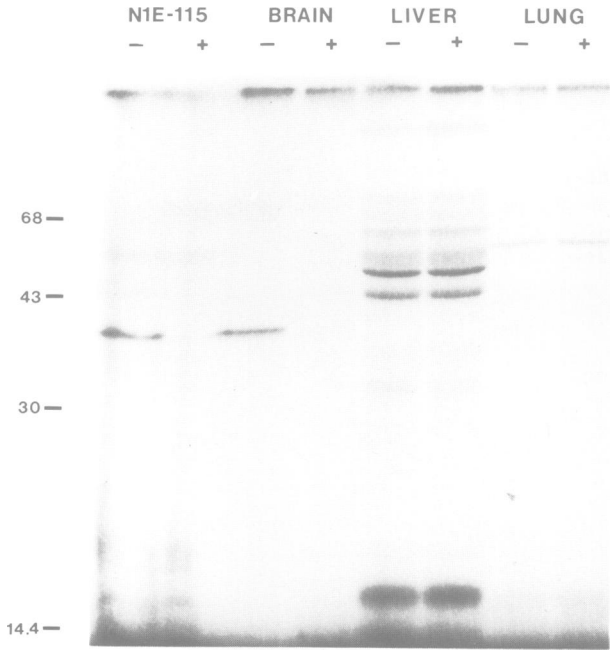


Fig. 7. Photoaffinity labeling of membranes prepared from different rat tissues. N1E-115 cells (2×10^6/ml) and membranes from rat brain, liver and lung were irradiated using [^{32}P]diazirine-LPA (0.8 nM) in the absence (–) or presence (+) of 1 μM oleoyl-LPA. Protein concentrations were 1.0, 1.7 and 1.3 mg/ml for brain, liver and lung membranes, respectively. 200 μg of membrane protein was loaded per slot.

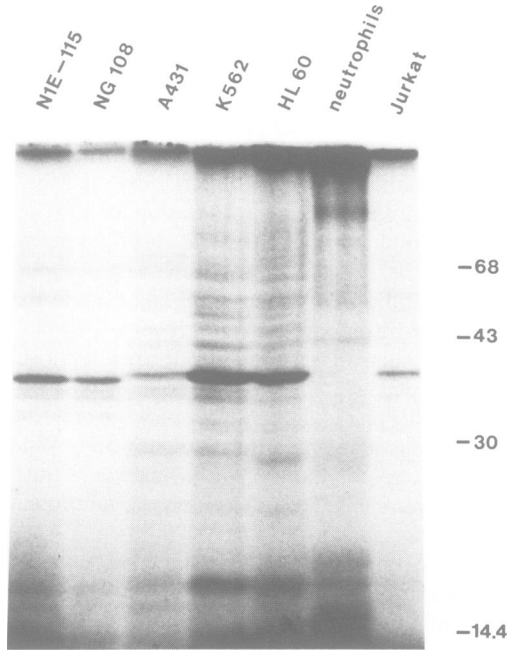


Fig. 8. Photoaffinity labeling of different cell lines and human neutrophils. Cells ($2-5\times10^6$/ml) were irradiated for 10 min in the presence of 1 nM [^{32}P]diazirine-LPA and analyzed by SDS–PAGE and autoradiography as described in Materials and methods.

Corven *et al.*, 1989; Jalink *et al.*, 1990; Plevin *et al.*, 1991; van der Bend *et al.*, 1992a,b), the precise site of action of LPA has remained elusive to date. Yet the early cellular responses to LPA have many of the hallmarks of receptor-mediated events. First, LPA action is cell type specific in that no early responses to LPA are observed in neutrophils (Jalink *et al.*, 1990). Second, the kinetics of LPA-induced signaling events, notably Ca^{2+} mobilization, are virtually indistinguishable from those evoked by an authentic receptor ligand such as bradykinin in the same cell type (Figure 2 and Jalink *et al.*, 1990). Third, responses to LPA are subject to homologous desensitization (Jalink *et al.*, 1990) and, fourth, microinjected LPA fails to mimic the action of extracellular LPA (Fernhout *et al.* 1992; K.Jalink, T. Eichholtz and W.H.Moolenaar, submitted), consistent with an extracellular site of action and suggesting that there is no apparent role for transbilayer 'flip-flop' or internalization of the lipid (although the possibility remains that later responses such as DNA synthesis may indeed involve such action). Moreover, catabolic breakdown of LPA cannot account for its biological activities (Van der Bend *et al.*, 1992a).

In this study, we have identified a 38–40 kDa membrane protein in a number of cell types that binds specifically a photoreactive LPA analogue, diazirine-LPA. This LPA binding protein is likely to represent a specific LPA receptor, mediating at least part of the various cellular responses to LPA. Its identification as a putative LPA receptor is supported by the following findings: (i) binding is specifically inhibited by bioactive 1-oleoyl-LPA in a concentration-dependent manner (IC_{50} ~10 nM), but not by other (lyso)phospholipids; (ii) the cross-linked protein is membrane bound, Triton soluble, and fully digested by trypsin treatment of intact cells; (iii) optimal diazirine-LPA binding is observed at nanomolar concentrations, whilst the concentration range at which unlabeled LPA inhibits the binding of the photoreactive LPA (2–200 nM) parallels the dose–response relationships for LPA-induced Ca^{2+} mobilization, inositol phosphate formation and neuronal shape changes (Jalink *et al.*, 1990; Plevin *et al.*, 1991; K.Jalink, T.Eichholtz and W.H.Moolenaar, submitted); (iv) binding is inhibited by the polysulfonated compound suramin, a 'broad-specificity' inhibitor of ligand–receptor interactions in general and an inhibitor of LPA biological activity in particular (van Corven *et al.*, 1992 and references therein); suramin is membrane impermeant and appears to act on proteins rather than lipids (Müller and Wolpert, 1976; van Corven *et al.*, 1992 and references therein); (v) no LPA binding protein is detectable in human neutrophils, which are physiologically unresponsive to LPA.

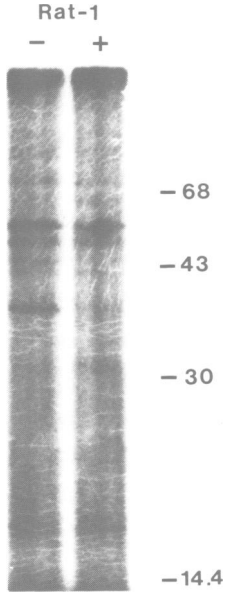


Fig. 9. Photoaffinity labeling of Rat-1 fibroblasts. Photolabeling of Rat-1 cells (5×10^6/ml) was carried out using 8 nM [^{32}P]diazirine-LPA (10 min irradiation) in the absence (−) or presence (+) of 1 μM oleoyl-LPA.

In the past, diazirine-containing fatty acids and phospholipids have mainly been used for topological and structural rather than functional studies of membrane proteins (Brunner, 1989). Consistent with a fairly uniform lateral distribution of these reagents within membranes and as a result of the high reactivity of the photogenerated carbene, all membrane constituents are usually labeled upon photolysis, albeit with vastly different efficiencies. Labeling of integral proteins was generally found to be very inefficient (Brunner, 1989). The present study now represents one of the rare examples where the technique of photoaffinity labeling (Chowdhry and Westheimer, 1979) has successfully been used to identify a putative membrane receptor for a bioactive phospholipid. A conceptually similar study is the photolabeling of the prostaglandin E_2 receptor in cardiac sarcolemmal vesicles using an azido ligand (Michalak *et al.*, 1990). Although it appears that only a small fraction of the total membrane-associated diazirine-LPA actually binds to the putative receptor protein, labeling of this protein is highly specific and far more efficient than any 'non-specific' protein labeling by free diazirine-LPA. This is not surprising since the specific labeling results from a 'quasi-intramolecular' reaction, a process expected to be far more efficient than intermolecular labeling. However, since the labeling efficiency of diazirine-LPA to its putative receptor is as yet unknown, the present results do not allow reliable estimates of the number of specific LPA binding sites per cell to be derived.

What may be the molecular structure of the putative LPA receptor? Given the GTP dependence of LPA-induced phosphoinositide hydrolysis and the pertussis toxin sensitivity of adenylate cyclase inhibition, one would predict the LPA receptor to be a member of the superfamily of seven-transmembrane-domain receptors, which couple to G proteins. Indeed, the apparent molecular mass of the putative LPA binding protein (38–40 kDa) falls into the range of that of known G protein-coupled receptors. There is precedent for lipid mediators binding to their cognate seven-transmembrane-domain receptor: the ether phospholipid platelet-activating factor and the bioactive arachidonate metabolite, thromboxane A2, each bind to a G protein-coupled receptor of predicted molecular mass 39 kDa and 37 kDa, respectively (Honda *et al.*, 1991; Hirata *et al.*, 1991). Obviously, direct proof that the 38–40 kDa LPA binding protein identified here is a G protein-coupled receptor awaits elucidation of its amino acid sequence. The present study provides a first step towards that goal.

Materials and methods

Materials

1-oleoyl-*sn*-glycero-3-phosphate (lysophosphatidic acid; oleoyl-LPA), 1,2-diacyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine; PC) (from egg yolk), 1,2-diacyl-*sn*-glycero-3-phosphate (phosphatidic acid; PA) (from egg

yolk), 1,2-diacyl-*sn*-glycero-3-phosphoinositol (phosphatidylinositol; PI) (from soybean), 1,2-diacylglycerol-*sn*-glycero-3-phosphoethanolamine (phosphatidylethanolamine; PE) (from bovine brain), 1,2-diacyl-*sn*-glycero-3-phosphoserine (phosphatidylserine; PS) (from bovine brain; additionally TLC-purified), 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine; LPC), 1-acyl-*sn*-glycero-3-phosphoserine (lysophosphatidylserine; LPS) (from bovine brain), 1-acyl-*sn*-glycero-3-phosphoethanolamine (lysophosphatidylethanolamine; LPE) (from bovine brain), 1-acyl-*sn*-glycero-3-phosphoglycerol (lysophosphatidylglycerol; LPG) (from egg yolk), 1-acyl-*sn*-glycero-3-phosphoinositol (lysophosphatidylinositol; LPI) (from soybean), 1,2-dioleoyl-*sn*-glycerol (DG) and neuraminidase (type V) were obtained from Sigma (St Louis, MO). Tunicamycin was obtained from Calbiochem (La Jolla, CA), 1-deoxymannojirimycin and endoglycosidase F/N-glycanase F from Boehringer (Mannheim, Germany), indo-1-acetoxymethylester from Molecular Probes (Oregon), suramin from Bayer (Leverkusen, Germany) and [γ-^{32}P]ATP (3000 Ci/mmol) from Amersham (UK).

Cell culture

Mouse N1E-115 neuroblastoma cells, mouse neuroblastoma×rat glioma NG108-15 cells, Rat-1 fibroblasts, human fibroblasts and human A431 carcinoma cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Jurkat T cells and HL60 cells were grown in RPMI1640 medium containing 10% FCS and K562 erythroleukemic cells in Iscove's modified Dulbecco's medium containing 10% FCS. Human neutrophils were freshly isolated as described (Kramer *et al.*, 1989). All cultured cells were harvested without using trypsin. N1E-115 and NG108-15 cells were suspended in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) by gently shaking the culture flasks, while other cell types were detached by scraping them in Ca^{2+}/Mg^{2+}-free PBS. Immediately before labeling experiments, suspended cells were thoroughly washed with PBS to remove residual serum proteins.

Preparation of crude membranes

Rat brain and lung tissue were homogenized with a Potter-Elvehjem tissue grinder in 250 mM sucrose, 20 mM Tris−HCl (pH 7.5), mouse N1E-115 neuroblastoma cells in PBS. Cell debris and nuclei were removed by centrifugation at 1500 *g* for 10 min and the supernatant fraction centrifuged at 100 000 *g* for 1 h to separate the cytosolic and membrane fractions. The crude membrane pellet was resuspended in homogenization buffer. Crude rat liver membranes were prepared according to the initial steps of the purification method of Emmelot *et al.* (1974).

Preparation of diazirine-LPA

Diazirine-derivatized fatty acid (11-{4-[3-(trifluoromethyl)-diazirinyl]phenyl}-undecanoic acid) was synthesized and purified according to Harter *et al.* (1988). Esterification of the cadmium chloride complex of glycerophosphocholine (42 mg; 0.096 mmol) with the diazirine-fatty acid (140 mg; 0.38 mmol), using dicyclohexylcarbodiimide (45 mg; 0.218 mmol) as the condensing agent and 4-(dimethylamino)pyridine (24 mg; 0.196 mmol) as the catalyst was performed essentially following the procedure of Sadownik *et al.* (1986). The reaction mixture (0.5 ml) was diluted with 6 ml of methanol/water (19:1, v/v) and treated with 1 g of Dowex 50×8 (H^+-form). The supernatant was concentrated and the residue subjected to silica gel column chromatography using chloroform/methanol (9:1, v/v) and chloroform/methanol/water (65:25:4, v/v/v) as the eluants. Fractions containing 1,2-bis(11-{4-[3-(trifluoromethyl)-diazirinyl]phenyl}-undecanoyl)-*sn*-glycero-3-phosphorylcholine (diazirine-PC) were pooled and concentrated *in vacuo*. The yield of diazirine-PC was 33 mg (36%).

Diazirine-PC was converted to diazirine-PA using phospholipase D (from cabbage; Sigma) according to Kates (1986). Extracted lipids were subsequently incubated with 70 units of phospholipase A_2 (from porcine pancreas; Sigma) in 0.1 M borate (pH 7.5) and 1 mM deoxycholate for 16 h at room temperature under argon. The diazirine-LPA formed was extracted according to Bligh and Dyer (1959) in the presence of 0.1 N acetic acid and separated on 2D-TLC (silica gel); first dimension: chloroform/methanol/0.88 N ammonia, 60:60:5, by vol (twice); second dimension: chloroform/methanol/acetic acid/H_2O, 50:30:4:4, by vol. Extraction of diazirine-LPA from silica was performed by suspending scraped material in chloroform/methanol/0.1 N acetic acid, 2:1:0.8, by vol. After removal of silica by centrifugation, chloroform extraction of diazirine-LPA was performed according to Bligh and Dyer (1959).

Preparation of [^{32}P]oleoyl-LPA, [^{32}P]diazirine-PA and [^{32}P]diazirine-LPA

Diazirine-PC (preparation described above) was converted to diazirine-DG with phospholipase C (from *Bacillus cereus*; Sigma) according to Kates (1986) followed by purification of diazirine-DG on a silica gel 60 TLC plate developed in hexane/methanol/diethylether (80:20:20, by vol).

1,2-dioleoyl-*sn*-glycerol (DG) and diazirine-DG (0.3−1 mg/ml) were converted to their ^{32}P-labeled phosphatidic acid (PA) analogues by incubation for 3 h at 30°C with DG kinase [1 mg protein, partially purified from cytosol of human white blood cells by DE-52 chromatography according to Schaap *et al.* (1990)] in the presence of 1 mg/ml phosphatidylserine, 1 mM deoxycholate, 10 mM $MgCl_2$, 50 mM Tris−HCl (pH 7.5), 10 mM NaF and 1.8 μM [^{32}P]ATP (270 Ci/mmol), in a total vol of 1 ml. Partial conversion to ^{32}P-labeled LPA forms was achieved by subsequent incubation of the reaction mixture with 70 units of phospholipase A_2 for 16 h at room temperature under argon. Lipids were extracted according to Bligh and Dyer (1959) in the presence of 0.1 N acetic acid and separated on 2D-TLC (silica gel) followed by extraction from the silica as described above for unlabeled diazirine-LPA. Diazirine-forms of LPA and PA migrated at almost the same position as oleoyl-LPA and -PA, respectively. Starting from [^{32}P]ATP and after the two enzymatic reactions, ^{32}P label recovered from the TLC plate was 5−10% for the diazirine-LPA fraction and 1−3% for the diazirine-PA fraction. Radiolabeled products were stored in chloroform/methanol (2:1; v/v) at −20°C under argon. Prior to use, radiolabeled LPA and PA analogues were dried under a stream of argon and suspended in PBS by a 1 min bath sonication.

Binding of [^{32}P]oleoyl-LPA to intact cells

Confluent, serum-deprived Rat-1 fibroblasts grown in 15 cm^2 dishes, or suspended, serum-deprived N1E-115 cells (in 1.5 ml vials) were washed with PBS. Next, cells were incubated with [^{32}P]oleoyl-LPA (0.05 nM in PBS) in the presence of varying concentrations of unlabeled oleoyl-LPA on ice (final volume 1.5 ml). After incubation, cells were washed once with 2 ml ice-cold PBS and scraped off the dish with a rubber policeman. N1E-115 cells (10^6 cells/ml) were centrifuged at 5000 *g* for 30 s and washed once. The amount of label in the scraped or pelleted cells was determined by liquid scintillation counting.

Photoaffinity labeling using [^{32}P]diazirine-LPA

After harvesting and extensive washing, suspended cells ($2-5\times10^6$ cells/ml) were incubated in 9 cm^2 dishes with 0.3−8 nM [^{32}P]diazirine-LPA in PBS (1 ml) at 0°C. The samples were irradiated at 0°C for 10 min using a CAMAG Standard UV light source (CAMAG, Switzerland) equipped with a low-pressure mercury lamp (8 W, maximal intensity at 350 nm) and a band-pass filter (200−450 nm). The distance from light source to cell suspension was 4 cm. Photolabeling experiments were also executed with [^{32}P]diazirine-PA as a probe. After photolysis, cells were centrifuged at 13 000 *g* for 30 s and taken up in either reducing or non-reducing sample buffer according to Laemmli (1970), followed by SDS gel electrophoresis (13% polyacrylamide) and autoradiography using XAR-5 films (Kodak) and an intensifying screen. Exposure times varied from 4 to 40 days. The amount of cellular material loaded per lane corresponded to $3-6\times10^5$ cells. Photolabeling of membranes, cytosol or serum was performed likewise with 0.8 nM [^{32}P]diazirine-LPA at protein concentrations specified in the figure legends.

Ca^{2+} measurements

Nearly confluent human A431 cells, grown on glass coverslips, were loaded with the fluorescent Ca^{2+} indicator indo-1 by incubating them with 5 μM indo-1-acetoxymethylester for 20 min at 37°C. Fluorescence monitoring, calibration procedures and other experimental details were similar to those described elsewhere (Jalink *et al.*, 1990).

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