Activation of protein kinase C by lysophosphatidic acid: dependence on composition of phospholipid vesicles

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Lysophosphatidic acid (LPA) has attracted recent attention as a major serum-derived regulator implicated in responses to vascular injury and inflammation, in tumour invasiveness and in neuronal signalling and remodelling. Although the possibility of a specific G-protein-coupled LPA receptor protein has been suggested, characterization of such a receptor is lacking. Since LPA can activate protein kinase C (PKC) pathways in many cells and PKC activators mimic many LPA effects, the possibility of more direct LPA effects on PKC was investigated. Phosphatidylcholine (PC)/phosphatidylserine (PS)/diacylglycerol (DAG) lipid vesicles of defined acyl chain composition were used to activate the enzyme. At total concentrations of saturated PC/PS+DAG vesicles (2–3 mM) that provided maximal PKC activation, 1–10 mol % [18:1]-LPA led to a further approx. 2-fold

activation of PKC α . At lower lipid concentrations, a greater increase was observed with LPA concentrations up to 16–20 mol %. Higher concentrations of LPA were inhibitory. The LPA activation of PKC was dependent on the presence of DAG, PS and Ca²⁺. [18:1]-Lysophosphatidylcholine produced similar PKC activation in PC/PS/DAG vesicles. [14:0]-LPA was less effective, and longer-chain saturated lysolipids were ineffective. In unsaturated PC/PS vesicles, very little to no effect of LPA was discernable. These results suggest that physiologically or pathologically relevant concentrations of LPA can contribute to PKC activation depending on the composition of the lipid membrane. We hypothesize that LPA may affect the formation of lipid domains that are recognized by the enzyme.

INTRODUCTION

Lysophosphatidic acid (LPA) has attracted recent attention as a serum-derived regulator implicated in responses to vascular injury and inflammation, in neurite retraction and neuronal signalling, and in tumour invasiveness (reviewed in ref. [1]). Controversy concerning its mechanism may be due, in part, to species-specific and cell-specific differences, and to stimulation of several different intracellular signalling pathways. Mitogenic effects in fibroblasts require high (1–100 μ M) LPA concentrations, are not affected by long-term treatment with phorbol esters, but are blocked with *Pertussis* toxin or suramin [2,3]. In contrast, rapid increases in intracellular Ca²⁺ require lower LPA concentrations [2,4–7], are blocked by phorbol ester treatment [2,4] and vary in their response to *Pertussis* toxin. More recently, tyrosine kinase cascades have also been implicated in the action of LPA. Activation of Ras, Raf and the mitogen-activated protein (MAP) kinase pathway have been demonstrated, as have activation of src and focal adhesion kinase (reviewed in ref. [1]).

It has been suggested on the basis of the following arguments that the Ca²⁺ and mitogenic effects of LPA are mediated by a G-protein-coupled receptor: (a) the effects are dependent on GTP [2,8]; (b) the kinetics and shape of the LPA-induced response are similar to those of similarly acting hormones [4]; (c) the LPA effect is subject to homologous desensitization [4,8]; (d) the LPA-induced phospholipid hydrolysis is cell-type specific, cells of haematopoietic origin failing to respond [4]; (e) the effects are blocked by the addition of suramin (reported to be membrane-impermeant [9]) outside the cells [3,6]; (f) the LPA effect on

Xenopus oocytes is not mimicked by intracellular LPA injection [5,6]. Although, to date, no success has been reported in cloning such a receptor, there is one report of a binding protein of 38–40 kDa identified by cross-linking with a [³²P]LPA analogue containing a photoactivatable acyl chain [10]. However, a similar LPA-binding protein was also observed in several haematopoietic cells [10] that failed to respond to LPA [4]. Problems with non-specific binding of LPA and potential metabolism make this work difficult but also weaken some of the arguments for a specific receptor.

Since LPA can lead to protein kinase C (PKC) activation in various cells [2] and phorbol ester activators of PKC can also stimulate tyrosine phosphorylation pathways [11–13], modulate G-proteins [14–17] and lead to mitogenic effects via activation of the MAP kinase pathway [18], we considered that LPA might exert some of its effects through PKC without the need for an additional specific receptor protein. PKC can also be activated or inhibited by suramin, depending on the concentration [19,20], can down-regulate a number of membrane receptors (reviewed in ref. [21]) and can stimulate or inhibit phospholipid hydrolysis in many cell types (reviewed in ref. [22]). PKC is affected by numerous products of phospholipid metabolism (reviewed in ref. [22]) and many other lipid-soluble molecules. Another phorbol-ester-binding protein, *n*-chimaerin, which has a p21^{rac} GTPase-activating protein domain in place of the kinase domain of PKC, was inhibited by LPA in a purified *in vitro* system and this inhibition required the lipid cofactors and the cysteine-rich phorbol-ester-binding region of *n*-chimaerin that is shared with PKC [23].

However, although low concentrations (< 20 μ M) of lyso-

Abbreviations used: LPA, lysophosphatidic acid; PKC, protein kinase C; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; DAG, diacylglycerol; DMPS, dimyristoyl-PS; DMPC, dimyristoyl-PC; DOPS, dioleoyl-PS; DOPC, dioleoyl-PC; DSC, differential scanning calorimetry; SUV, small unilamellar vesicle; MLV, multilamellar vesicle; LUV, large unilamellar vesicle; MAP kinase, mitogen-activated protein kinase.

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phosphatidylcholines (LPC) have been shown to activate PKC *in vitro* and higher concentrations to inhibit, very little effect of LPA was observed in those studies [24,25]. Similarly, LPC was effective in stimulating PKC pathways in haematopoietic cells including lymphocytes [26], HL60 cells [27] and mast cells [28], as well as in endothelial cells [29], all of which failed to respond to LPA; but LPC was not effective in stimulating the cells that respond to LPA with mitogenesis or increased Ca^{2+} [2]. Such cellular differences contribute to the argument for the presence of specific LPA or LPC receptors. However, Sasaki et al. [25] have reported differential responses of PKC isoenzymes to LPC, with PKC α , β , γ and δ being activated maximally at 4 μM LPC in the absence of Ca^{2+} and PKC α , β and γ being maximally activated at 10–20 μM phosphatidylcholine (PC) in the presence of Ca^{2+} followed by inhibition at higher concentrations, but inhibition only observed with PKC ϵ . Thus it is possible that expression of different PKC isoenzymes in different cells may account for the differential response to the different lysolipids.

Another possibility is that the effect of the lysolipids varies as a function of the differing membrane lipid composition of different cell types. In addition to changes in concentrations of its cofactors phosphatidylserine (PS), diacylglycerol (DAG) and Ca^{2+} , PKC is also sensitive to changes in physical properties of the membrane (reviewed in ref. [30]). A dependence on phospholipid acyl chain unsaturation was observed but saturated PC/PS vesicles could be rendered effective at very high concentrations of DAG [31]. Analysis of this saturated lipid system with various DAG concentrations by differential scanning calorimetry (DSC) and Fourier-transformed IR spectrometry suggests that the formation of DAG/PS-rich domains is associated with PKC activation [32]. Using vesicle systems with these defined compositions, we have re-examined the possibility that LPA may affect PKC more directly.

EXPERIMENTAL

Materials

Lysine-rich histone and bovine brain PS were from Sigma. LPA, LPC, dimyristoyl-PS (DMPS), dimyristoyl-PC (DMPC), dioleoyl-PC (DOPC), dioleoyl-PS (DOPS) and diacylglycerol (DAG) were from Avanti Polar Lipids. [^{32}P]ATP (7000 Ci/mmol) was obtained from ICN and adjusted to a specific radioactivity of 1.5–2.9 mCi/mmol for kinase assays. [^3H]Phorbol 12,13-dibutyrate (18.6 Ci/mmol) was from Dupont–NEN. Fetal calf serum was from Gibco and yeastolate and lactalbumin were from Difco. A polyclonal anti-PKC α antibody directed against the catalytic domain was obtained from Santa Cruz Biotechnology.

Preparation of lipids

Small unilamellar vesicles (SUVs) were prepared from the lipids just before assay. Purity of lipid stocks was confirmed by TLC and concentrations were determined by phosphate assay [33]. Lysophospholipids, phospholipids and DAG stocks in chloroform were mixed together in various molar ratios as indicated in the Figures, dried under a stream of N_2 gas and then under vacuum overnight. The samples were rehydrated with 20 mM Mops buffer, pH 7.5, in the dark, and sonicated to make SUVs. Multilamellar vesicles (MLVs) were prepared from the same stocks by vortexing rather than sonicating the preparations. For some experiments, large unilamellar vesicles (LUVs) were prepared by extruding the MLVs through a 100 nm membrane 31 times in a hand-held extruder (Avestin). Characterization of the LUVs by light scattering revealed an average diameter of approx.

120 $\pm$ 30 nm with a Gaussian distribution. Total lipid concentrations used in each experiment are given with the Figures.

DCS

DCS was performed on a Hart Scientific instrument with LPA-containing MLVs as indicated in the legend to Figure 7. Scan rate was 10°/h. Repeat scans were identical

Purification of PKC

PKC α was purified from Sf9 cells infected with PKC α -containing baculovirus generously provided by Dr. Peter Parker, Dr. Silvia Stabel and Dr. Dorian Fabbro. Enzyme was purified by sequential chromatography on Q-Sepharose and phenyl-Sepharose as described [34]. Purity was verified by PAGE followed by silver staining and Western blotting with isoenzyme-specific antibodies. Purified PKC was brought to 30% (v/v) glycerol and stored at -70°C . Enzyme concentration was determined by measuring binding of [^3H]phorbol 12,13-dibutyrate as described previously [35].

Assay of PKC activity

PKC activity was determined by its ability to transfer phosphate from [^{32}P]ATP to lysine-rich histone. The standard reaction mixture (75 μl total) contained 2–4 nM PKC, 0.2 mg/ml histone, 40 μM [^{32}P]ATP (2.5×10^3 – 5×10^3 c.p.m./pmol), 5 mM magnesium acetate, 300 μM CaCl_2 , 20 mM Mops buffer, pH 7.5, and vesicles of various composition (67 μM –6 mM). Reactions were started by the addition of the substrates to tubes containing enzyme and lipid vesicles and terminated after 5 min at 30 $^\circ\text{C}$ by spotting 60 μl on to P-81 ion-exchange paper (Whatman). Papers were washed in 50 mM NaCl to remove unchanged ATP and dried. Bound radioactivity was determined by Čerenkov counting.

RESULTS

To test the hypothesis that LPA might affect PKC activation in a manner that was dependent on the lipid composition of the membrane vesicle, we examined the effect of [18:1]-LPA on baculovirus-expressed PKC α in the DMPC/DMPS lipid system, the physical properties and PKC-activating ability of which are strongly dependent on the mol% of DAG. At near maximal DAG concentrations (15 mol%), LPA activated PKC, but the concentration required for maximal activation was dependent on the total lipid concentration. Maximal stimulation occurred at 10 μM LPA in 50 μM lipid but at 100 μM LPA in 500 μM or 3 mM lipid. Higher LPA concentrations were inhibitory.

Since the stimulation with LPA was less at the higher lipid concentrations, we asked whether LPA was simply replacing total lipid in activating the enzyme. Figure 1 shows that the addition of 25 μM LPA provided an approx. 2-fold greater PKC activation than maximally effective concentrations of SUVs. The increase was maximal between 2 and 16 mol% LPA and the stimulation was greater at suboptimal concentrations of lipid. In the absence of phospholipid vesicles, LPA did not activate PKC at any concentration (results not shown).

The concentration-dependence of the LPA effect was examined at maximal PKC-activating concentrations (3.2 mM) of DMPC/DMPS/DAG (60:25:15, molar ratio), and again, maximal activity occurred at 11–16 mol% LPA in both SUVs and MLVs (Figure 2). To determine whether similar-sized vesicle structures were formed at all LPA concentrations, LUVs were prepared at

Figure 1 Dependence of the LPA effect on PKC on total lipid concentration

PKC α was assayed with various concentrations of DMPC/DMPS/DAG (60:25:15, molar ratio) vesicles in the absence (○) or presence (●) of 25 μ M LPA. A representative experiment of three is shown. Values are means $\pm$ S.E.M. for duplicate determinations.

each lipid concentration and examined by light scattering for uniformity of size distribution. All preparations exhibited the same uniform Gaussian distribution. Again, maximal activity occurred at 11–16 mol% LPA, although the LUVs often produced increased total activation (Figure 2).

To determine whether LPA was replacing the PKC requirement for PS, the assays were carried out with varying mol% PS and at several LPA concentrations. Figure 3(A) shows that addition of 100 μ M (approx. 3 mol%) LPA provided greater activation than maximally effective concentrations of PS and that 200 μ M LPA (approx. 6 mol%) provided a minimal additional increase in the maximal activity, but significantly decreased the concentrations of PS required for maximal ac-

Figure 2 Effect of LPA concentration on PKC activity in different vesicle structures

PKC α activity was assayed in the presence of the indicated LPA concentrations in SUVs (●), MLVs (○) or LUVs (▲) all with 3.2 mM DMPC/DMPS/DAG (60:25:15, molar ratio). The results represent means $\pm$ S.E.M. for four or five (SUVs and MLVs) or two or three (LUVs) individual experiments, depending on the point.

Figure 3 Dependence of the LPA effect on DMPS concentration

(A) PKC α was assayed with 0 (□), 100 μ M (●) or 200 μ M (▲) LPA in DMPC/DMPS/DAG vesicles of various DMPS mol% as indicated. (B) PKC α was assayed with 0 (▲) or 25 mol% (●) DMPS in vesicles containing DMPC, 15 mol% DAG and the indicated concentrations of LPA. Total lipid concentration was 3.2 mM. The experiment in (A) is representative of three individual experiments and the one in (B) is representative of two. Data are presented as means $\pm$ S.E.M. for duplicate determinations.

tivation. As shown in Figure 3(B), LPA had no effect at all in the absence of PS.

Figures 4(A) and 4(B) show that LPA was similarly ineffective in the absence of DAG, nor did it significantly affect the concentration of DAG required. Figure 4(C) reveals that LPA failed to affect PKC activation in the unsaturated lipid (DOPC/DOPS) system. This system requires very little DAG for maximal PKC activity, but LPA did not affect this DAG requirement (Figure 4D). Similarly, LPA did not alter the Ca²⁺ requirement for activation of PKC α (results not shown).

The specificity of the LPA activation of PKC was examined by comparing several LPA and LPC species varying in acyl chain. Figure 5 shows that oleoyl (18:1) LPA and LPC provided similar maximal PKC activation, with LPC causing stimulation and inhibition at lower concentrations. The long-chain saturated LPC and LPA were minimally effective but the shorter-chain saturated (14:0) LPA was moderately effective.

Since minimal LPA effects were observed in previous reports that showed substantial effects of LPC [24,25], we compared the two lysolipids in the mixed acyl chain bovine PS used in previous studies as well as in the saturated and unsaturated PC/PS systems. As shown in Figure 6, both LPC and LPA caused a dramatic activation of PKC in the DMPC/DMPS system whereas neither was effective in the unsaturated lipid system. In

Figure 4 Dependence of LPA effect on DAG concentration and acyl-chain composition of the phospholipid

PKC α was assayed with the indicated concentrations of LPA without DAG ($\square$) or with two maximally effective DAG concentrations ($\circ$, $\bullet$) in saturated (A) or unsaturated (C) PC/PS vesicles. PKC α was also assayed at various DAG concentrations in the absence ($\circ$) or presence ($\bullet$) of 25 μM LPA in saturated (DMPC/DPMS; B) or unsaturated (DOPC/DOPS; D) PC/PS vesicles. Total PC/PS/DAG concentration was 67 μM . Values are means $\pm$ S.E.M. from duplicate determinations. Each experiment was replicated completely three to four times, depending on the experiment.

Figure 5 Effect of lysolipid headgroup and acyl-chain composition on activity of PKC

PKC α was assayed in DMPC/DMPS/DAG (60:25:15, molar ratio; 100 μM) vesicles with the indicated LPC or LPA at a concentration of 0 ($\square$), 10 μM ($\square$) or 25 μM ($\blacksquare$). Data are means $\pm$ S.E.M. for duplicate determinations. The experiment is representative of two performed.

bovine brain PS, LPC caused a 20% increase in PKC α activity, as observed previously [25], whereas the LPA effect was much less. As with LPA (Figure 2), LPC inhibited PKC α at high concentrations and was more potent than LPA in this inhibitory effect.

In order to investigate a possible LPA effect on the physical

Figure 6 Comparison of LPA and LPC effects on PKC in different phospholipid systems

PKC α activity was measured as a function of LPA (solid symbols and lines) or LPC (open symbols and dashed lines) concentration in DMPC/DMPS/DG (60:25:15, molar ratio; 100 μM) ($\bullet$, $\circ$), DOPC/DOPS/DAG (70:25:5, molar ratio; 100 μM) ($\blacksquare$, $\square$) or bovine brain PS (bbPS)/DAG (95:5, molar ratio; 100 μM) ($\blacktriangle$, $\triangle$) vesicles. Values are means $\pm$ S.E.M. for duplicate determinations. The experiment shown is a representative of two.

properties of the membrane, DMPC/DMPS/DAG (60:25:15, molar ratio) MLVs with and without PKC-activating concentrations of LPA were examined by DSC. Figure 7 shows that LPA

Figure 7 Effect of LPA on melting behaviour of DMPC/DMPS/DAG vesicles

MLVs (25 mM) composed of DMPC/DMPS/DAG (60:25:15, molar ratio) with the indicated concentrations of LPA (mol%) were scanned through the indicated temperatures in a Hart differential scanning calorimeter at a rate of 10°/h. Repeat scans did not differ. The third down scan from one of three independent experiments is shown.

significantly affected the melting behaviour of these lipid mixtures by broadening the main gel/liquid crystalline phase transition.

DISCUSSION

Whereas previous studies have revealed significant effects of LPC on PKC activity *in vitro*, very little PKC activation was observed with LPA [24,25]. In contrast, we observed a significant (≥ 2 -fold) increase in PKC activity with LPA (Figures 1 and 2). The explanation for the different effects is probably the acyl chain composition of the phospholipid system used to activate the enzyme. Only in saturated phospholipids was the effect dramatic. In fully unsaturated PC/PS systems, which produce maximal PKC activation with minimal DAG, LPA effects were minimal (Figures 4 and 6). The previous studies used bovine brain PS, which contains a mixture of acyl chains, largely unsaturated, to activate PKC. Using this system, we also observed about a 20% increase in maximal activity with LPC and a marginal effect of LPA.

As with the LPC activation of PKC characterized previously [24,25], we found that the LPA activation was dependent on the presence of DAG and did not change the concentrations of DAG required (Figure 4). The effect of LPA was also dependent on the presence of PS, although it could decrease significantly the concentration of PS required for maximal activity and, again, double the maximal activation at maximally effective concentrations of PS (Figure 3). This effect may be similar to that reported previously [36,37] in which other acidic lipids could replace part, but not all, of the requirement for PS.

Since purified PKC and defined lipid systems were used in the present study, the LPA effects observed cannot be attributed to binding to a separate LPA receptor. It is also unlikely that the effects are mediated by direct binding to PKC. First, LPA was ineffective in the absence of phospholipid PKC activators. Secondly, the biphasic LPA effects (Figure 2) cannot be explained by interaction with a single site. Thirdly, the concentration of LPA required was dependent on total lipid (Figure 1) and on the mol% of PS (Figure 3) and was not directly related to PKC concentration (results not shown). These results and the dependence on acyl-chain properties of both the phospholipid and the LPA (Figures 4–6) support the hypothesis that LPA affects PKC by altering the physical properties of the lipid. In support of this hypothesis, LPA was observed to broaden the

main (gel/liquid crystalline) phase transition observed by DSC when incorporated into DMPC/DMPS/DAG MLVs (Figure 7). These data are consistent with the enhanced formation of distinct lipid domains exhibiting overlapping phase transitions.

The difference in the effects of LPA in saturated and unsaturated phospholipid systems suggests that one role of LPA may be to contribute unsaturation to the phospholipid system. As shown previously [31], minimal DAG was required to activate the enzyme in unsaturated PC/PS vesicles, whereas activity could be achieved in saturated lipid systems only with large increases in the DAG content. Consistent with the importance of unsaturation is the observation that unsaturated LPA and LPC are most efficacious (Figure 5). Partial activation was observed with [14:0]-LPA but longer-chain saturated lysolipids were ineffective. This result differs from those showing very good activation of PKC with [16:0]-LPC [24,25]. Part of the explanation for this difference may be the different phospholipid systems and concentrations used {bovine PS at 10 $\mu\text{g}/\text{ml}$ [24] or 4 μM [25] compared with DMPC/DMPS at 100 μM (Figure 6)}. Some of the LPC effect observed previously may have been due to the increase in total lipid concentration.

The effectiveness of saturated lysolipids and differences in effectiveness of LPC and LPA may also depend on the background phospholipid composition and physical properties. Examination of the DMPC/DMPS/DAG system via DSC and Fourier-transformed IR spectroscopy is consistent with the formation of distinct PS- and DAG-enriched lipid domains, in association with activation of PKC [32]. An association of PKC activity with PS-substrate domains has also been visualized recently in large PC/PS/DAG vesicles [38]. We hypothesize that LPA and other lysolipids may alter the formation of these domains, and that their ability to do so may depend, in part, on the acyl chains of both the lysolipid and the surrounding phospholipid, and also on the headgroup charge of both. Thus, in a system comprised of only the optimal acidic phospholipid, PS, addition of more acidic headgroup in the form of LPA may not help, whereas LPC may promote domain formation more effectively. The effectiveness of saturated compared with unsaturated lysolipids may depend on their acyl-chain mismatch with the surrounding lipid. The ability of LPA or LPC to affect PKC activity in a given cell may therefore depend on the phospholipid composition and physical properties of that cell's membrane in addition to variables such as PKC isoenzyme expression and the generation of other activators such as Ca^{2+} and DAG.

The concentrations of LPA (1–3 mol%) that could activate PKC under some conditions *in vitro* were well within the range expected in physiological (or pathological) settings. Even the maximal effects of 11–16 mol% (300–500 μM at 3.2 mM total lipid) may have physiological significance if it is remembered that these *in vitro* lipid systems do not represent models of the whole cell membrane, but only of small subsets of lipid domains that may form within the whole membrane. We do not yet know the concentrations of LPA that may be found within specific lipid domains relevant to PKC activation in intact cells, but certainly these concentrations will be much higher than those relating LPA to total cellular phospholipids. Thus it is possible that direct activation of PKC may contribute to some cellular responses to LPA in the absence of a specific LPA receptor. The response observed in a given cell would depend on the PKC substrates (G-proteins, receptors, MAP kinase and other signalling pathway components) having access to the activated PKC isoenzymes in that cell.

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