Lysophospholipids activate ovarian and breast cancer cells

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We have investigated the effects of phospholipids on activation and proliferation of ovarian and breast cancer cells. Lysophosphatidic acid (LPA), lysophosphatidylserine (LPS) and sphingosylphosphorylcholine (SPC) all induce transient increases in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) in both ovarian and breast cancer cell lines. The ability of LPA, LPS and SPC to induce increases in $[\text{Ca}^{2+}]_i$ in ovarian and breast cancer cells is likely to be due to an interaction with cell-surface receptors as the increases in $[\text{Ca}^{2+}]_i$ were: (1) due to release of calcium from intracellular stores and not from transmembrane uptake due to changes in permeability; (2) blocked by lanthanum and suramin which do not enter cells; (3) blocked by phorbol esters which interrupt increases in $[\text{Ca}^{2+}]_i$ induced through a number of different receptors; and (4) not detected in freshly isolated peripheral blood mononuclear cells, indicating cell type specificity. In addition, increases in $[\text{Ca}^{2+}]_i$ induced by LPA, LPS and SPC in ovarian and breast cancer cells completely self-desensitized and cross-desensitized each other, but did not block increases in $[\text{Ca}^{2+}]_i$ induced by thrombin. Lysophosphatidylglycerol (LPG), but not other lysophospholipids, inhibited LPA- but not LPS- or SPC-induced increases in $[\text{Ca}^{2+}]_i$, suggesting that LPA may

interact with a different receptor(s) to LPS or SPC and that their downstream signalling pathways converge or interact. LPA, SPC and LPS also induced rapid increases in tyrosine phosphorylation of specific cellular proteins, including p125^{PAK} . Strikingly, LPA, but not LPS or SPC, induced activation of mitogen-activated protein (MAP) kinases. Despite an ability to activate similar intracellular signalling events, LPA, LPS and SPC exhibited markedly different effects on cell proliferation. Whereas LPA induced a significant increase in cell proliferation, LPS did not substantially alter cell proliferation and SPC inhibited cell proliferation. Surprisingly, phosphatidic acid (PA), which did not induce increases in $[\text{Ca}^{2+}]_i$, p125^{PAK} activation or activation of MAP kinases, did induce proliferation of ovarian cancer cells, albeit at higher concentrations than LPA. The discordance between sensitivity to LPG, early biochemical events stimulated, and the eventual proliferation response combine to suggest that LPA probably utilizes a different receptor from LPS, SPC and PA. Therefore ovarian and breast cancer cells are sensitive to the effects of a number of different phospholipids which may play a role in the growth of these tumour cells in the cancer patient and are thus potential targets for therapy.

INTRODUCTION

Ovarian cancer ranks second in incidence among gynaecological cancers. A poor prognosis for this disease results in ovarian cancer causing more deaths than any other cancer of the female reproductive system, accounting for an estimated 13 600 deaths in 1994 in the U.S.A. [1]. It is estimated that one of every 65 women will develop ovarian cancer by age 85, with over 60 % of patients dying from their disease [1]. This dismal outcome is due, at least in part, to the failure to detect the disease at an early stage, where the cure rate approaches 90 % [1]. An improved understanding of the mechanisms that regulate the growth of ovarian cancer cells may lead to an improved prognosis for individuals who develop this dismal disease.

Lipids have previously been demonstrated to be important bioactive mediators or second messengers that are generated in cells following stimulation of cell-surface receptors (reviewed in [2]). For example, glycolipid-derived messengers, such as 1,2-diacylglycerol (DAG), inositol phosphate, phosphatidic acid (PA), lysophospholipids and arachidonic acid are generated through the action of phospholipases C, D and A_2 on specific cellular glycolipids. Phospholipase C, D and A_2 activity is

frequently regulated via specific cell-surface receptors. These second messenger products have been shown to play important roles in a variety of biological functions [3–7]. Sphingolipid-derived messengers have been shown to be involved in vitamin D_3 , tumour necrosis factor α , γ -interferon, interleukin-1, dexamethasone and platelet-derived growth factor-induced cell activation (see [2] for review). Platelet-activating factor (PAF) [8,9] and lysophosphatidic acid (LPA) function extracellularly, activating cells through specific cell membrane receptors [2]. LPA exhibits potent growth factor activity towards fibroblasts with maximal activity occurring in the micromolar range [10,11]. In addition, LPA stimulates other cellular responses, such as platelet aggregation, smooth muscle contraction, changes in neuronal cell shape and Cl^- conductance activation (reviewed in [12–15]). Other lysophospholipids with marked structural specificity, particularly related to those derivatives of the phosphate at the glycerol *sn*-3 position, have also been shown to be active signalling molecules [16–21].

We report here that three phospholipids, LPA, lysophosphatidylserine (LPS) and sphingosylphosphorylcholine (SPC), stimulate a rapid and transient increase in cytosolic free calcium ($[\text{Ca}^{2+}]_i$) in ovarian and breast cancer cells. Calcium

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free calcium; DAG, 1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; Indo-1-AM, indo-1 acetoxymethyl ester; FCS, fetal-calf serum; IGF, insulin-like growth factor; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; MAP, mitogen-activated protein; MBP, myelin basic protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PA, phosphatidic acid; PAF, platelet-activating factor (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; PTX, pertussis toxin; SPC, sphingosylphosphorylcholine; TBS, Tris-buffered saline; TGF, transforming growth factor.

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changes induced by these phospholipids completely cross-desensitize each other. LPA-induced changes in $[Ca^{2+}]_i$, but not those induced by LPS or SPC, were blocked by lysophosphatidylglycerol (LPG). All three lysophospholipids stimulated tyrosine phosphorylation of a number of proteins including p125^{FAK}. However, LPA but not LPS or SPC induced activation of the mitogen-activated protein (MAP) kinases. While LPA stimulated proliferation, as assessed by [³H]thymidine incorporation, reduction of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye and colony formation, LPS had no significant effect on cell proliferation, and SPC inhibited proliferation. PA, in contrast, did not induce increases in $[Ca^{2+}]_i$, tyrosine phosphorylation, or MAP kinase activation. However, PA did induce a low but significant level of proliferation. The different early signals induced by each of the phospholipids combined with the differential inhibition by LPG suggests that LPA activates breast and ovarian cancer cells through a different receptor(s) and mechanism(s) from LPS, SPC and PA.

MATERIALS AND METHODS

Materials

Epidermal growth factor (EGF), acidic and basic fibroblast growth factor, insulin-like growth factor (IGF)-I, IGF-II, transforming growth factor (TGF) α and TGF β were from UBI (Lake Placid, NY, U.S.A.). Ionomycin, hexanolamine-PAF, lyso-PAF and PAF were from Calbiochem (San Diego, CA, U.S.A.). Indo-1 acetoxymethyl ester (Indo-1 AM) was from Molecular Probes (Eugene, OR, U.S.A.). LPA (synthetic oleoyl-LPA), LPS (from bovine brain, contains primarily stearic acid), SPC and PA (from egg-yolk lecithin) were purchased from Sigma (St. Louis, MO, U.S.A.), Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.), or Serdary Research Laboratories (London, Ontario, Canada). [³H]Thymidine (6.7 Ci/mmol) was from New England Nuclear (Lachine, Quebec, Canada). Fatty acid-free BSA was from Sigma. The anti-phosphotyrosine antibody 4G10, and anti-focal adhesion kinase (p125^{FAK}) monoclonal antibodies were from UBI (Lake Placid, NY, U.S.A.). Anti-(MAP kinase) antibodies were from Zymed (South San Francisco, CA, U.S.A.). The ECL kit was from Amersham (Arlington Heights, IL, U.S.A.). All other reagents were of the highest grade available and were from Sigma unless indicated otherwise.

Cell lines and media

HEY (a kind gift of R. Buick, Toronto, Ontario, Canada; [22]), OCC1 and OCC3 [23,24] ovarian cancer cells and SKBR3, MDA-MB-231, MDA-MB-453 and MDA-MB-468, (A.T.C.C.) breast cancer cell lines were cultured in complete medium which is RPMI 1640 (GIBCO, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) fetal-calf serum (FCS) (Flow Laboratory, Mclean, VA, U.S.A.), 2 mM glutamine (GIBCO) and 1×10^{-5} M mercaptoethanol. Cells were subcultured weekly and were cultured in RPMI 1640 without FCS for at least 20 h prior to use. Cells were harvested in PBS supplemented with 2 mM EDTA or in trypsin/EDTA solution (GIBCO/BRL).

Solutions/buffers

Buffer A was 140 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 10 mM glucose, 20 mM Hepes, pH 7.23. Buffer B was 10 mM Hepes, pH 7.4, containing 100 mM NaCl. Lysis buffer was 1% Nonidet P-40, 25 mM Hepes, pH 7.23, 150 mM NaCl, 50 μ M ZnCl₂, 50 μ M NaF, 50 μ M sodium vanadate, 0.5 mM EDTA and 1 mM PMSF. RIPA buffer was lysis buffer supplemented with 0.1% SDS, 0.1% deoxycholate, 1 mM sodium orthovanadate and

1 mM PMSF. TBS buffer contained 25 mM Tris, pH 7.6, and 150 mM NaCl.

$[Ca^{2+}]_i$ assays

$[Ca^{2+}]_i$ assays were performed as described previously [25]. Since non-specific perturbation of the cell-surface membrane can result in artifactual changes in $[Ca^{2+}]_i$ due to inward movement of calcium down its electrochemical gradient, we performed the experiments in a nominally calcium-free buffer (estimated calcium concentration of 50 nM). After equilibration to a stable baseline, 1.7 μ l of 1 M EGTA (final concentration 1 mM) was introduced to chelate residual extracellular calcium, giving an extracellular free calcium lower than 10 nM. Under these conditions, increases in $[Ca^{2+}]_i$ are due to release from intracellular stores.

[³H]Thymidine Incorporation

[³H]Thymidine incorporation was determined as described elsewhere [25]. All cultures were performed in 200 μ l of complete medium in 96-well, flat-bottomed plates. Thymidine incorporation was measured following a pulse with 1 μ Ci of [³H]thymidine (6.7 Ci/mM; New England Nuclear, Lachine, Quebec, Canada) by scintillation counting. All experiments were repeated at least three times.

Colony-forming cell assays

Colony-forming cell assays were performed as described in [25] except that the medium for the colony-forming cell assays was Hams F12/Dulbecco's modified Eagle's medium (DMEM) (1:1, v/v) supplemented with 0.1% fatty acid-free BSA and with insulin, transferrin, selenium (Gibco) as recommended by the manufacturer.

MTT assay

MTT (Sigma) dye reduction was used to measure the increase in cell numbers, essentially as described by Mosmann [26]. Cells were starved for 48 h in serum-free RPMI 1640 washed and cultured in Hams F12/DMEM (1:1, v/v) supplemented with 0.1% fatty acid-free BSA, insulin, transferrin and selenium as recommended by the manufacturer (Gibco BRL). Following a 72 h incubation in the presence or absence of the indicated concentrations of LPA, 10 μ l of MTT solution (5 mg of MTT/ml in water) was added and incubated at 37 °C for 4 h. An aliquot (100 μ l) of acid-isopropanol (0.04 M HCl in isopropanol) was added to each well and mixed by shaking on a plate shaker to dissolve the reduced MTT crystals. Relative cell number was obtained by scanning with an ELISA reader (Molecular Dynamics) with a 570 nm filter.

Immunoprecipitation and Immunoblotting

HEY or OCC1 ovarian cancer cells were cultured in complete medium RPMI 1640 with 10% (v/v) FCS and starved overnight of serum in RPMI 1640. 10^6 cells in 1 ml of RPMI 1640 were incubated with 3 μ M of LPA, LPS, SPC, or PA as indicated for various times at 37 °C. After incubation, the cells were immediately chilled on ice. Cells were then centrifuged and washed with cold PBS and lysed with 60–100 μ l of RIPA buffer. Samples were mixed with 5 $\times$ Laemmli buffer containing 5% mercaptoethanol, boiled for 5 min and separated on an SDS/10–15% PAGE gel. Separated proteins were transferred to Immobilon membranes (Millipore), which were blocked with 10% (w/v) milk powder in Tris-buffered saline (TBS) for 1 h. Membranes were incubated with anti-phosphotyrosine antibody (UBI) in

antibody binding buffer (5% milk and 0.1% Tween 20 in TBS) for 1 h and washed three times with TBS minus 0.1% Tween 20 before incubation with anti-(mouse IgG) antibody in antibody binding buffer for 1 h. Antibody binding was detected by an ECL kit (Amersham) using the manufacturer's recommended procedure.

For studies of activation of p125^{FAK}, 10⁷ cells (10⁶ cells/ml) were stimulated with various lipids (3 μ M) for 10 min. Cells were lysed in 100 μ l of RIPA buffer and the protein concentration determined using the Bio-Rad protein assay kit (Bio-Rad). Equal amounts of protein from each sample were incubated with 2 μ l of the anti-p125^{FAK} antibody at 4 °C overnight. Rabbit anti-(mouse IgG) pre-coupled Protein A-Sepharose was then added. The mixture was incubated for 1 h at 4 °C and centrifuged at 1000 g for 3 min. Immunoprecipitates were washed three times with 500 μ l of RIPA buffer, suspended in Laemmli buffer containing 5% mercaptoethanol, boiled for 5 min, and subjected to SDS/PAGE and immunoblotting as described above using anti-phosphotyrosine antibodies.

MAP kinase activity

Cells (10⁶) in 1 ml of RPMI 1640 were incubated with 3 μ M of LPA, LPS, SPC, PA or 25 ng/ml EGF for 10 min at 37 °C. After incubation, the cells were immediately chilled on ice, centrifuged and washed with cold PBS. Cells were lysed in 60 μ l of RIPA buffer. Protein content of each sample was determined using the Bio-Rad protein assay kit and an equal amount of protein was mixed with 5 $\times$ Laemmli sample buffer and loaded on to an SDS/10%-PAGE gel containing 0.25 mg/ml of myelin basic protein (MBP). The remainder of the procedure was performed as described by Yoshimasa et al. [27].

RESULTS

A subset of bioactive phospholipids stimulate increases in [Ca²⁺]_i in ovarian cancer cells

A number of different bioactive lipids and organic compounds have been shown to induce increases in [Ca²⁺]_i in several different

cell lineages. We therefore determined whether these compounds would induce increases in [Ca²⁺]_i in ovarian or breast cancer cell lines. The following compounds did not induce detectable increases in [Ca²⁺]_i due to release of calcium from intracellular stores in the HEY and OCC1 ovarian cancer cell lines or in the MDA-MB-231, MDA-MB-453 and MDA-MB-468 breast cancer cell lines: arachidonic acid, carbachol, ceramide, DAG, oleic acid, lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), LPG, lysophosphatidylinositol (LPI), lysoplasmalogenphosphatidylcholine, lysoplasmalogenphosphatidylethanolamine, lyso-PAF, PA, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (PS), PAF, *N*-oleoyl-D-sphingomyelin and sphingosine.

In contrast, LPA, LPS and SPC elicited a rapid, transient increase in [Ca²⁺]_i in HEY cells (Figure 1). Similar effects were observed with the OCC1 and OCC3 ovarian cancer cell lines and in the SKBR3, MDA-MB-231, MDA-MB-453 and MDA-MB-468 breast cancer cells in all the [Ca²⁺]_i assays described herein (results not shown). For clarity, unless otherwise stated, the results described are with the HEY or OCC1 ovarian cancer cell lines. The concentrations required to induce detectable increases in [Ca²⁺]_i were different, with LPA (oleoyl) inducing increases in [Ca²⁺]_i at concentrations of 200 nM, SPC at 100 nM and LPS at 5 μ M. TLC analysis of the preparations (data not shown) failed to detect significant contamination with the heterologous compounds, indicating that LPA, LPS or SPC were indeed able to increase [Ca²⁺]_i in ovarian cancer cells. It is unlikely that the effects on [Ca²⁺]_i are due to metabolism of these phospholipids into other active compounds, because there was virtually no lag time from addition of LPA, SPC and LPS to the observed increase in [Ca²⁺]_i (Figure 1).

The ability to increase [Ca²⁺]_i demonstrated marked structural specificity. Since PA, PS and sphingomyelin had no effect on [Ca²⁺]_i and the equivalent lysophospholipid forms LPA, LPS and SPC were active, the addition of a fatty acyl or amide chain to these lysophospholipids abrogates the ability to induce increases in [Ca²⁺]_i in ovarian and breast cancer cells. A free phosphate group or a serine derivative of the phosphate at the *sn*-3 position of a glycerol backbone was permissive, whereas glycerol, choline,

Figure 1 LPA, LPS and SPC cross-desensitize increases in [Ca²⁺]_i

(a–f) HEY cells (1 $\times$ 10⁷/ml) were incubated with 2.5 μ g/ml of Indo-1-AM for 30 min in RPMI 1640 and washed in RPMI 1640 three times. For assays, cells were suspended in 1.7 ml of Buffer A at 4 $\times$ 10⁵ cells/ml in a stirred, heated cuvette and were incubated until the baseline was obtained. EGTA (1 mM, final concentration) was added to chelate the residual extracellular free calcium. LPA (10 μ M), LPS (30 μ M) and SPC (10 μ M) were added as indicated.

Figure 2 The effect of lanthanum on $[Ca^{2+}]_i$ release induced by LPA and thrombin

HEY cells were loaded with Indo-1-AM as described in Figure 1 and equilibrated in calcium-free medium with EGTA. $LaCl_3$ (1 mM), LPA (10 μ M) and thrombin (0.1 unit) were added as indicated.

inositol, or ethanolamine were non-permissive as LPG, LPC, LPI and LPE did not increase $[Ca^{2+}]_i$ even when assessed at 10 times higher concentrations than LPA, LPS or SPC. A choline derivative of the *sn*-3 phosphate of SPC was permissive whereas the free phosphate or other derivatives were not, as ceramide, sphingosine and sphingosine 1-phosphate did not increase $[Ca^{2+}]_i$. This was in contrast to phospholipids with a glycerol backbone where a free phosphate group was permissive and the choline derivative was non-permissive. Alkyl- or alkenyl-containing phospholipids, such as PAF, lyso-PAF, plasmalogen, or lysoplasmalogen, were without detectable effect on $[Ca^{2+}]_i$ in HEY cells when assessed at 10 μ M.

Homologous and heterologous desensitization of increases in $[Ca^{2+}]_i$ in ovarian cancer cells

The $[Ca^{2+}]_i$ increases induced by LPA, LPS or SPC were transient, returning to baseline within 2 min (Figure 1). In all cases, an increase in $[Ca^{2+}]_i$ induced by addition of optimal concentrations of LPA, LPS or SPC resulted in homologous and heterologous desensitization, in that addition of a second bolus of the same or different lysophospholipid did not induce an increase in $[Ca^{2+}]_i$ (Figure 1). These results suggest that these phospholipids might share the same receptor(s) or that their downstream signalling pathways converge or interact.

LPA, LPS and SPC appear to stimulate ovarian cancer cells through specific cell-surface receptors

Lipids may alter cellular functions by intercalating into membranes and altering membrane fluidity or permeability. However, there is evidence that LPA may mediate its effects on cells by activation of a specific G-protein-linked receptor (reviewed in [12–15]). Indeed, the structurally similar PAF mediates its effects by binding to a specific high-affinity G-protein-linked receptor [8,9]. The family of G-protein-linked receptors is very large, comprising at least 200 identified receptors and 'orphan receptors' which may include the receptor(s) for LPA, LPS or SPC [28,29].

It is important to note that LPA, LPS and SPC increased $[Ca^{2+}]_i$ in medium with free calcium concentrations of < 10 nM

Figure 3 The effect of LPG on calcium release induced by LPA, SPC and LPS

(a) HEY ovarian cancer cells were loaded with Indo-1-AM as indicated in Figure 1 and equilibrated in calcium-free medium with EGTA. LPG (60 μ M), LPA (2 μ M), LPS (10 μ M), SPC (2 μ M) or thrombin (0.1 unit) were added as indicated. (b) Dose-dependent curve of the inhibitory effect of LPG on LPA activity. Different amounts of LPG were added before addition of LPA. The percentage of inhibition is calculated based on the activity of 2 μ M of LPA without pretreatment with LPG (100%).

(Figure 1 and all experiments described herein). At this extracellular calcium concentration, the increases in $[Ca^{2+}]_i$ could not be due to movement of calcium into the cell across the cell

Figure 4 Effect of lipids on proliferation of ovarian cancer cells

Thymidine incorporation was determined as described in the Materials and methods section. OCC1 (a) or HEY (b) cells were starved from serum for 20 h. Indicated concentrations of the lipids were added to 5×10^3 cells/well for 36 h in 96-well plates in RPMI 1640. [3 H]Thymidine (0.5 μ Ci) was added to each well and the cells were incubated for an additional 6 h. The Figure demonstrates mean S.E.M. of four separate experiments. (c) HEY ovarian cancer cells were plated in 96-well microtitre plates, starved of serum for 48 h and then treated with the indicated concentrations of synthetic oleoyl-LPA for 72 h. MTT was then added and cell proliferation determined by dye reduction as described in the Materials and methods section. Results

membrane (intracellular concentration of approximately 50 nM increasing to approximately 300 nM on stimulation). Therefore the observed increases in $[Ca^{2+}]_i$ are due to release from intracellular stores. This observation, combined with the structural specificity of the ligands, argues that the increases in $[Ca^{2+}]_i$ induced by LPA, LPS and SPC are unlikely to be due to non-specific perturbation of the cell membrane or due to changes in membrane integrity.

The ability of LPA, LPS and SPC to increase $[Ca^{2+}]_i$ in ovarian cancer cells was sensitive to a brief pretreatment of cells with lanthanum (Figure 2). Since lanthanum does not penetrate the cell membrane [10], this suggests that each of the lipids has an extracellular site of action, probably the presence of a specific receptor(s) in the cell membrane. We have found that thrombin also stimulated a similar increase in $[Ca^{2+}]_i$ in HEY cells (Figure 2). Lanthanum did not alter thrombin-induced changes in $[Ca^{2+}]_i$, indicating that the cells retain their ability to increase $[Ca^{2+}]_i$ due to release from intracellular stores and that the effect was not due to non-specific membrane perturbation induced by lanthanum (Figure 2). Furthermore, preincubation of cells with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), abrogated LPA-, LPS- and SPC-induced increases in $[Ca^{2+}]_i$ (data not shown). Phorbol esters, probably through activation of protein kinase C family members, prevent increases in $[Ca^{2+}]_i$ induced by activation of a number of different cell-surface receptors [30]. This provides further support that LPA, LPS and SPC increase $[Ca^{2+}]_i$ in ovarian cancer cells by interacting with a specific cell-surface receptor(s).

LPG has been shown to prevent binding of LPA to a putative cell-surface receptor in mouse NIE-115 neuroblastoma cells [31]. Preincubation of HEY cells with 2, 10, 30, or 60 μ M of LPG, LPE or LPI did not alter the ability of 2 μ M LPA to increase $[Ca^{2+}]_i$ in HEY cells (data not shown). However, pretreatment with 60 μ M LPG completely blocked $[Ca^{2+}]_i$ increases induced by 2 μ M LPA in HEY cells (Figure 3a). Pretreatment with LPG, in contrast, did not alter thrombin-induced increases in $[Ca^{2+}]_i$, indicating that LPG was not toxic to cells and that LPG-treated cells retain the ability to mobilize intracellular calcium in response to extracellular stimuli. LPG-mediated inhibition was dose-dependent with 1 μ M LPG resulting in detectable decreases and with 10 μ M LPG giving approximately 50% inhibition (Figure 3b). Thus a 5-fold molar excess of LPG results in 50% inhibition and a 30-fold molar excess of LPG results in 100% inhibition of LPA-induced increases in $[Ca^{2+}]_i$. Strikingly, LPG (60 μ M) did not alter increases in $[Ca^{2+}]_i$ induced by 10 μ M of LPS (LPS is less potent than LPA and SPC, so that higher concentrations were used), or 2 μ M of SPC (Figure 3a). Since LPG appears to be a competitive inhibitor for the LPA receptor, this suggests that neither LPS nor SPC interact with the LPA receptor or that they interact with a different stereochemistry which is not sensitive to inhibition by LPG.

LPA, LPS and SPC, but not other phospholipids, increased $[Ca^{2+}]_i$ in all ovarian and breast cancer cell lines tested. In contrast, these lysophospholipids induce increases in $[Ca^{2+}]_i$ in freshly prepared human peripheral blood mononuclear cells, whereas the mitogenic lectin phytohaemagglutinin induced an increase in $[Ca^{2+}]_i$ (data not shown). This further argues that LPA, LPS and SPC interact with a specific cell-surface receptor(s) present on breast and ovarian cancer cells which is not present on normal human mononuclear cells.

represent relative absorbance on an ELISA reader in arbitrary units. Optimal proliferation (10% FCS) was three times higher than that induced by optimal concentrations of LPA.

Table 1 Stimulation of anchorage-independent growth by LPA

SKOV-3, OCC1 or Rat-1 cells were plated in semi-solid media in the presence or absence of synthetic 12.5 μ M *sn*-1-oleoyl LPA or EGF (25 ng/ml) as indicated. Plates were examined to establish that clumps of cells were not present. Colony-forming cell number was determined 14 days later by assessing colonies with greater than 100 cells. Numbers represent colonies/ 10^5 cells for SKOV-3 and OCC1 and colonies/ 10^4 cells for RAT-1. Results represent means $\pm$ S.E.M. of three plates from one of three similar experiments.

Cell line	Medium alone	+ EGF	+ LPA
SKOV-3	10.0 $\pm$ 2.8	106 $\pm$ 13	496 $\pm$ 78
OCC1	2.0 $\pm$ 1.4	2 $\pm$ 2.8	172 $\pm$ 23
RAT-1	10.0 $\pm$ 0.4	53 $\pm$ 13	507 $\pm$ 64

Table 2 Effect of PMA, PTX and staurosporin on LPA-induced proliferation of HEY cells

HEY cells were starved from serum for 20 h and plated at 5×10^3 cells/well in 96-well plates in RPMI 1640. Synthetic oleoyl-LPA (10 μ M), PMA (10 nM), PTX (100 ng/ml) or staurosporin (1 μ M) were added as indicated. Thymidine incorporation was determined as described in Figure 4.

LPA	Thymidine incorporation (d.p.m.)			
LPA	Control	PMA	PTX	Staurosporin
—	19000 $\pm$ 2530	14500 $\pm$ 1225	17500 $\pm$ 2050	10050 $\pm$ 4100
+	65500 $\pm$ 3890	59800 $\pm$ 1870	42555 $\pm$ 980	9500 $\pm$ 1350

The effect of LPA, LPS and SPC on cellular proliferation

LPA increased cellular proliferation as assessed by [3 H]thymidine incorporation in completely serum-free medium in HEY and OCC1 ovarian cancer cells (Figures 4a and 4b). LPA-induced increases in [3 H]thymidine incorporation were first detectable at approximately 0.2 μ M and peaked at 10–20 μ M. LPA was sufficient to induce proliferation of ovarian cancer cells for at least 1 month in serum-free medium as assessed by cell counts (data not shown) and for shorter periods of times as assessed by MTT dye reduction (Figure 4c) and DNA content (data not shown). Indeed, LPA alone can increase both the size (data not shown) and number (Table 1) of colonies in soft agar. LPA was much more efficient than EGF in inducing colony formation in the SKOV-3 and OCC1 lines (note that HEY cells were not used in these experiments because they formed small colonies compared with SKOV-3 and OCC1 cells when cultured in serum-free medium). Indeed, OCC1 cells, which do not proliferate in response to EGF, respond to LPA with an 80-fold increase in colony-forming cell activity (Table 2). Rat 1 cells, which have previously been demonstrated to be responsive to LPA [10,11], were included as positive controls. In most experiments, LPS did not significantly alter [3 H]thymidine incorporation at low concentrations ($< 25 \mu$ M) and was growth inhibitory at high concentrations ($> 50 \mu$ M) (see Figure 4b). This suggests that increases in [Ca^{2+}]_i are not sufficient to induce cellular proliferation and that either LPA induces biochemical processes not activated by LPS or SPC or that negative 'signals' induced by LPS or SPC over-ride their growth promoting effect. Phorbol esters such as PMA, at concentrations which completely blocked LPA-induced increases in [Ca^{2+}]_i (data not shown), did not inhibit LPA-induced proliferation (Table 2), suggesting that at least in the presence of activation or down-regulation of protein-kinase C

and other intracellular enzymes by phorbol esters, LPA-induced increases in [Ca^{2+}]_i are not obligatory for cellular proliferation. PA, which did not induce increases in [Ca^{2+}]_i in ovarian cancer cells, increased [3 H]thymidine incorporation in both HEY and OCC1 ovarian cancer cells, in support of a lack of correlation in the ability to increase [Ca^{2+}]_i and proliferation (Figures 4a and 4b). The effects were not as marked as those of LPA and required much higher concentrations of PA (Figures 4a and 4b). The PA preparations utilized did not contain significant LPA contamination as assessed by TLC, also the PA preparations studied did not increase [Ca^{2+}]_i in ovarian cancer cells indicating that the preparations contained less than 0.5% LPA. However, as PA can be converted into LPA by the action of phospholipases, the growth-promoting effect of PA could be due to the action of LPA produced during the culture period.

It has been proposed that LPA activates cells through a G-protein-linked cell-surface receptor. Indeed, pretreatment with pertussis toxin (PTX; 100 ng/ml), which inactivates some G-protein subtypes [32], decreased LPA-induced cell proliferation by 30–40% in various experiments (Table 2). In our studies, treatment with PTX did not significantly inhibit EGF-induced cellular proliferation under conditions where it inhibited LPA-induced proliferation (data not shown), indicating a lack of non-specific effect of PTX (data not shown). The relatively non-specific kinase inhibitor staurosporin (1 μ M) [30,33] also markedly inhibits LPA-induced proliferation, indicating that intracellular kinase activity is required for the mitogenic activity of LPA (Table 2).

LPA, LPS and SPC induce tyrosine phosphorylation of specific intracellular substrates

Incubation of fibroblasts with LPA has been shown to induce tyrosine phosphorylation of a number of intracellular substrates including focal adhesion kinase and MAP kinase [33–35]. We therefore investigated the effects of LPA, LPS, SPC and PA on tyrosine phosphorylation in ovarian cancer cells. LPA and SPC induced a rapid increase in tyrosine phosphorylation of several proteins in the OCC1 (Figures 5a and 5b) and HEY (data not shown) ovarian cancer cell lines as assessed by Western blotting with anti-phosphotyrosine antibodies. LPS and PA had no detectable effect on tyrosine phosphorylation as assessed by Western blotting of total cell lysates with anti-phosphotyrosine antibodies (data not shown). EGF produced a marked increase in tyrosine phosphorylation of the EGF receptor and of other proteins with both unique and overlapping bands compared with LPA and SPC. Thus the effects of LPA, LPS and SPC on cellular proliferation did not correlate with the ability to induce increases in total cellular tyrosine phosphorylation. Indeed, SPC, which is growth inhibitory, induced a higher level of tyrosine phosphorylation than LPS. PA, which is growth stimulatory at high concentrations ($> 12.5 \mu$ M), did not induce detectable tyrosine phosphorylation even at concentrations which induce proliferation. This suggests a lack of correlation between the levels of total cellular tyrosine phosphorylation and the effect of LPA, LPS, SPC and PA and cellular proliferation.

The presence of a major tyrosine phosphorylated band at 125 kDa (Figures 5a and 5b), suggested that p125^{PAK} may be a target for kinases activated by the phospholipids. Indeed, following immunoprecipitation with anti-p125^{PAK} antibodies and Western blotting with anti-phosphotyrosine antibodies, LPA, LPS and SPC, but not PA, induced tyrosine phosphorylation of p125^{PAK} (data not shown). Strikingly, LPS, which did not induce detectable changes in tyrosine phosphorylation as indicated by Western blotting of total cell lysates with anti-phosphotyrosine

Figure 5 LPA and SPC induce tyrosine phosphorylation in OCC1 ovarian cancer cells: PTX and staurosporin inhibit MAP kinase activity induced by phospholipids

OCC1 ovarian cancer cells were cultured in complete medium consisting of RPMI 1640 supplemented with 10% FCS, glutamine and mercaptoethanol. Cells were starved of serum by an overnight incubation in RPMI 1640 medium. In (a), OCC1 cells (10^6 cell in 1 ml) were incubated with LPA ($3 \mu\text{M}$) for 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4) or 30 (lane 5) min at 37°C or with EGF (lane 6, 25 ng/ml) for 10 min. In (b), OCC1 cells (10^6 cell in 1 ml) were incubated with SPC ($3 \mu\text{M}$) for 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), 30 (lane 5), or 60 (lane 6) min at 37°C or with EGF (lane 7, 25 ng/ml) for 10 min. After incubation, the cells were immediately chilled on ice. Cells were then pelleted and washed with cold PBS and lysed with $100 \mu\text{l}$ of RIPA buffer. The proteins were separated on an SDS/12.5%-PAGE gel and tyrosine phosphorylation was analysed by immunoblotting using monoclonal 4G10 anti-phosphotyrosine antibodies and detected with an ECL detection kit as described in the Materials and methods section. In (c), OCC1 cells (10^7 /ml) were incubated with medium alone (lane 1), SPC ($3 \mu\text{M}$, lane 2), LPS ($3 \mu\text{M}$, lane 3), LPA ($3 \mu\text{M}$, lane 4), PA ($3 \mu\text{M}$, lane 5), or EGF (25 ng/ml) for 10 min and lysed in RIPA buffer. Proteins were separated by SDS/PAGE and MAP kinase activity assessed as indicated in the Materials and methods section. In (d), OCC1 cells (10^7 /ml) were incubated with medium (lanes 1, 3 and 5) or with LPA ($3 \mu\text{M}$; lanes 2, 4 and 6) for 10 min. Cells in lanes 3 and 4 were pretreated with PTX (100 ng/ml) for 2 h prior to addition of LPA, and cells in lanes 5 and 6 were pretreated with $1 \mu\text{M}$ staurosporin for 10 min prior to addition of LPA. Cells were lysed in RIPA buffer, proteins separated by SDS/PAGE and MAP kinase activity assessed as indicated in the Materials and methods section.

antibodies, demonstrated significant increases in tyrosine phosphorylation of p125^{FAK} (data not shown). This suggests that the immunoprecipitation assay which utilizes much higher

numbers of cells is more sensitive at detecting changes in tyrosine phosphorylation than is Western blotting of total cellular lysates. Regardless, similar to changes in $[\text{Ca}^{2+}]_i$, the ability to induce tyrosine phosphorylation of p125^{FAK} did not correlate with the ability to induce cellular proliferation.

MAP kinases which are regulated by both tyrosine and threonine phosphorylation [36] have been shown to be substrates for LPA signalling in fibroblasts [33,34]. LPA, but not LPS, SPC, or PA, activated MAP kinase, as assessed by a renaturation kinase assay (Figure 5c). Similar to cellular proliferation, the ability of LPA to activate MAP kinase was sensitive to both PTX and staurosporin (Figure 5d). In the renaturation kinase assay, a protein with apparent molecular mass of 68 kDa (Figure 5d) was phosphorylated even in control samples. The migration of this protein is similar to that of a major constitutively phosphorylated protein identified in the anti-phosphotyrosine Western blot analysis (Figures 5a and 5b), suggesting that it may be a tyrosine kinase which uses MBP as a substrate. Although the identity of the 68 kDa kinase is currently unknown, its activity in resting cells suggests that it does not account for LPA-induced cellular proliferation.

DISCUSSION

In recent years, lipids, in particular phospholipids such as LPA and PAF, have been shown to function in fibroblasts and other normal cell lineages as important intercellular signalling molecules. They exhibit growth factor-like properties including the presence of high-affinity receptors, specific signalling pathways, homologous and heterologous desensitization, structural specificity, and regulated production [12–15,37,38]. The role of the phospholipids on the activation and growth of malignant cells, however, has not been extensively explored.

We have demonstrated that three lysophospholipids, LPA, LPS and SPC, are potent inducers of calcium release from intracellular stores in ovarian and breast cancer cell lines. These lysophospholipids completely desensitize each other in calcium release, suggesting that they either share a common receptor or that their downstream signalling pathways interact or converge. This is supported by the ability of lanthanum (which does not penetrate the cell membrane [10]) and phorbol esters (which have been shown to desensitize receptor-mediated effects in other systems [30]) to prevent increases in $[\text{Ca}^{2+}]_i$ induced by LPA, LPS and SPC. The ability of LPA to competitively inhibit LPS, but not LPS or SPC, suggests that LPA binds to an independent receptor(s) from LPS and SPC.

We have shown that LPA, LPS and SPC induced very different effects on tyrosine phosphorylation, MAP kinase activation and cellular proliferation, despite their ability to induce similar patterns of increases in $[\text{Ca}^{2+}]_i$. In addition, PA induced cellular proliferation, but did not induce an increase in $[\text{Ca}^{2+}]_i$ or detectable tyrosine phosphorylation in ovarian cancer cells. These results suggest that the increase in $[\text{Ca}^{2+}]_i$ is not sufficient to explain the ability of these lipids to differentially modulate tyrosine phosphorylation and proliferation. This is supported by the observations that PMA inhibited LPA-induced calcium release, but not the mitogenic activity of LPA. The mitogenic activity of LPA and LPA-induced MAP kinase activation were sensitive to PTX (which inactivates some G-protein subtypes) and staurosporin (which is a relatively non-specific kinase inhibitor). In contrast, LPS and SPC did not induce MAP kinase activation or increase proliferation, suggesting that the ability to activate MAP kinase may be an obligatory part of the signalling pathway by which LPA induces cell proliferation. However, MAP kinase activation is not sufficient to explain the ability of

LPA to induce cellular proliferation as phorbol esters (potent activators of MAP kinases [39]) do not induce proliferation in ovarian cancer cells. Furthermore PA, which induces neither increases in $[Ca^{2+}]_i$ nor MAP kinase activation, induces cellular proliferation (see Figure 5). Therefore it appears likely that the appropriate combination of signals including increases in $[Ca^{2+}]_i$, tyrosine phosphorylation and MAP kinase activation, along with other interacting pathways, may be activated by LPA, but not by LPS or SPC. Alternatively, LPS, and in particular SPC, may activate specific inhibitory pathways that are not induced by LPA, accounting for the differences in ability to stimulate proliferation.

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