Lipid A stimulates phospholipase D activity in rat mesangial cells via a G-protein

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Stimulation of mesangial cells (MC) with the bacterial endotoxin Lipid A activated two enzymes involved in lipid metabolism. First, a phospholipase D hydrolyses phosphatidylethanolamine (PE) to phosphatidic acid (PA), followed by dephosphorylation of PA to 1,2-diacylglycerol (DAG) by PA phosphohydrolase. MC or microsomes from these cells were pre-labelled with [³H]glycerol. A 30–60 s stimulation with 10–100 ng of Lipid A/ml caused a decrease in [³H]glycerol in PE and increased radioactive glycerol in PA. The enzyme responsible for this hydrolysis preferred PE containing unsaturated acyl side chains. DAG was formed from PA within the first 1 min after Lipid A stimulation. Microsomes incubated with 25 mM-NaF to inhibit phospholipase C and to stimulate GTP-binding proteins also caused PE to be converted into PA. The [³H]glycerol and acyl mass of phosphatidylcholine, phosphatidylserine and phosphatidylinositol did not change with either Lipid A or NaF. Addition of guanosine 5'-[γ-thio]triphosphate to MC microsomes caused the rapid decrease in proportion of PE and increase in PA, followed by an increase in DAG unsaturated acyl mass. These data suggest the concurrent G-protein-dependent activation by Lipid A of a PE-directed phospholipase D and a PA phosphohydrolase.

INTRODUCTION

Bacterial endotoxins, the lipopolysaccharide complexes from Gram-negative cell walls, have many different effects on eukaryotic cells (Hamilton *et al.*, 1986) and mammalian organ systems (Morrison & Ryan, 1979). The core moiety of endotoxin, Lipid A, has been shown to affect monocytes and glomerular mesangial cells *in vitro* by increasing synthesis, secretion and myristoylation of interleukin-1 (Loppnow *et al.*, 1986; Aderem *et al.*, 1986a; Bursten *et al.*, 1988), prostanoid synthesis (Lovett *et al.*, 1988a), and protein acylation patterns (Aderem *et al.*, 1986b; Bursten *et al.*, 1988). Lipid A, in tandem with interleukin-1 and tumour necrosis factor, is responsible for the diffuse physiological effects associated with Gram-negative sepsis (Gombos *et al.*, 1967; Bessler & Henning, 1979; Kikeri *et al.*, 1986; Libby *et al.*, 1986). Endotoxin has been shown to alter renal blood flow (Henrich *et al.*, 1982), possibly by altering synthesis patterns of eicosanoids (Badr *et al.*, 1986).

It has been difficult to define the nature of initial endotoxin interaction with eukaryotic cells resulting in a signal response (Morrison & Rudback, 1981; Hamilton *et al.*, 1986). There is specific and rapid binding of Lipid A to a membrane protein in splenic lymphocytes (Goodman & Morrison, 1984; Lei & Morrison, 1988a,b), but the function of this protein is not known. A lipopolysaccharide-binding protein in serum, with a sequence similar to that of cholesterol ester transport protein, has also been described (Schumann *et al.*, 1990). The lipopolysaccharide-protein complex was found to bind to CD14, a monocyte surface antigen; antibodies against CD14 prevented lipopolysaccharide induction of monocyte tumour-necrosis-factor synthesis (Wright *et al.*, 1990). Lipid A may rapidly activate polyphosphoinositide hydrolysis in some cell systems (Prpic *et al.*, 1987), but also has

alternative effects on signalling in other cells, such as pre-B lymphocytes (Rosoff & Cantley, 1985), and may directly activate protein kinase C in platelets and renal cells (Wightman & Raetz, 1984; Grabarek *et al.*, 1988). Lipid A induction of interleukin-1 in WEHI pre-B lymphocytes is dependent on a pertussis-toxin-sensitive G-protein (Jakway & DeFranco, 1986). These results suggest that a rapid interaction of endotoxin with membrane proteins, including G-proteins, is necessary for cell activation.

G-protein regulation of phospholipase D (PLD), which catalyses the hydrolysis of phosphatidylcholine (PC) or phosphatidylethanolamine (PE) to phosphatidic acid (PA), has been implicated in rapid cell activation in endothelial cells, neutrophils and neuroblastoma/glioma cells (Martin, 1988; Martin & Michaelis, 1989; Reinhold *et al.*, 1990). Bocckino *et al.* (1987) demonstrated PLD activation by Ca²⁺-mobilizing hormones in rat hepatocytes that was regulated via GTP-binding proteins. The activation of PLD appears to follow several parallel pathways in neutrophils and other granulocytes (Reinhold *et al.*, 1990).

PA, the product of PLD, may serve as a signalling molecule, or may be converted into 1,2-diacylglycerol (DAG) by PA phosphohydrolase (Bocckino *et al.*, 1987; Cascales *et al.*, 1988; Martin, 1988; Martin & Michaelis, 1989). PA is an autocrine growth factor for fibroblasts (Moolenaar *et al.*, 1986), and directly promotes Ca²⁺ flux (Altin & Bygrave, 1987). Short-term increases in PA are associated with increases in phosphoinositide hydrolysis mediated through either a G-protein (Freissmuth *et al.*, 1989) or a second pathway leading to generation of DAG that is independent of phosphatidylinositol (PI) hydrolysis or Ca²⁺ flux (Kester *et al.*, 1988). Lyso-PA, the immediate precursor of PA, is also a mitogen for fibroblasts, and a possible activator of signalling pathways (van Corven *et al.*, 1989).

Abbreviations used: PLD, phospholipase D; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEth, phosphatidylethanol; PA, phosphatidic acid; PI, phosphatidylinositol; DAG, 1,2-diacylglycerol; MC, mesangial cells; PBS, phosphate-buffered saline; GTP[S], guanosine 5'-[γ-thio]triphosphate; *cis*-PnA, *cis*-parinaric acid; NBD-phalloidin, 7-nitrobenzo-2-oxa-1,3-diazole-phalloidin; f.a.b./m.s., fast-atom bombardment mass spectrometry.

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The purpose of the present study was to examine rapid effects of Lipid A interaction with intact rat mesangial cells (MC) and microsomes prepared from these cells. Lipid A causes activation of both PLD and PA phosphohydrolase in whole MC and microsomes, apparently regulated by separable G-protein activities. Stimulation of MC microsomes with dilinoleoyl PA also results in activation of PLD and PA phosphohydrolase. Lipid A may duplicate a PA-based amplification pathway that activates MC. This hypothesis is supported by experiments that show rapid cytoskeletal reorganization upon addition of either PA or Lipid A. Reorganization is blocked in the presence of pertussis toxin.

MATERIALS AND METHODS

Reagents

Proliferating rat MC were maintained in RPMI 1640 (Gibco, San Jose, CA, U.S.A.) containing 20% (v/v) fetal-bovine serum (Irvine Scientific, Irvine, CA, U.S.A.) supplemented with 300 μ g of glutamine/ml, 100 units of penicillin/ml, 100 μ g of streptomycin/ml, 2.5 μ g of vancomycin/ml (Sigma Chemical Co., St. Louis, MO, U.S.A.), and 5 μ g of transferrin/ml, 5 μ g of insulin/ml and 5 ng of selenium/ml (ITS Pre-mix; Collaborative Research, Boston, MA, U.S.A.). All media components were screened for the presence of exogenous endotoxin by using a *Limulus* amoebocyte-lysate assay sensitive to 100 pg of endotoxin/ml (E-toxate; Sigma). *cis*-Parinaric acid (*cis*-PnA; Molecular Probes, Eugene, OR, U.S.A.) was prepared as a 1 mM stock solution in ethanol with butylated hydroxytoluene as an antioxidant, and had a molar absorption coefficient of 78000 M⁻¹·cm⁻¹. NBD-phalloidin (7-nitrobenzo-2-oxa-1,3-diazole-phalloidin) for actin staining was from Collaborative Research.

Standard PC, PE, PI, phosphatidylserine (PS) and sphingomyelin, fatty-acid-free BSA, 1-palmitoyl-2-stearoyl-PA and 1-oleoyl-2-acetylgllycerol (OAG) were obtained from Sigma. 1-Palmitoyl-2-arachidonoyl-*sn*-PA, 1-palmitoyl-lyso-PC, 1-palmitoyl-lyso-PI and 1-palmitoyl-lyso-PE were obtained from Supelco (Bellefonte, PA, U.S.A.) Lyso-PA (1-palmitoyl species) was from Serdary Labs (London, Ontario Canada). 1,2-*sn*-Dilinoleoyl-PC was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Stock solutions of phospholipids were prepared by dissolving the compounds in phosphate-buffered saline (PBS; 120 mM-NaCl/2.7 mM-KCl/10 mM-sodium phosphate, pH 7.4) containing 0.5% (w/v) BSA, such that final concentrations of BSA in reaction volumes ranged from 0.002 to 0.04% (w/v). All phospholipids were re-characterized for identity and purity by t.l.c. and h.p.l.c. (Bursten & Harris, 1991). 1,2-*sn*-Dilinoleoyl-PC was converted into 1,2-*sn*-dilinoleoyl-PA with plant PLD, by using a modification of the protocol of Kates & Sastry (1969). This reaction was run at room temperature for 30 min in 100 mM-sodium acetate/100 mM-CaCl₂, pH 5.6, containing 25% (v/v) diethyl ether, and approx. 1 mg of PLD from cabbage [Sigma: 170 units (μ mol/h)/mg of solid]. The reaction was terminated by cooling, the ether was removed by a stream of nitrogen, and the aqueous suspension was then extracted in chloroform/methanol (2:1, v/v) by the method of Folch *et al.* (1957). Reaction products were separated by h.p.l.c. as described below. The dilinoleoyl-PA fraction was isolated, then repurified by h.p.l.c. and identified by t.l.c., g.l.c. analysis of acyl side chains, and mass spectrometry: >99.5% of acyl ester side chains were shown by g.l.c. to be linoleate. [2-³H]Glycerol (sp. radioactivity 5 Ci/mmol) and [1-¹⁴C]ethanol (27 mCi/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.).

Preparation of rat MC and rat MC microsomes

Primary cultures of rat MC were derived from the outgrowths

of collagenase-treated glomeruli as described in detail (Lovett *et al.*, 1983, 1987, 1988b). Exponential growth was maintained in growth medium in a humidified atmosphere containing 5% CO₂. MC appeared as homogeneous bundles of strap-like cells growing in an interwoven pattern. Immunofluorescence studies demonstrated prominent intracellular myosin fibrils and were negative for common leucocyte antigen, Ia antigens and Factor VIII (Lovett *et al.*, 1986). Rat MC also demonstrated actin-containing stress filaments when stained with NBD-phalloidin (Bursten *et al.*, 1991). MC were used between passages 4 and 10 for preparation of microsomes or for cell labelling experiments. Where indicated, cycling MC were treated with pertussis toxin (0.1–100 ng/ml) for 4 h before harvesting and microsomal preparation.

Microsomes were prepared from rat MC as reported previously (Lovett *et al.*, 1987). Confluent MC layers were washed in PBS at 4 °C, scraped, and pelleted by centrifugation at 400 g for 10 min. The pellets were suspended in 20 mM-sodium borate (pH 10.2)/10 mM-EDTA/pepstatin (1 μ g/ml)/2 mM-phenylmethanesulphonyl fluoride, followed by disruption of cells by 15–20 strokes with tight-fitting Dounce homogenizers. The broken-cell suspension was centrifuged at 50 g for 10 min. The supernatant was re-centrifuged at 15200 g for 25 min. The resultant microsomal preparation was washed in 20 mM-Hepes, pH 7.4, and used immediately or stored in liquid nitrogen at a concentration of 2–6 μ g of protein/ μ l. Protein was quantified by the Bradford (1976) method.

Whole-cell labelling studies

Rat MC were grown to near confluence in 75 cm² culture flasks in RPMI 1640 with additional elements as described above. The medium was removed, and the cell layers were washed three times at 37 °C in PBS. Fresh serum-free medium containing only antibiotics and 0.5% BSA was added. To label the glycerol moiety of cellular lipids, the cultures were incubated for 12 h at 37 °C with 4 μ Ci of [³H]glycerol/ml (sp. radioactivity 200 mCi/mol) as previously described (Lovett *et al.*, 1986, 1987). The labelling medium was removed, and the cells layers were washed twice with PBS at 37 °C. Fresh medium containing 0.5% BSA with or without 10–100 ng of Lipid A/ml was added. Flasks were harvested at sequential time periods by washing twice with PBS at 4 °C, followed by two washes with methanol at 4 °C. Precipitated cells were scraped from dishes. The lipids were extracted in chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957) and separated by h.p.l.c. as described below. Effluent fractions were analysed in two ways: the A₂₀₆ was monitored (unsaturated acyl side-chain content), and radioactivity was measured to determine [³H]glycerol labelling of each lipid fraction.

H.p.l.c. analysis

Preparations of rat MC microsomes (crude microsomal fraction containing plasma membranes; Lovett *et al.*, 1988a) (250–300 μ g of protein) were preincubated for 5 min at 37 °C in acyltransferase reaction buffer, consisting of 125 mM-Tris/HCl, pH 7.4, 5 mM-Na₂ATP, 5 mM-MgCl₂ and 0.1 mM-CoA. Experimental incubations included 10–100 ng of Lipid A/ml, 25 mM-NaF, 100 μ M-guanosine 5'-[γ -thio]triphosphate (GTP[S]), 100 nM-dilinoleoyl-PA, or 4 μ M-dilinoleoyl-PC. To determine sensitivity of G-protein(s) to pertussis toxin, microsomes were prepared from MC that were preincubated with pertussis toxin (0.1–100 ng/ml) for 4 h before harvest of cells. At indicated times, samples of the preparations were added to 8 ml of chloroform/methanol (2:1, v/v) to extract lipids. H.p.l.c. analysis was performed on a Gilson system 45 with a Waters μ -Porasil silica column (0.45 cm $\times$ 25 cm). The mobile phase consisted of a gradient of 1–9% water in hexane/propan-2-ol (3:4, v/v) run at

a flow rate of 1 ml/min. This was found to give resolution of DAG, PA, PI, PS, PC and sphingomyelin. With a shallower gradient, better separation of PA subfractions was obtained. Lipids in the column effluent were monitored at 206 nm, a wavelength of optimum absorption for unsaturated acyl side chains. Fractions found to contain large concentrations of these (i.e. PE and PA) were monitored for changes in relative concentration (Harris & Stahl, 1983). For experiments where a fluorescent side chain was covalently bound into the phospholipid fraction, fluorescence was monitored sequentially in the h.p.l.c. column effluent with excitation at 325 nm and emission at > 390 nm. H.p.l.c. fractions were also analysed for phosphorus content, amines and acyl esters by established methods (Kornberg & Pricer, 1953; Keenan *et al.*, 1968).

T.l.c.

Extracts of cellular or microsomal lipids isolated via h.p.l.c. were analysed by t.l.c. on Whatman K-6 silica gel G plates developed at 4 °C in chloroform / methanol / 0.9 % NaCl (20:10:1, by vol.), made visible by brief exposure to iodine vapour, and compared with purified standards (Goppelt & Resch, 1984). This system permits resolution of PC, PS, PI and PE in one dimension. PA was analysed on silica G plates by using the upper phase from a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (9:5:2:10, by vol.) (Billah *et al.*, 1981). Neutral lipids were separated from phospholipids by t.l.c. on silica G plates in light petroleum (b.p. 30–60 °C)/diethyl ether/acetic acid (50:50:1, by vol.).

Methyl esterification of lipids and g.l.c.

The acyl content of non-esterified fatty acid fractions, neutral lipids or phospholipids was determined by g.l.c. after esterification with BF₃/methanol. BF₃/methanol (Supelco) was added to the sample in hexane or pentane, and the sample was heated to 100 °C for 10 min. The reaction was stopped by adding $\frac{1}{4}$ vol. of water. After mixing, the fatty acid derivatives were extracted into the upper organic phase. Fatty acid methyl ester standards of myristate, palmitate, stearate, oleate, linoleate, linolenate, arachidate, arachidonate, eicosatrienoate, eicosapentaenoate and docosahexaenoate were obtained from Supelco. G.l.c. was performed on a Hewlett–Packard model 5790A g.l.c. apparatus with a 1.8 m (6 ft) $\times$ 3.2 mm ($\frac{1}{8}$ in) column packed with GP (3 %)/SP-231 (2 %) on 100/120 Chromasorb WAW with nitrogen as carrier gas. Analysis was by flame ionization with integration of peaks by an HP-3388 integrator. The oven temperature was programmed for 2 min at 190 °C, followed by a gradient to 220 °C at the rate of 2 °C/min.

Assay of phosphatidylethanol (PEth) formation

Formation of PEth by the transphosphatidylase activity of PLD was measured to confirm the presence of PLD activity (Kobayashi & Kanfer, 1987). The control reaction was performed with rat kidney microsomes (100–200 μ g of microsomal protein) in the presence of 120–220 mM-ethanol containing 50–100 nCi of [¹⁴C]ethanol in 0.1 M-sodium acetate (pH 5.6), 37.5 mM-CaCl₂, 0.5 mM-SDS and 1 mg of cabbage PLD. Phospholipids were extracted and separated by h.p.l.c. as described above. Labelled phospholipid fractions were identified as PEth and [¹⁴C]PEth by t.l.c. and mass spectroscopy. The retention time of PEth on h.p.l.c. was similar to that of PA. Rat MC microsomes were then stimulated with Lipid A as above or with 100 μ M-GTP[S], in the presence of 86 mM-[¹⁴C]ethanol. The reaction was stopped, and the lipids were extracted and separated by h.p.l.c.; 0.5 min fractions were collected and counted for ¹⁴C content. Identified PEth fractions were analysed by g.l.c. and mass spectroscopy to

determine (1) the acyl content of the phospholipid, and (2) that the masses of PEth moieties were those expected to be produced from the measured acyl-chain content of PE.

Fast-atom bombardment mass spectrometry (f.a.b./m.s.) of h.p.l.c.-isolated fractions

F.a.b./m.s. spectra were acquired with a VG 70 SEQ tandem hybrid instrument of EBqQ geometry (VG Analytical, Altrincham, Cheshire, U.K.). The instrument was equipped with a standard unheated VG f.a.b. ion source and a standard saddle-field gun (Ion Tech Ltd., Middlesbrough, U.K.) producing a beam of xenon atoms at 8 keV and 1 mA. The mass spectrometer was adjusted to a resolving power of 1000, and spectra were obtained at 8 kV and at a scan speed of 10 s/decade. In this study, all samples were applied to the f.a.b. target as a matrix in the positive-ion f.a.b./m.s., and triethanolamine was used as a matrix in the negative-ion f.a.b./m.s.

Whole-cell stimulation studies

These studies were performed with rat MC grown on coverslips. MC were grown to sub-confluency, and placed in rest medium containing 0.5 % BSA with antibiotics 12 h before the experiment. No fetal-bovine serum was used. To initiate the study, coverslips were washed five times in warm PBS and placed in medium containing one of the following: (a) control (rest) medium; (b) 50 nM-1,2-*sn*-dilinoleoyl-PC; (c) 15 nM-1,2-*sn*-dilinoleoyl-PA. After incubation for 5 min, coverslips were washed three times with cold PBS and prepared for fluorescence microscopy (Lovett *et al.*, 1990; Bursten *et al.*, 1991). For this analysis, two coverslips from each time point were immersed in 3.7 % (v/v) formaldehyde in PBS containing 50 μ g of 1-palmitoyl-lyso-PC/ml and 25 units of NBD-phalloidin/ml (Barak *et al.*, 1980), which binds to F-actin. Samples were washed three times in ice-cold PBS, dried, and fluorescence microscopy was performed.

Statistical analysis

Representative experiments performed two or more separate times are given for whole-cell labelling studies. In each case, phospholipid analyses were performed in groups of four to eight, and the data are presented as means $\pm$ 1 s.d. Other data were analysed by analysis of variance and multiple comparison techniques, with *P* < 0.05 considered significant (Tukey, 1949; Dunnett, 1964).

RESULTS

Whole-cell glycerol labelling and distribution of unsaturated mass

Rat mesangial cells were labelled with [³H]glycerol to examine possible transfers of the glycerol moiety between phospholipid species. After 60 s of stimulation with Lipid A, the [³H]glycerol content of PE in whole MC decreased by 80 %, whereas [³H]glycerol in PA species doubled (Fig. 1a). This decrease in [³H]glycerol in PE and increase in labelled PA continued for 2 min after stimulation. Other phospholipid species did not change [³H]glycerol content during this time (cf. legend to Fig. 1). The unsaturated acyl content of each phospholipid species was also measured in these cells for the same time course (Fig. 1b). After 60 s of Lipid A stimulation, there was a decrease in unsaturated acyl composition of PE species that was proportionate to the 80 % decrease in glycerol content. Unsaturated acyl chains in PA began increasing after 5 s of stimulation. After 30 s of stimulation the unsaturated acyl chains in PA increased by 200 %, whereas the [³H]glycerol content increased by 100 %. It

Fig. 1. Effect of Lipid A on whole-MC PA and PE metabolism

Confluent MC layers were pre-labelled with [^3H]glycerol in serum-free media (see the Materials and methods section). After removal of the labelling media, the washed cell layers were incubated from 0 to 2 min with serum-free media containing 100 ng of Lipid A/ml. Lipids from unstimulated cultures were analysed in parallel with stimulated cultures. At the indicated times, cells were washed with 4 ml of ice-cold methanol, then extracted into chloroform/methanol (2:1, v/v), and lipids were analysed by h.p.l.c. (see the Materials and methods section). Lipid species were detected and relative unsaturated masses determined by u.v.-absorption spectroscopy at 206 nm (λ_{max} for $\text{C}_{16}\text{--C}_{22}$ unsaturated acyl side chains). Fractions of effluent were collected, and analysed by β -scintillation counting. [^3H]Glycerol labelling of phosphatidylserine (1210 ± 150 d.p.m./100 μg of protein), PI (1750 ± 215 d.p.m./100 μg of protein) and PC (76400 ± 1455 d.p.m./100 μg of protein) and A_{206} for these phospholipids did not vary significantly during the incubation. The [^3H]glycerol d.p.m. values at zero time were within experimental error of the values shown for 5 s. (a) [^3H]Glycerol labelling of MC PA and PE: PA, control (□); PA + Lipid A (■); PE, control (△); PE + Lipid A (▲). (b) A_{206} of MC, PA and PE (relative unsaturated mass) relative to A_{206} of all lipids extracted from MC (i.e. % absorption of each fraction at the given time): PA, control (□); PA + Lipid A (■); PE, control (△); PE + Lipid A (▲).

appeared that, after 60 s of stimulation with Lipid A, there was a parallel decrease in PE glycerol and unsaturated acyl content. In contrast, after the same period of Lipid A stimulation, there was an increase in both PA glycerol and unsaturated acyl mass. This raised the possibility of conversion of PE into PA by PLD.

Lipid A and dilinoleoyl-PA stimulation of rat MC microsomes

To study rapid changes in membrane lipids caused by Lipid A, rat MC microsomes were stimulated with Lipid A. Stimulation with Lipid A resulted in rat MC microsome response similar to

Fig. 2. Effect of Lipid A and 1,2-*sn*-dilinoleoyl-PA on MC microsome lipid species

Confluent MC incubated in rest medium for 12 h were converted into microsomes, and then stimulated with 100 ng of Lipid A/ml or 1 μM -1,2-*sn*-dilinoleoyl-PA, a reaction product of lyso-PA acyl-transferase produced after Lipid A stimulation of MC for 3–10 s. In some cases, MC in rest medium were preincubated for 5 h with 0.1, 1, 3, 10 or 100 ng of pertussis toxin/ml before isolation of MC microsomal fractions, followed by stimulation with the reagents listed above. At indicated times, the reaction was stopped by addition of 8 ml of ice-cold chloroform/methanol (2:1, v/v), followed by vigorous shaking. Lipids were then extracted as described in the Materials and methods section and analysed by h.p.l.c. Lipid species were detected and their relative masses determined by u.v.-absorption spectroscopy at 206 nm. Kinetics of PA and PE species unsaturated acyl content in MC microsomes stimulated with the indicated reagents for 0–5 min are shown: PA, control (□); PA + Lipid A (▲); PE, control (△); PE + Lipid A (■).

Table 1. Pertussis-toxin pre-treatment of MC microsomes: effect on Lipid A- and dilinoleoyl-PA-induced changes

Confluent rat MC incubated in rest medium for 12 h were converted into microsomes and stimulated with 100 ng of Lipid A/ml or 1 μM -1,2-*sn*-dilinoleoyl-PA. The reaction was stopped with ice-cold chloroform/methanol (2:1, v/v); lipids were then extracted and separated by h.p.l.c. The relative mass of each lipid species was determined by u.v. absorption at 206 nm. Abbreviation: ptx, pertussis toxin. Significant change in lipids compared with control microsomes: * $P < 0.01$, † $P < 0.05$.

Condition	Lipids (% of A_{206})		
	PA	PE	DAG
Control microsomes (no ptx or Lipid A)	6.5	42.4	4.3
Microsomes + Lipid A or dilinoleoyl-PA	18.7*	21.3*	17.4*
Microsomes pretreated with ptx (0.1 ng/ml)†	7.3	41.5	3.7
Microsomes pretreated with ptx (0.1 ng/ml) + Lipid A‡	38.6*†	26.2*	6.2†
Microsomes pretreated with ptx (0.1 ng/ml) + dilinoleoyl-PA‡	35.2*†	28.4*	5.1†

‡ Mesangial cells in rest medium were preincubated for 4–5 h with 0.1–100 ng of pertussis toxin/ml before preparation of microsomes.

that seen in whole MC (Fig. 2). There was a decrease in PE unsaturated acyl mass beginning at 15–30 s, sustained for 5 min in the presence of Lipid A. Over the same time course, there was an increase in PA unsaturated acyl mass. This supported our

Fig. 3. Effect of GTP[S] on MC microsomal lipid distribution

MC were grown to confluency, rested for 12 h, and then converted into microsomes. They were then stimulated with 100 μ M-GTP[S], an activator of G-proteins, for 15 s (a), 30 s (b), 45 s (c) and 60 s (d). The reaction was stopped at the indicated times by addition of 8 ml of chloroform/methanol (2:1, v/v), followed by extraction of lipids and separation by h.p.l.c. Abbreviations: PS, phosphatidylserine; SM, sphingomyelin; Rel. Int. fluor.₍₃₂₅₋₃₈₀₎ refers to fluorescence with excitation at 325 nm and emission at 380 nm.

hypothesis that, when rat MC were stimulated with Lipid A, PLD was hydrolysing PE to produce PA.

To examine possible G-protein regulation of PLD activity, whole MC were treated with pertussis toxin (0.1–100 ng/ml) for 4 h before isolation of the microsomal fraction. Microsomes from control and pertussis-toxin-pre-treated MC were then stimulated with Lipid A or dilinoleoyl-PA, a PA previously shown to activate MC by inducing actin reorganization similar to that seen with Lipid A (Bursten *et al.*, 1991). 1,2-*sn*-DAG was measured to determine whether phospholipase C or PA phosphohydrolase was activated in these circumstances. After 30 s at 37 °C, control microsomes had the distribution of PA, PE and DAG shown in Table 1. Large increases in PA and DAG, and a large decrease in PE, were induced by Lipid A or dilinoleoyl-PA. Without a stimulant, microsomes pre-treated with pertussis toxin showed no differences from control microsomes in distribution of these lipids. However, microsomes from cells treated with pertussis toxin had a different distribution of lipids after stimulation with Lipid A or dilinoleoyl-PA. In these microsomes, no change in DAG distribution occurred, whereas the proportion of PA was greatly increased, and a decrease in PE was observed.

This suggested the following. (1) PE hydrolysis resulted in formation of PA rather than DAG, indicating activation of PLD rather than phospholipase C. The same results were obtained in the presence of 25 mM-NaF, which inhibits phospholipase C (results not shown). (2) Formation of 1,2-DAG resulted from activation of PA phosphohydrolase, which was blocked by pertussis-toxin preincubation (also cf. Bursten & Harris, 1991). (3) PE hydrolysis and PA formation were not blocked by pertussis-toxin preincubation. Both dilinoleoyl-PA and Lipid A appear to activate PLD.

Effect of GTP[S] on PE and PA species

GTP[S], a G-protein activator, was added to microsomes to examine (1) the role of lyso-PA acyltransferase in increasing the proportion of PA in membrane lipids, and (2) possible activation of PLD via non-pertussis-toxin-sensitive G-proteins. The fluorescent fatty acid *cis*-parinaric acid ($C_{18:4}$) was used to monitor the acyltransferase reaction. Fig. 3(a) shows a normal-phase h.p.l.c separation of lipids from microsomes that had reacted for 15 s with GTP[S] and *cis*-PnA. The upper tracing shows unsaturated acyl mass of phospholipid fractions, and the lower tracing shows

Table 2. Effect of ethanol on PA and PEth formation in MC microsomes

For PA, MC microsomes were incubated in the absence or presence of Lipid A (100 ng/ml), 1 μ M-dilinoleoyl-PA or 100 μ M-GTP[S]. The reaction was stopped by addition of cold chloroform/methanol (2:1, v/v). Lipids were extracted and separated on h.p.l.c. Relative unsaturated acyl mass was quantified at 206 nm. For PEth, MC microsomes were treated with the above reagents in the presence of 125 mM-ethanol. Statistics: * P < 0.05 for significant increase in PA compared with control sample; † P < 0.05 for significant decrease in PA compared with Lipid A-stimulated sample; ‡ P < 0.001 for significant increase in PEth.

Conditions	PA	PEth
Control (no additions)	5.3	—
+ Lipid A	23.2*	—
+ dilinoleoyl-PA	21.5*	—
Lipid A + ethanol	3.4†	36.7‡
+ dilinoleoyl-PA + ethanol	7.6†	34.9‡
+ GTP[S] + ethanol	6.2†	33.6‡

Fig. 4. F.a.b./m.s. of PA species isolated after h.p.l.c. separation

MC microsomes were stimulated with Lipid A for 1–5 min, followed by quenching of the reaction with ice-cold chloroform/methanol (2:1, v/v), lipid extraction, and separation by h.p.l.c. PA fractions formed at these times were isolated, re-purified by h.p.l.c., and divided into samples. One was treated with BF_3 /methanol, with formation of fatty acyl methyl esters from the acyl side chains. These were analysed by g.l.c. showing predominant side-chain composition. The other sample was analysed by f.a.b./m.s. as described in the Materials and methods section. From these data together, most probable identities of each m/z ratio were assigned. See the text for identities of predominant peaks.

fluorescence intensity due to *cis*-PnA content of each phospholipid. A large DAG fraction (retention time 4.5–5 min) was labelled with *cis*-PnA. This pattern was characteristic of normal-phase h.p.l.c. separation of lipids of these microsomes, and showed formation of fluorescent-labelled PA which was rapidly converted into DAG (Bursten *et al.*, 1989). After 30 s of stimulation (Fig. 3b), the PE fraction had begun to decrease, and the PA fraction to increase. After 45 s (Fig. 3c), the PE continued to decrease, but PA fractions had also begun to decrease and a new lipid (DAG-2) fraction was seen, with a retention time of 6–7 min. After 60 s (Fig. 3d), the PE fraction had nearly disappeared, the PA fractions had substantially diminished, and a large *cis*-PnA-unlabelled DAG fraction was found. PS, PC and sphingomyelin did not change within this time period. PI was not present in significant amounts.

PA can be synthesized by phosphorylation of DAG by the enzyme DAG kinase. To examine this possible synthetic route,

the above experiments were performed in the presence of the DAG kinase inhibitor R50922. The results (not shown) were the same in the presence or absence of this inhibitor, indicating that phosphorylation of DAG to PA is not the mechanism by which PA was formed.

These data indicate that in rat MC microsomes both a PE-directed PLD and a PA phosphohydrolase were activated via a G-protein. The sensitivity of PA phosphohydrolase activity to pertussis toxin, whereas the toxin had no effect on PLD activity, suggested two different G-protein activities.

Verification of PLD activity: formation of PEth

In the presence of > 20 mM-ethanol, PLD can catalyse a base-exchange reaction using ethanol rather than water, resulting in a phospholipid being converted into PEth rather than PA (Kobayashi & Kanfer, 1987). Formation of PEth is regarded as evidence of the action of PLD. In our h.p.l.c. separation PEth had a unique retention time of 11.5–12.5 min, so it could be distinguished from PA species. When microsomes were incubated with [^{14}C]ethanol and exogenous PLD, the fraction of retention time 11.5–12.5 min was the only fraction containing ^{14}C , other than the void volume.

The endogenous PLD in rat mesengial cells incubated in the presence of 86 mM-[^{14}C]ethanol generated 0.88 ± 0.09 nmol of PEth/min per mg of protein. Addition of 100 ng of Lipid A/ml to the microsomal suspension stimulated the PLD to an activity of 4.26 ± 0.27 nmol/min per mg. Addition of 100 μ M-GTP[S] to microsomes resulted in PLD activity of 5.16 ± 0.14 nmol/min per mg. The stimulation of this endogenous PLD in microsomes by Lipid A, dilinoleoyl-PA and GTP[S] is shown in Table 2. In the presence of ethanol the amount of PA decreased as PEth was generated in these samples. This occurred in an ethanol-dose-dependent manner (results not shown). These results suggested that the PA formed after the microsomes were stimulated was the result of the action of PLD and that this enzyme was associated with a G-protein, as GTP[S] caused significant stimulation of the enzyme.

Microsomes incubated with 86 mM-[^{14}C]ethanol and stimulated with Lipid A synthesized large amounts of the [^{14}C]PEth. In the presence of ethanol, PA synthesis after stimulation with Lipid A or dilinoleoyl-PA was decreased to control levels, whereas PEth formation was significantly increased (Table 2). These observations taken together indicate that Lipid A and dilinoleoyl-PA cause significant and sustained formation of PEth in the presence of ethanol. The presence of ethanol decreased PA formation, confirming PLD activation.

Mass spectrometry of PA fractions formed from PE

By using the h.p.l.c. solvent gradient II, PA and DAG fractions were separated on the basis of acyl-chain composition (cf. Fig. 3). The PA fractions formed during the 1–5 min decrease in PE fractions were separated, purified, and analysed by mass spectrometry (Fig. 4). The PA identity and purity was confirmed by t.l.c., indicating that there was no contamination by other lipids in these samples. By f.a.b./m.s., predominant mass peaks were found at 699 (1-oleoyl-2-linoleoyl-PA), 701 (1-oleoyl-2-oleoyl-PA and 1-stearoyl-2-linoleoyl-PA), 712 (1-*O*-palmitoleoyl-2-docosapentaenoyl-PA), 714, (1-*O*-palmitoyl-2-docosapentaenoyl-PA), 719 (1-*O*-stearoyl-2-eicosanoyl-PA) and 728 (1-stearoyl-2-eicosatrienoyl-PA). These fractions are enriched in 1-*O*-alkyl, 1-*O*-alkenyl and 2-unsaturated PAs, suggesting a selective preference of rat MC PLD for alkyl ether/unsaturated PE as a substrate. Mass peaks at 605, 619 and 733 are diglyceronium derivatives of the dominant PA species formed by loss of the phosphate moiety after xenon bombardment.

Effect of pertussis toxin on index of MC phenotypic change

We have previously reported that MC exposed to 15 nM-Lipid A or 15–50 nM-dilinoleoyl-PA undergo rapid shape change, membrane invagination and actin reorganization (Bursten *et al.*, 1991). Having shown that pertussis toxin blocked the hydrolysis of PA to DAG by PA phosphohydrolase, we examined the effect of pertussis toxin on rapid whole-MC phenotypic changes. Control MC stained with NBD-phalloidin showed the prominent stress fibres, whereas MC stimulated for 5 min with Lipid A or dilinoleoyl-PA showed rapid actin reorganization. Pre-treatment of cells with pertussis toxin blocked this effect of Lipid A after 5 min and 20 min of stimulation, as well as the effect of dilinoleoyl-PA after 5 min (results not shown). The blockade by pertussis toxin of both dilinoleoyl-PA- and Lipid A-induced actin reorganization confirms that both of these substances stimulated rat MC through similar G-protein-mediated pathways involving PLD and PA phosphohydrolase.

DISCUSSION

We have previously reported that Lipid A, the core moiety of bacterial endotoxin, activates a lyso-PA acyltransferase within

Scheme 1. Proposed mechanism of Lipid A action in mesangial cells

Lipid A stimulates activity of a lyso-PA acyltransferase, which transfers long-chain unsaturated fatty acids into the *sn*-2 position of lyso-PA synthesized by the action of PA-specific phospholipase A₂ (PLA₂) (linoleoyl-CoA is the prototypic fatty acyl chain shown). This creates a PA species (PA2) highly enriched with long-chain unsaturated fatty acyl groups in the *sn*-2 position: this PA species stimulates the activity of the lyso-PA acyltransferases, as is characteristic of acyltransferases (Szamel & Resch, 1981), and also activates a pertussis-toxin-insensitive G-protein, which in turn activates PLD. The PLD hydrolyses PE, creating a third PA species highly enriched in 1-*O*-alkyl, 1-plasmenyl and 2-unsaturated acyl groups (PA3). This PA is converted into a 1,2-*sn*-DAG species by means of a PA phosphohydrolase. ++ indicates greatly stimulated.

5 s of MC exposure to the phlogogen (Bursten *et al.*, 1989). Activation of this enzyme induced a rapid production of PA species with unsaturated acyl groups in the *sn*-2 position (also cf. Bazan *et al.*, 1984). In this paper we demonstrate that both Lipid A and a product of lyso-PA acyltransferase, 1,2-*sn*-dilinoleoyl-PA, activate a signalling pathway involving the conversion of PE into PA. This was followed by dephosphorylation of PA to produce 1,2-*sn*-diacylglycerol species (DAG) and, as suggested by mass spectrometry, 1,2-diradylglycerol species. The activations of PA phosphohydrolase and PLD were mediated by separable G-protein activities, the first sensitive to pertussis toxin (Fig. 2b) and the second resistant to pertussis toxin. GTP[S] caused a decrease in amount of PE species concomitant with increases in PA mass, followed by conversion of PA into DAG. This process could be observed in microsomes stimulated with GTP[S] (Fig. 3) and occurred at concentrations of NaF sufficient to inhibit phospholipase C (> 25 mM).

PLD has been implicated as an important signal-transducing enzyme in a variety of cells. It has been found to be activated by a variety of separate pathways, including protein kinase C and a pathway sensitive to blockade of ATP generation (Reinhold *et al.*, 1990). In the present study, incubation of microsomes with ethanol produced PEth after either Lipid A or dilinoleoyl-PA activation. Our data show that 1,2-*sn*-dilinoleoyl-PA is sufficient to activate PLD in rat mesangial microsomes, independent of Lipid A stimulation. Dilinoleoyl-PA is a product of the acylation/deacylation pathway that we have previously described (Bursten & Harris, 1991; also cf. Yamashita *et al.*, 1975; Billah *et al.*, 1981). Acylation of lyso-PA by its acyltransferase is dependent on availability of ATP (Lands & Hart, 1964). We suggest that the generation of the *sn*-2-unsaturated PA fraction by lyso-PA acyltransferase serves to activate two G-proteins and/or PLD, generating another new PA species. Activation of lyso-PA acyltransferase, and generation of 2-unsaturated PA species, may thus be the energy-sensitive pathway of PLD activation described by Reinhold *et al.* (1990). This would provide a mechanism for a limited-receptor system to amplify a signal, and generate a rapid cellular response by means of PA synthesis (Scheme 1). In addition, 1-ether-linked diradylglycerol species with *sn*-2-position arachidonate may serve as a precursor of eicosanoids (Chilton & Connell, 1988). The importance of these highly unsaturated PA and resulting diradylglycerol species in MC was demonstrated by the blockade of rapid MC phenotypic changes by low dose of pertussis toxin (0.1 ng/ml), which also blocked activation of PA phosphohydrolase.

The significance of PA as a cytokine has been described (Moolenaar *et al.*, 1986). Evidence has been presented implicating the immediate precursor of PA, and a substrate of lyso-PA, lysophosphatidate, as an important mediator of early cell signalling pathways (van Corven *et al.*, 1989). Our data support the role of PA as a central mediator in rapid glomerular MC activation, particularly by its hydrolysis to DAG. The whole-cell studies described suggest not only that PA may be an important mediator of the activity of Lipid A, but that some aspect of this activity is dependent on G-protein activation, as suggested in WEHI cells (Jakway & DeFranco, 1986).

These studies have shown that Lipid A activation of MC was connected with the synthesis of specific PA species (Bursten & Harris, 1991). We have previously suggested that activation of lyso-PA acyltransferase by Lipid A is sufficient to explain its effects, and that it need not directly stimulate more than one enzyme. Stimulation of lyso-PA acyltransferase by Lipid A results in production of a PA fraction that stimulates further PA production by PE hydrolysis. Thus a rapid amplification mechanism may be switched on by relatively small amounts of Lipid A, causing MC activation. The cellular consequences of PA

stimulation are of increasing interest, and worthy of further investigation.

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