Rapid Activation of Phosphatidate Phosphohydrolase in Mesangial Cells by Lipid A†

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ABSTRACT: Knowledge of rapid events in cell signaling initiated by lipid A, the core moiety of bacterial lipopolysaccharide, is limited. In the present study we have demonstrated that *cis*-parinaric acid (cis-PnA) rapidly labels 1,2-*sn*-diacylglycerol (DAG) subsequent to labeling of phosphatidic acid (PA). Stimulation of microsomal membranes with lipid A decreased the level of PA labeled with cis-PnA within 5 s and increased the proportion of fluorescent label in DAG. Lipid A stimulation of DAG synthesis at 5–15 s was inhibited by incubation of mesangial cells with pertussis toxin prior to isolation of microsomal membranes. Inhibition of DAG formation was accompanied by an accumulation of the mass and fluorescent label in the cis-PnA-labeled phosphatidic acid pool. GTPγS caused a decrease in labeled PA and an increase in labeled 1,2-DAG. We conclude that the PA pool was enlarged via the lipid A sensitive lyso-PA acyl transferase (lyso-PA-AT) and was decreased by a phosphatidate phosphohydrolase to form DAG. The phosphatidate phosphohydrolase was at least partly regulated by a pertussis-sensitive G-protein. Lipid A or 1,2-dilinoleyl-PA, a product of lyso-PA-AT, induced cell activation as monitored by actin reorganization and cellular shape changes. Pretreatment of cells with pertussis toxin prevented the morphological changes normally induced by lipid A or 1,2-dilinoleyl-PA. In contrast, 1-oleoyl-2-acetylglycerol induced rapid actin reorganization and shape change, presumably bypassing the pertussis blockade. We propose that specific pools of PA and PA-derived DAG are key elements in rapid signaling in mesangial cells and are independent of the PI cycle and phospholipase C.

Lipopolysaccharide (LPS) from Gram-negative bacterial cell walls and its active core, lipid A, have profound effects on a variety of cells (Morrison & Ryan, 1979). In macrophages, endothelial cells, B-lymphocytes, and glomerular mesangial cells (MC), these effects include induction of mRNA for interleukin-1 (IL-1) (Fuhlbrigge et al., 1987; Libby et al., 1986), increased secretion of IL-1 (Bakouche et al., 1987), prostanoid synthesis (Aderem et al., 1986a; Lovett et al., 1988a,b), and changes in both protein synthesis patterns (Hamilton et al., 1986) and protein acylation patterns (Aderem et al., 1986b; Bursten et al., 1988).

The early events that mediate LPS/lipid A effects on cells are not fully understood. Recent findings suggest a role for G-protein mediation in the Lipid A dependent induction of IL-1 in WEHI pre-B lymphocytes (Jakway & DeFranco, 1986). Phosphoinositide (PI)[1] turnover occurring in less than 10 min does not appear to be involved in stimulation of rat glomerular MC or murine lymphocytes by lipid A (Bursten et al., 1991; Kester et al., 1989; Rosoff & Cantley, 1985). Specific and rapid binding of lipid A to a membrane protein has been described, but the function of the protein is unknown (Lei & Morrison, 1988a,b).

PI-cycle events are not critical to rapid cell stimulation. Early activation of 1,2-*sn*-diacylglycerol (DAG) synthesis by lipid A or IL-1 does not involve the PI/phospholipase C (PLC) cycle in cultured MC (Kester et al., 1989). Alternate pathways have been suggested in other cell lines such as (a) synthesis of DAG from phosphatidylcholine (PC) and/or phosphatidylethanolamine (PE) in T lymphocytes utilizing a specialized PLC (Rosoff et al., 1988; Besterman et al., 1986) or (b) G-protein involvement of phospholipase D mediated (PLD) conversion of PC or PE to phosphatidic acid (PA) (Bocckino et al., 1987). PA represents a small percentage of total membrane phospholipid (PL) but may be of importance in cellular signal transduction. Insulin increases de novo PA synthesis in myocytes within 1–2 min of stimulation (Farese et al., 1987), followed by increased DAG synthesis not mediated by PLC. PA itself is an autocrine growth factor for certain cells (Moolenaar et al., 1986) and a factor active in promoting calcium flux (Altin & Bygrave, 1987). Lyso-PA, an immediate PA precursor, is a mitogen for fibroblasts and a possible activator of signaling pathways (van Corven et al., 1989). Short-term increases in PA are associated both with increases in phosphoinositide hydrolysis (van Corven et al., 1989) and with generation of 1,2-DAG independent of PI hydrolysis or Ca^{2+} flux (Farese et al., 1985).

A lyso-PA-directed acyl-CoA acyl transferase (lyso-PA-AT) in glomerular MC was stimulated within 5 s after addition of lipid A. Activity of this enzyme was monitored optically following the incorporation of *cis*-parinaric acid (cis-PnA), a fluorescent 18:4 free fatty acid, into endogenous lyso-

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[1] Abbreviations: AT, acyl transferase; CA-M kinase, calmodulin-associated kinase; cis-PnA, *cis*-parinaric acid (9,11,13,15-*cis*,*cis*,*cis*,*cis*-octadecatetraenoic acid); DAG or 1,2-DAG, 1,2-*sn*-diacylglycerols; lyso-PA AT, lyso-PA-directed acyl transferase; MC, mesangial cells; OAG, 1-oleoyl-2-acetylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PLC, phospholipase C; PLD, phospholipase D; Ptd Phydr, phosphatidate phosphohydrolase.

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phospholipids. This occurred irrespective of the presence of R50922, a DAG kinase inhibitor. In less than 5 s after initiation of the stimulus, lipid A in glomerular MC microsomes stimulates formation of unique PA fractions and DAG derived from these fractions. There is evidence that lipid A also promotes DAG catabolism by mechanisms other than DAG kinase.

Preincubation of MC with pertussis toxin prior to microsome preparation blocks dephosphorylation of PA to DAG. The possible importance of these PA-derived DAG fractions in MC activation was shown in growing cells. Addition of lipid A or 1,2-dilinoleoyl-PA to MC caused a shape change and actin reorganization, but these morphological changes were also prevented by preincubation with pertussis toxin. This suggested that lipid A affects early signal transduction by formation of phosphatidic acid and generation of DAG from this PA pool. Synthesis of DAG from PA was apparently dependent upon activation of a G-protein stimulated by lipid A, whereas lipid A stimulation of lyso-PA-AT was independent of G-protein mediation.

Materials and Methods

Materials. Proliferating MC were maintained in RPMI 1640 (Gibco) containing 20% fetal bovine serum (FBS) (Irvine Scientific, Irvine, CA) and supplemented with 300 μg/mL glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL vancomycin (Sigma Chemical Co., St. Louis, MO) and 5 μg/mL transferrin, 10^{-6} M insulin, and 5 ng/mL selenous acid (ITS Pre-mix, Collaborative Research, Waltham, MA). All media components were screened for the presence of exogenous endotoxin by using a *Limulus* amoebocyte lysate assay sensitive to 10–100 pg of endotoxin/mL (E-toxate, Sigma). *cis*-Parinaric acid (cis-PnA, Molecular Probes, Eugene, OR) was prepared as a 1 mM stock solution in ethanol with butylated hydroxytoluene as an antioxidant. A molar extinction coefficient (E) of 78 000 M^{-1} cm^{-1} was used to establish the concentration. NBD-phallacidin (7-nitrobenzo-2-oxa-1,3-diazol-4-yl-phallacidin) for actin staining was obtained from Molecular Probes (Junction City, OR).

Lipids. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin, essentially fatty acid free bovine serum albumin (BSA), 1-palmitoyl-2-stearoyl-PA, and 1-oleoyl-2-acetylglycerol (OAG) were obtained from Sigma. Fatty acid methyl ester (FAME) standards of myristate, palmitate, stearate, oleate, linoleate, linolenate, arachidate, arachidonate, eicosatrienoate, eicosapentaenoate, docosahexaenoate, 1-palmitoyl-2-arachidonoyl-*sn*-phosphatidic acid (PA), 1-palmitoyl-lyso-PC, and 1-palmitoyl-lyso-PI were obtained from Supelco (Bellefonte, PA). 1-Palmitoyl-lyso-PA was obtained from Serdary Labs (London, ON). Stock solutions of phospholipids were prepared by drying an organic solution with nitrogen and then suspending lipids by vortexing the compounds in phosphate-buffered saline without Ca^{2+} containing 0.5% (w/v) BSA, such that final concentrations of BSA in reaction volumes ranged from 0.002% to 0.04% (w/v). Lipids were dispersed in 500 nM stock solutions by active vortexing alternated with 15-s sonications on ice for a total time of sonication of 2–3 min. All phospholipids were characterized for purity via thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) as described below. Gas–liquid chromatography of methyl esters (GLC) was used to confirm acyl chain composition.

Cell Culture and Preparation of Microsomal Fractions. Rat mesangial cells (MC) were obtained from rat glomeruli as previously described (Lovett et al., 1983a,b). Exponential growth was maintained in medium as above in a 5% CO_2, humidified atmosphere. MC appeared as homogenoeus bundles of strap-like cells growing in an interwoven pattern (Lovett et al., 1983a,b, 1986, 1987). MC were used between the fourth and tenth passages at near confluency for preparation of microsomes or cell-labeling experiments (Lovett et al., 1988a; Harris & Stahl, 1983). Where indicated, cycling MC were treated with 0.1–100 ng/mL pertussis toxin for 4 h prior to harvesting and microsomal preparation. Microsomes were prepared from MC by washing confluent MC layers in PBS (4 °C), with scraping followed by cell pelleting via centrifugation at 400g for 10 min. The pellets were suspended in 20 mM sodium borate, pH 10.2, 10 mM EDTA, 1 μg/mL pepstatin, and 2 mM PMSF, followed by disruption of cells by 10 strokes with tight-fitting Dounce homogenizers. The broken cell suspension was centrifuged at 500g for 10 min and the supernate recentrifuged at 15 200 g for 25 min. The resulting microsomal preparation was washed twice in 20 mM HEPES, pH 7.4, and used immediately or stored in liquid nitrogen at a concentration of 2–6 μg of protein/μL. Microsomal protein was quantitated by the Bradford method (Bradford, 1976). The presence of lyso-PA-directed acyl transferase was assayed by published methods (Harris & Stahl, 1983).

Uptake of Fluorescent Label. Mesangial microsomes (250–300 μg of protein) were preincubated for 2 min at 37 °C in 3 mM ATP–Tris, 5 mM $MgCl_2$, and 0.1 mM CoA in 250 mM Tris-HCl buffer, pH 7.4, in a total volume of 0.50 mL (Harris & Stahl, 1983). For initiation of the reaction, *cis*-parinaric acid (cis-PnA) (final concentration 4 μM) was added in 15 μL. The final reaction volume was 500–520 μL. Following incubations at 5 and 15 s, samples were quenched by addition of 8 mL of chloroform/methanol (2:1), and phospholipids were extracted. Where indicated, lipid A (200 ng/mL from S. Minnesota 595A, Ribi, Butte, MT) or 100 μM GTPγS (Sigma, St. Louis, MO) was added. Lipid A was dispersed in a stock solution of 1 mg/mL containing 0.5% BSA as described for lipids above. In experiments with GTPγS, 20 mM NaF was added to inhibit possible PE-directed PLC activity. Each experiment was repeated in the presence of 100 μM R50922, the DAG kinase inhibitor.

High-Performance Liquid Chromatography. High-performance liquid chromatography (HPLC) analysis of phospholipids (PL) from microsomes was performed on total lipid extracts of the sample (Folch et al., 1957; Chen & Chan, 1984). Modified procedures for lipid separation (Chen & Kou, 1982) were followed by using a Gilson System 45 controlled by an Apple IIe computer. A normal-phase μ-Porasil (silica) 5 μm column (0.45 × 25 cm) was used with a solvent gradient of 1–9% water in hexane/2-propanol (3:4 v/v) and a flow rate of 1 mL/min. The column effluent was monitored at 206 nm with a Kratos detector, and fluorescence was monitored with a Gilson filter fluorometer (Model 121), with excitation monitored at 325 nm and emission monitored at >390 nm. Post-run data analysis employed a Gilson Data Master system. Computer imaging of data resulted in differential scaling of PL peaks despite similar PL concentrations. Therefore, peak areas were confirmed by phospholipid phosphorus determinations (Keenan et al., 1968).

Confirmation of HPLC fraction identity was achieved by collection of individual peaks and analysis for total organic phosphorus (Keenan et al., 1968), amines, and acyl esters (Kornberg & Pricer, 1953), according to published procedures (Harris & Stahl, 1983). Thin-layer chromatography (TLC) of lipids was run at 4 °C on Whatman K-6 silica plates de-

Table I: Percent Distribution of Exogenous *cis*-Parinaric Acid in Microsomal Phospholipids[a]

condition	time (s)	DAG	PA-1	PA-2	PA-3
(A) control	5	<5	32	1	37
	15	7	34	2	40
(B) lipid A	5	77	9	<0.5	12
	15	60	12	<0.5	13
(C) GTPγS	5	15	28	5	35
	15	42	16	2	24

[a] Distribution of exogenous cis-PnA covalently bound in phospholipids from mesangial cell microsomes incubated at 37 °C under the indicated conditions. Data are presented as percentages of the total integrated fluorescence area for each analysis, with each point in triplicate to $n = 3$ independent experiments. The significance of a change in fluorescence intensity of >10% ($p < 0.01$) is based on ANOVA. Incubations, lipid extraction, and HPLC analysis were performed as described under Materials and Methods. Integration was performed with the Data Master Gilson system with an Apple IIe computer. The fluorescence intensity (proportional to content of cis-PnA) is independent of the relative acyl mass as seen in Figures 1 and 2. The remaining *cis*-parinanic acid was found in the free fatty acid fraction except as specified in the text. The three PA fractions differ in acyl ester composition, as determined by GLC and mass spectrometry.

veloped in chloroform/methanol/0.9% NaCl (50:25:2.5 v/v) (Harris & Stahl, 1983). Only limited amounts of microsomal lipids were available, and, therefore, extracted lipids from sheep kidney microsomes, asolectin, and egg phosphatidylcholine (PC) hydrolyzed with commercial PLD and PLC were also run on HPLC and then analyzed to confirm PL identity. Where fractions were difficult to separate, fast-atom bombardment mass spectrometry was used to confirm combined peak identities (i.e., PA3 and PE).

Assay of Phosphatidate Phosphohydrolase (Ptd Phydr). Ptd Phydr activity was both indirectly and directly assayed as follows: Microsomes were equilibrated with 500 nM 1-palmitoyl-lyso-PA and 4 μM cis-PnA in the presence of acylation buffer for 10 min, allowing synthesis of cis-PnA-labeled PA at the *sn*-2 position. Microsomes were then divided into aliquots and stimulated with the indicated reagents (e.g., lipid A, GTPγS) for times ranging from 5 s to 5 min. The reaction was stopped by addition of 8 mL of chloroform/methanol (2:1), followed by extraction of lipids and separation on HPLC. The fluorescence intensity for each fraction was monitored as described, and the percentage conversion of fluorescent-labeled PA to fluorescent-labeled 1,2-*sn*-diacylglycerol was calculated from computer imaging/integration. This means of following Ptd Phydr activity was used in Table I.

For direct quantitation of Ptd Phydr activity, 300–600 μg of MC microsomes was placed in acylation buffer with cis-PnA and allowed to come to equilibrium, once again synthesizing *sn*-2-labeled parinoyl-PA. These PA fractions were then isolated by HPLC as above, repurified by HPLC, dried down with argon, and dispersed as previously described. 2-Parinoyl labeling was verified by formation of methyl esters and comparison of FAME concentrations by GLC (cf. below). PA concentration was calculated by using phospholipid phosphorus determinations and a stock solution of parinoyl-PA made at a concentration of 500 nM. 2-*sn*-Parinoyl-PA was then added to MC microsomes in constant amounts (5–100 nM, final concentration) in the presence and absence of 200 ng/mL lipid A and 100 μM GTPγS, and the conversion rate to 1,2-*sn*-diacylglycerol was calculated by using the extinction coefficient of *cis*-parinaric acid. Baseline rates of DG formation were found to be 0.1–0.5 nmol of DG/[min·(mg of protein)], compared to stimulated rates of 5–9 nmol of DG/[min·(mg of protein)], consistent with previously observed Ptd Phydr activity (Cascales et al., 1984).

Methyl Esterification of Lipids and Gas–Liquid Chromatography (GLC). The acyl content of free fatty acid fractions, neutral lipids, or phospholipids was determined by gas–liquid chromatography (GLC) after esterification with BF_3–methanol. BF_3–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 1/4 volume of water. After being mixed, the derivatized fatty acids were in the upper organic phase. GLC was performed on a Hewlett-Packard Model 5790A GLC with a 6 ft × 1/8 in. column packed with GP 3%, SP-23 1/2% on 100/120 Chromasorb WAW with nitrogen as a carrier gas. Analysis was by flame ionization with integration of peaks by an HP-3388 integrator. The oven temperature was programmed for the initial 2 min at 190 °C, followed by a gradient to 220 °C at the rate of 2 °C/min.

Fast-Atom Bombardment Mass Spectrometry (FAB/MS) of HPLC-Isolated Fractions. FAB/MS spectra were acquired with a VG 70 SEQ tandem hybrid instrument of EBqQ geometry (VG Analytical, Altrincham, U.K.). The instrument was equipped with a standard unheated VG FAB ion source and a standard saddle-field gun (Ion Tech Ltd., Middlesex, 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 FAB target as solutions of known concentrations. 2-Hydroxyethyldisulfide (2-HEDS) was used as a matrix in the positive ion FAB/MS, and triethanolamine was used as a matrix in the negative ion FAB/MS.

Whole Cell Stimulation. Whole cell stimulation of intact MC was conducted in the presence and absence of varying concentrations of pertussis toxin (10 ng/mL, 100 ng/mL). Rat MC were grown to subconfluency on coverslips (Lovett et al., 1988a,b), followed by placement in rest medium (Lovett et al., 1983b) for 16 h prior to the experiment. Half of the coverslips were maintained in rest medium containing pertussis toxin for 4 h prior to the experiment. At the initial time, MC coverslips were washed five times in warm PBS and placed in rest medium containing the following reagents: (1) control media; (2) 50 nM 1-palmitoyl-2-stearoyl-PA; (3) 1 nM 1-oleoyl-2-acetylglycerol; (4) 50 nM 1,2-*sn*-dilinoleoyl-PC; (5) 200 ng/mL lipid A; and (6) 1.5 nM 1,2-*sn*-dilinoleoyl-PA. Concentrations are based on previous determinations of activity for phlogogens (Lovett et al., 1988b). Following exposure for 5, 10, or 20 min in the described media, MC coverslips were washed three times with cold PBS and prepared for fluorescence microscopy (Bursten et al., 1991).

Two coverslips from each time point and preincubation status (i.e., with or without pertussis) were fixed and stained by immersion for 20 min in 3.7% formaldehyde in PBS containing 50 μg/mL 1-palmitoyl-lysophosphatidylcholine and 25 units/mL NBD-phallacidin (Barak et al., 1980). Coverslips were then washed three times with ice-cold PBS and dried, and fluorescence microscopy was performed as described previously (Barak et al., 1980; Lovett et al., 1987). A total of 10–15 areas, each containing 100–200 cells, were evaluated for actin reorganization (Bursten et al., 1987, 1991). The results were averaged and expressed as the percentage of cells demonstrating active reorganization; where necessary, the significance of differences was analyzed with an ANOVA statistical analysis program (StatGraphics; STSC, Rockville, MD).

Statistics. For analysis of lipid samples on HPLC by absorption at 206 nm or fluorescence emission at >390 nm,

repetition of experiments five to seven times showed that the control A_{206} for a given lipid fraction varied by no more than 1–2% of the total unsaturated acyl mass. Thus, the significance of the change was calculated as $p < 0.01$ for a change greater than 10% in the absorption or fluorescence emission by analysis of variance. Other data were analyzed by analysis of variance and multiple comparison techniques, with p values of <0.05 considered significant (Dunnett, 1964). Linear and curve fitting were performed with StatGraphics (STSC, Rockville, MD) and displayed with SigmaPlot (Jandel Scientific, Corte Madera, CA).

Results

Phospholipid Distributions of Microsomes Stimulated with Lipid A. This study was undertaken to determine if specific phospholipids in MC microsomes are involved in early cellular signaling events. Acyl transferase activity in MC microsomes was established by incubating microsomes with cis-PnA, CoA, $MgCl_2$, and ATP, followed by extraction and analysis of the PL fractions. The unsaturated acyl mass in each PL fraction was monitored at 206 nm during separation by HPLC.

The pattern of distribution of cis-PnA in PL's 5 s after its addition to MC microsomes under control conditions is shown in Figure 1A: the majority of cis-PnA label appears in PA. PA3, containing 37% of fluorescent label, forms a biphasic delta wave preceding the PE fraction in this experiment. Less than 10% of label was found in PE. A small amount of DAG containing cis-PnA is synthesized at 5 and 15 s (Table IA). These results were not altered by preincubation with 100 μM R50922 (data not shown) an inhibitor of DAG kinase, indicating that the labeled DAG is not the source of PA. Addition of lipid A caused a marked change in probe distribution (Figure 1B). Approximately 57% of the unsaturated acyl mass entered biphasic DAG peaks, with a 15–20-fold increase of fluorescent label in DAG ($p < 0.001$; Figure 1B, lower trace; Table I). The relative unsaturated acyl mass and fluorescence in the PA fractions decreased by 70% ($p < 0.001$; Figure 1A,B). After 5 s of stimulation with lipid A, there was a large increase of both the unsaturated acyl mass and the fluorescent label in DAG with a corresponding decrease in PA. Levels of other PL fractions showed little or no change from control. Lipid A apparently caused a shift in mass from PA to DAG.

Effect of Pertussis Toxin and GTPγS on MC Microsomal Lipids. These data suggested that DAG was generated from PA within the first 5 s after stimulation with lipid A via a phosphatidate phosphohydrolase (Ptd Phydr). To examine the possibility of G-protein mediation of Ptd Phydr, the effects of pertussis toxin preincubation of MC and GTPγS were studied. Microsomes prepared from cells incubated with 0.1 ng/mL pertussis toxin were unable to rapidly synthesize DAG (Figure 1C). PL analysis after a 5-s incubation with lipid A and cis-PnA had a >95% diminution in the DAG peak ($p < 0.01$) and a 5-fold increase of acyl mass in PA fractions ($p < 0.001$). The fluorescent label was present in these PA fractions (Figure 1C), but no fluorescent label was found in DAG. The fluorescent label also entered PE as well as PA3, as shown by the biphasic fluorescence peak. No change in mass was seen in PC, PS, or PI (data not shown). Microsomes stimulated with GTPγS showed a rapid increase of fluorescent label in DAG over 15 s, with a corresponding decrease in PA (Table IC). This suggested that at least part of the Ptd Phydr activity stimulated by lipid A was mediated via a G-protein.

Stimulation of PA synthesis and 1,2-DAG metabolism within 5 s after addition of lipid A appears to be an important element in the initial activation of PL metabolism (Figure 2A,B). The percentage change in DAG fractions at 5 s in

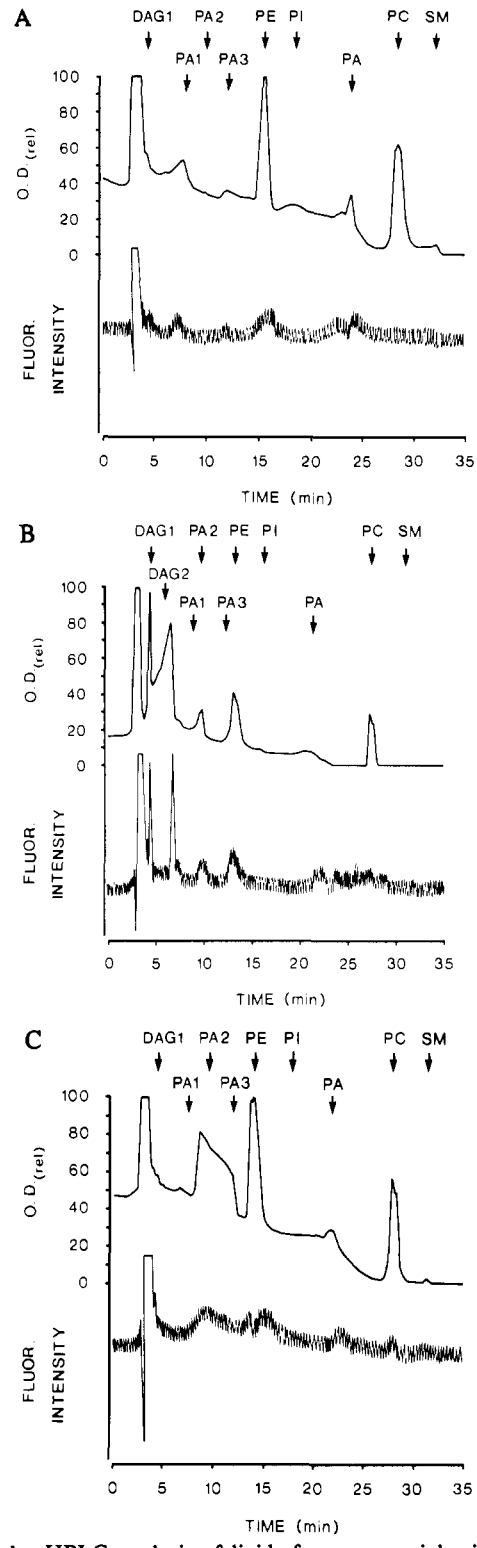

FIGURE 1: HPLC analysis of lipids from mesangial microsomes incubated 5 s with *cis*-parinaric acid and the specified reagents. The microsomal phospholipids were extracted and separated by HPLC (see Materials and Methods). The upper trace represents the lipid mass measured at 206 nm; the lower tracing shows fluorescence intensity due to incorporation of cis-parinaric acid. Four discrete PA peaks are denoted PA 1–3 and PA (R_f 24'). (A) controls. (B) 200 ng/mL lipid A. (C) Microsomes from MC pretreated for 4 h with 0.1 ng/mL pertussis toxin. Microsomes were stimulated 5 s with 200 ng/mL lipid A. Each panel is representative of $n = 2$–4 experiments. Computer scaling is different in panels A–C despite similar concentrations of PE and PC. R_f varies by 5–10%, and PL fluorescence determinations lag behind peak mass by 10–15 s. PA3 appears as a not fully separable delta wave preceding PE in panels A and B. The identify of PA3 was confirmed by mass spectrometry in these experiments.

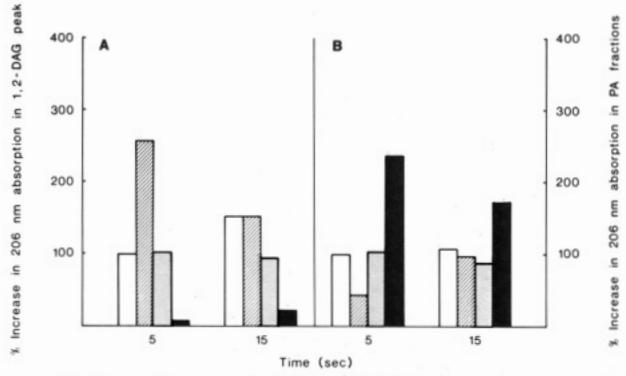

FIGURE 2: Percent increase in DAG and PA fractions from mesangial microsomes following stimulation with lipid A with and without pretreatment with pertussis toxin. Microsomes were treated either in control (rest) medium or in medium with 200 ng/mL lipid A for 5 or 15 s. Bars represent the following: (open) microsomes incubated in rest medium; (diagonal lines) microsomes incubated in medium containing 200 ng/mL lipid A; (dotted) microsomes from MC grown for 4 h in the presence of 0.1 ng/mL pertussis toxin, incubated in rest medium; (solid) microsomes from MC grown for 4 h in the presence of 0.1 ng/mL pertussis toxin, incubated in medium containing 200 ng/mL lipid A. (A) Percent increase in 206-nm absorption in summed 1,2-*sn*-diacylglycerol peaks. (B) Percent increase in 206-nm absorption in summed phosphatidic acid peaks. Each bar represents results from $n = 2$–4 experiments, with the variation in the mean percentage increase being <10% in all cases.

pertussis-treated and control microsomes is seen in Figure 2A. At 5 s, lipid A causes a 165% increase in DAG mass in control microsomes, which returned to lower levels after 15 s. Microsomes pretreated with pertussis toxin synthesized little DAG within 5 or 15 s after stimulation by lipid A, and DAG mass was reduced relative to pertussis pretreated microsomes not stimulated with lipid A. Control and pertussis-treated microsomes contained the same amount of DAG at 5 s. It appeared that a small amount of DAG was synthesized at a constant rate, independent of G-protein mediation, as shown by this similarity in DAG concentrations between control and pertussis pretreated microsomes (also cf. Table IA). However, the decrease in DAG in lipid A treated microsomes at 15 s, and the larger diminution in DAG in pertussis pretreated microsomes stimulated with lipid A, also indicated that lipid A stimulated DAG catabolism as well as DAG and PA synthesis.

The proportion of unsaturated lipid found in PA at 5 s was inversely related to that found in DAG (Figure 2B). At 5 and 15 s, unsaturated PA acyl mass in both control and pertussis-treated microsomes was similar. Lipid A stimulation of microsomes caused a decrease in PA mass at 5 s, with a return to initial levels at 15 s. However, lipid A stimulation of pertussis-treated microsomes showed a 150% augmentation in PA acyl mass at 5 s, with a 75% augmentation in mass at 15 s. This indicated that the accumulation in PA mass was due both to blockage of PA conversion to DAG by pertussis-toxin inhibition of G-protein-induced Ptd Phydr activity and stimulation by lipid A of lyso-PA acyl-CoA acyl transferase (cf. Figure 1C). Lipid A caused an increased total mass in (PA + DAG) or PA, even when DAG mass was decreased or absent, arguing against a role for DAG kinase. This suggested that enhanced PA production was not a consequence of shifting equilibria, with the fall in the acyl transferase end-product PA stimulating AT activity. Stability of acyl mass and phospholipid phosphorus mass in other PL fractions indicated that phospholipase D action was not a source of PA (cf. Figure 1B).

Effect of Pertussis Toxin and GTPγS on Lyso-PA Acyl Transferase. Since lipid A increased the conversion of PA to DAG, we examined potential G-protein mediation of lipid A stimulation of lyso-PA acyl transferase. Acyl transferase activity was determined in microsomes treated with GTPγS, an activator of G-proteins. There was no change in acyl transferase activity with GTPγS (Table II, column 4). Basal activity and lipid A stimulation of acyl transferase was not affected by pertussis toxin. There was no evidence for G-protein modulation of lyso-PA acyl transferase activity.

Table II: Relative Activity of Acyl-CoA Transferase in Mesangial Microsomes[a]

condition	−lipid A	+lipid A[b]
control MC microsomes[c]	1.0 ± 0.03[c]	1.55 ± 0.06
0.1 ng/mL pertussis toxin	1.1 ± 0.04	1.60 ± 0.05
10 ng/mL pertussis toxin	0.96 ± 0.04	1.50 ± 0.07
100 ng/mL pertussis toxin	1.1 ± 0.05	1.54 ± 0.05
100 μM GTPγS	1.06 ± 0.02	1.49 ± 0.08

[a] Relative enzyme activity of the acyl-CoA acyl transferase in mesangial microsomes in the indicated conditions ±lipid A (200 ng/mL) is shown. The enzyme activity was measured by the change in polarization of fluorescence of cis-PNA as this fatty acid was acylated to endogenous lysophospholipids in microsomes (100 μg/mL) in the presence of 250 mM Tris-HCl, 3 mM ATP, 5 mM $MgCl_2$, and 0.1 mM CoA, pH 7.4, at 37 °C (see Materials and Methods). A control curve was established by linear regression (averaged over three experiments). [b] $p < 0.05$ by comparing conditions without lipid A to conditions with lipid A, by using the chi-square test. No significant changes were found between conditions indicated in each row by the chi-square test either with or without lipid A. [c] The control curve was assigned the value of unity, and other curves were compared to this value.

Table III: Effect of Lipid A and GTPγS on the PA ⇒ DAG Conversion in MC Microsomes[a]

	time (s)		
additions	5	15	30
control	<0.1	<0.1	<0.1
lipid A[b]	5.4 ± 0.6	9.2 ± 1.3	7.3 ± 0.6
GTPγS[b]	3.5 ± 0.8	8.7 ± 1.9	5.4 ± 0.7

[a] Expressed as nanomoles of DAG/(mg of protein·min) ± SEM. Rates of conversion of 2-parinoyl-PA to labeled 1,2-*sn*-DAG, determined as described under Materials and Methods. [b] Microsomes were stimulated at 37 °C for the indicated times with either 100 ng/mL lipid A or 100 μM GTPγS. The reaction was quenched with 8 mL of chloroform/methanol (2:1 v/v), and lipids were extracted. Lipids were then separated via HPLC, and the mass of labeled DAG was quantitated.

Effect of Lipid A and GTPγS on Rates of DAG Formation. MC microsomes were treated with 200 ng/mL lipid A (≈125 nM) or 100 μM GTPγS in the presence of 2-parinoyl-PA for 5, 30, and 45 s, the reaction was terminated, and PL extracts were examined. This allowed the quantitation of rates of DAG formation from labeled PA (Table III). Control microsomes at 37 °C did not convert PA to DAG, whereas microsomes treated with lipid A or GTPγS showed rapid and persistent activation of Ptd Phydr. The observed rates [3.5–9.2 nM DAG/(mg of protein·min)] are comparable to those previously observed (Cascales et al., 1989).

To confirm that the source of the newly formed DAG fraction was PA, isolated PA and DAG fractions were analyzed by mass spectrometry. This technique indicated that the predominant PA constituents in 15-s control MC microsomes were 1-palmitoyl-2-oleoyl-PA, 1-palmitoyl-2-linoleoyl-PA, and 1-oleoyl-2-linoleoyl-PA. This combination of acyl groups was not present in significant amounts in other PL fractions. After stimulation of the microsomes, the mass/charge ratio of acyl groups found in DAG was essentially identical with that previously found only in PA (Table IV). This confirms the stimulated conversion of PA to DAG via dephosphorylation.

Table IV: Composition of Constituent PA and DAG Subfractions in MC Microsomes Determined by Fast-Atom Bombardment Mass Spectroscopy[a]

condition	time (s)	DAG	PA
(A) control	5	not detectable	1-palmitoyl-2-oleoyl (675); 1-palmitoyl-2-linoleoyl (673)
	15	not detectable	1-palmitoyl-2-oleoyl (675); 1-palmitoyl-2-linoleoyl (673); 1-oleoyl-2-linoleoyl (699); 1-linoleoyl-2-linoleoyl (697)
(B) +lipid A	5	1-palmitoyl-2-oleoyl (578); 1-palmitoyl-2-linoleoyl (576)	1-palmitoyl-2-oleoyl (675); 1-palmitoyl-2-linoleoyl (673)
	15	1-oleoyl-2-linoleoyl (602)	1-oleoyl-2-linoleoyl (699)

[a] Acyl chain composition of predominant PA and DAG subfractions from MC microsomes was determined. MC microsomes were incubated in acylation buffer for the indicated times, with and without stimulation. Lipid extraction and HPLC analysis and separation were performed as described under Materials and Methods. Isolated PA and DAG fractions from 2 to 3 experiments were analyzed via fast-atom bombardment mass spectrometry. Principal peaks corresponding to a characteristic mass/charge ratio are reported with the mass/change ratio in parentheses. The DAG mass/charge ratio represents formation of a carbonium ion in the *sn*-3 position following removal of PO_4^{3-} in FAB (detected by positive ion FAB).

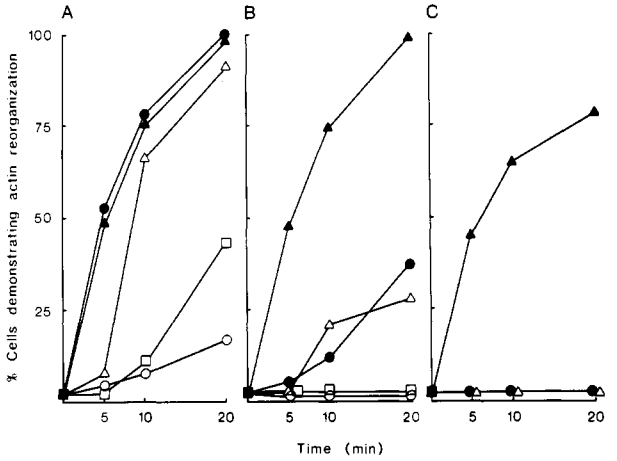

FIGURE 3: Analysis of actin reorganization in mesangia cells incubated with specified reagents. MC was stimulated for 5, 10, or 20 min, followed by simultaneous fixation and labeling with NBD-phallacidin (see Materials and Methods). Groups of 100 cells were counted on two discs for individual time points as specified, and the percentage of cells showing actin reorganization with shape change was determined. Data are from $n = 2$–4 experiments. Reagents: (■) rest medium (control); (○) 1,2-*sn*-dilinoleoylphosphatidylcholine; (□) 1-palmitoyl-2-stearoylphosphatidic acid; (△) 1,2-*sn*-dilinoleoyl-phosphatidic acid; (▲) 1-oleoyl-2-acetylglycerol; (●) 200 ng/mL lipid A. The first panel (A) shows MC preincubated in rest medium, the second panel (B) shows MC preincubated for 4 h with 10 ng/mL pertussis toxin, and the third panel (C) shows MC preincubated for 4 h with 100 ng/mL pertussis toxin.

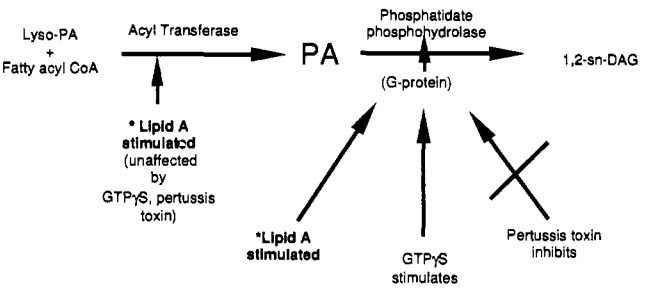

FIGURE 4: Proposed mechanism of activation of phospholipid metabolism in MC by lipid A. Lipid A stimulates activity of a lyso-PA acyl CoA acyl transferase, resulting in an increase in PA containing unsaturated acyl side chains. This PA is converted to 1,2-*sn*-DAG rapidly via phosphatidate phosphohydrolase. Activation of acyl transferase by lipid A was independent of G-protein mediation; increased lipid A activation of phosphatidate phosphohydrolase occurs via a G-protein.

Effects of Pertussis Toxin, 1,2-Dilinoleoyl-PA, and OAG on Whole MC Morphology. Lipid A causes actin reorganization and shape change in cultured rat glomerular MC within 5–10 min. These features are associated with phenotypic change (Bursten et al., 1991). Pertussis toxin was used to examine the role of the G-proteins stimulated by lipid A in these rapid phenotypic changes in MC. The response of MC to various putative cellular activators is presented in Figure 3A. After 12–18 h in medium containing no serum, subconfluent rat MC have a stellate and strap-like cell shape, with large numbers of intermediate stress fibers (Bursten et al., 1991). Stimulation of these cells with 200 ng/mL lipid A resulted in actin reorganization and shape change in 52% of MC within 5 min and 100% by 20 min (Figure 3A). This is similar to stimulation with 1-oleoyl-2-acetylglycerol (OAG), a protein kinase C activator. 1,2-*sn*-Dilinoleoyl-PA, a product of lyso-PA AT recently shown to be mitogenic for mouse mammary epithelium (Imagawa et al., 1989), was nearly as effective as lipid A in inducing actin reorganization and shape change in MC. PA containing predominantly palmitate and stearate acyl side chains was much less effective, inducing actin reorganization in <50% of MC after 20 min. 1,2-*sn*-Dilinoleoyl-PC was even less effective than PA containing saturated acyl chains, with <20% of MC showing a change after 20 min.

The effect of both lipid A and 1,2-*sn*-dilinoleoyl-PA on actin and cell shape after 20 min was greatly attenuated in MC pretreated for 4 h with 10 ng/mL pertussis toxin (Figure 3B): Lipid A had induced actin reorganization in 40% of MC, and 1,2-dilinoleoyl-PA induced actin reorganization in <30% of MC. No effect of saturated PA or 1,2-dilinoleoyl-PC was seen under these conditions. MC pretreated with 100 ng/mL pertussis toxin showed no actin reorganization or shape change after addition of either lipid A or 1,2-*sn*-dilinoleoyl-PA (Figure 3C). OAG, a direct activator of protein kinase C, was able to bypass pertussis blockage and allow the actin reorganization and shape change. This suggests that the DAG produced by Ptd Phydr from PA is involved in these early activation pathways via protein kinase C.

Discussion

Two rapidly activated phosphatidic acid metabolic pathways in MC microsomes have been identified (Figure 4). One pathway involves a lysophosphatidic acid directed acyl transferase that is stimulated within 5 s of addition of lipid A (Figure 1B, Table II). Phospholipid analysis of mesangial microsomes showed a large increase in the proportion of PA in the membrane within 30 s of stimulation with lipid A. Newly synthesized PA contained highly unsaturated acyl chains from endogenous sources and/or exogenous cis-PnA, consistent with the preference of the lyso-PA AT for unsaturated fatty acids (Yamashita et al., 1975). The lyso-PA acyl transferase activity was unaffected by preincubation of microsomes with pertussin toxin or addition of GTPγS. Lipid A stimulated this enzyme in the presence or absence of either G-protein modulation reaction. This suggests that the lipid A interaction with the acyl transferase is not mediated by a

G-protein. It has been recently reported that phosphorylation of lyso-PA acyl transferase caused a marked increase in its activity in parotid acinar cells (Soeling et al., 1989). A calmodulin-associated (CA-M) kinase had greatest phosphorylating activity toward the lyso-PA acyl transferase. This suggests that lipid A may stimulate lyso-PA acyl transferase by activating a kinase that phosphorylates AT. Transfer of unsaturated acyl side chains into PA via acyl transferase, which was not subject to G-protein regulation, indicates a separate cell activation pathway (Bursten et al., 1991; Szamel & Resch, 1981).

The second PA-related pathway involves the enzyme phosphatidate phosphohydrolase (Ptd Phydr) that dephosphorylates PA to form DAG (Figure 4). The formation of cis-PnA-labeled DAG fractions from labeled PA within 5 s of addition of an exogenous substrate provides evidence for activation of this enzyme. The products of lyso-PA AT and Ptd Phydr are significant during early MC activation as shown by the following: (1) after a 5-s incubation of microsomes, cis-PnA was found almost exclusively in PA and DAG; (2) Lipid A caused a 5- to 10-fold increase in unsaturated fatty acids found in PA and DAG; (3) in microsomes from pertussis-toxin treated cells, an accumulation of unsaturated acyl mass and label in PA, with reduced amounts entering DAG, suggested that DAG was derived from PA; (4) 1,2-dilinoleoyl-PA, a product of lyso-PA-AT (Table IV), stimulated rapid actin reorganization and shape change in rat MC (Figure 3).

This suggests that within 5 s lipid A stimulated PA formation via lyso-PA AT and the catabolism of this PA into DAG by Ptd Phydr. The increased proportion of PA after treatment with pertussis toxin, and the lack of effect of pertussis toxin treatment on lyso-PA-AT activity, also suggested that lipid A did not stimulate the acyl transferase. A proportion of Ptd Phydr activity was regulated by a G-protein, as demonstrated by stimulation with GTPγS. It has been shown that Ptd Phydr activity may be hormone sensitive (Pollard & Brindley, 1984). Ptd Phydr may also be tightly regulated by the cell via phosphorylation (Berglund et al., 1982) and/or possibly translocation (Cascales et al., 1984, 1988). Jakway and deFranco (1986) found that pertussis toxin blocked some of the cellular activation parameters of LPS in WEHI cells, which indicated G-protein activation is involved in lipopolysaccharide-mediated cell activation. We found that microsomes from cells treated with 0.1 ng/mL of pertussis toxin and lipid A were unable to convert PA to DAG over short time courses. Induction of this PA ⇒ DAG activity by GTPγS indicates G-protein interaction with Ptd Phydr.

The data suggested that lipid A also stimulated DAG catabolism: (1) After an initial increase in DAG levels, there was a decrease in DAG concentration after lipid A stimulation. This decrease in DAG levels also occurred in the presence of R50922, a DAG kinase inhibitor. (2) Pertussis pretreated microsomes not stimulated with lipid A demonstrated a constant low-level synthesis of DAG. (3) Pertussis pretreated microsomes stimulated with lipid A showed a significant decrease in DAG levels. In parotid acinar cells, specific protein phosphorylation increases DAG catabolism in parallel with lyso-PA AT activity (Soeling et al., 1989). Continued formation and accumulation of labeled PA in the absence of DAG formation, and in the presence of R50922, suggests that DAG kinase is not significantly involved in lipid A stimulated cascades in MC. DAG catabolism via DAG lipase is associated with cellular activation in vascular smooth muscle cells (Moore & Riordan, 1990), which are similar to MC. It is possible that lipid A was acting through a single kinase such as Ca-M kinase to stimulate lyso-PA AT activity and turnover of DAG.

No changes in PI were detected in MC microsomes after incubation with cis-PnA or cis-PnA with lipid A. This supports the findings that the PI/PLase C pathways were not activated in glomerular MC at early times after stimulation with phlogogens (Kester et al., 1989). During the first minute after addition of cis-PnA and/or lipid A, no fluorescent label entered phospholipids other than PA or PE. The appearance of cis-PnA in PE after lipid A stimulation of MC microsomes is consistent with a general increase in lipid metabolism, e.g., conversion of labeled PA ⇒ PE, after lipid A (Bursten and Harris, in preparation). Absence of change in relative PE mass argues against a PE-directed PLC, yet this activity cannot be completely excluded. In addition, no change in PC mass was detected over the first minute. PC-directed PLC and PLD do not appear to be involved in the initial signaling systems of glomerular MC.

The ability of lipid A to activate several distinct pathways within 5–15 s of MC stimulation suggests that it is mimicking the effect of an inherent autocrine molecule or causing unregulated production of such a molecule. The stimulation of lyso-PA AT by lipid A suggests that it is mimicking PA. The structural similarity between core monophosphoryl lipid A and PA has been noted (Matsuura et al., 1989). PA has a unique stimulatory end-product effect on lyso-PA AT (Ferber & Resch, 1977; Szamel & Resch, 1981). If lipid A is mimicking this property of PA in stimulating lyso-PA AT, it would be sufficient to explain its diffuse stimulatory effects on MC by the increased production of unsaturated PA.

The importance of PA as a cytokine has been described (Moolenaar et al., 1986). Additional evidence implicates lysophosphatidate as a mediator of numerous early cell signaling pathways (Van Corven et al., 1989). Our data support the role of PA as a central mediator in the rapid activation of glomerular MC, particularly via its conversion to DAG. The current whole cell studies of actin reorganization not only suggest that PA is involved in cell activation by lipid A but also suggest that some part of this activity is dependent on production of DAG and activation of a protein kinase C. Production of separate PA pools may be the result of stimulation of the lyso-PA acyl transferase, increasing polyene-enriched phospholipids. The lyso-PA AT has a preference for C18 and C20 polyunsaturated fatty acids as substrates (Yamashita et al., 1975). These PA pools may serve several functions: (1) as precursors of specialized DAG pools for protein kinase C activation (Coussens et al., 1986; Rando, 1988), (2) as a direct activator of protein kinase C (Ando et al., 1989), and (3) as a source of PA with unique biophysical properties. PA responds to changes in calcium with lateral phase separation (Eklund et al., 1988), and PA-enriched membrane regions may undergo phase separations that modulate protein function or calcium transport across membranes (Altin & Bygrave, 1987; Szamel & Resch, 1981). Since PA is a precursor for other phospholipids, PA pools containing long-chain unsaturated acyl moieties may serve as sources of generalized membrane fluidity enhancement. Changes in membrane enzyme activities may be observed after increases in phospholipid unsaturated acyl chains (Szamel & Resch, 1981). The importance of unsaturated acyl chains in the *sn*-2 position of PA in determining cell activation has been demonstrated previously in mouse mammary epithelium (Imagawa et al., 1989). It is also possible that enriching the *sn*-2 position of PA-derived DAG with unsaturated fatty acids may provide ligands for formation of other membrane components

such as cholesterol esters (via acyl-CoA:cholesterol AT) after release by DAG lipase (Moore & Riordan, 1990). Our data on actin reorganization demonstrated that very low concentrations of 1,2-dilinoleoyl-PA were effective in initiating phenotypic changes. Lyso-PA AT, acting as a membrane modulator of *sn*-2 unsaturation, may be a central regulating point of cell activation.

These results suggest a complex and rapid interaction of lipid A with PA metabolism, involving lyso-PA acyl transferase and phosphatidate phosphohydrolase in mesangial cells. Evidence that phosphatidic acid is an important regulator of cellular activation is increasing.

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REFERENCES

Aderem, A. A., Cohen, D. S., Wright, S. D., & Cohn, Z. A. (1986a) *J. Exp. Med. 164*, 165–176.

Aderem, A. A., Keum, M. M., Pure, E., & Cohn, Z. A. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5817–5821.

Altin, J. G., & Bygrave, F. (1987) *Biochem. J. 247*, 613–619.

Ando, Y., Jacobson, H. R., & Breyer, M. D. (1989) *Am. J. Physiol. 257*, F524–F530.

Bakouche, O., Koff, W. C., Brown, D. C., & Lachman, L. B. (1987) *J. Immunol. 139*, 1120–1126.

Barak, L. S., Yocum, R. R., Nothnagel, E. A., DeMarco, E. F., & Webb, W. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 980–984.

Berglund, L., Bjorkhen, I., & Einarsson, K. (1982) *Biochem. Biophys. Res. Commun. 105*, 288–295.

Besterman, J. M., Duronio, V., & Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6785–6789.

Bocckino, S. B., Blackmore, P., Wilson, P. B., & Exton, J. H. (1987) *J. Biol. Chem. 262*, 15309–15315.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–257.

Bursten, S. L., Lo, L., & Lovett, D. H. (1987) *Kidney Int. 31*, 314 (abstract).

Bursten, S. L., Locksley, R. M., Ryan, J. L., & Lovett, D. H. (1988) *J. Clin. Invest. 82*, 1479–1488.

Bursten, S. L., Stevenson, F., Torrano, F., & Lovett, D. H. (1991) *Am. J. Pathol.* (in press).

Cascales, C., Mangiapane, E. H., & Brindley, D. N. (1984) *Biochem. J. 219*, 911–916.

Cascales, C., Bosca, L., Martin, A., Brindley, D. N., & Cascales, M. (1988) *Biochim. Biophys. Acta 963*, 384–388.

Chen, S., & Chan, P. H. (1984) *Trans. Am. Neurochem. Soc. 15*, 191–198.

Chen, S.-H., & Kou, A. Y. (1982) *J. Chromatogr. 227*, 25–34.

Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., & Ullrich, A. (1986) *Science 233*, 859–866.

Eklund, K. K., Vuorinen, J., Mikkola, J., Virtanen, J. A., & Kinnunen, P. K. J. (1988) *Biochemistry 27*, 3433–3437.

Farese, R. V., Davis, J. S., Barnes, D., Standaert, M. L., Babischkin, J. S., Hock, R., Rosic, N. K., & Pollet, R. J. (1985) *Biochem. J. 231*, 269–278.

Farese, R. V., Konda, T. S., Davis, J. S., Standaert, M. L., Pollet, R. J., & Cooper, D. R. (1987) *Science 236*, 586–589.

Ferber, E., & Resch, K. (1977) in *The Lymphocyte: Structure and Function* (Marchalonis, J. J., Ed.) pp 593–630, M. Dekker, New York.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–504.

Fuhlbrigge, R. C., Chaplin, D. D., Kiely, J. M., & Unanue, E. R. (1987) *J. Immunol. 138*, 3799–3802.

Hamilton, T. A., Somers, S. D., Jansen, M. M., & Adams, D. O. (1986) *J. Cell. Physiol. 128*, 9–11.

Harris, W. E., & Stahl, W. (1983) *Biochim. Biophys. Acta 736*, 79–91.

Hosaka, K., Yamashita, S., & Numa, S. (1985) *J. Biochem. 77*, 501–509.

Imagawa, W., Bandyopadhyay, G. K., Wallace, D., & Nandi, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4122–4126.

Jakway, J. P., & DeFrano, A. L. (1986) *Science 234*, 743–746.

Keenan, R. W., Schmidt, G., & Tanaka, T. (1968) *Anal. Biochem. 23*, 555–566.

Kester, M., Simonson, M. S., Mene, P., & Sedor, J. R. (1989) *J. Clin. Invest. 83*, 718–723.

Kornberg, A., & Pricer, T. (1953) *J. Biol. Chem. 204*, 345–365.

Lei, M.-G., & Morrison, D. D. (1988a) *J. Immunol. 141*, 1006–1011.

Lei, M.-G., & Morrison, D. C. (1988b) *J. Immunol. 141*, 996–1005.

Libby, P., Ordovas, J. M., Akuger, K. R., Robbins, L. S., Birinyi, L. K., & Dinarello, C. A. (1986) *Am. J. Pathol. 124*, 179–186.

Lovett, D. H., Ryan, J. L., & Sterzel, R. B. (1983a) *J. Immunol. 131*, 2830–2834.

Lovett, D. H., Ryan, J. L., & Sterzel, R. B. (1983b) *Kidney Int. 23*, 342–349.

Lovett, D. H., Szamel, M., Ryan, J. L., Sterzel, R. B., Gemsa, D., & Resch, K. (1986) *J. Immunol. 136*, 3700–3705.

Lovett, D. H., Resch, K., & Gemsa, D. (1987) *Am. J. Pathol. 129*, 543–551.

Lovett, D. H., Bursten, S., Gemsa, D., Bessler, W., Resch, K., & Ryan, J. L. (1988a) *Am. J. Pathol. 34*, 472–484.

Lovett, D. H., Martin, M., Bursten, S., Szamel, M., Gemsa, D., & Resch, K. (1988b) *Kidney Int. 34*, 25–35.

Matsuura, M., Kojima, Y., Homma, J. Y., Shiba, T., & Kasumoto, S. (1984) in *Bacterial Endotoxin: Chemical, Biological, and Clinical Aspects* (Homma, J. Y., Kanegasaki, S., Luederitz, O., Shiba, J., & Westphal, O., Eds.) pp 61–79, Verlag Chemie, Wennheim, Germany.

Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J., & de Laat, S. W. (1986) *Nature 323*, 171–173.

Moore, F., & Riordan, J. F. (1990) *Biochemistry 29*, 228–233.

Morrison, D. C., & Ryan, J. L. (1979) *Adv. Immunol. 28*, 293–450.

Pollard, A. D., & Brindley, D. N. (1984) *Biochem. J. 217*, 461–469.

Rando, R. (1988) *FASEB J. 2*, 2348–2352.

Rosoff, P. M., & Cantley, L. C. (1985) *J. Biol. Chem. 260*, 9209–9215.

Rosoff, P. M., Savage, N., & Dinarello, C. A. (1988) *Cell 54*, 73–84.

Soeling, H. D., Fest, W., Schmidt, T., Esselmann, H., & Bachmann, V. (1989) *J. Biol. Chem. 264*, 10643–10648.
Szamel, M., & Resch, K. (1981) *J. Biol. Chem. 256*, 11618–11623.
Van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T., & Moolenaar, W. H. (1989) *Cell 59*, 45–54.
Yamashita, S., Nakaya, N., Miki, Y., & Numa, S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 600–603.

Identification of a Novel Amino Acid, *o*-Bromo-L-phenylalanine, in Egg-Associated Peptides That Activate Spermatozoa†

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ABSTRACT: Eight sperm-activating peptides containing a novel amino acid were isolated from the egg jelly of the sea urchin *Tripneustes gratilla*. Accurate mass measurement of the peptide in FAB mass spectrometry showed that the mass of the novel amino acid residue was 224.978. On the basis of the isotopic ion distribution and the degree of unsaturation, the mass value indicated that the elemental composition of the amino acid residue was $C_9H_8O_1N_1Br_1$, suggesting that the novel amino acid was bromophenylalanine. Proton NMR spectroscopy, amino acid analysis, and RP-HPLC with three synthetic isomers of bromophenylalanine demonstrated that *o*-bromophenylalanine was the novel amino acid. Derivatization of the amino acid with Marfey's reagent, (1-fluoro-2,4-dinitrophen-5-yl)-L-alanine amide (FDAA), further indicated that the amino acid was the L-isomer. In other sperm-activating peptides isolated from the egg jelly of the sea urchin, both *m*- and *p*-bromophenylalanines were discovered. The presence of *m*-bromophenylalanine has not been previously reported in natural products, while *p*-bromophenylalanine is found in theonellamide F, an antifungal bicyclic peptide from a marine sponge.

The egg-conditioned media (egg jelly) of sea urchins contain sperm-activating peptides (SAPs)[1] that have several effects on sea urchin spermatozoa. They cause stimulation of sperm respiration through intracellular alkalinization (Suzuki et al., 1981, 1984a; Hansbrough & Garbers, 1981; Repaske & Garbers, 1983), transient elevations of cAMP, cGMP, and Ca^{2+} concentrations in sperm cells (Garbers et al., 1982; Suzuki et al., 1984b; Lee & Garbers, 1986; Schackmann & Chock, 1986), and transient activation of the membrane form of guanylate cyclase (Ramarao & Garbers, 1985; Ward et al., 1986). Recently, it has been reported that SAP-I, Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly (Suzuki et al., 1981; Garbers et al., 1982), one of the sperm-activating peptides isolated from the egg jelly of sea urchins *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus*, promotes the acrosome reaction of *H. pulcherrimus* spermatozoa as a specific cofactor (Yamaguchi et al., 1988, 1989; Shimizu et al., 1990), SAP-IIA (Cys-Val-Thr-Gly-Ala-Pro-Gly-Cys-Val-Gly-Gly-Gly-Arg-Leu-NH_2) (Suzuki et al., 1984b), has been shown to act as a potent chemoattractant for spermatozoa of the sea urchin *Arbacia punctulata* (Ward et al., 1985).

The genetic code specifies only 20 amino acids as monomer building blocks in protein synthesis. Posttranslational modifications, such as phosphorylation, glycosylation, methylation and halogenation, of amino acids lead to additional diversity in the final protein products. In the past decade, many different SAPs have been purified and sequenced from the egg jelly of various species of sea urchins (Suzuki, 1990) but none of them has contained a modified amino acid except leucine amide in SAP-IIA. In the present study, we isolated eight SAPs that contain a novel modified amino acid from the egg jelly of the sea urchin *Tripneustes gratilla*. The structure of the novel amino acid was determined by FAB mass spectrometry, ^1H NMR spectroscopy, amino acid analysis, and RP-HPLC. We also found one more novel amino acid and another modified amino acid in some SAPs isolated from the egg jelly of the same species.

Experimental Procedures

Experimental Animals and Chemicals. The sea urchin *T. gratilla* was collected along the Okinawan coast of the East China Sea near Sesoko Marine Science Center, University of the Ryukyus. The sea urchin *H. pulcherrimus* was collected at the coast of Toyama Bay near Noto Marine Laboratory. Marfey's reagent, (1-fluoro-2,4-dinitrophen-5-yl)-L-alanine

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[1] Abbreviations: ACN, acetonitrile; *B/E*, mass spectrometric scan method where flux density (*B*)/deflecting voltage (*E*) = constant; Br-Phe, bromophenylalanine; cAMP, cyclic adenosine 3′,5′-monophosphate; CID, collision-induced dissociation; cGMP, cyclic guanosine 3′,5′-monophosphate; DDW, deionized and distilled water; FAB, fast atom bombardment; FDAA, (1-fluoro-2,4-dinitrophen-5-yl)-L-alanine amide; HPLC, high-performance liquid chlorotography; NMR, nuclear magnetic resonance; RP, reverse phase; SAP, sperm-activating peptide; TFA, trifluoroacetic acid.

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