Accumulation of Lysophosphatidylinositol in RAW 264.7 Macrophage Tumor Cells Stimulated by Lipid A Precursors*

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Lipid A (Fig. 1), the hydrophobic portion of lipopolysaccharide, serves to anchor lipopolysaccharide to the outer membrane of Gram-negative bacteria (1, 2). Lipid A is also responsible for many of the pathophysiological effects associated with Gram-negative infections, including endotoxin-induced shock (3, 4). Some of these events are mediated by macrophages, which are stimulated by lipopolysaccharide to produce and secrete prostaglandins (5–7), interleukin-1 (8), neutral proteinases (9), and tumor necrosis factor (10).

The mechanisms by which lipopolysaccharide activates macrophages are not well understood. One reason for this is that the structure of lipid A, now believed to be the active component of lipopolysaccharide (1, 2, 7), was unknown prior to 1983 (11–15). Recently, a monosaccharide precursor of lipid A was isolated from a mutant strain of Escherichia coli (11, 16) in our laboratory. This substance, N2,0-diacetylglucosamine 1-phosphate (11) (also termed lipid X)1 displays some of the biological activities of lipid A (7, 17–19); but lipid X is unique in that it is nontoxic to animals (20, 21). In some cases, it may actually protect against endotoxin-induced shock associated with Gram-negative bacteremia (21).

Lipid X (Fig. 1) possesses a formal structural resemblance to phosphatidic acid (22). Both have two long-chain fatty acyl moieties attached to a carbohydrate backbone, and both are phosphomonoesters (22). Because of the similarity of lipid X to classical glycerophospholipids (22, 23), we considered the possibility that lipids X and A might exert some of their effects on animal cells by perturbing glycerophospholipid metabolism. Consequently, we investigated the effects of lipid X and other lipid A precursors on the composition of membrane phospholipids in RAW 264.7 macrophage tumor cells, a cell line that responds to lipopolysaccharide by synthesizing various proteins including tumor necrosis factor (24, 25). We now report that the level of a minor phospholipid, tentatively identified as lysophosphatidylinositol, rises 4–8-fold after a 45-min exposure of these cells to lipid X. The lysophosphatidylinositol response is dose-dependent, is observed with much lower concentrations of the more biologically active disaccharide precursor IVA (Fig. 1 and Refs. 26–28), and is correlated with prostaglandin formation, possibly suggesting the involvement of a specific phospholipase A2 activity. The levels of the major glycerophospholipids, as well as lysophosphatidylcholine and lysophosphatidylethanolamine, are not significantly altered. The accumulation of lysophosphatidylinositol is an early response to lipid A precursors and lipopolysaccharide and may provide new molecular insights into the interaction of lipid A with animal cell membranes.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate, lipopolysaccharide W from E. coli 055:B5, A23187, phospholipase A2 (Najas rana), endotoxin-free bovine serum albumin, and most of the lipid standards were
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1 The abbreviations used are: lipid X (2,3-diacylglycerol 1-phosphate), N2,0-bis[(R)-3-hydroxytetradecanoyl]-0-0-glycosamine 1-phosphate; precursor IVa, O-[2-amino-2-deoxy-N2,0-bis[(R)-3-hydroxytetradecanoyl]-0-0-glucopyranosyl][1-6]-2-amino-2-deoxyN2,0-bis[(R)-3-hydroxytetradecanoyl]-0-0-glucopyranosyl]-1,4/-biophosphate; PBS, phosphate-buffered saline; PG, prostaglandin; HPLC, high pressure liquid chromatography; EGTA, (ethylenebis(oxyethyl)enedinitrilo)tetraacetic acid.

2 D. Golenbock, C. R. H. Raetz, and R. A. Proctor, manuscript in preparation.

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Cells were plated in 60-mm diameter tissue culture dishes at a density of 10^5 cells/dish and labeled with 32Pi (5-10 pCi/10^5 cells/dish). The labeled medium was removed, and F-12 medium containing 32Pi, 32P, [6,8,9,11,12,14,15-3H]arachidonic acid, and [2,3-H]inositol was purchased from Sigma. Lysophosphatidylinositol was purchased from Servadary (London, Ontario). Chemical synthesis of lipid X was obtained from Drs. I. Macher and F. Unger (Sandoz Inc., Vienna, Austria). Tissue culture medium was purchased from Sigma. 

Stimulation of Prostaglandin Formation—Cells were plated at 3 x 10^5 cells/35-mm diameter tissue culture dish and allowed to attach for 24 h, essentially as previously described for mouse peritoneal macrophages (32). medium was removed, and 1 ml of F-12 medium (with serum) containing 2 μCi of [3H]arachidonic acid was added. After 5 h, the labeled medium was removed, the cells were washed twice with 3 ml portions of serum-free F-12 medium, and 1 ml of serum-free medium, containing stimulant was added. Medium was recovered after 1 h and added to 3.75 ml of CHCl₃:MeOH (1:2, v/v) containing 300 μg of mouse liver lipid and 20 μg each of PGE₂, PGD₂, and PGI₂, eluted with an 

Solvent System—Several solvent systems were used during thin-layer chromatography. They are designated as follows: solvent system A, chloroform:methanol:acetic acid:H₂O (25:15:4:2, v/v); solvent system B, chloroform:methanol:acetic acid:H₂O (35:15:4:2, v/v); solvent system C, chloroform:methanol:acetic acid:H₂O (65:25:10, v/v); solvent system D, chloroform:methanol:triethyamine:H₂O (60:30:4:2, v/v); solvent system E, n-hexane:diethyl ether:acetic acid (95:5:1, v/v); solvent system G, chloroform:methanol:formic acid (88%) (60:30:10, v/v); solvent system F, ethyl acetate:methanol:acetic acid (95:5:1, v/v); and solvent system H, n-hexane:diethyl ether:pyridine:methanol:H₂O (50:50:20:3:1, v/v).

Isolation of Lipid X and IV₄ from Biological Sources—Lipid X and IV₄ were isolated from E. coli strain MN7 (11) and Salmonella typhimurium strain STi50 (26), respectively. Final purification was achieved by HPLC using an Alltech C₂₅ 10-μm reverse-phase column eluted with an acetonitrile:water:isopropl alcohol system reported previously (29).

Cell Lines and Culture Conditions—RAW 264.7 cells were obtained from the American Type Culture Collection. They were typically maintained in F-12 medium (GIBCO), supplemented with 10% fetal serum unless otherwise indicated. Cells were grown and all stimulation experiments were performed at 37°C, but F-12 medium without serum was used for the stimulation of cells unless otherwise indicated.

Stimulation of Lysophosphatidylinositol Labeling in RAW Macrophages—Cells were plated in 60-mm diameter tissue culture dishes at a density of 10^5 cells/dish and labeled with [3H]arachidonic acid, and [2,3-H]inositol. After incubation for 45 min, the medium was removed, and the cells were washed with 5 ml of phosphate-buffered saline (PBS) (30) and harvested by scraping in 0.8 ml of PBS. The cells were added to 3.0 ml of CHCl₃:MeOH (1:2, v/v) containing 300 μg of carrier lipid (extracted from mouse liver) and 25 μg of carrier lysophosphatidylinositol. After 15 min at room temperature, 1.0 ml of CHCl₃ and 1.0 ml of PBS were added to form two phases. After centrifugation at 600 x g, the upper phase was recovered. Care was taken not to include the interfacial material during removal of the upper phase. The upper phase was washed once with 2 ml of a neutral pre-equilibrated lower phase solution to remove any residual bulk lipids.

Scrubbed cells were resuspended in 0.1 ml of 0.1 M phosphate-buffered saline (PBS) (30) and harvested by scraping in 0.8 ml of PBS. The cells were added to 3.0 ml of CHCl₃:MeOH (1:2, v/v) containing 300 μg of carrier lipid (extracted from mouse liver) and 25 μg of carrier lysophosphatidylinositol. After 15 min at room temperature, 1.0 ml of CHCl₃ and 1.0 ml of PBS were added to form two phases. After centrifugation at 600 x g, the upper phase was recovered. Care was taken not to include the interfacial material during removal of the upper phase. The upper phase was washed once with 2 ml of a neutral pre-equilibrated lower phase solution to remove any residual bulk lipids.

After incubation for 45 min, the medium was removed, and the cells were harvested by scraping in 0.8 ml of PBS. The cells were added to 3.0 ml of CHCl₃:MeOH (1:2, v/v) containing 300 μg of carrier lipid (extracted from mouse liver) and 25 μg of carrier lysophosphatidylinositol. After 15 min at room temperature, 1.0 ml of CHCl₃ and 1.0 ml of PBS were added to form two phases. After centrifugation at 600 x g, the upper phase was recovered. Care was taken not to include the interfacial material during removal of the upper phase. The upper phase was washed once with 2 ml of a neutral pre-equilibrated lower phase solution to remove any residual bulk lipids.

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Radioactivity was measured by scintillation counting (31).

Varying conditions were studied in an arsenityl chloride (31) was added for counting. When an analysis of the entire phospholipid pool, including lysophosphatidylinositol, was desired, the cells were extracted directly under acidic conditions (16), without prior extraction under neutral conditions. In the latter case, the isolated lipids were separated by two-dimensional thin-layer chromatography system consisting of solvent system B in the first dimension and solvent system C in the second dimension.

Lysophosphatidylinositol was isolated from E. coli strain MN7 (11) and Salmonella typhimurium strain STi50 (26), and was added at 1 μg/ml to 1 ml cultures of 1 x 10^6 RAW 264.7 macrophages (32) for the times indicated. The medium was then removed, and acidified with HCl to pH 1.0. The lower phase was recovered after centrifugation at 600 x g for 10 min. The lower phase was dried under nitrogen and analyzed by thin-layer chromatography using solvent system F (32). Radioactive species were localized by fluorography at -65°C using ENHANCE (Du Pont-New England Nuclear), and prostaglandin species were identified by their migration with authentic standards. Radioactivity for each species was quantitated as described above.

Preparation of [32P]-Labeled Lysophosphatidylinositol—Cells were labeled for several generations with [32P] after harvesting the cells, purification of the labeled lipids, phosphatidylinositol was isolated by two-dimensional thin-layer chromatography (33). Phosphatidylinositol was extracted from the scrapings with CHCl₃:MeOH:H₂O (50:5:1), dried under nitrogen, and digested with phospholipase A₂ using a modification of the method of Trotter et al. (34). The reaction mixture was spotted directly on a thin-layer plate, which was developed in solvent system B, to separate lysophosphatidylinositol from other products.

Uptake and Metabolism of [%]Lipid X by RAW 264.7 Cells—[14]C]Lipid X (~10^6 cpm/nmol) was prepared as described previously (11) and was added at 1 μg/ml to 1 ml cultures of 1 x 10^6 RAW 264.7 macrophages (32) for the times indicated. The medium was then removed and acidified with HCl to pH 1.0. The cells were scraped into 1 ml of 0.1 M HCl. Both medium and cells were extracted by the method of Bligh and Dyer (see Ref. 16 and 35). [%]Lipid X and its metabolites were separated by thin-layer chromatography using solvent system G or H. Products were visualized by overnight autoradiography using Kodak X-AR-5 film and quantified by liquid scintillation counting (31).
Above acidic conditions, approximately phosphatidylinositol formation by lipid contribute to the observed extraction procedure described in the text is used, phosphatidylinositol fraction in the absence of lipid decomposition would contribute is removed prior to acidification, and its breakdown cannot broken down to the lyso-form (R. A. Zoeller, P. D. Wightman, M. S. Anderson, and C. R. H. Ftaetz, unpublished observations). This material, which migrated with authentic lysophosphatidylinositol, increased 3.5-fold in the lipid X-treated cells (Table I), rising from 0.2% of the total phospholipid in the absence of lipid X to 0.7% in its presence.

Selective Extraction of the Accumulated Lipid and Its Identification as Lysophosphatidylinositol—The putative lysophosphatidylinositol was recovered in the lower, chloroform phase only if the extractions were performed under acidic conditions. This prompted us to devise a more rapid assay for this material in radiolabeled macrophages, employing its partitioning properties as a function of pH. As detailed under “Experimental Procedures,” the radiolabeled phospholipids of the macrophages are initially extracted under neutral conditions. All the major diacylglycerophospholipids partition into the lower phase, whereas most of the putative lysophosphatidylinositol remains in the upper phase. When this material is recovered from the upper phase by acidification and washing with a fresh lower phase, it is relatively pure and can be separated from remaining contaminants by one-dimensional thin-layer chromatography, as illustrated in Fig. 3. Quantitation of the putative lysophosphatidylinositol recovered in this manner from cells labeled either with $^{32}$P, or $[3^H]$inositol is shown in Table II and is in good agreement with the results obtained by the direct, acidic extraction (Table I and Fig. 2).

The labeled compound that accumulates in the presence of lipid X migrated with authentic lysophosphatidylinositol in four thin-layer chromatography systems (solvent systems A–D). Furthermore, when authentic lysophosphatidylinositol was subjected to the same extraction protocol (Table III), it demonstrated partitioning properties identical to that of the unknown. The data presented above strongly suggest that this substance is lysophosphatidylinositol and that lysophosphatidylinositol accumulates in these cells during exposure to lipid X. It seems likely that the lysophosphatidylinositol is predominantly the 1-monoacyl isomer, but on the basis of the present data, it is not possible to exclude the idea that it arises in vivo as the 2-monoacyl isomer. It was not feasible to isolate enough material for mass spectrometry or NMR spectroscopy. However, the selective extraction method was very useful for monitoring the accumulation of lysophosphatidylinositol under various conditions provided the 71% yield (Table III) was taken into account.

**Table I**

| Phospholipid species | −Lipid X | +Lipid X |
|----------------------|---------|---------|
| Phosphatidylcholine   | 568 ± 62| 2,040 ± 8|
| Phosphatidylethanolamine | 1,242 ± 292| 1,544 ± 166|
| Phosphatidylinositol | 7,756 ± 1,841| 9,411 ± 2,415|
| Sphingomyelin         | 31,310 ± 729| 33,350 ± 2,033|
| Phosphatidylglycerol  | 25,585 ± 673| 22,618 ± 1,064|
| Phosphatidylinositol  | 120,099 ± 2,687| 121,883 ± 6,093|
| Phosphatidylethanolamine | 18,958 ± 1,243| 22,005 ± 931|
| Cardiolipin           | 7,756 ± 1,841| 9,411 ± 2,415|
| LysoPC                | 6,910 ± 588| 7,850 ± 749|
| LysoPE                | 845 ± 399| 992 ± 464|

Counting (Table I). In these initial experiments, the total phospholipid fraction was extracted under acidic Bligh-Dyer conditions (see Ref. 16). As shown in Fig. 2 and Table I, macrophages treated for 45 min with 5 μM lipid X (Fig. 2B) had essentially the same phospholipid composition as untreated control cells (Fig. 2A), with the exception of a slowly migrating component (indicated by the arrowheads in Fig. 2). This material, which migrated with authentic lysophospho-
Macrophage Activation by Lipid A

**TABLE I**

Partitioning properties of authentic lysophosphatidylinositol

|                      | cpm     | % of total label |
|----------------------|---------|-----------------|
| [32P]Lysophosphatidylinositol added to the extraction | 4744 ± 150 | 100 |
| Label recovered in the neutral lower phase | 925 ± 20 | 19 |
| Label recovered in the acidic lower phase | 3300 ± 157 | 71 |
| Unrecovered label | 468 ± 37 | 10 |

**FIG. 3.** Isolation of lysophosphatidylinositol using selective extraction and one-dimensional thin-layer chromatography. RAW cells were labeled, stimulated with lipid X, and harvested as described for Fig. 2. The cells were extracted under neutral conditions (see “Experimental Procedures”), and the upper phase was washed once with 2 ml of a pre-equilibrated neutral lower phase solution. The washed upper phase was then acidified with 50 μl of concentrated HCl and extracted twice using 2 ml of a pre-equilibrated acidic lower phase solution. The acidic lower phases were pooled, dried under N2, and chromatographed in solvent system A. The radiolabeled species were localized by autoradiography.

**TABLE II**

Detection of lysophosphatidylinositol accumulation using either [32P] or [3H]inositol

| Label associated with lysophosphatidylinositol | [32P] | [3H] |
|------------------------------------------------|-------|------|
| −Lipid X (cpm) | 1002 ± 57 | 144 ± 24 |
| +Lipid X (cpm) | 4404 ± 437 | 586 ± 52 |
| −Fold stimulation | 4.4 | 4.1 |

25 min. Chemically synthesized lipid X has essentially the same effect on the lysophosphatidylinositol pool (Table IV) as lipid X isolated from E. coli strain MN7 (11) and purified by HPLC (29) as described under “Experimental Procedures.”

In other biological systems that have been examined, the disaccharide precursor IVa or mature lipid A (Fig. 1) are much more potent agonists than is lipid X (17, 18, 26, 28, 35). In some systems, lipid X may even be an antagonist (21). Accordingly, HPLC-purified precursor IVa (29) and commercial lipopolysaccharide were examined, and both were also active in stimulating lysophosphatidylinositol release (Table IV). Interestingly, phorbol myristate acetate and the calcium ionophore A23187 mimicked the lipid A metabolites in stimulating lysophosphatidylinositol accumulation (Table IV).

**Effects of Cycloheximide and Calcium on Lysophosphatidylinositol Formation**—When cycloheximide was added to the
medium together with biologically derived lipid X, synthetic lipid X, or precursor IVₐ, the accumulation of lysophosphatidylinoositol was almost completely inhibited (Table IV). This inhibition was also observed when phorbol myristate acetate was used as the stimulator. On the other hand, the effect of lipopolysaccharide was only partially blocked by the addition of cycloheximide, and the action of the calcium ionophore A23187 was not changed by cycloheximide. The ability of A23187 to stimulate lysophosphatidylinositol accumulation prompted us to examine the requirement of the response for extracellular calcium. As demonstrated in Table V, the addition of EGTA to the medium at the time of stimulation resulted in a drastic reduction in lipid X-induced lysophosphatidylinositol formation.

**Stimulation of Prostaglandin Formation in RAW 264.7 Cells**—The stimulation of prostaglandin formation by resident peritoneal macrophages is dependent upon extracellular calcium (36) and can be inhibited by the addition of cycloheximide (37). Considering the similarities between this event and lysophosphatidylinositol formation, it was expected that the formation of lysophosphatidylinositol in these cells might be associated with the formation and release of prostaglandins. Therefore, cells which had been prelabeled with [³²P] arachidonic acid were treated with the same agonists that had been used to stimulate lysophosphatidylinositol. All agonists examined were also capable of stimulating prostaglandin release (Table VI). Commercial lipopolysaccharide and precursor IVₐ were the most effective, causing a 9–10-fold increase in the release of both PGD₂ and PGE₁.³ Lipid X, at the concentration used, was only a weak stimulator of prostaglandin release (Table VI); but in these experiments, lysophosphatidylinositol release was not quantitated simultaneously since the conditions for measuring prostaglandin release are somewhat different (see “Experimental Procedures”).

³ Prostaglandins D₁ and E₂ were identified solely by thin-layer chromatography, as described under “Experimental Procedures.” We do not know why RAW cells elaborate more PGD₁ than PGE₂, whereas mouse peritoneal macrophages generated mostly PGE₂.

### Table IV

**Accumulation of lysophosphatidylinositol in response to various stimuli and its sensitivity to cycloheximide**

| Stimulant | Increase in lysophosphatidylinositol labeling (fold) |
|-----------|---------------------------------------------------|
|           | -Cycloheximide +Cycloheximide                     |
| Control (no additions) | 1.0 ± 0.1 | 0.7 ± 0.2 |
| Biological lipid X (2.5 μM) | 5.9 ± 0.4 | 1.3 ± 0.3 |
| Synthetic lipid X (2.5 μM) | 4.4 ± 0.2 | 1.2 ± 0.1 |
| Disaccharide precursor IVₐ (2.5 μM) | 5.0 ± 0.3 | 1.2 ± 0.2 |
| Lipopolysaccharide (2 μg/ml) | 8.3 ± 0.2 | 3.6 ± 0.5 |
| A23187 (10⁻⁴ M) | 5.7 ± 0.7 | 4.6 ± 0.3 |
| Phorbol myristate acetate (10⁻³ M) | 5.6 ± 0.2 | 1.8 ± 0.1 |

### Table V

**Extracellular calcium is required for lysophosphatidylinositol accumulation**

| Stimulant | Label associated with lysophosphatidylinositol |
|-----------|------------------------------------------------|
|           | 0.3 mM CaClᵡ | 0.55 mM EGTA |
| -Lipid X (cpm) | 381 ± 19 | 399 ± 45 |
| +Lipid X (cpm) | 2286 ± 130 | 870 ± 88 |
| Fold stimulation | 6.0 | 2.2 |

### Table VI

**Prostaglandin release in stimulated RAW 264.7 cells**

| Stimulant | PGD₁ (cpm) | PGE₁ (cpm) |
|-----------|------------|------------|
| Control (no addition) | 1010 ± 210 | 279 ± 61 |
| Synthetic lipid X (2.5 μM) | 1770 ± 354 | 450 ± 95 |
| Biological lipid X (2.5 μM) | 2378 ± 269 | 512 ± 63 |
| IVₐ (2.5 μM) | 9614 ± 720 | 2039 ± 133 |
| Lipopolysaccharide (1 μg/ml) | 9281 ± 1730 | 1816 ± 349 |
| Phorbol myristate acetate (10⁻³ M) | 5725 ± 469 | 1190 ± 102 |
| A23187 (10⁻⁴ M) | 5368 ± 229 | 1218 ± 14 |

release, especially at lower concentrations. However, subtle differences were observed in the extent of stimulation in the micromolar range (Fig. 5). As shown in Fig. 5, IVₐ was several orders of magnitude more active than lipid X in stimulating both processes, whereas phosphatidic acid was inactive at all concentrations examined.

Lysophosphatidylinositol production preceded the release of both arachidonic acid and prostaglandin D₃ (Fig. 6). Cellular lysophosphatidylinositol levels peaked 30–45 min after stimulation with IVₐ. Arachidonic acid release leveled off after 45 min, when lysophosphatidylinositol levels began to decrease. Prostaglandin D₃ was released throughout the 90-min period.

### Arachidonic Acid Turnover during Stimulation by Lipid X—

The loss of [³²P]arachidonic acid from the various phospholipid species during stimulation by lipid X was also examined (Table VII). There was a significant loss of label from phosphatidylinositol and, possibly, from phosphatidylethanolamine, whereas phosphatidylcholine showed no change. The loss of label from both phosphatidylinositol and phosphatidylethanolamine was inhibited by the addition of cycloheximide, consistent with the view that arachidonate mobilization might account for the increased levels of prostaglandins recovered in the medium. Lipid X also caused a significant increase in diglyceride formation, as judged by [³²P]arachidonate labeling, that was not inhibited by the addition of cycloheximide. However, in a separate experiment in which lysophosphatidylinositol was isolated from RAW cells labeled with [³²P]arachidonate, control values and stimulated values were identical (data not shown).
Macrophage Activation by Lipid A

**Uptake and Metabolism of Lipid X by RAW 264.7 Macrophages during Stimulation**—To investigate the physical interactions of lipid X with RAW 264.7 cells, [14C]lipid X (1 pg/ml) was incubated with cultures of RAW 264.7 cells, and its time-dependent redistribution between medium and cells was monitored, as described in Fig. 7. [14C]Lipid X which became cell-associated was subsequently converted to a less polar species which migrated near the front in solvent system G (Fig. 8). Since only one new radiolabeled species was produced, it was unlikely that the modification was a deacylation of the glucosamine 1-phosphate ring. A second solvent system (solvent system H) was employed to resolve lipid X, (R)-3-hydroxymyristyl, and N²,O²-diacylglycosamine with Rₜ values of 0, 0.5, and 0.2, respectively. When the cell-associated [14C]lipid X was extracted and the products were resolved in solvent system H, the less polar metabolite migrated as a single product with an Rₜ of 0.2, near the front in solvent system H. This metabolite was subsequently converted to a less polar species which migrated near the front in solvent system G (Fig. 8). Since only one new radiolabeled species was produced, it was unlikely that the modification was a deacylation of the glucosamine 1-phosphate ring.

**DISCUSSION**

The lipid A moiety of lipopolysaccharide triggers many complex physiological responses in animal systems, including fever, shock, and the activation of certain immune cells (1–4). The response of macrophages is especially important since the prostaglandins are critically important mediators of the observed pathology (4–10). Recent evidence has implicated tumor necrosis factor, one of several specific proteins synthesized by lipid A-treated macrophages, as the causative agent of shock (10, 28). The increased production of tumor necrosis factor can be attributed to increased mRNA synthesis and more efficient translation of pre-existing mRNA (10, 39). However, very little is known about the initial interactions of lipid A with animal cell membranes (40) (receptors, second messengers, etc.) or about the pathways that ultimately lead to the synthesis of specific proteins (25, 41).

The RAW 264.7 macrophage tumor cell line (24) offers a relatively simple model system with which to probe lipid A–animal cell interactions. These cells can synthesize prosta-
values, as determined by the Student's t test. Footnote 0.10; Footnote

bands of interest were located by fluorography at -65°C, and the
three determinations. Probability values when compared to control
were determined as described under "Experimental Procedures." Bovine serum albumin was added to the medium

controls (control), 5 μg/l of biological lipid X, or biological lipid
X plus 5 μg/ml cycloheximide. After 60 min at 37°C, medium was removed, and the cells were washed with phosphate-buffered saline and harvested by scraping. The cells and medium were extracted in order to isolate glycerolipids and prostaglandin species, respectively. Prostaglandins were identified by thin-layer chromatography as described under "Experimental Procedures." The individual glycerolipid species were isolated by a two-step development of a one-dimensional thin-layer plate. Total cellular glycerolipids were spotted onto Silica Gel 60 plates and developed to 14 cm with solvent system B. The

distribution of [14C]arachidonic acid during stimulation by lipid X

RAW cells were labeled for 5 h with [3H]arachidonic acid (32). Medium was removed and replaced with serum-free medium containing no additions (control), 5 μg/l of biological lipid X, or biological lipid X plus 5 μg/ml cycloheximide. After 60 min at 37°C, medium was removed, and the cells were washed with phosphate-buffered saline and harvested by scraping. The cells and medium were extracted in order to isolate glycerolipids and prostaglandin species, respectively. Prostaglandins were identified by thin-layer chromatography as described under "Experimental Procedures." The individual glycerolipid species were isolated by a two-step development of a one-dimensional thin-layer plate. Total cellular glycerolipids were spotted onto Silica Gel 60 plates and developed to 14 cm with solvent system B. The

It is very difficult, at this point, to assess the relationship between the build-up of lysophosphatidylinositol and other phenomena associated with the stimulation of the RAW cells. Several factors suggest that lysophosphatidylinositol accumulation might occur in conjunction with prostaglandin formation. 1) All the compounds (i.e. lipid A precursors, phorbol, and A23187) that caused lysophosphatidylinositol accumulation also caused prostaglandin release. 2) To a first approximation, lysophosphatidylinositol accumulation correlated with prostaglandin release when different concentrations of lipid X and IV were used to stimulate the cells (Fig. 5). Furthermore, an analysis of the time course of lipid IV action revealed that prostaglandin accumulation does not precede the appearance of lysophosphatidylinositol (Fig. 6). 3) When cells that were labeled with [3H]arachidonic acid were stimulated, there was a significant decrease in [14C]associated with phosphatidylinositol (Table VII). Furthermore, fatty acid analysis of RAW 264.7 phospholipids (data not shown) revealed that arachidonic acid makes up 32% of the fatty acid mass of phosphatidylinositol, making this phospholipid a likely source of arachidonic acid. 4) Lysophosphatidylinositol

1 D. Golenbock, R. A. Zoeller, and C. R. H. Raetz, unpublished observations.

![Graph](image-url)

**Fig. 6. Time course of lysophosphatidylinositol accumulation, release of arachidonate, and PGD₂ formation in RAW cells stimulated with IVα.** Cells were doubly labeled with both 32P, (5 μCi/ml) and [3H]arachidonic acid as described for Fig. 5. The medium was removed, and the cells were washed twice with F-12 medium containing 0.5% endotoxin-free bovine serum albumin. Next, 2 ml of F-12 medium containing 0.5% endotoxin-free bovine serum albumin and 500 nM IVα, were added, and cells were incubated at 37°C. At various times, the medium and cells were harvested separately, as described for Fig. 5. Cellular lysophosphatidylinositol accumulation (solid lines), release of arachidonic acid (O—O), and PGD₂ release (Δ—Δ) were determined as described under "Experimental Procedures." Bovine serum albumin was added to the medium to enhance recovery of arachidonic acid, but it had little effect on prostaglandin recovery or lysophosphatidylinositol accumulation. All values represent the mean ± S.D. of three cultures. The solid lines represent values from stimulated cells, and the dashed lines represent control values.

**Table VII**

| Lipid X |
|---------|
| Control | Lipid X | Lipid X + cycloheximide |
|---------|
| 133.1 ± 15.9 | 132.9 ± 12.7 | 131.7 ± 26.9 |
| 58.2 ± 2.4 | 51.6 ± 2.8 | 67.0 ± 7.2 |
| 93.5 ± 3.7 | 82.3 ± 7.7 | 91.5 ± 13.0 |
| 5.7 ± 0.4 | 8.1 ± 1.1 | 7.0 ± 0.4 |
| PGE₂ | 0.61 ± 0.05 | 1.36 ± 0.06 |
| PGD₂ | 2.15 ± 0.17 | 5.70 ± 0.50 |

CPM (X 10⁻⁴)

![Graph](image-url)

**Fig. 7. Uptake of lipid X by RAW 264.7 macrophages.** [14C]Lipid X was added to RAW 264.7 macrophages for the times indicated, as described under "Experimental Procedures." Next, the medium was removed, and radioactivity associated with the medium (Δ), the cell monolayer (Ω), or their total (■) was quantified by liquid scintillation counting. Results depicted are the mean of three determinations. The standard deviations were less than 10% of the mean.
build-up triggered by lipid A precursors demonstrated many of the characteristics observed for arachidonate and prostaglandin release from macrophages, including the requirement for extracellular calcium, the sensitivity to cycloheximide, and the ability of A23187 to by-pass the cycloheximide inhibition (36, 37).

Although the pattern of lysophosphatidylinositol accumulation and prostaglandin release triggered by lipid A-like molecules in RAW cells is consistent with the predominant involvement of a phospholipase A₂, catalyzing the direct release of arachidonate from phosphatidylinositol, the data are only suggestive. We cannot exclude other sources of arachidonate to account for the observed prostaglandin formation. For instance, stimulation of the cells with lipid X caused a significant decrease in arachidonate from phosphatidylethanolamine and an increase in arachidonate-labeled diglyceride (Table VII), suggesting that a phospholipase C may be activated and that a role for protein kinase C must be considered (19). Furthermore, there may be direct effects of lipid A precursors on protein kinase C itself (19). Both phospholipase A₂ and C activities have been shown to exist in mouse macrophages (43, 44).⁵

The ability of cycloheximide to inhibit the accumulation of lysophosphatidylinositol triggered by lipid A precursors could be interpreted to mean that protein synthesis is required to initiate the phospholipase A₂ response. The requirement for extracellular calcium (Table V) and the ability of the calcium ionophore A23187 to by-pass the inhibition by cycloheximide (Table IV) suggest that the stimulation of the cells by lipid A precursors leads to the synthesis of a protein that facilitates calcium influx. Presumably, the increased intracellular calcium might then activate a phospholipase A₂, either directly or indirectly.

The possibility must be considered that lysophosphatidylinositol has some of its own functions within RAW cells. Lysophospholipids can destabilize phospholipid bilayers and may be fusigenic (45), suggesting that they might play a role in exocytosis. Perhaps, lysophosphatidylinositol facilitates the secretion of various proteins that accumulate in the medium of RAW cells exposed to endotoxin (25), just as lysophosphatidylinositol stimulates the release of insulin from pancreatic islet cells (46). Since we do not know whether the lysophosphatidylinositol that accumulates in vivo is the 1- or 2-monoacetyl derivative, it remains a possibility that phosphatidylinositol serves as the donor for the fatty acylation of certain membrane proteins, a process that is enhanced in the presence of lipid A precursors (47).

We do not know whether the uptake and dephosphorylation of lipid X (Figs. 7 and 8) are required for the stimulation of lysophosphatidylinositol and prostaglandin metabolism. A priori, lipid A precursors might be imagined to exert their pharmacological effects by interacting with surface proteins or enzymes, not necessitating bulk uptake or metabolism. In the absence of further information, it seems reasonable to suggest that dephosphorylation is part of a detoxification mechanism since the product of lipid X dephosphorylation is biologically inactive (7). It would be interesting to isolate RAW mutant lines that are unable to take up lipid X, using colony autoradiography (48), and to examine the effects of such mutations on the lysophosphatidylinositol response.

As in other, more complex cellular or animal systems (17, 18, 26, 28, 35), the monosaccharide lipid X was, at best, a much weaker agonist than the disaccharide IVₐ (Fig. 5). In some settings, especially in whole animals, lipid X is nontoxic and may even provide some protection against the lethal effects of lipopolysaccharide injection (20, 21). On the other hand, IVₐ, like endotoxin, can cause fatal shock (28, 35). We have not yet tested the possibility that nonstimulatory concentrations of lipid X (or related monosaccharide analogs) might antagonize the stimulation observed with nanomolar concentrations of IVₐ or lipopolysaccharide (Fig. 5). In any event, the fact that the RAW system responded to lipid A precursors in a manner that parallels other more complicated biological systems makes it ideally suited for probing the molecular basis of endotoxin physiology.

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⁵ Murine resident peritoneal macrophages (6, 36) respond less vigorously to lipopolysaccharide alone than do RAW 264.7 cells. However, "normal" macrophages do mobilize considerable amounts of arachidonate when incubated with phorbol myristate acetate (6, 36). Low concentrations of lipopolysaccharide prime normal macrophages to release even more arachidonate in the presence of phorbol myristate acetate (6, 36). We have not observed such synergism with RAW cells (data not shown), perhaps reflecting differences of normal and tumor cell membranes.
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