Bacterial lipopolysaccharide-stimulated GTPase activity in RAW 264.7 macrophage membranes

Timothy TANKE,* Jan-Willem VAN DE LOO,†§ Hyewhon RHIM,* Phillip S. LEVENTHAL,* Richard A. PROCTOR‡ and Paul J. BERTICS*||

Departments of *Physiological Chemistry, †Medical Microbiology/Immunology, and ‡Medicine, University of Wisconsin, 1300 University Avenue, Madison, Wisconsin 53706, U.S.A., and §Laboratory for Medical Microbiology, State University of Utrecht, Utrecht, The Netherlands

The molecular mechanisms surrounding the toxicity and high mortality rate that accompany the release of bacterial lipopolysaccharide (LPS) are unclear, although its potent activity suggests that an amplification system is involved. Because previous studies suggest that a guanine-nucleotide-binding protein (G-protein) may participate in LPS action, we have evaluated the effects of LPS on GTPase activity in membranes isolated from macrophage (RAW 264.7) and fibroblast (B82L) cell lines. LPS induced substantial GTPase activation (200–300% above basal), and kinetic analyses indicated that the maximal LPS-stimulated increase in velocity is observed within 15 min, that it is a low- K_m (for GTP) activity, that it can be enhanced by ammonium sulphate, and that it appears to be pertussis toxin-insensitive. Moreover, the LPS-enhanced GTPase activity was not antagonized by phosphatase/ATPase inhibitors such as *p*-nitrophenyl phosphate, ouabain, bafilomycin or *N*-ethylmaleimide, and in fact was potentiated by the addition of ATP or ADP. Conversely, the LPS precursor, lipid X, which can decrease the lethal effects of LPS, was found to dose-dependently inhibit the LPS-mediated stimulation of GTPase activity. Half-maximal inhibition was seen at the same lipid X/LPS ratio known to be effective *in vivo*, i.e. 1:1 (w/w). These effects appear to be specific because other phospholipids, detergents and glycosides neither stimulated basal, nor inhibited LPS-induced, GTPase activity. These data suggest the involvement of a GTPase in LPS action, and indicate that lipid X may act to directly antagonize LPS at this level.

INTRODUCTION

The pathophysiology of Gram-negative septicæmia can be largely reproduced by endotoxin, which is a lipopolysaccharide (LPS) that makes up the outer leaflet of the outer membrane of Gram-negative bacteria [1]. The release of mediators such as tumour necrosis factor- α (TNF) and interleukin-1 (IL-1) is thought to produce the toxicity associated with endotoxaemia [2–4]. However, despite the high mortality rate of endotoxic shock [5], relatively little is known about the biochemical mechanisms involved in LPS-induced events, e.g. TNF and IL-1 release, although an amplification system is suggested, given that nanogram quantities of LPS can produce severe toxicity in animals [6]. Most amplification pathways involve receptors and various enzyme cascades, thus allowing for several points of antagonism within the system. Although certain steps in LPS action are known, such as the stimulation of phosphoinositide hydrolysis, a transient increase in intracellular Ca^{2+} levels and the activation of protein kinase C and phospholipase A_2 [7–9], the lack of specific information on the cellular mechanisms involved in LPS action has impeded the development of a therapeutic blockade of its effects.

Because many of the events initiated by LPS can be mediated by agonist-stimulated guanine-nucleotide-binding protein (G-protein) activity [10,11], it has been postulated that the broad cellular responses to LPS may be amplified through interactions with certain G-proteins [12,13]. In support of this concept, pertussis toxin has been found to decrease the LPS-induced release of IL-1 from murine P388D1 macrophages [12]. Recent evidence has also indicated that the LPS response of the promonocytic cell line U937 is linked to the expression of the GTP-binding protein G_{i2} [13].

In general, G-proteins (and their associated GTPase activity) are stimulated via interaction with hormone-receptor complexes located in the plasma membrane, and this process may be blocked by antagonists that compete for receptor binding [11,12,14]. However, an endotoxin biosynthetic precursor and subunit, lipid X, decreases the mortality of animals challenged with LPS or Gram-negative bacteria [15,16], yet it is unlikely that this compound efficiently competes for a plasma membrane LPS-binding protein [17]. Therefore lipid X may act, at least in part, by directly interfering with LPS signal transduction, and an analysis of the cellular events modulated by LPS and lipid X should help to outline the pathways that determine the pathophysiology of endotoxic shock. In this paper, we provide evidence indicating that LPS stimulates a GTPase activity in macrophage and fibroblast membranes, and that this activity can be selectively antagonized by lipid X.

EXPERIMENTAL

Materials

$[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (3000 Ci/mmol) was purchased from Amersham; LPS (from *Escherichia coli*, serotype 0111:B4) was obtained from DIFCO Laboratories (Detroit, MI, U.S.A.) and Sigma (St. Louis, MO, U.S.A.); lipid X (free of contaminating byproducts [18]) and bisphospho-aza-lipid X were kindly obtained from Dr. Peter Stuetz, Sandoz Pharmaceutical Co. (Vienna, Austria). The cyclic GMP ^3H radioimmunoassay kit was purchased from Amersham. Unless otherwise indicated, all other reagents were obtained from Sigma and were of the highest quality available.

Cell culture and membrane preparation

The macrophage-like cell line RAW 264.7 was cultured as

Abbreviations used: LPS, lipopolysaccharide; TNF, tumour necrosis factor; IL-1, interleukin 1; G-protein, guanine-nucleotide-binding protein; FCS, fetal calf serum; p[NH]ppA, adenosine 5'-[β -imidotriphosphate]; EGF, epidermal growth factor.

|| To whom correspondence should be addressed.

described [17,19] using RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT, U.S.A.) containing < 0.1 ng/ml LPS. Mouse B82L fibroblasts were grown in Dulbecco's modified Eagle's minimal essential medium (Gibco Laboratories, Grand Island, NY, U.S.A.) containing 10% FCS [20], and cell membranes were prepared from the RAW 264.7 and B82L cells via differential centrifugation as detailed previously [20]. The membranes were resuspended in 20 mM-Hepes (pH 7.4), 1 mM-dithiothreitol, 1 mM-EDTA and 10 µg of leupeptin/ml. Aliquots were stored at -70 °C until assayed.

GTPase assay

Unless otherwise indicated, GTPase activity was measured as the release of [³²P]P_i from [γ -³²P]GTP, essentially as described earlier [21,22] using a 100 µl assay volume and a reaction buffer containing 20 mM-Hepes (pH 7.4), 0.1 mM-ATP, 0.2 mM-adenosine 5'-[β - γ -imidotriphosphate (p[NH]ppA), 5 mM-MgCl₂, 6–100 µg of membrane protein, 2 µM-[γ -³²P]GTP [(3–9) × 10³ c.p.m./pmol]), and the various additions listed in the legends to the Tables and Figures. The reactions were initiated by the addition of [γ -³²P]GTP, incubated at 30–37 °C for the indicated times (generally 15–30 min) and terminated by the addition of 500 µl of 5% trichloroacetic acid and 500 µl of acid-activated charcoal (0.1 g/ml) (Norite) in 5% trichloroacetic acid. The samples were mixed and centrifuged at 14000 g for 10 min, and a 550 µl aliquot of the supernatant was removed for scintillation counting.

RESULTS AND DISCUSSION

Influence of LPS on macrophage membrane GTPase activity

Although previous studies on G-protein involvement in LPS action used pertussis toxin [12], or involved the measurement of specific G-protein subunit expression [13], we sought to more directly evaluate the influence of LPS on possible G-protein function by examining agonist-enhanced GTPase activity, which is a hallmark of G-protein action [10,11]. Moreover, because macrophages are one of the key cell types in the release of toxic mediators following an LPS challenge [23], we first assessed the capacity of LPS to influence GTPase activity in membranes isolated from RAW 264.7 cells, which is a highly LPS-responsive macrophage cell line [24].

In these studies, GTPase activity was measured using a standard assay described previously [21,22]. The activity was determined using 2 µM-[γ -³²P]GTP in the presence of 0.1 mM-ATP and 0.2 mM of the non-hydrolysable ATP analogue p[NH]ppA in order to minimize non-specific nucleotidase activity in the membrane preparations. Moreover, because it has been reported that certain ions, such as ammonium, chloride and sulphate, can influence G-protein activity [25], we also examined the effects of ammonium sulphate on the ability of LPS to stimulate GTPase in the presence of 5 mM-MgCl₂. In addition, classical studies [21] on the activation of GTPase activity in isolated cell membranes have found that the maximal signal *in vitro* requires agonist (e.g. isoprenaline) concentrations much greater than the receptor K_a measured in intact cells, thereby suggesting inefficient coupling. Macrophages have been shown to possess LPS (lipid A) binding sites on their cell surface [17,26], and one of these appears to be similar to the macrophage scavenger receptors [27] which exhibit broad binding specificity and can rapidly internalize various lipids and lipid vesicles [28,29]. Thus macrophages may internalize LPS *in vivo*, perhaps yielding the high intracellular levels necessary for some of its biological effects. In fact, previous experiments *in vitro* have found that maximal LPS interaction with intracellular enzymes

such as lysozyme are seen at concentrations in the range 100–200 µg/ml [30]. Therefore higher LPS concentrations may be needed for *in vitro* or intracellular activation when compared with that required for intact cells.

As shown in Fig. 1(a), 300 µg of LPS/ml increased GTPase activity by ~30% in the absence of ammonium sulphate, whereas in the presence of an optimal amount of ammonium sulphate (250 mM), the LPS-induced GTPase activity was nearly 3-fold above the lowered basal rate. Under these conditions, GTPase activation was evident at 100 µg of LPS/ml (roughly 6 µM, assuming an average molecular mass of approx. 17000 kDa), and the maximal effect was seen with 300 µg of LPS/ml (Fig. 1b). In all experiments, the LPS dose-response curve was quite steep, with the highest activity occurring at around 200–300 µg of LPS/ml. When the time course of LPS-activated GTPase was studied (Fig. 1c), LPS was found to enhance the rate of GTP hydrolysis as early as 2 min after addition, and the effect was maximal by 15 min.

Influence of lipid X on LPS-stimulable GTPase

Because earlier work has suggested that lipid X can block LPS action *in vivo* [15,16], experiments were performed to determine whether lipid X could also antagonize LPS-stimulated GTPase activity *in vitro*. As shown in Fig. 2(a), lipid X was capable of dose-dependently inhibiting the LPS-enhanced GTPase activity. Using LPS concentrations of either 300 µg/ml or 1 mg/ml, lipid X exhibited half-maximal inhibition when the two agents were present at an approx. 23:1 molar ratio of lipid X to LPS (1:1 w/w). This was consistent with previous studies that found a similar lipid X/LPS ratio to be half-maximally effective at blocking LPS shock *in vivo* [15,16], indicating that the relative concentrations of these agents required for GTPase inhibition closely parallel those observed for the antagonism of LPS toxicity under pathophysiological conditions. Also, a synthetic lipid X derivative, bisphospho-aza-lipid X, was effective in blocking LPS-induced GTPase activity (Fig. 2b), but it has the advantage of being more water-soluble. Although the major influence of lipid X appeared to be on the inhibition of LPS-stimulated GTPase activity, lipid X was also capable of slightly decreasing basal activity (Fig. 2c), suggesting a possible direct interaction at the GTPase level. Furthermore, with regard to the reversibility of the lipid X inhibition of LPS-stimulated GTPase activity, we observed this inhibition regardless of whether LPS or lipid X was first incubated with the membrane preparation (results not shown).

Specificity of the LPS and lipid X effects

In view of the general lipophilicity of LPS and lipid X, other hydrophobic compounds were tested to demonstrate the specificity of the LPS- and lipid X-mediated effects on GTPase activation/inhibition. The data in Table 1 show that various phospholipids, i.e. phosphatidylcholine, phosphatidic acid and phosphatidylserine, did not stimulate basal, nor inhibit LPS-stimulated, GTPase activity in macrophage membranes. The detergent Triton X-100 did decrease basal GTPase activity, but it did not substantially interfere with the LPS-stimulated activity. These data suggest that the effects of LPS and lipid X are not strictly due to general detergent or other lipid interactions.

With respect to non-LPS-stimulated GTPase activity in this macrophage system, we observed that 10% FCS (< 0.1 ng of endotoxin/ml, by the *Limulus* amoebocyte lysate method) induced a large degree of GTPase activation (Table 1), whereas 3 mg of lipid X/ml inhibited the FCS-enhanced GTPase activity by ~50%. These results also suggest that lipid X may have direct effects, as it can apparently interfere with other agonist-enhanced GTPase activities.

Fig. 1. Characterization of the LPS-enhanced GTPase activity in RAW 264.7 macrophage membranes

GTPase was measured at 30 °C for 15 min as described in the Experimental section. (a) Influence of ammonium sulphate on LPS-induced GTPase activity. The samples were incubated with the indicated levels of ammonium sulphate with (●) or without (○) 300 μ g of LPS/ml (in 20 mM-Hepes, pH 7.4). (b) Dose-dependent stimulation of GTPase activity by LPS in the presence of 250 mM-ammonium sulphate. (c) Time course of LPS stimulation of GTPase. All samples contained 250 mM-ammonium sulphate and were incubated either with (●) or without (○) 300 μ g of LPS/ml for the time periods shown. In all cases (a–c), the data are presented as the means of duplicate determinations ($\pm$ ranges, most of which are contained within the data points), and similar results were obtained in two to five different experiments.

Fig. 2. Inhibition by lipid X and bisphospho-aza-lipid X of LPS-enhanced GTPase activity in RAW 264.7 membranes

The assays were performed for 15 min at 30 °C in the presence of 250 mM-ammonium sulphate. All points are the means of duplicate measurements. The data are shown as percentages of LPS-stimulated GTPase activity (typically 250–300 % of the basal rate), which was calculated according to the following equation:

$$\% = \frac{[\text{LPS-induced GTPase activity} (\pm \text{lipid X})] - (\text{Basal GTPase activity})}{\text{Basal GTPase activity}} \times 100$$

(a) Lipid X inhibition of LPS-stimulated GTPase. The samples contained 300 μ g (●) or 1000 μ g (○) of LPS/ml and the indicated levels of lipid X. (b) Comparison of the lipid X and bisphospho-aza-lipid X inhibition of LPS-enhanced GTPase. The reaction samples contained 300 μ g of LPS/ml and the indicated concentrations of lipid X (●) or bisphospho-aza-lipid X (□). (c) Effect of lipid X on basal GTPase activity.

Although these experiments were designed to minimize non-specific GTP hydrolysis by using very low GTP levels (2 μ M) and very high concentrations of ATPase/nucleotidase antagonists, i.e. 0.1 mM-ATP and 0.2 mM-p[NH]ppA, it was still possible that some of the observed effects were due to the activation of membrane phosphatases, e.g. alkaline phosphatase, or ATPases such as the abundant Na^+/K^+ -ATPase. However, the contribution of these enzymes may be discounted, based on the fact that the LPS-stimulated GTPase activity exhibited an insensitivity to high concentrations of the phosphatase inhibitor *p*-nitrophenylphosphate and the ATPase inhibitor ouabain, even in the presence of 0.1 mM-ATP and 0.2 mM-p[NH]ppA (Table 1).

To further characterize the specificity of the LPS-stimulated GTPase, we first assayed its activity in the absence of any other

nucleotides, and then compared this response with that found in reactions containing various ATPase/nucleotidase inhibitors (Table 2). If the GTPase activity is due to a generalized increase in nucleotidase-like activities, one would expect that the inclusion of these inhibitors would attenuate the LPS-stimulated activity. Otherwise, a specific GTPase would be predicted to be unaffected or even enhanced (less non-specific substrate hydrolysis) in the presence of these agents. Interestingly, we observed that in the absence of any added adenine nucleotides, there was very little detectable LPS-enhanced GTPase activity. However, when ATP, ADP or the non-hydrolysable ATP analogue p[NH]ppA was included in the incubation, there was a pronounced increase in LPS-stimulated GTPase activity. Moreover, the addition of the ATPase inhibitors ouabain, bafilomycin and *N*-ethylmaleimide

Table 1. Effectors of GTPase activity in RAW 264.7 membranes

The GTPase assays were performed at 30 °C using a 15 min incubation time in the presence of 250 mM-ammonium sulphate, 0.1 mM-ATP and 0.2 mM-p[NH]ppA. In the studies using *p*-nitrophenyl phosphate, the assays were performed at 37 °C using a 20 min incubation time. The results are expressed as percentages of the basal GTPase activity (set at 100%) in the absence of the added factor(s). The values presented are the means of duplicate determinations (all data within 10% of the mean), and similar results were obtained in at least two experiments.

Assay additions and concentrations	GTPase activity (% of basal)
Lipopolysaccharides	
300 µg of LPS/ml	270
300 µg of LPS/ml + 1 mg of lipid X/ml	137
1 mg of Lipid X/ml	58
Lipids and detergents	
100 µg of Phosphatidylcholine/ml	109
1 mg of Phosphatidylcholine/ml	110
100 µg of Phosphatidic acid/ml	102
1 mg of Phosphatidic acid/ml	92
1 mg of Phosphatidic acid/ml + 300 µg of LPS/ml	269
100 µg of Phosphatidylserine/ml	106
1 mg of Phosphatidylserine/ml	120
1 mg of Phosphatidylserine/ml + 300 µg of LPS/ml	291
0.03% Triton X-100	35
0.03% Triton X-100 + 300 µg of LPS/ml	256
Activators and inhibitors	
10% FCS	457
10% FCS + 3 mg of lipid X/ml	248
10% FCS + 300 µg of LPS/ml	489
1 mM-Ouabain	93
1 mM-Ouabain + 300 µg of LPS/ml	254
2 mM- <i>p</i> -Nitrophenyl phosphate	103
2 mM- <i>p</i> -Nitrophenyl phosphate + 300 µg of LPS/ml	321

did not block LPS-stimulated GTPase activity in the presence of ADP. Conversely, as expected, the very general phosphoryl-transferase inhibitor, sodium orthovanadate, did block activation by LPS.

Because the above inhibitor studies indicated a maximum LPS-stimulated GTPase activity in the presence of ATP and ATP analogues, we further investigated this interaction by measuring the ATP-dependence of the GTPase activity in macrophage membranes in the presence and absence of a maximal dose of LPS. As shown in Fig. 3, ATP alone effected a biphasic stimulation of GTPase activity, with the maximum effect being observed around 1–10 µM. This stimulation by ATP may be due to the action of purinergic receptors (i.e. the adenosine and ATP receptors), which are well-characterized to be coupled to various G-proteins [31–33]. When ATP and LPS are added together, there is a striking stimulation of GTPase activity at 1–10 µM-ATP, which is much greater than that seen with ATP or LPS alone. This synergistic stimulation by ATP plus LPS is also biphasic, with the inhibition of GTPase activity observed at very high ATP levels, probably resulting from competitive or ionic effects. These data suggest that LPS may interact in some fashion with the purinergic receptor signal transduction pathway, and that the LPS-mediated GTPase activation is not the result of a non-specific ATPase or other nucleotidase activity.

To assess whether the LPS/ATP-enhanced GTPase is a low- K_m form, which would be expected for a variety of large and

Table 2. Influence of ATPase inhibitors on LPS-stimulatable GTPase activity in RAW 264.7 membranes

The GTPase assays were performed at 37 °C using a 15 min incubation time in the presence of 250 mM-ammonium sulphate, 5 mM-MgCl₂ and 2 µM-[γ-³²P]GTP, with or without 300 µg of LPS/ml. The results are expressed as percentages of the basal GTPase activity in the presence of LPS for each of the added factor(s), which was calculated according to the following relationship:

$$\% = \frac{\text{LPS-stimulated GTPase activity in the presence of added inhibitor}}{\text{Basal GTPase activity in the presence of added inhibitor}} \times 100$$

The values presented are the average of duplicate measurements (all data within 20% of the mean), and similar results were obtained in two different experiments.

ATPase inhibitors	GTPase activity (% of basal)
No inhibitor added (300 µg of LPS/ml alone)	100
100 µM-ATP	209
100 µM-p[NH]ppA	414
30 µM-ADP	335
30 µM-ADP + 1 mM-ouabain	289
30 µM-ADP + 5 µM-bafilomycin	262
30 µM-ADP + 100 µM-sodium orthovanadate	132
30 µM-ADP + 100 µM- <i>N</i> -ethylmaleimide	315

Fig. 3. Influence of ATP on LPS-stimulated GTPase activity in RAW 264.7 macrophage membranes

GTPase activity was measured at 30 °C for 15 min as described in the Experimental section, except that 0.1 mM-ATP and 0.2 mM-p[NH]ppA were absent from the standard assay buffer. Instead, the samples were incubated with 250 mM-ammonium sulphate and the indicated concentrations of ATP, with (●) or without (○) 300 µg of LPS/ml (in 20 mM-Hepes, pH 7.4). The data are presented as the means of duplicate determinations ($\pm$ ranges, most of which are contained within the data points), and similar results were obtained in two different experiments.

small molecular mass G-proteins, as well as for various GTPase-activating protein (GAP)- and ADP-ribosylation factor-like proteins [10] (as opposed to a high- K_m form, such as a phosphatase), we assayed the GTPase activity using 0.2 and 50 µM-GTP essentially as described previously [34]. In view of the interactive effects of LPS and ATP, we measured high- and low- K_m GTPase in the absence of added ATP, as well as in the presence of a maximally activating dose of ATP (10 µM), and

Table 3. Effect of ATP and LPS on high- and low- K_m GTPase activity in RAW 264.7 membranes

The GTPase assays were performed at 30 °C using a 15 min incubation time, as described in the Experimental section. The samples contained ~ 5 µg of membrane protein/ml, 250 mM-ammonium sulphate, 5 mM-MgCl₂, [γ -³²P]GTP (~ 75000 c.p.m. were added to all assay tubes) and the indicated concentrations of GTP (0.2, 2 and 50 µM), in the presence or the absence of ATP (10 µM) and/or LPS (300 µg/ml). In these studies, using the standard assay conditions of 2 µM-GTP, 100 µM-ATP and 200 µM-p[NH]ppA [21,22], the GTPase activity in the absence of LPS was determined to be 0.31 ± 0.02 pmol of P_i released/min, whereas the addition of 300 µg of LPS/ml resulted in a GTPase activity of 0.63 ± 0.01 pmol of P_i released/min (203 % of the activity in the absence of LPS). The low- K_m GTPase was calculated by first subtracting the counts obtained in the presence of 50 µM unlabelled GTP (high- K_m GTPase) from those obtained in the presence of 0.2 µM-GTP [34]. The values presented are the means of duplicate measurements ± ranges, and similar results were obtained in two different experiments.

Assay additions	GTPase activity (pmol of P _i released/min)			
	+ 2 µM-GTP	Total GTPase (0.2 µM-GTP)	High- K_m GTPase (50 µM-GTP)	Low- K_m GTPase
No LPS, no ATP (basal)	1.2 ± 0.06	0.25 ± 0.01	16.6 ± 0.4	0.18 ± 0.01
300 µg of LPS/ml, no ATP	1.3 ± 0.01 (108 %)*	0.32 ± 0.01 (128 %)*	18.4 ± 0.9 (111 %)*	0.24 ± 0.01 (133 %)*
No LPS, 10 µM-ATP	1.7 ± 0.01	0.31 ± 0.01	17.6 ± 0.5	0.24 ± 0.01
300 µg of LPS/ml, 10 µM-ATP	2.5 ± 0.08 (147 %)* (208 %) [†]	0.48 ± 0.01 (155 %)* (192 %) [†]	18.7 ± 0.7 (106 %)* (113 %) [†]	0.41 ± 0.01 (171 %)* (228 %) [†]

* Results of LPS stimulation expressed as a percentage of the basal GTPase activity.

[†] Results of LPS stimulation expressed as a percentage of the 10 µM-ATP-stimulated GTPase activity.

Table 4. Effects of LPS and lipid X on the GTPase activity of membranes isolated from RAW 264.7 cells pre-treated with pertussis toxin

Intact RAW 264.7 cells were grown on 10 cm plates until near-confluent, and were then treated with 1 µg of pertussis toxin for 2.5 h at 37 °C. Membranes were prepared as described in the Experimental section, and GTPase assays were performed at 30 °C using a 15 min incubation time. The samples contained approx. 50 µg of membrane protein/ml, 250 mM-ammonium sulphate, 5 mM-MgCl₂, 10 µM-ATP, [γ -³²P]GTP (~ 73000 c.p.m./assay) and various concentrations of GTP (0.2, 2 and 50 µM) in the presence or the absence of LPS (300 µg/ml) and lipid X (1 mg/ml). In these experiments, GTPase activity was measured using the standard assay conditions of 2 µM-GTP, and in addition the low- K_m GTPase was calculated by subtracting the counts obtained in the presence of 50 µM unlabelled GTP from those obtained in the presence of 0.2 µM-GTP, as detailed in the legend to Table 3. The values presented are the means of duplicate measurements ± ranges, and similar results were obtained in two different experiments.

Assay additions	GTPase (pmol of P _i released/min)	
	Standard assay (2 µM-GTP)	Low- K_m GTPase
No LPS, no Lipid X	3.6 ± 0.2	0.28 ± 0.02
300 µg of LPS/ml, no lipid X	6.7 ± 0.1	0.43 ± 0.01
300 µg of LPS/ml, 1 mg of lipid X/ml	2.5 ± 0.1	0.17 ± 0.04

compared these results with those obtained using our standard assay conditions (2 µM-GTP, 0.1 mM-ATP, 0.2 mM-p[NH]ppA). As shown in Table 3, LPS and ATP stimulated a low- K_m GTPase activity by ~ 30 % and 70 % respectively, when added individually. This stimulation of a low- K_m activity was slightly greater than that estimated using the standard assay conditions. In addition, the combined effects of LPS and ATP on GTPase activity also appeared to represent an influence on a low- K_m component, with the stimulation calculated for the low- K_m activity (228 % of basal activity) being comparable with the stimulation (208 % of basal activity) measured using our standard

assay conditions. In summary, the LPS-stimulated GTPase activity appears to be specific for GTP, it exhibits a low K_m (for GTP), it is insensitive to various ATPase inhibitors, and in fact the LPS stimulation is potentiated rather than antagonized by adenine nucleotides.

Because these findings were consistent with G-protein activation, and because pertussis toxin-sensitive G-proteins have been implicated in the cellular response to LPS [12,13], we evaluated the ability of pertussis toxin treatment to inhibit the LPS-stimulated GTPase activity *in vitro*. Macrophage membranes (500 µg of protein) were ADP-ribosylated for 45 min in a 100 µl reaction volume at 30 °C using activated pertussis toxin (2 µg) and 50 µM-[α -³²P]NAD⁺ according to the method of Backlund *et al.* [35]. In untreated membranes, 300 µg of LPS/ml stimulated GTPase activity to 234 % of the basal rate, whereas in pertussis toxin-treated membranes, 300 µg of LPS/ml yielded an increase in GTPase activity to 253 % of the basal rate. These preliminary experiments indicated that ADP-ribosylation of macrophage membranes *in vitro* using activated pertussis toxin does not cause measurable inhibition of the LPS effects.

Although in the above studies ADP-ribosylation of a 40 kDa protein was verified using [α -³²P]NAD⁺, SDS/PAGE and autoradiography (results not shown), it is possible that the degree of ADP-ribosylation was insufficient for substantial blockade of GTPase activity or that certain cellular factors may be required for the pertussis toxin sensitivity observed in intact cells. To address this issue, intact RAW 264.7 macrophages were incubated with or without 1 µg of pertussis toxin/dish for 2.5 h at 37 °C, and then membranes were prepared and assayed for LPS-stimulated GTPase activity as described in the Experimental section. In untreated cell membranes, 300 µg of LPS/ml stimulated GTPase activity to 145 ± 2 % of the basal rate, whereas, with the membranes prepared from pertussis toxin-treated cells, 300 µg of LPS/ml yielded an increase in GTPase activity to 137 ± 7 % of the basal rate. These data reveal that pertussis toxin treatment of intact cells also does not substantially attenuate the LPS-enhanced GTPase activity. To further explore the effects of pertussis toxin treatment on LPS/lipid X action, we assayed the ability of lipid X to inhibit LPS/ATP-stimulated GTPase activity in membranes prepared from cells treated with

Fig. 4. Analysis of LPS-stimulated GTPase activity in mouse B82L fibroblast membranes

GTPase was assayed at 37 °C for 30 min. (a) Influence of ammonium sulphate on LPS-induced GTPase. The samples were incubated with (■) or without (□) 300 µg of LPS/ml and the indicated levels of ammonium sulphate. (b) Dose-dependent stimulation of GTPase activity by LPS in the presence of 250 mM-ammonium sulphate. (c) Time course of LPS-stimulated GTPase. All samples contained 250 mM-ammonium sulphate and were incubated either with (■) or without (□) 300 µg of LPS/ml for the time periods shown. In all cases (a–c), the data are presented as the means of duplicate determinations ($\pm$ ranges, most of which are contained within the points), and similar results were obtained in two different experiments.

pertussis toxin *in vivo*. The results shown in Table 4 indicate that LPS can still stimulate a low- K_m GTPase in these membranes, and that lipid X can block this stimulation.

Altogether, these results suggest that LPS/lipid X can act, at least in part, on a GTPase that is relatively pertussis toxin-insensitive, e.g. pertussis toxin-insensitive G-proteins, or on the receptor(s) to which they are coupled. The possibility that LPS may act on multiple G-protein pathways is compatible with previous studies indicating that various receptors, such as the M_1 muscarinic acetylcholine receptor [36], and cell mitogens, such as the lipid lysophosphatidate [37], can exert their actions via multiple G-proteins exhibiting both sensitivity and insensitivity to pertussis toxin. Finally, LPS may influence other pertussis toxin-insensitive GTP-binding proteins, such as GTPase-activating proteins, small-molecular-mass G-proteins or other nucleotidases [10], that can ultimately interact with a pertussis toxin-sensitive pathway within the cell. However, LPS did not activate guanylate cyclase, which can exhibit a GTPase-like activity [38], because there was no detectable LPS-enhanced cyclic GMP production in our membrane system, as measured using a cyclic [3 H]GMP radioimmunoassay. In these experiments, basal GTPase activity was determined to be 36.0 ± 0.43 pmol of 32 P released/10 min, and 300 µg of LPS/ml stimulated this release to 86.7 ± 0.6 pmol of 32 P released/10 min. While the LPS-stimulated increase in phosphate release was over 50 pmol/10 min, the cyclic GMP levels remained below the detection limit (< 0.5 pmol) under all conditions.

Cell specificity of LPS-stimulated GTPase activity

Previous studies on LPS action have focused primarily on its role in macrophage activation [7–9,23], although other cell types appear to be LPS-sensitive [17]. To assess the cell specificity of the LPS/lipid X modulation of GTPase activity, we used membranes isolated from mouse B82L fibroblasts, which are less sensitive to LPS toxicity than macrophages. The LPS sensitivity of the B82L cells was tested by determining the influence of LPS on cell viability and epidermal growth factor (EGF) binding. Rapidly growing mouse B82L cells [20] were exposed for 24 h at 37 °C to LPS levels ranging from 1 ng/ml to 100 µg/ml. No cytotoxicity was observed, as evidenced by cell density, nor were any substantial effects detected on EGF binding (results not shown).

As shown in Fig. 4, when LPS was incubated with B82L fibroblast membranes, we observed an LPS-stimulatable GTPase activity that exhibited a similar ammonium sulphate sensitivity, a somewhat higher dose response (maximal activation at ~ 1 mg/ml) and a slower time course of activation when compared with that in macrophage membranes. In addition, lipid X could block LPS-enhanced GTPase activity in the fibroblast membranes, i.e. 300 µg of LPS/ml resulted in a GTPase activity that was 294 % of the basal rate, whereas 300 µg of LPS/ml plus 3 mg of lipid X/ml resulted in a GTPase activity that was 204 % of the basal rate.

A major question concerning the biochemical actions of LPS centres on the possible involvement of a plasma membrane binding protein or 'receptor' [17,26]. Whether the initial cellular responses to LPS are mediated by a specific receptor, or whether LPS can directly interact with other cellular components (e.g. G-proteins, hormone receptor systems, etc.), is unknown. In the latter case, the primary role of a membrane binding protein may be for internalizing LPS, thereby potentiating its toxicity. Macrophages are highly responsive to LPS and accordingly possess a high number of LPS binding sites [17]; in contrast, other cell types such as the CHO-K1 and L929 fibroblast cell lines are less sensitive to LPS, and they appear to have few, if any, LPS receptors [17]. The macrophage membranes used here appear to be more sensitive to LPS than the fibroblast membranes (as demonstrated by the LPS dose-response curves and time courses in Figs. 1 and 4). However, given the differences in the LPS sensitivity *in vivo* of these cell types, yet the similar magnitude (-fold stimulation) of the response in both membrane preparations, it is possible that these effects may result from direct action at the level of the GTPase, rather than strictly via specific receptor-mediated events. Differential sensitivity of these cells may result from different rates of LPS binding or uptake, or from differing levels of cellular components such as certain G-proteins.

The observation that LPS stimulates a low- K_m GTPase activity in macrophage and fibroblast membranes suggests that certain G-proteins may be involved in its signal transduction. The ability of lipid X to specifically interfere with this effect of LPS appears to directly relate to the capacity of lipid X to protect against LPS toxicity *in vivo*, and this observation may partially explain the molecular mechanisms involved in their respective biological actions.

These studies were supported by a Shaw Scholar Award from the Milwaukee Foundation and NIH grant CA-47881 (P.J.B.), and by awards from the University of Wisconsin Medical and Graduate Schools (P.J.B. and R.A.P.) and the Department of Medicine (R.A.P.).

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Received 21 November 1990/18 March 1991; accepted 2 April 1991