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Activation of cyclic nucleotide phosphodiesterase by a monosaccharide precursor of *Escherichia coli* lipid A

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The *E. coli* lipid A precursor lipid X (N^2,O^3-diacylglucosamine 1-phosphate) activates calmodulin-dependent cyclic nucleotide phosphodiesterase in a noncooperative, calcium-independent manner by increasing its V_{max} and decreasing its K_m for substrate. The glycolipid produces half-maximal activation at 11 μg/ml and does not further enhance activation by calcium-calmodulin. Lipid X activation of phosphodiesterase requires the presence of the O^3-linked hydroxymyristoyl residue. These findings suggest that lipid X could produce some of its biological effects by modulating intracellular cAMP levels.

Lipopolysaccharide; Lipid A precursor; Cyclic-nucleotide phosphodiesterase; cyclic AMP

1. INTRODUCTION

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative microorganisms, produces a variety of pathophysiological effects in mammals. The lipid A portion of LPS is responsible for most of its stimulatory effects on B-lymphocytes, neutrophils, and macrophages [1]. Relatively little is known of the biochemical basis for these effects. Chemically defined lipid A precursors are useful model compounds for studying the biochemical mechanisms and structural requirements of lipid A-induced responses. Lipid X, a monosaccharide isolated from *Escherichia coli* mutants [2], is a potent B-cell mitogen [3] and induces spreading, prostaglandin E_2 synthesis, and tumor necrosis factor production by macrophages [4,5].

Lipid X (10–150 μg/ml) has been shown to activate purified protein kinase C by substituting for phosphatidylserine [6]. Since this enzyme, along with the cyclic AMP-dependent protein kinase system, is a major pathway for signal transduction in immune cells [7], it has been suggested that protein kinase C mediates some of the effects of lipid X. Here, we provide evidence that lipid X may also influence cyclic AMP-dependent processes by stimulating the hydrolysis of cyclic nucleotide second messengers by phosphodiesterase.

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2. MATERIALS AND METHODS

E. coli lipid X, dephosphorylated lipid X (dephospho-X) and N^2-monoacylglucosamine 1-phosphate were obtained from Lipidex. Histone type IIa and 4-methylumbelliferyl phosphate were purchased from Sigma. Methylanthraniloyl-cyclic GMP was obtained from Molecular Probes. All other chemicals were reagent grade.

Cyclic nucleotide phosphodiesterase and calcineurin were purified from bovine brain as described by Sharma et al. [8]. Calmodulin was purified from bovine brain by the procedure of Gopalakrishna and Anderson [9]. Phosphodiesterase was assayed fluorometrically as described by Johnson et al. [10]. The assay (1 ml volume) contained 10 mM Mops, pH 7.0, 8 μM methylanthraniloyl-cyclic GMP, 5 mM $MgCl_2$, 90 mM KCl and 200 μM EGTA. Lipid X and its analogs were dispersed in this buffer by bath sonication. Calcineurin was assayed with the fluorescent substrate 4-methylumbelliferyl phosphate as described by Anthony et al. [12].

3. RESULTS AND DISCUSSION

Dispersed lipid X (1–50 μg/ml) stimulated cyclic

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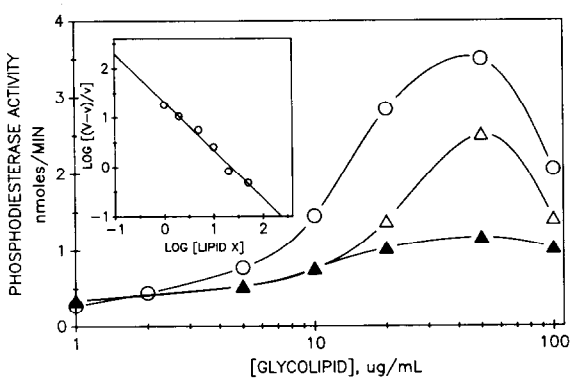

Fig.1. Activation of phosphodiesterase (3.4 μg/ml) by lipid X (○), dephospho-X (△) and monoacylglucosamine 1-phosphate (▲). (Inset) Hill plot of phosphodiesterase activation by lipid X.

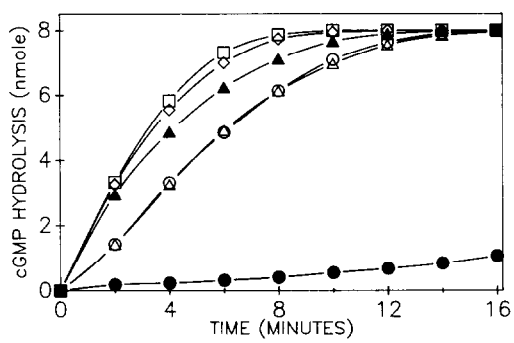

Fig.2. Comparison of lipid X- and calcium/calmodulin-stimulated phosphodiesterase activity and their additive effects. Phosphodiesterase (1.4 μg/ml) was monitored as a function of time under the following conditions: (●) unstimulated, (○) lipid X (40 μg/ml) with EGTA (200 μM), (△) lipid X (40 μg/ml) with calcium (0.73 mM), (▲) lipid X (40 μg/ml) with calcium and calmodulin (56 nM), (◇) lipid X (15 μg/ml) with calcium and calmodulin (56 nM), and (□) calcium and calmodulin (56 nM).

nucleotide phosphodiesterase in a dose-dependent manner in the absence of calcium (fig.1). Activation by lipid X was noncooperative with a Hill coefficient of 0.98 (fig.1, inset). Lipid X produced half-maximal activation at 11 μg/ml and stimulated phosphodiesterase 16-fold over basal activity at 50 μg/ml. Dephospho-X (50 μg/ml) stimulated phosphodiesterase 11.3-fold. Deacylated lipid X (N^2-monoacylglucosamine 1-phosphate, 50 μg/ml) produced only 5.3-fold stimulation, suggesting that the O^3-acyl group is an essential structural requirement for activation by

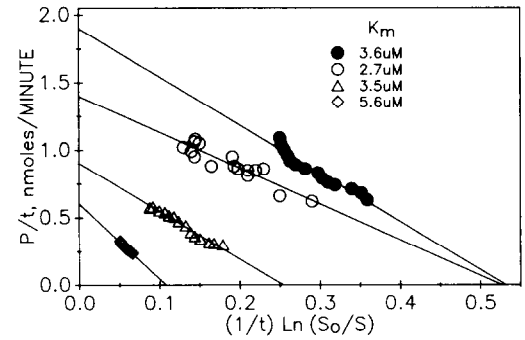

Fig.3. Effects of calcium/calmodulin, lipid X and dephospho-X on the K_m and V_{max} of phosphodiesterase. Hydrolysis of methylanthraniloyl-cyclic GMP by phosphodiesterase (0.5 μg/ml) was assayed at 25°C in the presence of lipid X (50 μg/ml) (○), dephospho-X (50 μg/ml) (△), and calcium (0.73 mM)/calmodulin (56 nM) (●). Unstimulated substrate hydrolysis (◇) by phosphodiesterase (5.0 μg/ml) was monitored in the absence of calcium. The method of Walker and Schmidt [11] was used to estimate K_m and V_{max}.

lipid X. Interestingly, stimulation of phosphodiesterase occurred at lipid X concentrations similar to those which activate B-lymphocytes and macrophages [3,4]. Further, the structural requirements for phosphodiesterase stimulation parallel those for activation of immune cells. Selective O^3-deacylation of lipid X abolishes its ability to activate B-lymphocytes and macrophages. Dephospho-X is incapable of macrophage activation, but is partially mitogenic toward B-lymphocytes [3,4].

The time course of cyclic nucleotide hydrolysis in the presence of lipid X (40 μg/ml) and EGTA (200 μM) was not affected by the addition of calcium (0.73 mM), indicating that activation by lipid X is calcium-independent (fig. 2). Simultaneous stimulation by lipid X and saturating amounts of calcium-calmodulin was not additive. Although the degree of stimulation produced by lipid X and calcium-calmodulin was greater than that produced by lipid X alone, it was less than that obtained by maximal stimulation with calcium-calmodulin (56 nM). These findings suggest that calmodulin and lipid X activate phosphodiesterase by similar, noncomplementary mechanisms.

Certain fatty acids, phospholipids and gangliosides can partially activate phosphodiesterase in a

calcium-independent manner by increasing its V_{max} [13–15]. In contrast, stimulation by lipid X produced catalytically favorable changes in both K_m and V_{max} (fig.3). Lipid X (50 μg/ml) decreased the K_m from 5.6 to 2.7 μM, and increased the V_{max} by 27-fold. Dephospho-X decreased the K_m to 3.5 μM, but increased V_{max} by only 17-fold. In comparison, stimulation by calcium-calmodulin (56 nM) decreased the K_m to 3.6 μM and increased V_{max} by 37-fold. With regard to its effect on kinetic constants, lipid X stimulates phosphodiesterase in a manner more similar to calmodulin than other lipids.

We examined the effect of lipid X on calcineurin, a calmodulin-dependent phosphatase which interacts with acidic phospholipids and undergoes significant changes in its activity [16]. Lipid X (50 μg/ml) had negligible effects on calcineurin, producing only 1.2-fold stimulation in the presence of 0.73 mM calcium (not shown). This finding suggests that activation of phosphodiesterase by lipid X is a somewhat selective interaction which does not occur with all calmodulin-dependent enzymes.

The biochemical mechanisms involved in mediating the complex effects of LPS on inflammatory cells have not been fully explained. Its structurally defined precursor, lipid X, has been shown to activate protein kinase C in vitro. Because of the complexity of the LPS molecule and its biological effects, other mechanisms may also be involved. Our observation that lipid X stimulates calmodulin-dependent phosphodiesterase in vitro may account for some of its biological activities. Internalization of the relatively small, lipophilic lipid X molecule could potentially disrupt cyclic AMP-dependent processes by stimulation of intracellular cyclic AMP hydrolysis. Since increased cyclic AMP levels have an antagonistic effect on activation of inflammatory cells [7], lipid X could possibly promote or potentiate cellular activation by this mechanism. The findings of this study suggest that some of the immunostimulatory effects of lipid X could be mediated by interaction with phosphodiesterase. Because lipid X may represent the minimal essential structure for the biological activity of lipid A, this study also provides insight into the biochemistry of the cellular response to lipid A.

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