

Modulation of Human Platelet Protein Kinase C by Endotoxic Lipid A

Jadwiga Grabarek, Sheila Timmons, and Jacek Hawiger

Division of Experimental Medicine, Department of Medicine, New England Deaconess Hospital and Harvard Medical School, Boston, Massachusetts 02215

Abstract

Lipid A is the toxic principle of lipopolysaccharide of gram-negative bacteria, which causes a spectrum of changes in blood cells and vascular cells. We now report that human platelets are directly stimulated by endotoxic lipid A that activates protein kinase C. Rapid phosphorylation of a human platelet protein of M_r 47,000, a marker of protein kinase C activation, accompanies secretion of [^{14}C]serotonin and aggregation triggered by endotoxic lipid A. These events are time and concentration dependent, with phosphorylation reaching maximum in 2 min and the concentration of lipid A causing a 50% effect (EC_{50}) between 12 and 15 μM . Phospholipase C activation in lipid A-stimulated platelets was not observed as judged by a lack of generation of [^3H]diacylglycerol in [^3H]arachidonic acid-labeled platelets and a lack of generation of [^{32}P]phosphatidic acid in [^{32}P]labeled platelets. Lipid A did not induce formation of TXA_2 as measured by radioimmunoassay for TXB_2 .

The stimulation of human platelets and activation of protein kinase C by endotoxic lipid A was blocked by lipid X, a structural precursor of lipid A. Lipid X also blocked the stimulation of human platelets by phorbol 12-myristate 13-acetate, suggesting that lipid A, lipid X and phorbol ester share reactive site(s) on the human platelet membrane. Although lipid X inhibited thrombin-induced phosphorylation of P47 it did not suppress secretion of [^{14}C]serotonin, indicating the role of protein kinase C-independent pathways in platelet stimulation by thrombin. The inhibitory effect of lipid X did not involve generation of cyclic AMP in human platelet membrane preparations.

These results indicate that human platelets are stimulated by endotoxic lipid A, a naturally occurring biologic modifier of protein kinase C. Due to the widespread presence of this enzyme in blood cells, vascular cells, and neurons, its modulation by lipid A may represent a significant mechanism underlying hematologic and circulatory derangements observed in endotoxic shock in humans.

Introduction

Thrombocytopenia, disseminated intravascular coagulation, and shock are complications of bacteremia due to endotoxin-

producing gram-negative rods, a cause of significant morbidity and mortality with an estimated 18,000 deaths in the United States per year (1). It has been established that the toxic principle of endotoxin is lipid A, which is responsible for induction of fever, macrophage activation, mitogenic lymphocyte stimulation, complement activation, hemorrhagic skin necrosis, and gelation of *Limulus* amoebocyte extract (2, 3). Lipid A is composed of a disaccharide with amide- and ester-linked fatty acid residues. The latter consist of hydroxymyristic, lauric, palmitic, and myristic acids. The presence of these fatty acids and phosphate group is required for the biologic activity of lipid A (4). Its precursor, lipid X, is diacyl-*N*-glucosamine 1-phosphate containing hydroxymyristoyl residues (5). Lipid A with the myristoxymyristic acid-containing part resembles phorbol 12-myristate 13-acetate (PMA) (Fig. 1). PMA is a known agonist of platelets (6, 7) and an activator of protein kinase C (8).

Protein kinase C is a phospholipid- and calcium-dependent enzyme of apparent M_r 82,000 that occurs in tissues of a large number of species, ranging from annelida to mammalia (9–11). The inactive form of the enzyme is particularly susceptible to activation by diacylglycerol generated by the action of phospholipase C (12). Then, protein kinase C forms a quaternary complex with diacylglycerol, calcium, and phosphatidylserine in the membrane (13). The enzyme is also activated by the calcium-dependent neutral protease (calpain), present in platelet cytoplasm (14). Protein kinase C phosphorylates seryl and threonyl but not tyrosyl residues in cellular proteins (15). In human platelets the main protein phosphorylated by kinase C is a protein of M_r 40,000–47,000, which was initially described by Lyons et al. (16) and established as a marker of protein kinase C activation in platelets by Takai and colleagues (17). The recent report that this protein is a specific phosphatase for inositol triphosphate (IP_3),¹ inositol triphosphate 5'-phosphomonoesterase, (18), indicates its important role in regulation of the intracellular level of IP_3 , a key messenger for mobilization of calcium from intracellular stores (19). Phosphorylation of P47 is observed in response to several known agonists of platelets such as thrombin, collagen, epinephrine, and platelet activating factor (16, 20–22).

We observed that mutant Re595 of *Salmonella minnesota*, bearing lipid A and a unique sugar, 2-keto-3-deoxyoctonate (KDO), but deficient in oligosaccharides, stimulates human platelets. This stimulation was accompanied by phosphorylation of P47 (23). Wightman and Raetz reported that both lipid A and its precursor, lipid X, activate protein kinase C in a murine macrophage cell line (24). In this study we show that lipid A, but not lipid X, stimulates human platelets. Modulation of protein kinase C in human platelets by lipid A is an important attribute of this toxic principle of lipopolysaccha-

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Address reprint requests to Dr. Hawiger, Division of Experimental Medicine, New England Deaconess Hospital, 185 Pilgrim Road, Boston, MA 02215.

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1. Abbreviations used in this paper: IP_3 , inositol triphosphate; KDO, 2-keto-3-deoxyoctonate; PA, phosphatidic acid; P47, platelet protein of molecular weight 47,000; PRP, platelet-rich plasma; TEA, triethylamine.

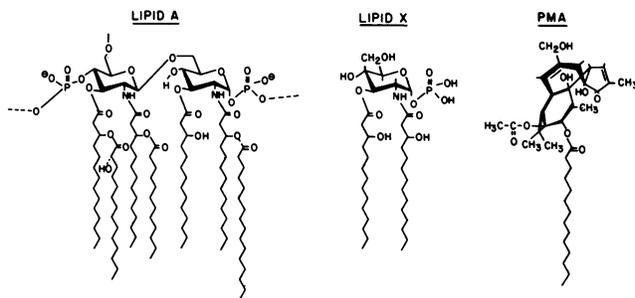


Figure 1. Structure of the endotoxic lipid A, its precursor lipid X, and of PMA derived from references cited in the text (4–6).

ride, which can be of potential significance in the mechanism of vascular changes in endotoxic shock.

Methods

Preparation of human platelets. Blood was drawn from healthy volunteers who had not taken aspirin or any other medication for the preceding 8 d. Platelets were separated from plasma proteins by stepwise albumin gradient centrifugation and Sepharose 2B gel filtration of platelet-rich plasma using modified Tyrode buffer, pH 7.35 (25). All experiments were performed with platelets suspended in modified Tyrode buffer without phosphate and calcium, pH 7.35.

Preparation of lipid A, lipid X, and PMA. Lipid A (Calbiochem-Behring Corp., La Jolla, CA), prepared by acid hydrolysis of LPS from *Salmonella minnesota* Re595 and containing less than 0.1% KDO, and lipid X (Lipidex, Middleton, WI) were suspended in distilled water (2 mg/ml). Triethylamine (TEA) was added to have final concentration 0.1%. The concentration of TEA in the platelet test sample was from 0.001 to 0.005%. PMA was dissolved in DMSO (10 mg/ml) and diluted to have 100 ng PMA/1 μ l DMSO. The concentration of DMSO in the platelet incubation mixture did not exceed 0.5%. Parallel controls of diluents were run in the platelet incubation mixture for all experiments.

Secretion of [¹⁴C]serotonin from human platelets. Platelet-rich plasma (PRP) was loaded with [¹⁴C]serotonin (0.05 μ Ci/ml) and then platelets radiolabeled with serotonin were separated from free amine and from plasma proteins as described previously (26). Secretion of [¹⁴C]serotonin was measured at 37°C with stirring for the specified times in the presence of imipramine (1.5 μ g/ml). The reaction was stopped by chilling the platelets on ice and adding 0.3% *p*-formaldehyde in 2 mM EDTA before centrifugation in a microcentrifuge for 15,000 *g* for 2 min (27). Secretion was calculated as previously described (26).

Platelet aggregation was done at 37°C with stirring according to the method of Born (28), using platelets separated from plasma proteins as described above and monitored in a dual channel aggregometer (Payton Associates, Buffalo, NY). Aggregation was measured using percent maximum transmission (T_{max}) and slope value, which represented the change per one minute along a tangent line to the steepest increase in light transmission (25).

Phosphorylation of human platelet proteins. Human platelets, separated from plasma proteins, were incubated with ³²PO₄ (50 μ Ci/ml) for 60 min at room temperature. After removal of free ³²PO₄ by Sepharose 2B gel filtration, the platelets were incubated with agonists, lipid A, lipid X, PMA, thrombin or diluent control, for various times at 37°C with stirring. The incubation mixture was immediately chilled on ice, and then spun down in a microcentrifuge at 15,000 *g* for 2 min. Platelet pellets were solubilized in 20 μ l of 5% SDS in 0.15 M NaCl and an equal volume of Laemmli solubilizing solution (29). Samples with 2-mercaptoethanol (10%) were boiled for 2 min and applied to SDS-

containing polyacrylamide gradient (5–15%) slab gels for electrophoresis according to the method of Laemmli (29). Molecular weight markers (Bio-Rad Laboratories, Richmond, CA) were simultaneously run. The Coomassie Blue-stained gels were dried, exposed to Kodak (Rochester, NY) X-Omat-AR film using a Cronex lighting plus intensifying screen (Dupont, Wilmington, DE), and developed in a Kodak RPX-Omat processor. The autoradiographs were scanned at 600 nm using a Response spectrophotometer (Gilford Instruments, Oberlin, OH) equipped with gel scan accessories and software that allowed quantitation of the integrated area under the peak (30).

[³²P]Phosphatidic acid generation and detection. Labeling of human platelets with ³²PO₄ was performed as above using siliconized glassware throughout all steps. The platelets (2 \times 10⁸/ml) were incubated with agonists for various times at 37°C with stirring. Incubations were terminated by adding 3.75 vol of ice-cold chloroform/methanol (1/2, vol/vol), followed by 1 vol of chloroform and 1 vol of 1 M NaCl. After centrifugation at 700 *g* for 3 min, the lower chloroform fraction was removed and the upper aqueous phase of each sample was washed twice with 2 vol of chloroform (31). The pooled lower phases were evaporated and redissolved in 30 μ l of chloroform for application to Whatman linear K preadsorbent silica gel (20 \times 20 cm) TLC plates. To every sample 15 μ g of standard unlabeled phosphatidic acid was added. A TLC solvent system of chloroform/methanol/water/ammonium hydroxide (60:38:4:2, vol/vol) was used for resolution of phospholipids, which were visualized with iodine vapor and autoradiography was performed as above (31). The spots at the points of the phosphatidic acid standard were scraped into a liquid scintillation vial, methanol was added to elute the [³²P]phosphatidic acid, and its radioactivity was counted by liquid scintillation using Aquasol-2.

[³H]Diacylglycerol generation and detection. Platelet-rich-plasma (PRP) from 100 ml of human blood was incubated with [³H]-arachidonic acid (³H-AA) (220 Ci/mmol) in final concentration 34 μ Ci/30 ml PRP at 37°C for 60 min. ³H-AA-labeled platelets were separated from plasma proteins as described above using siliconized glassware in all steps. Samples of platelets (2 \times 10⁸ platelets/ml) were incubated with different agonists for various times at 37°C with stirring. Incubation was stopped by adding 3.75 vol of ice-cold chloroform/methanol (1/2, vol/vol). Lipid extractions were done as described in the phosphatidic acid detection method. After drying, the samples were dissolved in 30 μ l of CHCl₃ and 10 μ g of 1,2-diolein (Sigma Chemical Co., St. Louis, MO) was added to each sample as a standard. Lipids were separated on the TLC plates characterized above using a two solvent system described by Skipski et al. (32). The first developing solvent was isopropyl ether/acetic acid (96/4, vol/vol) and the second developing solvent was petroleum ether/diethyl ether/acetic acid (90/101, vol/vol). Phospholipids were localized by iodine vapor, the spots corresponding to 1,2-diacylglycerol were scraped, eluted with methanol, and [³H]-1,2-diacylglycerol was measured by liquid scintillation counting.

Thromboxane B₂ formation. The aqueous phase from samples prepared for measurement of [³²P]phosphatidic acid and [³H]-diacylglycerol was evaporated and resuspended in radioimmunoassay buffer. Samples were assayed for TXB₂ using Amersham's radiolabeled TXB₂ immunoassay system.

Adenylate cyclase activity in platelet membrane preparation. Platelets were isolated as described above and disrupted by nitrogen cavitation (Parr bomb) at 500 psi for 30 min (platelets were kept on ice). Disrupted material was pelleted by centrifugation at 49,000 *g* for 1 h at 4°C. The membrane pellet was resuspended in a small volume (200–220 μ l) of ice-cold Tyrode buffer, pH 7.35, providing a source of Mg²⁺. Adenylate cyclase was assayed by measuring the conversion of α -[³²P]ATP to [³²P]cAMP. All reactions were carried out in a final volume of 50 μ l containing 20 μ l of platelet membrane in Tyrode's buffer, pH 7.35, 3.7 mM phosphoenolpyruvate, 0.1 mM cAMP, 0.6 mM ATP, 0.1 U pyruvate kinase, 0.5 U myokinase, and 1 μ Ci α -[³²P]-ATP. Each membrane preparation was assayed with control buffer, or 50 nM PGI₂, or 0.5 mM lipid X. Reaction mixtures were incubated at

37°C for indicated periods of time and the reaction was stopped by adding 1 ml of an ice-cold solution containing 0.36 mM ATP, 0.3 mM cAMP, and 0.03 μ Ci [3 H]cAMP (25,000 cpm). Samples were spun down in an Eppendorf centrifuge at 10,000 rpm for 5 min. [32 P]cAMP in the supernatant was isolated by sequential Dowex AG 50W-X4 and alumina chromatography as described by Salomon et al. (33). Recovery from the columns was monitored using [3 H]cAMP as the internal standard and the results were corrected for percent of recovery.

Reagents. [14 C]Hydroxytryptamine, binoxalate (serotonin), [3 H]-arachidonic acid, α -[32 P]ATP, and [3 H]cAMP were purchased from New England Nuclear (Boston, MA); phosphorus-32 as orthophosphate and [125 I]-TX B₂ Assay System were obtained from Amersham, Arlington Heights, IL; phorbol 12-myristate 13-acetate, phosphatidic acid, 1,2-diolein, phosphoenolpyruvate, pyruvate kinase (Type III from rabbit muscle), ATP, and cAMP from Sigma Chemical Co.; Dowex AG 50W-X4 from Bio-Rad Laboratories; alumina (aluminum oxide, neutral for chromatography) from Baker; lipid A from Calbiochem (La Jolla, CA) and lipid X from Lipidex (Middleton, WI). PGI₂ was a gift from Dr. John Pike at the Upjohn Co. All other chemicals were reagent grade.

Results

Effect of endotoxic lipid A on human platelet aggregation, [14 C]serotonin secretion and phosphorylation of p47 as compared to the effect of PMA. Upon addition of lipid A (35 μ M) to human platelets separated from plasma proteins, aggregation was observed which reached maximum within 8 min. At the end of 10 min 50% of [14 C]serotonin was secreted from platelets (Fig. 2, upper panel). For comparison, a pattern of response to PMA (0.3 μ M) is shown in the lower panel.

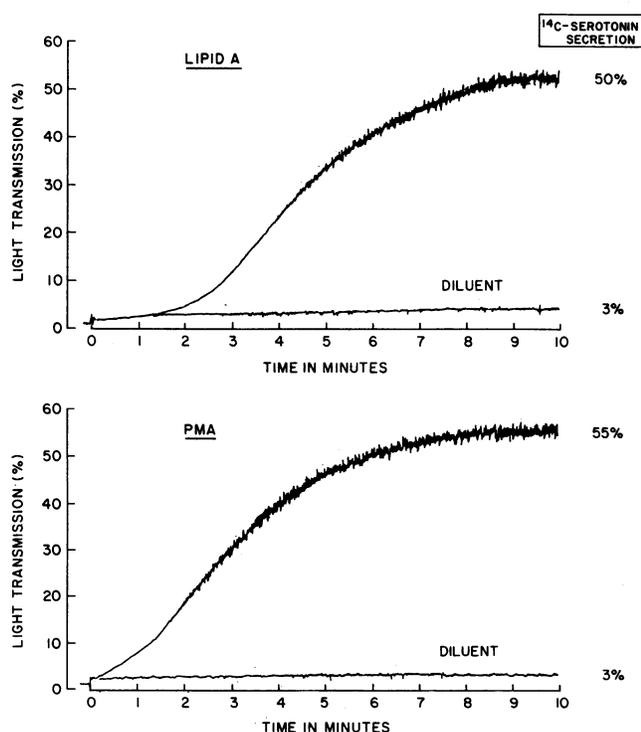


Figure 2. Aggregation and [14 C]serotonin secretion of human platelets (2×10^8 /ml) induced by 35 μ M lipid A (upper panel) as compared to 0.3 μ M PMA (lower panel). The diluent control for lipid A was 0.003% TEA and for PMA it was 0.5% DMSO in the incubation mixture.

Stimulation of platelets with a number of agonists, PMA in particular, induces activation of protein kinase C, which then phosphorylates proteins of M_r 40,000–47,000 in addition to other proteins (16, 20, 34). Therefore, phosphorylation experiments were done to examine the role of protein kinase C in activation of human platelets by lipid A. When lipid A (35 μ M) was added to human platelets prelabeled with 32 PO₄, a protein of apparent M_r 47,000 (P47) was prominently phosphorylated. The phosphorylation was observed within 5 s and reached maximum between 2 and 5 min (Fig. 3, upper panel). A similar pattern of rapid phosphorylation was observed with 0.3 μ M PMA (Fig. 3, lower panel). With both agonists a protein of M_r 20,000, corresponding to myosin light chain was also phosphorylated.

Stimulation of human platelets by lipid A was concentration dependent. Half maximal stimulation of platelets in terms of [14 C]serotonin secretion and aggregation was ob-

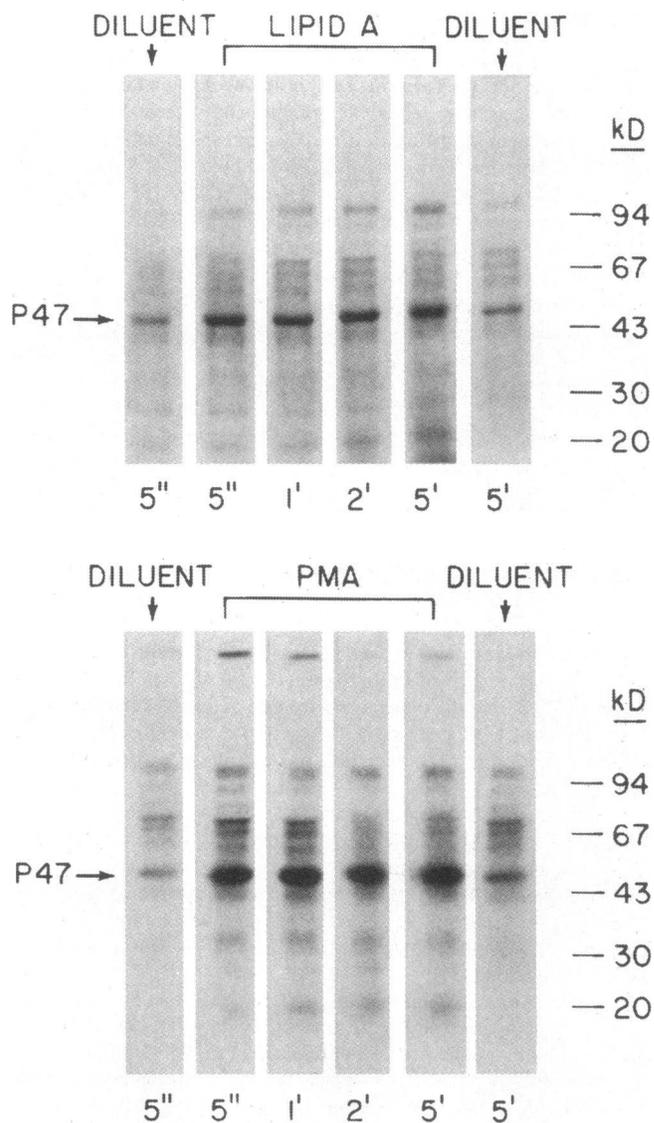


Figure 3. Autoradiograms showing phosphorylation of platelet proteins induced by 35 μ M lipid A (upper panel) or by 0.3 μ M PMA (lower panel) at the given time intervals. P47 indicates a protein of M_r 47,000.

served at lipid A concentration of 12 μM (Fig. 4, upper panel). The concentration of PMA inducing half-maximal stimulation of human platelets was 0.2 μM (Fig. 4, lower panel). When saturating concentration of lipid A was added to human platelets, together with saturating concentration of PMA, there was no significant increment in the observed effect (results not shown).

The time course of phosphorylation induced in lipid A-stimulated platelets was examined in more detail and the extent of radioactivity in P47 was expressed as percent of maximum integrated peak area representing the radioactive band. Software from the Response spectrophotometer equipped with gel scanning accessories was used and the values observed in diluent controls were subtracted. Cumulative data from time course of phosphorylation (Fig. 5) indicate that phosphorylation of P47 was rapid, reaching a peak in 2 min and then gradually decreasing during the 15-min incubation period. During this time secretion of [^{14}C]serotonin was progressing and reached maximum between 15 and 20 min.

Lack of an effect of endotoxic lipid A on phospholipase C and thromboxane synthetase-mediated generation of lipid metabolites. To examine whether the activating effect of endotoxic lipid A on platelet protein kinase C required the activation of phospholipase C, platelets labeled with [^3H]arachidonic acid or $^{32}\text{PO}_4$ were stimulated with lipid A for various time intervals and the formation of [^3H]diacylglycerol or [^{32}P]phosphatidic acid was measured. Lipid A did not induce production of [^3H]diacylglycerol nor generation of [^{32}P]-

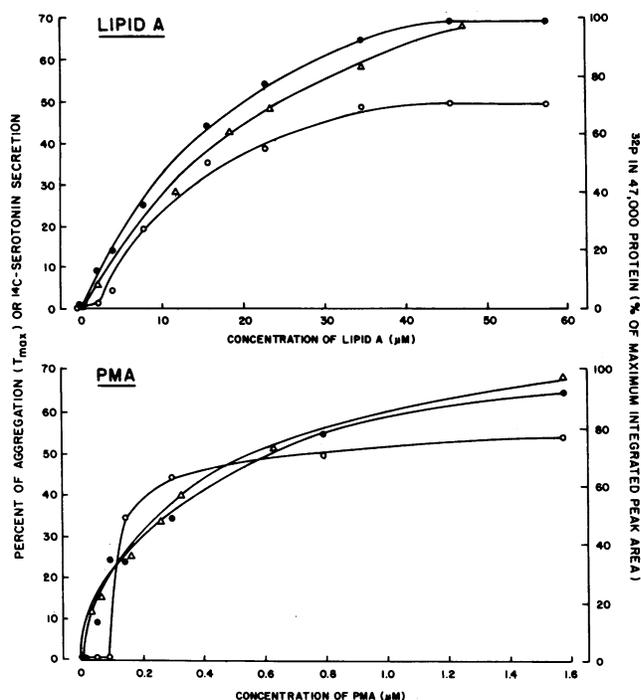


Figure 4. The response of human platelets ($2 \times 10^8/\text{ml}$) to increasing concentrations of lipid A (upper panel) or of PMA (lower panel) as measured by platelet aggregation (○) and [^{14}C]serotonin secretion (●) at 10 min, and phosphorylation of P47 (Δ) measured at 1 min. Data points for phosphorylation were obtained after subtracting the values observed in the controls containing platelets and diluents. Data represent mean values of two independent experiments.

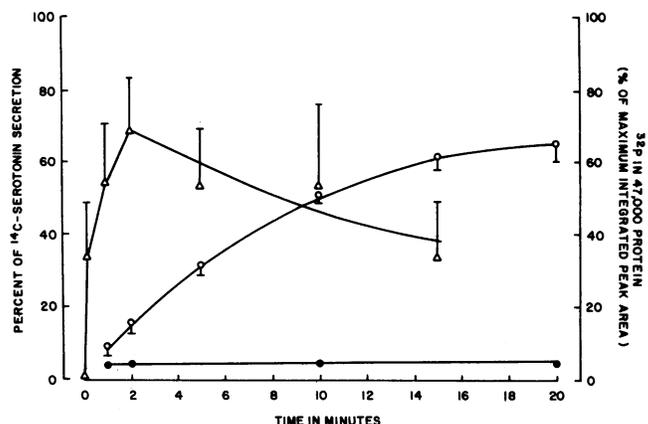


Figure 5. Time course of phosphorylation of a protein of M_r 47,000 (Δ) and [^{14}C]serotonin secretion (○) in platelets stimulated with lipid A (35 μM). Data for phosphorylation represent the mean value of four independent experiments after subtracting values observed in diluent controls. Diluent controls contained 0.003% TEA (●). Data for [^{14}C]serotonin secretion represent mean values of three independent experiments. Bars indicate the standard error.

phosphatidic acid (Fig. 6). In contrast, thrombin induced rapid and transient formation of diacylglycerol followed by the generation of phosphatidic acid (Fig. 6). Thrombin also caused production of TXB_2 , whereas lipid A-stimulated platelets did not produce TXB_2 measured by radioimmunoassay (results not shown).

Lipid X inhibits stimulation of human platelets by endotoxic lipid A and PMA. Biosynthesis of endotoxic lipid A involves a series of structural precursors. One of them, derived from *Escherichia coli*, is lipid X (N^2, O^3 -diacylglucosamine 1-phosphate), which was shown to share some biologic properties of lipid A, exemplified by mitogenic stimulation of B lymphocytes and gelation of the *Limulus* lysate (5, 35) but not toxicity to chick embryos and pyrogenicity in rabbits (36). In our experiments with human platelets, lipid X did not induce [^{14}C]serotonin secretion (Fig. 7). However, lipid X preincubated with platelets for 5 min at room temperature without stirring inhibited [^{14}C]serotonin secretion from human platelets induced by lipid A or PMA (Fig. 7). The inhibitory effect of lipid X was concentration-dependent. 50% of inhibition of the lipid A-mediated secretion of [^{14}C]serotonin was observed at almost equimolar concentration of lipid X and complete inhibition was achieved at 4–5 M excess of lipid X. Secretion of [^{14}C]serotonin from platelets by PMA was also inhibited, whereas that induced by thrombin (0.02 U/ml) was not blocked by lipid X in concentration range tested (Fig. 7).

Using similar conditions, lipid X inhibited activation of protein kinase C in human platelets stimulated with lipid A, PMA, and thrombin, as shown by suppressed phosphorylation of P47 (Fig. 8). Since a rise in platelet cyclic AMP blocks several stimulatory pathways in platelets (37), it was important to examine whether lipid X influences adenylate cyclase in a human platelet membrane preparation. As shown in Fig. 9, lipid X did not activate adenylate cyclase, whereas prostaglandin I_2 (prostacyclin) did. Thus, the inhibitory effect of lipid X on lipid A-induced secretion of [^{14}C]serotonin and phosphorylation of P47 does not appear to be mediated by an increase in cyclic AMP.

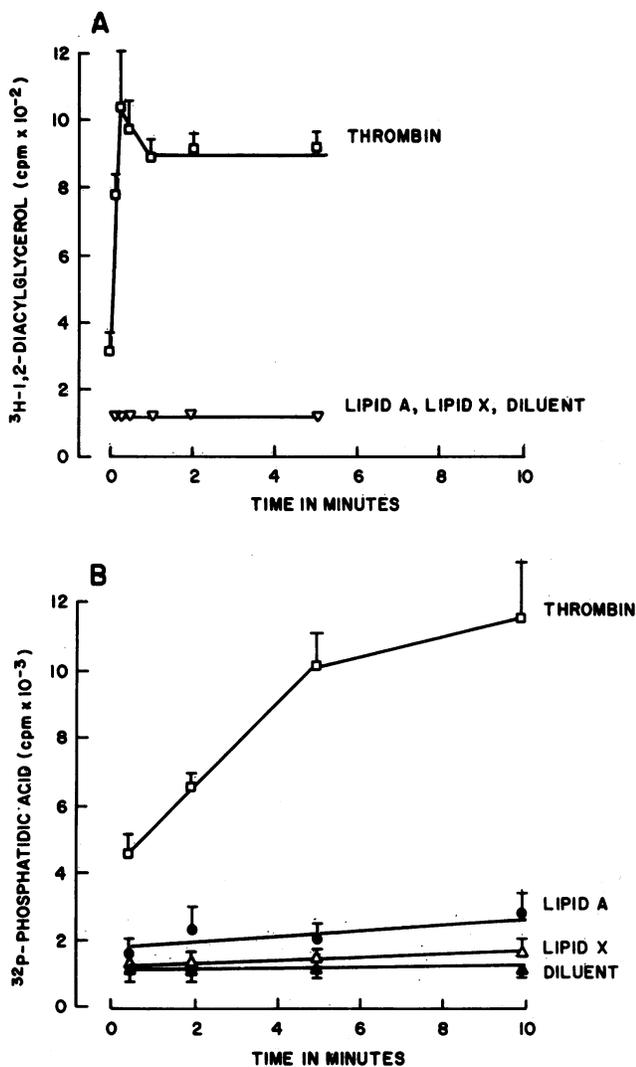


Figure 6. (A) Time course of 1,2-³H-diacylglycerol formation in human platelets challenged with thrombin (0.17 U/10⁸ platelets) (□), lipid A (35 μM) (▽), lipid X (110 μM) (▽), or diluent control containing 0.003% TEA (▽). (B) Time course of [³²P]phosphatidic acid generation in human platelets challenged with thrombin (0.17 U/10⁸ platelets) (□), lipid A (35 μM) (●), lipid X (110 μM) (Δ), or diluent controls containing 0.003% TEA (▲). Data points represent mean values of three independent experiments. Bars indicate the standard error.

Discussion

Our results provide the initial evidence that lipid A isolated from endotoxic lipopolysaccharide stimulates human platelets as measured by secretion of [¹⁴C]serotonin and aggregation. Both reactions are progressive, reaching maximum within 15 min, and they are preceded by a rapid phosphorylation of a platelet protein of apparent *M_r* 47,000, representing activation of protein kinase C (21). Lipid A preparations obtained from hydrolysis of lipopolysaccharide extracted from the mutant Re595 of *Salmonella minnesota* are heterogenous, composed of eight species of different mobilities in a TLC system attributed to different degrees of substitution of fatty acids (38, 39). Chromatographically isolated fractions which were character-

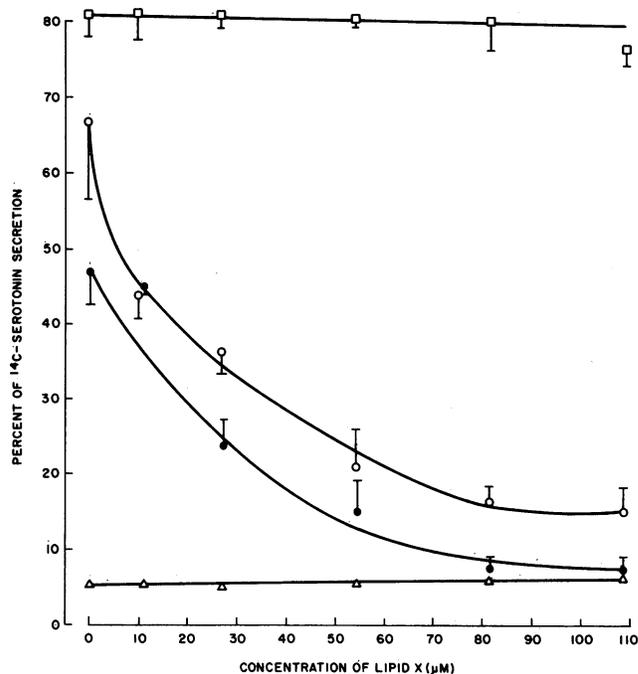


Figure 7. Effect of lipid X on secretion of [¹⁴C]serotonin from human platelets. Lipid X and platelets incubated for 10 min at 37°C with stirring (Δ); lipid X added to platelets for 5 min at room temperature with stirring before challenge with 22 μM lipid A (●); with 0.3 μM PMA (○); and with 0.02 U/ml thrombin (□). Secretion of [¹⁴C]serotonin was measured after 10 min incubation of platelets with agonists at 37°C with stirring. Data points represent mean values of three independent experiments. Bars indicate the standard error.

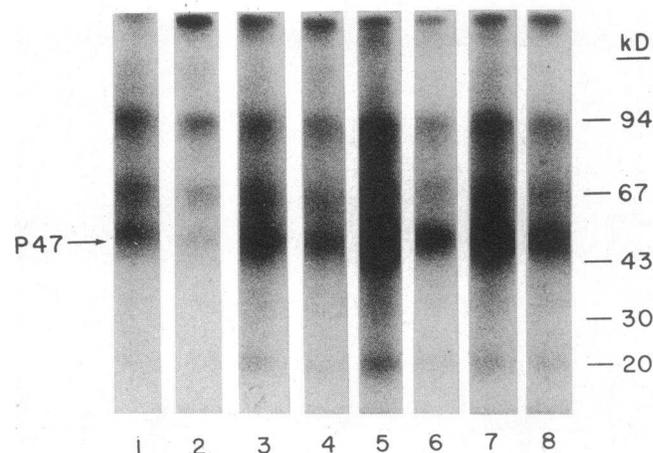


Figure 8. Effect of lipid X on phosphorylation of a protein of *M_r* 47,000 (P47) in human platelets challenged with lipid A, PMA, or with thrombin. Platelets were preincubated for 5 min with lipid X or diluent before challenge with stimuli. After addition of agonists, the reaction mixture was incubated at 37°C for 1 min with stirring. Lane 1, platelets with diluent (0.003% TEA); lane 2, platelets with lipid X (110 μM); lane 3, platelets with lipid X (110 μM) and lipid A (22 μM); lane 4, platelets with lipid X (110 μM) and PMA (0.3 μM); lane 5, platelets with PMA (0.3 μM); lane 6, platelets with lipid X (110 μM) and PMA (0.3 μM); lane 7, platelets with thrombin (0.02 U/ml); lane 8, platelets with lipid X (110 μM) and thrombin (0.02 U/ml).

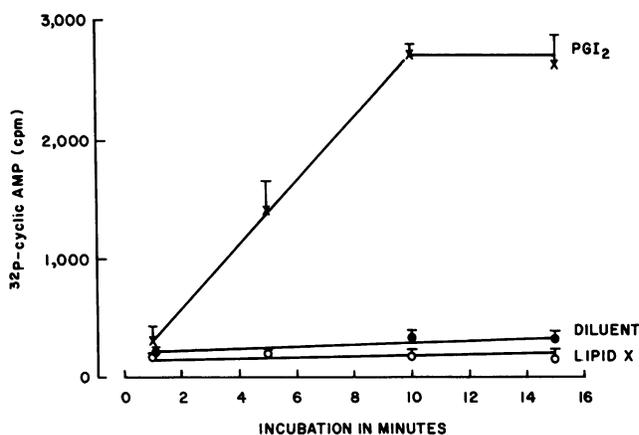


Figure 9. Adenylate cyclase activity as measured by α - ^{32}P cyclic AMP in platelet membranes challenged with PGI_2 (50 nM) (x), lipid X (0.5 mM) (○), or diluent (0.003% TEA) (●) at 37°C for the indicated times. Data points represent mean values of three independent experiments. Bars indicate the standard error.

ized by fast atom bombardment mass spectroscopy are also stimulatory toward human platelets.²

The mechanism through which endotoxic lipid A modulates protein kinase C in human platelets can involve a direct interaction with the enzyme in a manner similar to the acidic phospholipid, phosphatidyl serine, or an indirect interaction through activation of phospholipase C and subsequent generation of diacylglycerol, a known activator of protein kinase C (40–42). Neither production of ^3H diacylglycerol nor ^{32}P -phosphatidic acid was observed in lipid A-stimulated platelets, indicating a lack of phospholipase C activation in human platelets. Moreover, we did not observe generation of TXA_2 . Thus, its role in early mediation of the stimulatory effect of lipid A on human platelets seems unlikely.

Activation of protein kinase C in human platelets in response to endotoxic lipid A occurs almost immediately since phosphorylation of P47 reaches a high level within the first 30 s. This time course is similar to that induced by PMA, which is known to intercalate membrane phospholipids and form a complex with protein kinase C translocated to the membrane (13, 43). The concentration of lipid A inducing phosphorylation of P47 is ~ 25 –50 times higher than PMA. The more complex structure of lipid A than that of PMA (Fig. 1) may require a higher concentration of the former to intercalate the platelet membrane. In experiments using a murine macrophage cell-line RAW264.7, a similar range of concentrations of lipid A, and its monosaccharide precursor lipid X, to those used in our study were inducing activation of protein kinase C and hydrolysis of phosphatidylinositol with a concomitant increase in synthesis of prostaglandins (24, 44).

The important findings in our studies, distinct from those in the murine macrophage cell line, are that lipid X does not activate protein kinase C in human platelets but rather it inhibits the lipid A-induced activation of protein kinase C. Moreover, lipid X does not stimulate generation of diacylglyc-

erol, PA, or TXB_2 . Lipid X resembles structurally a “half molecule” of lipid A (Fig. 1). With a 1:1 stoichiometry it inhibits 50% of the ^{14}C serotonin secretion induced by lipid A. It also inhibits PMA-induced ^{14}C serotonin secretion but not that induced by thrombin. This finding is consistent with the view that thrombin can activate platelets by induction of additional stimulatory pathways independent of protein kinase C (37). Hence, ^{14}C serotonin secretion induced by thrombin in the presence of lipid X involves other pathways such as cyclooxygenase and phospholipase C activation, which are not affected by lipid X. Lipid X did not inhibit the thrombin-induced generation of phosphatidic acid. Moreover, lipid X did not stimulate adenylate cyclase in platelet membrane preparations. Therefore, secretion of ^{14}C serotonin induced by thrombin was not inhibited by lipid X which blocks selectively platelet protein kinase C. It appears that lipid X interacts with the same component of the human platelet membrane as that involved in the interaction with endotoxic lipid A and phorbol ester. Experiments with saturable concentrations of these two activators seem to support such an interpretation. Phorbol ester binds in a saturable manner to mink lung cells and murine BALB/3T3 clone A31 cells (45) and the possibility of a specific saturable binding site for both phorbol ester and lipid A on the human platelet membrane cannot be excluded.

Although we do not have evidence that protein kinase C in human platelets is directly accessible to endotoxic lipid A, the following points indicate that this is possible. (a) As demonstrated in other cells, the enzyme is translocated to the membrane (13, 43). (b) PMA can activate protein kinase C directly, without inducing phosphatidyl inositol turnover (34). (c) Affinity-purified protein kinase C from human platelets is activated by endotoxic lipid A.³

Endotoxic lipid A, as a biologic modifier of protein kinase C, causes stimulation of this enzyme which in platelets phosphorylates a phosphatase controlling the IP_3 level (18). IP_3 mobilizes intracellular calcium (19). In turn, sustained phosphorylation of an IP_3 -specific phosphatase by lipid A-activated protein kinase C can result in deranged calcium fluxes and abnormal stimulus-response coupling. The list of cells which are targets for endotoxic lipopolysaccharide includes, in addition to blood platelets, granulocytes, monocytes, lymphocytes, erythrocytes and endothelial cells (2, 10, 46). All of them possess protein kinase C while its highest reported tissue concentration is in the spleen, brain, and ganglia (9, 11). During experimentally induced endotoxemia a lipid A-like substance reappears in the circulation 24 h after injection of endotoxin (3). Moreover, higher titers of antibodies, directed against the lipid A-containing core of gram-negative bacteria, protected patients from irreversible changes of endotoxic shock in gram-negative bacteremia (47). Modulation of protein kinase C in human platelets by endotoxic lipid A indicates that this pivotal cellular regulatory enzyme is a target for the toxic principle of bacterial lipopolysaccharide.

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3. Romano, M., and J. Hawiger, manuscript in preparation.

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