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Down-Modulation Through Protein Kinase C-α of Lipopolysaccharide-Induced Expression of Membrane CD14 in Mouse Bone Marrow Granulocytes

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ABSTRACT. We have previously shown that stimulation of mouse bone marrow granulocytes (BMC) by lipopolysaccharide (LPS) induces the expression of CD14. We found here that phorbol 12-myristate 13-acetate (PMA) blocks this LPS effect. The aim of this study was to investigate the mechanism by which PMA can block the LPS signaling pathway in BMC. The unmodified binding of a radiolabeled LPS in PMA-treated cells indicated that the PMA effect was not the consequence of a shedding or an internalization of the LPS receptor, but was rather due to a biochemical event that follows the interaction of LPS with its receptor. The observations that a selective activator of protein kinase C (PKC)-α (sapintoxin D) mimics the PMA effect, whereas a selective PKC-α inhibitor (Ro-320432) antagonizes this effect, suggest a regulatory role of PKC-α in the LPS signaling pathway in mouse BMC. BIOCHEM PHARMACOL **60**;12:1837–1843, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. lipopolysaccharide; PKC; bone marrow; CD14; granulocytes

LPS,§ a major component of the cell wall of gram-negative bacteria, is a potent stimulant that acts on a wide variety of cell types by triggering the production of mediators or by modulating the expression of cell-surface constituents. This leads to severe pathological processes in the host and explains the efforts at understanding the mechanisms of these cell responses. There exists an abundant literature on LPS-induced signaling in monocytes/macrophases [1] in which the major LPS receptor is CD14 [2], but in other LPS-responsive cells that do not constitutively express CD14, such as bone marrow granulocytes [3], the mechanism of action of LPS remains elusive. Understanding the mechanism of stimulation of bone marrow granulocytes by LPS would deserve, however, close scrutiny, because these cells are the precursors of neutrophils, which are indispensable for efficient host defense against invading microorganisms.

As for other cell activators (hormones, cytokines, and growth factors), the effects induced by LPS are mediated by a variety of enzymes, which are often regulated by their own phosphorylation and dephosphorylation. Because PKC is an important signal transducer in mammalian cells [4], its involvement in LPS responses has been early and actively investigated, but the literature on this topic is controversial. PKC activation by LPS has been documented in several cell types including monocytes [5], macrophages [6], endothelial cells [7], and vascular smooth muscle cells [8]. After exposure to LPS, 68-kDa [9] and 66-kDa [6] PKC substrates have been shown to undergo increased protein phosphorylation and plasma membrane translocation in murine peritoneal macrophages. One complication in these studies is the fact that PKC is not a single entity, but a family of at least twelve closely related serine/threonine isoenzymes [10] that differ in the extent of their dependence on calcium and phospholipids, as well as in their tissue and subcellular distribution. The various isoenzymes may thus exert specific functions. In this regard, a translocation of PKC-β to the cytoskeleton of LPS-stimulated murine peritoneal macrophages has been reported [11], and PKC-α [8] and PKC-ζ [12] have been implicated in LPS signaling in vascular smooth muscle cells and in the mouse macrophage cell line J774, respectively. Controversial findings have been obtained with PKC-ε: using isoform specific antibodies, Shapira *et al.* [13] found that PKC-ε is the major PKC isoform involved in the activation of mouse macrophages with LPS, whereas Diaz-Guerra *et al.* [14] found that PKC-ε is not involved in the expression of the inducible form of nitric oxide synthase induced by LPS in the mouse macrophage cell line RAW 264.7.

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§ *Abbreviations:* BMC, bone marrow cells; CPT-cAMP, sodium 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate; DOG, 1,2-di-octanoyl-*sn*-glycerol; FACS, fluorescence-activated cytometry sorter; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; LPS-Bp, LPS from *Bordetella pertussis*; LPS-Sc, LPS from *Salmonella enterica* serovar choleraesuis; ^{125}I-LPS, LPS-Sc radiolabeled with ^{125}I; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; and TNF-α, tumor necrosis factor-α.

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In contrast to the results mentioned above, various macrophage responses to LPS have been reported to occur independently of PKC activation. This is particularly the case for the production of TNF-α [15], the enhancement of arachidonic acid metabolism [16], the inhibition of proliferation [17], and the down-regulation of colony-stimulating factor-1 receptors [18]. More recently, it has been reported that LPS-induced nuclear factor-κB activation and release of interleukin-6, interleukin-8, and TNF-α in human alveolar macrophages is PKC-independent [19].

As regards the activation of BMC with LPS, we have shown in previous studies that stimulation of these cells with nanomolar concentrations of LPS triggers the expression of membrane CD14 [20] and that this cell response to LPS cannot be blocked by specific PKC inhibitors [21]. However, our observation that PKC is not directly involved in LPS-induced stimulation of BMC does not preclude the possibility of cross-talk between PKC and the LPS-induced pathway. Actually, in other cell systems, modulations of LPS responses by PKC activators have been found, but here again, opposite overall effects have been reported. For example, overexpression of PKC-α strongly inhibits some LPS-induced functions in the RAW 264.7 mouse macrophage cell line [22], but enhances other responses to LPS in vascular smooth muscle cells [8].

In view of these controversial results and the importance of bone marrow granulocytes as neutrophil precursors, we decided to examine herein the influence of PMA, a widely used PKC activator, on the LPS-induced expression of CD14 in BMC.

MATERIALS AND METHODS

Animals and Cell Culture

Eight- to ten-week-old female C3H/HeOU mice were from the Breeding Center of the Pasteur Institute. BMC were collected from mouse femurs. Culture medium was RPMI-1640 (Sigma Chemical Co.) containing 20 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin.

Reagents

LPS-Sc and LPS-Bp were prepared from *Salmonella enterica* serovar choleraesuis (serotype 6_2,7,14) and from *Bordetella pertussis* (vaccinal strain 1414), respectively, by the phenol–water extraction procedure [23]. PMA, dibutyl phthalate, and dinonyl phthalate were purchased from Sigma Chemical Co. The PKC inhibitors Ro-318220 and GF-109203X and the PKC activators sapintoxin D and DOG were from Calbiochem. The PKC-α inhibitor Ro-320432 and CPT-cAMP were from Biomol Research Laboratories.

Labeled Reagents

The rat anti-mouse CD14 monoclonal antibody (rmC5-3 mAb) was from PharMingen. Biotin-labeled goat anti-rat immunoglobulin antibody and peroxidase-labeled streptavidin were from Southern Biotechnology Associates. A radiolabeled derivative of LPS-Sc (^{125}I-LPS) was prepared as described previously [24]. CNBr-activated LPS-Sc was first coupled to tyramine, and the tyramine-labeled LPS (Tyr-LPS) was then iodinated with ^{125}I by the chloramine-T method. After extensive dialysis, the radiolabeled LPS was separated from residual iodine by precipitation with ethanol (five volumes) at −20° for 30 min. The precipitate was recovered by centrifugation (10 min, 900 × *g*). The pellet containing the labeled endotoxin (2.1×10^6 cpm/μg) was suspended in water (1 mL) and stored at −30°. Aliquots were thawed, diluted in binding buffer, and sonicated before use in binding assays. We showed in a previous study that this preparation of ^{125}I-LPS interacts with the specific LPS-binding site on BMC [24].

SDS–PAGE and Western Blot Analysis of CD14

BMC were pelleted and lysed by incubation (45 min, 4°) in lysing buffer consisting of 300 mM NaCl, 50 mM Tris pH 7.5, 1% cholamidopropyl dimethylammoniopropane sulfonate (CHAPS), 10 μg/mL of aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM iodoacetamide, and 2 μg/mL of pepstatin. The cell lysate was centrifuged (10 min at 12,500 × *g*), and the supernatant was mixed with the same volume of the sample loading buffer (4% SDS and 20% glycerol in 0.05 M Tris–HCl, pH 6.8) and boiled for 5 min. An aliquot was analyzed by SDS–PAGE in 10% polyacrylamide slab gels according to the method of Laemmli. Molecular mass markers (Rainbow marker, Amersham) from 14.3 to 220 kDa were run in parallel. Gels were fixed in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol) and proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) with a semi-dry blotting system at 45 v for 1 hr. Membranes were blocked by incubation for 18 hr at 20° with 2% BSA in PBS and incubated (1 hr, 20°) with the rat anti-mouse antibody rmC5-3 (1:1000 in PBS–2% BSA). The blots were washed with PBS–0.1% Tween 20, incubated for 1 hr at 20° with a biotin-labeled goat anti-rat antibody (1:2500 in the same buffer), rewashed, and incubated with peroxidase-labeled streptavidin (1:20,000 in PBS containing 2% non-fat milk). After extensive washing, sites with peroxidase activity were detected by chemiluminescence with the Super Signal system (Pierce) according to the guidelines of the manufacturer.

LPS-Binding Assay

The LPS-binding capacity of BMC was determined as described previously [24]. The binding of ^{125}I-LPS to BMC was carried out at 37° for 120 min in a binding medium (BMI) of RPMI-1640 containing 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM EDTA, 5 mM 2-deoxyglucose, 2 mM NaF, and 10 mM NaN_3. BMC (5 ×

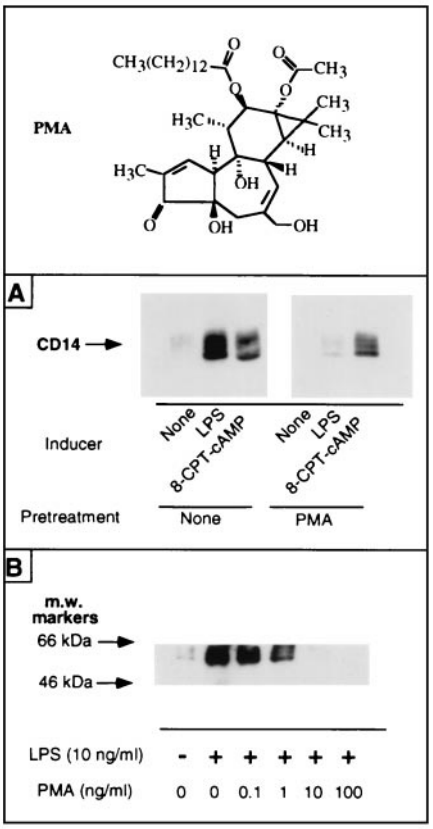

FIG. 1. Influence of PMA on CD14 expression induced by LPS or CPT-cAMP in BMC. (A) Comparison of two CD14 inducers LPS and CPT-cAMP. BMC (1.5×10^6 cells/mL) were first incubated (30 min, 37°) with or without PMA (100 ng/mL) and were then exposed to LPS (10 ng/mL), CPT-cAMP (50 μM), or culture medium alone in the absence of serum. (B) Influence of various concentrations of PMA. BMC (1.5×10^6 cells/mL) were first incubated (30 min, 37°) with different concentrations of PMA and then exposed to 10 ng/mL of LPS (3 hr, 37°). In experiments A and B, cell lysates were analyzed for CD14 by SDS–PAGE and Western blotting, using the anti-mouse CD14 monoclonal antibody rmC5-3. Molecular mass markers are shown on the left.

10^6 cells in 1-mL polystyrene tubes) were preincubated for 60 min in BMI (total volume of 400 μL) in the presence or absence of a 10- or 20-fold excess of unlabeled LPS-Bp used as a inhibitor, and further incubated for 60 min in the presence of ^{125}I-LPS (5 μg/mL). To remove unbound ligand, the cells were resuspended and layered on cold mixtures (0°, 200 μL) of 30% dinonyl phthalate/70% dibutyl phthalate (density of 1.025) in 1.5-mL conical microcentrifuge tubes. After centrifugation for 3 min at $10{,}000 \times g$ and removal of the supernatant, the tips of the tubes containing the cell pellets were cut off, and the radioactivity was measured in a gamma counter (Kontron MR 480C). Assays were done in five replicates.

RESULTS

Influence of PMA on LPS-Induced Expression of CD14

We previously showed that stimulation of BMC with LPS or with cAMP analogs induces the expression of membrane-bound CD14 [25]. Therefore, to examine the influence of PMA on LPS stimulation of BMC, we analyzed by Western blot the expression of CD14 on BMC exposed successively to PMA and LPS, as compared to cells exposed to LPS alone. Treatments with the cAMP analog CPT-cAMP were also carried out for comparison. The results in Fig. 1A confirm our previous findings that both LPS and CPT-cAMP induce the expression of membrane CD14. The results also show that after treatment for 30 min with 100 ng/mL of PMA, the cells were still responsive to CPT-cAMP, but their response to LPS was considerably down-modulated. In a second experiment, the dose of PMA required for this down-modulation was examined. We found (Fig. 1B) that the down-regulation of the LPS effect was only partial with 1 ng/mL of PMA, but was complete with 10 ng/mL of this PKC activator.

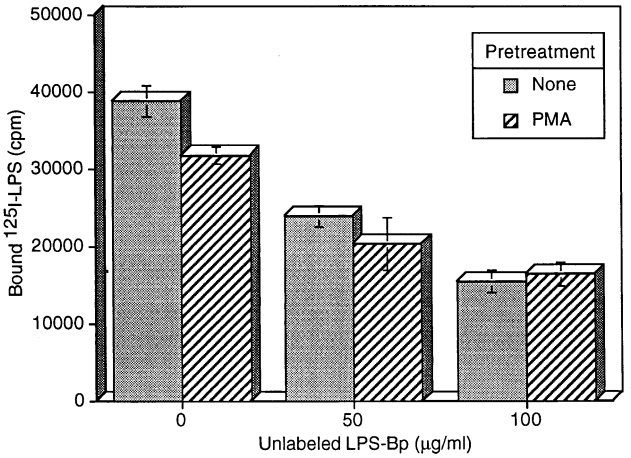

FIG. 2. Influence of PMA on the LPS-binding capacity of BMC. BMC (6×10^7 cells) were incubated (30 min, 37°) in the presence or absence of PMA (20 ng/mL), washed in binding medium, incubated (1 hr, 37°) with various amounts of unlabeled LPS-Bp, and reincubated (1 hr, 37°) with ^{125}I-LPS in the presence of LPS-Bp. Washings and incubations were carried out in binding medium. Bound ligand was measured after centrifugation of the cells through phthalate. Data represent means ± SD of five determinations.

Absence of Influence of PMA on the LPS-Binding Sites of BMC

One hypothesis that might explain the down-modulation induced by PMA on the LPS response could be a down-regulation of membrane LPS receptors induced by PMA. It was demonstrated previously, for instance, that PMA induces a shedding of various cell surface molecules such as CD62L (also termed "L-selectin") [26, 27] and FcγRIIIB [28] in neutrophils, TNF-RI in airway epithelial cells [29], and an epidermal growth factor-like molecule in kidney cells [30]. Therefore, the blocking of the LPS effect induced by PMA could be due to a loss of the constitutive LPS receptor. To examine this possibility, we analyzed the influence of PMA on the binding of a radiolabeled LPS (^{125}I-LPS-Sc). In order to analyze binding sites directed

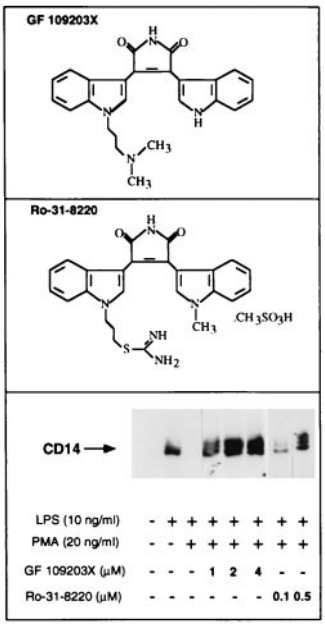

FIG. 3. Influence of PKC inhibitors on PMA-induced inhibition of the response to LPS. BMC (1.5 × 10^6 cells/mL) pretreated with the indicated amount of the PKC inhibitor (15 min, 37°) were incubated with 20 ng/mL of PMA (30 min, 37°) and then exposed to 10 ng/mL of LPS (3 hr, 37°). Cell lysates were analyzed for CD14 by SDS–PAGE and Western blotting as described in the legend to Fig. 1.

against the biologically active (lipid A) region of LPS, the experiment was performed with a radiolabeled derivative of LPS-Sc (^{125}I-LPS) in the presence or absence of a 10- or 20-fold excess of an unlabeled heterologous LPS (LPS-Bp). The results in Fig. 2 show that the PMA treatment of the BMC induced only a small decrease in the specific LPS-binding capacity of the cells (23,388 ± 2,402 cpm in untreated cells; 15,384 ± 1,839 cpm in PMA-treated cells). This moderate (34%) loss of specific LPS-binding sites can hardly account for the almost complete inhibition of responsiveness to LPS in the PMA-treated cells.

Involvement of PKC in the PMA-Induced Inhibition of the Response to LPS

Since PMA does not considerably influence the number of LPS-binding sites, its effect on the LPS response is likely due to its influence on downstream biochemical events of the LPS signaling pathway. Because PMA is a well-known activator of PKC, we examined whether PKC inhibitors could block PMA-induced inhibition and thus restore the response to LPS. We used two specific inhibitors of PKC: GF-109203X [31] and Ro-318220 [32]. Again, the responsiveness of the cells to LPS was visualized by Western blot detection of membrane CD14. The results in Fig. 3 show that GF-109203X (at concentrations of 1 μM and higher) and Ro-318220 (at concentrations of 0.1 μM and higher) were both able to restore the LPS response blocked by PMA.

Down-modulation of the Response to LPS through Activation of PKC-α

To determine if PKC activation is sufficient for inhibition of the LPS effect, we used two other PKC activators, sapintoxin D and DOG. We found that preincubation of

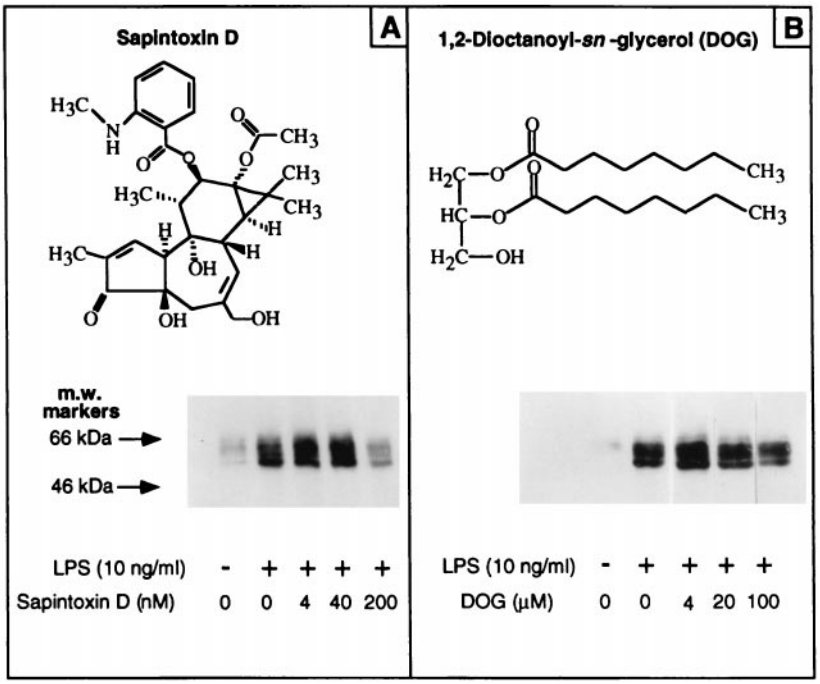

FIG. 4. Influence of PKC-α activators on the response to LPS. BMC (1.5 × 10^6 cells/mL) were first incubated (30 min, 37°) with the indicated amounts of sapintoxin D (A) or DOG (B) and were then exposed to 10 ng/mL of LPS (3 hr, 37°). Cell lysates were analyzed for CD14 by SDS–PAGE and Western blotting as described in the legend to Fig. 1.

the cells for 30 min with 200 nM sapintoxin D markedly inhibited the LPS-induced expression of CD14 (Fig. 4A), whereas preincubation with DOG did not (Fig. 4B). It is noteworthy that sapintoxin D is a semisynthetic fluorescent derivative of phorbol which, at concentrations lower than 1 μM, is a selective activator of PKC-α [33], whereas DOG has lower affinity for PKC-α than for other PKC isoenzymes [34]. Therefore, the observations of Fig. 4 strongly suggest that the inhibition of the LPS effect induced by phorbol derivatives (PMA, sapintoxin D) is mediated by the activation of PKC-α.

To confirm this hypothesis, we examined whether a PKC-α inhibitor could reverse the effect of PMA on LPS-induced expression of CD14. We used compound Ro-320432, a cell-permeable and selective inhibitor of PKC-α [35]. The results in Fig. 5 show that the inhibitory effect of PMA on the response to LPS was dose dependently reversed by pre-exposure to Ro-320432.

DISCUSSION

Several studies have established that PMA stimulates some granulocyte functions, but decreases other responses [36].

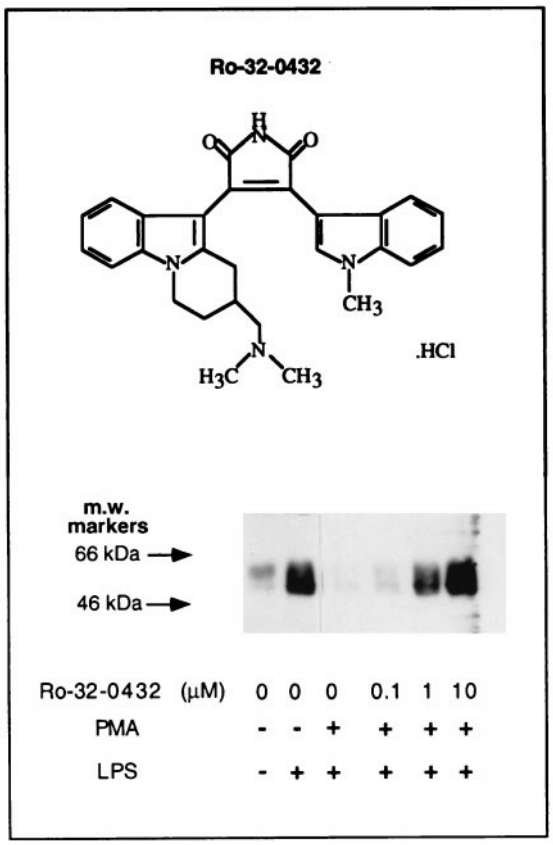

FIG. 5. Influence of a PKC-α inhibitor on the PMA effect. BMC (1.5 × 10^6 cells/mL) pretreated with the indicated amount of Ro-320432 (15 min, 37°) were incubated with 20 ng/mL of PMA (30 min, 37°) and then exposed to 10 ng/mL of LPS (3 hr, 37°). Cell lysates were analyzed for CD14 by SDS–PAGE and Western blotting as described in the legend to Fig. 1.

The present data show that PMA inhibits the expression of membrane CD14 resulting from the activation of bone marrow granulocytes exposed to LPS. Concerning the mechanisms of this inhibition, there are two possibilities: (i) a down-regulation of the LPS receptor by internalization or shedding; and (ii) an inhibition of the biochemical events that follow the interaction of LPS with its receptor.

The first hypothesis was attractive because: (i) we have shown previously that a low-affinity LPS receptor distinct from CD14 is constitutively present on BMC [24]; (ii) L-selectin has been reported to be a low-affinity LPS receptor in neutrophils [37]; and (iii) it has been shown that PMA induces the shedding of L-selectin and other membrane proteins in granulocytes [26] and the internalization of the transferrin receptor in HL-60 cells [38]. We found, however, that the specific binding of LPS is very moderately reduced by PMA treatment. Therefore, the possibility that the LPS receptor becomes unavailable or inefficient for binding after a biochemical event induced by LPS can be excluded.

The second and more likely mechanism is that PMA blocks one of the biochemical events of the cascade of LPS signaling, downstream of the interaction of LPS with its receptor. This effect of PMA can be mediated by the phosphorylation of a specific protein. It has been reported that PMA can induce the serine phosphorylation of a myristoylated alanine-rich C kinase substrate (MARCKS) via activation of PKC [39] or inhibit the tyrosine phosphorylation of several proteins in human neutrophils [40]. We found that the inhibitory effect of PMA on the LPS response was abolished by two specific PKC inhibitors, thus suggesting that the inhibition is indeed mediated by PKC. The PKC isoenzyme involved in this inhibitory effect is apparently the α isoform, since sapintoxin D, which selectively activates PKC-α, blocks the cell response to LPS, whereas DOG, which mainly activates other PKC isoforms, does not. Furthermore, the inhibitory effect of PMA can be reversed by treatment with Ro-320432, a selective PKC-α inhibitor.

Therefore, our results indicate the existence of a cross-talk between activation of PKC-α and activation of the LPS signaling pathway. This may suggest, at the physiological level, the possibility of a regulatory role of PKC-α in the response of some cell types to LPS. Several problems remain, however, to be solved. It would be interesting, for example, to determine if the PKC-α that regulates the LPS response is localized in a particular cell site, to identify the protein that is phosphorylated by this PKC isoenzyme, and to determine at what level of the LPS signaling cascade PKC-α exerts its inhibitory effect. Current studies to answer these questions are in progress.

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