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ACTIVATION OF PROTEIN KINASE C FROM B LYMPHOCYTES BY LIPID A

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The effect of lipid A, a residue of the lipopolysaccharide molecule, on protein kinase C from B lymphocytes has been studied. Lipid A activates and promotes the translocation of protein kinase C from the soluble to the particulate membrane fraction in a cell-free system reconstituted with purified enzyme and membranes isolated from B lymphocytes. These results demonstrate that the activating effect of lipopolysaccharide on protein kinase C from B cells is due to the lipid moieties of this molecule.

Bacterial lipopolysaccharides elicit a wide range of biological effects including activation of macrophages (1,2) and B lymphocytes (3). This activation produces growth and differentiation of the B cells and the process can be followed by the induction of RNA and DNA synthesis. Although the molecular basis for the policlonal B lymphocyte activation by lipopolysaccharide (LPS) remains unclear, evidence has been reported suggesting that activation of protein kinase C (PKC) is involved in the proliferative response to LPS (3,4). However, LPS does not seem to stimulate either phosphatidylinositol turnover or Ca^{2+} mobilization in resting B cells (5).

The chemistry of the LPS molecule obtained from various Gram-negative bacteria is well known. Lipid A, a moiety of LPS obtained by mild acid hydrolysis, has been purified and retains some of the biological properties of the complete LPS molecule, among which is the capacity to induce B lymphocyte proliferation (6). In the case of stimulation of macrophages by LPS it has been reported that a precursor of lipid A (lipid X) increases phosphatidylinositol turnover and activates partially purified PKC from these cells (7). In this paper we present evidences suggesting that lipid A activates PKC purified from B lymphocytes or other tissues, and also promotes translocation of this enzyme from cytosol to membranes acting synergistically with other effectors.

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MATERIAL AND METHODS

Chemicals. [γ-^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear. Histone H1, phosphatidylserine (PS), oleoyl acetylglycerol (OAG), phorbol dibutyrate (PDBu) and lipid A (from S. minnesota) were from Sigma. LPS was from Difco. Lipid A from E. coli was prepared as described (8). Lipids were suspended at 1 mg/ml in chloroform:methanol (19:1), dried under N_2 and resuspended by sonication. Other chemicals were from Merck or Boehringer.

Purification of B cells. Cells were prepared from 8 to 10 B6/C57 mice, aged 3 to 4 months. Spleen cells were released by disrupting the organ, and resuspended in 20 ml of balanced salt solution (BSS). After centrifuging at 300xg for 10 min, the red cells were lysed by incubation at 4 °C for 5 min in 25 ml of BSS containing 130 mM of NH_4Cl instead of NaCl. After two washes with 40 ml of BSS, the cells were counted, resuspended at $25x10^6$ cells/ml and incubated with monoclonal anti Thy-1 for 45 min at room temperature. T-cells were lysed by incubation with rabbit complement for 45 min at 37 °C. After two washes with BSS the B cells were pelleted.

Purification of PKC. Cell pellets were homogenized at 4 °C in a Potter with 2 ml of a medium containing 2 mM EGTA, 2 mM EDTA, 10 mM ß-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 µg of leupeptin and 10 mM PIPES, pH 6.6. The homogenate was centrifuged at 140.000xg for 30 min to yield a supernatant form which PKC was purified by DE52-chromatography. After loading, the column (0.3x5 cm) was washed with 10 ml of homogenization medium supplemented with 0.2 mM EGTA, 10 mM ß-mercaptoethanol, 1 mM PMSF, 100 mM NaCl and 10 mM HEPES, pH 7.5 (buffer A). The purification was followed by protamine-agarose chromatography (9). The membranes obtained after centrifugation of the cell homogenates were resuspended in 2 ml of homogenization medium suppemented with 0.1% Nonidet P-40 and sonicated. After centrifugation as previously described, the resulting pellet was resuspended in 2 ml of buffer A plus 50 µg of leupeptin. The supernatant contained the membrane-bound activity.

Assay of PKC. The histone kinase activity of PKC was measured at 30 °C in an incubation volume of 150 µl. The assay mixture contained in final concentrations 20 µM ATP (0.3 µCi), 1 mM Mg acetate, 5 mM ß-mercaptoethanol, 50 µg of histone H1, 20-50 µl of sample, 20 mM HEPES, pH 7.5 and except otherwise indicated, 0.6 mM $CaCl_2$, 10 µg of PS and 2 µg of OAG. The incorporation of [^{32}P]phosphate into histone was linear for at least 20 min under these conditions. The reaction was stopped by the addition of 2 ml of 5% (w/v) trichloroacetis acid, 10 mM H_3PO_4 at 0 °C. The radioactivity retained on GF/C glass fiber fiters after filtration was determined by counting the oven-dried filters in 2 ml of scintillation fluid. PKC activity was determined after subtracting the incorporation in the abscence of Ca^{2+}, PS and OAG. A unit of PKC was defined as incorporating 1 pmol of phosphate into histone H1 per minute. Endogenous phosphorylation was negligible. Proteins were determined as described by Bradford (10).

RESULTS

PKC was purified from B cells following standard procedures (11). The purification was necessary because the activity present in cell extracts exhibits a markedly lowered response to Ca^{2+} and phospholipids stimulation when compared with the purified enzyme. Figure 1A shows the activation of PKC by lipid A purified from E. coli, at different

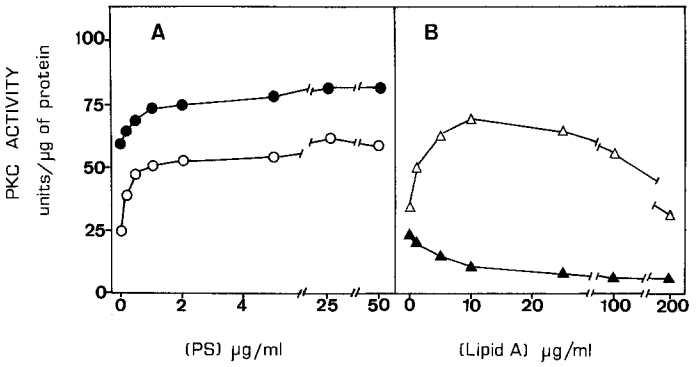


Figure 1. Activation by lipid A of PKC partially purified from B lymphocytes. (A) Effect of PS on PKC activity in the presence of 1 µg/ml of OAG. Control (○); 10 µg/ml lipid A (●). (B) Dose-response curve for lipid A; 5 µg/ml PS, 2 µg/ml OAG (△); 0.5 µg/ml PS, 0.05 µg/ml OAG (▲).

concentrations of PS and in the presence of saturating concentrations of OAG. The stimulation produced by lipid A was higher in the absence of PS which suggests that this lipid may replace the phospholipid requirement of PKC. However, even at saturating concentrations of PS the stimulatory effect of lipid A was present, indicating that other sites of action exist for this effector. Similar results were obtained when the complete LPS molecule was used. The maximal stimulation of PKC by lipid A was obtained at 10 µg/ml. Higher concentrations of lipid A resulted in inhibition of the enzyme activity (figure 1B). When the enzyme was assayed at subsaturating concentrations of OAG and PS a dose-dependent inhibition by lipid A was observed. The activation of PKC by lipid A was also present when highly purified enzyme from rat brain or liver was used (Table 1). Accordingly, activation by lipid A and lipid X of PKC from macrophages has been reported (7).

To study the ability of lipid A to promote translocation of PKC from cytosol to membranes, an *in vitro* test was used. Partially purified PKC from B cells (DE52 chromatography) was incubated with washed lymphocyte

Table 1. Effect of lipid A on PKC from rat brain and liver. The enzyme was purified by protamine-agarose chromatography. The incubation volume was 170 µl. Results are means ± S.E.M. for 3 different experiments.

Addition	PKC activity, units/µg of protein	
	Brain	Liver
0.2 µg OAG	1930 ± 180	73 ± 12
0.2 µg OAG, 5 µg lipid A	3980 ± 120	145 ± 32
0.2 µg OAG, 2 µg PS	4420 ± 315	210 ± 21
0.2 µg OAG, 2 µg PS, 5 µg lipid A	7580 ± 342	397 ± 27

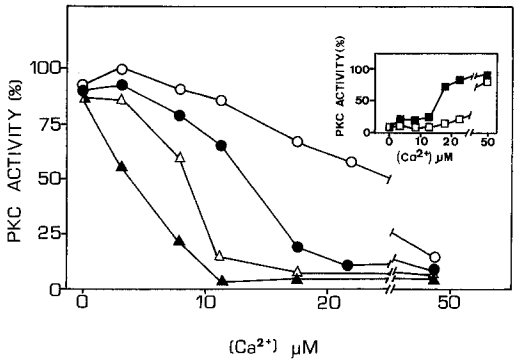
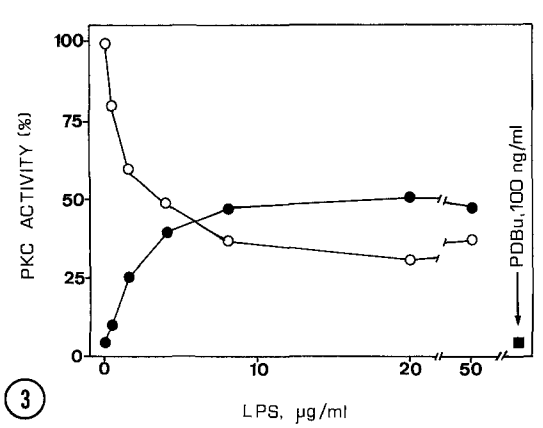


Figure 2. Effect of lipid A and effectors on PKC distribution. The enzyme purified from B cells was incubated for 10 min in the presence of membranes and effectors of the enzyme. After centrifugation to sediment the membranes, the soluble activity was measured. Filled symbols are given for the presence of 10 μg/ml of lipid A. Control (○,●); 0.5 μg/ml PS (△, ▲). Inset, extracted membrane-bound activity corresponding to controls incubated in the absence (□) or presence of lipid A (■).

membranes in the presence of different effectors that have been recognized as modulators of translocation (12). After centrifuging at 140,000xg for 20 min PKC activity was measured in the supernatant (soluble fraction) and in the pellet (membrane-bound fraction) after extraction with Nonidet P-40 and sonication as described. As shown in figure 2 when the enzyme was incubated in the presence of increasing concentrations of Ca^{2+}, lipid A markedly stimulated the translocation of PKC from the soluble fraction to the membranes. This effect was enhanced by the presence of PS or OAG (data not shown) in the incubation medium. In all cases when lipid A was present a shift in the sensibility to Ca^{2+}-induced translocation was observed.

Soluble enzyme and membranes were also incubated in the presence of LPS. As shown in figure 3 the maximal translocation induced by LPS was only 70% of the translocation promoted by phorbol dibutyrate, an analogue of diacylglycerol (13), or lipid A. The non-specific binding of LPS to biological membranes may explain the incomplete translocation obtained with LPS. The half-maximal decrease of PKC activity from the cytosol, parallel to the increase in the membrane-bound fraction obtained with 2 μg/ml of LPS, demonstrated that both changes in PKC activity are related. The time-course of the translocation induced by lipid A is shown in figure 4 and the process takes only a few minutes to be completed. The inhibition of Ca^{2+}-dependent proteases by leupeptin (50 μg/ml) and PMSF (1 mM) was necessary to obtain quantitative results in the translocation assay.



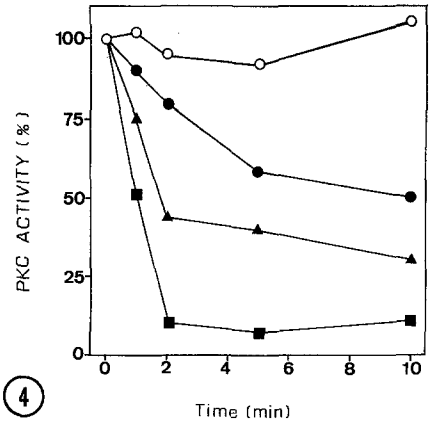


Figure 3. Effect of LPS on PKC distribution in a cell-free system. PKC was purified from B lymphocytes and incubated for 10 min in the presence of membranes. After centrifugation at 145,000xg for 20 min, the supernatant and the pellet were analysed for PKC activity. Soluble activity (○), membrane-bound activity (●). Soluble activity in the presence of 100 ng/ml PDBu (■). The specific activity of PKC was 70 units/µg of protein.

Figure 4. Time-course of the distribution of PKC after exposure to lipid A. The soluble activity was measured after incubation of the enzyme with different concentrations of lipid A and in the presence of B cell membranes. Lipid A was used at 0 (○), 2 (●), 5 (▲) and 20 µg/ml (■) respectively. The specific activity of PKC was 70 units/µg of protein.

DISCUSSION

Activation of PKC seems to be one of the cellular responses elicited after exposure of B cells to the policlonal mitogen LPS. Thus, highly specific inhibitors of this enzyme block the biological action of LPS on B cells (14).

According to our results lipid A generated by splitting the LPS molecule either from *E. coli* or from *S. minnesota*, activated PKC isolated from B cells, rat brain or liver, by substituting, at least in part, for the requirements of phospholipids. Moreover, lipid A promoted the translocation of PKC from soluble to membrane-bound fractions. The same pattern of activation and translocation of PKC was obtained with the complete molecule of LPS, although with quantitative differences. Taken together, these results clearly showed that LPS derived moieties were able to activate and translocate PKC regardless of the generation of phosphatidylinositol derived messengers, diacylglycerol and Ca^{2+}, which produce activation of PKC. This is specially important in the case of B lymphocyte stimulation by LPS where no changes in phosphoinositide turnover has been observed.

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