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ANTIGEN-INDUCED PHOSPHOLIPASE D ACTIVATION IN RAT MAST CELLS IS INDEPENDENT OF PROTEIN KINASE C

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Summary: In the present study, we investigated the involvement of protein kinase C (PKC) in antigen (Ag, DNP-*Ascaris suum*)-induced phospholipase D (PLD) activation of rat peritoneal mast cells. Phorbor myristate acetate (PMA) as well as Ag activated PLD as inferred by phosphatidylethanol (PEt) production. PKC inhibitors, staurosporine and H-7, however, failed to suppress PMA-stimulated PLD activation, suggesting that PLD activation by PMA is independent of PKC. By contrast, Ag-stimulated PLD activity was significantly reduced by staurosporine and slightly by H-7. Surprisingly, the inhibitors inhibited Ag-stimulated phospholipase C (PLC), correlated to the inhibition of PLD. These observations lead us to conclude that in Ag-stimulated mast cells 1,2-diacylglycerol (DG) formed by PLC directly or indirectly stimulates PLD, independently of PKC.

Cross-linking by Ag of IgE receptors in mast cells induces secretion of chemical mediators, such as histamine, leukotrienes and prostaglandins, through biochemical reactions that are initiated by second messengers [1]. To generate second messengers, phospholipid metabolism, especially phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown by PLC and cleavage of arachidonyl phospholipids by phospholipase A_2, plays crucial roles.

Of recent great interest is phosphatidylcholine (PC) breakdown by PLD [2]. PLD hydrolyzes mainly PC, yielding phosphatidic acid (PA) and

Abbreviations: Ag, antigen; DNP-As, 2,4-dinitrophenyl-conjugated *Ascaris suum* extract; PMA, phorbor 12-myristate 13-acetate; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; PC, phosphatidylcholine; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PEt, phosphatidylethanol; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DG, 1,2-diacylglycerol; H-7, 1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine.

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choline, and it also catalyzes transphosphatidylation by which PEt is produced in the presence of ethanol [3].

It has been demonstrated that PLD is activated by various agonists in many mammalian cells. Although the detailed mechanism of PLD activation is not established, several reports suggest the involvement of PKC. Several experimental observations indicate that a PKC activator PMA activates PLD. However, the effects of PKC inhibitors on PLD activation are controversial. Recently, Gruchalla et al. [4] have demonstrated that Ag stimulation of rat mast cells activates PLD. However, it was not described whether PMA stimulates PLD and PKC is involved in Ag activation of PLD in mast cells. In the present study, we investigated whether PKC is involved in PLD activation by PMA and Ag in rat peritoneal mast cells.

MATERIALS AND METHODS

Preparation of antigen and antiserum

The crude extract of nematode worm *Ascaris suum* was prepared by the method of Strejan and Campbell [5]. The extract was conjugated with DNP-sulphonic acid by the method of Eisen et al. [6]. To obtain the anti-[DNP-*Ascaris suum* (DNP-As)] sera, rats were immunized by the method of Tada and Okamura [7].

Isolation of rat peritoneal mast cells

Mast cells were obtained from the peritoneal cavity of Wistar rats and purified by a Percoll-gradient method of Wells and Mann [8]. The buffer used is consisting of 137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg gelatin/ml, 5 units heparin/ml, 10 mM Hepes (pH 7.4). Mast cell preparations were about 95 % pure as assessed by Toluidine Blue staining. More than 95 % of cells were viable as judged by Trypan Blue uptake.

Lipid Metabolism

Purified mast cells were incubated with [^{3}H]myristic acid (2 μCi/10^6 cells per 0.1 ml) in the buffer containing 0.1 % BSA for 3 h at 30°C. The mast cells were sensitized with anti DNP-As serum by adding for the last 1 h of labeling period. The mast cells ($2.5x10^5$ cells/assay) were stimulated with 1 μg Ag /ml in the presence of 30 μg of phosphatidylserine (PS)/ml or 10 nM of PMA at 37°C. After stimulation, lipids were extracted by the method of Bligh and Dyer [9]. The individual phospholipids were separated by two-dimensional TLC and the radioactivity of each lipid was determined in a liquid scintillation counter described previously [10].

Measurement of inositol 1,4,5-trisphosphate (IP_3)

Mast cells were sensitized with anti-serum for 1 h at 30°C. Cells ($6x10^6$ cells/0.1 ml/assay) were pretreated with 0.1 μM staurosporine or 500 μM H-7, and then stimulated with 1 μg Ag/ml at 37°C for 15 s at which time IP_3 formation reached the maximum. The reactions were terminated by addition of 20 μl of 20% perchloric acid. IP_3 formed was measured by Amersham IP_3 assay kit.

Materials

[9, 10-^{3}H(N)]Myristic acid (specific activity 39.3 Ci/mmol), and [^{32}P]orthophosphate (carrier-free) were purchased from New England

Nuclear. Staurosporine and H-7 were obtained from Kyowa Medix Co. LTD (Tokyo, Japan) and Seikagaku Kogyo Co. LTD (Tokyo, Japan), respectively. PS was obtained from Serdary Research Laboratories (London, Ontario, Canada). Percoll and Silica-gel H60 plates were products of Pharmacia and Merck, respectively. PEt was prepared by the method of Yang et al. [3].

RESULTS AND DISCUSSION

When rat mast cells were incubated with [^{3}H]myristic acid at 30°C for 3 h, the label was predominantly incorporated into PC (73.8 % of total radioactivity in lipids, 6.7×10^5 d.p.m./10^6 cells). The radioactivity distributed in phosphatidylethanolamine and other phospholipids, such as PS, PI, and PA, were 3.8 % and less than 1 %, respectively (data not shown). Upon stimulation of the labeled cells with Ag (1 μg/ml), [^{3}H]PA formation was increased in a time-dependent manner, reaching a maximum (4.8 % of total radioactivity) at 60 s, and then decreased gradually by prolonged incubation (Fig. 1). Of all [^{3}H]phospholipids, only [^{3}H]PC was decreased with a concurrent [^{3}H]PA formation (data not shown). In the presence of 0.4% ethanol, [^{3}H]PA formation upon Ag stimulation was diminished. Instead, [^{3}H]PEt was produced with the similar time course to that of [^{3}H]PA formation in the absence of ethanol. Thus we confirmed that Ag stimulates PLD activity in rat peritoneal mast cells.

It has been reported that PLD in many cells is stimulated by a PKC activator PMA [11, 12]. In rat mast cells, stimulation of PMA for 10 min also stimulated [^{3}H]PA and [^{3}H]PEt formation in the absence and the presence of 0.4% ethanol, respectively, in a dose-dependent manner, reaching a plateau at 10 nM PMA (data not shown). Thus, PMA as well as Ag stimulated PLD activity in rat peritoneal mast cells. Another PKC activator, 1-oleoyl 2-acetylglycerol, also activated PLD (data not shown).

Although these observations appear to indicate the involvement of PKC in PLD activation, this is not conclusive because the effects of PKC inhibitors on the activation are controversial among several reports. To investigate whether PLD activation is regulated by PKC, effects of PKC inhibitors, staurosporine [13] and H-7 [14], on PLD-induced [^{3}H]PA formation by PMA and Ag stimulation were examined. When mast cells were pretreated with 0.1 μM staurosporine and 500 μM H-7, protein phosphorylation enhanced by 10 nM PMA was inhibited completely by the former and moderately by the latter (data not shown). Under these conditions, the inhibitors failed to inhibit PMA-induced [^{3}H]PA formation (Fig. 2). These results suggest that PLD activation by PMA in rat peritoneal mast cells is independent of PKC. Taken together with the report that PMA and DG stimulate PLD partly through PKC activation and also directly in HL-60 granulocytes [15], we postulate that DG formed by Ag-stimulated PLC or PMA stimulates PLD directly or indirectly, independent of PKC

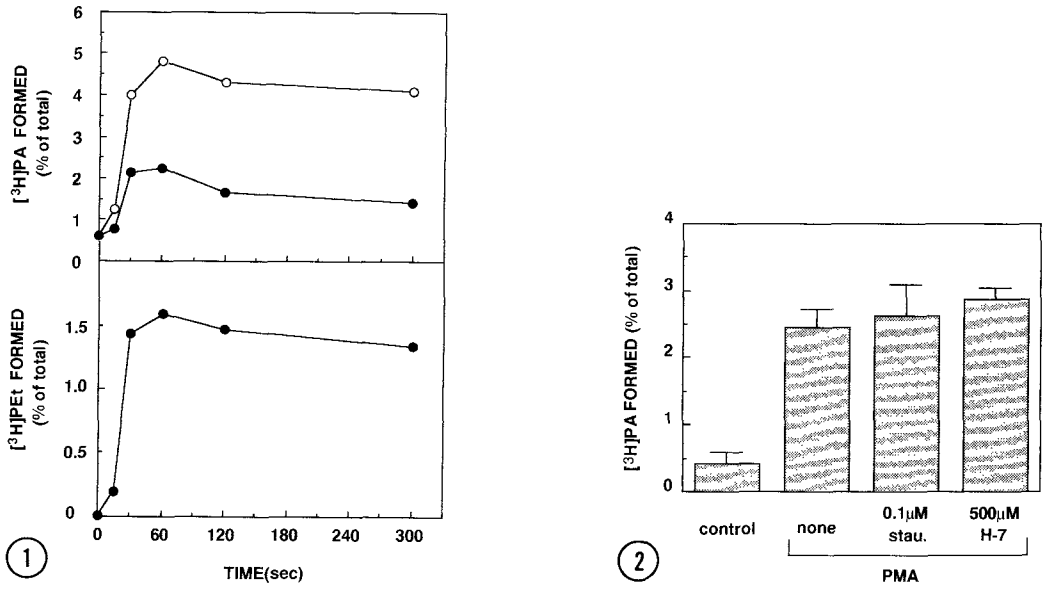


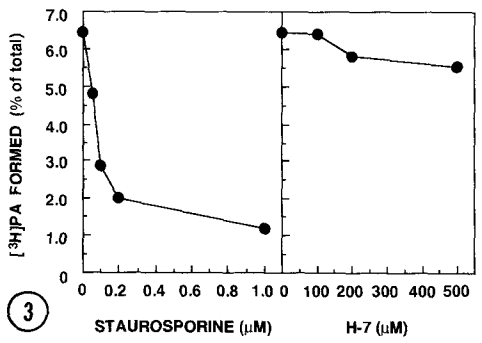
Fig. 1. Ag stimulation of [^{3}H]PA and [^{3}H]PEt formation in rat peritoneal mast cells. The labeled and sensitized mast cells were stimulated with 1 μg DNP-As/ml at 37°C for the indicated times in the presence (●) or absence (○) of 0.4% ethanol. Each value is the mean of duplicate determinations from one of two experiments with similar results.

Fig. 2. Effects of PKC inhibitors on PMA-stimulated [^{3}H]PA formation. The labeled mast cells were preincubated with 0.1 μM staurosporine (stau.) or 500 μM H-7 for 6 min, and then stimulated with 10 nM PMA for 10 min at 37°C. Each value is the mean ± SD of three experiments performed in duplicate.

activation, in rat mast cells. However, Ag-induced [^{3}H]PA formation is inhibited by staurosporine in a dose-dependent manner, with the inhibition being almost complete at 1 μM of staurosporine (Fig. 3). [^{3}H]PA formation induced by Ag was slightly but definitively inhibited by H-7 at concentrations more than 200 μM (Fig. 3). These results, taken together with the idea that PMA or DG stimulates PLD independently of PKC, led us to speculate that staurosporine and H-7 prevent PLC activation induced by Ag stimulation, resulting in the suppression of DG formation, thereby attenuating PLD activation.

To test this assumption, we have examined whether staurosporine and H-7 inhibit Ag-induced PLC activation. IP_3, product by PLC, can be quantitatively measured by Amersham IP_3 assay kit. As shown in Fig. 4, IP_3 formation stimulated by Ag was considerably inhibited by the pretreatment with staurosporine and slightly diminished in H-7-pretreated cells. Thus, PKC inhibitors suppressed the Ag-induced PLC activation, and the suppression seems to be correlated to the decrease of [^{3}H]PA formation.

From these observations we hypothesized PLD activation mechanism by Ag in rat peritoneal mast cells and the inhibition mechanism by PKC



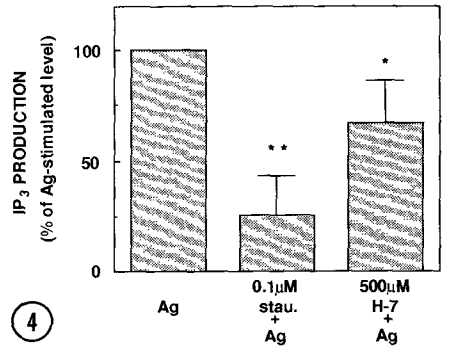


Fig. 3. Effects of PKC inhibitors on Ag-stimulated [^{3}H]PA formation. Mast cells were labeled and sensitized as described in Materials and Methods. The cells were preincubated with staurosporine or H-7 at the indicated concentrations for 6 min at 37°C, and then stimulated with 1 μg DNP-As/ml for 60 s at 37°C. Each value is the mean of duplicate determinations from one of two experiments with similar results.

Fig. 4. Effects of PKC inhibitors on Ag-stimulated IP_3 formation. The sensitized mast cells were preincubated with 0.1 μM staurosporine or 500 μM H-7 for 6 min, and stimulated with 1 μg DNP-As/ml for 15 s at 37°C. Results are shown as percentage of Ag-stimulated IP_3 level. Each value is the mean ± SD of three experiments performed in duplicate. The asterisks indicate significant differences (* $p<0.05$, ** $p<0.01$).

inhibitors as follows: 1) Ag induces DG formation through PLC activation, and, in turn, the formed DG activates directly or indirectly PLD, independently of PKC, and 2) staurosporine and H-7 inhibit not only PKC activation but also Ag-stimulated PLC activation, resulting in the attenuation of DG formation, thereby inhibiting Ag-stimulated PLD activation. The mechanism for staurosporine inhibition of Ag-stimulated PLC activation is unknown. To prove the proposed concept of PKC-independent activation mechanism of PLD in rat mast cells, further experiments with isolated membranes will be required.

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