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Phosphatidylcholine-specific phospholipase D-derived 1,2-diacylglycerol does not initiate protein kinase C activation in the RBL 2H3 mast-cell line

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We examined the role of phosphatidylcholine-specific phospholipase D (PC-PLD) in the IgE-dependent activation of protein kinase C (PKC) in RBL 2H3 cells (a model for mast-cell function). Cells were sensitized with mouse monoclonal anti-trinitrophenol (TNP) IgE (0.5 μg/ml) and were then triggered with an optimal concentration (10 ng/ml) of TNP–ovalbumin conjugate (TNP–OVA). This resulted in an immediate biphasic increase in the production of 1,2-diacylglycerol (DAG) and activation of PKC. The initial increase in DAG production reached a peak within 30 s, and the second phase reached a plateau within 5 min after stimulation. TNP-OVA-induced PC-PLD activation followed the initial increase in DAG formation in response to IgE-receptor cross-bridging, but coincided with the second peak. Phosphatidic acid (PA), derived from the PC-PLD pathway, is metabolized to DAG by the action of PA phosphohydrolase (PAPase). Propranolol (0.3 mM), which inhibits PAPase, blocked the IgE-dependent increase in DAG, activation of PKC, and subsequently degranulation. The PKC inhibitor staurosporine (0.1 μM) inhibited the second, but not first, peak of DAG accumulation, reversed PKC translocation after 10 min and inhibited subsequent mediator release. Taken together, these results demonstrate that PC-PLD does not initiate, but may play a latent role in, IgE-dependent DAG production, PKC activation and mediator release from RBL 2H3 cells.

INTRODUCTION

Mast cells and the related basophils play a pivotal role in the pathogenesis of allergic reactions such as asthma. These reactions are a consequence of the release of granule-associated mediators, such as histamine and 5-hydroxytryptamine, mediators synthesized *de novo*, such as platelet-activating factor and arachidonic acid metabolites, and cytokines, such as interleukins 3–5 (Plaut *et al.*, 1989), tumour necrosis factor (Ohno *et al.*, 1990) and granulocyte/macrophage colony-stimulating factor (Wodnar-Filipowicz *et al.*, 1989), when these cells are activated. In general, the initiating event in degranulation is the cross-bridging of receptor-bound IgE molecules by a specific antigen, although other endogenous compounds, such as adenosine (Church *et al.*, 1983; Lohse *et al.*, 1987; Gilfillan *et al.*, 1990*b*), C5a (Hugli & Muller-Eberhard, 1978), substance P (Fewtrell *et al.*, 1982) and cytokines (Schleimer *et al.*, 1981; Subramanian & Bray, 1987; Morita *et al.*, 1987; Haak-Frendscho *et al.*, 1988), may also play a physiological role in mast-cell secretion. Studies have suggested that the events linking cross-bridging of IgE receptors to degranulation involve G-protein-activated phospholipase C (PLC) with subsequent phosphoinositide (PtdInsP_2) hydrolysis to yield inositol trisphosphate and diacylglycerol (DAG) (Cunha-Melo *et al.*, 1987; Kennerly, 1987). These two 'second messengers' transduce the signal by liberating Ca^{2+} from intracellular storage sites (Beaven & Cunha-Melo, 1988) and activating protein kinase C (PKC) (White *et al.*, 1985; Nagao *et al.*, 1987), respectively.

PKC activation appears to be a pivotal step in IgE-dependent mediator release from mast cells and basophils (Ali *et al.*, 1989; Gilfillan *et al.*, 1990*a*; Warner & MacGlashan, 1990). The extent to which PtdInsP_2 hydrolysis influences DAG levels, and thus activation of PKC, in activated mast cells has been questioned. In this context, when mass is taken into consideration, the amount of DAG formed on cell activation is in excess of that which can be accounted for by hydrolysis of PtdInsP_2 alone (Kennerly, 1987; Exton, 1990). In addition, the fatty acid composition of DAG formed on cross-bridging of IgE receptors shows greater similarity to that of phosphatidylcholine (PC) than to PtdIns (Kennerly, 1987). It has been suggested that breakdown of PC in mast cells catalysed by PC-specific phospholipase D (PC-PLD) may help to explain the apparent disparity between DAG mass and PtdInsP_2 hydrolysis. PC-PLD primarily cleaves PC to yield phosphatidic acid (PA), which can be subsequently dephosphorylated by PA phosphohydrolase (PAPase) to form DAG. PC-PLD is activated in response to antigen in a number of mast-cell models (Kennerly, 1990; Lin *et al.*, 1991*a*), and it has been reported that this pathway may account for most of the DAG produced on cell activation (Exton, 1990). We previously reported that PC-PLD activation appeared to be required for the release of both granule-associated mediators and those synthesized *de novo* in RBL 2H3 cells (Lin *et al.*, 1991*a*), a model for mast-cell function. The precise role that PC-PLD plays in PKC activation, and thus in mediator release, remains to be established, however. The aim of this study was therefore to determine the role of PC-PLD in the regulation of DAG levels and PKC activation in a rat mast-cell (RBL 2H3) line. Preliminary results from this study have been previously published in abstract form (Lin & Gilfillan, 1990, 1991).

METHODS AND MATERIALS

Secretion experiments

RBL 2H3 cells were maintained as described previously (Gilfillan *et al.*, 1990*a*). Cells used for secretion experiments were grown to confluency and then dislodged from the plastic with a

Abbreviations used: Anti-TNP IgE, mouse monoclonal anti-trinitrophenol IgE; TNP–OVA, trinitrophenol–ovalbumin; PC, phosphatidylcholine; PA, phosphatidic acid; PEtOH, phosphatidylethanol; DAG, 1,2-diacylglycerol; AA, arachidonic acid; MA, myristic acid; PC-PLD, PC-specific phospholipase D; PLC, phospholipase C; PAPase, PA phosphohydrolase; PKC, protein kinase C; MEM, minimal essential medium; GPI, glycosylphosphatidylinositol.

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trypsin (0.05%)/EDTA (0.02%) solution (Gibco, Grand Island, NY, U.S.A.). After rinsing, the cells were plated at a density of 1×10^6 cells/35 mm-diameter tissue-culture dish (Falcon; Becton Dickinson, Lincoln Park, NJ, U.S.A.) in Eagle's minimal essential medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal-bovine serum (Hyclone, Logan, UT, U.S.A.), 100 units of penicillin/ml and 100 μg of streptomycin/ml (Gibco) containing anti-trinitrophenol (TNP) IgE (0.5 μg/ml) for 17–20 h and cultured in a humidified atmosphere of air/CO_2 (19:1) at 37 °C. The cells were than rinsed thoroughly with Hepes buffer (137 mM-NaCl, 2.7 mM-KCl, 0.4 mM-Na_2HPO_4, 5.6 mM-glucose, 10 mM-Hepes, 1.8 mM-$CaCl_2$ and 1.3 mM-$MgSO_4$, pH 7.4), followed by addition of 1 ml of this buffer to each dish. After a 5 min preincubation, the cells were triggered with TNP-ovalbumin (TNP–OVA; 0–10 ng/ml). At pre-determined times, the incubation medium was carefully removed and centrifuged at 200 *g* for 5 min to remove any free-floating cells. Distilled water was added to the cells remaining attached to the dish, and the cellular material was removed by freeze–thawing and then scraping with a rubber policeman.

The supernatant and lysate were then appropriately diluted with distilled water and then mixed with an equal volume of $HClO_4$ (0.8%). The histamine content of the $HClO_4$ supernatant was measured by an automated spectrofluorimetric assay (Siraganian, 1974).

Cell labelling

To label the lipid fractions of the RBL 2H3 cells with [^{3}H]myristic acid (MA), the cells were initially plated and sensitized with anti-TNP IgE as described under 'Secretion experiments'. After incubation for 17–20 h, the cells were rinsed twice and then MEM (1 ml) containing [^{3}H]MA (1 μCi/ml) was added. After incubation for 1 h at 37 °C, the cells were again rinsed, the MEM was replaced with fresh medium, and the cells incubated for another 1 h. At this point, the cells were rinsed again and the medium was replaced with 1 ml of the Hepes buffer described above. The cells were then activated with TNP–OVA as described under 'Secretion experiments'. The experiments were terminated by rapidly removing the Hepes buffer and then adding 1 ml of methanol. The cells were scraped from the plate, and then the methanol/cell extract from each tissue-culture dish was placed in separate extraction tubes. Lipids were extracted and separated as described below.

Quantification of DAG mass

Cellular lipids were extracted as described above and solubilized in 20 μl of octyl β-D-glucoside/cardiolipin solution (7.5% octyl β-D-glucoside/5 mM-cardiolipin in 1 mM- diethylenetriaminepenta-acetic acid) by sonication in a bath sonicator for 15 s. DAG was assayed as described by Preiss *et al.* (1986). DAG kinase (Lipidex, Westfield, NJ, U.S.A.) (10 μl), 20 mM-dithiothreitol (10 μl), sample (20 μl) and reaction buffer (100 mM-NaCl/25 mM-$MgCl_2$/2 mM-EGTA, pH 6.6) (10 μl) were mixed and the reaction was initiated by addition of 10 mM-[γ-^{32}P]ATP (100000–500000 c.p.m./nmol; 10 μl). After incubation for 30 min at 25 °C, the reaction was terminated by adding chloroform/methanol (1:2, v/v) (3 ml), followed by chloroform (1 ml) and 1 M-NaCl (1 ml). The lower phase was washed once with 1% $HClO_4$ (2 ml), and dried under N_2. The samples then were separated by t.l.c. as described under 'Lipid extraction and separation'. The amount of DAG present in the sample was calculated from the sample volumes and the specific radioactivity of ATP employed (Preiss *et al.*, 1986).

Lipid extraction and separation

Lipids were extracted from the cell/methanol or aqueous fractions by the procedure described by Bligh & Dyer (1959). The lipid-containing chloroform phase was removed and dried down under N_2. The resulting residues were re-dissolved in chloroform and the individual lipids were separated by the following t.l.c. techniques.

(1) PA labelled with ^{32}P (from the DAG mass assays). The residues were spotted 1 cm from the base of Si250 silica-gel t.l.c. plates (Baker, Phillipsburg, NJ, U.S.A.). The plates were then developed in the solvent chloroform/methanol/acetic acid (13:3:1, by vol.). Standard PA was included on each lane and the relative position of PA was determined by exposure to iodine vapour. After the iodine had evaporated, the areas corresponding to PA standard were scraped into scintillation vials and the radioactivity was measured directly on the gel in Aquasol (New England Nuclear, Boston, MA, U.S.A.) containing 3% water.

(2) DAG labelled with [^{3}H]MA. The residues were spotted on Si250 silica-gel t.l.c. plates as described above and the lipids were separated by a slight adaptation of the technique described by Cochran *et al.* (1987). Essentially, the plates were developed to a height of 8 cm in chloroform/methanol/acetic acid/water (65:45:1:4, by vol.), dried, then redeveloped to the top of the plate in hexane/diethyl ether/formic acid (45:30:2, by vol.). After location by exposure to iodine vapour, the areas corresponding to standard DAG were scraped into scintillation vials and the radioactivity was determined as described above.

(3) Phosphatidylethanol (PEtOH) labelled with [^{3}H]MA. The residues were spotted on Si250 silica-gel t.l.c. plates as described above and the plates were developed in the organic phase of ethyl acetate/iso-octane/acetic acid/water (11:5:2:10, by vol.) as described by Anthes *et al.* (1989). After location by exposure to iodine vapour, the areas corresponding to standard PEtOH were scraped into scintillation vials and the radioactivity determined as described above.

Sample preparation for PKC assay

RBL 2H3 cells were plated at a density of 8×10^6/100 mm-diameter tissue-culture dish (Falcon) with anti-TNP IgE (0.5 μg/ml) for 17–20 h. After rinsing, the cells were triggered with TNP–OVA (10 ng/ml). At pre-determined times, the Hepes buffer was removed and cells were washed twice with buffer A (20 mM-Tris/HCl, 2 mM-EGTA, 1 mM-EDTA, 0.1 μg of phenylmethanesulphonyl fluoride/ml and 100 μg of leupeptin/ml, pH 7.5) at 4 °C. The cells were scraped into 2 ml of ice-cold buffer A and the cell suspension was disrupted by sonication for 20 s (Heat Systems, New York, NY, U.S.A.) at 4 °C. The homogenates were centrifuged at 100000 *g* for 1 h (4 °C) to separate cytosol and membrane fractions (White & Metzger, 1988). The membrane pellet was then resuspended in buffer A containing 0.153% Triton X-100 (1 ml). The cytosol and the solubilized membrane fractions were applied to DE52 DEAE-cellulose columns (1 ml packed volume) previously equilibrated with buffer B (20 mM-Tris/HCl, 2 mM-EGTA, 1 mM-EDTA, 0.1 mg of phenylmethanesulphonyl fluoride/ml, pH 7.5). After washing with 2×3 ml of buffer B, PKC was eluted with 2 ml of buffer B containing 0.1 M-NaCl. Leupeptin (25 μg/ml final concn.) was added to the eluate before the PKC assay. For immunoblot analysis of PKC, the membrane pellet was resuspended in buffer A and then sonicated for 20 s. This buffer contained 0.6% CHAPS rather than Triton X-100 to allow concentration of the protein and subsequent electrophoresis. The homogenates were centrifuged at 100000 *g* for 45 min at 4 °C. The supernatants from the membrane fractions and the cytosolic fractions were concentrated with a micro-concentrator (Amicon, Beverly, MA, U.S.A.).

Immunoblot analysis of PKC

Translocation of PKC activity from cytosol to membrane was evaluated by immunoblot analysis. Membrane or cytosolic proteins were separated by SDS/PAGE (10% gels), then transferred to nitrocellulose membranes. Non-specific binding was blocked by incubating the membrane with 3% gelatin in TBS (20 mM-Tris/500 mM-NaCl, pH 7.5) for 60 min at 37 °C. The membranes were incubated with mouse monoclonal anti-(rat-PKC) antiserum (1:100 dilution) for 4 h at 37 °C and then washed with 0.05% Tween 20 in TBS. The antiserum used recognizes PKC α and β isoforms. After incubation for 60 min with alkaline phosphatase rabbit anti-mouse IgG2a (1:100 dilution), the immunoreactive bands were made visible by the alkaline phosphatase reaction (Engvall & Perlmann, 1972).

Determination of PKC activity

PKC activity in the cells was determined by an adaptation of the protocol described by White & Metzger (1988). Samples (100 μl) of the solubilized membrane or cytosolic fractions were added to a reaction mixture consisting of 20 mM-potassium glutamate with potassium Pipes buffer (20 mM, pH 7.5), containing 10 mM-magnesium acetate, 2 mM-$CaCl_2$, 100 μg of histone III-S/ml, 50 μg of leupeptin/ml, 1 mg of phosphatidylserine/ml, 0.1375 mg of 1,2-diolein/ml and 100 μM-[^{32}P]ATP (1200 c.p.m./pmol). The reaction was initiated by addition of 25 μl of [^{32}P]ATP into the mixture and was allowed to proceed for 5 min at 30 °C. The histone phosphorylation was stopped by adding 25% trichloroacetic acid (1 ml). The entire reaction mixture was then filtered through a 0.45 μm-pore Millipore filter and washed with 5% trichloroacetic acid (3 × 2 ml). The filter paper was then counted for ^{32}P radioactivity. PKC activity was defined as the difference between ^{32}P incorporated in the presence and absence of phosphatidylserine and 1,2-diolein, and was expressed as pmol of ^{32}P incorporated into histone/min per 10^7 cells.

Statistics and data handling

All data were normalized to cell number. Unless otherwise indicated, the results are presented as means ± S.E.M. and, where appropriate, were analysed statistically by Student's two-tailed *t* test for paired samples.

Materials

RBL 2H3 cells were obtained from Dr. Hydar Ali (Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, U.S.A.) and mouse anti-TNP IgE and TNP–OVA were obtained from Dr. John Hakimi, Department of Immunopharmacology, Hoffmann–La Roche, Nutley, NJ, U.S.A. [^{32}P]ATP and [^{3}H]MA were purchased from New England Nuclear (Boston, MA, U.S.A.). Mouse monoclonal anti-(rat PKC) antiserum was obtained from Amersham (Arlington Heights, IL, U.S.A.). Alkaline phosphatase rabbit anti-mouse IgG2a was from Zymed (San Francisco, CA, U.S.A.). Other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

RESULTS

Time course of IgE-dependent PKC translocation in RBL 2H3 cells

PKC translocation in RBL 2H3 cells was initially assessed by immunoblot analysis and subsequently by measuring the activity of the membrane-associated PKC. In cells sensitized with anti-TNP IgE (0.5 μg/ml), TNP–OVA (10 ng/ml) produced a rapid increase in membrane-associated PKC (Fig. 1). Although this was associated with a decrease in cytosolic PKC (results not shown), the decrease in this fraction was slight, owing to the much higher mass of PKC in the cytosolic fraction compared with the membrane fraction of the unstimulated RBL 2H3 cells. To assess accurately the kinetics of translocation, the activity of the membrane-associated PKC was measured, by histone III-S phosphorylation in the presence of phosphatidylserine and 1,2-diolein, over the duration of the experiment (Fig. 2). As described above, there was a rapid and immediate increase in membrane-associated PKC activity from a pre-stimulus level of 0.46 ± 0.09 to 1.12 ± 0.16 pmol/min per 10^7 cells after 5 min. The membrane-associated PKC activity then remained elevated for the duration of the experiment (30 min).

Kinetics of IgE-dependent DAG production and histamine release in RBL 2H3 cells

The kinetics of IgE-dependent PKC translocation, described above, was compared with the relative time courses of IgE-

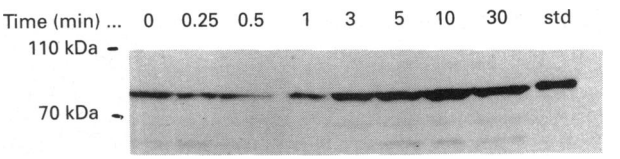

Fig. 1. Translocation of PKC in RBL 2H3 cells in response to TNP–OVA

A typical example (n = 3) is shown of the membrane PKC of control and treated RBL 2H3 cells; std, PKC standard from rat brain. Cells were sensitized and then triggered with TNP–OVA (10 ng/ml). The experiments were then terminated at the indicated times, and membrane and cytosolic fractions (results not shown) of the cells were prepared as described in the Methods and materials section. The proteins were then separated by SDS/PAGE, and immunoblot analysis was conducted as described in the Methods and materials section.

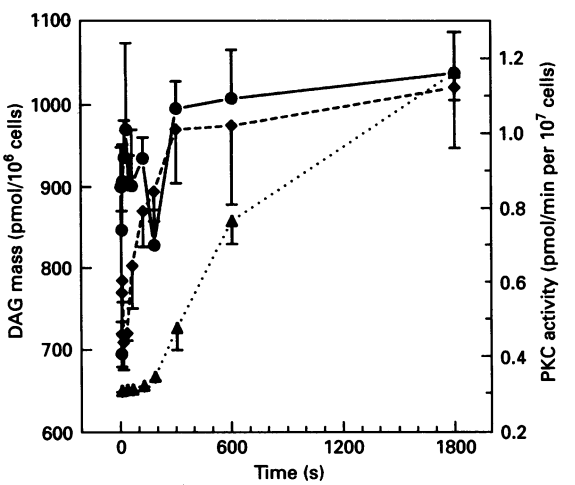

Fig. 2. Time course of IgE-dependent DAG production, PKC activation and release of histamine in RBL 2H3 cells

●, DAG mass; ◆, membrane-associated PKC activity; ▲, histamine release, range 10–500 ng/10^6 cells. The results are means ± S.E.M. of 4–5 experiments. A representative result from the DAG mass experiments has been previously published (Lin *et al.*, 1991*a*). The cells were sensitized with anti-TNP IgE (0.5 μg/ml) before triggering with TNP–OVA (10 ng/ml). For histamine-release studies, the experiments were terminated at the indicated times and the medium was assayed for histamine content as described in the Methods and materials section. DAG mass was assayed by the techniques described in the Methods and materials section. The PKC experiments were conducted by a similar protocol to that described in the legend to Fig. 1; however, activity was measured by the technique described in the Methods and materials section.

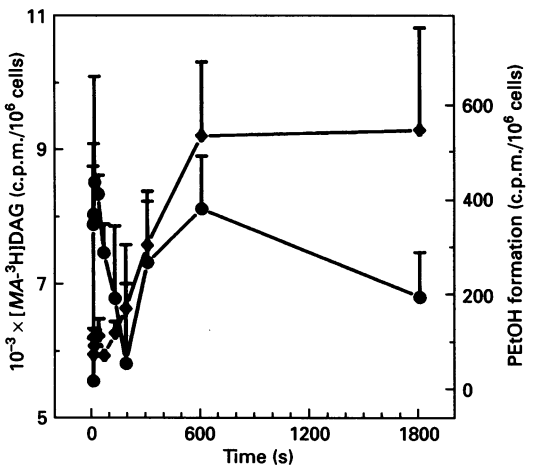

Fig. 3. Time course of IgE-dependent [^{3}H]DAG production (●) and PC-PLD activity (◆) in [^{3}H]MA-labelled RBL 2H3 cells

The results are the means ± S.E.M. of 4–5 experiments. The cells were sensitized with anti-TNP IgE (0.5 μg/ml), then labelled with [^{3}H]MA. In experiments in which PC-PLD was assayed, the cells were exposed to ethanol (0.5%) for 5 min before addition of TNP–OVA (10 ng/ml). At pre-determined times, the experiments were terminated by rapidly removing the incubation medium and then adding methanol to the cells. [^{3}H]PEtOH and [^{3}H]DAG formation was then determined as described in the Methods and materials section.

dependent DAG production, PC-PLD activation and mediator release in RBL 2H3 cells. When the cells were triggered under experimental conditions identical with those described above, there was a rapid biphasic increase in DAG formation (Fig. 2). The initial increase reached a maximum within 30 s after addition of TNP–OVA, and this was followed by a slower, more prolonged, increase in DAG mass which reached a plateau after 5 min and then remained elevated for the duration of the experiment (30 min). The initial increase in DAG production preceded the increase in membrane-associated PKC activation. The addition of TNP–OVA (10 ng/ml) to cells sensitized with anti-TNP IgE (0.5 μg/ml) induced the release of histamine and peptidoleukotrienes (results not shown) after a lag phase of approx. 3 min (Fig. 2). This increase in mediator release followed the initiation of PKC translocation and both phases of DAG production. After the lag phase, there was a rapid increase in the release of both categories of mediators, and maximal release was observed 10 min after addition of TNP–OVA.

Comparison of the relative kinetics of IgE-dependent activation of PC-PLD and DAG production

In our previous study (Lin *et al.*, 1991*a*), we were unable to detect the initial increase in DAG production when monitored by labelling the cells with [^{14}C]arachidonic acid, although the secondary DAG increase was observed. This probably reflected the fact that DAG produced during the initial phase does not contain AA. For subsequent studies described below, therefore, we wished to confirm that both DAG phases could be observed with other radiolabelled precursors. When [^{3}H]MA was used to label the cellular lipids, both phases of DAG production were observed (Fig. 3). The time course of these responses was also similar to that of DAG mass production. To monitor PC-PLD activation, we therefore utilized this label in our studies. To determine if both phases of DAG production, and hence PKC activation, are dependent on PC-PLD activation, we examined the time course of PC-PLD activation in response to TNP–OVA. PC-PLD catalyses a transphosphatidylation reaction which results in the formation of PEtOH when ethanol is included in the culture medium (Kobayashi & Kanfer, 1987). In cells labelled with [^{3}H]MA and then triggered with TNP–OVA (10 ng/ml) in the presence of 0.5% ethanol, there was a rapid increase in PEtOH formation (Fig. 3), which followed the initial phase of DAG production and activation of PKC, but preceded the second increase in DAG formation and histamine release. The time course was identical with that of the formation of PA and other indices of PC-PLD activation which we previously described in RBL 2H3 cells (Lin *et al.*, 1991*a*).

On a temporal basis, therefore, the above data indicate that PKC translocation is not dependent on PC-PLD activation. From our studies, however, it is evident that translocation of PKC is maintained throughout the duration of the experiments. This maintained translocation is dependent on the sustained elevation of DAG levels in the cells. Our previous studies (Lin *et al.*, 1991*a*) have demonstrated that the secondary phase of DAG production is PC-PLD-dependent. It is therefore possible that the maintenance of PKC translocation and subsequent histamine release is dependent on the secondary PC-PLD-dependent phase of DAG production. Below, we describe experiments to investigate this possibility.

Effect of staurosporine, propranolol and ethanol on the production of DAG, activation of PKC and release of histamine in response to TNP-OVA

To explore the possibility that PC-PLD may play a latent role in IgE-dependent PKC activation in RBL 2H3 cells, we have examined the effect of inhibiting the PC-PLD pathway on the production of DAG and activation of PKC. Propranolol blocks the conversion of PA into DAG by inhibiting PAPase (Koul & Hauser, 1987), and thus can be used to explore the role of DAG generated by the PC-PLD/PAPase pathway in cell function. In previous studies (Lin *et al.*, 1991*a*) we have fully characterized the concentration-dependency of the actions of propranolol, and on the basis of these studies we have conducted the experiments using an optimal concentration (300 μM). When added before TNP–OVA, propranolol inhibited the IgE-dependent increase in DAG production (Fig. 4). We examined the effects of propranolol and other inhibitory agents on PKC activation at 10 min, as this point was a time where the second phase of DAG production was maximal, and examining the responses at 10 min rather than at earlier time points allows time for any DAG produced during the first phase to be metabolized. Using the conditions described above, propranolol decreased the translocation of PKC (Fig. 5).

As described above, addition of ethanol to the cells results in the preferential synthesis of PEtOH at the expense of DAG production, when the reaction is catalysed by PC-PLD. Again, we have fully characterized the concentration-dependency of these effects (Lin *et al.*, 1991*a*), and on the basis of these studies we have selected a concentration of ethanol that results in the maximal formation of PEtOH, with no effect on other parameters such as Ca^{2+} flux. We previously demonstrated that addition of ethanol to mast cells resulted in inhibition of DAG production and histamine release in RBL 2H3 cells when triggered by TNP–OVA. When ethanol was added before TNP–OVA, PKC translocation was reversed when assayed during the secondary PC-PLD-dependent secondary phase of DAG production (Fig. 5).

To explore the possibility that PC-PLD activation and the latent DAG production may be dependent on the initial increase in PKC, we examined the effect of the PKC inhibitor staurosporine on IgE-dependent DAG production. When added before TNP-OVA, staurosporine inhibited the second, but not the first, phase of DAG production in response to TNP–OVA (Fig. 4),

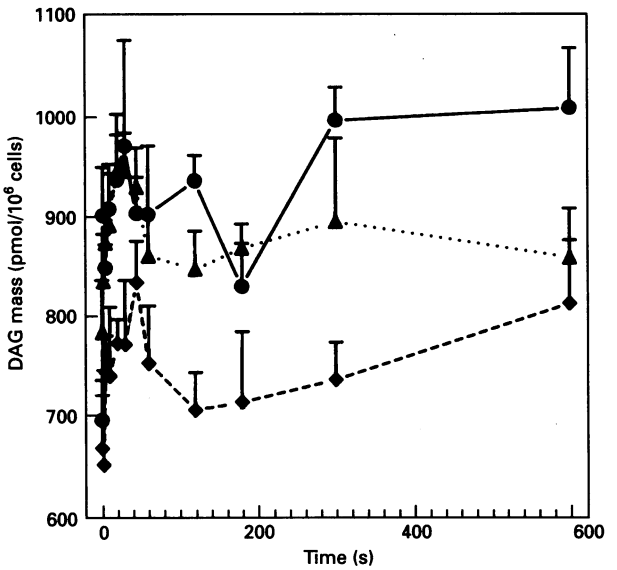

Fig. 4. Effect of propranolol and staurosporine on the production of DAG mass in response to TNP–OVA

●, TNP–OVA (10 ng/ml) alone; ◆, TNP–OVA + propranolol (0.3 mM); ▲, TNP–OVA + staurosporine (0.1 μM). The results are means ± S.E.M. of 4 experiments. The agents were added to sensitized RBL 2H3 cells 5 min before addition of TNP–OVA, and the experiment was then conducted as described in the legend to Fig. 2.

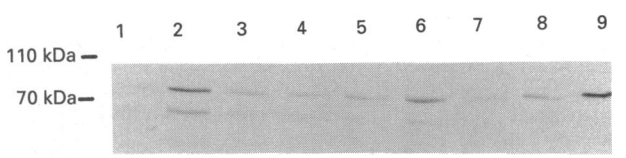

Fig. 5. Effect of staurosporine, propranolol and ethanol on PKC translocation in RBL cells triggered with TNP–OVA

The figure shows membrane-associated PKC. Cells were sensitized and then triggered with TNP–OVA (10 ng/ml) for 10 min. Inhibitors were added 5 min before TNP–OVA. The cytosolic and membrane fractions of the cells were prepared as described in the Methods and materials section. The proteins were then separated by SDS/PAGE (10% gel), and immunoblot analysis was conducted as described in the Methods and materials section. Lanes: 1, unstimulated cells; 2, TNP–OVA (10 ng/ml); 3, staurosporine (1 μM); 4, TNP–OVA + staurosporine (1 μM); 5, propranolol (0.3 mM); 6, TNP–OVA + propranolol (0.3 mM); 7, ethanol (1%, v/v); 8, TNP–OVA + ethanol (1%, v/v); 9, rat PKC.

and subsequently PKC translocation (Fig. 5). Staurosporine, propranolol and ethanol added alone, at the concentrations used in this study, had no effect on PKC translocation (Fig. 5).

Finally, propranolol (300 μM) inhibited IgE-dependent histamine (control 408.2 ± 91.3, treated 11.2 ± 3.2 ng/10^6 cells; $n = 4$, $P < 0.05$) and peptidoleukotriene (control 2693.4 ± 606.4, treated 10.1 ± 3.5 pg/10^6 cells; $n = 4$, $P < 0.05$) release. Similarly, ethanol (1%) also significantly inhibited the IgE-dependent release of histamine (control 409.3 ± 83.9, treated 47.1 ± 6.9 ng/10^6 cells; $n = 7$, $P < 0.005$) and leukotriene (control 1506.7 ± 309.9, treated 9.7 ± 3.4 pg/10^6 cells; $n = 5$, $P < 0.01$). We have previously demonstrated that staurosporine also inhibits histamine and leukotriene C_4 release (Gilfillan *et al.*, 1990*b*).

Taken together, therefore, these data suggest that PC-PLD-dependent DAG formation does not initiate PKC activation in RBL 2H3 cells. Although the reagents used in this study are not necessarily specific, the results suggest that PC-PLD may play a latent role in the maintenance of DAG levels and PKC activation. These results also point to the possibility that the activity of PKC may be autoregulated by the secondary PKC-dependent DAG production.

DISCUSSION

Cross-bridging of IgE receptors on the surface of RBL 2H3 cells results in the rapid translocation of PKC from the cytosol to the membrane (White & Metzger, 1988). Other stimuli for mediator release from mast cells, including phorbol 12-myristate 13-acetate, concanavalin A and compound 48/80, also produce translocation of PKC in rat peritoneal mast cells (Nagao *et al.*, 1987). Activation of membrane-associated PKC appears to be an important regulatory step in IgE-dependent mediator release from mast cells and basophils. In this context, down-regulation of PKC by long-term exposure to phorbol 12-myristate 13-acetate results in inhibition of IgE-dependent degranulation of RBL 2H3 cells (Ali *et al.*, 1989). Additionally, IgE-dependent mediator release from mast cells (White & Zembryki, 1989), basophils (Warner & MacGlashan, 1990) and RBL 2H3 cells (Gilfillan *et al.*, 1990*b*) is inhibited by preincubation with PKC inhibitors such as staurosporine, K252a and H7.

PKC activation in cells is directly regulated by the relative levels of DAG (Nishizuka, 1984). In response to antigen, there is a rapid elevation of DAG levels in mast cells (Kennerly, 1990) and RBL 2H3 cells (Lin *et al.*, 1991*a*). Studies have suggested that PC-PLD-catalysed PC breakdown may be a more important source of DAG than phosphoinositides in activated mast cells (Kennerly, 1990). Activation of PC-PLD may therefore be an important regulatory step for both degranulation and arachidonic acid release. The exact role that PC-PLD plays in DAG production and PKC activation is, however, unknown. The purpose of this study was therefore to investigate the role of PC-PLD in the regulation of DAG levels, and thus in PKC activation and mediator release in RBL 2H3 cells.

When RBL 2H3 cells were stimulated with TNP–OVA, we observed a rapid increase in membrane-associated PKC which reached a maximum within 5 min and then remained elevated for the 30 min duration of the experiment. These results were observed both by immunoblot analysis of the protein and by direct measurement of PKC activity. PKC activation in RBL 2H3 cells can therefore be considered as an initiating stage during the first 0–5 min, followed by a maintenance phase over the subsequent duration of the experiment. The elevated membrane PKC activity is similar to that previously reported by White & Metzger (1988); however, the time course of the response in their study was markedly different. In the study of White & Metzger (1988), maximal membrane-associated PKC activity was observed between 15 and 30 s, after which PKC activity essentially returned to pre-stimulus levels. The reasons for the differences between the results of this and our study are unclear; however, White & Metzger (1988) suggested that the transient activation of PKC observed in their study may reflect a return of DAG levels to pre-stimulus values. It is clear in our study, however, that these levels are maintained for the duration of the experiment.

In response to TNP–OVA, we observed a rapid biphasic increase in DAG mass. The initial increase in DAG production coincided with the activation of PKC. The resting DAG levels were approx. 700 pmol/10^6 cells, which agrees with our previous study (Lin *et al.*, 1991*a*), but is substantially higher than that reported in rat mast cells (Kennerly, 1990). The net increase, however, is similar in magnitude to that reported in rat mast cells

(Kennerly, 1990). As the initial increase in DAG was not observed in that study, we could not discount the possibility that the initial increase in DAG production coincided with PC-PLD activation, as monitored by the formation of PEtOH. In our present study, we have been able to monitor the biphasic increase in DAG production, using [^{3}H]MA to label the cells. A similar biphasic increase in [^{3}H]MA-labelled DAG in antigen-triggered RBL 2H3 cells has recently been reported by Nakashima *et al.* (1991). We have therefore been able to utilize this approach to determine the exact temporal relationship between PC-PLD activation and DAG production.

When cells labelled with [^{3}H]MA were incubated in ethanol and triggered with TNP–OVA, there was a time-dependent increase in PC-PLD activation, as assessed by [^{3}H]PEtOH formation. This increase in [^{3}H]PEtOH formation followed the initial increase in DAG production and PKC activation, but preceded the initiation of the second phase of DAG production. The time course of [^{3}H]PEtOH formation, however, was identical with the indices of PC-PLD activation previously described. These results demonstrate that the initial increase in DAG production and subsequent activation of membrane-associated PKC activity in triggered RBL 2H3 cells is not dependent on PC-PLD activation. Recent studies on the signal-transduction events associated with the interleukin 2 receptor have indicated that DAG formation may occur by the hydrolysis of MA-rich glycosylphosphatidylinositol (GPI) molecules (Merida *et al.*, 1990; Eardley & Koshland, 1991). Preliminary data from our laboratory have suggested a similar mechanism contributing to the initial IgE-dependent phase of DAG production in RBL 2H3 cells, although in RBL 2H3 cells PA appears to be the initial product (Lin *et al.*, 1991*b*).

As described above, we have previously determined that the secondary phase of IgE-mediated DAG formation is dependent on PC-PLD activation. We therefore examined the possibility that the PC-PLD-dependent DAG production may act as a maintenance signal for PKC activation. To explore this possibility, we examined the effects of two agents previously demonstrated to inhibit the PC-PLD/PAPase pathway on DAG formation and PKC activation in RBL 2H3 cells. Propranolol blocks the conversion of PA into DAG by inhibiting PAPase (Koul & Hauser, 1987), and ethanol results in the preferential synthesis of PEtOH at the expense of DAG production, when the reaction is catalysed by PC-PLD. When propranolol was added to RBL 2H3 cells, the formation of DAG and activation of PKC in response to TNP–OVA was inhibited. Surprisingly, the initial phase of DAG production was also inhibited by propranolol. This was associated with an increase in PA levels (P. Lin & A. M. Gilfillan, unpublished work), suggesting that the initial phase of DAG is also largely derived from PA. As described in the previous paragraph, preliminary data from our laboratory suggests that this is largely derived by the action of GPI-specific PLD on GPI molecules (Lin *et al.*, 1991*b*). Using 0.5% ethanol, which we previously demonstrated inhibited the DAG production via the PLD/PAPase pathway, we also observed inhibition of IgE-dependent membrane-associated PKC activity. Although it is a possibility that these agents may be working via a non-specific mechanism, on the basis of our previous and the above results, it is possible that maintenance of antigen-stimulated DAG levels and PKC activity may be dependent on IgE-dependent PC-PLD activation.

Finally, evidence indicates in a number of systems that PC-PLD activation is regulated by PKC (Billah & Anthes, 1990). Therefore, to examine the possibility that the second DAG peak was dependent on PKC-mediated activation of PC-PLD, we examined the effect of the PKC inhibitor staurosporine on the above parameters. Staurosporine was observed to inhibit the secondary, but not the initial, phase of DAG production and to reverse PKC translocation. As the mechanism of action of staurosporine does not involve inhibition of PKC translocation in itself, but depends on interaction with the ATP-binding site, this demonstrates that the maintenance phase of PKC translocation is dependent on the initial activation of PKC. As we (Lin & Gilfillan, 1991*a*) and, recently, others (Nakashima *et al.*, 1991) have demonstrated, PC-PLD activity in RBL 2H3 cells appears to be regulated by PKC activation, it is likely that PC-PLD activation may be an integral link between both phases of DAG production.

In conclusion, the above results demonstrate that PC-PLD activation does not initiate the IgE-dependent increase in intracellular DAG levels and PKC activation in RBL 2H3 cells. PC-PLD activation, however, may play a role in the maintenance of PKC activation by producing a secondary increase in DAG levels. It is likely that the initial activation of PKC in response to antigen in turn regulates PC-PLD activity to maintain PKC activation. The secondary increase in DAG levels, and hence maintained PKC activity, appears to be obligatory for subsequent mediator release.

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