J. Biochem. 120, 616-623 (1996)

Importance of the Phospholipase D-Initiated Sequential Pathway for Arachidonic Acid Release and Prostaglandin D_2 Generation by Rat Peritoneal Mast Cells[1]

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Received for publication, May 9, 1996

The association of prostaglandin D_2 (PGD_2) production as well as arachidonic acid release with the phospholipase D (PLD)-linked mechanism was studied in rat peritoneal mast cells. Stimulation of mast cells with cross-linking of the high-affinity Fc receptor for IgE caused increases in the release of arachidonic acid and PGD_2, which are suppressed almost completely by ethanol or RHC 80267, a diacylglycerol lipase inhibitor. Ethanol did not influence inositol phosphate release in response to an antigen. An increase in diacylglycerol, that is inhibited by propranolol, was observed, with a peak within 1 min. Antigen stimulation induced little production of lysophosphatidylcholine, while ionomycin as a control markedly induced the production. However, the phospholipase A_2 (PLA_2) activity in the cytosol of antigen-stimulated cells increased to the level in ionomycin-stimulated cells. The addition of the ADP-ribosylation factor-containing fraction prepared from bovine brain, that is known to specifically activate PLD, to permeabilized mast cells in the presence of GTPγS, apparently increased arachidonic acid and PGD_2 release, but not in the presence of ethanol. Furthermore, arachidonic acid release by an antigen was enhanced by melittin, that activates PLA_2, but PGD_2 production was not. These results suggest that antigen-stimulated PGD_2 production as well as arachidonic acid release are strongly associated with the sequential PLD-linked pathway.

Key words: arachidonic acid, mast cell, phospholipase A_2, phospholipase D, prostaglandin D_2.

Receptor-stimulated arachidonic acid liberation from membrane phospholipids is the initial step in the generation of eicosanoids in a variety of cells and tissues. It is generally accepted that one of the enzymes responsible for the liberation is the cytosolic form of phospholipase A_2 (PLA_2), which has a molecular mass of 85 kDa and preferentially hydrolyzes phospholipids with arachidonic acid at the *sn-2* position (*1-3*). Type II (secretory) PLA_2, however, that has a molecular mass of 14 kDa and is secreted extracellularly from cellular granules, is also suggested to be responsible for arachidonic acid liberation in some cells, although the enzyme exhibits no specificity for phospholipids with arachidonic acid at the *sn-2* position (*1-3*).

Previous studies demonstrated detectable activity of diacylglycerol (DAG) lipase in human platelets, which participates in the release of arachidonic acid on thrombin stimulation (*4*). Therefore, it has been considered that DAG is formed through the hydrolytic action of phospholipase C on inositol phospholipids, that are enriched in arachidonic acid, and that the sequential actions of phospholipase C and DAG lipase comprise the major pathway for the liberation of arachidonic acid by platelets (*4, 5*) or mast cells (*6*). However, the pathway has been shown to represent the source of only a minor amount of the total arachidonic acid released by platelets (*7-9*), although in mast cells the mechanism responsible for arachidonic acid release remains unclear.

Recently, evidence has accumulated that arachidonic acid liberation and subsequent eicosanoid production are associated with the DAG lipase action on DAG, which is formed from phosphatidylcholine through the actions of phospholipase D (PLD) and then phosphatidate phosphohydrolase (PAPase) upon stimulation (*10-12*). This was indicated by the finding that upon stimulation in the presence of ethanol, eicosanoid formation and arachidonic acid liberation decrease in parallel with an increase in phosphatidylethanol (PEt) due to the PLD-catalyzed transphosphatidylation reaction. Recent studies in our laboratory demonstrated that about half the arachidonic acid released and almost all the prostaglandin D_2 (PGD_2) formed arise through the sequential pathway of PLD-PAPase-DAG lipase, the activation of which is dependent on the intracellular Ca^{2+} concentration in Ca^{2+} ionophore-stimu-

[1] This work was supported by a Grant-in-Aid for Scientific Research (No. 06672213) from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: APMSF, *p*-(amidinophenyl)methanesulfonyl fluoride; ARF, ADP-ribosylation factor; DAG, diacylglycerol; DNP-BSA, dinitrophenol-bovine serum albumin conjugate; FceRI, high-affinity Fc receptor for IgE; GTPγS, guanosine 5'-[γ-thio]triphosphate; lysoPC, lysophosphatidylcholine; lysoPS, lysophosphatidylserine; PAPase, phosphatidate phosphohydrolase; PEt, phosphatidylethanol; PGD_2, prostaglandin D_2; PLA_2, phospholipase A_2; PLD, phospholipase D.

lated rat peritoneal mast cells (*13, 14*). These observations suggest that DAG lipase activity, in addition to PLA_2 activity, also plays a physiological role in arachidonic acid liberation upon stimulation.

The present study was undertaken to further characterize the physiological roles of PLD activation followed by the sequential PAPase-DAG lipase pathway, in comparison with that of PLA_2, in arachidonic acid liberation and PGD_2 formation by rat peritoneal mast cells. The results obtained indicate that arachidonic acid liberation and PGD_2 formation after activation of the high-affinity Fc receptor for IgE (FcεRI) by cross-linking are fully dependent on the sequential PLD-PAPase-DAG lipase pathway.

MATERIALS AND METHODS

Materials—Guanosine 5′-[γ-thio]triphosphate (GTPγS) was obtained from Boehringer Mannheim GmbH (Germany). *p*-(Amidinophenyl)methanesulfonyl fluoride (APMSF), dithiothreitol, isonicotinic acid hydrazide, and propranolol were from Wako Pure Chemical Industries (Osaka), thymidine and NAD were from Kohjin (Tokyo), ionomycin and cholera toxin were from Calbiochem (La Jolla, CA), melittin, BSA (fraction V), and ADP-ribose were from Sigma (St. Louis, MO), 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane (RHC 80267) was from Biomol Research Laboratories (Plymouth Meeting, PA), leupeptin was from Peptide Institute (Osaka), heparin was from Novo Nordisk (Denmark), and lysophosphatidylserine (lysoPS) was from Avanti Polar Lipids (Alabaster, AL). Monoclonal anti-2,4-dinitrophenyl mouse immunoglobulin E (anti-DNP IgE) was from BioMakor (Israel). [^{3}H]Arachidonic acid (100 Ci/mmol), [^{3}H]choline chloride (79.2 Ci/mmol), 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine (57 mCi/mmol), and [α-^{32}P]NAD (800 Ci/mmol) were obtained from New England Nuclear (Boston, MA). The prostaglandin D_2 assay kit and myo-[2-^{3}H]inositol (18.3 Ci/mmol) were from Amersham (Buckinghamshire, UK). DNP-BSA, as an antigen, was prepared by conjugating an average of seven 2,4-dinitrophenyl groups with one molecule of BSA. Phosphatidylethanol (PEt) was prepared according to the method of Comfurius and Zwaal (*15*). Other reagents were obtained from commercial sources.

Isolation of Rat Peritoneal Mast Cells—Mast cells were harvested from the peritoneal cavities of Wistar rats of 8 weeks of age and purified essentially according to the method of Sullivan *et al.* (*16*). Briefly, male rats were decapitated, and then intraperitoneally injected with 25 ml of a medium consisting of 150 mM NaCl, 3.7 mM KCl, 3 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 6 mM glucose, 1 mg/ml BSA, 1 mg/ml gelatin, and 10 U/ml heparin (pH 6.8). The peritoneal exudate was collected by aspiration and centrifuged at 50 × *g* for 6 min at 4°C. The pellet obtained was suspended in the same medium, layered over a 31.5% bovine serum albumin/saline solution, and then centrifuged at 300 × *g* for 10 min at 4°C. The pellet was washed twice and then suspended in the same medium. The purity of mast cells obtained was more than 90%.

Prelabeling and Sensitization of Mast Cells—The purified mast cells (5×10^6 cells/ml) were incubated with [^{3}H]arachidonic acid (10 μCi/ml), [^{3}H]choline chloride (40 μCi/ml), or myo-[2-^{3}H]inositol (200 μCi/ml) in the presence or absence of anti-DNP IgE (1 μg/ml) at 37°C for 2 h. The cells were washed three times and then suspended in the medium without heparin.

Measurement of Lipid Metabolism—[^{3}H]Arachidonic acid- or [^{3}H]choline-labeled mast cells (5×10^5 cells/ml) were treated with various inhibitors in the presence of 1 mM $CaCl_2$ and 80 μM BW755C (3-amino-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline, a dual inhibitor of cyclooxygenase and lipoxygenase), and stimulated with DNP-BSA plus lysoPS, ionomycin or melittin at 37°C for appropriate times. The reaction was terminated by adding ice-cold chloroform/methanol/HCl (200 : 200 : 1, v/v/v). Lipids were extracted and separated by TLC on a Silica Gel G plate (Merck, Germany) with the following development systems: for the analysis of lysophosphatidylcholine (lyso-PC), chloroform/methanol/water (65 : 35 : 6, v/v/v); for the analysis of arachidonic acid, phosphatidic acid and phosphatidylethanol, the combination of chloroform/methanol/7 M NH_4OH (65 : 35 : 7.3, v/v/v) for the first dimension and chloroform/methanol/acetic acid/water (60 : 30 : 8 : 4, v/v/v/v) for the second dimension; and for the analysis of DAG, diethyl ether/petroleum ether/acetic acid (80 : 80 : 1.5, v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was determined by liquid scintillation counting.

For the determination of PGD_2 formation, unlabeled mast cells (5×10^5 cells/ml) were treated with various inhibitors in the presence of 1 mM $CaCl_2$, and then stimulated with DNP-BSA plus lysoPS, ionomycin or melittin at 37°C for appropriate times. The reaction was terminated by the addition of an ice-cold buffer consisting of 140 mM NaCl, 1 mM EGTA, and 10 mM Hepes (pH 7.4), and then the mixture was centrifuged at 600 × *g* for 10 min at 4°C. The amount of PGD_2 in the supernatant was measured by radioimmunoassaying with a commercial assay kit.

ADP-Ribosylation Factor (ARF)-Dependent Phospholipase D Activation—A fraction containing ARF was prepared from bovine brain by the method of Brown *et al.* (*17*). Briefly, bovine brain tissue (300 g) was homogenized in 600 ml of a buffer consisting of 1 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, 5 μM GDP, 100 μM APMSF, 100 μM leupeptin, and 20 mM Tris-HCl (pH 7.3). The lysate was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was centrifuged at 150,000 × *g* for 30 min at 4°C. The supernatant obtained was fractionated by adding a 1.5-fold volume of a saturated ammonium sulfate solution, followed by centrifugation at 12,000 × *g* for 10 min at 4°C. The pellet obtained was dialyzed at 4°C for 20 h against a dialysis buffer consisting of 1 mM EDTA, 5 mM $MgCl_2$, 5 μM GDP, and 20 mM Tris-HCl (pH 7.3). The dialyzed sample was loaded onto a DEAE-Sephacel column (Pharmacia Biotech, Sweden) preequilibrated with the dialysis buffer, and the absorbed proteins were eluted (0.3 ml/min) with the dialysis buffer supplemented with 100 mM NaCl. The activity of ARF in the fraction was measured as the auto-ADP-ribosylation of cholera toxin. Each fraction was incubated with 10 μM GTPγS, 1 mM EDTA, 10 mM thymidine, 1 mM ADP-ribose, 15 mM isonicotinic acid hydrazide, 2.5 mM $MgCl_2$, 2.5 μM NAD, 50 μCi/ml [α-^{32}P]NAD, and 50 mM potassium phosphate (pH 7.5) at 30°C for 1 h in the presence of 100 μg/ml cholera toxin, which had been preactivated with 50 mM dithiothreitol in 50 mM potassium phosphate (pH 7.5) at 30°C for 20 min.

The reaction was terminated by adding 2% SDS, 2% β-mercaptoethanol, 10% glycerol, 1 mM EDTA, 0.01% bromophenol blue, and 100 mM Tris-HCl (pH 6.8). Proteins in the mixture were solubilized at 100°C for 3 min, and then subjected to SDS-PAGE. The auto-ADP-ribosylation of cholera toxin was visualized by autoradiography. With this procedure, we confirmed that the fraction from the DEAE-Sephacel column contained a factor which caused the auto-ADP-ribosylation of cholera toxin in a GTPγS-dependent manner, indicating the existence of ARF in the fraction (data not shown).

For the determination of arachidonic acid release and PEt formation induced by the ARF-containing fraction, [^{3}H]-arachidonic acid-labeled mast cells (5×10^5 cells/ml) were added to a medium consisting of 137 mM NaCl, 2.7 mM KCl, 4 mM $MgCl_2$, 50 μM ATP, 50 μM $CaCl_2$, and 20 mM Hepes (pH 7.4) containing 10 μg/ml saponin and 80 μM BW755C, with or without 1% ethanol, 20 μM GTPγS, or the ARF-containing fraction. For the determination of PGD_2 formation, non-labeled mast cells (5×10^5 cells/ml) were added to the same medium except for the omission of BW755C. The cell suspension was further incubated for 20 min. After the reaction had been terminated, the amounts of [^{3}H]PEt, [^{3}H]arachidonic acid, and PGD_2 were determined as described above.

Determination of Inositol Phosphates—The determination of inositol phosphates released was performed by the method of Berridge *et al.* (*18*). Briefly, [^{3}H]inositol-labeled mast cells (5×10^5 cells/ml) were treated with or without 1% ethanol in the presence of 1 mM $CaCl_2$ and 10 mM LiCl, and then stimulated with DNP-BSA plus lysoPS at 37°C for appropriate times. The reaction was terminated by the addition of a one-fifth volume of 20% (v/v) $HClO_4$, and the mixture was allowed to stand for 30 min at 4°C and then centrifuged at 150,000×*g* for 30 min. The supernatant obtained was neutralized with 5 M KOH, and then centrifuged to remove the precipitated $KClO_4$. The supernatant was loaded onto a 2 ml AG1-X8 anion exchange column (200–400 mesh, formate form, Bio-Rad, Hercules, CA). Free inositol and phosphatidylinositol were sequentially washed out with ten bed volumes of H_2O and 5 mM sodium tetraborate/60 mM ammonium formate, respectively. Inositol phosphates were eluted with 10 ml of 100 mM formic acid/1 M ammonium formate, and the radioactivity in the eluate was determined by liquid scintillation counting.

Assay for PLA_2 Activity—Mast cells (5×10^5 cells/ml) were stimulated with DNP-BSA plus lysoPS or ionomycin at 37°C for 2 or 5 min, respectively. The reaction was terminated by the addition of a one-third volume of 400 mM Tris-HCl (pH 8.5) containing 11 mM EGTA. The mixture was centrifuged at 600×*g* for 5 min at 4°C. The pellet was washed, suspended in 100 mM Tris-HCl (pH 8.5) containing 2 mM EGTA, 2 mM Na_3VO_4, 100 μM APMSF, 100 μM leupeptin, and 20 mM β-glycerophosphate, and then lysed by N_2 cavitation. The lysate was centrifuged at 100,000×*g* for 30 min at 4°C. The supernatant (400 μg protein/ml) pretreated with 5 mM dithiothreitol was incubated with 3 μM 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine, 2 mM $CaCl_2$, 2 mM Na_3VO_4, and 100 mM Tris-HCl (pH 8.5) at 37°C for 30 min. [^{14}C]Arachidonic acid liberated was determined according to the method of Sundaram *et al.* (*19*). The protein concentration in the lysate was determined with a commercial assay kit, with BSA as a standard.

RESULTS

FcεRI-Dependent Responses of Mast Cells—Stimulation of [^{3}H]arachidonic acid-labeled mast cells with an antigen caused time-dependent increases in arachidonic acid release and PGD_2 formation, as shown in Fig. 1. The formation of PGD_2 seemed to reach a plateau at 2 min after antigen stimulation, while arachidonic acid release increased continuously up to 10 min.

PLD-Linked Release of Arachidonic Acid and PGD_2 upon Antigen-Stimulation—We showed in our previous study that about half the total arachidonic acid release and almost all the PGD_2 formation are associated with a PLD-linked mechanism upon stimulation with ionomycin, since they are inhibited by ethanol (*14*). In the present work, we examined the behavior of antigen-stimulated release of free arachidonic acid and PGD_2 as to ethanol. As shown in Fig. 2, ethanol induced concentration-dependent suppression of the release, that became complete at 0.5% ethanol. Under the conditions used, PEt, that is produced *via* the PLD-catalyzed transphosphatidylation reaction, increased and phosphatidic acid decreased with increasing concentrations of ethanol. These results indicate that the release of free arachidonic acid and PGD_2 upon antigen-stimulation occur *via* the PLD-linked mechanism.

DAG, an intermediate which is responsible for arachidonic acid release, is supposed to be generated by PLD followed by PAPase activation, while it is also produced through inositol phospholipid-specific phospholipase C activation. However, the results in Fig. 3 indicate that total inositol phosphates released from [^{3}H]inositol-labeled mast cells on stimulation with antigen were not affected by ethanol, supporting our idea that arachidonic acid release is associated with the PLD-linked pathway. Furthermore, we confirmed DAG generation *via* the PLD-PAPase-linked pathway. As shown in Fig. 4, stimulation of [^{3}H]arachido-

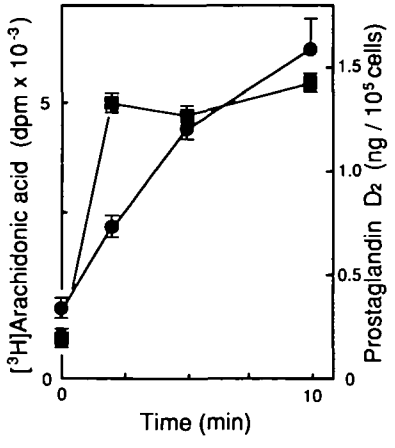

Fig. 1. **Arachidonic acid liberation and PGD_2 formation induced by DNP-BSA.** [^{3}H]Arachidonic acid-labeled or non-labeled mast cells were stimulated with 10 μg/ml DNP-BSA and 5 μg/ml lysoPS at 37°C for the indicated times. Arachidonic acid liberated (●) and PGD_2 formed (■) were determined as described under "MATERIALS AND METHODS." Each point represents the mean ± SEM for three separate experiments.

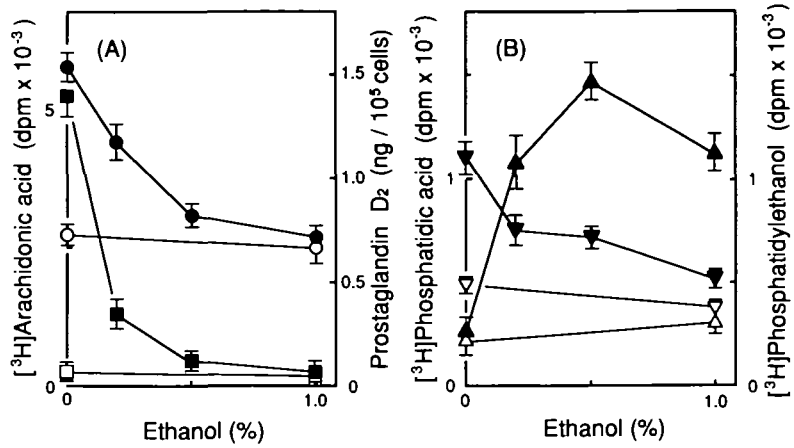

Fig. 2. **Effects of ethanol on DNP-BSA-stimulated responses.** [^{3}H]Arachidonic acid-labeled or non-labeled mast cells were treated with various concentrations of ethanol at 37°C for 10 min, and then stimulated with (closed symbols) or without (open symbols) 10 μg/ml DNP-BSA and 5 μg/ml lysoPS for 10 min. Arachidonic acid liberated (A, ●, ○), PGD_2 formed (A, ■, □), phosphatidic acid formed (B, ▼, ▽), and phosphatidylethanol formed (B, ▲, △) were determined as described under "MATERIALS AND METHODS." Each point represents the mean±SEM for three separate experiments.

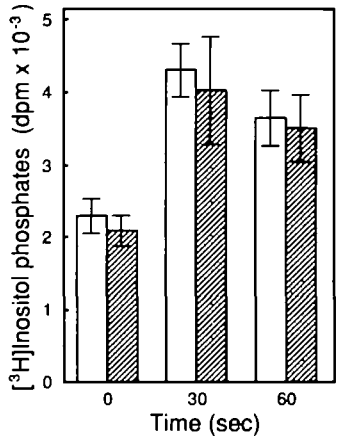

Fig. 3. **Effect of ethanol on DNP-BSA-stimulated inositol phosphate release.** [^{3}H]Inositol-labeled mast cells were treated with (hatched bars) or without (open bars) 1% ethanol at 37°C for 10 min, and then stimulated with 10 μg/ml DNP-BSA and 5 μg/ml lysoPS for the indicated times. Total inositol phosphates released were determined as described under "MATERIALS AND METHODS." Each bar represents the mean±SEM for three separate experiments.

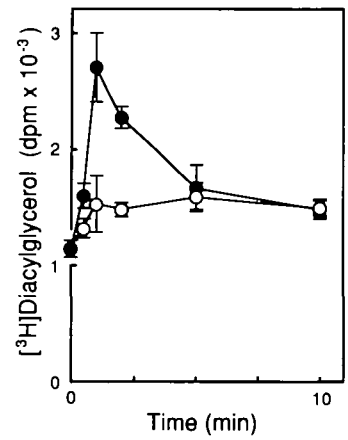

Fig. 4. **Effect of propranolol on DNP-BSA-stimulated DAG formation.** [^{3}H]Arachidonic acid-labeled mast cells were treated with (○) or without (●) 300 μM propranolol at 37°C for 15 min, and then stimulated with 10 μg/ml DNP-BSA and 5 μg/ml lysoPS for the indicated times. Diacylglycerol generated was determined as described under "MATERIALS AND METHODS." Each point represents the mean±SEM for three separate experiments.

nic acid-labeled mast cells with an antigen resulted in a time-dependent increase in DAG that peaked within 1 min and decreased after 5 min incubation. Under the conditions used, propranolol, a PAPase inhibitor, apparently suppressed the DAG increase.

To confirm the involvement of DAG lipase in the release of free arachidonic acid and PGD_2, the effect of RHC 80267, a DAG lipase inhibitor, was investigated. The results in Table I show that the inhibitor prevented the release in a dose-dependent manner. The inhibition of arachidonic acid release and PGD_2 synthesis was approximately 60 and 90% at 20 μM RHC 80267, respectively. On the other hand, RHC 80267 at 50 μM had no effect on antigen-stimulated PEt formation (data not shown) or melittin-stimulated arachidonic acid release (Table II). These data suggest that the sequential pathway of PLD-PAPase-DAG lipase is fully responsible for almost all the antigen-stimulated release of free arachidonic acid and PGD_2.

Antigen-Stimulated PLA_2 Activation—The results in Fig. 2 and Table I suggest a lack of involvement of PLA_2 activation in the release of free arachidonic acid and PGD_2 on stimulation with an antigen. Therefore, to examine this possibility we measured lysoPC generation as a result of PLA_2 activation in response to an antigen. However, only

TABLE I. **Effects of RHC 80267 on arachidonic acid liberation and PGD_2 formation.** [^{3}H]Arachidonic acid-labeled or non-labeled mast cells were treated with various concentrations of RHC 80267 at 37°C for 15 min, and then stimulated with 10 μg/ml DNP-BSA and 5 μg/ml lysoPS for 10 min. Arachidonic acid liberated and PGD_2 formed were determined as described under "MATERIALS AND METHODS." Data represent the means±SEM for three separate experiments.

Treatment	[^{3}H]Arachidonic acid (dpm)	Prostaglandin D_2 (pg/10^5 cells)
Control	2,756±198	59±11
DNP-BSA	5,780±242	1,392±100
+RHC 80267 (5 μM)	4,895±427	1,146±125
(10 μM)	4,789±336[a]	916±110[a]
(20 μM)	3,972±228[b]	157±21[b]
(50 μM)	3,225±240[b]	94±9[b]

[a] $p<0.05$ and [b] $p<0.02$, relative to DNP-BSA-stimulated cells.

TABLE II. **Effect of RHC 80267 or ethanol on melittin-induced arachidonic acid liberation.** [^{3}H]Arachidonic acid-labeled mast cells were treated with 50 μM RHC 80267 or 2% ethanol at 37°C for 15 or 10 min, respectively, and then stimulated with 4 μg/ml melittin at 37°C for 10 min. Arachidonic acid liberated was determined as described under "MATERIALS AND METHODS." Data represent the means±SEM for three separate experiments.

Stimulation	[^{3}H]Arachidonic acid (dpm)
Control	2,894±165
Melittin	5,258±543
Melittin+RHC 80267	4,981±699
Melittin+ethanol	5,652±319

little generation of lysoPC was induced by antigen stimulation, while time-dependent generation of the lipid was observed in response to ionomycin as a control agonist, as shown in Fig. 5. Since the antigen and ionomycin at the concentrations used in this experiment released nearly equal amounts of arachidonic acid (data not shown), this result indicates the insufficiency of PLA_2 activation or of the PLA_2 hydrolytic action on membrane phospholipids upon antigen stimulation. It is shown in Table III, however, that there was no difference in PLA_2 activity between the cells stimulated with the antigen and those with ionomycin. These results suggest the lack of susceptibility of PLA_2 to membrane phospholipids in spite of its increased activity in response to the antigen. Furthermore, ionomycin- or antigen-stimulated lysoPC formation is not affected by ethanol, indicating that the hydrolytic action of PLA_2 on membrane phospholipids is insensitive to ethanol (data not shown). Taken together, these results support the idea that the PLD-linked mechanism is involved in the ethanol-sensitive arachidonic acid release and PGD_2 generation.

PLD Activation Resulting in the Release of Arachidonic Acid and PGD_2—Recently, small G-proteins, such as the ARF or Rho family (*17, 20-23*), and other cytosolic factors (*24-26*) were found to be involved in the modulation of PLD activity. To confirm the strong relevance of the release of arachidonic acid and PGD_2 to PLD activation upon stimulation, we studied the effect of PLD activation induced by adding a fraction containing ARF prepared from bovine brain cytosol on their release from permeabilized mast cells. We confirmed that this fraction possesses adequate ARF activity (see "MATERIALS AND METHODS"). The results obtained are presented in Fig. 6. The addition of the ARF-containing fraction to mast cells in the presence of saponin and GTPγS caused dose-dependent increases in the release of arachidonic acid and PGD_2. In the presence of ethanol, the release of arachidonic acid was significantly suppressed and that of PGD_2 almost completely suppressed, while PEt formation increased remarkably. In the absence of GTPγS, these responses were evidently reduced. These results suggest the enhanced activation of PLD on the addition of the ARF-containing fraction, resulting in the release of arachidonic acid and PGD_2, and thus the existence of a strong correlation between the PLD-initiated pathway and their release.

Effects of Melittin on the Release of Free Arachidonic Acid and PGD_2—Melittin, a polypeptide obtained from honey bee venom, is known to cause the activation of PLA_2 and the release of arachidonic acid in rat peritoneal mast cells (*27*). We examined whether or not the activation of PLA_2 by melittin results in an increase in antigen-induced

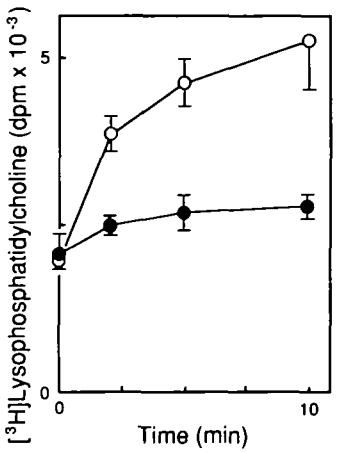

Fig. 5. **Lysophosphatidylcholine formation induced by DNP-BSA and ionomycin.** [^{3}H]Choline-labeled mast cells were stimulated with 10 μg/ml DNP-BSA plus 5 μg/ml lysoPS (●) or 200 nM ionomycin (○) at 37°C for the indicated times. Lysophosphatidylcholine formed was determined as described under "MATERIALS AND METHODS." Each point represents the mean±SEM for three separate experiments.

TABLE III. **Increase in cytosolic PLA_2 activity on stimulation.** Mast cells were stimulated with 10 μg/ml DNP-BSA plus 5 μg/ml lysoPS or 200 nM ionomycin at 37°C for 2 or 5 min, respectively. The cytosolic PLA_2 activity in the supernatant of the cells was determined as described under "MATERIALS AND METHODS." Data represent the means±SEM for three separate experiments.

Stimulation	PLA_2 activity (pmol/min/mg protein)
Control	7.3±0.2
DNP-BSA	10.6±1.5*
Ionomycin	9.9±0.6*

*$p<0.05$, relative to unstimulated cells (Control).

PGD_2 generation. As shown in Table IV, melittin induced a significant increase in arachidonic acid release, but very little generation of PGD_2. Under the experimental conditions, melittin caused the generation of a significant amount of lysoPC, but not the formation of PEt in the presence of ethanol (data not shown). Ethanol also did not affect the melittin-induced formation of arachidonic acid or lysoPC (data not shown). Furthermore, combined stimulation with melittin and the antigen had an additive effect on the arachidonic acid release, but it failed to increase the PGD_2 generation in comparison with the stimulation with the antigen alone. These results suggest that melittin can potentiate PLA_2 activation to release arachidonic acid but that the activation may not result in PGD_2 formation.

DISCUSSION

The present work provides evidence that PGD_2 is synthesized from the arachidonic acid which is released through the DAG lipase action on DAG, that is formed *via* the sequential actions of PLD and PAPase upon cross-linking of FcεRI in rat peritoneal mast cells. The participation of the PLA_2 pathway was not observed in arachidonic acid release or subsequent PGD_2 generation. These observations are explained by the following results: the increases in arachidonic acid release and PGD_2 formation were inhibited almost completely by ethanol (Fig. 2), and they were also

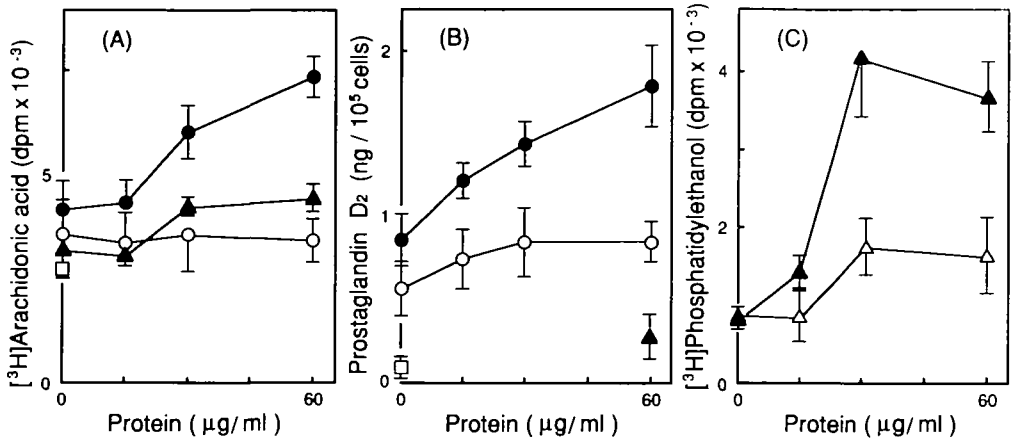

Fig. 6. **ARF-dependent stimulation of lipid metabolism.** [³H]-Arachidonic acid-labeled or non-labeled mast cells were treated with (triangles) or without (circles) 1% ethanol at 37°C for 2 min, and then incubated with (closed symbols) or without (open symbols) 20 μM GTPγS in the presence of 10 μg/ml saponin and various concentrations of the ARF-containing fraction for 20 min. Arachidonic acid liberated (A), PGD_2 formed (B), and phosphatidylethanol formed (C) were determined as described under "MATERIALS AND METHODS." Each point represents the mean±SEM for three separate experiments. □, intact cells.

TABLE IV. **Effects of combined stimulation with melittin and DNP-BSA on arachidonic acid liberation and PGD_2 formation.** [³H]Arachidonic acid-labeled or non-labeled mast cells were stimulated with or without 4 μg/ml melittin, 10 μg/ml DNP-BSA plus 5 μg/ml lysoPS, or both at 37°C for 10 min. Arachidonic acid liberated and PGD_2 formed were determined as described under "MATERIALS AND METHODS." Data represent the means±SEM for three separate experiments.

Treatment	[³H]Arachidonic acid (dpm)	Prostaglandin D_2 (pg/10⁵ cells)
Control	3,145±260	94±26
Melittin	7,182±89	178±10
DNP-BSA	5,572±283	1,155±67
Melittin+DNP-BSA	10,344±609	1,137±132

suppressed by RHC 80267, a specific inhibitor of DAG lipase (Table I). The suppression by ethanol of arachidonic acid release as well as PGD_2 formation was indicated to result from the decrease in PLD-catalyzed phosphatidic acid generation *via* the transphosphatidylation reaction of PLD. Ethanol did not exert an effect on the release of inositol phosphates on inositol phospholipid-specific phospholipase C activation (Fig. 3). In addition, antigen-stimulated DAG generation is prevented by propranolol, an inhibitor of PAPase (Fig. 4). These results support the idea that the receptor-stimulated PLD activation triggers the arachidonic acid release. Since RHC 80267 did not have an inhibitory effect on antigen-stimulated PEt formation (data not shown) or melittin-induced arachidonic acid release (Table II), and also ethanol did not affect ionomycin-stimulated lysoPC formation (data not shown) or melittin-induced arachidonic acid release (Table II), these compounds are suggested to exhibit their specific actions described above under the experimental conditions used here.

In the previous work we showed that upon stimulation with ionomycin about half the total amount of arachidonic acid released is linked to PLA_2 activation (*14*). The present results indicate that PLA_2 is not responsible for arachidonic acid release on antigen stimulation, since lysoPC is apparently generated upon ionomycin stimulation, whereas it is generated only slightly upon stimulation with an antigen, the concentration of which used can induce the same extent of arachidonic acid release as that by ionomycin (Fig. 5). However, the PLA_2 activity in the presence of dithiothreitol increased significantly in a mast cell lysate upon stimulation with an antigen as well as ionomycin (Table III). These results indicate that the PLA_2 is substantially activated but fails to hydrolyze membrane phospholipids upon antigen stimulation.

Although the mechanism underlying the insufficiency of the hydrolytic action of PLA_2 remains unknown, the PLA_2 detected in the cytosol above is considered to be of the cytosolic type, because the activity of the secretory type of PLA_2 is blocked by dithiothreitol (*28*). Receptor-stimulated activation of the cytosolic type of PLA_2 was reported to be regulated through its phosphorylation and Ca^{2+} dependent translocation from the cytosol to the membrane (*1-3*). In addition, we reported recently that in platelets a factor, such as G-protein, is further required for the hydrolytic action of cytosolic PLA_2 on membrane phospholipids after phosphorylation and translocation to membranes of the enzyme (*29*). Therefore, it is suggested by the results obtained that the failure of PLA_2 to release arachidonic acid upon antigen stimulation may be due to insufficient translocation of the enzyme from the cytosol to the membrane, or to inadequate contribution of the G-protein on membranes. Recently, interesting evidence was reported that cytosolic PLA_2 can be translocated from the cytosol to the nuclear envelope in response to a Ca^{2+} ionophore or an antigen in rat basophilic leukemia cells (*30*). Further studies are needed in this regard.

We presented here evidence that the specific PLD activation on the addition of the fraction containing ARF obtained from bovine brain cytosol resulted in marked generation of PGD_2 (Fig. 6). Under the conditions used, the PGD_2 formation and arachidonic acid release were suppressed by ethanol, with a concomitant increase in PEt formation. The existence of adequate ARF activity in the fraction was confirmed by the finding that the fraction clearly induced the auto-ADP-ribosylation of cholera toxin (see "MATERIALS AND METHODS"). Since ARF has been

reported to be a regulating factor for PLD activation (*17, 21*), these results clearly indicate that arachidonic acid released through the DAG lipase action on DAG, which is formed through the actions of PLD and then PAPase, is utilized effectively for PGD_2 synthesis.

On the other hand, melittin, that can activate PLA_2 (*27*), did not enhance PGD_2 formation stimulated by an antigen, nevertheless it caused apparent arachidonic acid release and also enhanced antigen-stimulated release (Table IV). Taking the above together, we propose the possibility that the arachidonic acid released through the sequential pathway of PLD-PAPase-DAG lipase may be an effective precursor for PGD_2 in mast cells, while that released by PLA_2 may not. This possibility well explains the present results obtained for antigen-stimulated mast cells.

Several investigators have reported that the PLA_2 responsible for eicosanoid production is of the cytosolic type in cultured mast cells (*31-33*). This suggestion was made on the basis of that cytosolic PLA_2 activity increased with the phosphorylation of the enzyme after cross-linking of FcεRI. However, whether or not the activated enzyme actually hydrolyzes membrane phospholipids has not been confirmed. The secretory type of PLA_2 has also been reported to contribute to the release of arachidonic acid and eicosanoids by mast cells (*34, 35*). The contribution of the secretory type of PLA_2, however, has been assumed from the results of an experiment in which the purified or recombinant enzyme was added extracellularly. Hence, such an experimental result may not reflect the effect of intact secretory PLA_2.

The mechanism by which the PLA_2 pathway is not responsible for PGD_2 synthesis could not be elucidated in the present work. It has been reported that antigen stimulation of cultured mouse mast cells leads to the mobilization of at least two kinds of arachidonic acid, one is the free acid released extracellularly and the other is that released intracellularly, which is utilized as a precursor for leukotriene (*36*). Therefore, it is considered that the release of arachidonic acid into the intracellular fluid may be linked to the sequential PLD pathway. On the other hand, it was reported recently that agonist-stimulated production of prostanoids is linked to the increased activity of an enzyme, such as cyclooxygenase, in cultured mouse mast cells (*37*) or synovial cells (*38*). These observations lead us to assume that PLD-linked enhancement of the activities of enzymes involved in the metabolism from arachidonic acid to PGD_2 may occur, although regulation by PLD of enzymes such as cyclooxygenase and PGD_2 synthetase is unknown. However, our results showing that combined stimulation with melittin and an antigen did not enhance the antigen-stimulated PGD_2 production, in spite of the additional supply of free arachidonic acid by melittin, may not support the possibility. Further studies are needed to determine the mechanism.

In conclusion, the present work has shown that antigen-stimulated arachidonic acid release and PGD_2 formation are fully dependent on the activation of the sequential actions of PLD-PAPase-DAG lipase in rat peritoneal mast cells. This was confirmed by the experimental finding that specific activation of PLD induced by the ARF-containing fraction resulted in apparent PGD_2 formation as well as arachidonic acid release. The fact that melittin-induced activation of PLA_2 did not result in the enhancement of antigen-stimulated PGD_2 formation may support this idea. These results suggest that the PLD-initiating pathway contributes to eicosanoid production in rat peritoneal mast cells.

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