A major role for phospholipase A₂ in antigen-induced arachidonic acid release in rat mast cells

Kouji YAMADA,* Yukio OKANO,* Kiyoshi MIURA† and Yoshinori NOZAWA*

*Department of Biochemistry and †Third Department of Internal Medicine, Gifu University School of Medicine, Tsukasamachi-40, Gifu, Japan

Cross-linking of IgE receptors by antigen stimulation leads to histamine release and arachidonic acid release in rat peritoneal mast cells. Investigators have reported a diverse distribution of [³H]arachidonate that is dependent on labelling conditions. Mast cells from rat peritoneal cavity were labelled with [³H]arachidonic acid for different periods of time at either 30 or 37 °C. Optimum labelling was found to be after 4 h incubation with [³H]arachidonate at 30 °C, as judged by cell viability (Trypan Blue uptake), responsiveness (histamine release) and distribution of radioactivity. Alterations in ³H-radioactivity distribution in mast cells labelled to equilibrium were examined on stimulation with antigen (2,4-dinitrophenyl-conjugated *Ascaris suum* extract). The results indicated that [³H]arachidonic acid was lost mainly from phosphatidylcholine and, to a lesser extent, from phosphatidylinositol. A transient appearance of radiolabelled phosphatidic acid and diacylglycerol indicated phosphatidylinositol hydrolysis by phospholipase C. Pretreatment with a phospholipase A₂ inhibitor, mepacrine, substantially prevented the antigen-induced liberation of [³H]arachidonic acid from phosphatidylcholine. It can be thus concluded that, in the release of arachidonic acid by antigen-stimulated mast cells, the phospholipase A₂ pathway, in which phosphatidylcholine is hydrolysed, serves as the major one, the phospholipase C/diacylglycerol lipase pathway playing only a minor role.

INTRODUCTION

Mast cells have been shown to activate a sequence of cellular events that causes secretion in response to various stimulants [1]. Cross-linking of membrane-bound receptors for antibody (or IgE) induces mobilization of Ca²⁺, enhanced phospholipid metabolism and release of histamine, leukotrienes and prostaglandins, all of which play a role in acute allergic reactions. Leukotrienes and prostaglandins are derived from arachidonic acid released from phospholipids, and therefore its release plays a crucial role in the regulation of leukotriene and prostaglandin synthesis. Two main pathways for arachidonic acid release in activated cells have been proposed: phospholipase C/diacylglycerol lipase [2] and phospholipase A₂ [3].

Although there have been many studies of these pathways, namely in platelets [2,4–6], neutrophils [7], macrophages [8,9], pancreatic acinar cells [10], RBL-2H3 cells [11], and NIH-3T3 cells [12], a consensus has not yet emerged regarding the relative contribution of the two pathways of arachidonate mobilization. We have previously suggested that the phospholipase A₂ pathway may play a major role in arachidonic acid liberation in mast cells stimulated with compound 48/80 or the ionophore A23187 [13,14]. However, no studies to date have investigated arachidonic acid mobilization in antigen-stimulated mast cells.

Although it is conceivable that arachidonate-release pathways may differ with the type of cell or stimuli, there are several other explanations for the controversy about the primary source of the released arachidonate, one of

which may be the lack of specific inhibitors for either phospholipase C or phospholipase A₂. In addition, the [³H]arachidonate labelling conditions have to be taken into consideration. Colard *et al.* [15] revealed that [¹⁴C]-arachidonic acid was first incorporated into diacyl phosphatidylcholine (PC) and then transferred to alkenylacyl phosphatidylethanolamine (PE) during labelling. Garcia-Gil & Siraganian [11] showed that the distribution of [¹⁴C]arachidonic acid in RBL-2H3 cells which had been labelled for 2 h was different from that observed in cells labelled for a longer period. These findings indicated that the apparent differences in the primary source of released arachidonic acid could be attributed partly to the different periods of labelling, and partly also the fact that the conditions for obtaining equilibrium labelling may be different for different cells.

The aim of the present work was to examine arachidonic acid release in equilibrium-labelled mast cells after stimulation with antigen, and also to determine the relative contribution of the phospholipase A₂ and phospholipase C/diacylglycerol lipase pathways in arachidonate mobilization.

MATERIALS AND METHODS

Preparation of antigen and antiserum

An extract of the nematode worm *Ascaris suum* was prepared by the method of Strejan & Campbell [16]. This crude extract was conjugated with 2,4-dinitrophenyl-(DNP)sulphonic acid by the method of Eisen *et al.* [17].

Abbreviations used: BW755C, 3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline; DNP-As, 2,4-dinitrophenyl-conjugated *Ascaris suum* extract; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; NEFA, non-esterified fatty acid; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol.

To obtain the anti-[DNP-*Ascaris suum* (DNP-As)] serum, rats were immunized by the method of Tada & Okamura [18]. Briefly, rats were injected with 0.5 ml of a mixture of 1 mg of DNP-As protein with 10^{10} killed *Bordetella pertussis* cells in the four footpads. Rats were boosted 5 days later with 0.5 mg of DNP-As protein injected into the back muscle. On the third day after the second injection, anti-DNP-As serum was separated from blood obtained from the abdominal aorta under pentobarbital anaesthesia. The antiserum was freeze-dried and stored at -20°C . A passive cutaneous anaphylactic test performed at 48 h to detect IgE antibody showed a titre of 1:512.

Isolation of rat mast cells

Mast cells were obtained from the peritoneal cavity of Wistar rats and purified by a Percoll-gradient method [19]. Briefly, rats were exsanguinated by decapitation and then 20 ml of ice-cold THG medium [NaCl (137 mM)/ KCl (2.7 mM)/ NaH_2PO_4 (0.4 mM)/ CaCl_2 (1.8 mM)/ MgCl_2 (1 mM)/glucose (5.6 mM)/Hepes (10 mM)/gelatin (1 mg/ml)/heparin (5 units/ml), pH 7.4] was injected into the peritoneal cavity. The trunk of the rat was massaged more than 100 times, and the medium was recovered by aspiration and centrifuged (150 g, 5 min, 4°C). The pellet was resuspended in 1.6 ml of THG medium. The cell suspension was mixed with 3.6 ml of Percoll and 0.4 ml of 10-fold-concentrated phosphate-free THG, overlaid with 1 ml of THG, and then centrifuged (200 g, 15 min, 4°C). Mast cells were obtained from the pellet, washed twice, and resuspended in THG medium. Mast-cell preparations were about 95% pure as assessed by Toluidine Blue staining, and more than 95% of cells were viable as judged by Trypan Blue uptake.

Lipid metabolism

Purified mast cells were incubated in THG medium containing anti-DNP-As serum and [^3H]arachidonic acid ($2\ \mu\text{Ci}/10^6$ cells per 0.1 ml) for the indicated periods at 30 or 37°C , and then washed with THG medium without antiserum. The mast-cell suspension (3×10^5 cells/0.48 ml) was stimulated by exposing it to $20\ \mu\text{l}$ of DNP-As ($25\ \mu\text{g}/\text{ml}$) for the indicated periods of time at 37°C . Stimulation was carried out in the presence of $10\ \mu\text{g}$ of phosphatidylserine (PS)/ml. The reactions were terminated by the addition of 2 ml of chloroform/methanol (1:2, v/v), and lipid extraction was performed by the method of Bligh & Dyer [20]. The individual phospholipids were separated by two-dimensional t.l.c. on silica-gel H 60 plates impregnated with magnesium acetate (2.5%, w/v), using chloroform/methanol/13.5 M- NH_3 /water (65:35:6:1, by vol.) in the first dimension, and chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.) in the second dimension [4]. The neutral lipids were analysed on borate (0.4 M)-impregnated silica-gel H 60 plates in chloroform/acetone (24:1, v/v) [4]. Spots were identified by their co-migration with authentic standards. The areas corresponding to individual lipids were scraped into vials and radioactivity was determined in a liquid-scintillation counter (Packard MINAXI 4000), with toluene, Triton-X-100, water, PPO (2,5-diphenyloxazole), POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] (2800 ml, 700 ml, 175 ml, 12 g and 0.84 g respectively) [4] as scintillant.

Histamine assay

At the end of the labelling period, a portion of the mast-cell suspension was assayed for measurement of spontaneous histamine release. The remaining portion was washed twice and suspended to a density of 2×10^5 cells/0.48 ml in the THG medium in the presence of PS ($10\ \mu\text{g}/\text{ml}$). The cells were then stimulated with DNP-As ($1\ \mu\text{g}/\text{ml}$) for the indicated periods and the reaction was terminated by adding 1 ml of ice-cold 10 mM-EGTA-containing THG medium. Histamine in the supernatant and the pellet was determined by the method of Shore *et al.* [21], and percentage release was expressed as the ratio of histamine in the supernatant to the sum of that in the supernatant and pellet.

Materials

[5,6,8,9,11,12,14,15- ^3H]Arachidonate (sp. radioactivity 210 Ci/mmol) was obtained from Amersham International. PS was obtained from Serdary Research Laboratories (London, Ontario, Canada). Mepacrine was purchased from Nakarai Chemicals Ltd., Kyoto, Japan. BW755C was obtained from Teikoku Zoki Co. (Tokyo, Japan). Silica-gel H 60 plates and Percoll were products of Merck and Pharmacia respectively.

RESULTS AND DISCUSSION

When mast cells were incubated with [^3H]arachidonate, the label was predominantly incorporated into phospholipids, with PC and PI displaying the highest radioactivity. A significant labelling was also observed in neutral lipids. The time sequence of the changes in the distribution of [^3H]arachidonate radioactivity in various lipid fractions was investigated when mast cells were labelled at either 30 or 37°C (Fig. 1). The radioactivity of [^3H]arachidonate in the total lipid progressively increased in cells incubated at 37°C , whereas with cells incubated at 30°C a plateau was reached at 4 h. In the 37°C -incubated cells, except for phosphatidic acid (PA), the ^3H radioactivity in other phospholipid fractions gradually increased up to 6 h of incubation. The radioactivity in the non-esterified-fatty-acid (NEFA) fraction was markedly decreased as phospholipids acquired radioactivity.

By contrast, in 30°C -incubated cells, ^3H radioactivity incorporation into various phospholipids reached a plateau by 4 h after a gradual increase. In addition, the level of radiolabelled NEFA remained stable during the incubation at 30°C . These results indicated that the 4 h incubation at 30°C was the more appropriate for equilibrium labelling in further studies.

We then investigated histamine release from cells labelled for various periods of time. In the 30°C -incubated cells the spontaneous release was less than 10% up to 4 h and, when cells were labelled for 8 h at 30°C , stimulation with antigen DNP-As resulted in more than 60% histamine release, indicating a good responsiveness to antigenic stimulation. By contrast, in 37°C -incubated cells, the spontaneous release increased with time, reaching as high as 70% at 8 h, the stimulation-induced histamine release being decreased in a reciprocal manner. The time course of histamine release in response to DNP-As was examined in cells that had been incubated for 4 h at 30°C . Histamine release was observed to be about half-maximal at 5 s and reached a maximum

Fig. 1. $[^3\text{H}]\text{Arachidonate}$ incorporated into total and individual lipids of the cells incubated for various periods at 30 or 37 °C

Mast cells were labelled with $[^3\text{H}]\text{arachidonic acid}$ in the presence of anti-DNP-As suspended in THG medium at 30 °C (●) or 37 °C (○). At the indicated times, each cell suspension was washed twice and the lipids extracted immediately. Each value is the mean of duplicate determinations from one of two experiments, each showing similar results.

Fig. 2. Time course of $[^3\text{H}]\text{arachidonic acid}$ radioactivity in the NEFA fraction of mast cells stimulated with DNP-As

Mast cells were incubated with $[^3\text{H}]\text{arachidonic acid}$ in the presence of anti-DNP-As suspended in THG medium for 4 h at 30 °C, washed twice, and then exposed to DNP-As (1 $\mu\text{g}/\text{ml}$) in the presence of PS (10 $\mu\text{g}/\text{ml}$) for the indicated times at 37 °C. (a) shows $[^3\text{H}]\text{arachidonic acid}$ radioactivity in the NEFA fraction of antigen-stimulated cells, examined by neutral-lipid t.l.c. (●), and the ^3H radioactivity recovered in the neutral-lipid fraction, which includes arachidonate metabolites (▲) separated by two-dimensional t.l.c. for phospholipids. (b) shows the $[^3\text{H}]\text{arachidonate}$ radioactivity in the NEFA fraction separated by neutral-lipid t.l.c. in the antigen-stimulated cells, which had been treated with 100 μM -BW755C for 2 min at 37 °C. Each value is the mean of duplicate determinations from one of two similar experiments. The bars indicate the range.

(64%) at 60 s. This response is compatible with the typical profile of degranulation observed in freshly prepared mast cells, indicating that cell responsiveness to antigen was well preserved during the long-term labelling (4 h) at 30 °C. Cell viability was also examined by the Trypan Blue-exclusion test. The viability of 37 °C-incubated cells was inversely proportional to the level of spontaneous release of histamine. For example, at 4, 6 and 8 h of incubation, the spontaneous release was 20, 30, and 70 % respectively, whereas the viability was about 90, 70 and 20 % respectively. On the other hand, more than 90 % of cells were viable after 6 h incubation at 30 °C.

On the basis of these results [labelling pattern (Fig. 1), histamine-release activity and cell viability], we chose the $[^3\text{H}]\text{arachidonate}$ labelling conditions of 4 h at 30 °C to examine arachidonate mobilization.

Arachidonic acid liberation in mast cells stimulated with DNP-As at 1 $\mu\text{g}/\text{ml}$ was investigated in the presence of PS (10 $\mu\text{g}/\text{ml}$). The ^3H radioactivity in the NEFA fraction separated by neutral-lipid t.l.c. was low (Fig. 2a). This implies that the released arachidonic acid could have been immediately metabolized via lipoxygenase or cyclo-oxygenase. This was confirmed by the experiment using BW755C, an inhibitor of both lipoxygenase and cyclo-oxygenase [22]. The ^3H radioactivity in NEFA exhibited a transient increase in the mast cells pretreated with 100 μM -BW755C (Fig. 2b). Since the neutral-lipid fraction separated by two-dimensional t.l.c. contained arachidonic acid and its metabolites, the changes in ^3H radioactivity of this fraction reflected the overall level of released arachidonate.

Fig. 3 illustrates the changes in $[^3\text{H}]\text{arachidonate}$ distribution in various lipids on stimulation with DNP-

Fig. 3. Time course of the changes in the $[^3\text{H}]$ arachidonic acid content of individual lipids in mast cells stimulated with DNP-As

Mast cells were incubated with $[^3\text{H}]$ arachidonic acid in the presence of anti-DNP-As suspended in THG medium for 4 h at 30 °C, washed twice, and then exposed to DNP-As (1 $\mu\text{g}/\text{ml}$) in the presence of PS (10 $\mu\text{g}/\text{ml}$) for the indicated times at 37 °C. Each value is the mean of duplicate determinations from one of two similar experiments. The bars indicate the range. The radioactivity incorporated in the total-lipid fraction was 1.1×10^5 d.p.m. The radioactivities at zero time for PC, PI, PE, PS, PA, TG, DG and MG were 6.1×10^4 , 2.2×10^4 , 1.2×10^4 , 6.9×10^3 , 1.7×10^3 , 2.9×10^3 , 5.1×10^2 and 5.2×10^2 d.p.m. respectively.

Fig. 4. $[^3\text{H}]$ Arachidonate radioactivity in individual lipids in mast cells stimulated (or not stimulated, C) with DNP-As (Ag) in the presence (M) or absence (C, control) of mepacrine

Mast cells were labelled with $[^3\text{H}]$ arachidonate in the presence of anti-DNP-As suspended in THG medium for 4 h at 30 °C and then washed twice. The labelled cells were preincubated for 5 min at 37 °C in the presence (right column in each case) or absence (left two columns in each case) of 0.2 mM-mepacrine, and then stimulated with DNP-As (1 $\mu\text{g}/\text{ml}$) for 1 min (right two columns in each case). In the right-most panel the stippled columns show DG and the open columns show PA. Each value is the mean of duplicate determinations from one of two similar experiments. The bars indicate the range.

As (1 $\mu\text{g}/\text{ml}$) in the presence of PS (10 $\mu\text{g}/\text{ml}$). The radioactivities in PC and PI decreased abruptly within 60 s after stimulation. The loss of $[^3\text{H}]$ arachidonate from PC was much greater than that from PI. There were

some transient increases in DG and PA. These findings lead us to predict that (i) PC hydrolysis is due to involvement of phospholipase A_2 and (ii) PI is degraded by phospholipase C [14]. In contrast, PE showed an

increase on stimulation, which may reflect some transacylation, a phenomenon that has been observed in other cells [15,23]. It is therefore conceivable that, in antigen-stimulated mast cells, arachidonic acid is liberated through two routes, namely the phospholipase A_2 and the phospholipase C/diacylglycerol lipase pathways, and that the former may make a major contribution to the phospholipid changes occurring on stimulation. We previously suggested that arachidonic acid release could be mediated by phospholipase A_2 and that the major source was PC in mast cells stimulated with concanavalin A [14].

To assess the role played by phospholipase A_2 , a commonly used inhibitor of the enzyme, namely mepacrine [24], was used in the present study. Mast cells were incubated with 0.2 mM-mepacrine for 5 min, and then stimulated with DNP-As (1 μ g/ml) for 1 min (Fig. 4). Mepacrine pretreatment barely affected antigen-induced PI hydrolysis and the production of both DG and PA (which are the immediate metabolites in the phospholipase C pathway). However, antigen-induced PC hydrolysis was decreased in mepacrine-pretreated cells, and the antigen-induced [3 H]arachidonate release was decreased to 35%. These results suggest that PC serves as a substrate for phospholipase A_2 , and that the arachidonate released by antigen induction is mainly derived from PC via phospholipase A_2 . On the other hand, an appreciable decrease of 3 H radioactivity in PC in the presence of mepacrine could indicate two possibilities. One is that the concentration (0.2 mM) of the drug exerts incomplete inhibition of phospholipase A_2 ; since higher concentrations of mepacrine have been found to inhibit phospholipase C [25], we have used only 0.2 mM-mepacrine in the present study. An alternative possibility is the novel activity of a PC-specific phospholipase C producing DG, as has been observed in other cells [26,27]. We have not yet identified this pathway in antigen-stimulated cells.

In summary, mast cells equilibrium-labelled with [3 H]arachidonate for 4 h at 30 °C preserve good responsiveness to the antigen. On receptor-induced stimulation by antigen, arachidonic acid is released mainly by phospholipase A_2 action upon PC, and the remainder originates from PI by a sequential action of phospholipase C and DG lipase.

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