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CONCERTED STIMULATION OF PI-TURNOVER, Ca^{2+}-INFLUX AND HISTAMINE RELEASE IN ANTIGEN-ACTIVATED RAT MAST CELLS

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SUMMARY: Phospholipid metabolism in rat mast cells activated by antigen was examined with reference to phosphatidylinositol (PI) turnover. Upon antigen stimulation, histamine release from passively sensitized mast cells with IgE was potentiated by adding phosphatidylserine (PS). The addition of antigen to [^{3}H]glycerol-prelabeled and sensitized mast cells induced a marked loss of radioactivity of PI and a concurrent accumulation of 1,2-diacylglycerol (DG) and phosphatidic acid (PA) within 5 to 60 sec. Furthermore, this antigen-induced PI breakdown was enhanced in the presence of Mg^{2+}. Histamine release occurred in parallel with PI breakdown. On the other hand, the transient Ca^{2+} influx into mast cells, as measured by uptake of $^{45}Ca^{2+}$, was found to occur quickly after cells were activated by antigen, which was concerted with PI breakdown. These results suggest that enhanced PI turnover may be an important step in the biochemical sequence of events leading to release of histamine, and that not only Ca^{2+} but also Mg^{2+} appears to take a part in stimulus-response coupling in rat mast cells.

It is generally known that phospholipid metabolism is markedly enhanced when secretory cells are activated by various stimulating factores (1-5). Especially, receptor-mediated phosphatidylinositol (PI) turnover in many tissues has been reported to be a primary event for calcium mobilization and consequent secretory reaction (6).

Mast cells have receptors for immunoglobulin E (IgE) on their sulface and crosslinking of IgE with antigen, concanavalin A or anti-IgE is an effective stimulus for secretion of histamine (7-8). Although not as widely studied as phospholipid methylation (9-10), PI turnover has also been described to be associated with mast cell activation, for example, enhanced incorporation of radioactive labels [^{32}P]- and [^{3}H]-inositol into PI (11-13).

Abbreviations: PI; phosphatidylinositol, PA; phosphatidic acid, PS; phosphatidylserine, DG; 1,2-diacylglycerol, DNP-As; dinitrophenylated *Ascaris* extracts, IgE; immunoglobulin E, BSA; bovine serum albumin.

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Our previous study with rat peritoneal mast cells has shown that marked *de novo* PI synthesis rather than its breakdown and Ca^{2+} influx were induced by compound 48/80- and A23187-stimulation (14). The present experiments were undertaken to examine phospholipid metabolism during IgE-mediated histamine release from rat mast cells.

The results obtained from this work indicate that the rapid PI breakdown would be involved in Ca^{2+} influx in mast cells stimulated with IgE-directed antigen and that the rate of PI turnover is affected by Mg^{2+} ion. Taken together with our previous data (14), evidence was presented that the membrane phospholipid metabolism would exert different responses depending upon the kind of stimulant.

MATERIALS AND METHODS

Isolation of rat mast cells: Mast cells were obtained from peritoneal cavity of Wistar rats and then purified using a BSA density-gradient method according to the procedure of Sullivan *et al.* (15). Finaly, cells were suspended in a buffered salt solution (150 mM NaCl, 3.7 mM KCl, 3.0 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 1.0 mM $CaCl_2$, 0.5-5.0 mM $MgCl_2$, 5.6 mM dextrose, 0.1 % gelatine and 10 units/ml heparine, pH 6.8) (15). All glasswares were siliconized before used.

Preparation of antigen and antiserum: Extracts from *Ascaris suum* were prepared by the method of Strejan and Campbell (16). Crude *Ascaris suum* extracts were coupled with 2,4-dinitrophenyl (DNP) sulfonic acid by the method of Eisen *et al.* (17). To obtain the anti-DNP antibody, rats were immunized by injecting the combination of 1 mg protein of DNP-coupled *Ascaris* extracts (DNP-As) and 10^{10} *Bordetella pertusis* in the four footpads. Five days later rats were boosted with 0.5 mg of protein of DNP-As alone in the back muscle. Antisera was isolated from the blood obtained by abdominal aorta puncture 3 days after the last injection. Sera were freeze-dryed and stored at -20°C. Forty-eight-hour passive cutaneous anaphylactic (PCA) reaction in rat to detect IgE antibody was positive at a titer of 1 : 1024 for anti-DNP antibody. On the other hand, the PCA titer of antisera was abolished by warming for 30-60 min at 56°C.

Lipid metabolism: Purified mast cells were incubated with [^{3}H]glycerol (100 μCi/10^6 cells) in the presence of anti DNP-As diluted 2-fold with buffered solution for 60 min at 37°C. After radiolabeling and passive sensitization, cells were washed two times with the above buffered solution and resuspended in the solution to a final concentration of 2-4 x 10^5 cells/ml. Mast cell suspension (1.2 x 10^5 cells/0.45 ml) was preincubated at 37°C for 5 min, and then cells were activated by the exposure to DMP-As (0.1-10 μg/ml, protein content) containing PS (0.1-10 μg/ml) for indicated time at 37°C. The reaction was terminated by the addition of 1 ml of ice-cold 10 mM EDTA-buffer and then centrifuged at 150 x g for 10 min. The supernatant and pellets were retained for the determination of histamine and the extraction of lipids, respectively. The cell lipids were extracted by the method of Bligh and Dyer (18). The phospholipids were separated by two dimensional thin layer chromatography on Slica gel 60 plates, impregnated with magnesium acetate (2.5 %, w/v), using chloroform/methanol/13.5 N ammonia water (65 : 35 : 5.5, v/v) in the 1st dimension, and chloroform/acetone/methanol/acetic acid/water (3 : 4 : 1 : 1 : 0.5, v/v) in the 2nd dimension (19). The neutral lipids were analyzed on Slica gel G plates containing borate (0.4 M) in a solvent system of chloroform/acetone (96 : 4, v/v) (20). Spots were identified by comigration with

authentic standards. The areas corresponding to individual lipids were scrapped into vials and the radioactivity was determined in a liquid scintillation counter (Beckman LS 7500) with toluene/Triton X-100/water/2,5-diphenyloxazole/2,2-*p*-phenylen-*bis*-(5-phenyloxazole)(800 ml : 200 ml : 50 ml : 3.3 g : 0.2 g) (21).

Ca^{2+} influx: To examine the $^{45}Ca^{2+}$ incorporation into mast cells, passively sensitized mast cells (2 x 10^5 cells/0.45 ml) were preincubated with $^{45}Ca^{2+}$ (2 μCi) at 37°C for 5 min, and then cells were activated by DNP-As 0.05 ml (10 μg /ml, protein content) containing PS (10 μg/ml) and reaction was stopped as described above. After centrifugation at 150 x g for 10 min at 4°C, the resulting pellet was washed two times with the ice-cold 10 mM EDTA-buffer. The radioactivity of $^{45}Ca^{2+}$ incorporated into cells was then determined in the scintillation counter with toluene/Triton X-100/2,5-diphenyloxazole/2,2-*p*-phenylene-*bis*-(5-phenyloxazole) (1000 ml : 500 ml : 4 g : 0.2 g) (21).

Histamine assay: Histamine released into extracellular medium was measured fluoremetrically by the modification (15) of the method of Shore *et al.* (22). The released histamine was expressed as a percentage of the total cell histamine. Total histamine in a separate sample of cells was determined after heated at 100°C for 10 min. Spontaneous (i.e. non-activated) release of histamine was < 8 % of total histamine.

Materials: [2-^{3}H]Glycerol (10 Ci/mmol) and $^{45}CaCl_2$ (12 Ci/g Ca) were obtained from New England Nuclear and Amersham, respectively. BSA (fraction V) was purchased from Sigma. Slica gel 60 plates were product of Merk. All other chemicals were of the highest reagent grade.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of phosphatidylserine (PS) on antigen-induced histamine release from sensitized mast cells. Although the mode of action of PS is not fully clarified, PS has been reported to enhance the entry of Ca^{2+} into the

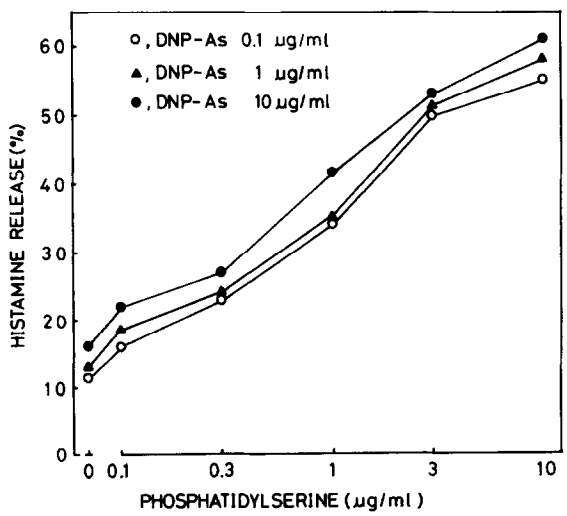

Fig. 1 Phosphatidylserine (PS) requirement for IgE-mediated histamine release from rat mast cells. Mast cells were preincubated with anti DNP-As antibody for 60 min at 37°C and then cells were washed two times. Mast cell suspension (1.2 x 10^5 cells/0.45 ml) was incubated with DNP-As 0.05 ml (0.1-10 μg/ml) containing PS (0.1-10 μg/ml) for 10 min. ○ ; DNP-As 0.1 μg/ml, ▲ ; DNP-As 1 μg/ml, ● ; DNP-As 10 μg/ml. Each value is the mean of three determinations.

cells (23) without affecting binding of the ligand to its receptors (24), which leads to the contention that the site of action of PS is at the level of Ca^{2+}-gating mechanism (25). IgE-mediated histamine release from rat mast cells was appreciably enhanced by PS in a dose-dependent manner over a range of concentrations from 0.1 to 10 μg/ml. A final concentration of 10 μg/ml PS was used in all subsequent experiments.

The time course of histamine release was examined. When the antigen was added to sensitized mast cells, a rapid histamine release was observed at 5 to 20 sec after stimulation and reached a plateau with further incubation (Fig. 2a, open symbol).

The PS requirement for histamine release was shown by Sullivan *et al.* (15) using Mg^{2+} free medium. On the other hand, Hirata *et al.* (26) have demonstrated

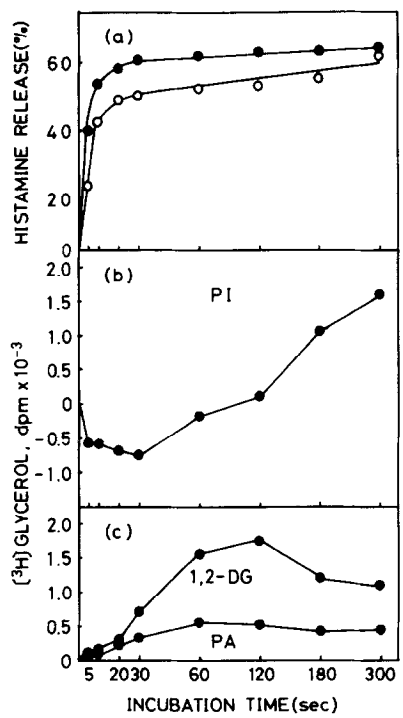

Fig. 2 Time course of histamine release (a) and phospholipid metabolism (b,c) in IgE-mediated rat mast cells. Mast cells pretreated with [^{3}H]glycerol and anti DNP-As antibody were exposed to DNP-As (10 μg/ml) containing PS (10 μg/ml) at 37°C for indicated time. After reaction was terminated by adding ice-cold 10 mM EDTA-buffer, cells were separated by centrifugation. The resulting supernatants and pellets were used for the determination of histamine and for analysis of lipid, respectively. Results are expressed as the difference of radioactivity of stimulation *vs* control. Fig. 2a) ○; Mg^{2+} 0 mM, ●; Mg^{2+} 0.5 mM. Fig. 2b, 2c) ●; Mg^{2+} 0.5 mM. Each value is the mean of three determinations.

with concanavalin A-activated mast cells that phospholipid methylation is potentiated by the presence of Mg^{2+}. This prompted us to examine whether antigen-stimulation may enhance histamine release and phospholipid metabolism. As shown in Fig. 2a, addition of 0.5 mM Mg^{2+} induced a rapid histamine release, which was greater than that observed in the absence of Mg^{2+}. Furthermore, in an attempt to assess breakdown of PI, experiments were performed in which mast cells were prelabeled with [^{3}H]glycerol. The PI breakdown was induced in the absence of Mg^{2+}, but this was markedly enhanced by adding of Mg^{2+} (0.5-5.0 mM) (Fig. 3). At 30 sec after antigen-stimulation, the percentage loss of radioactivity from PI in the presence of Mg^{2+} (0, 0.5, 1.0, 5.0 mM) was 9.5, 29.8, 58.3 and 67.6 %, respectively. The PI resynthesis following the antigen-induced PI breakdown, which was reflected as the increased radioactivity in PI, was observed by addition of Mg^{2+} (0, 0.5 mM) at 60 sec after stimulation. In contrast, the addition of high concentration of Mg^{2+} (1.0, 5.0 mM) reduced PI resynthesis. At the present moment, there is no plausible explanation for the mechanism by which PI breakdown and histamine release are affected by Mg^{2+}. Because the breakdown and resynthesis of PI and the enhanced phospholipid methylation (26) was observed at 0.5 mM Mg^{2+}, this dose was used in subsequent experiments. The PS requirement for IgE-mediated histamine release was not affected by the presence of 0.5 mM Mg^{2+}.

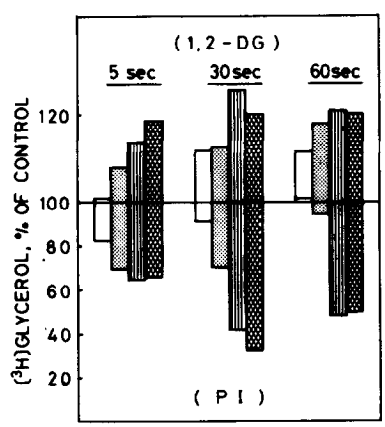

Fig. 3 Effect of Mg^{2+} on 1,2-DG and PI changes in antigen-stimulated rat mast cells. After reaction termination (by adding chloroform : methanol = 1 : 2), cell lipids were extracted. The other conditions were described under the legend to Fig. 2. □; 0 mM Mg^{2+}, ▨; 0.5 mM Mg^{2+}, ▥; 1 mM Mg^{2+}, ▩; 5 mM Mg^{2+}.

Time-sequential relationship between kinetics of histamine release and metabolism of phospholipids was in detail examined for the same cell preparation. As demonstrated in Fig. 2b, PI indicated a biphasic change. A marked decrease of radioactivity in PI was seen at 5 sec after addition of antigen and reached a maximum at 30 sec. The initial loss of PI was subsequently followed by its re-synthesis via so-called PI cycle. While at 30 sec after stimulation the decrease of radioactivity in PI was 750 dpm, the radioactivity in 1,2-DG, produced from PI by its specific phospholipase C, was increased to a corresponding degree and reached a maximum at 120 sec, and then progressively decreased with further incubation (Fig. 2c). The increase of radioactivity in PA, a product of phosphorylation of the resultant 1,2-DG by 1,2-DG kinase, was 344 dpm within 30 sec, and reached a peak at 60 sec (Fig. 2c). The rate of initial rise in 1,2-DG was much greater than that observed in PA, which suggests that a pathway PI → 1,2-DG → PA was activated. Thus, rat mast cell activation induced by receptor-mediated antigen is coupled with augmented turnover of PI. The previous study (14) from this laboratory has dealt with phospholipid metabolism in rat mast cells stimulated by compound 48/80 or A23187 in the absence of Mg^{2+}, and has demonstrated that little or no significant PI turnover was induced by either stimulant. Rather *de novo* biosynthesis of PI was more appreciated than PI breakdown. Taken together, evidence was presented that the membrane phospholipid metabolism would exert different responses depending upon the nature of stimulant.

The importance of Ca^{2+} as an intracellular mediator of response mechanisms elicited by external stimuli is well documented. To ascertain whether the PI breakdown is associated with Ca^{2+} influx, Ca^{2+} incorporation into mast cell was measured using $^{45}Ca^{2+}$. The time course for $^{45}Ca^{2+}$ influx induced by antigen showed a biphasic response (Fig. 4). A transient rapid increase was observed to be induced within 5 sec after antigen addition, and then $^{45}Ca^{2+}$ influx was gradually enhanced over a range of time from 30 to 300 sec. However, the mechanism and physiological significance of the latter Ca^{2+} influx is not known. These findings collectively indicated that PI breakdown occurs in accordance with the initial Ca^{2} influx and the histamine release in antigen-stimulated mast cells.

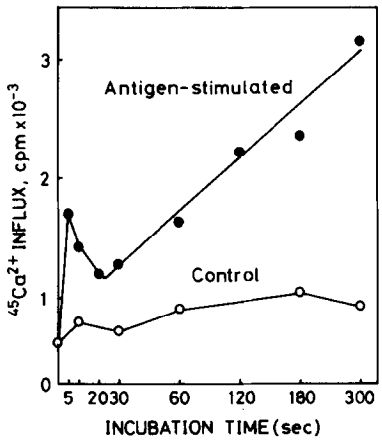

Fig. 4 Time course of IgE-mediated $^{45}Ca^{2+}$ influx into mast cells. Mast cells (2×10^5 cells/0.45 ml) sensitized with anti DNP-As antibody were preincubated with $^{45}Ca^{2+}$ (2 μCi) at 37°C for 5 min, and then cells were activated with DNP-As 0.05 ml (10 μg/ml) containing PS (10 μg/ml) at 37°C for indicated time. After reaction termination (by adding ice-cold 10 mM EDTA-buffer), cells were separated by centrifugation. $^{45}Ca^{2+}$ radioactivity in resulting pellets was measured. ○; Control, ●; DNP-As 10 μg/ml. Each value is the mean of two experiments performed in triplicate.

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