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Stimulation of phospholipid methylation, Ca^{2+} influx, and histamine release by bridging of IgE receptors on rat mast cells

(methyltransferase/anti-receptor antibodies/methyltransferase inhibitors)

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ABSTRACT **Normal rat mast cells were stimulated by antibodies against IgE receptors (anti-RBL) or by anti-IgE, and [^{3}H]methyl group incorporation into phospholipids, ^{45}Ca uptake, and histamine release were examined. Anti-RBL or its F(ab′)$_2$ fragments and anti-IgE induced an increase in the incorporation of [^{3}H]methyl into phospholipids, in ^{45}Ca influx, and in histamine release. By contrast, Fab′ monomer fragments of anti-RBL induced none of these reactions. The transient increase of [^{3}H]methyl incorporation in lipids peaked within 15 sec after the addition of either anti-RBL or anti-IgE and fell to basal level in 30 sec. This was then followed by an influx of ^{45}Ca that increased to a maximum in 2 min and by histamine release that reached a maximum in 3 min. Inhibition of phospholipid methylation resulted in an inhibition of ^{45}Ca influx and histamine release. These findings demonstrate that phospholipid methylation in rat mast cells is induced by bridging of IgE receptors on the cell surface and that increased methylation of phospholipids sets the stage for an influx of Ca^{2+} and subsequent release of histamine.**

Mast cells and basophilic granulocytes bear specific receptors for IgE (1–4), and the bridging of cell-bound IgE molecules by either multivalent antigen or divalent anti-IgE antibody initiates the release of various chemical mediators from these cells. Recent studies by Ishizaka *et al.* (5–7) have shown that bridging of IgE receptors on mast cells by antibodies against IgE receptor or its F(ab′)$_2$ fragments induces an increase of ^{45}Ca influx into normal mast cells, and this process is accompanied by histamine release. The binding of monomer Fab′ fragments of the antibody to the receptors failed to trigger these reactions. The results indicated that bridging of IgE receptors rather than polymerization of cell-bound IgE molecules is responsible for Ca^{2+} influx and histamine release from mast cells.

Concanavalin A (Con A) can stimulate histamine release in rat mast cells (8). Siraganian and Siraganian (9) suggested that Con A-induced histamine release is due to bridging of cell-bound IgE molecules by the lectin. More recently, Hirata *et al.* (10) have shown that membrane phospholipids of rat mast cells are methylated in the early stage of Con A-induced histamine release. The phospholipid methylation is stimulated when rat mast cells are treated with the lectin in the absence of Ca^{2+}. The subsequent addition of Ca^{2+} liberates histamine only when phospholipids are methylated. These findings suggested the possibility that direct bridging of IgE receptors may induce phospholipid methylation prior to Ca^{2+} influx. We now show that divalent antireceptor antibodies stimulate phospholipid methylation in rat mast cells and that the prior activation of methyltransferases is involved in the Ca^{2+} influx process and histamine release.

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MATERIALS AND METHODS

Antireceptor Antibodies and Anti-Immunoglobulin. The anti-receptor antibody preparation used in the present experiments was the same material previously described (5). Briefly, antibodies against IgE receptors on rat basophilic leukemia (RBL) cells were raised by immunization of a rabbit with immune precipitates composed of receptor–IgE complexes and rabbit anti-IgE. Antibodies against cell surface components were purified by using RBL cells, and were rendered specific for mast cells by appropriate absorptions. Analysis of membrane components that bound to the antibody preparation (anti-RBL) by $NaDodSO_4$ gel electrophoresis showed that the major antibodies in anti-RBL were anti-receptor antibodies. Further analysis indicated that the antibodies were directed toward the binding site for IgE in the receptor molecules (8). Previous studies have shown that the anti-receptor antibodies in anti-RBL are responsible for histamine release and ^{45}Ca uptake (6, 7). The F(ab′)$_2$ fragments of anti-RBL were obtained by pepsin digestion and these fragments were split into Fab′ monomer by reduction and alkylation (6). Because of the low protein concentration of purified anti-RBL (1.6 mg/ml), an 8-fold excess of normal rabbit IgG was added to the antibody preparation before digestion.

Monoclonal rat IgE (IR 162) and the IgG fraction of a goat antiserum specific for rat IgE (anti-IgE) were those described (7). The concentration of anti-IgE antibody in the goat antiserum was 3.25 mg/ml.

Purification of Mast Cells. Mast cells were obtained from peritoneal cells of Sprague–Dawley rats (Holtzman, Madison, WI) by the method of Bach and colleagues (9) with slight modifications (10). Purity of mast cell preparations was in the range 89–92%. Viability of the cells was >97% as assessed by trypan blue exclusion.

Measurement of ^{45}Ca Influx. The method described by Foreman *et al.* (11) was followed. Purified mast cells were suspended in Tyrode's solution (pH 7.0) containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (Hepes), 5 mM 2-(*N*-morpholino)-ethanesulfonic acid (Sigma), and 0.5 g of gelatin per liter. Phosphatidylserine (50 μg/ml; Supelco, Bellefonte, PA) was dispersed in the solution by sonication. One hundred microliters of Versilube F50 silicone oil (General Electric, Waterford, NY) was placed in the bottom of Microfuge tubes; 40 μl of Tyrode's solution containing ^{45}Ca (3 μCi/ml; 1 Ci = 3.7×10^{10} becquerels; Amersham) and 10 μl of an appropriate concentration of either anti-RBL or anti-IgE was layered on top of the silicone oil. The Microfuge tubes were warmed at 37°C, and then 50 μl of a mast cell suspension containing 1–2 × 10^5 cells was

Abbreviations: RBL cells, rat basophilic leukemia cells; Con A, concanavalin A.

added to each tube. Unless otherwise specified, tubes were incubated for 5 min at 37°C before centrifugation. In kinetic studies, ^{45}Ca uptake was stopped by addition of 100 μl of Tris-A-EDTA buffer‡ at pH 7.6 to the cell suspension. The tubes were centrifuged for 1 min at 10,000 × *g* in a Beckman 152 Microfuge. The bottom of the tubes containing a cell pellet was cut off, and the pellet was dissolved in 1 ml of Triton X-100 (10 mg/ml) by vigorous shaking. Radioactivity in 100 μl of the solution was measured in a scintillation spectrometer (Searle, Des Plaines, IL). The coefficient of variation of ^{45}Ca influx for stimulated cells was 6%. Unless otherwise specified, an increase in ^{45}Ca uptake was expressed by the difference between radioactivity bound to antibody-stimulated cells and that bound to unstimulated cells.

Determination of Phospholipid Methylation. Purified mast cells were suspended in Tyrode's solution containing 2 μM-L-[*methyl*-^{3}H]methionine (11 mCi/mmol; New England Nuclear), phosphatidylserine (50 μg/ml), and 0.05% gelatin. After incubation for 30 min at 37°C, 50-μl aliquots of the cell suspension containing 1–2 × 10^5 mast cells were incubated with an appropriate concentration of anti-RBL or anti-IgE preparation. The reaction was stopped by addition of 0.5 ml of ice-cold 10% (wt/vol) trichloroacetic acid containing 10 mM L-methionine, and tubes were centrifuged at 18,000 × *g* at 0°C for 10 min. The precipitates were washed with 10% trichloroacetic acid and then extracted with 3 ml of chloroform/methanol, 2:1 (vol/vol) (12). The chloroform phase was washed twice with 1.5 ml of 0.1 M KCl in 50% methanol. For the measurement of phospholipid methylation, 1 ml of chloroform phase was transferred to a counting vial and evaporated to dryness in an oven at 80°C. The residue was dissolved in 10 ml of Hydrofluor and the radioactivity was determined in a scintillation spectrometer. Identification of methylated phospholipids was carried out by thin-layer chromatography as described (12).

Determination of Products in Mast Cells from [*methyl*-^{3}H]Methionine. After incubation with 2 μM L-[*methyl*-^{3}H]-methionine, mast cells were washed at 4°C with ice-cold saline and then treated with 1.0 ml of 10% trichloroacetic acid containing 10 mM methionine. After centrifugation, 0.5 ml of supernatant was lyophilyzed and applied to cellulose plates. Development was with 60% ethanol. The precipitates were extracted with 2 ml of chloroform/methanol, 2:1 (vol/vol), and filtered through Whatman 3 MM filter paper. The filter papers were washed 4 times with 0.3 ml of 1% $NaDodSO_4$; these $NaDodSO_4$ eluates were extracted with 2 ml of 80% phenol. The DNA and RNA were precipitated by adding 2 ml of ethanol to 1 ml of phenol extract and chilling at −20°C overnight. The protein was precipitated by the addition of 3 ml of 10% trichloroacetic acid to 0.1 ml of the $NaDodSO_4$ solution.

Histamine Release. Histamine release experiments were carried out with the same preparations of cells used for the measurement of ^{45}Ca uptake and phospholipid methylation. Purified mast cells or peritoneal cells were suspended in Tyrode's solution containing 50 μg of phosphatidylserine per ml. Aliquots (0.2 ml) of the cell suspension containing 5–10 × 10^4 mast cells were incubated at 37°C with an optimal concentration of either anti-RBL or anti-IgE for maximal histamine release. The reaction was stopped by adding an equal volume of Tris-A-EDTA buffer, and histamine content in the supernatant was measured by the automated technique of Siraganian (13).

‡ Tris-A-EDTA buffer, pH 7.6, contains 0.025 M Tris, 0.12 M NaCl, 5 mM KCl, 0.01 M EDTA, and 0.2 g of human serum albumin (Nutritional Biochemicals) per liter.

Chemicals. 3-Deaza-adenosine was obtained from Southern Research Institute (Birmingham, AL). S-Isobutyryl-3-deaza-adenosine was synthesized as described (14). L-Homocysteine thiolactone was from Sigma.

RESULTS

Stimulation of Phospholipid Methylation by Bridging of IgE Receptors. Aliquots of purified normal mast cell suspensions were preincubated with L-[*methyl*-^{3}H]methionine and then challenged with 1.5 μg of anti-RBL per ml, an optimal concentration for maximal histamine release. The addition of anti-RBL caused a transient uneven incorporation of [^{3}H]-methyl into phospholipids. Maximal incorporation of [^{3}H]-methyl into the lipid fraction of the cells was observed within 10–15 sec after the challenge. Incubation of normal mast cells with 20 μg of normal rabbit IgG failed to increase phospholipid methylation.

To examine whether the bridging of IgE receptors is necessary for the enhancement of phospholipid methylation, normal mast cells preincubated with L-[*methyl*-^{3}H]methionine were challenged with either $F(ab')_2$ or Fab' monomer fragments of anti-RBL at 50 μg/ml, and the kinetics of the incorporation of methyl group were determined. $F(ab')_2$ fragments induced a marked increase in the incorporation of [^{3}H]methyl within 10 sec, reached maximum at 15 sec, and declined within 30 sec (Fig. 1). Because the stimulation of normal mast cells by the same concentration of the $F(ab')_2$ fragments of anti-RBL also induced an increase in ^{45}Ca influx and histamine release (7), the time courses of these reactions were studied using the same cell suspensions. ^{45}Ca uptake reached a plateau at 2 min; the maximal histamine release was obtained between 2 and 3 min (Fig. 1). When normal mast cells were challenged with Fab' monomer fragment of anti-RBL at 50 μg/ml, however, no significant increase in phospholipid methylation was observed. As reported (7), the monovalent fragment failed to induce either ^{45}Ca uptake or histamine release (Fig. 1). These results indicate that phospholipid methylation precedes Ca^{2+} influx and histamine release.

Similar experiments were carried out to study whether the bridging of cell-bound IgE induces phospholipid methylation. Normal mast cells were incubated with 100 μg of monoclonal

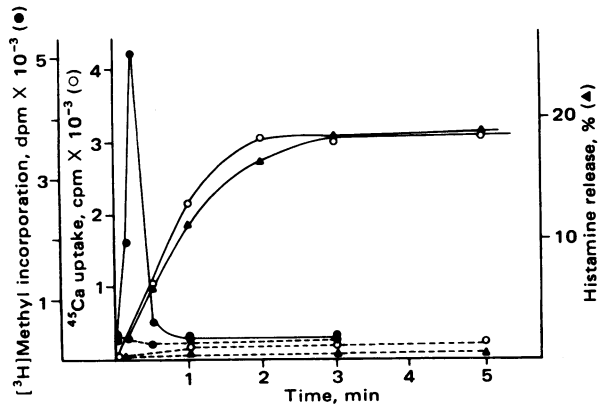

FIG. 1. Kinetics of [^{3}H]methyl incorporation (●), ^{45}Ca uptake (○), and histamine release (▲) induced by anti-RBL. The same preparation of purified mast cells was used for all measurements. The cells were challenged by either $F(ab')_2$ fragments (——) or Fab' fragments (- - -) of anti-RBL at 50 μg/ml. Each point is an average of duplicate measurements. [^{3}H]Methyl incorporation and ^{45}Ca uptake by unstimulated cells were 41 ± 8 dpm/1.2 × 10^5 cells and 592 ± 39 cpm/10^6 cells, respectively. Spontaneous histamine release from the cells was 5%. These values were subtracted from experimental values. Five separate experiments of the same design gave similar results.

rat IgE per ml for 30 min at 4°C to saturate IgE receptors. After washings, the cells were preincubated with L-[*methyl*-^{3}H]-methionine and then challenged with a concentration (1–2 μg/ml) of anti-IgE optimal for histamine release. Anti-IgE also enhanced incorporation of [^{3}H]methyl into the lipid fraction and ^{45}Ca influx. The sequence of phospholipid methylation, ^{45}Ca uptake, and histamine release observed in this system was similar to that obtained by challenging unsensitized mast cells with the F(ab')$_2$ of anti-RBL (Fig. 2). The results indicated that bridging of IgE receptors, either directly by divalent antireceptor antibodies or indirectly through receptor-bound IgE and anti-IgE, is necessary for the stimulation of phospholipid methylation in the plasma membrane of mast cells.

Effect of Inhibitors of Methyltransferases on ^{45}Ca Influx and Histamine Release. An apparent correlation among the stimulation of phospholipid methylation, enhancement of Ca^{2+} uptake, and histamine release suggested strongly that phospholipid methylation is associated with the process of opening Ca^{2+} channels. We examined the effect of inhibitors of *S*-adenosyl-L-methionine-mediated methylations on ^{45}Ca influx and histamine release. Purified mast cells were preincubated in 1–30 μM of *S*-isobutyryl-3-deaza-adenosine for 1 hr at 4°C and then were challenged with anti-RBL. [^{3}H]Methyl incorporation into the lipid fraction, ^{45}Ca uptake, and histamine release all were inhibited in a similar dose-dependent manner (Fig. 3). The correlation coefficient (r^2) between the inhibition of phospholipid methylation and the inhibition of ^{45}Ca uptake was 1.00; that between the inhibitions of ^{45}Ca influx and histamine release was 0.99 (Fig. 4). The results suggest close association among phospholipid methylation, Ca^{2+} influx, and histamine release. We also examined the effect of 3-deazaadenosine on the anti-RBL-induced reactions; both phospholipid methylation and an increase in ^{45}Ca influx were inhibited by 3-deaza-adenosine in a dose-response fashion in the range 0.1 to 6.4 μM. Inhibition of these reactions was significantly enhanced in the presence of 1–10 nM of L-homocysteine thiolactone, which alone had no inhibitory effect on either phospholipid methylation or ^{45}Ca influx.

Experiments were carried out to show that methyltransferases are involved in IgE-mediated ^{45}Ca influx and histamine release. Mast cells were saturated with monoclonal rat IgE and challenged with anti-IgE in the presence of *S*-isobutyryl-3-deaza-adenosine; [^{3}H]methyl incorporation into phospholipids, ^{45}Ca influx, and histamine release were inhibited at 1–10 μM. Dose–response curves of inhibition were identical to those observed in anti-RBL-induced reactions.

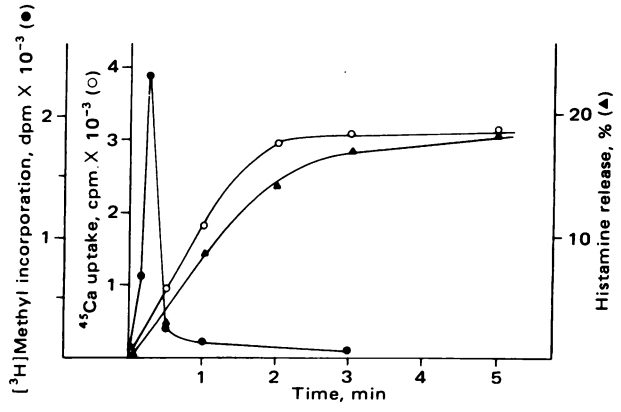

FIG. 2. Kinetic studies of [^{3}H]methyl incorporation (●), ^{45}Ca uptake (○), and histamine release (▲) induced by anti-IgE. Purified mast cells were incubated with rat IgE (100 μg/ml) at 4°C for 30 min, washed, and then challenged by anti-rat IgE antibody (1.6 μg/ml). [^{3}H]Methyl incorporation and ^{45}Ca uptake by unstimulated cells were 73 ± 9 dpm/1.1 X 10^5 cells and 220 ± 16 cpm/10^6 cells, respectively. Spontaneous histamine release from the cells was 4%. These values were subtracted from the experimental values. Three more experiments of the same design gave similar results.

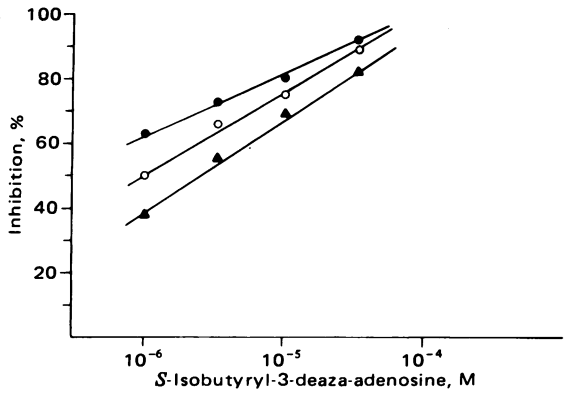

FIG. 3. Inhibition of anti-RBL-induced [^{3}H]methyl incorporation (●), ^{45}Ca uptake (○), and histamine release (▲) by *S*-isobutyryl-3-deaza-adenosine. Purified mast cells were preincubated with inhibitor at 4°C for 1 hr, and then challenged with anti-RBL (2 μg/ml). Each point is an average of duplicate measurements. The [^{3}H]methyl incorporation, ^{45}Ca uptake, and histamine release in the absence of inhibitor were 2094 ± 66 dpm/1.1 X 10^5 cells, 2837 ± 106 cpm/10^6 cells, and 19%, respectively. Representative results from four separate experiments are shown.

Identification of Methylated Products. To exclude the possibility that *S*-isobutyryl-3-deaza-adenosine inhibits the other transmethylating reactions, the main products, in mast cells, from L-[*methyl*-^{3}H]methionine were analyzed. Representative results are shown in Table 1. Free methionine represented approximately 80% of the total trichloroacetic acid-soluble radioactivity as measured by thin-layer chromatography. The radioactive *S*-adenosyl-L-methionine was rapidly utilized after the stimulation with anti-RBL or anti-IgE, probably for phospholipid methylation. When mast cells were pretreated with *S*-isobutyryl-3-deaza-adenosine at a concentration that inhibited ^{45}Ca influx and histamine release, only phospholipid methylation was blocked. No significant difference could be detected in the amounts of [^{3}H]methyl incorporation into nucleotides, *S*-adenosylmethionine, and proteins.

Chromatographic analysis of methylated phospholipids on silica gel G plates revealed that the formation of monomethyl and dimethyl phosphatidylethanolaminine and phosphatidylcholine markedly increased upon stimulation with anti-RBL.

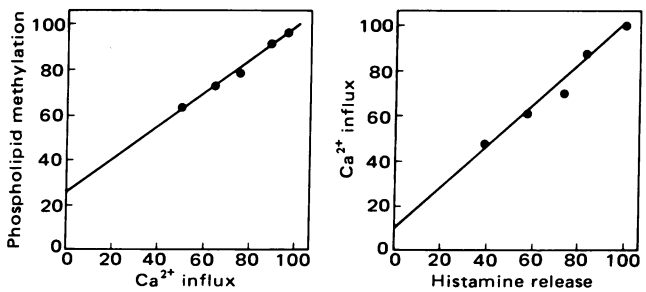

FIG. 4. Correlation between phospholipid methylation and ^{45}Ca influx (*Left*) and between ^{45}Ca influx and histamine release (*Right*). Phospholipid methylation, ^{45}Ca influx, and histamine release were measured in the absence and presence of *S*-isobutyryl-3-deaza-adenosine at various concentrations; linear regression analysis was performed to obtain a best fit line.

Table 1. Incorporation of [^{3}H]methyl into mast cell components after stimulation with anti-RBL

Conditions*	Acid-soluble dpm		Acid-insoluble dpm		
	Total	*S*-Ado-Met	Lipids	Nucleotides	Proteins†
0 time	10,756	1252	101	51	6930
Anti-RBL	7,169	104	1143	61	6665
Anti-RBL + inhib.	10,889	1201	304	59.5	7125

The variation in [^{3}H]methyl incorporation was wide from one preparation to another. Within the same experiment, SEM was approximately ± 5%. Precipitation was with trichloroacetic acid.

* The cells were preincubated with 2 μM L-[*methyl*-^{3}H]methionine for 30 min at 37°C. The reaction was started by the addition of anti-RBL and terminated by addition of ice-cold saline. The incubation was for 15 sec. inhib, 6.4 μM *S*-isobutyryl-3-deaza-adenosine.

† These values include both direct incorporation of methionine into proteins and methylation of arginine and lysine residues of proteins. Carboxymethylation of proteins could not be detected in a significant amount under the present conditions.

Also, [*methyl*-^{3}H]lysophosphatidylcholine, produced from phosphatidylcholine by the action of phospholipase A_2, was detectable at 15 sec after the stimulation.

DISCUSSION

Data presented in this paper show that divalent anti-receptor antibodies or anti-IgE cause a rapid increase and decrease in phospholipid methylation in rat mast cells. The peak in methylation was achieved in 15 sec, and this process was followed by an influx of Ca^{2+} into mast cells and histamine release. Binding of monovalent fragments of the anti-receptor antibodies to IgE-receptors did not stimulate any of the three processes, indicating that bridging of IgE receptors is required to stimulate this sequence of events. Inhibition of methyltransferase reactions by *S*-isobutyryl-3-deaza-adenosine prevented an increase in ^{45}Ca influx and histamine release. Among the many methylation reactions, phospholipid methylation is most vulnerable to inhibition by this inhibitor as shown here and elsewhere (14). Because phospholipid methylation precedes Ca^{2+} influx and histamine release and all three reactions are blocked by methyltransferase inhibitors, it is likely that increased phospholipid methylation sets the stage for Ca^{2+} influx and subsequent histamine release. This idea explains the previous findings on Con A-induced histamine release from normal mast cells by Hirata *et al.* (10). They demonstrated that Con A causes stimulation of phospholipid methylation in the absence of Ca^{2+}, and subsequent addition of Ca^{2+} to the Con A-treated mast cells resulted in the disappearance of methylated phospholipids and release of histamine. More recent experiments on RBL cells sensitized with rat IgE extended this finding (unpublished data); challenge of the sensitized RBL cells with anti-IgE increased phospholipid methylation, and the decrease in methylphospholipid was associated temporally with the liberation of arachidonic acid. Inhibition of phospholipid methylation in this system blocked both arachidonic acid liberation and histamine release.

Hirata and Axelrod (15) have shown that phospholipids are methylated by two enzymes in plasma membrane. The first enzyme converts phosphatidylethanolamine, which faces the cytoplasmic side of the membrane, to phosphatidyl-*N*-monomethylethanolamine; the second enzyme adds two methyl groups to form phosphatidylcholine which is oriented to the outer surface of the membrane. It was found that the process of methylation and translocation of the phospholipids changes the viscosity of the cell membrane (16). In rat reticulocytes, an increased lipid methylation and membrane fluidity facilitated the lateral mobility and coupling of the receptors with adenylcyclase (17). Furthermore, the present experiments indicated an important role of methyltransferases in opening Ca^{2+} channels. Preliminary experiments indicate that phospholipid methylation also influences Ca^{2+} influx into lymphocytes, neuroblastoma cells, and fibroblasts (unpublished data).

Activation of phospholipid methylation by bridging of IgE receptors on mast cells strongly suggests that IgE receptors are in close association with membrane methyltransferases. The possibility still remains, however, that the bridging of IgE receptors may first activate some other enzyme(s) which subsequently activates methyltransferases. Our more recent experiments have shown that activation of phospholipid methylation by IgE receptor bridging was inhibited by increasing the intracellular cyclic AMP level. Considering that the intracellular cyclic AMP level in normal mast cells increases upon bridging of cell-bound IgE by anti-IgE (18), the topographical relationship among IgE receptors, methyltransferases, and β-adrenergic receptors in the mast cell membrane has to be elucidated for further understanding of biochemical process of cell activation.

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