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INHIBITION OF PHOSPHOLIPID METHYLATION BY AN ANTI-ALLERGIC AGENT, NCO-650, DURING HISTAMINE RELEASE

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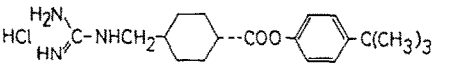
Abstract—Antigen, anti-IgE and concanavalin A (Con A) induced an increase in both the incorporation of the ^{3}H-methyl moiety into phospholipids and histamine release. Maximal incorporation of the ^{3}H-methyl moiety into the lipid fraction of the cells was observed within 15 sec and 1 min after being challenged with antigen (100 μg/mL) and anti-IgE (200 μg/mL) respectively. However, the methylated phospholipid decreased rapidly. The addition of Con A (10 μg/mL) also increased phospholipid methylation, which reached a maximum at 5 min after challenge. *Trans*-4-guanidinomethylcyclohexanecarboxylic acid *p-tert*-butylphenyl ester hydrochloride (NCO-650; 27 μM) strongly inhibited the incorporation of the ^{3}H-methyl moiety into phospholipid by antigen, anti-IgE and Con A. The IC_{50} values of NCO-650 for phospholipid methylation in response to antigen, anti-IgE and Con A were 1.5, 4.7 and 1.1 μM respectively. Although the Ca^{2+}-ionophore A23187 did not induce phospholipid methylation, it caused histamine release.

Mast cells and basophils have specific receptors towards IgE. It has been shown that either multivalent antigen or divalent anti-IgE antibody bridges with IgE on the cell membrane with subsequent release of histamine from the cell [1]. Siraganian and Siraganian [2] have reported that concanavalin A (Con A)-induced histamine release is due to the lectin-cell bound IgE bridging. Moreover, Hirata *et al.* [3] have reported that membrane phospholipids are methylated in the early stage of histamine release from rat peritoneal mast cells by Con A.

Studies by Ishizaka *et al.* [4] have demonstrated a transient increase in phospholipid methylation of up to 120-fold during IgE-dependent activation of rat mast cells.

We reported that *trans*-4-guanidinomethylcyclohexanecarboxylic *p-tert*-butylphenyl ester hydrochloride (NCO-650) is a strong and specific inhibitor for trypsin and pH 7 tryptase [5] and that it inhibits markedly histamine release from rat peritoneal mast cells induced by antigen, Con A, the Ca^{2+}-ionophore A23187 and compound 48/80 [6]. NCO-650 has no action on H_1- and H_2-receptors. In the present study we examined phospholipid methylation activities in rat peritoneal mast cells in response to antigen, anti-IgE and Con A, and the effect of NCO-650 on these processes.



Structure of NCO-650

MATERIALS AND METHODS

Mast cells were prepared as described by Saeki [7], and purified mast cells according to the method of Németh and Röhlich [8]. The purity of the mast cell preparation was in the range of 90%. Viability of the cells was >97% as assessed by trypan blue exclusion. Rats were immunized by an intramuscular injection of 0.1 mL of 1% egg albumin in saline followed by a subcutaneous injection of 1 mL of Bordetella pertussis vaccine (containing 2×10^{10} heat-killed Bordetella cells/mL). After 2 weeks, the rats were killed, and sera that gave a precipitin line against anti-rat IgE were used for the preparation of passively sensitized mast cells. Purified mast cells from normal rats were incubated with immunized rat sera for 1 hr at 4° [6]. Each secretagogue was incubated with mast cells at 37° in the presence or absence of NCO-650. Tyrode–Hepes solution contained 124 mM NaCl, 4.0 mM KCl, 1.0 mM $CaCl_2$, 10 mM $NaHCO_3$, 5.6 mM glucose, 0.64 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (Hepes), 0.05% gelatin, 50 mg of phosphatidylserine and 50 mg of bovine serum albumin (BSA)/L. BSA was omitted from the solution in experiments on histamine release. At intervals, the reaction was stopped by the addition of 2 mL of Tris-EDTA buffer (25 mM Tris, 120 mM NaCl, 5 mM KCl, 1 mM EDTA and 0.2 g of human serum albumin/L). Then the cells were separated from the released histamine by centrifugation at 1300 *g* for 10 min at 4°. Histamine remaining in the cells was released by disrupting the cells with 100% trichloroacetic acid (TCA) (final concentration 10% TCA) and centrifugation at 1500 *g* for 15 min at 4°. Histamine was determined fluorometrically [9]. The amount of

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histamine released was expressed as the percentage of the total histamine present in unstimulated cells.

Determination of phospholipid methylation. Phospholipid methylation was determined as described by Ishizaka *et al.* [10]. Purified mast cells were suspended in Tyrode's solution containing 2 μM-L-[*methyl*-^{3}H]methionine (200 mCi/mmol), phosphatidylserine (50 μg/mL) and 0.05% gelatin. After incubation for 30 min at 37°, 50-μL aliquots of the cell suspension containing 2×10^5 cells were incubated with 10 μL of an appropriate concentration of histamine releasers. The reaction was stopped by the addition of 500 μL of ice-cooled 10% TCA containing 10 mM L-methionine, and the tubes were centrifuged at 18,000 *g* for 10 min at 10°. The precipitates were washed with 10% TCA and then extracted with 3 mL of chloroform/methanol (2:1 v/v). The chloroform phase was washed twice with 1.5 mL of 0.1 M KCl in 50% methanol. A 1-mL fraction of the chloroform phase was transferred to a counting vial and evaporated to dryness in an oven at 80°. The residue was dissolved in 10 mL of scintillation solution (2.1 L xylene, 0.9 L NS 210, 12 g PPO,* 0.6 g POPOP and 330 mL methanol, and the radioactivity was determined in a liquid scintillation spectrometer (Packard, model 3385). Identification of methylated phospholipids was carried out by thin-layer chromatography as described by Hirata *et al.* [11]. The cell suspensions were incubated with an inhibitor for 5 min before the addition of the various histamine releasers. Tyrode's solution contained 124 mM NaCl, 4.0 mM KCl, 1 mM $CaCl_2$, 10 mM $NaHCO_3$, 0.64 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 5.6 mM glucose, 5 mM Hepes, 5 mM morpholinoethane sulfonic acid, 0.5 g of gelatin/L and phosphatidylserine (50 μg/mL), and was dispersed in the solution by sonication.

Identfication of phospholipid methylation products. Identification of methylated phospholipids was carried out by thin-layer chromatography as described by Hirata *et al.* [11]. The chloroform layer obtained from the chloroform/methanol extracts was evaporated under reduced pressure, and the residue was dissolved in 50 μL of chloroform/methanol (2:1, v/v). Then 10 μL of each solution was applied to a TLC plate (LK5DF, 250 μm thickness, Whatman), and developed for 4 hr with *n*-propanol/propionic acid/chloroform/water (3:2:2:1, by vol.). Lipid spots identified with iodine staining and corresponding to known R_f values of authentic standards (PMME, PDME, PC and LPC) were scraped into scintillation vials, and dispersed by sonication (Sonifer, model B-185, Bronson) with 400 μL of methanol and 8 mL of scintillation solution. ^{3}H-Methyl-label was determined by scintillation spectrometry.

NCO-650 was obtained from the Nippon Chemiphar Co., Ltd. Anti-IgE was purchased from Miles Laboratories. The Ca^{2+}-ionophore A23187 was purchased from Calbiochem. Phosphatidylserine and egg albumin were purchased from the Sigma Chemical Co. L-(*methyl*-^{3}H)Methionine (200 mCi/mmol) was obtained from New England Nuclear. NS 210 (polyethyleneglycolether) was purchased from Nippon Yushi Ltd., Tokyo.

* Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; PC, phosphatidylcholine; and LPC, lysophosphatidylcholine.

RESULTS

The effect of NCO-650 on phospholipid methylation activity in rat peritoneal mast cells was examined after the addition of an optimal concentration of antigen (egg albumin; 100 μg/mL).

At 15 sec after the addition of antigen, the incorporation of the ^{3}H-methyl moiety into the phospholipid reached a maximum, and phospholipid methylation decreased rapidly within 1 min. However, with NCO-650 (27 μM) the incorporation of the ^{3}H-methyl moiety into the membrane phospholipid was inhibited markedly (Fig. 1). The inhibition by NCO-650 of methylation was 87.0 ± 1.5% at 15 sec after the addition of antigen. At 27 μM, the inhibition by NCO-650 of antigen-induced histamine release from rat mast cells at 5 min was 78.8 ± 1.7%. The magnitude of this inhibitory effect was quite similar to the inhibition of phospholipid methylation by NCO-650 (Fig. 1).

Figure 2 shows the effect of NCO-650 on phospholipid methylation after the addition of an optimal concentration of anti-IgE (200 μg/mL). At 1 min after the addition of anti-IgE, the incorporation of the ^{3}H-methyl moiety reached a maximum and then decreased rapidly after 2 min. Maximum methylation induced by anti-IgE was later than that by antigen, and histamine release by anti-IgE was slower than that by antigen. Therefore, there seemed to be some correlation between the time course of methylation and that of histamine release. NCO-650 (27 μM) markedly inhibited the methylation induced by anti-IgE; the inhibitory effect was 70.1 ± 1.2% at 1 min after addition.

Histamine release stimulated by anti-IgE resembled that stimulated by Con A. NCO-650 inhibited the histamine release induced by anti-IgE, and its inhibitory effect at 5 min was 83.3 ± 1.85% (Fig. 2). The methylation in response to Con-A (10 μg/mL) was found to be slower than that caused by antigen or anti-IgE (Fig. 3). At 4 min after being challenged by Con A, the incorporation of the ^{3}H-methyl moiety reached a maximum and then decreased rapidly after 5 min. Histamine release in response to Con A was much slower than that caused by antigen or anti-IgE. In the presence of NCO-650 (27 μM), phospholipid methylation in response to Con A was suppressed extensively, and the inhibitory effect was 87.0 ± 2.3% at 4 min after challenge (Fig. 3). NCO-650 also concentration-dependently inhibited the histamine release induced by Con A, and its inhibitory effects at 10 min was 77.4 ± 1.6% at 27 μM (Fig. 3).

The Ca^{2+}-ionophore A23187 did not induce phospholipid methylation (unpublished observation. Takei *et al.*).

We examined the methylation products in chloroform/methanol extract by thin-layer chromatography after activation of mast cells with antigen, anti-IgE and Con A. Figure 4 shows the results of maximal phospholipid methylation after addition of

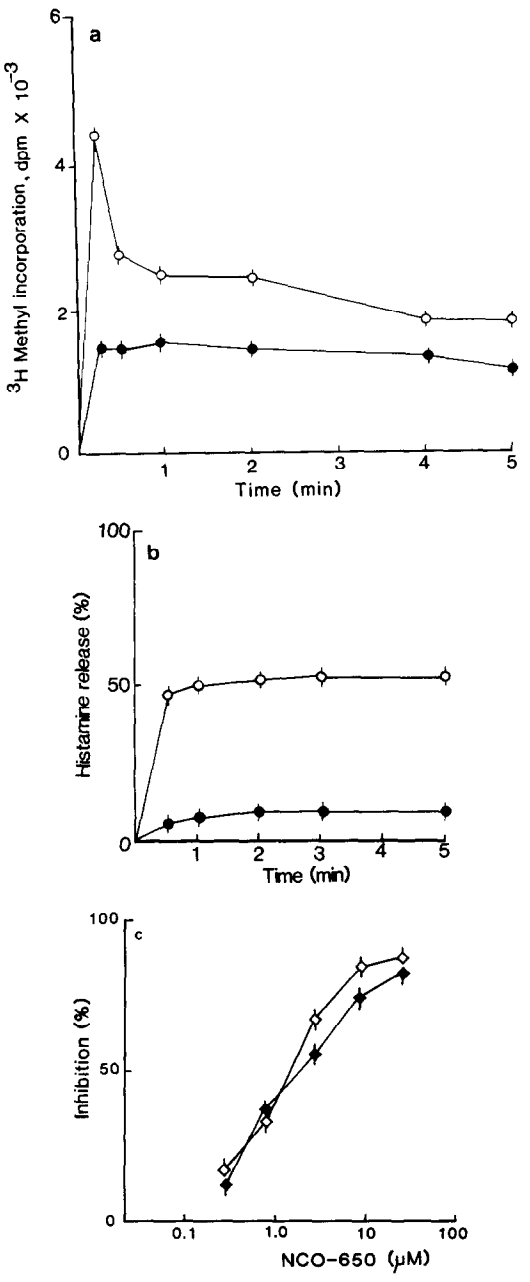

Fig. 1. Effect of NCO-650 on the kinetics of ^{3}H-methyl incorporation (a) and histamine release (b) induced by antigen (100 μg/mL) in rat mast cells. Key: (○) without NCO-650, and (●) with NCO-650 (27 μM). (c) Concentration–response curve for the inhibition of antigen-induced ^{3}H-methyl incorporation (◇), and histamine release (◆) by NCO-650. ^{3}H-Methyl incorporation by unstimulated cells was 53 ± 8 dpm/5 × 10^5 cells. Spontaneous histamine release from mast cells was 5.5 ± 0.12%, and this value was subtracted from each experimental value. Each value is the mean (±SE) of five experiments.

antigen (20 sec), anti-IgE (30 sec) and Con A (3 min). As compared to the control, the ^{3}H-methyl moiety was obviously incorporated into phospholipid of the mast cells challenged with histamine releasers.

NCO-650 (27 μM) strongly inhibited ^{3}H-methyl incorporation into phospholipid.

The inhibitory effects of NCO-650 on the phospholipid methylation by anti-IgE at 2.7, 8.1 and 27 μM were 40.0 ± 1.3, 61.2 ± 2.1 and 80.0 ± 2.0% respectively. The phospholipid methylation in response to antigen or Con A were also concentration-dependently inhibited by NCO-650.

The IC_{50} of values NCO-650 for the phospholipid methylation induced by antigen, anti-IgE and Con A were 1.5, 4.2 and 1.1 μM respectively. Furthermore, the IC_{50} values of NCO-650 for histamine release induced by antigen, anti-IgE and Con A were also in the micromolar range, being 1.9, 3.6 and 4.6 μM respectively.

When the cells were transferred to Mg^{2+}-free buffer, histamine release induced by anti-IgE was not affected significantly in comparison to the control sample (Fig. 5). In the Mg^{2+}-free buffer, the incorporation of ^{3}H-methyl into phospholipid by anti-IgE was greatly inhibited in comparison to the control sample. Similar results were obtained from the incorporation of the ^{3}H-methyl moiety into phospholipid and histamine release induced by antigen and Con A.

DISCUSSION

This paper reports that NCO-650 strongly and concentration-dependently inhibited phospholipid methylation in rat peritoneal mast cells induced by antigen, anti-IgE and Con A.

Previously, we reported the strong inhibitory effect of NCO-650 on histamine release by various secretagogues such as antigen, anti-IgE, Con A and the Ca^{2+}-ionophore A23187 [6]. Moreover, we found a trypsin-like protease (tryptase) with an optimal pH of 7.0 in mast cells and suggested the involvement of a trypsin-like protease in histamine release [5].

Siraganian and Siraganian [2] suggested that binding of anti-IgE to the membranous IgE receptor causes phospholipid methylation with subsequent Ca^{2+} influx, arachidonic acid release and histamine release. It is questionable that phospholipid methylation is associated with histamine release from mast cells. Benyon *et al.* [12] reported unsuccessful attempts to demonstrate increased lipid methylation following IgE-dependent stimulation of rat mast cells. Based on the experimental protocol of Ishizaka *et al.* [10], we attempted and confirmed a transient increased in ^{3}H-methyl incorporation in the chloroform extracts from the Con A-, anti-Ige-, and antigen-activated mast cells and identified the methylation and degradation products PMME, PDME, PC and LPC by thin-layer chromatography. The major difference between our method and that of Benyon *et al.* was the addition of 10% TCA to stop the reaction followed by centrifugation. We applied 18,000 *g*, whereas they applied 1200 *g* to the sample. It is rather difficult to explain the discrepancy based only on the difference in centrifugation. However, many other researchers have also reported a positive methylation reaction. Hirata *et al.* [3] and Morita and Siraganian [13] have reported no increase in phospholipid methylation in rat mast cells by

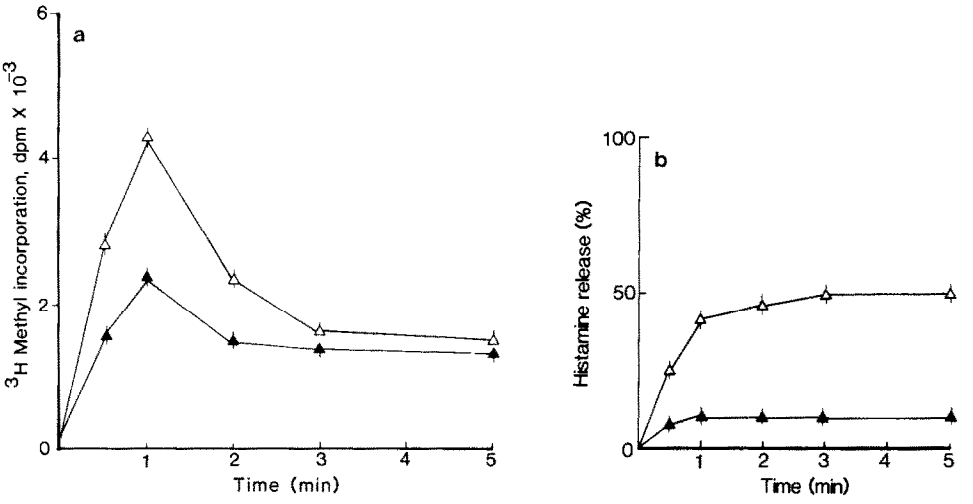

Fig. 2. Effect of NCO-650 on the kinetics of ^{3}H-methyl incorporation (a) and histamine release (b) induced by anti-IgE (200 μg/mL) in rat mast cells. Key: (△) without NCO-650, and (▲) with NCO-650 (27 μM). ^{3}H-Methyl incorporation by unstimulated cells was 50 ± 5 dpm/5 × 10^5 cells. Spontaneous histamine release from mast cells was 5.8 ± 0.21%, and this value was substracted from each experimental value. Each value is the mean (±SE) of five experiments.

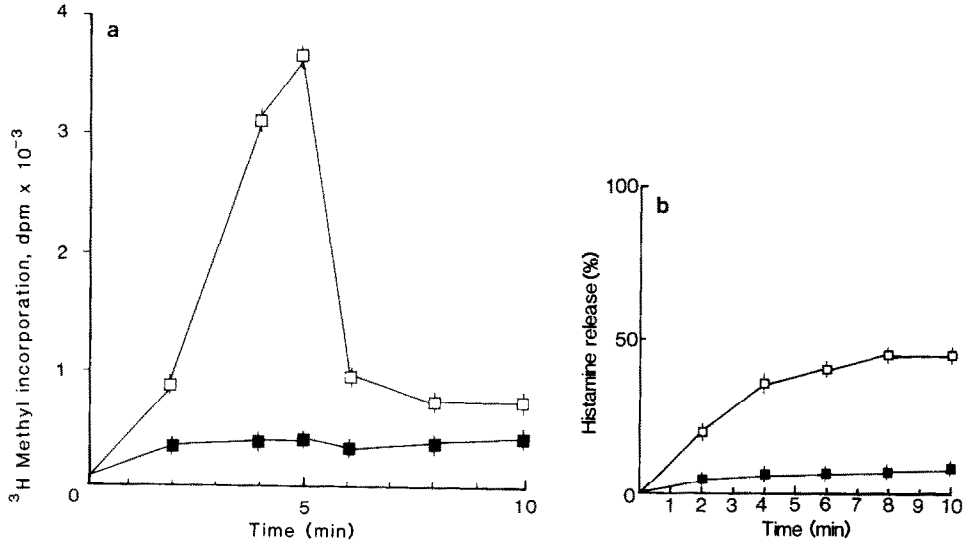

Fig. 3. Effect of NCO-650 on the kinetics of ^{3}H-methyl incorporation (a) and histamine release (b) induced by Con A (10 μg/mL) in rat mast cells. Key: (□) without NCO-650, and (■) with NCO-650 (27 μM). ^{3}H-Methyl incorporation by unstimulated cells was 57 ± 8 dpm/5 × 10^5 cells. Spontaneous histamine release from mast cells was 6.5 ± 0.21%, and this value was subtracted from each experimental value. Each value is the mean (±SE) of five experiments.

compound 48/80 or A23187. Furthermore, accumulated evidence shows that enzymes and methanisms involved in histamine release are different depending on the stimulus [14]. We have also shown that phospholipid methylation did not occur with compound 48/80 or A23187, although these drugs induced histamine release under the same experimental conditions. We have reported strong inhibitory effects of NCO-650 on histamine release induced by compound 48/80 and A23187 [6]. In our present study, the percentage of histamine release induced by antigen, anti-IgE or Con A in the absence of Mg^{2+} was not significantly different from that induced by the respective agents, in the presence of Mg^{2+}. In contrast, methylation of phospholipid by antigen, anti-IgE or Con A markedly diminished in the absence of Mg^{2+}. The results suggest that phospholipid methylation is not essential for histamine release. However, one cannot exclude the possibility that a small amount of phospholipid methylation may be sufficient for proceeding with the biochemical pathway for histamine release.

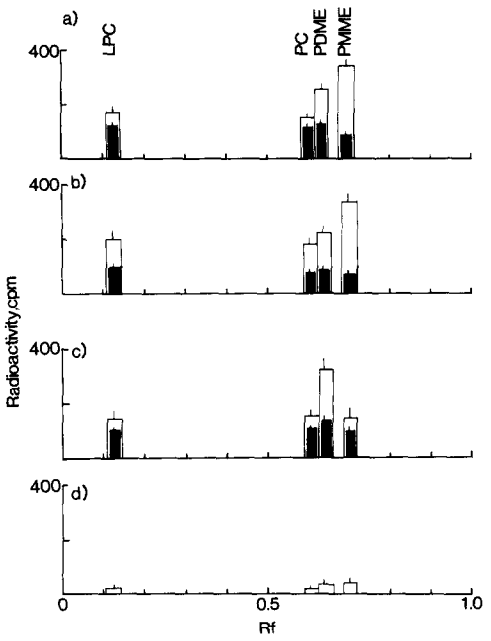

Fig. 4. Chromatographic pattern of the ^{3}H-methylated phospholipids on a TLC plate, and the effect of NCO-650 on phospholipid methylation. (a) Challenged with 100 μg/mL of antigen (egg albumin) and incubated for 20 sec. (b) Challenged with 200 μg/mL of anti-IgE and incubated for 30 sec. (c) Challenged with 10 μg/mL of Con A and incubated for 3 min. (d) Unchallenged mast cells. Blank column: without NCO-650; Black column: with NCO-650. Values are means ± SE for five experiments.

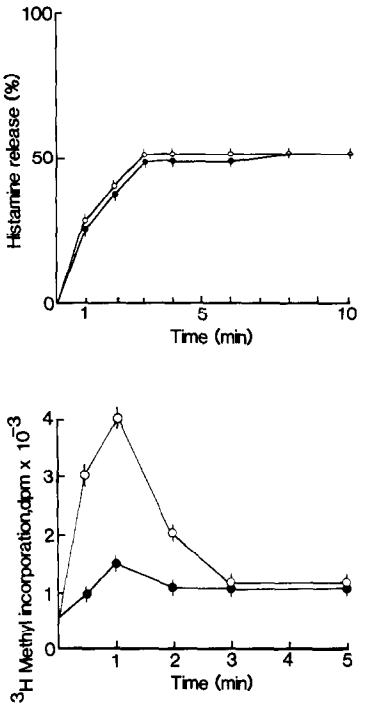

Fig. 5. Kinetics of histamine release and ^{3}H-methyl incorporation induced by anti-IgE (200 μg/mL). Key: (○) normal Hepes–Tyrode, and (●) Mg^{2+}-free Hepes–Tyrode. Spontaneous histamine release from mast cells was 5.5 ± 0.11%, and this value was subtracted from each experimental value. ^{3}H-Methyl incorporation by unstimulated cells was 52 ± 5 dpm/5 × 10^5 cells. Each value is the mean (±SE) of five experiments.

The possible participation of protease(s), probably a serine esterase, in the early stage of antigen-induced activation of rat mast cells for histamine release was first described by Austen and Brocklehurst [15].

Ishizaka *et al.* [16] suggested that the serine protease participated in the early stage of histamine release, particularly in phospholipid methylation and cyclic AMP (cAMP) formation from inhibition experiments with substrates or inhibitors for trypsin and chymotrypsin, and that the protease is activated by receptor binding before activation of methyltransferase.

The IC_{50} values of NCO-650 on phospholipid methylation in response to antigen, anti-IgE and Con A showed low values of 1.1 to 4.2 μM, and these values were similar to those of histamine release [6]. We have reported that chymase and trypsin-like protease with an optimal pH of 7, pH 7 tryptase (tryptase), are purified from rat peritoneal mast cells, and that NCO-650 strongly inhibits only the activity of this trypsin-like protease [5]. These results suggested that tryptase participates in histamine release.

The inhibitory effect of NCO-650 on phospholipid methylation induced by antigen, anti-IgE and Con A was not due to its cytotoxic action. The effect of NCO-650 disappeared entirely after the cells were washed. The purity of NCO-650 was above 99%; hence, it is not possible that another non-specific inhibitor of methylation was included. Therefore, the effect of NCO-650 is specific.

In conclusion, NCO-650 strongly and concentration-dependently inhibited not only histamine release from rat peritoneal mast cells induced by antigen, anti-IgE and Con A, but also phospholipid methylation induced by antigen, anti-IgE and Con A. Since NCO-650 is a specific inhibitor of trypsin-like enzyme, it is suggested that trypsin-like protease is involved in the histamine release process.

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