Study of the Mechanism of Inhibitory Action of Tranilast on Chemical Mediator Release

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Abstract—We investigated the mechanism of inhibitory action of tranilast on chemical mediator release by antigen-antibody reactions. Tranilast (10^{-5}–10^{-3} M) inhibited antigen (DNP-Ascaris)-induced histamine release from sensitized purified rat mast cells (PMC), but did not show an obvious influence on intracellular cyclic AMP. \(^{45}\text{Ca}\) uptake into PMC induced by antigen (300 \(\mu\)g/ml) was obviously suppressed by tranilast (10^{-6}–10^{-3} M). Tranilast (10^{-4} M) inhibited antigen-induced histamine release from and \(^{45}\text{Ca}\) uptake into PMC independently of the presence or absence of glucose in the medium. On the other hand, 2-deoxyglucose (10^{-2} M) markedly inhibited both responses in the absence but not in the presence of glucose. Tranilast slightly inhibited Ca-induced contraction of guinea pig taenia coli, but had no influence on aggregation of rabbit platelets. Verapamil (10^{-6}–10^{-4} M) had no effect on antigen-induced histamine release, but it markedly suppressed Ca-induced contraction and platelet aggregation. From these results, we suggest that the mechanism of inhibitory action of tranilast on the release of antigen-induced chemical mediator from mast cells involves the suppression of Ca uptake, but that its mode of action is apparently different from those of 2-deoxyglucose and verapamil.

N-(3, 4-dimethoxycinnamoyl) anthranilic acid (tranilast), one of the anti-allergic drugs, has been reported to inhibit the release of chemical mediators such as histamine and slow-reacting substance of anaphylaxis (SRS-A) by antigen-antibody reactions (1–9). Therefore, the clinical efficacies of tranilast on bronchial asthma (10), allergic dermatitis (11) and allergic rhinitis (12) are believed to be due to the inhibition of release of chemical mediators.

Although the effect of tranilast is distinguished from the drugs which increase intracellular cyclic AMP (cAMP), disodium cromoglycate (DSCG) which increases intracellular cAMP and inhibits the Ca uptake into cells and papaverine which acts on an energy-requiring process in the histamine release (5–7), the mechanisms of the inhibitory action of tranilast on the release of chemical mediators induced by the antigen-antibody reactions are not clarified yet. The concentrations at which tranilast inhibits the release of chemical mediators are approx. 10^{-6} to 10^{-3} M in various in vitro studies (3–9). Accordingly, the mechanisms of the inhibitory action of tranilast may involve the suppression of the same pathway involved in the release of chemical mediators.

It is generally known that mast cells bear IgE receptors and cross-linking of cell-bound IgE antibody molecules by multivalent ligands triggers the release of a variety of chemical mediators. The biochemical studies of the mechanisms of mast cell activation revealed that bridging of IgE receptors induces phospholipid methylation, a transient increase in cAMP, mobilization of intracellular Ca and Ca influx and enhancement of phosphatidylinositol turnover (13–15).
Therefore, we investigated the effect of tranilast on the antigen-induced changes in intracellular cAMP level and Ca movement using rat purified mast cells (PMC).

Materials and Methods

Preparation of the suspension of PMC and peritoneal exudate cells (PEC) of rats

a) Rat PMC: Murine hybridoma which secretes monoclonal IgE antibodies of anti-2, 4-dinitrophenyl (DNP) specificity was obtained by a fusion of X653Ag8 tumor cells and spleen cells from DNP-ascaris-hyperimmunized BALB/c mice. The supernatants of murine hybridoma give a threshold reaction of the passive cutaneous anaphylaxis (PCA) in the rat at a dilution of 1/2000. The supernatant was injected into the pleural and peritoneal cavities of male Wistar rats. After 16 hr, animals were exsanguinated by decapitation, and the exudate cells were harvested from the pleural and peritoneal cavities with a medium of the following composition: 150 mM NaCl, 3.7 mM KCl, 3.0 mM Na2HPO4·12H2O, 3.5 mM KH2PO4, 0.9 mM CaCl2·2H2O, 5.6 mM glucose, 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO, U.S.A.) and 5 units/ml heparin, pH 6.9. The suspension of sensitized rat PMC was incubated for 5 min at 37°C, 200 or 300 ug/ml of DNP-Ascaris was added as an antigen, and then the cells were incubated for a further 30 min. The reaction mixture was centrifuged at 35000×g for 10 min at 0°C and layered over 5 ml of 38% BSA solution as described by Sullivan et al. (16). The cell suspension was centrifuged at 35000×g for 10 min at 0°C and layered over 5 ml of 38% BSA solution as described by Sullivan et al. Subsequently, mast cells with a purity approx. 90% were obtained from the BSA layer.

b) Rat PEC: Male Wistar rats were sacrificed by a blow on the head and exsanguination by decapitation. Rat PEC suspension was prepared as described by Nakazawa et al. (5) using a phosphate-buffered salt solution (PBS) containing 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2·6H2O, 5.6 mM glucose, 5 units/ml heparin and 5% (v/v) sodium-potassium phosphate buffer (0.1 M, pH 7.2).

Assay of histamine and cAMP

The suspension of sensitized rat PMC was incubated for 5 min at 37°C, 200 or 300 ug/ml of DNP-Ascaris was added as an antigen, and then the cells were incubated for a further 30 min. The reaction mixture was cooled in an ice bath and then centrifuged, the separated supernatant was mixed with 4% HClO4 and boiled for 3 min. After centrifugation, the histamine content of the supernatant was measured by the method described by Shore et al. (17). The cell pellet was frozen in liquid nitrogen: the freezing and thawing procedure was repeated 3 times, and finally, the material was lyophilized. The lyophilized was dissolved in distilled water. The cAMP level was measured using a cAMP Assay Kit (Yamasa, Chushi, Japan).

Assay of 45Ca uptake

Rat PMC was suspended in the Tyrode's solution containing 5.0 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES, Sigma, St. Louis, MO, U.S.A.), 5.0 mM 2-(N-morpholino)-ethanesulfonic acid (MES, Sigma), 0.1% BSA and 5 units/ml heparin, pH 6.9. The suspension of rat PMC was set on silicon oil (d=1.05, Aldrich, Milwaukee, WI, U.S.A.) for 5 min at 37°C as described by Ishizaka et al. (13). Three ug/ml 45CaCl2 (Amersham, Buchinghamshire, England) and 300 or 400 ug/ml of DNP-Ascaris was simultaneously added to the cell suspension, and the reaction mixture was further incubated for 5 min at 37°C. Tris-AEDTA buffer, pH 7.6 (0.025 M Tris, 0.12 M NaCl, 5 mM KCl, 0.01 M EDTA and 0.02% human serum albumin (Miles, Elkhart, IN, U.S.A.)), was added to the mixture and then it was centrifuged to remove the supernatant. The radioactivities of 45Ca in the cell pellets were measured by a scintillation counter (Tri-Carb 4640, Packard, Downers Grove, IL, U.S.A.).

Ca-induced contraction of guinea pig taenia coli

The taenia coli was isolated from a male guinea pig. The preparation was mounted at a load of 1 g in the Krebs’ solution not containing Ca (Ca(-) Krebs) and incubated at 28°C under aeration. After incubating for more than 1 hr, 1 mM of CaCl2 was added to the incubation medium. The contraction was isometrically recorded using force-displacement transducer (Model SB-1T, Nihon Kohden, Tokyo, Japan).

Aggregation of rabbit platelets

Rabbit blood was collected from the auricular artery and mixed with one-tenth volume of 3.8% sodium citrate. From each
blood sample, platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation. Collagen (Horm, Munchen, W. Germany) as coagulant was added to PRP (0.45 ml). Platelet aggregation was monitored using a platelet aggregation tracer (Model PAT-4A, Nikokizai, Tokyo, Japan).

Test drugs
Tranilast (Kissei, Matsumoto, Japan), verapamil (Sigma, St. Louis, MO, U.S.A.), DSCG (Fujisawa, Osaka, Japan), 2-deoxyglucose (Nakarai, Kyoto, Japan) and antimycin A (Boehringer Mannheim, Mannheim, W. Germany). Tranilast was dissolved in 1% NaHCO₃ and other drugs were dissolved in the incubation medium or 0.9% saline. All test drugs were diluted to the desired concentration by the incubation medium or 0.9% saline. Drugs were added 1 min before the addition of antigen when testing for the effect on cAMP level, 45Ca uptake and histamine release, and they were added 1 min before the addition of collagen when testing for the effect on platelet aggregation. When testing for the effect on Ca-induced contraction of guinea pig ileum, drugs were added 5 min before the addition of CaCl₂.

Results
Effect of tranilast on the release of histamine: As shown in Fig. 1, the histamine release from sensitized rat PMC by 300 μg/ml of DNP-Ascaris was 40%. Tranilast at 10⁻⁶ to 10⁻³ M suppressed the histamine release in a dose-dependent manner. The IC₅₀ of tranilast is approx. 10⁻⁴ M. DSCG (10⁻³ M) inhibited the release of histamine by about 55%.

Effect of tranilast on the release of histamine and cAMP content in rat PMC: Figure 2 shows the relationship between the release of histamine and the intracellular cAMP level in sensitized rat PMC. When 300 μg/ml of DNP-Ascaris was added to rat PMC, the histamine release was induced. The release reaction was rapid until 1 min after the antigen addition, and then it proceeded slowly. On the other hand, the intracellular cAMP decreased immediately after the antigen addition, then increased to a peak at 15 sec. The cAMP level restored to the initial control value 30 sec after and remained at this level afterwards. Although 10⁻⁶ to 10⁻³ M of tranilast markedly inhibited the release of histamine from rat in a dose-dependent manner, it had no apparent influence on the change in the intracellular cAMP.

Effect of tranilast and antimycin A on ⁴⁵Ca uptake into rat PMC: Figure 3 shows the effect of tranilast and antimycin A on the ⁴⁵Ca uptake into rat PMC by 300 μg/ml of
DNP-Ascaris. The \(^{45}\)Ca uptake into the cells increased 5 min after the addition of antigen to about 2 times the value in the absence of antigen. Tranilast at concentrations of \(10^{-6}\) to \(10^{-3}\) M markedly suppressed the \(^{45}\)Ca uptake in a dose-dependent manner, but did not influence the spontaneous \(^{45}\)Ca uptake (Fig. 4). Antimycin A at a concentration of \(10^{-5}\) M obviously decreased the antigen-induced \(^{45}\)Ca uptake into the cells.

**Effect of glucose on the inhibition of histamine release and \(^{45}\)Ca uptake by tranilast:** The degree of antigen-induced histamine release was similar regardless of the presence or absence of glucose in the medium (data not shown). The antigen-induced histamine release from and \(^{45}\)Ca uptake into rat and PEC occurred in spite of the absence of glucose in the medium. As shown in Fig. 5, \(10^{-4}\) M of tranilast suppressed histamine release and \(^{45}\)Ca uptake by 70 to 80% independently of the presence or absence of glucose. On the other hand, whereas 2-deoxyglucose, one of the metabolic inhibitors, slightly suppressed by 15 to 35% the histamine release and \(^{45}\)Ca uptake in the presence

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**Fig. 2.** Effects of tranilast on the histamine release from (upper panel) and cyclic AMP (cAMP) content of (lower panel) the sensitized purified rat mast cells after a challenge with 300 \(\mu\)g/ml of DNP-Ascaris as the antigen. Each value indicates the mean of 4 or 5 experiments. (○): control, (●): \(10^{-5}\) M of tranilast, (▲): \(10^{-4}\) M of tranilast, (■): \(10^{-3}\) M of tranilast.
of glucose, it markedly inhibited both responses in the absence of glucose (Fig. 5).

Effect of tranilast and verapamil on the histamine release, Ca-induced contraction and platelet aggregation: Figure 6A shows the effect of tranilast and verapamil on the histamine release induced by 200 µg/ml of DNP-Ascariis from sensitized rat PEC. Tranilast (10^{-4} to 10^{-3} M) markedly inhibited the histamine release, but 10^{-6} to 10^{-4} M of verapamil had no influence on the histamine release.

Fig. 3. Effect of tranilast and antimycin A (antimycin) on the 45Ca uptake into the sensitized purified rat mast cells after a challenge with 300 µg/ml of DNP-Ascariis as the antigen. Each value indicates the mean of 8 to 10 experiments. Vertical bars show the S.E. of the mean. Spon: spontaneous uptake, Cont: control, * and **: significant difference from spontaneous uptake and control at P<0.01, respectively (Student's t-test).

Fig. 4. Effect of tranilast on the spontaneous 45Ca uptake into the sensitized purified rat mast cells. Each value indicates the mean of 8 experiments. Vertical bars show the S.E. of the mean.

Fig. 5. Effects of tranilast and 2-deoxyglucose on the histamine release from and 45Ca uptake into the sensitized rat mast cells in the medium with glucose [G(+)] or without glucose [G(-)] after a challenge with 400 µg/ml of DNP-Ascariis as the antigen.
CaCl$_2$ (1 mM) caused a marked contraction of the guinea pig taenia coli immersed in the Ca(−) Krebs’ solution (data not shown). As shown in Fig. 6B, tranilast at $10^{-4}$ M did not influence the Ca-induced contraction. However, at a concentration of $3 \times 10^{-4}$ M or higher, it slightly inhibited the contraction. On the other hand, $10^{-8}$ to $10^{-5}$ M of verapamil markedly reduced the contraction in a dose-dependent manner.

The aggregation of platelets in rabbit PRP induced by 10 to 20 µg/ml of collagen was hardly inhibited by tranilast even at $10^{-3}$ M. On the other hand, verapamil markedly suppressed it at a concentration of $10^{-4}$ M (Fig. 6C).

**Discussion**

In early studies, β-stimulants and phosphodiesterase inhibitors which increase intracellular cAMP were shown to inhibit the anaphylactic release of histamine and SRS-A from mixed cell systems (18, 19). Therefore, it was noted that intracellular cAMP plays an important role in release of chemical mediators. Recent biochemical studies of the mechanisms of mast cell activation have suggested that IgE-receptor bridging enhances membrane phospholipid methylation and subsequently activates adenylate cyclase, followed by the increase in cAMP and activation of cAMP-dependent protein kinase and modulation of the releasing process of chemical mediators (20-22). However, mediator releases induced by immunological and non-immunological stimulations were observed to be either suppressed, unaffected, or enhanced by cAMP-elevating stimulus (23). The role of cAMP in chemical mediator release is not known yet. In our present study, tranilast had no significant effect on intracellular cAMP although the antigen-induced histamine release. A reciprocal correlation between the intracellular cAMP level and histamine release from rat PEC as described by Kubota et al. (6) was not seen in the present study using rat PMC. The time course of the change in the intracellular cAMP, as shown in Fig. 2, did not coincide with that described by Ishizaka and Ishizaka (20, 21). A role of cAMP in the antigen-induced release of chemical mediators from mast cells appears to be unclear in this study. However, our data support which
described that the mechanism of inhibitory action of tranilast, unlike β-stimulants and phosphodiesterase inhibitors, has no relation to changes in intracellular cAMP level occurring in the process of release of chemical mediators (24).

Calcium ion is necessary in the process of anaphylactic histamine release (25, 26). The essential event which initiates the release of chemical mediators is an increase in the level of free calcium ions in the cytosol which may be derived from the extracellular environment or mobilized from intracellular Ca stores. Therefore, it is suggested that the Ca uptake across the plasma membrane acts as a trigger for the releasing process. The antigen-antibody reaction-induced histamine release from mast cells was inhibited up to 90% by inhibitors of glycolysis and oxidative phosphorylation, whereas the calcium influx under similar conditions is inhibited only by approx. 30% (14, 25). A combination of antimycin A (inhibitor of oxidative phosphorylation) and lanthanum (a competitive antagonist for Ca) reduced Ca uptake by 60% (14, 25). It appears that metabolic inhibitors suppress the energy-requiring Ca uptake into mast cells for the granule extrusion, and the inhibition of histamine release by lanthanum is the results of a competition between external membrane-bound Ca and lanthanum at an extracellular site which is thought to be the Ca channel (27, 28). In our study, effects of antimycin A on the antigen-induced histamine release (data not shown) from and Ca uptake into mast cells were similar to results described above (14). On the other hand, tranilast suppressed the Ca uptake by about 90% (Fig. 3). Further, tranilast was apparently different from 2-deoxyglucose (an inhibitor of glycolysis) in the mode of inhibitory action on the antigen-induced histamine release and Ca uptake (Fig. 5). Therefore, we suggest that the inhibition of Ca uptake into mast cells by tranilast is different from that of metabolic inhibitors and lanthanum, and that tranilast suppresses the energy-requiring and cell membrane-bound Ca uptake into mast cells in antigen-antibody reactions.

The effect of verapamil, one of the voltage-dependent Ca channel blockers, on the release of chemical mediators in immediate hypersensitive reactions is not clear yet (29). Indeed, verapamil did not suppress the IgE-dependent histamine release in our study. Effects of verapamil on the histamine release, the Ca-induced contraction and the platelet aggregation are obviously different from those of tranilast. Therefore, the Ca permeability through the plasma membrane of mast cells seems to be different from that pertinent to smooth muscle contraction and platelet aggregation, and the mechanism of Ca uptake in mast cells may not be related to the voltage-dependent Ca channels.

It has been suggested that the intracellular Ca source is utilized in the histamine release from mast cells (30). Recently, it was demonstrated that the dissociation of arachidonic acid from membrane phospholipids is induced by the activation of not only phospholipase A_2 but also phospholipase C. The latter is one of the enzymes catalyzing the phosphatidylinositol cycle and requires Ca, especially intracellular Ca (31). Therefore, to examine the mechanism of the inhibitory action of tranilast on the release of arachidonic acid and histamine, it will be necessary to investigate the effect of this drug on the mobilization of intracellular Ca in the future.

IgE-receptor bridging initiates a metabolism of membrane phospholipid which is followed by the activation of Ca channel and uptake of extracellular Ca. The entered Ca activates phospholipase A_2. It is also possible that IgE-receptor bridging causes an activation of phospholipase C which requires intracellular Ca and catalyzes a phosphatidylinositol cycle. Both processes may lead to dissociation of arachidonic acid from membrane phospholipids and a sequence of other events in histamine release, SRS-A and prostaglandin (PG) generation (13, 25, 32). Tranilast had no significant influence on the activity of phospholipase A_2 (5) and arachidonic acid converting enzyme (9). Thus, we suggest that the mechanism of the inhibitory action of tranilast on chemical mediator release is different from that of phospholipase A_2 inhibitors, metabolic inhibitors and Ca channel blockers, but involves the suppression of Ca influx into the cells occurring in the process of release of chemical mediators such as histamine, SRS-A and PGs.
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