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Original Paper

Anti-Allergic Drugs Tranilast and Ketotifen Dose-Dependently Exert Mast Cell-Stabilizing Properties

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Key Words
Tranilast • Ketotifen • Mast cell-stabilizing properties • Rat peritoneal mast cells • Exocytosis • Membrane capacitance • Plasma membrane deformation

Abstract
Background: Anti-allergic drugs, such as tranilast and ketotifen, inhibit the release of chemokines from mast cells. However, we know little about their direct effects on the exocytotic process of mast cells. Since exocytosis in mast cells can be monitored electrophysiologically by changes in the whole-cell membrane capacitance (Cm), the absence of such changes by these drugs indicates their mast cell-stabilizing properties. ***Methods:*** Employing the standard patch-clamp whole-cell recording technique in rat peritoneal mast cells, we examined the effects of tranilast and ketotifen on the Cm during exocytosis. Using confocal imaging of a water-soluble fluorescent dye, lucifer yellow, we also examined their effects on the deformation of the plasma membrane. ***Results:*** Relatively lower concentrations of tranilast (100, 250 μM) and ketotifen (1, 10 μM) did not significantly affect the GTP-γ-S-induced increase in the Cm. However, higher concentrations of tranilast (500 μM, 1 mM) and ketotifen (50, 100 μM) almost totally suppressed the increase in the Cm, and washed out the trapping of the dye on the surface of the mast cells. Compared to tranilast, ketotifen required much lower doses to similarly inhibit the degranulation of mast cells or the increase in the Cm. ***Conclusions:*** This study provides electrophysiological evidence for the first time that tranilast and ketotifen dose-dependently inhibit the process of exocytosis, and that ketotifen is more potent than tranilast in stabilizing mast cells. The mast cell-stabilizing properties of these drugs may be attributed to their ability to counteract the plasma membrane deformation in degranulating mast cells.

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Introduction

Tranilast and ketotifen are among the most potent anti-allergic drugs widely used in the treatment of allergic disorders, such as allergic conjunctivitis, chronic rhinitis, urticaria and asthma [1, 2]. Tranilast, whose therapeutic efficacy as an anti-fibrotic or anti-cancer reagent has also been demonstrated [3, 4], was originally developed as a mast cell stabilizer, since it exerts inhibitory effects on the release of chemokines [5, 6]. On the other hand, ketotifen is one of the second-generation antihistamine drugs that exert anti-allergic effects primarily by antagonizing histamine H1 receptors [2]. However, later studies using human conjunctival mast cells additionally revealed a mast cell-stabilizing property of ketotifen [7, 8], consistent with its higher potency in the treatment of allergic disorders. So far, by measuring the amount of chemokines released from rat peritoneal mast cells, such as histamine and β-hexosaminidase, previous studies have suggested the superiority of ketotifen to tranilast in the properties of mast cell stabilization [9-11]. However, due to the limitations of the methods used in molecular biology [12], the effects of these drugs on the exocytotic process of mast cells have not directly been examined. Recently, by monitoring the changes in whole-cell membrane capacitance (Cm) in rat peritoneal mast cells, we provided electrophysiological evidence for the first time that drugs, such as olopatadine and chlorpromazine, inhibited the process of exocytosis, and thus exerted mast cell-stabilizing properties [13, 14]. From our results, mast cells derived from peritoneal serosa required relatively lower doses of these drugs than those derived from human conjunctival mucosa [15-17]. However, these mast cells showed almost identical biochemical features in that they showed more wrinkles on their cell surface and released secretory granules by the external or internal stimuli for exocytosis [13, 14]. In the present study, employing this standard patch-clamp whole-cell recording technique in rat peritoneal mast cells, we examined the effects of tranilast and ketotifen on the changes in the Cm in order to determine the mechanism by which they stabilize mast cells. Here, we provide electrophysiological evidence for the first time that both tranilast and ketotifen dose-dependently inhibit the process of exocytosis, and that ketotifen is more potent than tranilast in stabilizing mast cells. We also show that the mast cell- stabilizing properties of these drugs can be ascribed to their ability to counteract the plasma membrane deformation in degranulating mast cells.

Materials and Methods

Cell Sources and Preparation

Male Wistar rats more than 25 weeks old, supplied by Japan SLC Inc. (Shizuoka, Japan), were deeply anaesthetized with isoflurane and then killed by cervical dislocation. The protocol for animal use was approved by the Animal Care and Use Committee of Tohoku University Graduate School of Medicine. As described in our previous studies [13, 14], peritoneal mast cells were obtained by washing the peritoneal cavity with standard external (bathing) solution containing (in mM): NaCl, 145; KCl, 4.0; $CaCl_2$, 1.0; $MgCl_2$, 2.0; HEPES, 5.0; bovine serum albumin, 0.01 % (pH 7.2 adjusted with NaOH). They were maintained at room temperature (22-24°C) for use within 8 hours. The mast cell suspension was scattered in a chamber placed on the headstage of an inverted microscope (Nikon, Tokyo, Japan). As we carefully showed in our previous studies [13, 14, 18], single mast cells were easily distinguishable from other cells by their intracellular inclusion of secretory granules [13, 14] or the positive staining for toluidine blue [18].

Quantification of Mast Cell Degranulation

Tranilast, purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), was separately dissolved at final concentrations of 100, 250, 500 μM and 1 mM. Ketotifen fumarate, purchased from Sigma Aldrich Co. (St. Louis, MO, USA), was also dissolved in the external solution at final concentrations of 1, 10, 50 and 100 μM. After mast cells were incubated in these solutions or a solution containing no drug, exocytosis was externally induced by compound 48/80 (Sigma-Aldrich; final concentration 10 μg/ml) [13, 14]. Bright-field images were acquired from randomly selected 0.1-mm^2 fields of view (10 views from 6 of each condition),

as described in our previous studies [13, 14]. Degranulated mast cells were defined as cells associated with ≥ 8 granules outside the cell membrane, as described previously [13, 14, 19] . These mast cells were then counted and their ratio to all mast cells was calculated.

Electrical Setup and Membrane Capacitance Measurements

We conducted standard whole-cell patch-clamp recordings using an EPC-9 patch-clamp amplifier system (HEKA Electronics, Lambrecht, Germany) as described previously [13, 14, 20, 21]. The patch pipette resistance was 4-6 MΩ when filled with internal (patch pipette) solution containing (in mM): K-glutamate, 145; $MgCl_2$, 2.0; Hepes, 5.0 (pH 7.2 adjusted with KOH). One-hundred μM guanosine 5'-*o*-(3-thiotriphosphate) (GTP-γ-S) (EMD Bioscience Inc., La Jolla, CA, USA) were additionally included in the internal solution to induce exocytosis in the mast cells [13, 14, 22, 23]. After a giga-seal formation on mast cells scattered in the external solutions containing no drug, the different concentrations of tranilast (100, 250, 500 μM and 1 mM) or ketotifen (1, 10, 50 and 100 μM), we applied suction briefly to the pipette to rupture the patch membrane and to dialyze the cells with GTP-γ-S. The series resistance of the whole-cell recordings was maintained below 10 MΩ during the experiments. To measure the membrane capacitance of the mast cells, we employed a sine plus DC protocol using the Lock-in amplifier of an EPC-9 Pulse program. An 800-Hz sinusoidal command voltage was superimposed on the holding potential of -80 mV. The membrane capacitance (Cm), membrane conductance (Gm) and series conductance (Gs), were continuously recorded during the whole-cell recording configuration. All experiments were carried out at room temperature.

Lucifer Yellow Trapping on the Cell Surface

After the mast cells were incubated in the external solutions containing no drug, the different concentrations of tranilast (100, 250, 500 μM and 1 mM) or ketotifen (1, 10, 50 and 100 μM), exocytosis was externally induced by compound 48/80 (10 μg/ml). Then, the cells were incubated for 5 min at room temperature in the external solution containing a hydrophilic fluorescent dye, lucifer yellow [13, 14, 20, 24] (Wako, Osaka, Japan; final concentration 10 μM), and washed thoroughly 2 or 3 times with dye-free external solutions. Fluorescent images were taken using a TE 2000-E Nikon Eclipse confocal microscope (Nikon, Tokyo, Japan).

Statistic Analyses

Data were analyzed using PulseFit software (HEKA Electronics, Lambrecht, Germany) and Microsoft Excel (Microsoft Corporation, Redmond, Wash., USA) and reported as means ± SEM. Statistical significance was assessed by two-way ANOVA followed by Dunnett's or Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Effects of tranilast and ketotifen on degranulation from rat peritoneal mast cells

Inhaled anesthetics, such as sevoflurane or isoflurane, were demonstrated to depress the activity of mast cells or inhibit the process of exocytosis in synaptic vesicles by reducing the Ca^{2+} influx into the cells [25, 26]. In the present study, however, despite the use of isoflurane during the initial isolation of rat peritoneal mast cells, compound 48/80 (10 μg/ml) induced degranulation in 79.8 ± 3.4 % of the mast cells observed (n=6; Fig. 1A). In human, the serum concentration of tranilast ranges between 30 and 300 μM when orally administered for the treatment of systemic diseases [27, 28]. However, in *in vitro* studies using rat peritoneal mast cells, doses as high as 1 mM tranilast were required to effectively elicit its inhibitory property on the release of histamine [9, 29]. Since doses as low as 1 or 10 μM tranilast failed to affect the degranulation of mast cells (data not shown), we tried doses starting from 100 μM up to 1 mM in the present study (Fig. 1A). Relatively lower doses, such as 100 and 250 μM, did not significantly affect the ratio of degranulating mast cells (Fig. 1A). However, higher doses, such as 500 μM and 1 mM, markedly decreased the numbers of degranulating mast cells (500 μM, 27.1 ± 3.0 %; 1 mM, 21.7 ± 4.4 %; n=6, P<0.05; Fig. 1A). These results were consistent with previous findings that tranilast, as a mast cell stabilizer, dose-dependently inhibited the release of histamine from mast cells [9, 29].

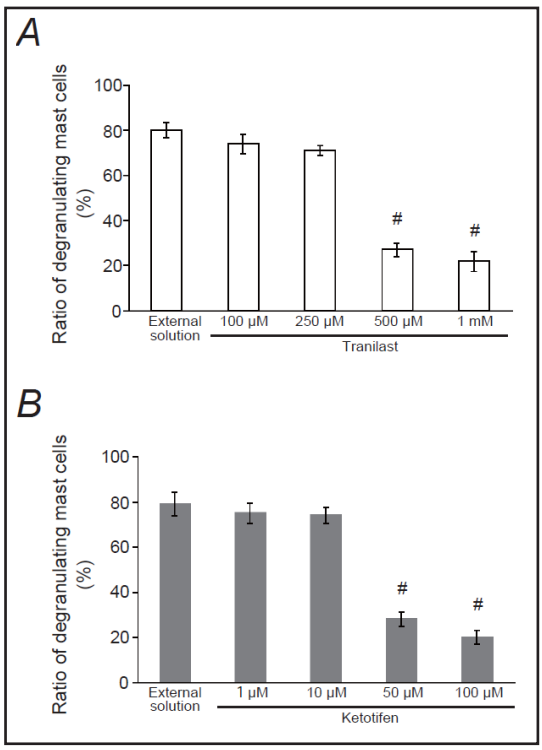

Fig. 1. Effects of anti-allergic drugs on mast cell degranulation. Effects of different concentrations of tranilast (100 μM, 250 μM, 500 μM or 1 mM) *(A)*, and ketotifen (1 μM, 100 μM, 50 μM or 100 μM) *(B)*. After the mast cells were incubated in the external solutions containing no drug or either drug, exocytosis was induced by compound 48/80. The numbers of degranulating mast cells are expressed as percentages of the total mast cell numbers in selected bright fields. $^{\#}P<0.05$ vs. incubation in the external solution alone. Values are means ± SEM (n=6: 10 views from 6 of each condition). Differences were analyzed by ANOVA followed by Dunnett's or Student's t test.

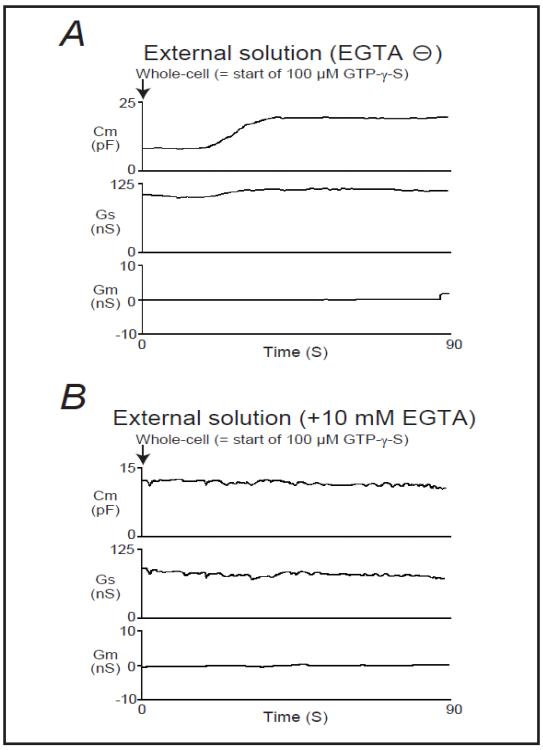

Fig. 2. Changes in mast cell membrane capacitance, series and membrane conductance during exocytosis. The whole-cell recording configuration was established in single mast cells and dialysis with 100 μM GTP-γ-S was started in the absence (*A*) or the presence (*B*) of 10 mM EGTA in the patch pipette internal solution. Membrane capacitance, series and membrane conductance were monitored for at least 90 sec. N=5 for each trace. Cm, membrane capacitance; Gs, series conductance; Gm, membrane conductance.

As we recently demonstrated with olopatadine [14], several second-generation antihistamine drugs are also known to exert mast cell stabilizing properties [30, 31]. Among them, since ketotifen is regarded as a relatively stronger mast cell stabilizer [32], we additionally examined the effects of this drug on the degranulation of mast cells (Fig. 1B). According to previous *in vivo* studies, the serum concentration of ketotifen ranges from 10 to 100 nM in humans when orally administered for the treatment of systemic allergic reactions [33, 34]. However, in *in vitro* studies using rat peritoneal mast cells or human conjunctival mast cells, doses as high as 100 μM ketotifen were required to effectively elicit its inhibitory property on histamine release [7, 8, 35]. Since doses more than 1 mM were cytotoxic enough to cause membrane lysis [36], we tried doses starting from 1 μM up to 100 μM in the present study (Fig. 1B). Relatively lower doses of ketotifen, such as 1 and 10 μM, did not significantly affect the ratio of degranulating mast cells (Fig. 1B). However, similar to the effects of tranilast (Fig. 1A), higher doses of ketotifen, such as 50 and 100 μM, markedly decreased the numbers of degranulating mast cells (50 μM, 28.0 ± 3.2 %; 100 μM, 19.6 ± 2.9 %; n=6, $P<0.05$; Fig. 1B). From these results, as we previously demonstrated with olopatadine [14], ketotifen dose-

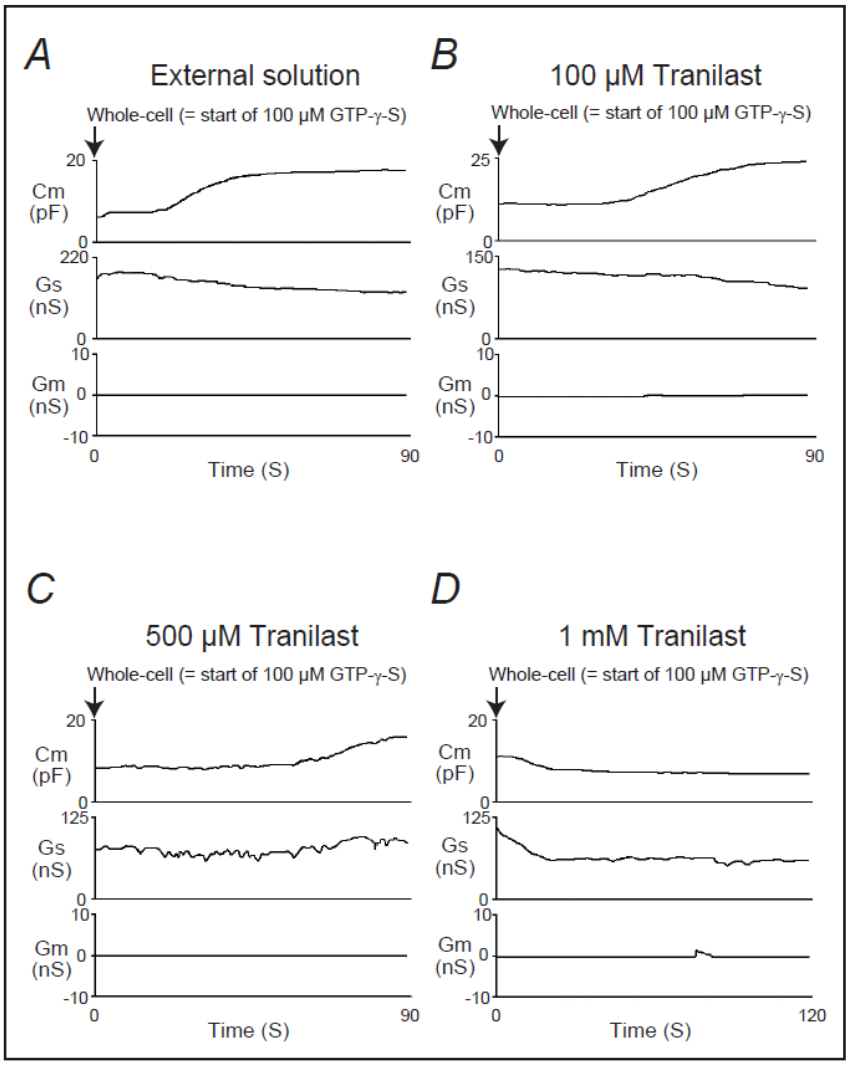

Fig. 3. Tranilast-induced changes in mast cell membrane capacitance, series and membrane conductance during exocytosis. After the mast cells were incubated in the external solutions containing no drug (*A*), 100 μM (*B*), 500 μM (*C*) or 1 mM tranilast (*D*), the whole-cell recording configuration was established in single mast cells and dialysis with 100 μM GTP-γ-S was started. Membrane capacitance, series and membrane conductance were monitored for at least 90 sec. *N*=5 for each trace. Cm, membrane capacitance; Gs, series conductance; Gm, membrane conductance.

Table 1. Summary of changes in membrane capacitance in different concentrations of tranilast-containing solutions. Values are means ± SEM. *Cm* = membrane capacitance. #$p < 0.05$ vs. *Cm* before GTP-γ-S internalization. *$p < 0.05$ vs. Δ*Cm* in external solution

Agents	N	*Cm* before GTP-γ-S Internalization (pF)	*Cm* after GTP-γ-S Internalization (pF)	*ΔCm* (pF)
External solution	5	8.58 ± 0.88	18.9 ± 1.97#	10.3 ± 1.39
100 μM Tranilast	5	8.49 ± 1.57	17.8 ± 3.49#	9.26 ± 2.33
250 μM Tranilast	5	8.55 ± 0.40	8.6 ± 1.12#	10.1 ± 1.34
500 μM Tranilast	5	8.60 ± 0.73	13.9 ± 1.96	5.29 ± 1.48*
1 mM Tranilast	5	9.79 ± 0.58	9.17 ± 1.76	-0.61 ± 1.86*

dependently inhibited the degranulation from rat peritoneal mast cells. However, compared to tranilast, much lower doses of ketotifen were required to exert similar inhibitory effects (Fig. 1B vs. A), indicating the higher potency of ketotifen than tranilast as a mast cell stabilizer.

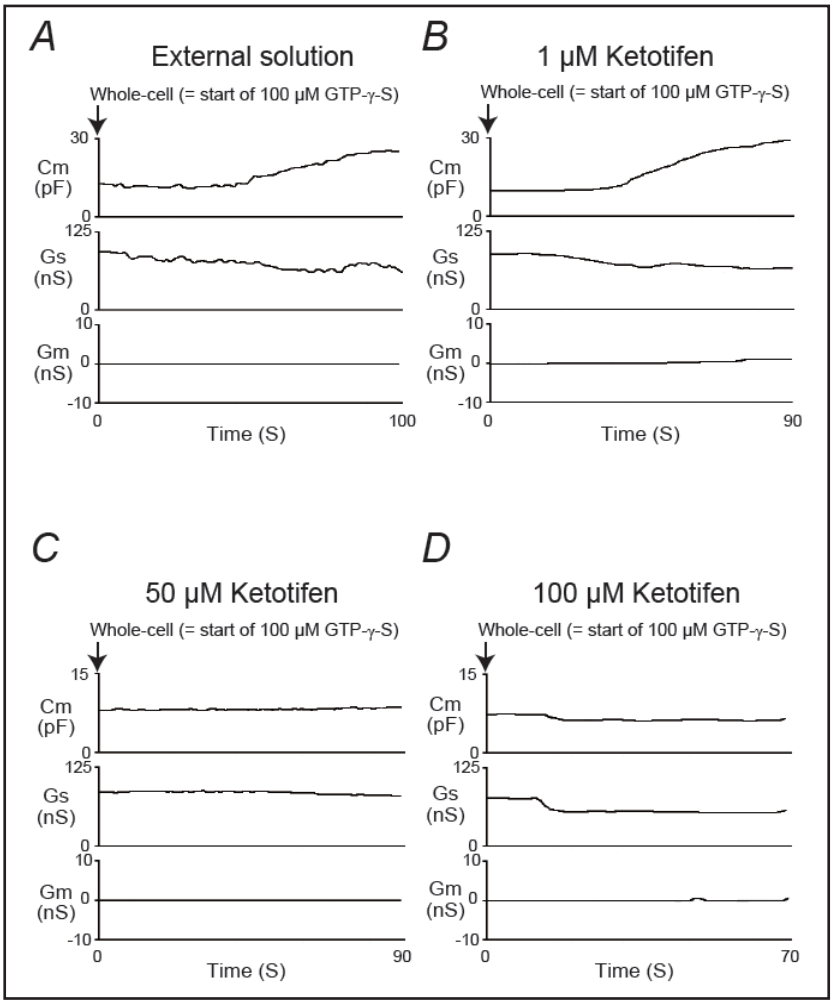

Fig. 4. Ketotifen-induced changes in mast cell membrane capacitance, series and membrane conductance during exocytosis. After mast cells were incubated in the external solutions containing no drug (*A*), 1 μM (*B*), 50 μM (*C*) or 100 μM ketotifen (*D*), the whole-cell recording configuration was established in single mast cells and dialysis with 100 μM GTP-γ-S was started. Membrane capacitance, series and membrane conductance were monitored for at least 70 sec. *N*=5 for each trace. Cm, membrane capacitance; Gs, series conductance; Gm, membrane conductance.

Table 2. Summary of changes in membrane capacitance in different concentrations of ketotifen-containing solutions. Values are means ± SEM. *Cm* = membrane capacitance. #$p < 0.05$ vs. *Cm* before GTP-γ-S internalization. *$p < 0.05$ vs. Δ*Cm* in external solution

Agents	N	*Cm* before GTP-γ-S Internalization (pF)	*Cm* after GTP-γ-S Internalization (pF)	*ΔCm* (pF)
External solution	5	9.92 ± 0.62	26.0 ± 2.36#	16.0 ± 1.97
1 μM Ketotifen	5	8.91 ± 0.83	24.8 ± 2.89#	15.9 ± 2.17
10 μM Ketotifen	5	8.15 ± 0.52	20.2 ± 2.87#	12.0 ± 2.64
50 μM Ketotifen	5	8.68 ± 0.42	9.47 ± 1.97	0.80 ± 2.09*
100 μM Ketotifen	5	7.12 ± 1.34	8.25 ± 2.85	1.12 ± 1.90*

Effects of tranilast and ketotifen on whole-cell membrane capacitance in rat peritoneal mast cells

In megakaryocytes or thymus lymphocytes, microscopic changes in the cell surface area were induced by the membrane invagination during thrombopoiesis [37] or by the accumulation of lipophilic drugs into the lipid bilayers [38]. In our previous studies, such microscopic changes in these cells were most precisely monitored by measurement of the

whole-cell membrane capacitance (Cm) [20, 21, 39-44]. In mast cells, it was also established that the increase in the Cm reflects the degranulating process during exocytosis [13, 14, 45, 46]. Therefore, in the present study, to determine the effects of tranilast and ketotifen on mast cell exocytosis, we incubated the cells in tranilast- or ketotifen- containing external solutions and examined the changes in the Cm (Fig. 3 and 4). The effects of different concentrations of tranilast and ketotifen on the Cm, Gs and Gm are shown in these figures and the numerical changes in the parameters are summarized in Table 1 and 2.

Consistent with previous findings [13, 14, 22, 23], internal application of GTP-γ-S to mast cells incubated in the external solution induced a more than 2-fold increase in the Cm (from 8.42 ± 0.97 to 19.8 ± 4.92 pF, n=5, P<0.05; Fig. 2A), which occurred within 30 to 60 s after the establishment of the whole-cell recording configuration (Fig. 2A). However, the addition of 10 mM EGTA, a specific chelator for calcium ions, into the patch pipette solution almost totally inhibited the increase in the Cm (from 9.57 ± 0.91 to 10.4 ± 1.38 pF, n=5, P>0.05; Fig. 2B), which supports the previous evidence that the exocytotic process is primarily dependent on changes in the intracellular Ca^{2+} concentration ([Ca^{2+}]) [22, 47]. In the following experiments, therefore, to examine the effects of the drugs on the Ca^{2+}-dependent increase in the Cm, we used external solutions without containing EGTA (Fig. 3 and 4).

When mast cells were incubated with relatively lower concentrations of tranilast (100 and 250 μM), the increase in the Cm was not significantly different from that of the mast cells incubated in the external solution alone (Fig. 3B vs. A, Table 1). However, incubation of mast cells with 500 μM tranilast significantly suppressed the incremental effect of GTP-γ-S on the Cm (Fig. 3C, Table 1), and incubation with 1 mM tranilast almost totally inhibited the increase in the Cm (Fig. 3D, Table 1). Similar to tranilast, the lower concentrations of ketotifen, such as 1 and 10 μM, failed to affect the increase in the Cm (Fig. 4B, Table 2), showing an identical pattern to that of the external solution alone (Fig. 4A, Table 2). However, incubation with relatively higher concentrations of ketotifen (50 and 100 μM) almost completely suppressed the increase in the Cm (Fig. 4C and D, Table 2). These results provided electrophysiological evidence for the first time that both tranilast and ketotifen inhibit the process of exocytosis in a concentration-dependent manner, strongly supporting our findings shown in Figure 1. Additionally, similar to these findings (Fig. 1), much lower doses of ketotifen than tranilast were required to suppress the increase in the Cm (Fig. 4 vs. 3). These results also supported our findings shown in Figure 1 that ketotifen exerts mast cell-stabilizing properties more potently than tranilast.

Effects of tranilast and ketotifen on exocytosis-induced membrane surface deformation in mast cells

In our previous study, drugs such as chlorpromazine, salicylate and olopatadine altered the plasma membrane curvature in rat peritoneal mast cells [13, 14], and thus modulated the process of exocytosis. In the present study, since tranilast and ketotifen both inhibited the exocytotic process of mast cells (Fig. 1, 3, 4), drug-induced changes in the membrane architecture would also affect this process. Therefore, after incubating mast cells in tranilast- or ketotifen-containing solutions, we externally induced exocytosis in the cells and examined the effects of these drugs on the plasma membranes (Fig. 5A). By the external addition of compound 48/80, mast cells incubated in the external solution showed more wrinkles on their cell surface and released secretory granules as a result of exocytosis (Fig. 5Ab vs. a). Mast cells incubated in relatively lower concentrations of tranilast (100, 250 μM; Fig. 4Ac, d) or ketotifen (1, 10 μM; Fig. 5Ag, h) were not different from those incubated in the external solution alone (Fig. 5Ab). However, mast cells incubated in relatively higher concentrations of tranilast (500 μM, 1 mM; Fig. 5Ae, f) or ketotifen (50, 100 μM; Fig. 5Ai, j) did not show any findings suggestive of exocytosis, such as the wrinkles on their cell surface and the release of secretory granules.

To determine whether the wrinkles observed in the degranulating mast cells (Fig. 5Ab, c, g and h) represented membrane surface deformation as a result of exocytosis, we then used

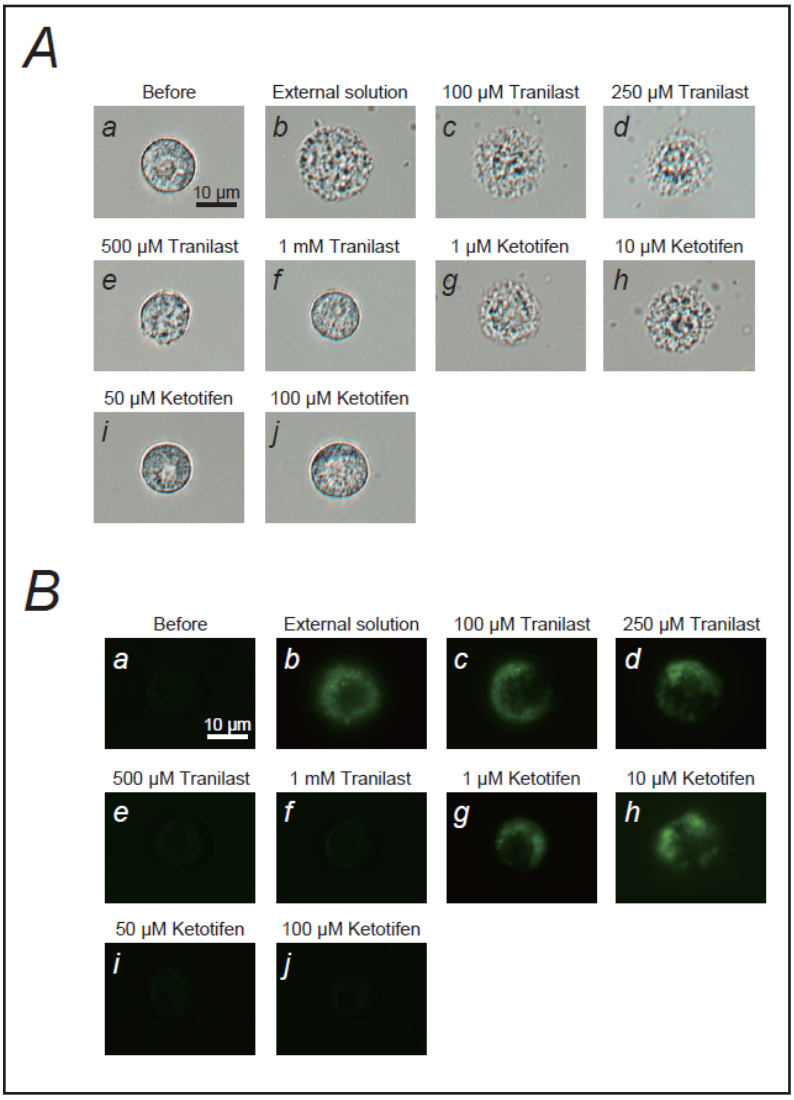

Fig. 5. Effects of anti-allergic drugs on exocytosis-induced membrane surface deformation in mast cells. *A*: Differential-interference contrast (DIC) microscopic images were taken before (*a*) and after exocytosis was externally induced by compound 48/80 in mast cells incubated in the external solutions containing no drug (*b*), 100 μM tranilast (*c*), 250 μM tranilast (*d*), 500 μM tranilast (*e*), 1 mM tranilast (*f*), 1 μM ketotifen (*g*), 10 μM ketotifen (*h*), 50 μM ketotifen (*i*) and 100 μM ketotifen (*j*). *B*: The trapping of the fluorescent dye (lucifer yellow) on the cell surface was detected before (*a*) and after exocytosis was externally induced in mast cells incubated in the external solutions containing no drug (*b*), 100 μM tranilast (*c*), 250 μM tranilast (*d*), 500 μM tranilast (*e*), 1 mM tranilast (*f*), 1 μM ketotifen (*g*), 10 μM ketotifen (*h*), 50 μM ketotifen (*i*) and 100 μM ketotifen (*j*).

lucifer yellow (Fig. 5B), a water-soluble fluorescent dye that is retained in the invaginated folds created in the plasma membranes [14, 20, 24]. In mast cells that were treated with external solution alone (Fig. 5Bb), lower concentrations of tranilast (100, 250 μM; Fig. 5Bc, d) or ketotifen (1, 10 μM; Fig. 5Bg, h), lucifer yellow was trapped partially on the cell surface area. Since the dye, which is normally membrane-impermeable [48], was completely absent in the cells before exocytosis was induced (Fig. 5Ba), the staining indicated its retention in the opened pores formed by exocytosis [13, 14, 49]. However, after incubating mast cells in relatively higher concentrations of tranilast (500 μM, 1 mM; Fig. 5Be, f) or ketotifen (50, 100 μM; Fig. 5Bi, j), the dye was almost completely washed out. These results indicated that both tranilast and ketotifen inhibited the formation of the invaginated folds when they exerted mast cell stabilizing abilities, and suggested that these drugs counteracted the membrane surface deformation induced by exocytosis.

Discussion

In previous studies, by measuring the amount of chemokines released from mast cells, the mast cell stabilizing properties of anti-allergic drugs were indirectly determined [5-9,

29]. However, in addition to their exocytotic release of chemokines, such as histamine and serotonin, mast cells are known to produce various kinds of pro-inflammatory cytokines or growth factors [50]. Therefore, to precisely determine the ability of a drug to stabilize mast cells, the release of all these substances must be evaluated, or the exocytotic process itself monitored, directly in mast cells. In previous patch-clamp studies of mast cells, the degranulating process during exocytosis was continuously monitored by measurement of the whole-cell Cm [13, 14, 22, 23]. In the present study, employing this electrophysiological approach, we provided direct evidence for the first time that the anti-allergic drugs tranilast and ketotifen dose-dependently inhibit the process of exocytosis, and thus exert mast cell-stabilizing properties. Additionally, ketotifen required much lower doses than tranilast to similarly inhibit the degranulation of mast cells or the increase in the Cm (Fig. 1, 3, 4). Therefore, based on our present and recent findings [14], second-generation antihistamine drugs, such as ketotifen and olopatadine, may be more effective than tranilast in stabilizing mast cells.

In addition to the pathogenesis of allergic or inflammatory disorders, mast cells are also involved in the development of fibrosis in many organs, including kidney, skin, lung and liver [51-53]. In certain pathological conditions, such as chronic inflammation, mast cells produce fibroblast growth factors and thus facilitate the progression of organ fibrosis [50]. Therefore, for the treatment or protection against such fibrosis, several pharmacological approaches to inhibit the activation of mast cells have been suggested, including the use of mast cell stabilizers [54, 55] or chemokine inhibitors [56, 57]. Recently, using rat models of chronic renal failure (CRF), we demonstrated that mast cells proliferated *in situ* within the fibrotic peritoneum and increased their production of fibroblast-activating factors [18]. Since treatment with tranilast actually ameliorated the progression of peritoneal fibrosis, mast cells were thought to be responsible for the progression of peritoneal fibrosis in CRF. In the present study, similar to tranilast, ketotifen also dose-dependently inhibited the process of exocytosis and thus exerted mast cell-stabilizing properties. Of note, ketotifen required much lower doses than tranilast, indicating its higher potency as a mast cell stabilizer. Given such pharmacological properties of ketotifen, the administration of this drug could also be useful in the treatment of organ fibrosis in addition to its primary efficacy for allergic diseases. Recently, several animal studies have actually shown the therapeutic efficacy of ketotifen in the treatment of post-traumatic dermal or joint capsular fibrosis [58, 59].

In the present study, as we recently demonstrated with chlorpromazine or olopatadine [13, 14], both tranilast and ketotifen prevented plasma membrane deformation in rat peritoneal mast cells (Fig. 5). In many types of secretory cells, the exocytotic process can be modulated by mechanical stimuli, such as changes in the membrane tension, shear stress, hydrostatic pressure and compression [60]. Therefore, the counteracting effects of tranilast and ketotifen on the plasma membrane deformation in degranulating mast cells were thought to contribute to their inhibitory effects on the exocytotic process. From our results, the internal application of EGTA, which chelates calcium ions and thus inhibits their transport in the cells, completely halted the process of exocytosis (Fig. 2). Therefore, as demonstrated in previous patch-clamp studies [22, 47], a rise in the $[Ca^{2+}]_i$ was considered to be the primary trigger of exocytosis in mast cells. Recently, Cruse et al. further demonstrated in human lung mast cells that the rise in $[Ca^{2+}]_i$ was dependent on the activity of Ca^{2+} activated K^+ channels (Kc_a 3.1), which provide the driving force for Ca^{2+} influx through store-operated calcium channels (SOCs) [61]. Previous studies revealed that the activity of SOCs, such as canonical transient receptor potential 1 (TRPC1), and Kc_a -channels was mechanically modified by stretching of the plasma membranes [62, 63]. In the present study, since tranilast and ketotifen both counteracted the exocytosis-induced deformation of the membrane surface (Fig. 5), these drugs were thought to induce membrane stretch in mast cells. Then, such mechanical stimuli to the membranes would influence the activity of the ion channels expressed in mast cells. Consequently, such induced changes in the $[Ca^{2+}]_i$ may largely contribute to the tranilast- or ketotifen-induced inhibition of exocytosis.

Previous studies demonstrated that physical stimuli, such as high temperature, can also induce mast cell degranulation through the activation of transient receptor potential vanilloid 2 (TRPV2) [64]. In *in vitro* experiments using rat peritoneal mast cells, the amount of histamine released from mast cells was actually dependent on temperature [65]. Therefore, in the present study, if the experiments were conducted at body temperature (35-37°C), which is higher than the room temperature (22-24°C), the mast cell-stabilizing properties of tranilast or ketotifen could be more blunted. Additionally, in normal or pathological conditions, certain peptides or proteins that systemically circulate in the plasma, such as anaphylatoxins (complement peptides), kinins, cytokines or various neuropeptides, can also trigger exocytosis in mast cells [66, 67]. On the other hand, arachidonic acid products or chondroitin sulfate, which are released from inflamed tissue, exert inhibitory effects on the degranulation of mast cells [68, 69]. In our present *in vitro* study, since we used rat peritoneal mast cells isolated into the external solutions, which were completely free from these proteins or peptides, we did not need to consider their influence. However, in *in vivo* conditions, such as in humans or in experimental animals, the effects of these proteins or peptides on the exocytotic process of mast cells have to be considered. In such conditions, the mast cell-stabilizing properties of tranilast or ketotifen could substantially be modified, representing the technical limitations of our present *in vitro* study.

In summary, this study provided electrophysiological evidence for the first time that tranilast and ketotifen dose-dependently inhibit the process of exocytosis, and that ketotifen is more potent than tranilast in stabilizing mast cells. The mast cell-stabilizing properties of these drugs could be ascribed to their counteracting effects on the plasma membrane deformation in degranulating mast cells.

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Disclosure Statement

The authors declare no conflicts of interest.

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