Olopatadine Inhibits Exocytosis in Rat Peritoneal Mast Cells by Counteracting Membrane Surface Deformation

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Key Words
Olopatadine \textbullet Mast cell stabilizing properties \textbullet Rat peritoneal mast cells \textbullet Exocytosis \textbullet Membrane capacitance \textbullet Plasma membrane deformation

Abstract

\textbf{Background/Aims:} Besides its anti-allergic properties as a histamine receptor antagonist, olopatadine stabilizes mast cells by inhibiting the release of chemokines. Since olopatadine bears amphiphilic features and is preferentially partitioned into the lipid bilayers of the plasma membrane, it would induce some morphological changes in mast cells and thus affect the process of exocytosis. \textbf{Methods:} Employing the standard patch-clamp whole-cell recording technique, we examined the effects of olopatadine and other anti-allergic drugs on the membrane capacitance (Cm) in rat peritoneal mast cells 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. \textbf{Results:} Low concentrations of olopatadine (1 or 10 \textmu M) did not significantly affect the GTP-\gamma-S-induced increase in the Cm. However, 100 \textmu M and 1 mM olopatadine almost totally suppressed the increase in the Cm. Additionally, these doses completely washed out the trapping of the dye on the cell surface, indicating that olopatadine counteracted the membrane surface deformation induced by exocytosis. As shown by electron microscopy, olopatadine generated inward membrane bending in mast cells. \textbf{Conclusion:} This study provides electrophysiological evidence for the first time that olopatadine dose-dependently inhibits the process of exocytosis in rat peritoneal mast cells. Such mast cell stabilizing properties of olopatadine may be attributed to its counteracting effects on the plasma membrane deformation in degranulating mast cells.

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**Introduction**

Olopatadine is one of the most potent anti-allergic drugs that are widely used in the treatment of allergic disorders, such as seasonal pollinosis, chronic rhinitis, urticaria and allergic conjunctivitis. Olopatadine was originally developed as a second-generation antihistamine drug [1], which was intended to exert its anti-allergic effects primarily by antagonizing histamine H1 receptors. Additionally, studies using human conjunctival mast cells further demonstrated that this drug also exerts inhibitory effects on the release of chemokines [2-4]. Concerning the mechanisms by which drugs exert such mast cell stabilizing properties, previous studies indicated that the drug-induced changes in the intracellular Ca

\[^{2+}\] concentration ([Ca

\[^{2+}\] ]i) are primarily responsible [5, 6]. However, later studies also revealed the involvement of G proteins [7] or mechanical stimuli to the plasma membranes [8] in modulating the process of exocytosis in mast cells. In our recent study, chlorpromazine, a positively charged membrane amphipath, changed the plasma membrane curvature in rat peritoneal mast cells [9], and the induced mechanical stretch of the membranes significantly inhibited the process of exocytosis. Since olopatadine, a relatively cationic and lipophilic antihistamine, shares common pharmacological features with those of the membrane amphipaths [10], this drug should also induce some morphological changes in mast cell plasma membranes, and thus may affect their exocytotic process. To test this, employing the standard patch-clamp whole-cell recording technique in rat peritoneal mast cells [9, 11, 12], we examined the effects of olopatadine on the membrane capacitance. By confocal imaging of a hydrophilic fluorescent dye that is trapped on the cell surface [9, 13], we also examined the effects of this drug on the plasma membrane deformation. Here, using rat peritoneal mast cells, we provide electrophysiological evidence for the first time that olopatadine dose-dependently inhibits the process of exocytosis. We also show that such mast cell stabilizing properties of olopatadine can be ascribed to its counteracting effects on 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 anesthetized 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. 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) as described in our previous study [9]. 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). Single mast cells were easily distinguishable from other cells by their intracellular inclusion of secretory granules [9].

**Quantification of Mast Cell Degranulation**

Olopatadine hydrochloride, kindly provided by Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan), was separately dissolved in the external solution at final concentrations of 1, 10, 100 μM and 1 mM, respectively. Fexofenadine hydrochloride, purchased from LKT Laboratories, Inc. (St. Paul, Min., USA), and loratadine, from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), were also separately dissolved in the external solution at the final concentrations of 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, St. Louis, MO, USA; final concentration 10 μg/ml) [9]. 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 [9, 14]. Degranulated mast cells were defined as cells associated with ≥8 granules outside the cell membrane, as described previously [15]. 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 [9, 16]. The patch pipette resistance was 4–6 MΩ when filled with internal (patch pipette) solution containing (in mM): K-glutamate, 145; MgCl₂, 2.0; Hepes, 5.0 (pH 7.2 adjusted with KOH). One-hundred μM guanosine 5’-o-(3-thiotriophosphate) (GTP-γS) (EMD Bioscience Inc., La Jolla, CA, USA) was additionally included in the internal solution to induce exocytosis in the mast cells [9, 11, 12]. After a giga-seal formation on mast cells scattered in the external solutions containing no drug, the different concentrations of olopatadine (1, 10, 100 μM and 1 mM), 100 μM fexofenadine or loratadine, we applied suction briefly to the pipette to rupture the patch membrane and to dialyze the cells with GTP-γS. 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), as well as membrane conductance (Gm) and series conductance (Gs), was 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 olopatadine (1, 10, 100 μM and 1 mM), 100 μM fexofenadine or loratadine, 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 [9, 13] (Wako, Osaka, Japan; final concentration 10 μM), and were washed 2 to 3 times thoroughly with dye-free external solutions. Fluorescent images were taken using a TE 2000-E Nikon Eclipse confocal microscope (Nikon, Tokyo, Japan).

Electron Microscopy
The mast cells, incubated in the external solutions containing no drug or 1mM olopatadine for 10 min were fixed with 4% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 2 h at room temperature. After being trimmed into small pieces, the above specimen containing the cells was postfixed in 1% osmium tetroxide for 1 h at 4°C, rinsed in PBS, dehydrated in a graded series of alcohol and propylene oxide, and finally embedded in epoxy resin. Ultrathin (80 nm) sections were prepared on an ultramicrotome (Ultracut R, Leica, Heerbrugg, Switzerland) with a diamond knife and then were stained with uranyl acetate and lead citrate, and viewed using an electron microscope (JEM-1200, JOEL, 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 olopatadine on degranulation from rat peritoneal mast cells
In the absence of olopatadine, compound 48/80 (10 μg/ml) induced degranulation in 80.5 ± 1.1 % of the mast cells observed (n=6). In human, the physiological plasma concentration of olopatadine ranges from 1 to 10 μM when orally administered for the treatment of systemic allergic reactions [17-19]. On the other hand, when topically administered for allergic conjunctivitis, doses as high as 559 to 653 μM olopatadine were required to effectively elicit its inhibitory properties on histamine release from human conjunctival mast cells [2-4]. Since doses as high as 10 mM did not cause any cytotoxic effects in a previous in vitro study [20], we tried doses starting from 1 μM up to 1 mM in the present study. Relatively lower concentrations of olopatadine, such as 1 and 10 μM, did not
100 μM and 1 mM markedly decreased the numbers of degranulating mast cells (100 μM, 17.1 ± 1.8%; 1 mM, 10.6 ± 0.55%; n=6, P<0.05; Fig. 1A). From our results, olopatadine, which is also known as a mast cell stabilizer [21], actually inhibited the degranulation from rat peritoneal mast cells in a concentration-dependent manner. These results were consistent dose-dependently inhibited the release of histamine [2-4].

Besides olopatadine, some anti-allergic drugs are also known to exert mast cell stabilizing properties in addition to their antihistaminic properties [22, 23]. Among them, fexofenadine exerts relatively lower [23, 24], whereas loratadine exerts higher mast cell stabilizing properties than olopatadine does [22, 25]. Therefore, we also examined the effects of these drugs on the degranulation of mast cells (Fig. 1B). As expected, fexofenadine did not significantly affect the ratio of degranulating mast cells (76.1 ± 0.85 % vs. external solution alone: 81.4 ± 1.4 %, n=6, Fig. 1B), but loratadine markedly decreased the number of degranulating mast cells (11.3 ± 0.26 %, n=6, P<0.05).

**Effects of olopatadine 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 membrane invagination during thrombopoiesis [26] or by the accumulation of lipophilic drugs into the lipid bilayers [27]. In our previous studies, such microscopic changes in these cells were most precisely monitored by measurement of the whole-cell membrane capacitance (Cm) [16, 28-31]. In mast cells, it was also established that the increase in the Cm well indicates the degranulating process during exocytosis [9, 32, 33]. Therefore, in the present study, to determine the effects of olopatadine on mast cell exocytosis, we incubated the cells in olopatadine-containing external solutions and examined the changes in the Cm (Fig. 2). The effects of different concentrations of olopatadine on the
Fig. 2. Olopatadine-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), 10 μM (B), 100 μM (C) or 1 mM olopatadine (D), the whole-cell recording configuration was established in single mast cells and the dialysis with 100 μM GTP-γ-S was started. Membrane capacitance, series and membrane conductance were monitored for at least 2 min. N=6 for each trace. Cm, membrane capacitance; Gs, series conductance; Gm, membrane conductance.

Table 1. Summary of changes in membrane capacitance after internal application of GTP-γ-S. Values are means ± SEM. Cm = membrane capacitance.

| Agents          | Cm before GTP-γ-S Internalization (pF) | Cm after GTP-γ-S Internalization (pF) | ΔCm (pF) |
|-----------------|----------------------------------------|---------------------------------------|----------|
| External solution | 9.78 ± 1.26                             | 30.5 ± 4.15                           | 20.7 ± 3.03 |
| 1 μM Olopatadine | 10.7 ± 0.31                             | 31.6 ± 3.85                           | 20.9 ± 3.69 |
| 10 μM Olopatadine| 10.2 ± 0.86                             | 28.0 ± 2.29                           | 17.8 ± 2.09 |
| 100 μM Olopatadine| 10.2 ± 0.87                             | 12.6 ± 2.18                           | 2.40 ± 2.61 |
| 1 mM Olopatadine | 8.06 ± 0.79                             | 9.38 ± 1.54                           | 1.32 ± 1.11 |
| 100 μM Fexofenadine| 9.37 ± 0.61                             | 24.4 ± 1.79                           | 15.0 ± 1.79 |
| 100 μM Loratadine| 9.36 ± 1.32                             | 11.8 ± 1.61                           | 2.43 ± 0.42 |

Cm, Gs and Gm are shown in the figure and the numerical changes in the parameters are summarized in Table 1. Consistent with previous findings [9, 11, 12], internal application of GTP-γ-S to mast cells incubated in the external solution induced a more than 3-fold increase in the Cm (from 9.78 ± 1.26 to 30.5 ± 4.15 pF; n=6, P<0.05; Table 1), which occurred within 30 to 100 s after the establishment of the whole-cell recording configuration (Fig. 2A).

Incubation of mast cells with relatively lower concentrations of olopatadine (1 or 10 μM) did not significantly affect this increase in the Cm (Fig. 2B, Table 1). However, incubation
Effects of olopatadine on exocytosis-induced membrane surface deformation in mast cells.

In our recent study, amphiphilic drugs, such as salicylate and chlorpromazine, changed the plasma membrane curvature in rat peritoneal mast cells [9], which modulated the process of exocytosis. In the present study, since olopatadine inhibited the exocytotic process in mast cells (Fig. 1, 2), the drug-induced morphological changes in the cells would also affect this process. Therefore, after incubating mast cells in fexofenadine, loratadine or olopatadine-containing solutions, we externally induced exocytosis in the cells and examined the effects of this drug on the plasma membranes (Fig. 3A). 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. 3 Ab vs. a). mast cells incubated in 100 μM fexofenadine- (Fig. 3 Ac) or 1 and 10 μM olopatadine-containing solutions (Fig. 3 Ae, f) were not different from those incubated in the external solution alone (Fig. 3 Ab). However, in mast cells incubated in 100 μM loratadine- (Fig. 3Ad) or 100 μM and 1 mM olopatadine (h).

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with 100 μM or 1 mM olopatadine almost completely suppressed the incremental effect of GTP-7-S on the Cm (Fig. 2C and D, Table 1). As previously shown in degranulating mast cells [9, 11, 33], the Gs tended to decrease after the establishment of the whole-cell recording configuration (Fig. 2A-D), reflecting the gradual increase in the series resistance between the pipette electrodes and the cells interior. These results provide electrophysiological evidence for the first time that olopatadine inhibits the process of exocytosis in a concentration-dependent manner, which strongly supported our findings obtained from Fig. 1.

We also examined the effects of loratadine and fexofenadine on the Cm (Table 1), since these drugs inhibited or failed to inhibit the degranulation of mast cells (Fig. 1B). In mast cells incubated with 100 μM fexofenadine, the Cm also significantly increased (from 9.37 ± 0.61 to 24.4 ± 1.79 pF; n=6, P<0.05; Table 1). However, in mast cells incubated with 100 μM loratadine, such increase in the Cm was almost totally suppressed (from 9.36 ± 1.32 to 11.8 ± 1.61 pF; n=6; Table 1).

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Fig. 4. Electron microscopic images of olopatadine-induced membrane surface deformation in mast cells. Thin-section electron micrographs of mast cell plasma membranes after incubating the cells in the external solutions containing no drug (A) or 1 mM olopatadine (B).

Fig. 5. Illustrations of the olopatadine-induced plasma membrane curvature and the proposed effects on mast cell exocytosis. A: Positively charged (+; cationic) olopatadine is partitioned preferentially into the negatively charged inner leaflets of the lipid bilayers. It induced inner leaflet expansion and generated inward membrane bending. B: On the “curved regions” of the membranes, where secretory granules started to dock or fuse into the cellular membranes, such induced inward membrane stretch mechanically counteracts the further docking or fusion forces of the secretory granules. This closes the opened pores, thereby inhibiting the further process of exocytosis. In contrast, on the “flat part” of the membranes, where the secretory granules are about to dock or fuse, the inward stretch of the membranes may rather facilitate the fusion process of the granules. However, olopatadine, which exerts membrane stabilizing effects, halts the further progression of exocytosis.

Olopatadine-containing solutions (Fig. 3Ag,h), the findings suggestive of exocytosis, such as the wrinkles on their cell surface and the release of secretory granules, were totally absent.

To determine whether the wrinkles observed in the degranulating mast cells (Fig. Ab,ce,f) represented membrane surface deformation as a result of exocytosis, we then used lucifer yellow (Fig. 4B), a water-soluble fluorescent dye, that is retained in the invaginated folds created in the plasma membranes [13]. In mast cells that were treated with external solution alone (Fig. 3Bb), fexofenadine (Fig. 3Bc) or relatively lower concentrations of olopatadine solution (1 and 10 μM; Fig. 3Be and f), lucifer yellow was trapped partially on the cell surface area. Since the dye, which is normally membrane-impermeable [34], was completely absent in the cells before exocytosis was induced (Fig. 3Ba), the staining indicated its retention in the opened pores formed by exocytosis [35]. However, after incubating mast cells in loratadine- (Fig. 3Bd) or 100 μM and 1 mM olopatadine-containing solutions (Fig. 3Bg,h), the dye was completely washed out. These results indicated that olopatadine
inhibited the formation of the invaginated folds, suggesting that this drug counteracted the membrane surface deformation induced by exocytosis.

**Electron microscopic images of olopatadine-induced membrane surface deformation in mast cells**

To examine the direct effect of olopatadine on the membrane surface deformation in mast cells (Fig. 3B), we took the electron microscopic images of mast cells after incubating them in the external solutions containing no drug or 1 mM olopatadine (Fig. 4). Mast cells incubated in the external solution alone showed the flat surface of the cellular membranes which consisted of the lipid bilayers (Fig. 4A). However, in mast cells incubated in the olopatadine-containing external solution, there were some inward invaginations in the cellular membranes (Fig. 4B, arrows), suggesting that the drug induced the inward membrane bending in mast cells.

**Discussion**

In previous in vitro studies using human conjunctival mast cells, olopatadine was demonstrated to exert inhibitory effects on the release of histamine [2-4], in addition to its primary properties as a histamine receptor antagonist [1]. Mast cells that are derived from mucosal tissues, including conjunctiva, are known to produce larger amounts of chemical mediators than those from serosal tissues, such as the peritoneal cavity [36]. Therefore, in previous studies, mast cells derived from human conjunctiva required extremely high doses of olopatadine to effectively elicit their inhibitory properties on exocytosis [2-4]. In the present study, using rat peritoneal mast cells, we provide electrophysiological evidence for the first time for the mast cell-stabilizing properties of olopatadine (Fig. 2). Additionally, from our results, mast cells derived from peritoneal serosa required relatively lower doses of olopatadine than those derived from conjunctival mucosa [2-4]. According to previous in vivo studies, serosal-type mast cells are frequently involved in the development of fibrosis in many organs, including kidney, skin, lung and liver [37-39]. From our results, since olopatadine exerted inhibitory effects on exocytosis more effectively in mast cells derived from serosal tissues, this drug may also be used as an anti-fibrotic agent for mast cell-triggered organ sclerosis.

In the present study, as we recently demonstrated with chlorpromazine [9], olopatadine prevented plasma membrane deformation in rat peritoneal mast cells (Fig. 3). In many types of secretory cells, the exocytic process can be modulated by mechanical stimuli, such as changes in the membrane tension, shear stress, hydrostatic pressure and compression [8]. Therefore, the counteracting effect of olopatadine on the plasma membrane deformation in degranulating mast cells was thought to contribute to its inhibitory effect on the exocytic process. Structurally, since olopatadine has both a carboxylic functional group and a tertiary amino group, this drug exists in different ionic forms depending upon the pH of the solution [19]. Since the pKa of the groups are 4.18 and 9.79, respectively, olopatadine usually exists as a zwitterion in the pH under a physiological condition [40]. However, since olopatadine was conjugated with hydrochloride in the present study, the external solution containing higher concentrations of olopatadine tended to be more acidic. Therefore, as is usually the case with the other anti-allergic reagents [10], olopatadine was thought to be relatively cationic under the condition applied in the present study. Additionally, in previous studies using erythrocyte membranes, lipophilic olopatadine actually interacted directly with lipid layers of the plasma membranes [20, 41]. Therefore, this drug was thought to share similar pharmacological features with those of membrane amphipaths, and thus easily partitions into cellular membranes [20]. As previously demonstrated with chlorpromazine in both mast cells [9] and erythrocytes [42], a positively charged membrane amphipath, such as cationic olopatadine, would preferentially partition into the inner leaflet of the plasma membranes.
and generate inward membrane bending (Fig. 5A), which was actually demonstrated by
electron microscopy in the present study (Fig. 4). On the “curved regions” of the membranes,
where secretory granules started to dock or fuse into the cellular membranes (Fig. 5B top),
such induced inward stretch would mechanically counteract the further docking or fusion
forces of the secretory granules, as we recently demonstrated with membrane amphipaths
[9]. Consequently, this closes the opened pores of the mast cell membrane surface and
thereby inhibits the further process of exocytosis (Fig. 5B bottom). In contrast, on the “flat
part” of the membranes, where the secretory granules are about to dock or fuse (Fig. 5B top),
the inward stretch of the membranes may rather facilitate the soluble N-ethylmaleimide-
sensitive factor attachment protein receptor (SNARE)-induced fusion process of the granules
(Fig. 5B bottom), as previously demonstrated at neuronal synapses [43]. However, since
anti-allergic drugs, including olopatadine, are known to exert membrane stabilizing effects
by decreasing the fluidity of the membranes [44], it would halt the further progression of
exocytosis at this stage (Fig. 5B bottom).

According to previous patch-clamp studies, a rise in the \([\text{Ca}^{2+}]\) was one of the main
triggers of exocytosis in mast cells [7, 11]. Recently, Cruse et al. further demonstrated in
human lung mast cells that the rise in the \([\text{Ca}^{2+}]\) was dependent on the activity of \(\text{Ca}^{2+}\)-
activated K\(^{+}\) channels (Kc, 3.1), which provide the driving force for \(\text{Ca}^{2+}\) influx through store-
operated calcium channels (SOCs) [45]. Previous studies revealed that the activity of SOCs,
such as canonical transient receptor potential 1 (TRPC1), and Kc\(^{+}\)-channels was mechanically
modified by the stretch of the plasma membranes [46, 47]. From our results, olopatadine
actually generated inward membrane stretch in mast cells (Fig. 4), and thus counteracted the
exocytosis-induced deformation of the membrane surface (Fig. 5). Therefore, this drug was
thought to influence the activity of these channels through such mechanical stimuli to the
membranes. As a result, such induced changes in the \([\text{Ca}^{2+}]\), may also have additional effects
on the olopatadine-induced inhibition of exocytosis.

In summary, this study provided electrophysiological evidence for the first time that
oloapatadine dose-dependently inhibits the process of exocytosis in rat peritoneal mast cells.
Such mast cell-stabilizing properties of olopatadine could be ascribed to its 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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