Original Paper

Amphipaths Differentially Modulate Membrane Surface Deformation in Rat Peritoneal Mast Cells During Exocytosis

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Key Words

Salicylate • Chlorpromazine • Exocytosis in mast cells • Membrane capacitance • Plasma membrane deformation

Abstract

Background/Aims: Salicylate and chlorpromazine exert differential effects on the chemokine release from mast cells. Since these drugs are amphiphilic and preferentially partitioned into the lipid bilayers of the plasma membranes, they would induce some morphological changes in mast cells and thus affect the process of exocytosis. **Methods:** Employing the standard patch-clamp whole-cell recording technique, we examined the effects of salicylate and chlorpromazine on the membrane capacitance (Cm) during exocytosis in rat peritoneal mast cells. Using confocal imaging of a water-soluble fluorescent dye, lucifer yellow, we also examined their effects on plasma membrane deformation of the cells. **Results:** Salicylate dramatically accelerated the GTP- γ -S-induced increase in the Cm immediately after its application, whereas chlorpromazine significantly suppressed the increase. Treatment with salicylate increased the trapping of the dye on the cell surface, while treatment with chlorpromazine completely washed it out, indicating that both drugs induced membrane surface deformation in mast cells. **Conclusion:** This study demonstrated for the first time that membrane amphipaths, such as salicylate and chlorpromazine, may oppositely modulate the process of exocytosis in mast cells, as detected by the changes in the Cm. The plasma membrane deformation induced by the drugs was thought to be responsible for their differential effects.

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Introduction

Salicylate is one of the most commonly used nonsteroidal anti-inflammatory drugs (NSAIDs) that relieve fever, pain and inflammatory symptoms [1]. Since NSAIDs inhibit the production of cytokines from lymphocytes [2-4], they are also used as immunomodulatory

drugs [5, 6]. However, in some situations, such as in food-dependent exercise-induced anaphylaxis (FDEIA) [7, 8], salicylate and its acetyl derivative, aspirin, can stimulate chemokine release from mast cells, triggering adverse immunological reactions [9, 10]. On the other hand, chlorpromazine, one of the most traditionally used antipsychotic drugs [11], has also been recognized as an anti-allergic agent, because it inhibits histamine release from mast cells [12, 13]. Concerning the mechanisms by which these drugs exert their effects, the studies indicate that the drug-induced changes in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) are primarily responsible [9, 12]. Plus, previous studies revealed additional mechanisms that promote the Ca^{2+} -dependent exocytotic pathways, such as the involvement of G proteins [14] and the mechanical stimuli to the membranes [15]. Therefore, these mechanisms would also contribute to the salicylate- or chlorpromazine- induced modulation of the exocytotic process. Since both salicylate and chlorpromazine are amphiphilic [16], they are preferentially partitioned into the lipid bilayers of the plasma membranes. In our recent study, aspirin, a synthetic derivative of salicylate, changed the plasma membrane curvature in rat megakaryocytes [17], which affected the process of thrombopoiesis. Because megakaryocytes share common membranous features with degranulating mast cells in that they have many blebs on their cell surface [18], salicylate and chlorpromazine would also induce some morphological changes in mast cells and thus may affect their exocytotic process. To test this, employing the standard patch-clamp whole-cell recording technique in rat peritoneal mast cells [19, 20], we examined the effects of salicylate and chlorpromazine on the membrane capacitance. By confocal imaging of a hydrophilic fluorescent dye that is trapped on the cell surface [21], we also examined the effects of these drugs on plasma membrane deformation. Here, we show for the first time that membrane amphipaths, such as salicylate and chlorpromazine, may differentially modulate the process of exocytosis in mast cells, as detected by changes in the membrane capacitance. We also show that the plasma membrane deformation induced by the drugs is responsible for their differential effects.

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. 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 [22]. 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 (Fig. 1A, left), which were released from the cells by the external addition of compound 48/80 (Sigma-Aldrich, St. Louis, MO, USA; final concentration 10 $\mu\text{g}/\text{ml}$) (Fig. 1A, right).

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 [17, 23]. The patch pipette resistance was 4–6 M Ω 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'- α -(3-thiotriphosphate) (GTP- γ -S) (EMD Bioscience Inc., La Jolla, CA, USA) was additionally included in the internal solution to induce exocytosis in the mast cells [19, 20]. After a giga-seal formation, 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 M Ω during the experiments. To measure the membrane

Fig. 1. External or internal induction of exocytosis in rat peritoneal mast cells. **A:** Differential-interference contrast (DIC) microscopic images of mast cells before (left) and after (right) compound 48/80 was externally applied. **B:** After establishing the whole-cell recording configuration, the cells were internally dialyzed with 100 μ M GTP- γ -S. Membrane capacitance, series and membrane conductance were monitored for at least 200 s. $N=5$ for each trace. C_m , membrane capacitance; G_s , series conductance; G_m , membrane conductance.

capacitance of the mast cells, we employed a sine plus DC protocol using the Lock-in amplifier of the EPC-9 Pulse program. An 800-Hz sinusoidal command voltage was superimposed on the holding potential of -80 mV. The membrane capacitance (C_m), as well as membrane conductance (G_m) and series conductance (G_s), was continuously recorded during the whole-cell recording configuration. All experiments were carried out at room temperature.

Drug Delivery

Salicylate and chlorpromazine, purchased from Wako Pure Chem Ind. (Osaka, Japan), were separately dissolved in the external solution at the final concentration of 0.3, 3 mM and 100 μ M, respectively. These amphiphilic drugs can be relatively hydrophilic depending on their doses and the conditions of the solvent water, such as its pH and temperature [24, 25]. With the doses we used and the external solution we prepared in the present study (pH 7.2, at room temperature), the drugs were easily dissolved in the solution without the need of lipophilic drug vehicles, such as dimethyl sulfoxide (DMSO). Therefore, as a control, we applied the external solution alone to the cells, and compared with those containing either drug. We delivered the solutions by the standing hydrostatic pressure of 3 cmH₂O from a nearby pipette as described previously [17, 26, 27]. The mast cells were exposed to the drugs for 20 s in the middle of exocytosis induced by GTP- γ -S.

Quantification of Mast Cell Degranulation

After exocytosis was externally induced by compound 48/80 (10 μ g/ml), the mast cells were incubated in the external solutions containing no drugs, 3 mM salicylate, or 100 μ M chlorpromazine. Bright-field images were acquired from randomly selected 0.1-mm² fields of view (10 views from 6 of each condition), as described in our previous study [23]. Degranulated mast cells were defined as cells associated with ≥ 8 granules outside the cell membrane as described previously [13]. These mast cells were then counted and their ratio to all mast cells was calculated.

Fig. 2. Amphipath-induced changes in mast cell membrane capacitance, series and membrane conductance during exocytosis. After establishing the whole-cell recording configuration and starting the dialysis with 100 μ M GTP- γ -S, external solutions containing no drugs (A), 0.3 mM salicylate (B), 3 mM salicylate (C) or 100 μ M chlorpromazine (D) were delivered for 20 sec to single mast cells. Membrane capacitance, series and membrane conductance were monitored for at least 2 min. $N=5$ for each trace. Cm, membrane capacitance; Gs, series conductance; Gm, membrane conductance.

Lucifer Yellow Trapping on the Cell Surface

After exocytosis was externally induced by compound 48/80 (10 μ g/ml), the mast cells were incubated for 5 min at room temperature in external solution containing a hydrophilic fluorescent dye, lucifer yellow [21] (Wako, Osaka, Japan; final concentration 10 μ M). Salicylate or chlorpromazine was then added to the external solution at the final concentration of 3 mM or 100 μ M, respectively. After incubating the cells for another 5 min, they were washed 2 to 3 times thoroughly with dye-free external solution containing either drug. 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 $\pm$ 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 salicylate and chlorpromazine on whole-cell membrane capacitance in rat peritoneal mast cells during exocytosis

Consistent with previous findings [19, 20], internal application of GTP- γ -S to mast cells induced a more than 3-fold increase in the membrane capacitance (Cm) (from 10.1 ± 0.55 to 32.6 ± 0.99 pF, $n=6$, $P<0.05$) without significant effects on the Gm and Gs (Fig. 1B). The increase in the Cm occurred within 30 to 120 s after the establishment of the whole-cell recording configuration, which then reached a plateau that remained for the rest of the observation period. It has been well established by previous studies that the increase in the

Table 1. Summary of changes in membrane capacitance after application of salicylate and chlorpromazine

Agents	N	Cm before drug application (pF)	Cm after drug application (pF)	ΔCm (pF)
External solution (control)	5	24.9 $\pm$ 0.82	28.0 $\pm$ 0.87 [#]	3.08 $\pm$ 0.61
0.3 mM Salicylate	5	25.8 $\pm$ 0.73	52.0 $\pm$ 2.37 [#]	26.2 $\pm$ 3.09*
3 mM Salicylate	5	25.2 $\pm$ 2.89	57.4 $\pm$ 4.29 [#]	36.7 $\pm$ 3.91*
100 μ M Chlorpromazine	5	26.5 $\pm$ 1.99	24.6 $\pm$ 1.39	-1.92 $\pm$ 0.77*

Values are means $\pm$ SEM. Cm = membrane capacitance. [#]p < 0.05 vs. Cm before drug application.

*p < 0.05 vs. ΔCm after application of the external solution.

Cm in mast cells correlates with the degranulating process of exocytosis [28, 29]. Therefore, in the present study, to determine the effects of the membrane amphipaths on mast cell exocytosis, we applied external solution, containing either salicylate or chlorpromazine, to the cells during exocytosis and examined the changes in the Cm (Fig. 2).

Since physiological or therapeutic levels of salicylate range from 0.1 to 1 mM [9, 30], we started with 0.3 mM of this drug in the present study. At higher concentrations than 3 mM, salicylate is known to cause necrosis or apoptosis of the cells [31]. In the present study, however, since we delivered the drugs to single, isolated mast cells by a puff application method, there was the possibility of partial and insufficient exposure of the drugs to the cells [17, 26, 27]. Therefore, in addition to 0.3 mM, we also tried a significantly higher concentration at 3 mM. On the other hand, the physiological concentration of chlorpromazine [32] and its doses to effectively elicit pharmacological effects on single mast cells were much lower than salicylate [12]. In recent *in vitro* studies using outer hair cells and red blood cells [16], 100 μ M chlorpromazine most effectively exerted contrastive effects to salicylate that ranges from 0.5 to 10 mM on the membrane curvature. Therefore, in the present study, we applied the same dose of chlorpromazine to mast cells.

The effects of salicylate and chlorpromazine on the Cm, Gs and Gm are shown in Figure 2 and the numerical changes in the parameter are summarized in Table 1. By simply applying the external solution alone to the mast cells during exocytosis (Fig. 2A), we confirmed that our procedure of reagent application, a puff application by constant hydrostatic pressure with a nearby pipette, was without significant effect on the GTP- γ -S-induced increase in the Cm (Fig. 2A, Table 1). However, the pipette inclusion of 0.3 mM salicylate significantly accelerated the increase in the Cm immediately after the application (Fig. 2B, Table 1), and the effect of this drug was more marked with 3 mM (Fig. 2C, Table 1). In contrast, the pipette inclusion of chlorpromazine significantly suppressed the Cm increase (Fig. 2D, Table 1), and lowered the parameter after the application (Table 1). As previously shown in degranulating mast cells [19, 29], the Gs tended to decrease with a GTP- γ -S internalization (Fig. 2A), reflecting the gradual increase in the series resistance between the pipette electrodes and the cells interior. However, the rate of decrease was not significantly affected by the application of salicylate or chlorpromazine (Fig. 2B-D). Additionally, these drugs did not influence the Gm either (Fig. 2B-D), maintaining its levels below 10 nS during the whole observation period. Since the Gm value usually correlates with the ionic permeability of the membrane [33], the doses of the drugs used in the present study were not considered to damage the membranes of the cells [19]. Therefore, the results obtained from the Cm measurement indicated that salicylate facilitated the process of exocytosis in mast cells, whereas chlorpromazine inhibited it.

Effects of salicylate and chlorpromazine on mast cell degranulation

From our results, salicylate and chlorpromazine differentially affected the Cm values in mast cells (Fig. 2). Since membrane capacitance is an indicator of exocytosis in mast cells [28, 29], we actually examined the effects of these drugs on mast cell degranulation (Fig. 3). Without the drugs, compound 48/80 induced degranulation in 56.9 $\pm$ 5.9 % of the mast cells observed (n=6). Salicylate markedly increased the ratio of degranulating mast cells, indicating that it induced degranulation in almost all the cells (93.2 $\pm$ 6.8 %, n=6, P<0.05).

Fig. 3. Effects of amphipaths on mast cell degranulation. After exocytosis was induced by compound 48/80, the mast cells were incubated in the external solutions containing no drugs, 3 mM salicylate, or 100 μ M chlorpromazine. The numbers of degranulating mast cells were expressed as percentages of the total mast cell number in selected bright fields. * $P < 0.05$ vs. incubation in the external solution alone. Values are means $\pm$ SEM ($n = 6$: 10 views from 6 of each condition). Differences were analyzed by ANOVA followed by Dunnett's or Student's t test.

In contrast, chlorpromazine significantly decreased the number of degranulating mast cells ($39.4 \pm 6.1\%$, $n = 6$, $P < 0.05$). These results strongly supported our hypothesis obtained from Fig. 2 that salicylate facilitates the process of exocytosis, whereas chlorpromazine inhibits it.

Effects of salicylate and chlorpromazine on membrane surface deformation in mast cells during exocytosis

In our recent study, aspirin, a synthetic derivative of salicylate, changed the plasma membrane curvature in rat megakaryocytes [17], which affected the process of thrombopoiesis. In the present study, since salicylate and chlorpromazine were thought to modulate the exocytotic process in mast cells (Fig. 2, 3), the drug-induced morphological changes would contribute to this process, as was the case with megakaryocytes. Therefore, after inducing exocytosis in mast cells by compound 48/80, we incubated the cells in either salicylate or chlorpromazine containing solution and examined their effects on the plasma membranes (Fig. 4A). Compared with mast cells incubated in the external solution alone (Fig. 4A, top left), those incubated in the 0.3 or 3 mM salicylate-containing solutions apparently had more wrinkles on their cell surface (Fig. 4A, top right and bottom left). However, the cells incubated in the chlorpromazine-containing solution were not much different from those incubated in the external solution alone (Fig. 4A, bottom right).

To determine whether the wrinkles represented membrane surface deformation induced by the drugs, we then used lucifer yellow (Fig. 4B), a water-soluble fluorescent dye, that is retained in the invaginated folds created in the plasma membranes [21]. In mast cells that were not treated with the drugs, lucifer yellow was trapped only slightly in the peripheral plasma membranes (Fig. 4B, top left), indicating its retention only in the opening pores formed by exocytosis [34]. However, after incubating mast cells in the salicylate-containing solution (0.3 and 3 mM), the dye was trapped thoroughly on the whole cell surface (Fig. 4B, top right and bottom left), whereas it was completely washed out in the chlorpromazine-containing solution (Fig. 4B, bottom right). These results indicated that salicylate increased the number of invaginated folds, whereas chlorpromazine decreased it, suggesting that both drugs induced membrane surface deformation in degranulating mast cells.

Discussion

Previous *in vitro* studies have examined the effects of salicylate and chlorpromazine on mast cell exocytosis by simply measuring the amounts of chemokines released from the cells [9, 12, 35, 36]. However, in these studies, the drugs were applied to the cells either before exocytosis was initiated or after it was completed. Therefore, the results were inconsistent and largely dependent on the experimental procedure. In the present study, employing an authentic electrophysiological approach [19, 20], we applied the drugs for the first time during exocytosis in mast cells, and thus directly monitored their effects on the membrane capacitance (Fig. 2), which actually correlated with those on mast cell degranulation (Fig. 3). Based on our results, salicylate facilitated the process of exocytosis, whereas chlorpromazine

Fig. 4. Images of amphipath-induced membrane surface deformation in mast cells during exocytosis. Mast cells were exposed to compound 48/80 and exocytosis was initially induced. *A*: Differential-interference contrast (DIC) microscopic images were taken after incubation in the external solutions containing no drugs (top left), 0.3 mM salicylate (top right), 3 mM salicylate (bottom left), or 100 μM chlorpromazine (bottom right). *B*: The trapping of the fluorescent dye (lucifer yellow) on the cell surface was detected after incubation in the external solutions containing no drugs (top left), 0.3 mM salicylate (top right), 3 mM salicylate (bottom left), or 100 μM chlorpromazine (bottom right).

Fig. 5. Illustrations of the amphipath-induced plasma membrane curvature and the proposed effects on mast cell exocytosis. *Aa*: Negatively charged salicylate is partitioned preferentially into the positively charged outer leaflets of the lipid bilayers. It induced outer leaflet expansion and generated outward membrane bending. *Ab*: Such induced outward membrane stretch mechanically reinforces the docking or fusion process of the secretory granules into the cellular membranes, facilitating the process of exocytosis. *Ba*: In contrast, positively charged chlorpromazine is partitioned preferentially into the negatively charged inner leaflets of the lipid bilayers. It induced inner leaflet expansion and generated inward membrane bending. *Bb*: Such induced inward membrane stretch counteracts the fusion forces of the secretory granules and closes the opened pores, thus inhibiting the process of exocytosis.

inhibited it. These findings supported the clinical evidence that salicylate can be a trigger of acute allergic reactions, such as anaphylaxis [7, 8], while chlorpromazine is a potent inhibitor of allergic reactions [11].

In the present study, as we recently demonstrated with aspirin in megakaryocytes [17], salicylate and chlorpromazine differentially induced plasma membrane deformations in mast cells (Fig. 4). In many cell types, the exocytotic process can be modulated by mechanical stimuli, such as changes in the membrane tension, shear stress, hydrostatic pressure and compression [15]. Therefore, the amphipath-induced plasma membrane deformations in mast cells were thought to contribute to their differential effects on the exocytotic process. As we schematized in Figure 5, salicylate, a negatively charged amphipath, partitions preferentially into the outer leaflet of the plasma membrane and generates outward membrane bending [16] (Fig. 5Aa). Such induced outward membrane stretch would mechanically reinforce the docking or fusion process of the secretory granules into the cellular membranes (Fig. 5Ab), which may thereby facilitate the process of exocytosis. On the other hand, chlorpromazine, a positively charged amphipath, partitions preferentially into the inner leaflet and generates inward membrane bending [16] (Fig. 5Ba). In such a case,

the inward stretch of the membranes would counteract the fusion forces of the secretory granules and close the opened pores (Fig. 5Bb), which may inhibit the process of exocytosis.

Patch-clamp studies have revealed that a rise in the $[Ca^{2+}]_i$ is the main trigger of exocytosis in mast cells [14, 19]. Recently, Cruse et al. further demonstrated in human lung mast cells that the rise in the $[Ca^{2+}]_i$ was dependent on the activity of Ca^{2+} activated K^+ channels ($K_{Ca} 3.1$), which provide the driving force for Ca^{2+} influx through store-operated calcium channels (SOCs) [37]. According to previous studies, the activity of SOCs, such as canonical transient receptor potential 1 (TRPC1), and K_{Ca} -channels was mechanically modified by stretching the plasma membranes [38, 39]. From our results, since salicylate and chlorpromazine generated bidirectional membrane stretch in mast cells (Fig. 5), they were thought to influence the activity of the channels. Consequently, such induced changes in the $[Ca^{2+}]_i$ may also have additional effects on the amphipath-induced modulation of exocytosis.

In summary, this study demonstrated for the first time that the membrane amphipaths, salicylate and chlorpromazine, may differentially modulated the process of exocytosis in mast cells, as detected by changes in the membrane capacitance. The plasma membrane deformation induced by the drugs was thought to be responsible for their differential effects.

Declaration of interest

The authors declare no conflicts of interest.

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