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Original Paper

Pyridoxine Synergistically Potentiates Mast Cell-Stabilizing Property of Ascorbic Acid

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Key Words
Ascorbic acid • Pyridoxine • Exocytosis • Mast cells • Membrane capacitance (Cm) • Mast cell-stabilizing property

Abstract
Background/Aims: Besides their physiological properties, vitamins, such as vitamin C (ascorbic acid) and B_6 (pyridoxine), ameliorate the symptoms of allergic disorders. Because exocytosis in mast cells can be detected electrophysiologically by the changes in the membrane capacitance (Cm), its continuous monitoring in the presence of these vitamins would determine their mast cell-stabilizing, anti-allergic properties. ***Methods:*** Employing the whole-cell patch-clamp technique in rat peritoneal mast cells, we examined the effects of ascorbic acid and pyridoxine on the degranulation of mast cells and the increase in the Cm during exocytosis. ***Results:*** Both ascorbic acid and pyridoxine dose-dependently suppressed the GTP-γ-S-induced increase in the Cm and inhibited the degranulation from mast cells. Surprisingly enough, relatively low concentrations of pyridoxine (1, 2 mM) synergistically enhanced the suppressive effect of 2 mM ascorbic acid on mast cell degranulation. ***Conclusion:*** These results provided electrophysiological evidence for the first time that ascorbic acid and pyridoxine inhibited the process of exocytosis in a dose-dependent manner. At relatively lower concentrations, these vitamins were not enough to stabilize mast cells. However, such concentrations of pyridoxine synergistically potentiated the mast cell-stabilizing property of ascorbic acid.

Introduction

Vitamins are essential nutrients that the body needs to function properly [1]. Among them, vitamin C (ascorbic acid) and B_6 (pyridoxine) are water-soluble vitamins that play roles in immune function, protein metabolism, growth and organ development [1]. Besides these physiological properties, previous studies revealed their roles in ameliorating the symptoms

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of allergic disorders, such as anaphylaxis and bronchial asthma, indicating their anti-allergic properties [2-4]. To elucidate the mechanisms underlying the anti-allergic properties of these vitamins, previous studies tried to evaluate the amount of histamine released from mast cells [2, 5-7]. However, since mast cells also release other chemical mediators or various types of growth factors [8], the exocytotic process itself must be monitored directly to determine the ability of vitamins in stabilizing mast cells. In our previous studies, by continuously monitoring the changes in whole-cell membrane capacitance (Cm) in mast cells, we provided electrophysiological evidence for the first time that anti-allergic drugs, anti-microbial drugs, corticosteroids and catecholamines exert mast cell-stabilizing properties [9-13]. Recently, we have additionally revealed that food constituents, such as caffeine and catechin, inhibit the process of exocytosis in mast cells in a dose-dependent manner [14]. In the present study, employing the standard patch-clamp whole-cell recording technique in rat peritoneal mast cells, we examined the effects of vitamins, such as ascorbic acid and pyridoxine, on the changes in the Cm to quantify their ability to stabilize mast cells. Here, this study provides electrophysiological evidence that ascorbic acid and pyridoxine inhibit the process of exocytosis in a dose-dependent manner for the first time. This study also shows that low concentrations of pyridoxine synergistically potentiate the mast cell-stabilizing property of ascorbic acid, which may be attributable to the synergistic interaction of their antioxidant properties.

Materials and Methods

Cell Sources and Preparation

Male Wistar rats no less than 25 weeks old were purchased from CLEA Japan Inc. (Tokyo, Japan). We profoundly anaesthetized the rats with isoflurane and sacrificed them by cervical dislocation. The protocols for the use of animals were approved by the Animal Care and Use Committee of Miyagi University. As we previously described [9-12, 15], we washed rat peritoneum using standard external (bathing) solution which consists of (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) and isolated mast cells from the peritoneal cavity. We maintained the isolated mast cells at room temperature (22-24 °C) for about 8 hours until use. The suspension of mast cells was spread on a chamber placed on the headstage of an inverted microscope (Nikon, Tokyo, Japan). Mast cells were easily distinguished from other cell types since they included characteristic secretory granules within the cells [9-15].

Quantification of Mast Cell Degranulation

Ascorbic acid, purchased from Wako Pure Chem Ind. (Osaka, Japan), and pyridoxine hydrochloride, from Kanto Chemical Co., Inc. (Tokyo, Japan), were separately dissolved in the external solution at final concentrations of 1, 2, 5 and 10 mM. After we incubated mast cells in these solutions or a solution without the substances, exocytosis was externally induced by compound 48/80 (Sigma-Aldrich Co., St. Louis, MO, USA; final concentration 10 μg/ml) [9-15]. We obtained bright-field images from randomly chosen 0.1-mm^2 fields of view (10 views from each condition), as described previously [9-15]. We counted the number of degranulated mast cells (definition; cells surrounded by more than 8 granules outside the cell membrane) and calculated their ratio to all mast cells.

Electrical Setup and Membrane Capacitance Measurements

As we described in our previous studies [9-13, 15], we employed an EPC-9 patch-clamp amplifier system (HEKA Electronics, Lambrecht, Germany) and conducted standard whole-cell patch-clamp recordings. Briefly, we maintained the patch pipette resistance between 3-5 MΩ when plugged with internal (patch pipette) solution which consists of (in mM): K-glutamate, 145; $MgCl_2$, 2.0; Hepes, 5.0 (pH 7.2 adjusted with KOH). We added 100 μM guanosine 5'-*o*-(3-thiotriphosphate) (GTP-γ-S) (Sigma Aldrich Co.) into the internal solution to endogenously induce exocytosis in mast cells [9-13, 15]. We induced a giga-seal formation on a single mast cell spread in the external solutions with or without different concentrations of ascorbic acid or pyridoxine hydrochloride (1, 2, 5 and 10 mM). Then we briefly sucked the pipette to rupture

the patch membrane and perfused GTP-γ-S into the cells. To monitor the membrane capacitance of mast cells, we conducted a sine plus DC protocol employing the Lock-in amplifier of an EPC-9 Pulse program. We superimposed an 800-Hz sinusoidal command voltage on the holding potential of -80 mV. We continuously monitored the membrane capacitance (Cm), membrane conductance (Gm) and series conductance (Gs) during the whole-cell recording configuration. We performed all experiments at room temperature.

Statistical 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. A value of $p<0.05$ was considered significant.

Results

Effects of ascorbic acid and pyridoxine on degranulation of rat peritoneal mast cells

Mast cells incubated in the external solution alone or 1 mM ascorbic acid showed many wrinkles on their cell surface and released secretory granules as a consequence of exocytosis (Fig. 1Ab, c vs. a). However, in the case of 2 mM or higher concentration of ascorbic acid, such findings suggestive of exocytosis were partially or completely suppressed (Fig. 1Ad, e, f). Quantitatively, 1 mM ascorbic acid did not affect the numbers of degranulating mast cells (Fig. 1B). However, 2 mM ascorbic acid significantly decreased the number of degranulating mast cells (external solution, 97.6 ± 0.71 % vs. 2 mM, 51.5 ± 5.03 %; n=10, $p<0.05$; Fig. 1B). Five and 10 mM ascorbic acid further reduced the numbers of degranulating cells (5 mM, 7.64 ± 1.41 %; 10 mM, 4.82 ± 1.22 %; n=10, $p<0.05$; Fig. 1B).

Like these effects of ascorbic acid (Fig. 1), 1 mM pyridoxine did not affect the degranulation of mast cells (Fig. 2Ab, c vs. a) and the numbers of them were almost comparable to those incubated in the external solution alone (Fig. 2B). However, 2 mM or higher concentration of pyridoxine remarkably suppressed the process of exocytosis (Fig. 2Ad, e, f) and significantly suppressed the numbers of degranulating mast cells (external solution, 97.5 ± 0.80 % vs. 2 mM, 73.9 ± 8.50 %; n=10, p<0.05; Fig. 2B). Of note, 5 and 10 mM pyridoxine showed a marked reduction in the numbers of degranulating mast cells (5 mM, 11.3 ± 0.75 %; 10 mM, 5.82 ± 1.27 %; n=10, $p<0.05$; Fig. 2B).

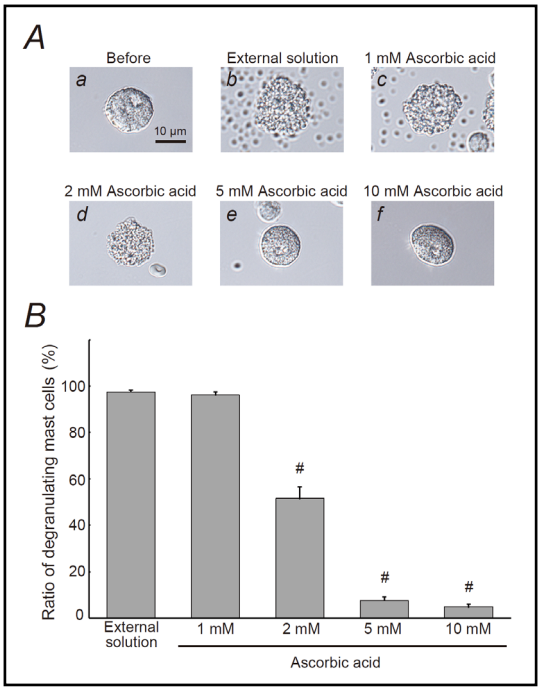

Fig. 1. Effects of ascorbic acid on mast cell degranulation. A: Differential-interference contrast (DIC) microscopic images were taken before (a) and after exocytosis was externally induced by compound 48/80 in mast cells incubated in the external solutions containing no ascorbic acid (b), 1 mM ascorbic acid (c), 2 mM ascorbic acid (d), 5 mM ascorbic acid (e) and 10 mM ascorbic acid (f). B: After the mast cells were incubated in the external solutions containing no ascorbic acid or different concentrations (1, 2, 5 and 10 mM) of ascorbic acid, exocytosis was induced by compound 48/80. The numbers of degranulating mast cells were 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. Differences were analyzed by ANOVA followed by Dunnett's t test.

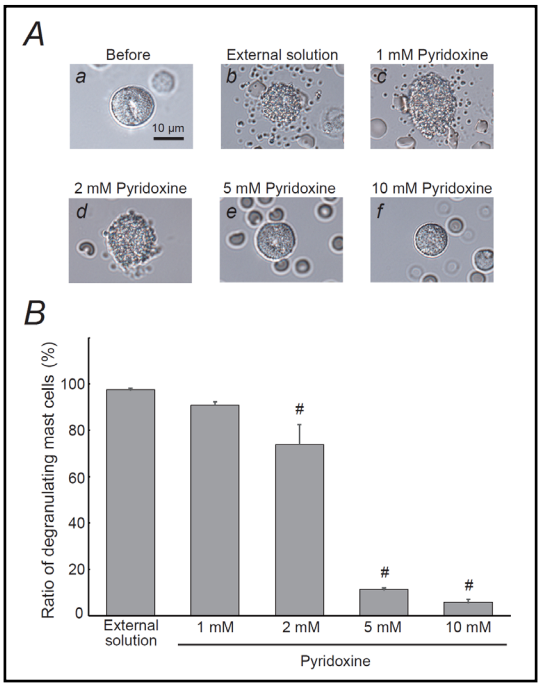

Fig. 2. Effects of pyridoxine on mast cell degranulation. A: Differential-interference contrast (DIC) microscopic images were taken before (a) and after exocytosis was externally induced by compound 48/80 in mast cells incubated in the external solutions containing no pyridoxine (b), 1 mM pyridoxine (c), 2 mM pyridoxine (d), 5 mM pyridoxine (e) and 10 mM pyridoxine (f). B: After the mast cells were incubated in the external solutions containing no pyridoxine or different concentrations (1, 2, 5 and 10 mM) of pyridoxine, exocytosis was induced by compound 48/80. The numbers of degranulating mast cells were 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. Differences were analyzed by ANOVA followed by Dunnett's t test.

Effects of ascorbic acid and pyridoxine on whole-cell membrane capacitance in rat peritoneal mast cells

In our previous studies, microscopic changes in megakaryocyte or lymphocyte membranes were exactly monitored by measuring the whole-cell membrane capacitance (Cm) [16-24]. Of note, in mast cells, the process of degranulation during exocytosis was continuously monitored using the degree of the increase in the Cm [9-13, 15, 25, 26]. Hence, in our study, to investigate the effects of ascorbic acid or pyridoxine on the process of exocytosis, we pre-incubated mast cells in ascorbic acid- or pyridoxine- containing external solutions and monitored the changes in Cm (Fig. 3 and 4). In these figures, we showed the effects of 1, 2, 5 and 10 mM ascorbic acid (Fig. 3) and pyridoxine (Fig. 4) on the Cm, series conductance (Gs) and membrane conductance (Gm). Table 1 summarizes the changes in the Cm.

Reflecting the endogenous induction of exocytosis [9-13, 15, 27, 28], the internal addition of GTP-γ-S into mast cells markedly increased the value of Cm (from 7.85 ± 1.44 to 30.5 ± 5.11 pF, n=5, $p<0.05$; Table 1). When mast cells were pre-incubated with 1 or 2 mM ascorbic acid, the addition of GTP-γ-S tended to increase the Cm similarly to that of mast cells pre-incubated with the external solution alone (Fig. 3A, B). However, compared to the external solution alone, 2 mM ascorbic acid significantly suppressed the increase in the Cm (ΔCm) (external solution, 22.7 ± 4.39 vs. 2 mM, 8.87 ± 3.22 pF; n=16, $p<0.05$; Table 1). In the case of relatively higher concentrations (5, 10 mM) of ascorbic acid, the GTP-γ-S-induced increase in the Cm was markedly suppressed (Fig. 3C and D; 5 mM, -0.93 ± 1.35 pF, n=6, $p<0.05$; 10 mM, -0.34 ± 1.22 pF, n=7, $p<0.05$; Table 1).

Similarily to the effects of ascorbic acid (Fig. 3), in mast cells pre-incubated with 1 or 2 mM pyridoxine, the addition of GTP-γ-S tended to increase the Cm (Fig. 4A, B). However, compared to the external solution alone, 2 mM pyridoxine significantly suppressed the increase in the Cm (external solution, 22.7 ± 4.39 vs. 2mM, 12.8 ± 1.65 pF; n=13, $p<0.05$; Table 1). In the case of higher concentrations (5, 10 mM) of pyridoxine, the GTP-γ-S-induced increase in the Cm was markedly suppressed (Fig. 4C and D; 5 mM, 0.65 ± 1.34 pF, n=6, $p<0.05$; 10 mM, 0.56 ± 1.43 pF, n=6, $p<0.05$; Table 1). These findings suggest that vitamins, such as ascorbic acid and pyridoxine, inhibit the exocytotic process of mast cells in a dose-dependent manner.

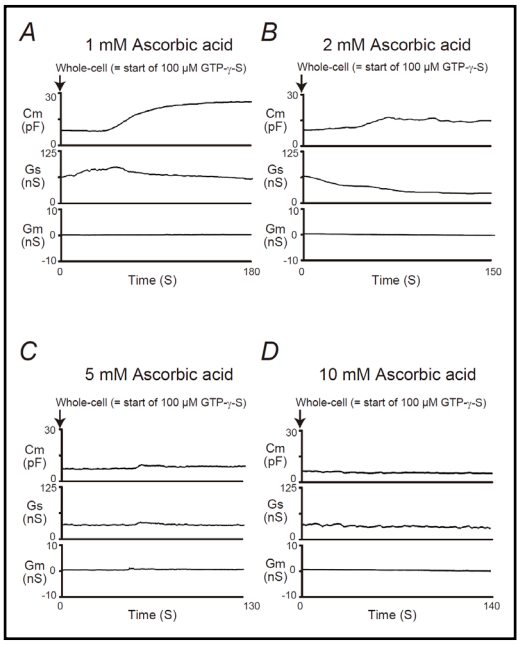

Fig. 3. Ascorbic acid-induced changes in mast cell membrane capacitance, series and membrane conductance during exocytosis. After the mast cells were incubated in the external solutions containing 1 mM (A), 2 mM (B), 5 mM (C) or 10 mM ascorbic acid (D), the whole-cell recording configuration was established 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 120 sec. Cm, membrane capacitance; Gs, series conductance; Gm, membrane conductance.

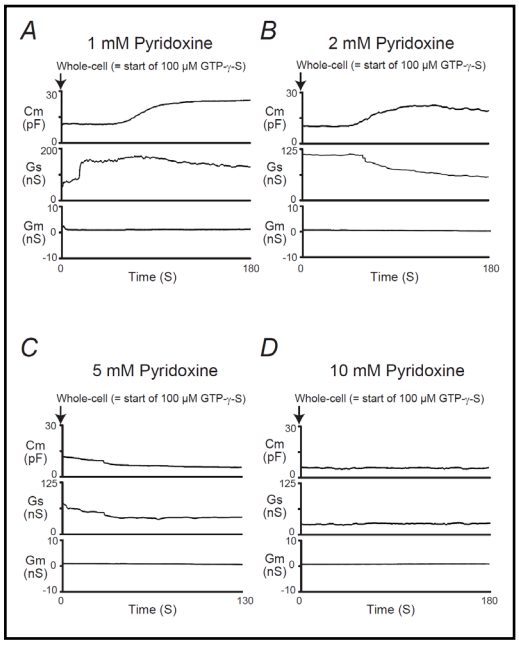

Fig. 4. Pyridoxine-induced changes in mast cell membrane capacitance, series and membrane conductance during exocytosis. After the mast cells were incubated in the external solutions containing 1 mM (A), 2 mM (B), 5 mM (C) or 10 mM pyridoxine (D), the whole-cell recording configuration was established 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 120 sec. Cm, membrane capacitance; Gs, series conductance; Gm, membrane conductance.

Table 1. Summary of changes in membrane capacitance in external solutions containing ascorbic acid or pyridoxine. Values are means ± SEM. Cm = membrane capacitance. $p < 0.05$ vs. Δ*Cm* in external solution

Substances	N	*Cm* before GTP-γ-S internalization (pF)	*Cm* after GTP-γ-S internalization (pF)	*ΔCm* (pF)
External solution (control)	5	7.85 ± 1.44	30.5 ± 5.11	22.7 ± 4.39
1 mM Ascorbic acid	6	10.7 ± 0.65	29.6 ± 2.85	18.9 ± 2.94
2 mM Ascorbic acid	16	7.28 ± 0.73	16.1 ± 3.61	8.87 ± 3.22*
5 mM Ascorbic acid	6	8.77 ± 1.21	7.84 ± 1.03	-0.93 ± 1.35*
10 mM Ascorbic acid	7	7.14 ± 1.51	6.80 ± 1.33	-0.34 ± 1.22*
1 mM Pyridoxine	9	10.8 ± 0.55	30.7 ± 4.18	19.9 ± 4.24
2 mM Pyridoxine	13	9.40 ± 0.39	22.2 ± 1.65	12.8 ± 1.65*
5 mM Pyridoxine	6	8.43 ± 0.72	9.07 ± 1.91	0.65 ± 1.34*
10 mM Pyridoxine	6	7.31 ± 0.84	7.87 ± 1.21	0.56 ± 1.43*

In Fig. 3A and 4A, the Gs initially increased immediately after the rupture the patch membrane, which preceded the increase in the Cm (Fig. 3A and 4A). This reflected the decrease in the series resistance due to the generation of the electrical connection between the pipette electrodes and the cells interior [27]. Then, as previously demonstrated in degranulating mast cells [26, 27], the Gs tended to decrease with the internalization of GTP-γ-S, reflecting the gradual increase in the series resistance. However, the Gs was not largely affected for the rest of the observation period, suggesting that the cells were accurately voltage-clamped during the measurement of the Cm.

Combined effects of ascorbic acid with low concentrations of pyridoxine on degranulation of rat peritoneal mast cells

As shown in Fig. 2 and 4, the suppressive effects of relatively lower concentrations of pyridoxine (1, 2 mM) on the degranulation of mast cells and the increase in the Cm were much smaller than those of relatively higher concentrations of pyridoxine (5, 10 mM) (Fig. 2, 4). However, we recently revealed that lower concentrations of food substances, such as catechin, could synergistically potentiate the mast cell-stabilizing property of caffeine [14]. To investigate the similar additive therapeutic efficacy of low dose substances, we examined the effects of 1 or 2 mM pyridoxine on the ascorbic acid-induced inhibition of exocytosis (Fig. 5).

Consistent with our results shown in Fig. 1, 2 mM ascorbic acid partially halted the process of exocytosis in mast cells (Fig. 5A b vs. a) and significantly but not markedly reduced the number of degranulating mast cells (control, 97.4 ± 0.70 % vs. 2 mM ascorbic acid, 45.7 ± 7.38 %; n=10, p<0.05; Fig. 5B). However, surprisingly enough, in the presence of 1 or 2 mM pyridoxine, the exocytotic process of mast cells was almost completely halted (Fig. 5Ac, d vs. b). Regarding the numbers of degranulating mast cells, they were more markedly decreased than those with 2 mM ascorbic acid alone (2 mM ascorbic acid + 1 mM pyridoxine, 2.19 ± 0.81 %, n=10, p<0.05; 2 mM ascorbic acid + 2 mM pyridoxine, 2.53 ± 1.08 %, n=10, p<0.05; Fig. 5B). These findings suggest that the inhibitory effect of ascorbic acid on exocytosis was augmented, and that lower concentrations of pyridoxine synergistically potentiated the mast cell-stabilizing property of ascorbic acid.

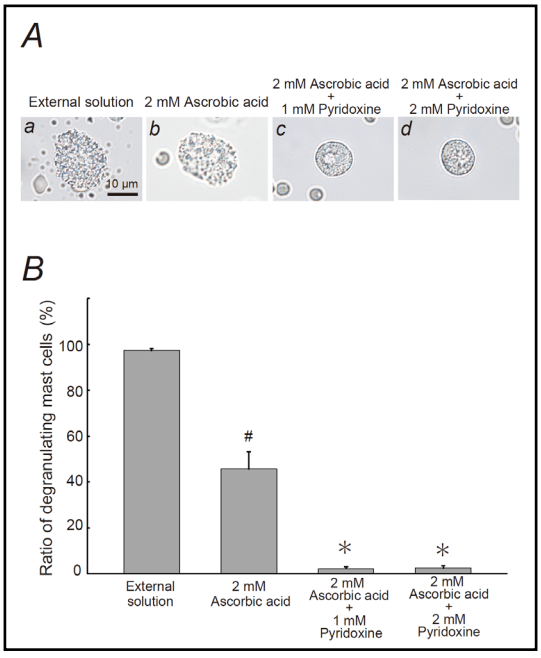

Fig. 5. Effects of low concentrations of pyridoxine on 2 mM ascorbic acid-induced inhibition of mast cell degranulation. A: Differential-interference contrast (DIC) microscopic images were taken after exocytosis was externally induced by compound 48/80 in mast cells incubated in the external solutions containing no substances (a), 2 mM ascorbic acid alone (b), 2 mM ascorbic acid in the presence of 1 mM pyridoxine (c) and 2 mM ascorbic acid in the presence of 2 mM pyridoxine (d). B: After exocytosis was induced in mast cells incubated in the external solutions containing no substances, 2 mM ascorbic acid alone, 2 mM ascorbic acid in the presence of 1 or 2 mM pyridoxine, the numbers of degranulating mast cells were expressed as percentages of the total mast cell numbers in selected bright fields. $^{\#}p$<0.05 vs. incubation in the external solution alone. *p<0.05 vs. incubation in the external solution containing 2 mM ascorbic acid. Values are means ± SEM. Differences were analyzed by ANOVA followed by Tukey's test.

Discussion

Regarding the mechanisms underlying the anti-allergic properties of vitamins, previous studies evaluated the amount of histamine released from mast cells [2, 3, 5-7]. In these studies, both ascorbic acid and pyridoxine reduced the serum concentration of histamine in allergic patients or suppressed the release of histamine from isolated mast cells. Concerning the mechanisms, ascorbic acid was considered to prevent the formation of histamine or facilitate its degradation [29, 30]. However, besides histamine, mast cells release other chemical mediators, such as leukotrienes or β-hexosaminidase, and produce various types of inflammatory cytokines or growth factors [8]. In this regard, to exactly determine the ability of vitamins in stabilizing mast cells, we have to evaluate the release or production of all these substances. Alternatively, we have to directly monitor the process of exocytosis itself in mast cells. In previous patch-clamp studies, degranulating process of mast cells during exocytosis was continuously monitored as a gradual increment of the whole-cell Cm [15, 27, 31]. Employing this electrophysiological approach, we have revealed the suppressive effects of several drugs or food constituents on the process of exocytosis in mast cells [9, 10, 12-14]. In these studies, the mast cell-stabilizing properties of the drugs or substances were quantified as the suppressive amount of the increasing Cm (ΔCm) [9, 10, 12, 13]. In the present study, employing the same approach, we provided direct evidence for the first time that vitamins, such as ascorbic acid and pyridoxine, dose-dependently inhibited the process of exocytosis, and thus exerted mast cell-stabilizing properties. Both compound 48/80 and GTP-γ-S directly activate guanine nucleotide-binding regulatory proteins (G proteins) in mast cells, which IgE-independently increases the intracellular Ca^{2+} concentration and thus triggers exocytosis [31]. In the present study, mass effects of vitamins on mast cell stabilization were morphologically analyzed by the ratio of degranulating mast cells (Fig. 1 and 2). On the other hand, using isolated single cells, the potency of vitamins on mast cell stabilization and its time-course changes were continuously quantified by the measurement of Cm (Fig. 3 and 4). Therefore, in our experiments, clusters of mast cells were externally exposed to compound 48/80 to evaluate their degranulation, while single mast cells were intracellularly exposed to GTP-γ-S to evaluate the time-course changes in the Cm.

In our recent study, popular beverage components, such as caffeine and catechin, also inhibited the process of exocytosis and thus exerted mast cell-stabilizing properties [14]. In this study, low concentrations of catechin synergistically potentiated the mast cell-stabilizing property of caffeine. Concerning the mechanisms, caffeine-induced decrease in the intracellular reactive oxygen species (ROS) level was thought to enhance the mast cell-stabilizing ability of catechin [32]. In mast cells, as we demonstrated in patch-clamp studies [10], an increase in the intracellular Ca^{2+} concentration is the primary trigger of exocytosis [33]. In several *in vitro* studies, the decrease in the intracellular ROS in mast cells also directly attenuated the high-affinity IgE receptor (FcεRI)-dependent Ca^{2+} signaling pathways and thus modulated the process of exocytosis [34, 35]. Since both ascorbic acid and pyridoxine are potent antioxidants that scavenge ROS [36-39], they would decrease the intracellular ROS. Such mechanisms were thought to underlie their mast cell-stabilizing properties demonstrated in the present study (Fig. 1 to 4). Generally, in the mixture of food constituents or natural products, including vitamins, herbs, teas and plant extracts, their antioxidant activities are known to be intensified by their synergic interactions [40, 41]. This may be the underlying mechanism of our additional finding that lower concentrations of pyridoxine synergistically potentiated the mast cell-stabilizing property of ascorbic acid (Fig. 5).

Besides allergic disorders, mast cells also contribute to the pathogenesis of fibrosis in various organs, such as kidney, skin, lung and liver [42-44]. In chronic inflammation, mast cells generate fibroblast growth factors and thereby stimulate the progression of organ fibrosis [8]. Therefore, in the treatment or prevention of organ fibrosis, suppressing the mast cell activity by mast cell stabilizers [45, 46] or chemokine inhibitors [47, 48] has been the useful pharmacological approach. In our previous study, using rat models with

chronic renal failure (CRF), we revealed that mast cells proliferated *in situ* within the fibrotic peritoneum by increasing the synthesis of fibroblast-activating factors [49]. In this study, the administration of tranilast, which was proven to be a potent mast cell stabilizer in our patch-clamp study [10], actually attenuated the progression of peritoneal fibrosis. Therefore, the activated mast cells within the CRF rat peritoneum were considered to be responsible for the progression of peritoneal fibrosis [49]. In the present study, similarly to tranilast, high doses of ascorbic acid and pyridoxine also dose-dependently suppressed the process of exocytosis and thus exerted mast cell-stabilizing properties (Fig. 1 to 4). Of note, the combination of these vitamins synergistically enhanced such properties with low doses (Fig. 5), indicating their usefulness as the potent mast cell stabilizer. Given such functional properties of ascorbic acid and pyridoxine, the administration of these vitamins could also be beneficial in the treatment of organ fibrosis, besides their fundamental efficacies for allergic disorders. In this regard, recent studies have actually shown the therapeutic efficacies of these vitamins in patients with liver fibrosis or animal models with lung fibrosis [50, 51].

Conclusion

This study provided electrophysiological evidence for the first time that vitamins, such as ascorbic acid and pyridoxine, inhibited the process of exocytosis in a dose-dependent manner. Although at relatively lower concentrations, these vitamins may not be enough to stabilize mast cells, such concentrations of pyridoxine may have the synergistically potentiating effects on the mast cell-stabilizing property by ascorbic acid.

Acknowledgements

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

IK and YS performed the experiments and analyzed the data. IK designed the experiments, interpreted the results and wrote the paper. TT provided logistical support and advised on the project. All authors read and approved the final manuscript.

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Statement of Ethics

This study was performed in accordance with the guide for the care and use of laboratory animals of Miyagi University, which included ethical considerations. The protocols for the use of the animals were approved by the Animal Care and Use Committee of Miyagi University (Protocol numbers: 2020-04-1, 2021-05-2, 2022-02-01).

Disclosure Statement

The authors declare no conflicts of interest.

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