

Short Communication

Anti-allergic Substances Contained in the Pollen of *Cryptomeria japonica* Possess Diverse Effects on the Degranulation of RBL-2H3 Cells

Yukiko Morimoto¹, Tadashi Yasuhara², Akiko Sugimoto³, Atsuko Inoue¹, Izumi Hide¹, Mitoshi Akiyama⁴, and Yoshihiro Nakata^{1,*}

¹Department of Pharmacology, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, Japan

²Tokyo University of Agriculture, Tokyo 156-8502, Japan

³Department of Molecular Design, Division of Biofunctional Molecules, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo 101-0062, Japan

⁴Department of Clinical Radiology, Faculty Health Sciences, Hiroshima International University, Hiroshima 724-0695, Japan

Received April 7, 2003; Accepted May 10, 2003

Abstract. Following prolonged exposure to some of the flavonoids with RBL-2H3 cells, secretion of hexosaminidase, a granule constituent, stimulated by an immunologic was enhanced. RBL-2H3 cells do not normally respond to polybasic secretagogues, but as reported here, they do so after prolonged exposure. Effect of flavonoids on secretion of hexosaminidase was also investigated. Of the thirteen flavonoids, quercetin and fisetin were the most potent inhibitors. A structure-activity study indicated that the position, number, and substitution of the hydroxy group of the B ring and saturation of the C2-C3 bond are important factors affecting flavonoid inhibition of secretory granules in RBL-2H3 cells.

Keywords: pollenosis, flavonoid, RBL-2H3 cell

While investigating anti-allergic substances from the extracts of the pollen of *Cryptomeria japonica*, these experiments have found several active substances. One of these has been identified as apigenin with histamine release inhibition assay using RBL-2H3 cells activated by antigen. In 1983, Ohmoto and Yoshida (1) reported that the pollen of *Cryptomeria japonica* contained nine flavonoids including apigenin, luteolin, and naringenin, but did not describe their physiological roles as polyphenolic compounds, which are present in plants and vegetables. It has been reported that flavonoids serve a multiplicity of functions in plant physiology (2) and have been shown to possess anti-inflammatory, anti-allergic, antiviral, and even mutagenic and anti-carcinogenic activities (3–5). In the present study, after RBL-2H3 cells were subjected to prolonged exposure to some of the flavonoids, the sensitivity of RBL-2H3 cells was examined by various stimuli including polybasic secretagogues such as compound 48/80, mastoparan, and

substance P; and we compared the direct effect of the thirteen flavonoids of different chemical classes on secretion of hexosaminidase from RBL-2H3 cells induced by antigen (2,4-dinitrophenylated BSA, DNP-BSA). Some flavonoids are known to inhibit histamine release from human basophiles (6) and a rat basophilic leukemia cell (RBL-2H3) (7, 8). However, there is no relationship between flavonoid structure and inhibition of secretory granules in RBL-2H3 cells. We also reported here a relationship to provide a basis for the further design of anti-allergic drugs.

RBL-2H3 cells were grown in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 37°C humidified incubator with an atmosphere of 95% air and 5% CO₂. A 96-well plate of the cultured cells (5 × 10⁴ cells/0.1 ml per well) was used for the degranulation assay. After sensitization of RBL-2H3 cells with 0.5 µg/ml of DNP-specific IgE and washing out, cells were incubated with various concentrations of flavonoids as indicated in the text, and then stimulated with antigen. DNP-

*Corresponding author. FAX: +81-82-257-5314
E-mail: ynakata@hiroshima-u.ac.jp

specific IgE treatment was omitted for polybasic secretagogues such as compound 48/80, mastoparan, and substance P. Secretion was determined by the amount of hexosaminidase release, a granule constituent. Hexosaminidase was subsequently assayed in medium as described previously (9) and its secretion used as a marker of degranulation. Briefly, 50 μ l samples of the medium and 50 μ l of the substrate (5 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide in 0.2 M citrate, pH 4.5) were incubated in a 96-well plate to yield the chromophore, *p*-nitrophenol. The absorbance of the colored product was assessed at 405 nm using a microplate reader. Values for hexosaminidase release were expressed as a percentage of the total hexosaminidase as determined by the cells lysed in 0.1% Triton X-100. DNP-specific monoclonal IgE, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, and flavonoids were from Sigma Chemical Co. (St. Louis, MO, USA). Mastoparan and substance P were from Peptide Institute, Inc., Osaka. Statistical analysis of the different treatment groups was carried out by one-way analysis of variance (ANOVA), followed by Student's *t*-test.

The cells were exposed to 30 μ M of flavonoids for 24 or 48 h. After washing the flavonoids out with RPMI medium, cells were stimulated with antigen, compound 48/80, mastoparan, and substance P. In the case of DNP-BSA, secretion of hexosaminidase increased to some extent with the treatment of flavonoids after 24 h of treatment. However, 48-h exposure to genistein enhanced secretion about two-fold (194.9%) and was followed by luteolin (191.4%), fisetin (176.3%), quercetin (171.2%), kaempferol (171.1%), and apigenin (152.6%), in decreasing order of enhancement, as shown in Fig. 1a.

Since RBL-2H3 cells do not normally respond to the basic secretagogues such as compound 48/80, mastoparan, or substance P, the study attempted to examine sensitization of flavonoid-treated RBL-2H3 cells to the basic secretagogues. Compound 48/80 (10 μ g/ml) had little effect on hexosaminidase secretion; less than 5% of total content in the RBL2H3 cell treated with DMSO (vehicle) for 48 h. In contrast, 30 μ M luteolin and genistein increased hexosaminidase secretion significantly by about 13.7% (vs 3.5% for the control) and 7.2% (vs 1.9% for the control), respectively, as shown in Fig. 1b. In the case of mastoparan used at the relatively high concentration of 30 μ M, genistein and luteolin increased hexosaminidase secretion by approximately 45.6% and 39.8% compared with 4.4% for the control, respectively. Finally, when stimulating RBL-2H3 cells treated with flavonoids for 48 h by 50 μ M substance P, luteolin and genistein increased hexosaminidase secretion significantly by about 11.1% and 5.56% compared

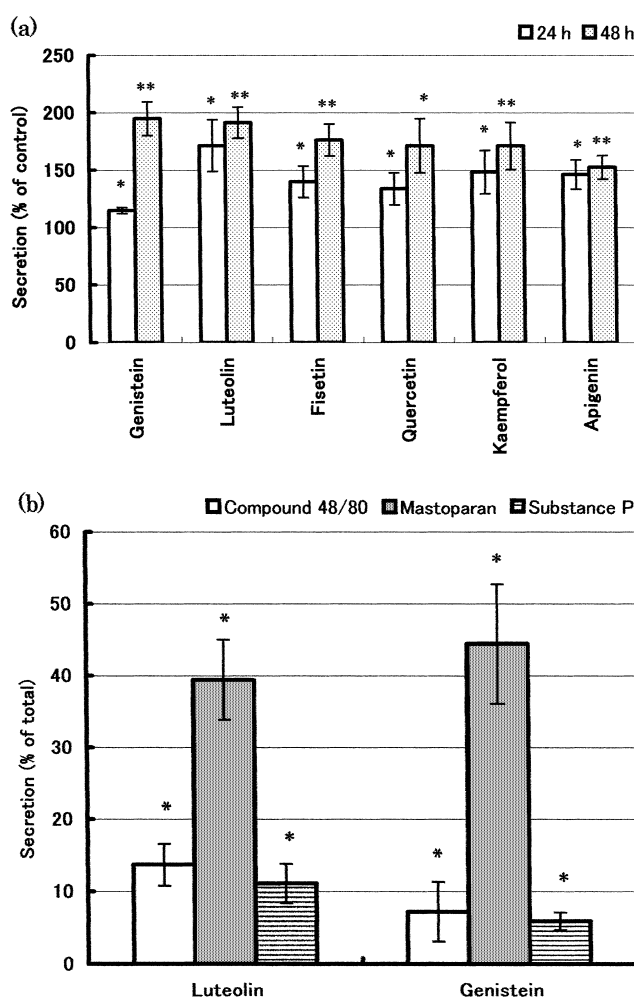


Fig. 1. Comparison of secretory responses to antigen or the various polybasic secretagogues. a: Acquired sensitivity to immunologics in flavonoid-treated RBL-2H3 cells. RBL-2H3 cells (5×10^4 cells/0.1 ml per well) were incubated in either vehicle (DMSO for the control) or 30 μ M of various flavonoids with 0.5 μ g/ml of DNP-specific IgE for 24 or 48 h in normal medium. The cells were washed twice with Siraganian medium and then stimulated with 20 ng/ml antigen (2,4-dinitrophenylated BSA, DNP-BSA) for 15 min at 37°C to measure the percent release of hexosaminidase as described in the text. Values are the mean $\pm$ S.E.M. from nine experiments with three cultures. **P*<0.05; ***P*<0.01 vs the control. The control values are 30–35% of the total contents. b: Acquired sensitivity to compound 48/80, mastoparan, and substance P in RBL-2H3 cells that had been treated with 30 μ M luteolin or 30 μ M genistein for 48 h. The cells were washed twice with Siraganian medium and then stimulated with 10 μ g/ml compound 48/80, 30 μ M mastoparan or 50 μ M substance P for 10 min at 37°C to measure the percent release of hexosaminidase as described in the text. Values are the mean $\pm$ S.E.M. from 9 experiments with three cultures. **P*<0.05, compared to the respective control. The control values of luteolin are 3.5% for compound 48/80, 4.4% for mastoparan, and 1.7% for substance P; and genistein's control values are 1.9% for compound 48/80, 4.4% for mastoparan, and 1.7% for substance P.

with 1.7% for the control, respectively, as shown in Fig. 1b. RBL-2H3 cells do not normally respond to

mastoparan, compound 48/80, and substance P, but the present study revealed that the cultured RBL-2H3 cell can indeed be made to respond to them by prior exposure to the various types of flavonoids. This finding in part is in accord with that of Senyshyn et al. (8), who showed that quercetin induces a substantial increase in the expression of G_{i3} proteins. They suggested that this increase accounts for the acquired sensitivity to polybasic mast cell secretagogues due to the over-expression of G_{i3} proteins. Indeed the wasp peptide venom, mastoparan, is known to induce histamine release from mast cells (10). It was previously shown that mastoparan activates GTP-binding protein directly (11, 12) to bypass conventional G-protein coupled receptors. In mast cells which are specialized secretory cells that secrete a variety of inflammatory mediators by regulated exocytosis, activation of G_{i3} proteins provides a sufficient signal for exocytosis (13). Therefore, the proposed target of mastoparan is G_{i3} proteins. However, regarding intact cells, the activation of G_{i3} proteins is not always needed to activate a cell. For example, Ca^{2+} -dependent secretion

from intact cells, as activated by Ca^{2+} ionophores or aggregation of $Fc\epsilon RI$, is G_{i3} -independent. An over-expression of the active G_{i3} mutant failed to induce exocytosis and arachidonic acid release stimulated by IgE, suggesting that the expression of active G_{i3} proteins, the target of mastoparan, is insufficient alone to elicit hexosaminidase release from RBL-2H3 cells (14). It remains unknown how flavonoids sensitize RBL2H3 cells. Based on the results of this study, however, it would appear that various secretagogues utilize or activate biochemical pathways leading to secretion that differ subtly from one stimulus to another, since certain flavonoids against one or more stimuli may not be effective against others.

Middleton and Drzewiecki reported that inhibition of histamine release by flavonoids varies depending on the stimulus and the particular structure of the flavonoid (6). The effect of thirteen flavonoids on hexosaminidase secretion, as stimulated by an antigen, was examined. In this study, various concentrations of flavonoids were added 10 min before stimulation of DNP-BSA. Flavo-

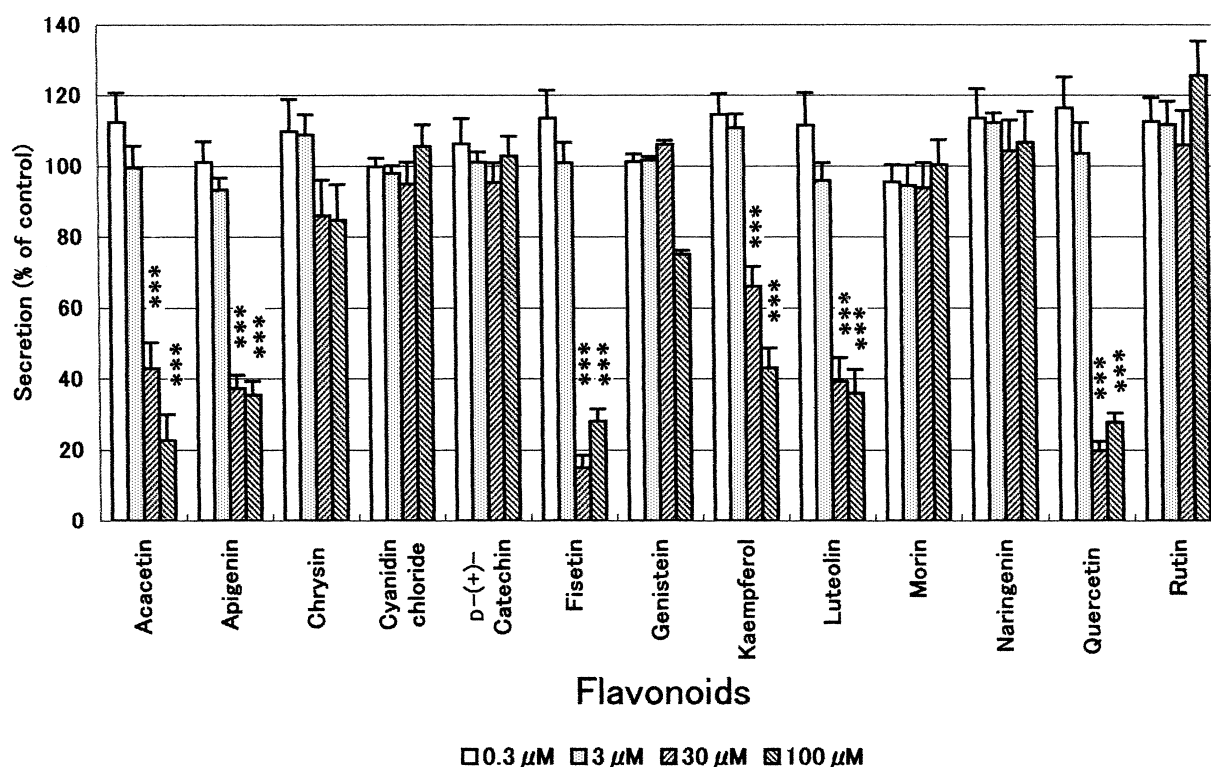


Fig. 2. Effects of flavonoids on the release of hexosaminidase in RBL-2H3 cells stimulated by the immunologic. RBL-2H3 cells in 96-well plate (5×10^4 cells/0.1 ml per well) were incubated overnight with 0.5 $\mu g/ml$ of DNP-specific IgE. Following a washing step, the cells were incubated with various concentration of flavonoid, as indicated in the text, for 10 min, and then stimulated with antigen (2,4-dinitrophenylated BSA, DNP-BSA) for 15 min. The reaction was stopped by the addition of cold medium, and hexosaminidase was assayed in the medium as described in the text. Values for hexosaminidase released in the medium are expressed as the mean $\pm$ S.E.M. from 3 or 4 experiments of the percentage of the total hexosaminidase, which was determined in the cells lysed in 0.1% Triton X-100. *** $P < 0.001$ vs the control.

noids have themselves neither stimulatory nor inhibitory effects. As shown in Fig. 2, fisetin and quercetin were the most potent inhibitors with IC_{50} values of 17.5 and 16.0 μM , respectively, while apigenin, luteolin, and acacetin were also effective inhibitors, with IC_{50} values of 35.0, 39.5, and 40.6 μM , respectively. Kaempferol was less potent (IC_{50} value of 66.7 μM) in comparison. Therefore, the inhibitory activity is dependent on flavo-

noid structures. The six flavonoids that displayed an inhibitory effect against hexosaminidase release showed considerable chemical structural similarities (Fig. 3). First, saturation of the C2-3 bond is required to sustain activity, because naringenin, D-(+)-catechin and cyanidin chloride did not show the inhibitory effects. Second, isoflavone structure, for example, genistein in this study, is not involved in the activity. Third, the 4'

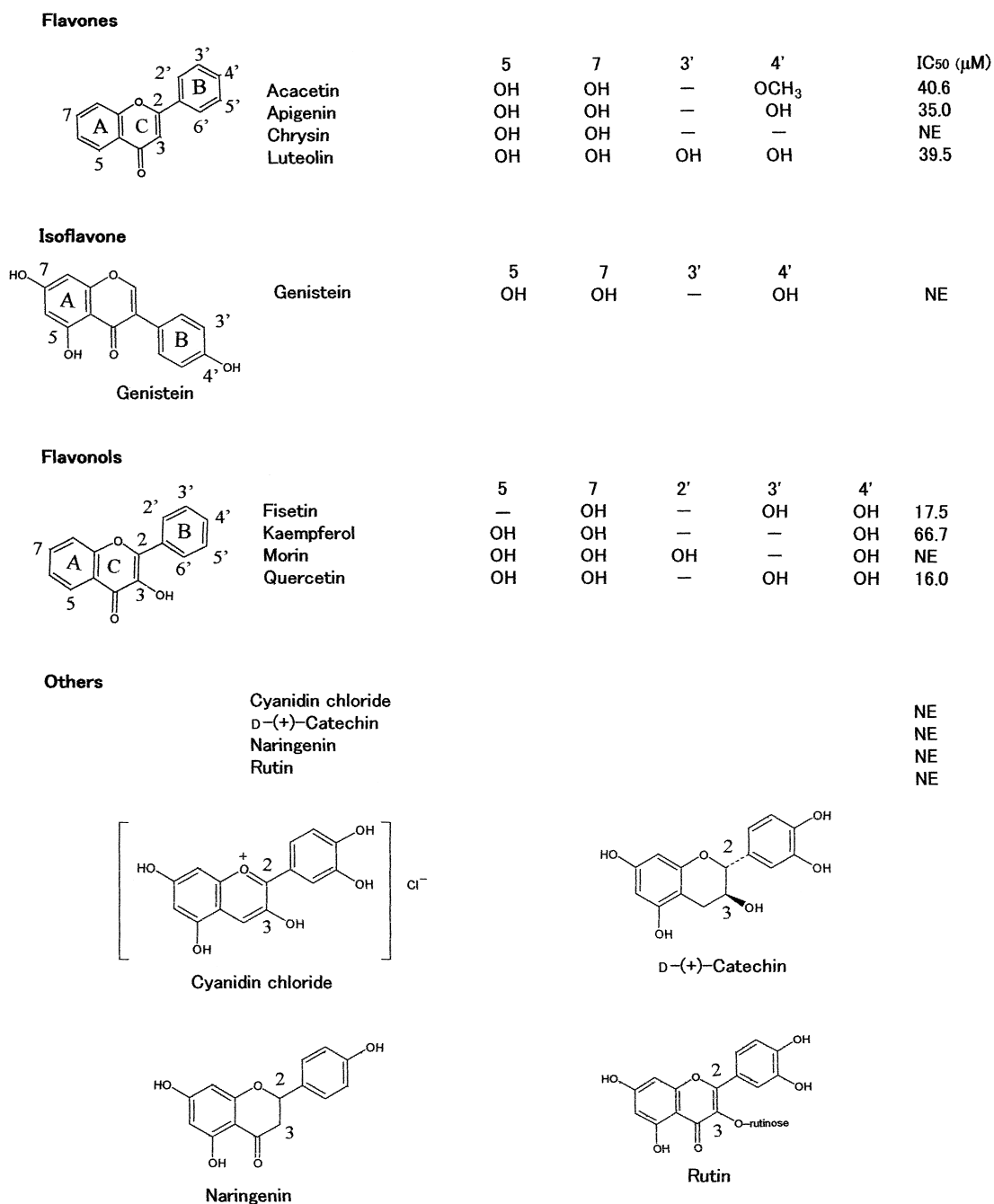


Fig. 3. Structure of flavonoids used in these experiments and their effects on the release of hexosaminidase in RBL-2H3 cells stimulated by the immunologic substance. NE represents no effect at 100 μM of flavonoids.

position of the B ring required an hydroxy or methoxy group because chrysin, which has no substituent at the 4' position of the B ring, exhibited no activity. Furthermore, an -OH group at the 3' position of the B ring is necessary to maintain activity due to the fact that morin, which has an -OH group at the 2' position of the B ring, did not show any inhibitory activity, whereas luteolin had activity, indicating that the number of -OH groups in the B ring did not correlate with the inhibitory effect of histamine release when using RBL-2H3 cells. The comparison of inactive flavonoids with those of the active ones in this study showed the presence of a 2-double bond on the C ring and the position of hydroxy in the B ring (i.e., flavonols and flavones). Beyond these two structural classes, the most potent inhibitors have 3' and 4' -OH groups on the B ring. Elimination of the 3' -OH substitution reduces the inhibitory potency within these chemical classes, as shown by comparing luteolin to apigenin and quercetin to kaempferol or morin. These results coincide with a previous paper by Agullo et al. (4) who reported that quercetin, luteolin, apigenin, and kaempferol inhibit more or less phosphatidylinositol 3-kinase, tyrosine kinase, and protein kinase C (PKC) in vitro at varying amounts. The mechanism of flavonoid inhibition of PKC is not known. Flavonoids may also play a significant role in specificity of inhibition, and could help to provide a basis for the further design of specific inhibitors of secretory granules in a mast cell.

These results suggest that flavonoid derivative contained in pollen may induce phenotypic changes or sensitization to various activators which may be involved in the mechanisms to cause pollenosis.

Acknowledgment

We thank Robert Crawford for a critical reading of the manuscript.

References

- 1 Ohmoto T, Yoshida O. Constituents of Pollen. XI. Constituents of *Cryptomeria japonica* D. Don. Chem Pharm Bull (Tokyo). 1983;31:919-924.
- 2 Harborne JB, Mabry TJ, Mabry H. The Flavonoids. New York: Academic Press; 1975.
- 3 Middleton E Jr, Kandaswami C. Effects of flavonoids on immune and inflammatory cell functions. Biochem Pharmacol. 1992;43:1167-1179.
- 4 Agullo G, Gamet-Payraastre L, Manenti S, et al. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochem Pharmacol. 1997;53:1649-1657.
- 5 Ranelletti FO, Ricci RR, Larocca LM, et al. Growth-inhibitory effect of quercetin and presence of type II estrogen-binding sites in human colon-cancer cell line and primary colorectal tumors. Int J Cancer. 1992;50:486-492.
- 6 Middleton E, Drzewiecki G. Flavonoid inhibition of human basophil histamine release stimulated by various agents. Biochem Pharmacol. 1984;21:3333-3338.
- 7 Trnovsky J, Letourneau R, Haggag E, Boucher W, Theoharides TC. Quercetin-induced expression of rat mast cell protease II and accumulation of secretory granules in rat basophilic leukemia cells. Biochem Pharmacol. 1993;46:2315-2326.
- 8 Senyshyn J, Baumgartner RA, Beaven MA. Quercetin sensitizes RBL-2H3 cells to polybasic mast cell secretagogues through increased expression of Gi GTP-binding proteins linked to a phospholipase C signaling pathway. J Immunol. 1998;160:5136-5144.
- 9 Hide I, Toriu N, Nuike T, et al. Suppression of TNF- α secretion by azelastine in rat mast (RBL-2H3) cell line: evidence for differential regulation of TNF- α release, transcription, and degranulation. J Immunol. 1997;159:2932-2940.
- 10 Hirai Y, Yasuhara T, Yoshida H, Nakajima T, Fujino M, Kitada C. A new mast cell degranulating peptide mastoparan in the venom of *Vespula lewisii*. Chem Pharm Bull. 1979;27:1942-1944.
- 11 Higashijima T, Burnier J, Ross EM. Regulation of Gi and Go by mastoparan, related amphiphilic peptides and hydrophobic amines. J Biol Chem. 1990;265:14176-14186.
- 12 Aridor M, Traub LM, Sagi-Eisenberg R. Exocytosis in mast cells by basic secretagogues: evidence for direct activation of GTP binding protein. J Cell Biol. 1990;111:909-917.
- 13 Aridor M, Rajmilevich G, Beaven MA, Sagi-Eisenberg R. Activation of exocytosis by the heterotrimeric G protein G $_{i3}$. Science. 1993;262:1569-1572.
- 14 Zussman A, Hermuet S, Sagi-Eisenberg R. Stimulation of Ca $^{2+}$ -dependent exocytosis and arachidonic acid release in cultured mast cells (RBL-2H3) by a GTPase-deficient mutant of G $_{\alpha i3}$. Eur J Biochem. 1998;258:144-149.