

## **CGRP, a neurotransmitter of enteric sensory neurons, contributes to the development of food allergy due to the augmentation of microtubule reorganization in mucosal mast cells**

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### **ABSTRACT**

Neuro-immune interaction in the gut is substantially involved in the maintenance of intestinal immune homeostasis and the pathology of intestinal immune diseases. We have previously demonstrated that mucosal mast cells and nerve fibers containing CGRP, a neurotransmitter of intrinsic enteric sensory neurons, are markedly increased and exist in close proximity to each other in the colon of food allergy (FA) mice. In the present study, a CGRP-receptor antagonist BIBN4096BS significantly alleviated allergic symptoms in the murine FA model. In addition, the elevated numbers of mucosal mast cells in the proximal colon of FA mice were significantly decreased in that of BIBN4096BS-treated FA mice. Thus, we investigated the effects of CGRP on calcium-independent process in degranulation of mucosal mast cells since CGRP increases intracellular cAMP levels, but not  $\text{Ca}^{2+}$  concentration. CGRP did not alter a calcium ionophore A23187-increased cytosolic  $\text{Ca}^{2+}$  concentration in mucosal-type bone marrow-derived mast cells (mBMMCs), but did augment microtubule reorganization in resting and A23187-activated mBMMCs. Furthermore, CGRP alone failed to cause the degranulation of mBMMCs, but CGRP significantly enhanced the degranulation of mBMMCs induced by A23187. Together, these data indicate that CGRP-enhanced microtubule reorganization augments IgE-independent/non-antigenic stimuli-induced mucosal mast cell degranulation, thereby contributing to the development of FA.

Food allergy (FA) is a serious public health concern that is estimated to affect approximately 5% of children and 3–4% of adults in developed countries (18). The pathogenic mechanisms underlying FA are not fully understood despite the epidemic prevalence and its life-threatening potential, and there is neither a curative drug treatment nor an effective means of prevention (3). Therefore, a deeper understanding of the pathology of FA is needed.

Mast cells play a central role as effector and conductor cells in the development of various allergic diseases via the release of various inflammatory mediators, such as proteases, eicosanoids, biogenic amines, cytokines and chemokines (1). High affinity IgE receptor ( $\text{Fc}\epsilon\text{RI}$ )-mediated mast cell degranulation process can be divided into the following two process as the calcium-independent and microtubule-dependent translocation of granules to the plasma membrane and the calcium-dependent fusion of granules with the plasma membrane for exocytosis (14). Mast cells are classified as mucosal mast cells or connective tissue mast cells. It is well known that mucosal mast cells are morphologically, biochemically and functionally distinct from connective tissue mast cells. We have previously demonstrated that our murine FA model exhibits several features

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such as allergic diarrhea, antigen-specific IgE hyperproduction and mucosal mast cell hyperplasia after oral food antigen exposure, and this model has provided evidence that mucosal mast cells, but not connective tissue mast cells are primarily responsible for the pathology of the FA model (21).

The enteric nervous system plays a pivotal role in the maintenance of mucosal integrity, and this function depends on the rapid alarm supplied by sensory neurons in the intestine (7). Accordingly, the interaction of mucosal mast cells with sensory neurons is crucial for understanding of the pathology of FA. Calcitonin gene-related peptide (CGRP) is presumed to be a neurotransmitter of intrinsic sensory neurons in the mouse enteric nervous system (17). Furthermore, our previous data show that intrinsic sensory neurons are more deeply involved in the pathology of FA than extrinsic sensory neurons and that CGRP-immunoreactive nerve fibers are specifically increased in the colonic mucosa of FA mice with the development of FA (11). Notably, CGRP-immunoreactive nerve fibers are juxtaposed with mucosal mast cells in the colonic mucosa of FA mice (11).

In this paper, we investigated the effects of CGRP on the activation of mucosal mast cells *in vitro* using mucosal-type bone marrow-derived mast cells (mBMMCs) and *in vivo* using the murine FA model to elucidate the pathophysiological contribution of neuro-immune interactions to the development of FA. CGRP activates cAMP/protein kinase A (PKA) signal transduction pathway, thereby increasing intracellular cAMP levels, but not  $\text{Ca}^{2+}$  concentration, and the  $\text{Ca}^{2+}$ -independent/microtubule-dependent pathway plays a critical role in mucosal mast cell degranulation (14).

Therefore, this study focuses on the effect of CGRP on the  $\text{Ca}^{2+}$ -independent/microtubule-dependent pathway and the additional effect of CGRP on the calcium-dependent degranulation of mucosal mast cells using a calcium ionophore to activate only the calcium-dependent pathway.

## MATERIALS AND METHODS

**Ethics statement.** This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Animal Experiment Committee at the University of Toyama approved all of the animal care procedures and experiments (authorization no. S-2009 INM-9).

**Animals.** Male BALB/c mice (4–9 weeks old) were

purchased from Japan SLC (Shizuoka, Japan). All mice were housed in the experimental animal facility at the University of Toyama and were provided free access to food and water.

**Reagents.** Calcium ionophore A23187, colchicine, ovalbumin (OVA, fraction V) and mouse monoclonal anti- $\beta$ -tubulin-Cy3-conjugated antibodies (C4585) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fura-2 AM was purchased from Dojindo (Kumamoto, Japan). Recombinant murine stem cell factor (SCF), interleukin-3 (IL-3), interleukin-9 (IL-9) and recombinant human transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) were purchased from Peprotech (London, UK). CGRP ( $\alpha$ -CGRP) was purchased from Peptide Institute (Osaka, Japan). BIBN4096BS was purchased from Tocris Bioscience (Bristol, UK). BD cytofix/cytoperm kit (No. 554715) was purchased from BD bioscience (San Diego, CA, USA). Mouse monoclonal anti-dinitrophenyl (DNP) IgE was purchased from Yamasa Corporation (Tokyo, Japan). Rabbit polyclonal anti-Receptor activity-modifying protein 1 (RAMP1) antibodies (sc-11379) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sheep anti-mouse mast cell protease-1 (mMCP-1: a marker of mouse mucosal mast cells) antibodies were purchased from Moredun Scientific (Midlothian, UK). Normal donkey serum, Cy3-conjugated donkey anti-sheep IgG and Cy3-conjugated donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

**A murine model of ovalbumin-induced allergic symptoms.** The murine model of FA was initiated as described previously (21). Briefly, male BALB/c mice (5 weeks old) were sensitized twice, 2 weeks apart, via an intraperitoneal injection of 100  $\mu\text{g}$  OVA in the presence of 2.0 mg aluminum hydroxide gel adjuvant. Two weeks later, the mice received repeated oral administrations of 50 mg OVA every other day. The symptom of allergic diarrhea resulting from FA was assessed by visually monitoring the mice for up to 60 min following the oral OVA challenge.

Morphological analysis of mucosal mast cells was carried out as previously described (11, 21). Briefly, one hour after the 6th oral OVA challenge, the proximal colon was excised. The proximal colon was fixed with 4% paraformaldehyde (w/v) in 0.1 M sodium phosphate buffer (pH 7.3), immersed for 18 h at 4°C, cryoprotected with 30% sucrose in 0.01 M phosphate-buffered saline (PBS) and embedded in optimal cutting tissue (OCT) compound. Frozen sec-

tions (25  $\mu$ m) were cut at  $-20^{\circ}\text{C}$  using a cryostat microtome (Leica Microsystems, Nussloch, Germany). The sections were soaked for 18 h at  $4^{\circ}\text{C}$  in 0.01 M PBS containing 0.3% Triton X-100 and exposed to normal donkey serum (1 : 10) for 30 min at room temperature. The sections were then exposed to sheep anti-mMCP-1 antibodies (1 : 5,000) for 18 h and incubated with Cy3-conjugated donkey anti-sheep IgG (1 : 1,000) for 2 h at room temperature. The preparations were examined using a fluorescence microscope (IX71 system; Olympus, Tokyo, Japan) with a U-MWIG3 filter set (Olympus) and photographed using an Olympus digital camera (DP70; Olympus). mMCP-1-immunoreactive cells were measured using ImageJ software (NIH Image J, Ver. 1.43.u).

Expression of mMCP-1 mRNA in the proximal colon was measured using real-time PCR as previously described (11, 21). The following primer pairs were used: mMCP-1, forward 5'-CCATCTGAAGATCATCACGGACA-3' and reverse 5'-ACATCATGAGCTCCAAGGGTGAC-3'; GAPDH, forward 5'-TGAACACAGTCCATGCCATC-3' and reverse 5'-GACGGACACATTGGGGGTAG-3'. Target mRNA levels were normalized to those of GAPDH as an internal control in each sample. The results are expressed as the ratio relative to vehicle-treated mice average.

**Cell culture of mBMMCs.** The mBMMCs were prepared from the femurs of BALB/c mice as previously described (9) with slight modification. Briefly, the bone marrow cells were cultured in RPMI-1640 medium supplemented with 40 ng/mL SCF, 20 ng/mL IL-3, 5 ng/mL IL-9 and 1 ng/mL TGF- $\beta_1$  for 3 weeks at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere.

**Immunocytochemistry in mBMMCs.** For immunocytochemistry of CGRP receptors, samples of mBMMCs were allowed to attach to MAS-coated slide glasses (Matsunami, Osaka, Japan) for 30 min at room temperature, washed with the culture medium and fixed for 20 min at  $4^{\circ}\text{C}$  with BD cytofix/cytoperm kit. The mBMMCs were then incubated with rabbit polyclonal anti-RAMP1 antibodies (1 : 50) for 60 min and exposed to Cy3-conjugated donkey anti-rabbit IgG (1 : 400) for 2 h at room temperature.

For immunocytochemistry of microtubule, samples of mBMMCs were stimulated with CGRP (10  $\mu$ M) for 60 min followed by further culture (72 h) in the regular medium. The mBMMCs were allowed to attach to MAS-coated slide glasses (Matsunami) for 30 min at  $37^{\circ}\text{C}$ , washed with the culture medium and stimulated with A23187 (1  $\mu$ M) for 5 min. Af-

terward, the mBMMCs were rinsed with a modified Brinkley Buffer 1980 (pH 6.8, 80 mM PIPES, 1 mM  $\text{MgCl}_2$ , 5 mM EGTA), fixed for 20 min at  $37^{\circ}\text{C}$  with 4% paraformaldehyde in modified Brinkley Buffer 1980 and permeabilized for 4 min with 0.5% Triton X-100. The mBMMCs were incubated with mouse monoclonal anti- $\beta$ -tubulin-Cy3-conjugated antibodies (1 : 500) for 60 min at room temperature.

The preparation was examined using a confocal laser-scanning microscope (LSM700; Carl Zeiss Japan, Tokyo, Japan). The cell spreading area was measured using ImageJ software.

**Intracellular calcium mobilization in mBMMCs.** The mobilization of intracellular  $\text{Ca}^{2+}$  was examined as described previously (8). Briefly, activated mBMMCs were loaded with 5  $\mu$ M Fura 2-AM in a loading buffer for 30 min. The fluorescence at 340 and 380 nm was measured using a fluorescence spectrophotometer intracellular  $\text{Ca}^{2+}$  measurement system (Model F-4500; Hitachi, Tokyo, Japan), and the background-corrected 340 : 380 ratio was calibrated.

**Effect of CGRP on mBMMCs.** Samples of mBMMCs were stimulated with CGRP (10  $\mu$ M) for 60 min followed by further culture (72 h) in the regular medium. The cells were stimulated with 1  $\mu$ M A23187 for 30 min. Degranulation was assessed by measuring  $\beta$ -hexosaminidase release, which has been described previously (9). The extent of degranulation was calculated by dividing the  $\beta$ -hexosaminidase activity in the supernatant by the sum of the activity in the supernatant and cell lysate.

**Statistical analysis.** The results are expressed as the means  $\pm$  SE. Statistical comparisons were performed using Student's unpaired *t*-test or one-way repeated measures ANOVA followed by Dunnett's post-hoc test with Microsoft Excel analysis tools or the chi-square test in SPSS software (version 19; IBM, Somers, NY). *P* values  $< 0.05$  were considered statistically significant.

## RESULTS

### *A CGRP-receptor antagonist alleviates allergic symptoms in a murine FA model.*

BIBN4096BS is a selective and high-affinity antagonist of CGRP receptor, of which the selectivity is due to a specific interaction with the extracellular region of the RAMP1 subunit of the receptor (10). BIBN4096BS (1 and 3 mg/kg/day) or vehicle was subcutaneously injected into the FA mice at daily

intervals. Allergic diarrhea began after the third OVA challenge in vehicle-treated mice (Fig. 1). BIBN4096BS attenuated the induction of allergic di-

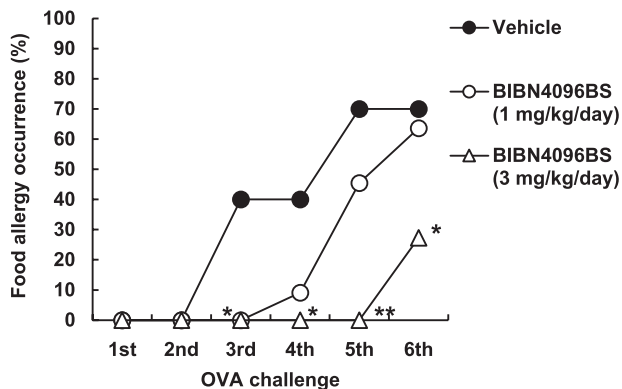


**Fig. 1** Suppressive effect of CGRP-receptor antagonist treatment on allergic symptom in FA mice. BIBN4096BS, a CGRP-receptor selective antagonist, was administered (1 and 3 mg/kg/day) subcutaneously at daily intervals for 11 days in the FA mice. Mice presenting profuse liquid stool were recognized as food allergy-positive animals. Significant differences were observed in the occurrence of FA (\* $P < 0.05$ , \*\* $P < 0.01$  vs. Vehicle,  $n = 10$ –11 mice per group).

arrhea at 1 and 3 mg/kg (Fig. 1; \* $P < 0.05$ , \*\* $P < 0.01$  vs. vehicle-treated mice).

As shown in Fig. 2A and 2B, immunohistochemical staining with the mMCP-1 antibodies revealed that the number of mucosal mast cells was dramatically increased in the proximal colon of FA mice, which was significantly decreased in that of BIBN4096BS (3 mg/kg)-treated FA mice (vehicle  $89.8 \pm 23.7$  cell/area ( $\text{mm}^2$ ) vs. BIBN4096BS  $29.8 \pm 6.3$  cell/area ( $\text{mm}^2$ ), \* $P < 0.05$ ).

As shown in Fig. 2C, the upregulated expression of mMCP-1 mRNA in the proximal colon of vehicle-treated mice was significantly suppressed in that of BIBN4096BS (3 mg/kg)-treated FA mice (vehicle  $1.00 \pm 0.56$  vs. BIBN4096BS  $0.35 \pm 0.17$ , \* $P < 0.05$ ).

#### *CGRP augments microtubule reorganization in mBMMCs.*

CGRP receptor is composed of two components, calcitonin receptor-like receptor (CRLR) and RAMP1 to induce cytosolic cAMP accumulation, but not  $\text{Ca}^{2+}$  mobilization, and RAMP1 associating with CRLR determines the specificity to CGRP (10). Immunocytochemical staining with the antibodies to

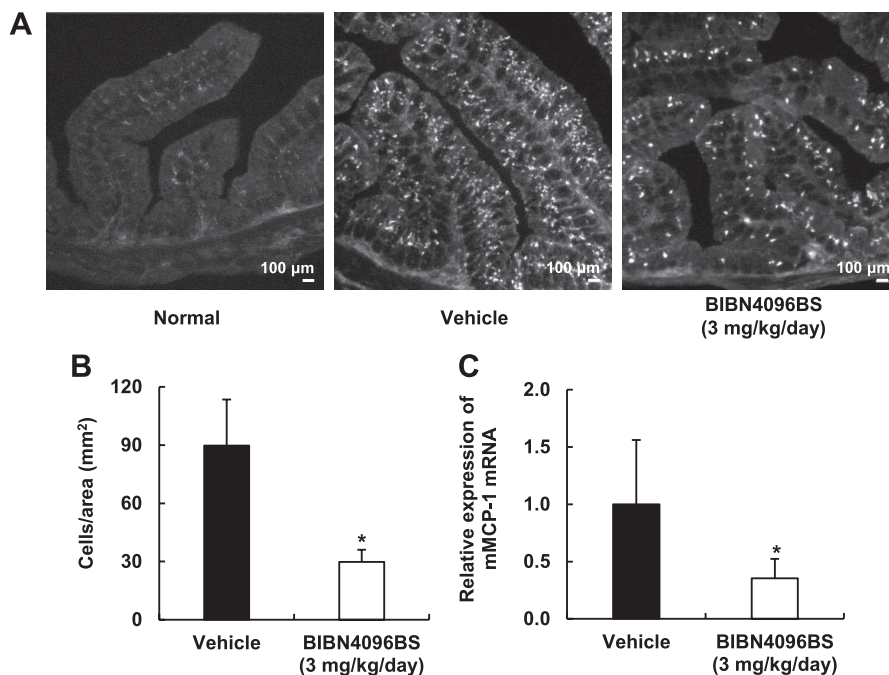


**Fig. 2** Suppressive effects CGRP-receptor antagonist treatment on increased number of mucosal mast cells and upregulation of mMCP-1 mRNA expression in the proximal colon of FA mice. (A) The proximal colons of normal mice, FA mice and FA mice treated with the subcutaneous administration of BIBN4096BS were stained with mMCP-1 antibodies. Scale bars represent 100  $\mu\text{m}$ . (B) The number of mMCP-1-immunoreactive cells was analyzed with ImageJ software. \* $P < 0.05$  vs. FA mice ( $n = 4$ –5 mice per group). (C) mMCP-1 mRNA expression in the proximal colon from FA mice and BIBN4096BS-treated FA mice was measured by real-time PCR. Relative mRNA level of mMCP-1 was normalized to GAPDH expression. \* $P < 0.05$  vs. FA mice ( $n = 10$ –11 mice per group).

RAMP1 revealed the presence of RAMP1 immunoreactivities on almost all mBMMCs in the resting state (Fig. 3A). Furthermore, in the resting state, most of the immunoreactivities were located within mBMMCs and primarily appeared as small clumps throughout their cytoplasm (Fig. 3A). Thus, the present experiments were designed to examine how CGRP, a potent cAMP-dependent neurotransmitter, affects mucosal mast cells via the calcium-independent pathway in mast cell activation using a calcium ionophore.

A calcium ionophore A23187 prompted a brisk increase in cytosolic  $\text{Ca}^{2+}$  concentration in mBMMCs, but CGRP failed to affect A23187-evoked cytosolic  $\text{Ca}^{2+}$  concentrations in mBMMCs (Fig. 3B).

In contrast, CGRP did cause microtubule reorganization in resting mBMMCs, which induced a flattening and spreading of resting mBMMCs (Fig. 3C). Therefore, we categorized these activated spreading

cells ( $> 150 \mu\text{m}^2$ ) into three groups by cell area, as shown in Fig. 4A, to assess the microtubule reorganization in mBMMCs. CGRP alone significantly elevated the proportion of mBMMCs with a large cell area ( $> 150 \mu\text{m}^2$ ) ( $**P < 0.01$  vs. Non-A23187/vehicle) (Fig. 4B). In addition, A23187 significantly elevated the proportion of mBMMCs with a large cell area ( $> 150 \mu\text{m}^2$ ) ( $**P < 0.01$  vs. Non-A23187/vehicle) (Fig. 3C and 4B). Furthermore, CGRP significantly upregulated the proportion of mBMMCs with a large cell area even when mBMMCs were activated by A23187 ( $> 150 \mu\text{m}^2$ ) ( $**P < 0.01$  vs. A23187/vehicle) (Fig. 3C and 4B).

*A microtubule depolymerizer inhibits FcεRI-mediated, but not A23187-stimulated, mBMMC degranulation.*

To evaluate the effect of the microtubule reorganization on mucosal mast cell degranulation, we investi-

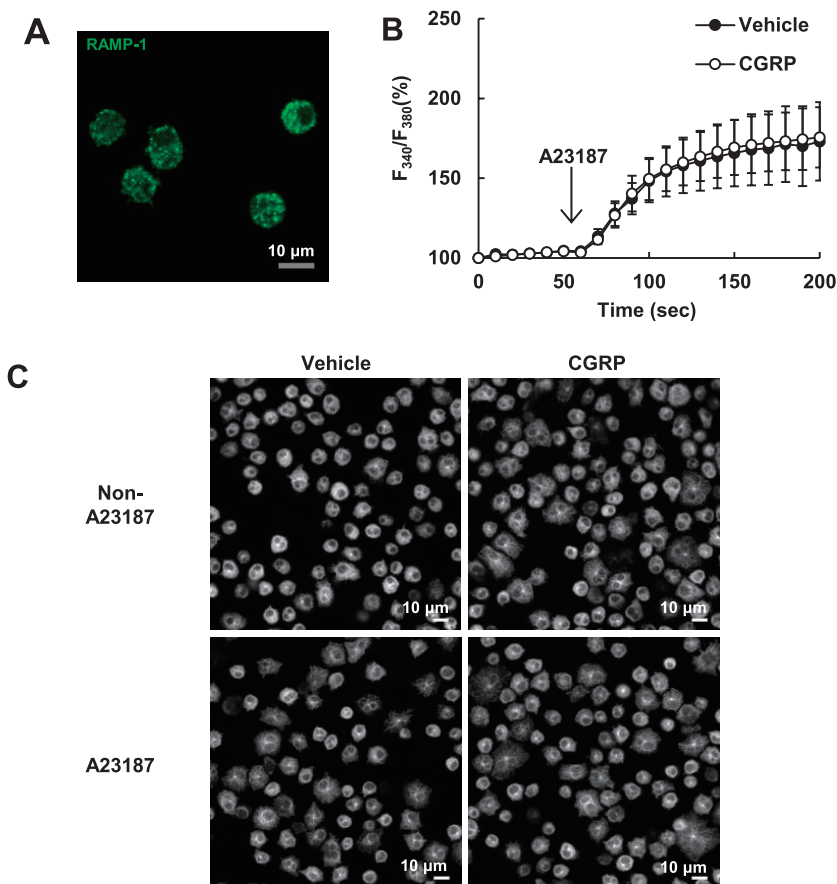


**Fig. 3** Morphological changes in mBMMCs after CGRP stimulation. (A) Immunocytochemistry for RAMP1 subunit of CGRP receptor in mBMMCs. In the resting state, almost all mBMMCs expressed RAMP1 immunoreactivity. (B) CGRP failed to affect A23187-evoked cytosolic  $\text{Ca}^{2+}$  concentrations in mBMMCs. The data shown are from 3 independent experiments. (C) Morphological changes induced by microtubule reorganization in mBMMCs were determined by immunocytochemical staining with antibodies to  $\beta$ -tubulin. Scale bar = 10  $\mu\text{m}$ .

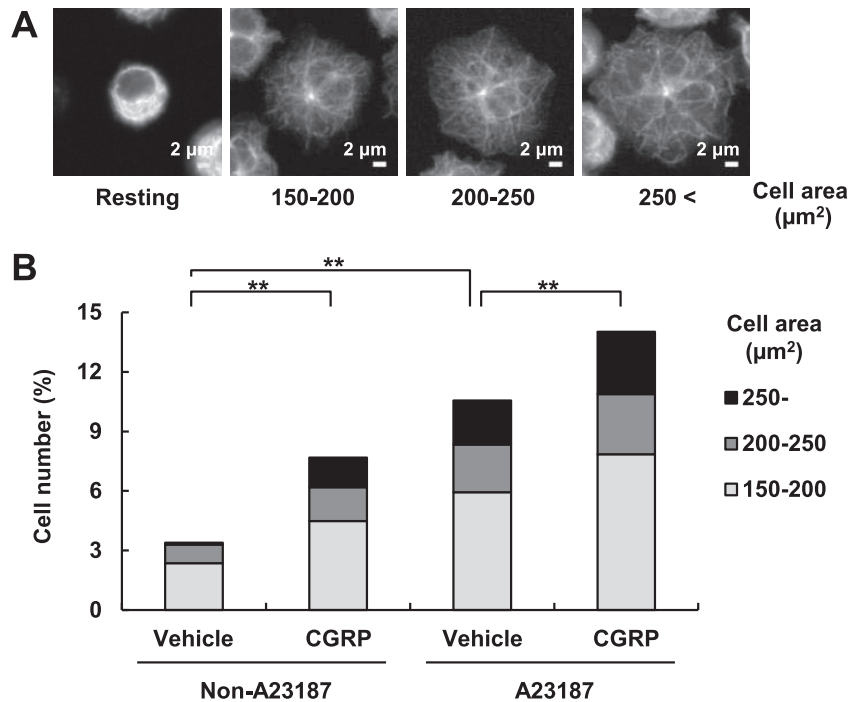


**Fig. 4** Effect of CGRP on microtubule reorganization in mBMMCs. **(A)** Representative images depending on the cell area of activated spreading mBMMCs (resting, 150–200  $\mu\text{m}^2$ , 200–250  $\mu\text{m}^2$ , > 250  $\mu\text{m}^2$ ). Scale bar = 2  $\mu\text{m}$ . **(B)** The photographs of mBMMCs were digitized, and the area of individual cells was quantified. All of activated cells (> 150  $\mu\text{m}^2$ ) in each group were divided into 3 categories according to the cell area ( $\mu\text{m}^2$ ). At least 200 cells were calculated in each group of 3 independent experiments. The diagram shows the mean activated cell distribution in the total cells in 3 independent experiments. \*\*, significant difference with  $P < 0.01$ .

gated whether a microtubule depolymerizer colchicine affects the degranulation of mucosal mast cells induced by stimuli for calcium-dependent pathway (calcium ionophore) and stimuli for both calcium-dependent and calcium-independent pathway (IgE/antigen). Samples of mBMMCs were incubated with colchicine (1–100  $\mu\text{M}$ ) for 15 min. Colchicine only slightly inhibited A23187-stimulated mBMMC degranulation (Fig. 5A;  $**P < 0.01$  vs. vehicle), but clearly suppressed Fc $\epsilon$ RI-mediated mBMMC degranulation in a concentration-dependent manner compared with vehicle treatment (Fig. 5B;  $*P < 0.05$ ,  $**P < 0.01$  vs. vehicle).

#### *CGRP augments A23187-induced degranulation of mBMMCs.*

We investigated whether CGRP-enhanced microtubule reorganization augmented the calcium ionophore-induced  $\text{Ca}^{2+}$ -dependent degranulation of mucosal mast cells. CGRP alone did not induce  $\beta$ -hexosaminidase release (vehicle  $0.5 \pm 0.1\%$ , CGRP  $0.7 \pm 0.2\%$ ). However, CGRP did significantly augment A23187-induced, but not Fc $\epsilon$ RI-mediated, degranulation (vehicle  $33.9 \pm 1.6\%$  vs. CGRP  $45.3 \pm$

$2.0\%$ ,  $**P < 0.05$ ) (Fig. 6).

## DISCUSSION

In the present study, we tested the hypothesis that the neuro-immune interaction between mucosal mast cells and enteric intrinsic sensory neurons containing CGRP contributes to the development of FA. This study demonstrated that CGRP-receptor antagonist alleviated allergic symptoms in the murine FA model. Furthermore, CGRP augmented the microtubule reorganization in the resting and calcium ionophore-activated mBMMCs and significantly enhanced the calcium ionophore-induced degranulation of mBMMCs. Therefore, we suggest that CGRP augments IgE/antigen-independent stimuli-induced mucosal mast cell activation through the enhancement of the microtubule reorganization and thereby contributes to the development of FA.

Recently, it has become increasingly clear that the immune system and nervous system are integrated, and a close cross-talk occurs between these systems (19). In particular, it is well known that sensory neurons participate in neurogenic inflammation, and

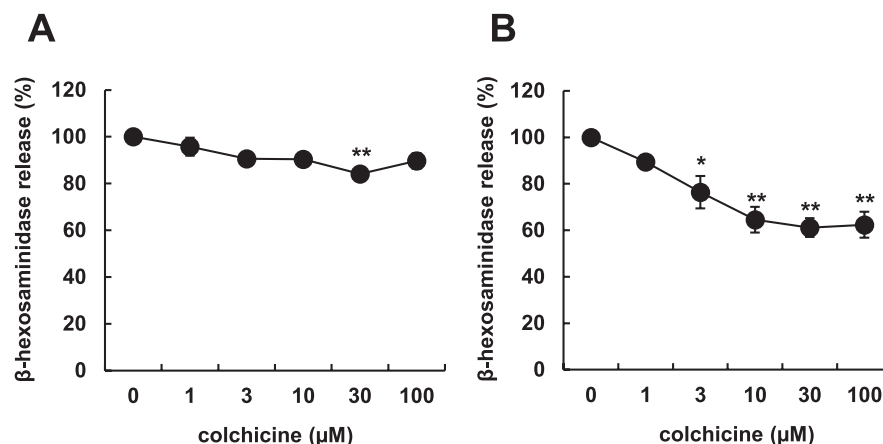


**Fig. 5** Effect of a microtubule depolymerizer, colchicine, on mBMMC degranulation. **(A)** Samples of mBMMCs were preincubated in different doses of colchicine (0–100  $\mu$ M) at 37°C for 15 min and then exposed to 1  $\mu$ M A23187 for 30 min, and degranulation was assessed. **(B)** Samples of mBMMCs were sensitized with 1.5  $\mu$ g/mL anti-DNP IgE for 6 h at 37°C and preincubated in different doses of colchicine (0–100  $\mu$ M) at 37°C for 15 min. The release of  $\beta$ -hexosaminidase was induced by the addition of 100 ng/mL DNP-bovine serum albumin (BSA) for 60 min, and degranulation was assessed. The data shown are from 3 independent experiments performed in triplicate (\* $P$  < 0.05, \*\* $P$  < 0.01 vs. vehicle).

the local release of neurotransmitters (*e.g.*, substance P and CGRP) from terminals of sensory neurons is critical to the development of neurogenic inflammation. However, the mechanisms and pathways whereby the nervous system may be involved in beneficial or harmful outcomes in immune diseases, particularly in allergy, remain obscure (19).

Mast cells are now considered as conductor cells as well as effector cells in allergic reactions, and play a critical role in the development of allergic diseases. We have also demonstrated that mucosal mast cells can orchestrate the allergic and inflammatory responses in the mucosal immune environment of the FA mouse colon (21). The interaction between mast cells and nerves is gaining increasing attention. Mast cell mediators sensitize sensory neurons, which, in turn, activate mast cells via the release of biogenic substances from sensory nerve terminals (19). However, the cross-talk between enteric neurons and mucosal mast cells remains largely unexplored in FA.

We therefore investigated the pathophysiological role of CGRP in the FA model. The administration of BIBN4096BS, the CGRP-receptor-selective antagonist, ameliorated the allergic symptoms in the FA mice and suppressed serious mastocytosis in the colon of FA mice. These results suggest that CGRP, through close communication between the mucosal mast cells and CGRP-containing sensory neurons, contributes to the development of FA.

Furthermore, we investigated the pathophysiological effects of CGRP on mucosal mast cells using

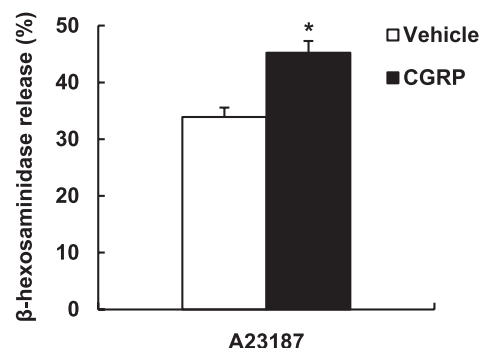


**Fig. 6** Augmentation effects of CGRP on A23187-induced degranulation in mBMMCs. After CGRP stimulation for 60 min, mBMMCs were cultured again for 72 h, then exposed to 1  $\mu$ M A23187 for 30 min, and degranulation was assessed. The data shown are from 3 independent experiments performed in triplicate (\*\* $P$  < 0.01 vs. vehicle).

mBMMCs. First, we verified the expression of the CGRP-specific receptor subunit RAMP1. In the resting state, most of RAMP1-immunoreactivities were located in small clumps inside the cells, suggesting that there is some RAMP1 internalization occurring in the absence of CGRP. However, CGRP alone failed to induce  $\beta$ -hexosaminidase release in this study. In addition, CGRP activates cAMP/PKA signal transduction pathway, thereby increasing intracellular cAMP levels, but not  $\text{Ca}^{2+}$  concentration. Fc $\epsilon$ RI-mediated signaling pathway can be dissected into calcium-dependent and calcium-independent pathways in mast cells, and Fc $\epsilon$ RI stimulation triggers the formation of microtubules and subsequently

microtubule-dependent translocation of granules to the plasma membrane in a calcium-independent manner in mast cells (14). Therefore, this study focused on the effect of CGRP-activated  $\text{Ca}^{2+}$ -independent/microtubule-dependent pathway on mucosal mast cells using a calcium ionophore to activate only the calcium-dependent pathway. Notably, CGRP significantly enhanced the A23187-induced, but not Fc $\epsilon$ RI-mediated, degranulation of mBMMCs, whereas CGRP did not alter the A23187-evoked cytosolic  $\text{Ca}^{2+}$  concentrations in mBMMCs. These results suggest that CGRP activates mBMMCs through a cytosolic  $\text{Ca}^{2+}$ -independent mechanism.

The  $\text{Ca}^{2+}$ -independent/microtubule-dependent pathway plays a critical role in mast cell degranulation (14). Fc $\epsilon$ RI-mediated rat basophilic leukemia (RBL)-2H3 mast cell-like cell activation causes a dynamic reorganization of microtubules that is associated with dramatic morphological changes, including cell spreading (22), and subsequent degranulation (2). In addition, the fusion of granules with the plasma membrane at exocytosis accounts for the cell spreading and increased plasma membrane area (6), and the cell spreading is the critical component of degranulation (16). Therefore, cell spreading during mast cell activation is closely related to degranulation.

We found that colchicine, which induces the depolymerization of microtubules, significantly inhibited Fc $\epsilon$ RI-mediated mBMMC degranulation. This result is consistent with a previous report that shows that the microtubule depolymerizers nocodazole and colchicine inhibit Fc $\epsilon$ RI-mediated degranulation in BMMCs and RBL-2H3 mast cell-like cells (12, 15). In contrast, colchicine exhibited only marginal effects on the calcium ionophore-induced mBMMC degranulation, which is consistent with previous studies in RBL-2H3 mast cell-like cells and rat peritoneal mast cells (12, 13). Therefore, calcium-independent dynamic microtubule networks are required for Fc $\epsilon$ RI-mediated mast cell degranulation. These experimental results can be interpreted as follows. There are two secretory vesicle pools in mast cells: the reserve secretory vesicle pool and the readily releasable secretory vesicle pool (20). Calcium ionophores quickly induce the degranulation of readily releasable secretory vesicles through a calcium-dependent fusion of granules with the plasma membrane. However, Fc $\epsilon$ RI-mediated degranulation requires both the  $\text{Ca}^{2+}$ -independent/microtubule-dependent translocation of granules to the plasma membrane and the calcium-dependent fusion of granules with the plasma membrane.

We hypothesized that CGRP stimulation augments A23187-induced activation of mBMMCs through the  $\text{Ca}^{2+}$ -independent/microtubule-dependent translocation of granules to the plasma membrane because CGRP alone did not elevate cytosolic  $\text{Ca}^{2+}$  concentrations and induce degranulation in mBMMCs. Consistent with this hypothesis, CGRP alone significantly elevated the proportion of spreading cells in mBMMCs compared with vehicle-treated mBMMCs under non-A23187-stimulated conditions, suggesting that CGRP induced microtubule reorganization in mBMMCs. However, these conditions were insufficient to induce degranulation. Furthermore, A23187 triggered degranulation in vehicle-treated mBMMCs, accompanied by a significant increase in the proportion of spreading cells. Notably, CGRP significantly augmented A23187-induced degranulation and cell spreading in mBMMCs. These data suggest that CGRP reorganizes the microtubules in mBMMCs, thereby augmenting IgE/antigen-independent mBMMC degranulation. Taken together, this study indicates that a microtubule network is required for mast cell degranulation, and its results provide the first evidence of prominent effects of CGRP on the degranulation of mBMMCs via the regulation of microtubule reorganization.

In the present study, the administration of the CGRP-receptor antagonist resulted in a significant suppression of allergic diarrhea and mastocytosis in the FA mice. FA is caused by IgE-dependent and IgE-independent mechanisms involving mast cells, eosinophils and other immune cells, and functional receptors/channels other than Fc $\epsilon$ RI also play a very important role in food allergic reactions (4, 5). In addition, food antigen-caused allergic reactions can be divided into acute and late phases: the acute phase is caused by activation of mast cells and basophils with release of various allergic and inflammatory mediators, and the late phase reactions starting within 2–24 h after allergen challenge feature a cellular infiltration of mast cells, granulocytes (basophils and eosinophils) and lymphocytes into the allergic sites (4, 5). Therefore, it is suggested that CGRP is greatly involved in IgE-independent release of various allergic and inflammatory mediators and thereby causes serious mastocytosis and allergic symptoms in the murine FA model.

In conclusion, these novel findings inspire a new appreciation for the pathology of neuro-immune interactions in FA, and the results provide a possible target for novel therapeutic agents against FA.

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