

Microtubule Disruption Suppresses Allergic Response through the Inhibition of Calcium Influx in the Mast Cell Degranulation Pathway¹

Tatsuya Oka, Masatoshi Hori,² and Hiroshi Ozaki

Mast cells are secretory cells that release their granules, which contain inflammatory mediators. Some recent data suggested that cytoskeletons play a role in this process. However, the role of microtubules in Ca^{2+} signaling has not yet been well defined. In this study, we demonstrate that the microtubule cytoskeleton is important to maintain Ca^{2+} influx in the degranulation pathway of mast cells, using the microtubule depolymerizers nocodazole and colchicine. The microtubule depolymerizers inhibited Ag-induced degranulation in RBL-2H3 cells and bone marrow-derived mast cells. When the cells were stimulated with Ag in the presence of the microtubule depolymerizers, the Ca^{2+} influx was decreased without affecting Ca^{2+} release from the endoplasmic reticulum (ER). Capacitative Ca^{2+} entry, which was induced by inhibitors of Ca^{2+} -ATPase in the ER membrane, thapsigargin and cyclopiazonic acid, was also decreased by nocodazole. Fluorescent probe analysis demonstrated that nocodazole disrupted microtubule formation and changed the cytoplasmic distribution of the ER. The microtubule depolymerizers attenuated the passive cutaneous anaphylaxis reaction in back skin of Sprague Dawley rats. These results suggest that the microtubule cytoskeleton in mast cells is important to maintain Ag-induced capacitative Ca^{2+} entry, which is responsible for degranulation and the allergic response. *The Journal of Immunology*, 2005, 174: 4584–4589.

Mast cells are secretory cells that undergo an extensive release of their granular content of inflammatory mediators within a few minutes following stimulation (1). Mast cells are found in variety of tissues, particularly in association with blood vessels. Mast cells release vasoactive mediators, such as histamine and serotonin, which increase vasopermeability (2). In mast cells, the cross-linking of IgE-bound FcεRI by allergen initiates biochemical cascades (1). This process, termed degranulation, involves regulated changes in the concentration of Ca^{2+} in the cytoplasm ($[\text{Ca}^{2+}]_i$)³. When Ca^{2+} signaling is stimulated with allergen, Ca^{2+} enters the cytoplasm from two general sources, namely, Ca^{2+} release from intracellular stores, i.e., from the endoplasmic reticulum (ER), and Ca^{2+} influx from the extracellular space across the plasma membrane (3, 4). An important initiating step is Ca^{2+} release from the ER by the binding of inositol 1,4,5-trisphosphate (IP_3) to IP_3 receptors in the ER membrane (5, 6). Subsequently, the depletion of Ca^{2+} in the ER activates a signaling pathway leading to the opening of Ca^{2+} channels in the plasma

membrane. This regulatory mechanism of Ca^{2+} influx is a process known as capacitative Ca^{2+} entry (CCE), and it is mediated by store-operated channels (SOCs) (7). Among the pathways responsible for the increase in $[\text{Ca}^{2+}]_i$, because the initial transient Ca^{2+} release in the absence of external Ca^{2+} is not able to induce degranulation, CCE is more important for allergen-mediated degranulation (8). Although considerable attention has been focused on the nature of the signal linking the depletion of Ca^{2+} in the ER to the opening of SOCs, its regulatory mechanism remains unknown (3). Thus far, at least two general mechanisms have been proposed to explain how signaling is transferred from the ER to the SOCs in the plasma membrane (3, 9, 10). One model describes a type of direct conformational coupling between the IP_3 receptors in the ER membrane and the SOCs in the plasma membrane (protein-protein interaction) (9, 11). The other model describes a diffusible calcium influx factor (CIF), which is released from the ER after Ca^{2+} depletion (diffusible messenger) (12). The exact mechanism of such communication remains controversial.

Microtubules are one of the most important components in the cytoskeleton. Microtubules are generally recognized to play a number of roles in mitosis, in the maintenance of organelle structures, in cell mobility, and in granular transport. As regards mast cell degranulation, the role played by microtubules in granular transport has been studied (13–15). Apart from their role in granular transport, it is well known that the microtubules are necessary for maintaining membrane-enclosed organelles at particular positions. It has been shown that the structures of the microtubules and the ER are highly interdependent (16, 17). Considering that signaling between the ER and the plasma membrane is important for opening the SOCs, and that an increase in $[\text{Ca}^{2+}]_i$ is prerequisite for mast cell degranulation, we hypothesized that the microtubules might modulate degranulation through Ca^{2+} signaling regulation.

In this study, we investigated the effect of microtubule depolymerizers as antiallergic agents in the PCA reaction, and we further demonstrated that these agents inhibited the allergic response

Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Tokyo, Japan

Received for publication October 20, 2004. Accepted for publication February 9, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists, by the Program for Promotion of Basic Research Activities for Innovative Biosciences, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture and Sciences.

² Address correspondence and reprint requests to Dr. Masatoshi Hori, Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan. E-mail address: ahori@mail.ecc.u-tokyo.ac.jp

³ Abbreviations used in this paper: $[\text{Ca}^{2+}]_i$, concentration of Ca^{2+} in the cytoplasm; ER, endoplasmic reticulum; IP_3 , inositol 1,4,5-trisphosphate; CCE, capacitative Ca^{2+} entry; SOC, store-operated channel; CIF, Ca^{2+} influx factor; BMMC, bone marrow-derived mast cell; AUC, area under the curve; DPX, *p*-xylene-bis-pyridinium bromide; PCA, passive cutaneous anaphylaxis; HSA, human serum albumin.

through a novel effect on the Ca^{2+} influx pathway in mast cell degranulation.

Materials and Methods

Mast cells

RBL-2H3 cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (18). Bone marrow-derived mast cells (BMMCs) were obtained according to a previously described method (19). Briefly, bone marrow cells of male C57BL/6J mice (Charles River) were cultured in RPMI 1640 supplemented with 100 μM 2-ME, 10% FBS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10 $\mu\text{g}/\text{ml}$ recombinant mouse IL-3 (Sigma-Aldrich). More than 90% of the cells were identified as immature mast cells 4 wk after the initiation of the culture. The cells were used at 6–10 wk of culture. Both types of cells were incubated overnight with 50 ng/ml anti-DNP IgE (Sigma-Aldrich) before use.

β -Hexosaminidase degranulation

We measured the release of β -hexosaminidase as an index of mast cell degranulation using a previously described method (18). In brief, RBL-2H3 cells or BMMCs (2×10^5 cells/tube) were washed with HEPES-buffered solution (125.4 mM NaCl, 11.5 mM glucose, 5.9 mM KCl, 1.2 mM MgCl_2 , 1.5 mM CaCl_2 , 10 mM HEPES; pH 7.4). The cells were stimulated at 37°C under gentle rotation. The supernatants were transferred to a 96-well plate. The rest of the supernatants were removed, and then Triton X-100 solution (0.5%) was added to the cells. The extracts were transferred to a 96-well plate. To each well, 1.3 mg/ml *p*-nitrophenyl-*N*-acetyl- β -D-glucosamide (Sigma-Aldrich) in 0.04 M sodium citrate (pH 4.5) was added. The plates were incubated at 37°C for 1 h. Glycine (0.2 M; pH 10.0) was then added to each well. The absorbance at 405 nm (OD) of each well was measured with multilabel counter (PerkinElmer). The percentage of degranulation was calculated using the following formula: % Degranulation = $\text{OD}_{\text{supernatant}} / (\text{OD}_{\text{supernatant}} + \text{OD}_{\text{Triton X-100}}) \times 100$

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was measured using fura-PE3/AM (TEF Laboratories) according to a method described previously (18). In brief, RBL-2H3 cells or BMMCs on glass coverslips in HEPES-buffered solution were loaded with 5 μM fura-PE3/AM for 40 min in a dark room at 37°C. Every 3 s, the fluorescence of an image at 340 nm (F_{340}) was divided by the fluorescence of an image at 380 nm (F_{380}) to provide a resultant ratio (F_{340}/F_{380}) by means of a fluorescence imaging system (Hamamatsu Photonics). When the extracellular Ca^{2+} was removed, Ca^{2+} -free HEPES-buffered solution (containing 0.5 mM EGTA instead of 1.5 mM CaCl_2) was used. In the mast cells, the changes in $[\text{Ca}^{2+}]_i$ were oscillatory, asynchronous, and irregular, and varied tremendously among cells. In this type of cell, measuring the area under the Δ ratio per time curve (area under the curve (AUC)) was effective for quantifying the response (20): Δ ratio = ratio – ratio_{min}; ratio_{min} was ratio measured in the Ca^{2+} -free HEPES-buffered solution.

Fluorescent staining

To visualize the ER, RBL-2H3 cells grown on glass coverslips or glass-bottomed dishes and BMMCs loosely attached to glass coverslips in HEPES-buffered solution were loaded with 1 μM ER-Tracker Blue-White *p*-xylene-bis-pyridinium bromide (DPX) (Molecular Probes) for 30 min in a dark room at 37°C. To visualize the ER in living cells, the RBL-2H3 cells on glass-bottomed dishes were observed under microscopy at 37°C. For the ER and microtubule cytoskeleton double-staining, the cells were fixed with 3.7% formaldehyde in HEPES-buffered solution for 10 min at 37°C, and then were permeabilized with 0.2% Triton X-100 for 15 s. Afterward, the cells were incubated with an FITC-labeled mouse monoclonal anti- α -tubulin Ab (Sigma-Aldrich) at a 1/100 dilution and 1 μM ER-Tracker for 30 min at 37°C. The images were captured using a fluorescence imaging system (Plantron). The microscope was equipped with excitation (FITC, 490 nm; DPX, 365 nm) and emission (FITC, 520 nm; DPX, 400 nm) filters.

Passive cutaneous anaphylaxis (PCA) reaction

We tested the effect of microtubule depolymerizers on mast cell degranulation *in vivo* using PCA reaction (21, 22). Male Sprague Dawley (SD) rats at 8 wk, each weighing ~300 g (Charles River), were anesthetized with 50 mg/kg sodium pentobarbital (i.p.). Anti-DNP IgE (200 ng/ml) and experimental molecules in 0.05 ml of normal saline were applied to the back skin of the rats by intradermal injection. Two hours later, 1% Evans blue (Sigma-Aldrich) and Ag, 1 mg/ml DNP-human serum albumin (HSA) (Sigma-Aldrich), in 1 ml of normal saline was injected via the tail vein (i.v.).

Immediately thereafter, 0.05 ml of 10 ng/ml compound 48/80, a mast cell secretagogue, was applied to the back skin of rats as a positive control. Thirty minutes later, the rats were sacrificed by exsanguination while under the anesthetic, and the skin was removed, turned over, and photographed. Identical circular skin areas (~1 cm²) were then cut out, and the extravasated Evans blue was extracted by incubating the skin samples in 99% *N,N*-dimethylformamide for 24 h at 55°C. The supernatant was collected by centrifugation and OD₆₅₀ was measured with a multilabel counter. The percentage of extravasation was calculated, taking saline- and compound 48/80-induced Evans blue extravasation as 0 and 100%, respectively. Animal care and treatment were conducted in accordance with the institutional guidelines of University of Tokyo, which are consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Other chemicals

Nocodazole, colchicine, thapsigargin, and cyclopiazonic acid were purchased from Sigma-Aldrich. DiOC₆ (3) was purchased from Molecular Probes.

Statistical analysis

The results of the experiments are expressed as the means $\pm$ SE. Statistical evaluation of the data was performed by unpaired Student's *t* test for comparisons between two groups, and by ANOVA followed by Dunnett's test for comparisons between more than two groups. A value of *p* < 0.05 was regarded as significant.

Results

Microtubule depolymerizers inhibit mast cell degranulation

First, we examined the effects of nocodazole and colchicine, both of which are microtubule depolymerizers, on mast cell degranulation. Intracellular β -hexosaminidase was released by DNP-HSA (0.1–100 ng/ml) stimulation for 15 min in a concentration-dependent manner in RBL-2H3 cells, and 10 ng/ml DNP-HSA induced submaximal degranulation (data not shown). In RBL-2H3 cells, nocodazole (Fig. 1A) and colchicine (B) inhibited the 10 ng/ml DNP-HSA-induced degranulation in a concentration-dependent manner. These microtubule depolymerizers also inhibited degranulation caused by higher concentration of DNP-HSA (30 ng/ml DNP-HSA, $42.8 \pm 1.0\%$; with 3 μM nocodazole, $17.9 \pm 1.8\%$;

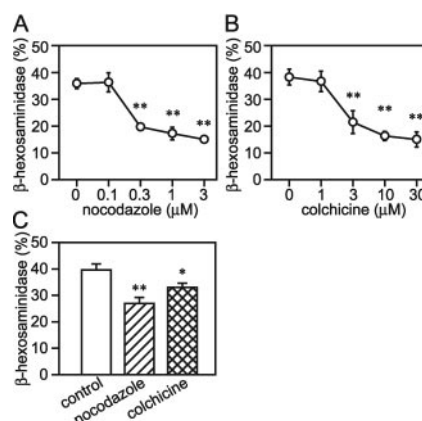


FIGURE 1. Effects of microtubule depolymerizers on mast cell degranulation. A and B, RBL-2H3 cells were preincubated with nocodazole (A) or colchicine (B) for 15 min at 37°C, and then were stimulated with 10 ng/ml DNP-HSA for 15 min. C, BMMCs were preincubated without (control) or with microtubule depolymerizers (3 μM nocodazole or 30 μM colchicine) for 15 min at 37°C, and then were stimulated with 10 ng/ml DNP-HSA for 15 min. The percentages of released β -hexosaminidase were calculated. The spontaneous release of β -hexosaminidase (no Ag) was $6.4 \pm 0.6\%$ (RBL-2H3 cells) or $1.9 \pm 0.2\%$ (BMMCs), respectively. The results are expressed as the mean $\pm$ SE of 5–10 experiments. Significantly different from the control: *, *p* < 0.05; **, *p* < 0.01.

with 30 μM colchicine, $28.2 \pm 1.5\%$). In BMMCs, the microtubule depolymerizers (3 μM nocodazole or 30 μM colchicine) inhibited 10 ng/ml DNP-HSA-induced degranulation as well (Fig. 1C).

Microtubule depolymerizers inhibit CCE

Because it was known that an increase in $[\text{Ca}^{2+}]_i$ is prerequisite for mast cell degranulation, we investigated whether or not the microtubule depolymerizers would affect the Ca^{2+} signaling. In RBL-2H3 cells, both nocodazole and colchicine inhibited DNP-HSA-induced $[\text{Ca}^{2+}]_i$ increase (Fig. 2, A and C). In BMMCs, nocodazole inhibited DNP-HSA-induced $[\text{Ca}^{2+}]_i$ increase as well (Fig. 2, B and D).

Ca^{2+} enters the cytoplasm from two general sources, namely, Ca^{2+} release from the ER and Ca^{2+} influx across the plasma membrane. First, we investigated whether or not microtubule disruption would affect Ca^{2+} release from the ER. In the absence of external Ca^{2+} (with 0.5 mM EGTA), stimulation with 10 ng/ml DNP-HSA induced a transient increase in $[\text{Ca}^{2+}]_i$ due to Ca^{2+} release from the ER (Fig. 3A). Nocodazole and colchicine had no effect on the DNP-HSA-induced Ca^{2+} release (Fig. 3, A and B). In addition, the remaining Ca^{2+} in the ER released by thapsigargin after stimulation with DNP-HSA was not affected by treatment with the microtubule depolymerizers (Fig. 3C), suggesting that the total amount of Ca^{2+} in the ER remained unaffected by disruption of the microtubules.

We then investigated whether or not microtubule disruption would exert an effect on the other source of $[\text{Ca}^{2+}]_i$ increase, namely, Ca^{2+} influx. Readmission of external Ca^{2+} after the depletion of Ca^{2+} in the ER induced sustained Ca^{2+} influx (Fig. 4). Nocodazole (Fig. 4A) and colchicine (B) significantly inhibited the

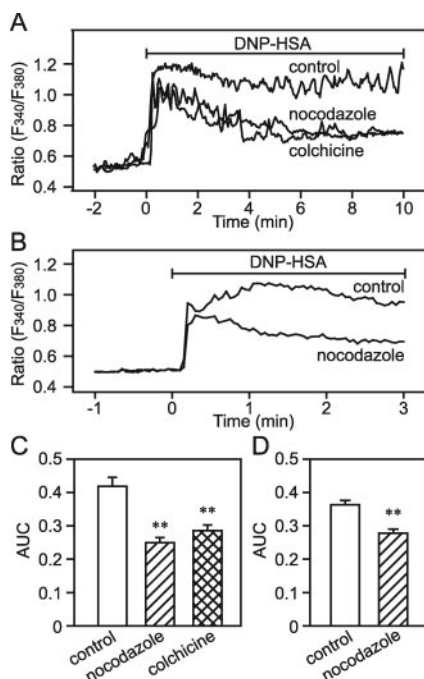


FIGURE 2. Effects of microtubule depolymerizers on DNP-HSA-induced $[\text{Ca}^{2+}]_i$. A and B, Typical recordings of the increase in $[\text{Ca}^{2+}]_i$ in fura-PE3-loaded RBL-2H3 cells (A) or BMMCs (B). The cells were preincubated without (control) or with microtubule depolymerizers (3 μM nocodazole or 30 μM colchicine) for 15 min at 37°C, and were subsequently stimulated with 10 ng/ml DNP-HSA. C, AUC for 10 min after DNP-HSA stimulation in A. D, AUC for 3 min after DNP-HSA stimulation in B. The results are expressed as mean $\pm$ SE of 49–119 cells in four to five independent experiments. **, Significantly different from the control with $p < 0.01$.

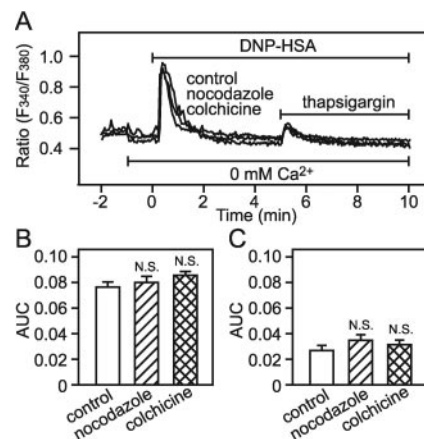


FIGURE 3. Effects of microtubule depolymerizers on Ca^{2+} release from the ER. A, Typical recordings of the increase in $[\text{Ca}^{2+}]_i$ in fura-PE3-loaded RBL-2H3 cells. The cells were preincubated without (control) or with microtubule depolymerizers (3 μM nocodazole or 30 μM colchicine) for 15 min at 37°C. The external solution was replaced with Ca^{2+} -free HEPES-buffered solution for the last 1 min of preincubation. The cells were then stimulated with 10 ng/ml DNP-HSA for 5 min, followed by stimulation with 1 μM thapsigargin in the absence of external Ca^{2+} . B and C, AUC for 5 min after DNP-HSA (B) or thapsigargin (C) stimulation in A. The results are expressed as the mean $\pm$ SE of 46–52 cells in three independent experiments. N.S., Not significantly different from control.

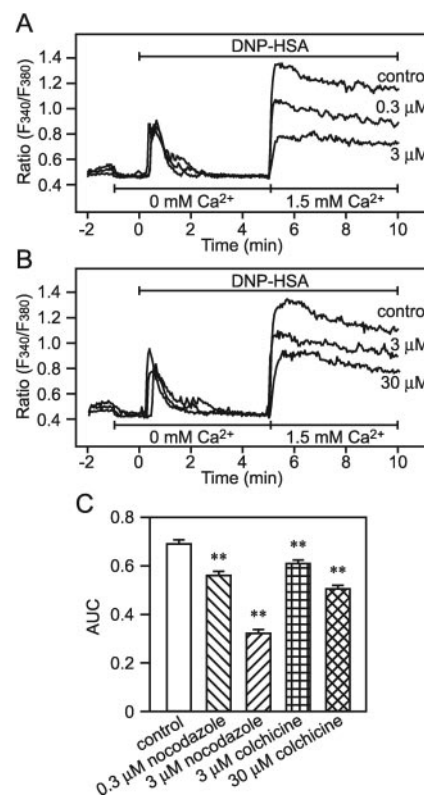


FIGURE 4. Effects of microtubule depolymerizers on Ca^{2+} influx. A and B, Typical recordings of the increase in $[\text{Ca}^{2+}]_i$ in fura-PE3-loaded RBL-2H3 cells. The cells were preincubated without (control), or with 0.3–3 μM nocodazole (A), 3–30 μM colchicine (B) for 15 min at 37°C. The external solution was replaced with Ca^{2+} -free HEPES-buffered solution for the last 1 min of preincubation. The cells were then stimulated with 10 ng/ml DNP-HSA for 5 min, followed by the readmission of external Ca^{2+} (1.5 mM). C, AUC for 5 min after the addition of external Ca^{2+} in A and B. The results are expressed as the mean $\pm$ SE of 54–81 cells in four to six independent experiments. **, Significantly different from the control with $p < 0.01$.

influx of Ca^{2+} after the depletion of Ca^{2+} induced by DNP-HSA stimulation in a concentration-dependent manner (C). We further investigated whether or not the Ca^{2+} influx that was inhibited by the microtubule depolymerizers was CCE. Inhibitors of sarco-ER Ca^{2+} -ATPase, thapsigargin or cyclopiazonic acid, induce Ca^{2+} depletion in the ER and cause CCE, and therefore these drugs have been used as a tool to activate CCE (3, 23). Nocodazole significantly inhibited thapsigargin-induced CCE (Fig. 5, A and C) and cyclopiazonic acid-induced CCE (B and D). In addition, we examined the time dependency of nocodazole treatment (Fig. 5, B and D). Fifteen minutes of pretreatment with nocodazole (i.e., a total of 23-min treatment before Ca^{2+} readmission) reduced CCE more potently than 2 min of pretreatment (i.e., 10 min treatment before Ca^{2+} readmission).

Microtubule disruption changes the distribution of the ER

As mentioned in the introduction, the ER is important for Ca^{2+} signal transduction, because this organelle is a store for Ca^{2+} release and triggers signaling to induce CCE. Therefore, we investigated the effects of microtubule depolymerizers on the distribution of the ER. In RBL-2H3 cells in the resting state, microtubules were nucleated from a microtubule-organizing center, the centrosome, which is typically located in the cytoplasm next to the nucleus (Fig. 6A, left panel). From this focal point, the cytoplasmic microtubules emanated in a star-like conformation. The ER was also located in accordance with the microtubules near the centrosome (Fig. 6A, middle panel). However, when the RBL-2H3 cells

were treated with nocodazole, the microtubules did not assume the star-like conformation (Fig. 6B, left panel). Moreover, the pattern of the distribution of ER was drastically altered. The ER was found to be dispersed throughout the cytoplasm (Fig. 6B, middle panel). Similar changes in the conformational patterns of the microtubules and the ER were observed in BMMCs. In BMMCs in the resting state, the cytoplasmic microtubules emanated in the star-like conformation (Fig. 6C, left panel) and the ER was also located in accordance with the microtubules (C, middle panel). After the nocodazole treatment, not only the conformation of the microtubules (Fig. 6D, left panel), but also the pattern of ER distribution was drastically altered (D, middle panel). We further investigated the time-dependent changes in ER distribution induced by treatment with nocodazole in an individual cell. The ER began to disperse throughout the cytoplasm at least 15 min after nocodazole treatment (Fig. 7).

We then examined the effects of DNP-HSA stimulation on microtubules and ER distribution. There was increased spreading of the cells over the substratum after the DNP-HSA stimulation (Fig. 8). In RBL-2H3 cells after the DNP-HSA stimulation, microtubules emanated in the star-like conformation (Fig. 8A, left panel), and the ER was located in accordance with the microtubules (A, middle panel). When the RBL-2H3 cells were pretreated with nocodazole, the microtubules did not assume the star-like conformation (Fig. 8B, left panel), and the ER was found to be dispersed throughout the cytoplasm (B, middle panel). Similar changes were observed in BMMCs. In BMMCs after the DNP-HSA stimulation, the microtubules emanated in the star-like conformation (Fig. 8C, left panel) and the ER was located in accordance with the microtubules near the centrosome (C, middle panel). When the BMMCs were pretreated with nocodazole, the conformation of the microtubules was drastically altered (Fig. 8D, left panel), and the ER was found to be dispersed throughout the cytoplasm (D, middle panel). The patterns of distribution of both microtubules and the ER were not found to have changed significantly after DNP-HSA stimulation.

In this study, we also verified the observed ER distribution using another fluorescent probe, DiOC₆ (3), which is also used for staining the ER (17). We confirmed the distribution of ER in the central region of RBL-2H3 cells in the resting state, whereas following nocodazole treatment, the ER was dispersed throughout the cytoplasm (data not shown).

Microtubule depolymerizers attenuate the PCA reaction

Finally, we examined the effects of nocodazole and colchicine on the PCA reaction in vivo. The intradermal injection of IgE (50–300 ng/ml) induced the extravasation of Evans blue in a concentration-dependent manner, and 200 ng/ml IgE induced submaximal extravasation of the dye (data not shown). Nocodazole (Fig. 9, A and C) and colchicine (B and D) attenuated the PCA reaction in a concentration-dependent manner.

Discussion

Microtubules are now recognized as crucial to the mast cell degranulation. Although inhibitory effects of microtubule depolymerizers on mast cell degranulation have been observed, most studies have focused primarily on the effects of such depolymerizers on the process of granular transport (13–15). In the present study, we confirmed that two microtubule depolymerizers, nocodazole and colchicine, reduced degranulation in RBL-2H3 cells and BMMCs (Fig. 1). Moreover, we observed additional interesting effects of these agents on ER distribution and Ca^{2+} signaling.

When the mast cells were treated with nocodazole, the ER was dispersed throughout the cytoplasm, along with the disruption of

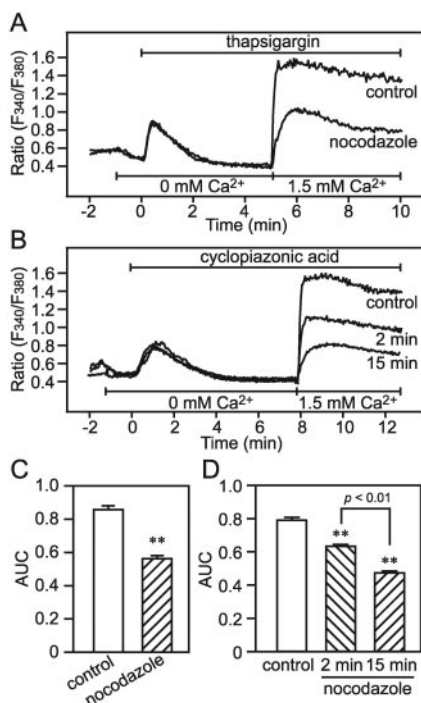


FIGURE 5. Effects of nocodazole on CCE. A and B, Typical recordings of the increase in $[\text{Ca}^{2+}]_i$ in fura-PE3-loaded RBL-2H3 cells. The cells were preincubated without (control) or with 3 μM nocodazole for 2 min (only in B) or 15 min at 37°C. The external solution was replaced with Ca^{2+} -free HEPES-buffered solution for the last 1 min of preincubation. The cells were then stimulated with 1 μM thapsigargin for 5 min (A) or 10 μM cyclopiazonic acid for 8 min (B), followed by the readmission of external Ca^{2+} (1.5 mM). C and D, AUC for 5 min after the addition of external Ca^{2+} in A (C) or in B (D). The results are expressed as the mean $\pm$ SE of 66–137 cells in four to nine independent experiments. **, Significantly different from the control with $p < 0.01$.

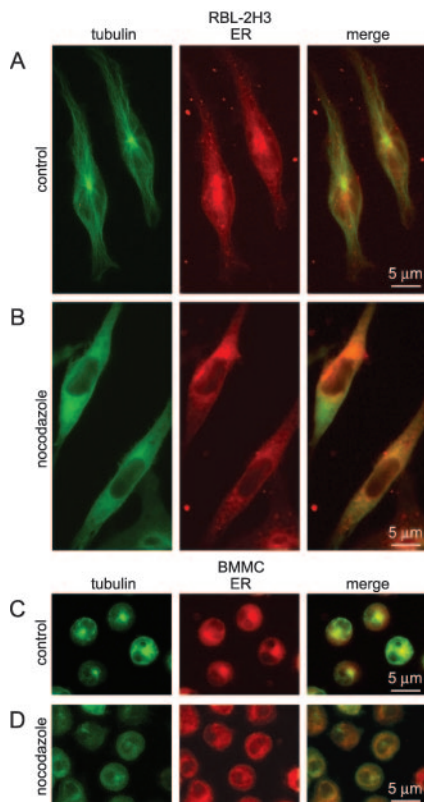


FIGURE 6. Effects of microtubule depolymerizers on the distribution of microtubules and the ER. RBL-2H3 cells (A and B) or BMMCs (C and D) were preincubated without (A and C) or with 3 μ M nocodazole (B and D) for 15 min at 37°C. The cells were double-labeled for tubulin (green) or ER (pseudocolored; red). These figures are typical images from four experiments.

the star-like microtubule conformation (Figs. 6–8). However, the microtubule depolymerizers did not affect the Ca^{2+} release induced by DNP-HSA, nor was the total amount of Ca^{2+} stored in the ER affected (Fig. 3). The other important source of $[\text{Ca}^{2+}]_i$ increase is Ca^{2+} influx from the extracellular space. The disruption of microtubules inhibited the Ag-induced Ca^{2+} influx (Fig. 4). We also verified that nocodazole reduced the Ca^{2+} influx, a process known as CCE, which was induced by thapsigargin and cyclopiazonic acid (Fig. 5). As shown in Fig. 5, B and D, 15 min of pretreatment with nocodazole reduced CCE more potently than 2 min of pretreatment. Nocodazole changed the distribution of the ER (Fig. 7) and also reduced CCE (Fig. 5, B and D) in a time-depen-

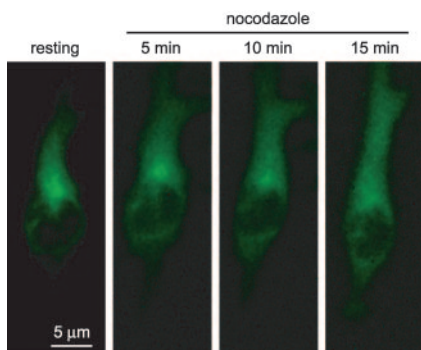


FIGURE 7. Time-dependent changes in ER distribution after microtubule disruption. RBL-2H3 cells were incubated with 3 μ M nocodazole for 0, 5, 10, or 15 min at 37°C. These figures are typical images of an individual cell from among four experiments.

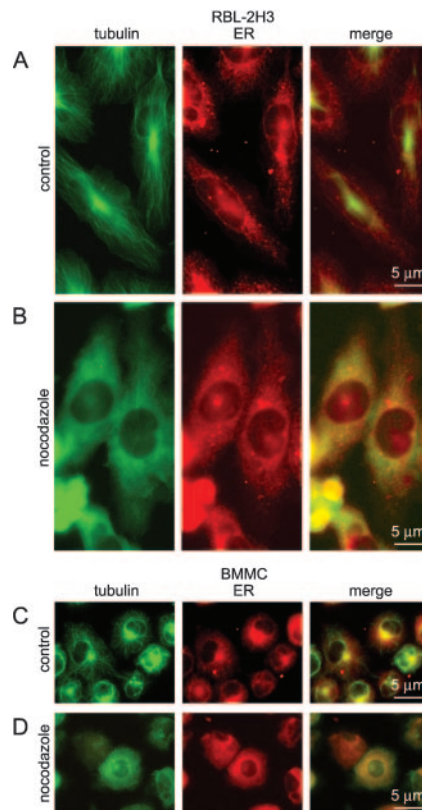


FIGURE 8. Effects of microtubule depolymerizers on the distribution of microtubules and the ER after Ag stimulation. RBL-2H3 cells (A and B) or BMMCs (C and D) were preincubated without (A and C) or with 3 μ M nocodazole (B and D) for 15 min, and the cells were subsequently stimulated with 10 ng/ml DNP-HSA for 10 min at 37°C. The cells were double-labeled for tubulin (green) or ER (pseudocolored; red). These figures are typical images from four experiments.

dent manner. These results suggest that, although the ER redistribution had no effect on Ca^{2+} release mediated by IP_3 , it did alter the potential of CCE. It is generally recognized that mast cells release their granules in proportion to increases in $[\text{Ca}^{2+}]_i$ (8, 24). The microtubule depolymerizers used here inhibited degranulation (Fig. 1) in accordance with the decrease in $[\text{Ca}^{2+}]_i$ (Fig. 2). Considering that the inhibition of degranulation (Figs. 1, A and B) parallels the inhibition of Ca^{2+} influx (Fig. 4), the effect of these

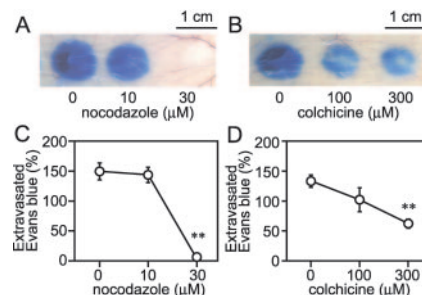


FIGURE 9. Effects of microtubule depolymerizers on PCA reaction. Nocodazole (A and C) and colchicine (B and D) were injected into the back skin of rats simultaneously with IgE injections (i.e., 2 h before Evans blue and DNP-HSA i.v.). A and B, Typical photographs of the PCA reaction from among eight tests. C and D, Analytical data regarding the extravasated Evans blue. The results are expressed as the mean $\pm$ SE of eight experiments. **, Significantly different from the control with $p < 0.01$.

agents on the Ca^{2+} influx might be one of the main mechanisms for the inhibition of degranulation.

One main question is how the disruption of microtubules inhibits CCE. As mentioned in the introduction, two general mechanisms have been proposed for CCE activation: a conformational coupling model (protein-protein interaction) and a CIF model (diffusible messenger). Our results suggest that the ER has to be located at a particular position to transfer a signal to the plasma membrane, where the targets (SOCs) are located. Therefore, our data appear to provide support for the conformational coupling model. However, we cannot rule out the CIF model, because CIF-producing stores, i.e., the ER (12), might need to be located at a particular position near the SOCs in the plasma membrane. Although we were unable to elucidate the entire process of CCE activation in mast cells, the present findings strongly suggest that intact ER distribution is important for the induction of CCE. In addition to the elucidation of the mechanism, the molecular identification of native SOCs remains a major subject for further research. However, the molecular identity of native SOCs in intact cells has not yet been clarified. We expect that the evidence of the involvement of microtubules in the opening of native SOCs will provide crucial leads for more rapid molecular identification of individual channels.

As discussed above, our findings suggest that the decrease in $[\text{Ca}^{2+}]_i$ by microtubule depolymerizers probably attenuates mast cell degranulation. To date, there have been several studies demonstrating that CCE plays an important role in mast cell degranulation in vitro. However, the CCE system has not been recognized as a clinical target in the treatment of allergic diseases. In this study, we examined the effects of CCE-targeting drugs on the mast cell-dependent allergic response in vivo, and observed a marked effect in the PCA reaction (Fig. 9). Thus, we expect that the use of CCE-targeting agents, including microtubule depolymerizers, will exhibit great potential in the treatment of allergic diseases in the future.

In summary, the ER of mast cells is maintained at particular positions by microtubules, and the microtubule depolymerizers used here were found to alter proper ER arrangement. As a consequence, these agents inhibited CCE, and subsequently reduced degranulation, and attenuated the allergic response in vivo. This is the first report demonstrating that microtubule depolymerizers could inhibit the allergic response; these drugs were found to exert a novel inhibitory effect on the Ca^{2+} influx pathway through CCE in the process of mast cell degranulation.

Disclosures

The authors have no financial conflict of interest.

References

1. Metcalfe, D. D., D. Baram, and Y. A. Mekori. 1997. Mast cells. *Physiol. Rev.* 77:1033.
2. Galli, S. J. 1993. New concepts about the mast cell. *N. Engl. J. Med.* 328:257.
3. Parekh, A. B., and R. Penner. 1997. Store depletion and calcium influx. *Physiol. Rev.* 77:901.
4. Lee, R. J., and J. M. Oliver. 1995. Roles for Ca^{2+} stores release and two Ca^{2+} influx pathways in the FcεRI-activated Ca^{2+} responses of RBL-2H3 mast cells. *Mol. Biol. Cell* 6:825.
5. Berridge, M. J. 1993. Inositol trisphosphate and calcium signalling. *Nature* 361:315.
6. Oka, T., K. Sato, M. Hori, H. Ozaki, and H. Karaki. 2002. Xestospongine C, a novel blocker of IP_3 receptor, attenuates the increase in cytosolic calcium level and degranulation that is induced by antigen in RBL-2H3 mast cells. *Br. J. Pharmacol.* 135:1959.
7. Putney, J. W., Jr. 1986. A model for receptor-regulated calcium entry. *Cell Calcium* 7:1.
8. Neher, E. 1991. Ion influx as a transduction signal in mast cells. *Int. Arch. Allergy Appl. Immunol.* 94:47.
9. Berridge, M. J. 1995. Capacitative calcium entry. *Biochem. J.* 312:1.
10. Putney, J. W., Jr., and G. S. Bird. 1993. The inositol phosphate-calcium signaling system in nonexcitable cells. *Endocr. Rev.* 14:610.
11. Irvine, R. F. 1990. "Quantal" Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates: a possible mechanism. *FEBS Lett.* 263:5.
12. Randriamampita, C., and R. Y. Tsien. 1993. Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature* 364:809.
13. Malkinson, F. D. 1982. Colchicine: new uses of an old, old drug. *Arch. Dermatol.* 118:453.
14. Gillespie, E., R. J. Levine, and S. E. Malawista. 1968. Histamine release from rat peritoneal mast cells: inhibition by colchicine and potentiation by deuterium oxide. *J. Pharmacol. Exp. Ther.* 164:158.
15. Smith, A. J., J. R. Pfeiffer, J. Zhang, A. M. Martinez, G. M. Griffiths, and B. S. Wilson. 2003. Microtubule-dependent transport of secretory vesicles in RBL-2H3 cells. *Traffic* 4:302.
16. Dabora, S. L., and M. P. Sheetz. 1988. The microtubule-dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts. *Cell* 54:27.
17. Terasaki, M., L. B. Chen, and K. Fujiwara. 1986. Microtubules and the endoplasmic reticulum are highly interdependent structures. *J. Cell Biol.* 103:1557.
18. Oka, T., K. Sato, M. Hori, H. Ozaki, and H. Karaki. 2002. FcεRI cross-linking-induced actin assembly mediates calcium signalling in RBL-2H3 mast cells. *Br. J. Pharmacol.* 136:837.
19. Oka, T., M. Hori, A. Tanaka, H. Matsuda, H. Karaki, and H. Ozaki. 2004. IgE alone-induced actin assembly modifies calcium signaling and degranulation in RBL-2H3 mast cells. *Am. J. Physiol.* 286:C256.
20. Narenjkar, J., S. J. Marsh, and E. S. Assem. 1999. The characterization and quantification of antigen-induced Ca^{2+} oscillations in a rat basophilic leukemia cell line (RBL-2H3). *Cell Calcium* 26:261.
21. Garvey, J. 1977. Antibody-mediated hypersensitivity. In *Methods in Immunology*. Addison-Wesley, Reading, MA, p. 451.
22. Wershil, B. K., G. T. Furuta, J. A. Lavigne, A. R. Choudhury, Z. S. Wang, and S. J. Galli. 1995. Dexamethasone or cyclosporin A suppress mast cell-leukocyte cytokine cascades: multiple mechanisms of inhibition of IgE- and mast cell-dependent cutaneous inflammation in the mouse. *J. Immunol.* 154:1391.
23. Thastrup, O., P. J. Cullen, B. K. Drobak, M. R. Hanley, and A. P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} -ATPase. *Proc. Natl. Acad. Sci. USA* 87:2466.
24. Sullivan, R., L. S. Price, and A. Koffer. 1999. Rho controls cortical F-actin disassembly in addition to, but independently of, secretion in mast cells. *J. Biol. Chem.* 274:38140.