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ROLE OF MICROTUBULES ON Ca^{2+} RELEASE FROM THE ENDOPLASMIC RETICULUM AND ASSOCIATED HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS

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Abstract—In order to study the role of cytoskeletons on histamine release from mast cells, the effects of cytoskeleton-inhibiting agents were investigated. Since neither colchicine, vinblastine nor cytochalasin D was effective in inhibiting the IP_3 formation, it is possible that neither microtubules nor microfilaments of rat peritoneal mast cells participate in the initial membrane events of the histamine release. However, both colchicine and vinblastine, but not cytochalasin D, were effective in inhibiting Ca^{2+} release from the intracellular Ca store. It was accordingly suggested that the microtubules, rather than microfilaments, are intimately related to the Ca^{2+} releasing process from the endoplasmic reticulum. The fluorescence intensity of the mast cells stained with FITC-labeled anti-tubulin antibody reflects the amount of tubulin polymers inside the cell, and colchicine treatment decreased the fluorescence intensity, indicating that colchicine is effective in depolymerizing the microtubules of rat mast cells. By contrast, the amount of tubulin polymer in the mast cells increased by compound 48/80, indicating that the rearrangement of microtubules took place in the mast cells, leading to histamine release. When permeabilized mast cells were exposed to potassium antimonate solution, microtubules attached themselves to the endoplasmic reticulum and many Ca antimonate dots were observed. From the present results, it was concluded that microtubules play an important role in the processes leading to Ca^{2+} release from the intracellular Ca store and subsequent histamine release.

It has been suggested that both microfilaments and microtubules are in some way related to histamine release from mast cells, since the cytoskeleton-inhibiting agents, cytochalasins and colchicine, are effective in inhibiting histamine release [1, 2]. The electron microscopic observations indicated that in rat mast cells, the granules and other organelles are densely connected to each other by the network of microfilaments and microtubules [3, 4]. The morphological changes of the cytoskeletons in association with histamine release are also reported [4]. By means of rhodamine-labeled phalloidine, an F-actin specific dye, it was revealed that the elongation of actin filaments takes place in association with degranulation so as to push the granules out of the cell [5]. In addition, in the repair process after degranulation, the re-uptake of extruded granules was performed by the filamentous structure [6]. These observations suggest that microfilaments in the mast cell play a leading role in the process of exocytosis. By contrast, the function of microtubules in histamine release is not recognized as clearly as in the case of microfilaments. In addition, although it is known that an increase in intracellular Ca^{2+} concentration is prerequisite for the histamine release from mast cells, the role of cytoskeletons in association with the increase in intracellular Ca^{2+} concentrations is not known. It has been shown that the structures of microtubules and endoplasmic reticulum are highly interdependent [7]. We have already shown that the endoplasmic reticulum is the most plausible Ca store in the mast cells in association with histamine release [8]. Therefore, it seems worthwhile to elucidate whether or not microtubules participate in the process leading to an increase of intracellular Ca^{2+} concentration. To examine this point in relation to histamine release, the present investigation was performed.

MATERIALS AND METHODS

Histamine release from rat mast cells. Rat peritoneal mast cells were isolated from the abdominal cavity of male Wistar rats (250–300 g) and purified by means of Percoll density gradient centrifugation with purity of 95% or higher. The mast cells were suspended in RPMI-1640 medium and incubated for 2 hr at 37° in the presence or absence of cytoskeleton-inhibiting agents in 5% CO_2 in humidified air. Thereafter, 0.5 μg/mL of compound 48/80 was added and incubation was continued for another 10 min. The reaction was terminated by chilling the test tube in an ice-cold bath. After centrifugation at 500 *g* for 10 min at 4°, the histamine contents in the supernatant and the cell pellet were measured separately by means of a fluorometric assay.

^{45}Ca uptake in the rat peritoneal mast cells induced by compound 48/80. Rat peritoneal mast cells purified with Percoll density gradient centrifugation were suspended in a physiological buffered solution

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(in mM; NaCl 154, KCl 2.7, $CaCl_2$ 0.9, HEPES* 5; pH 7.4: PBS) containing various concentrations of cytoskeleton-inhibiting agents and incubated at 37° for 100 min. Thereafter, 1.35 μCi of $^{45}CaCl_2$ was added and incubation was continued for another 20 min. Subsequently, 0.5 μg/mL of compound 48/80 was added and incubated for 10 min. The reaction was terminated by placing the test tube in an ice-cold bath. After repeated centrifugations at 1000 *g* for 10 min at 4°, the cells were washed with PBS and the radioactivity of the cell pellet was measured using a liquid scintillation counter (Aloka, LSC-700) [9, 10].

Measurement of inositol-1,4,5-trisphosphate (IP_3) in rat mast cells. Purified rat mast cells were suspended in RPMI-1640 medium containing cytoskeleton-inhibiting agents at various concentrations and incubated at 37° for 2 hr in a CO_2 incubator (5×10^6 cells, 200 μL/tube). After centrifugation, the medium was replaced by 150 μL of Ca-free PBS containing 0.1 mM EGTA, and 50 μL of compound 48/80 was added to make the final concentration of 0.5 μg/mL. Five seconds later, the reaction was terminated by an addition of 50 μL of 10% PCA (ice-cold) and the mixture was placed in an ice-bath for 20 min. After centrifugation at 2000 *g* for 10 min at 4°, 200 μL of the supernatant was neutralized with 1.5 N KOH solution containing 60 mM HEPES and centrifuged at 1000 *g* for 10 min. IP_3 contents in the supernatant were measured using the IP_3 assay kit (Amersham).

Changes in the intracellular Ca^{2+} concentrations measured with quin 2. Isolated rat peritoneal mast cells were incubated with 5 μM of quin 2/AM dissolved in a Ca-free PBS at 37° for 30 min. Thereafter, the cells were washed twice with the same buffer. Subsequently, the quin 2-loaded mast cells were preincubated with various concentrations of cytoskeleton-inhibiting agents at 37° for 1 hr. The cells were washed again with Ca-free PBS and the changes in fluorescence intensity of quin 2-loaded individual mast cell were determined after exposure to 0.35 μg/mL of compound 48/80, using a fluorescence microscope (XF, Nikon) connected to a video-intensified microscopy system (ARGUS-100, Hamamatsu) [11].

Fluorescence intensity measurement of tubulin-stained mast cells. Rat peritoneal mast cells placed on cover slips were cultured in RPMI-1640 medium, containing various concentrations of colchicine, for 2 hr, and another 10 min of incubation was performed in the presence or absence of compound 48/80 (0.5 μg/mL). The cells were washed with a stabilization buffer (in mM; PIPES 100, EGTA 1, $MgSO_4$ 1, GTP 1, polyethylene glycol 6000 4%; pH 6.9) containing 0.2% Triton X-100, and fixed with 3.7% of formaldehyde supplemented with 5 mM of EGTA for 10 min at room temperature [12]. The fixed cells were washed with ice-cold PBS and incubated with anti-tubulin sheep IgG for 1 hr at 37°. The cells were washed twice with PBS, and incubated with FITC-labeled anti-sheep IgG mice serum for 45 min at 37°. The specimens were washed with PBS and the fluorescence intensity of individual mast cells was measured by means of a photon counter (P-1, Nikon) connected to a fluorescence microscope (Nikon, XF).

Electron microscopy of the localization of intracellular calcium store in rat peritoneal mast cells. To identify the intracellular Ca^{2+} store morphologically, Ca^{2+} in the mast cells was precipitated with potassium antimonate, and electron microscopy was carried out, according to the method of Wick and Hepler [13]. Rat peritoneal mast cells were incubated in KG buffer (137 mM potassium glutamate, 3 mM ATP, 4 mM $MgSO_4$, 50 μM EGTA, 10 mM HEPES; pH 6.8) containing 5 μg/mL of β-escin for 5 min at 37° to permeabilize the cell membranes, and centrifuged at 40 *g* for 5 min at 4°. The cell pellet was prefixed with 3% glutaraldehyde solution containing 2% potassium antimonate for 10 min at 4°. Thereafter, the cell pellet was cut into small pieces, and fixed in the same solution for 4 hr at 4°. The specimens were washed with 5 mM HEPES (pH 7.4) buffer for 18 hr at 4°, and postfixed with 1% osmium tetroxide for 30 min at 4°. After dehydration in a graded alcohol series, the fixed cells were embedded in Epon 812 resin, thin-sectioned, and observed by means of a transmission electron microscope (TEM; Hitachi H-500).

Chemicals. The following compounds were used in this study: RPMI-1640 medium (Sigma), cytochalasin D (Sigma), colchicine (Sigma), vinblastine (Sigma), lumicolchicine (Sigma), compound 48/80 (Wellcome), IP_3 assay kit (Amersham), quin 2/AM (Dojindo), anti-tubulin sheep IgG (affinity purified, Caabco), FITC-conjugated anti-sheep IgG mice serum (affinity purified, Jackson Immunotech), β-escin (Fluka), potassium antimonate (Aldrich). Other chemicals used were all reagent grade and purchased from commercial sources.

Statistical analysis. A one-way analysis of variance with Dunnett's test was used to determine the statistical significance.

RESULTS

Inhibitory effects of cytoskeleton-inhibiting agents on the histamine release from rat peritoneal mast cells induced by compound 48/80

The effects of cytoskeleton-inhibiting agents on the histamine release from rat peritoneal mast cells are shown in Fig. 1. Both cytochalasin D, vinblastine and colchicine effectively inhibited the histamine release induced by compound 48/80; cytochalasin D was the most effective. By contrast, lumicolchicine was totally ineffective even at higher concentrations. Although lumicolchicine is an analog of colchicine, it is effective in neither interfering with the polymerization of the microtubules nor in affecting the function of the microtubules. Consequently, it was assumed that the inhibitory effect of colchicine may be exerted by inhibiting the polymerization of microtubules.

* Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PBS, physiological buffered solution; IP_3, inositol-1,4,5-trisphosphate; EGTA, ethyleneglycol bis(β-amino-ethylether)-*N,N,N',N'*-tetraacetic acid; PCA, perchloric acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); FITC, fluoresceinisothiocyanate; TEM, transmission electron microscope; PI, phosphatidylinositol; ER, endoplasmic reticulum.

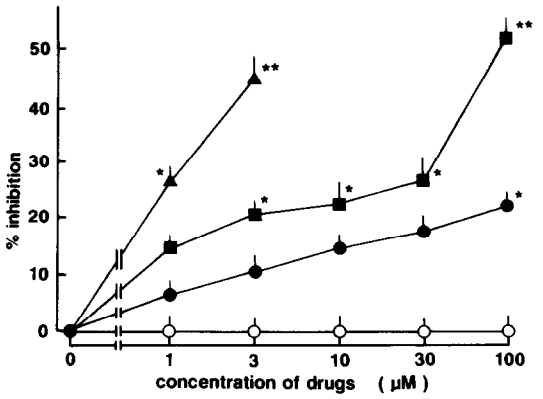

Fig. 1. Effects of cytoskeleton-inhibiting agents on the histamine release from rat peritoneal mast cells induced by compound 48/80 (0.5 μg/mL). (●) colchicine, (■) vinblastine, (○) lumicolchicine, (▲) cytochalasin D. * and ** represent $P < 0.05$ and $P < 0.01$, respectively. Each point represents the mean ± SEM (N = 5). Histamine release in the control experiments was 71.4 ± 1.9% (N = 9).

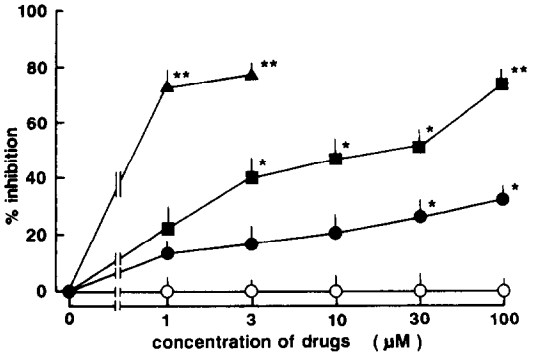

Fig. 2. Effects of cytoskeleton-inhibiting agents on the ^{45}Ca uptake of rat peritoneal mast cells induced by compound 48/80 (0.5 μg/mL). (●) Colchicine, (■) vinblastine, (○) lumicolchicine, (▲) cytochalasin D. * and ** represent $P < 0.05$ and $P < 0.01$, respectively. Each point represents the mean ± SEM (N = 5).

Effects of cytoskeleton-inhibiting agents on the ^{45}Ca uptake of rat peritoneal mast cells induced by compound 48/80

In order to study the inhibitory mechansim of cytoskeleton-inhibiting agents on histamine release from mast cells, the effects of these compounds on ^{45}Ca uptake were investigated. As shown in Fig. 2, the ^{45}Ca uptake into rat mast cells elicited by compound 48/80 was dose-dependently inhibited by separate pretreatments of cytochalasin D, vinblastine and colchicine, at concentrations higher than 1 μM.

Effect of cytoskeleton-inhibiting agents on the IP_3 formation of rat peritoneal mast cells

It is known that when mast cells are stimulated by histamine releasers, phosphatidylinositol (PI) breakdown in the cell membrane is elicited so as to liberate IP_3 at the early stage of the cell activation. Since IP_3 acts as a phospholipid-derived second messenger capable of releasing Ca^{2+} from intracellular Ca store, the activation of signal transduction in the cell membrane may be reflected by the IP_3 formation. In order to study the influence of cytoskeletons on the IP_3 formation, rat mast cells were stimulated with compound 48/80. In the resting stage, the IP_3 content of mast cells was 0.12 ± 0.05 pmol/10^6 cells. However, when the cells were stimulated with compound 48/80 for 5 sec, the IP_3 content markedly increased to 3.78 ± 0.07 pmol/10^6 cells, which is more than a 30-fold increase compared to the control level. Neither of the cytoskeleton-inhibiting agents tested was effective in inhibiting the IP_3 formation (Fig. 3).

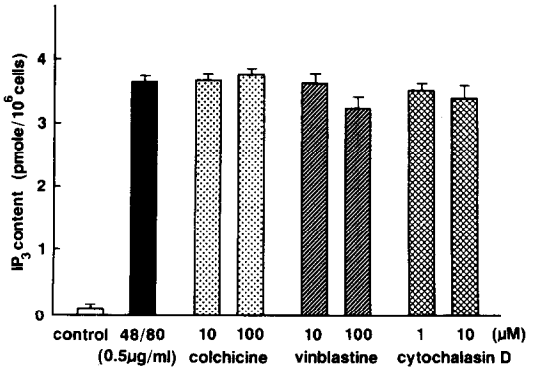

Fig. 3. Effects of cytoskeleton-inhibiting agents on the IP_3 formation of rat peritoneal mast cells induced by compound 48/80 (0.5 μg/mL) (N = 5).

Changes in intracellular Ca^{2+} concentration of mast cells

The effects of cytoskeleton-inhibiting agents on the Ca^{2+} release from the intracellular Ca store were determined by means of a video-intensified microscopy system. When the quin 2-loaded mast cells were stimulated with compound 48/80 in a Ca-free medium, the fluorescence intensity of the cell increased promptly and reached maximum level within a few seconds, indicating that Ca^{2+} was released from the intracellular Ca store. Thereafter, the fluorescence intensity gradually decreased to the control level within 30 sec after stimulation (Fig. 4). The inhibitory effects of cytoskeleton-inhibiting agents on the Ca^{2+} release from the intracellular Ca store are summarized in Table 1. Although cytochalasin D did not alter the Ca^{2+} release from the intracellular Ca store, both vinblastine and colchicine effectively inhibited it at concentrations higher than 1 μM.

Effects of colchicine and compound 48/80 *on the fluorescence intensity of tubulin-stained mast cell*

The fluorescence image of rat mast cells stained with an anti-tubulin antibody is shown in Fig. 5. Intense fluorescence was observed at the cell periphery and filamentous structures were seen

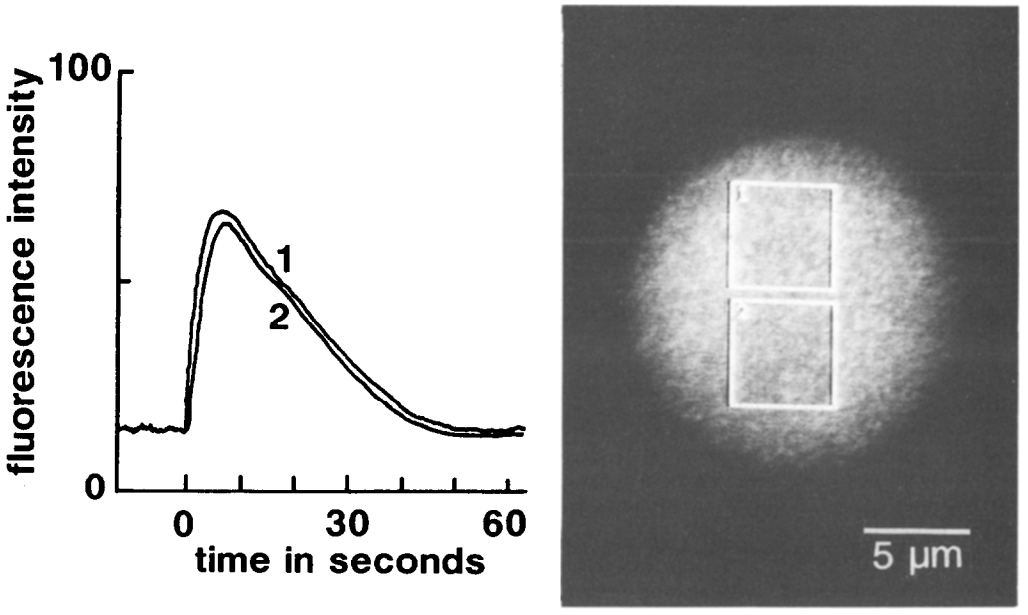

Fig. 4. Ca^{2+} release from the intracellular Ca store induced by compound 48/80. Target areas for the fluorescence intensity measurement were indicated as squares on the fluorescence image of the mast cell.

Table 1. Inhibitory effects of cytoskeleton-inhibiting agents on the Ca^{2+} release from intracellular Ca store induced by compound 48/80

Compounds (μM)	Fluorescence increase (arbitrary units)	% Inhibition
Control	41.2 ± 2.2	—
Cytochalasin D		
1	40.3 ± 1.3	2.18
10	39.6 ± 1.7	3.88
Colchicine		
1	20.3 ± 1.8*	50.73
10	14.7 ± 1.4*	64.32
100	7.6 ± 1.5*	81.55
Vinblastine		
1	25.7 ± 1.6*	37.62
10	13.9 ± 2.9*	66.26
100	6.4 ± 0.8*	84.47

The fluorescence intensities of quin 2-loaded mast cells stimulated with compound 48/80 were measured at 7 sec after stimulation.

Each value represents mean ± SEM (N = 50).

* Indicates statistical significance in $P < 0.05$.

inside the cell. When the mast cells were treated with compound 48/80, the microtubules seemed to be disrupted in various places and a cluster of microtubule fragments were observed (Fig. 5b).

Figure 6 shows the changes in the amount of microtubules in mast cells exposed to colchicine or compound 48/80. In this experiment, after permeabilization with Triton X-100, the cells were loaded with an anti-tubulin antibody. The process of permeabilization leads to a drastic loss of tubulin monomers, so that the fluorescence intensity of the cell stained with the anti-tubulin antibody corresponds to the amount of polymerized form of tubulin. The fluorescence intensity of mast cells treated with colchicine decreased in a dose-dependent fashion, indicating that colchicine acts to inhibit the polymerization of the microtubules. By contrast, in the mast cells treated with compound 48/80, the fluorescence intensity increased significantly and dose-dependently, indicating that an increase in polymerization was elicited. When the mast cells treated with 10 μM of colchicine were stimulated with 0.5 μg/mL of compound 48/80, the fluorescence intensity of the tubulin-stained mast cell was slightly lower than that of the control level, but higher than that of the cells treated with colchicine alone.

TEM observation of the localization of intracellular Ca and microtubules

When permeabilized mast cells were exposed to potassium antimonate solution, potassium antimonate entered into the cytoplasm and interacted with Ca^{2+} to precipitate it as Ca antimonate. After this treatment, the particles of Ca antimonate appeared as black dots on the endoplasmic reticulum (Fig. 7).

DISCUSSION

It has been suggested that both microfilaments and microtubules play some role in the histamine release from mast cells, since various cytoskeleton-inhibiting agents are effective in suppressing the histamine release [1, 2]. Electron microscopic observation of the internal structure of mast cells revealed that various organellas, most evidently

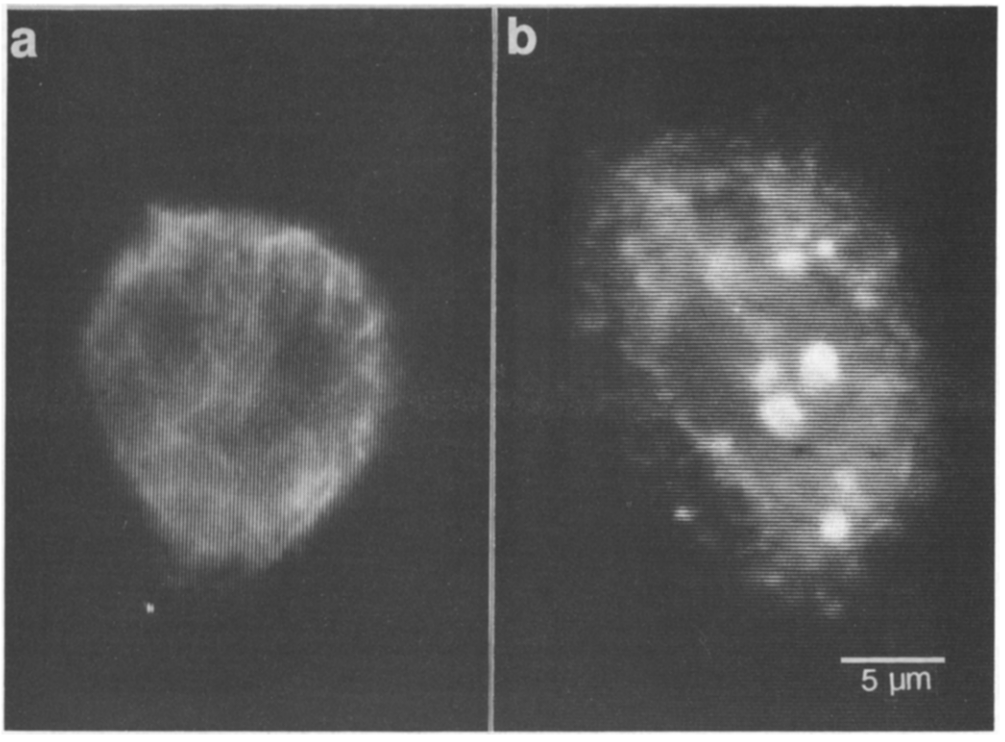

Fig. 5. Immunofluorescence microscopic image of rat peritoneal mast cells stained with anti-tubulin antibody. (a) Control, (b) stimulated with compound 48/80 (0.5 μg/mL).

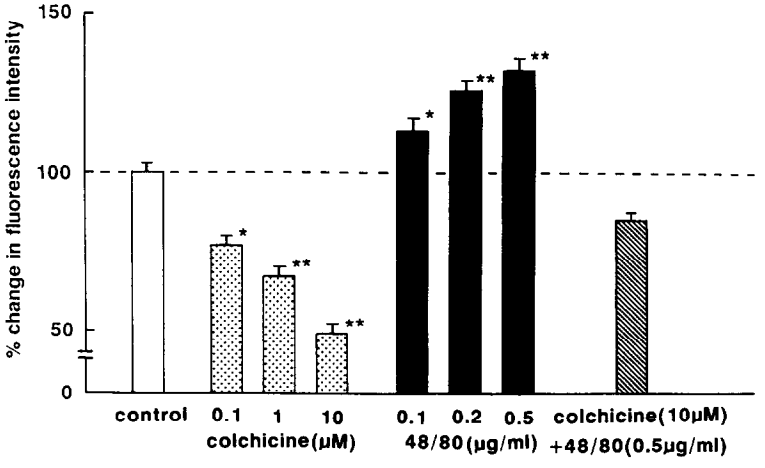

Fig. 6. Effects of colchicine and compound 48/80 on the fluorescence intensity of tubulin-stained rat peritoneal mast cells. In each column, over 500 cells were measured. * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

in the granules, are densely interconnected by cytoskeletons [3, 4]. In the previous paper, we reported that microfilaments participate not only in the exocytotic process but also in the retraction of the extruded granules [5, 6]. In the present experiment, it was shown that cytochalasin D, colchicine and vinblastine effectively inhibited the histamine release from mast cells due to compound 48/80. The results are in agreement with those previously reported [1, 2]. Since lumicolchicine did not affect the histamine release, it was assumed that the inhibitory effect of colchicine was exerted by its specific action on microtubules. When the concentration of vinblastine was increased from 30 to 100 μM, the effect of vinblastine in inhibiting histamine release increased remarkably as shown in Fig. 1. Since it is known that vinblastine promotes the formation of tubulin paracrystals very efficiently at 50 μM, the potent histamine release inhibition elicited by vinblastine at 100 μM may be caused by

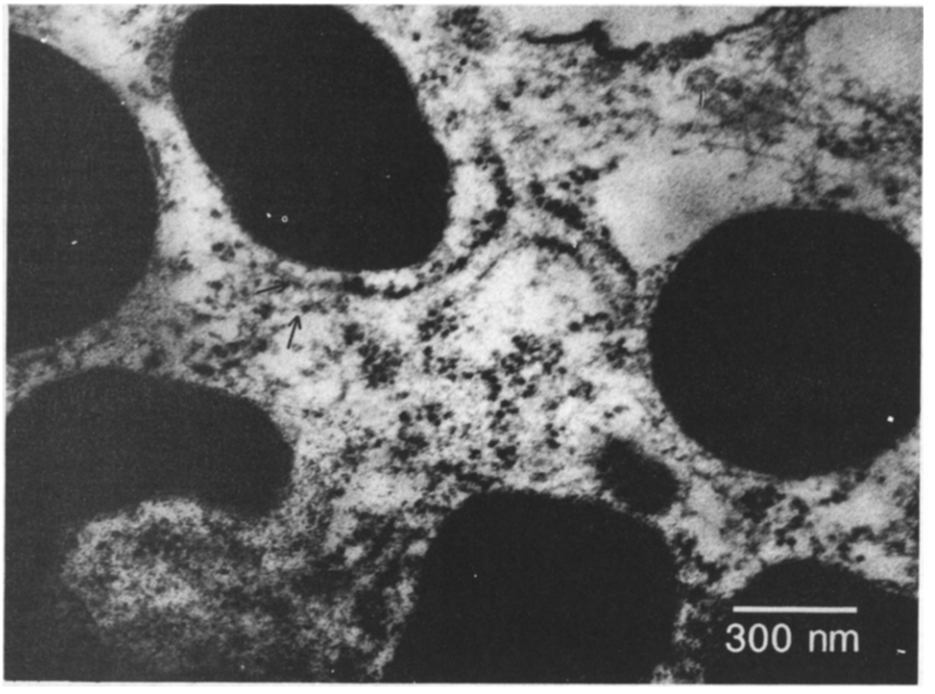

Fig. 7. TEM appearance of the inside structures and Ca stores of the rat peritoneal mast cells. The precipitates of Ca antimonate were observed as small black dots on the surface of endoplasmic reticulum, where microtubules are attached (arrows).

the paracrystalline formation of cytoplasmic tubulin [14].

It is known that an increase in the intracellular Ca^{2+} concentration is prerequisite for the histamine release from mast cells [15]. At least two pathways are proposed to increase intracellular Ca^{2+} concentrations: an increase in Ca^{2+} transport from an extracellular medium, or an increment of Ca^{2+} release from the intracellular Ca store [15]. In the former, it is suggested that a receptor-operated Ca channel may be of importance [16], while in the latter, a rapid formation of IP_3 and the resulting Ca^{2+} release from the endoplasmic reticulum may lead to histamine release [8]. Since none of the cytoskeleton-inhibiting agents used in the present study affected the IP_3 production of mast cells induced by compound 48/80, it became clear that both microfilaments and microtubules may not participate in the process(es) of IP_3 production in the early stage of the cell activation.

For the measurement of intracellular Ca^{2+} concentrations, Fura 2/AM is widely used in many types of cells. However, it has been reported that ester-loaded Fura 2 was accumulated significantly in the secretory granules and that the fluorescence was lost during exocytosis [17]. Furthermore, Highsmith *et al.* [18] reported that Fura 2/AM may be converted to Fura 2 via the intermediate form called Fura 2′. Fura 2′ is a compound which is lipophilic and fluorescent even in the absence of an interaction with Ca^{2+}. Since these peculiar fluorescences unrelated to intracellular Ca^{2+} were noticed, Fura 2/AM was not employed in the present experiment. In connection with this, when ^{45}Ca uptake into mast cells under stimulation with compound 48/80 was tested preliminarily according to the silicon oil method [19], it was noticed that mast cells spun through silicon oil were often damaged and the values were scattered diversely. Furthermore, when the effects of colchicine and vinblastine on histamine release were compared with those of Ca^{2+} release from the intracellular store, the extents of inhibition exerted by these two drugs were evident in the latter case. This can be ascribed to the differences in the preincubation with anti-microtubule agents and the incubation in the presence of compound 48/80. In histamine release the incubation was performed in the presence of Ca^{2+} for 10 min, while in Ca^{2+} release from the store the incubation was not extended beyond 60 sec. Moreover, the concentration of compound 48/80 used in histamine release was 0.5 μg/mL, while in the Ca^{2+} release experiment 0.35 μg/mL was employed. These differences may explain the dissociation of the effects of anti-microtubule agents on histamine release and Ca^{2+} release from the intracellular store.

In the present experiment, mast cells were stimulated with compound 48/80. However, it is known that in IgE-dependent histamine release the increase in intracellular Ca^{2+} concentration is exclusively dependent on the Ca uptake from the extracellular medium. As shown in Fig. 2, anti-microtubule agents inhibit the ^{45}Ca influx significantly and dose-dependently in the histamine release induced by compound 48/80. The identical mechanism may be exerted more effectively in inhibiting the histamine release from sensitized mast cells.

On the other hand, Ca^{2+} release from the

intracellular Ca store elicited by exposure to compound 48/80 was effectively inhibited by both colchicine and vinblastine, but not by cytochalasin D. Since these two compounds were not effective in inhibiting IP_3 production, it was assumed that the endoplasmic reticulum of mast cells treated with any of the anti-microtubule agents became irresponsive to IP_3, and that microtubules may be important in regulating the function of the intracellular Ca store. From the morphological observation it has been suggested that the structures of the endoplasmic reticulum and the microtubules are highly interdependent [7]. Also, it was demonstrated that in electron microscopy, the microtubules are linked to the endoplasmic reticulum, where many black dots of Ca antimonate were observed as indicated in Fig. 7. From these findings, it was proposed that microtubules play a critical role in histamine release by releasing Ca^{2+} into the cytosol from the intracellular Ca store: the endoplasmic reticulum.

Since anti-microtubule agent (colchicine) is not easily incorporated into the cells, the incubation should be extended up to 2 hr. Thus, in the study of the histamine release from mast cells, the experiment must be carried out in the presence of extracellular Ca^{2+} (RPMI-1640 medium). Therefore, colchicine definitely inhibited intracellular Ca^{2+} release, certain amounts of Ca were incorporated from the extracellular medium. Ca uptake and histamine release were inhibited similarly by colchicine, as shown in Figs 1 and 2.

As shown in Fig. 6, the fluorescence intensity of the microtubules in the mast cells treated with colchicine decreased in a dose-dependent fashion, clearly indicating that colchicine acts to depolymerize the microtubules. By contrast, the fluorescence intensity of microtubules in the mast cells treated with compound 48/80 increased dose-dependently. However, as shown in Fig. 5, disruption of the microtubules was elicited by stimulation with compound 48/80. These observations seem to suggest that after exposure to compound 48/80, a transient fragmentation of the microtubules takes place, while tubulin polymerization occurs sequentially. In accordance with this, the colchicine pretreatment prevented an increase in the fluorescence intensity of mast cells induced by compound 48/80, but the fluorescence intensity was higher than that of the cells treated with colchicine alone. This may indicate that depolymerization may be reversed as a consequence of an excessive incorporation of Ca^{2+} into mast cells in association with the histamine release induced by compound 48/80. It was reported that monomeric tubulin became polymeric in the presence of the millimolar order of ATP and Ca^{2+} at the micromolar range [20]; such changes in ATP and Ca^{2+} concentrations are frequently encountered in the cytosol of the activated mast cells [11].

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