Effects of Calcium-Binding Proteins on Histamine Release from Permeabilized Rat Peritoneal Mast Cells

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Among the calcium-binding proteins from chicken gizzard smooth muscle, smooth muscle calcium-binding protein 11 (SMCaBP 11), which is an EF-hand type calcium-binding protein, can prevent Ca^{2+} dependent histamine release from permeabilized rat peritoneal mast cells. This activity appears to be specific since other calcium-binding proteins (CaBPs), including h-calcimedin, l-calcimedin, calmodulin and S100, had no inhibitory action. Approximately 40% of the histamine release was inhibited by SMCaBP 11 at 48 nM and the half-maximal inhibition occurred at 20 nM. Immunological techniques revealed that permeabilized mast cells elicited by Ca^{2+} also released actin into the medium, but not tubulin. The addition of SMCaBP 11 prevented the secretion of actin from the mast cells, though the distribution of F-actin was only slightly affected. These findings suggest that SMCaBP 11 may modulate Ca^{2+} and actin-mediated exocytosis from permeabilized mast cells.

Keywords mast cell; calcium-binding protein; histamine release; actin; tubulin; permeabilization

Cytoskeletal compartments have been considered to be associated with the exocytosis of mast cells.^{1–3)} Immunocytochemical techniques demonstrated that microfilaments are located at the periphery of granules and the plasma membrane. Several drugs such as colchicine, vinblastine and cytochalasins inhibited the histamine release from mast cells.^{3,4)} The intracellular calcium level in mast cells is an important second messenger for leading the release of histamine and other substances.^{5,6)} The effects of calcium concentration changes appear to be mediated by CaBPs. The Ca^{2+} -CaBPs complex becomes activated and can affect cytoskeletal structures and enzyme activities.^{7,8)} Chakravarty and co-workers reported that the calmodulin concentration in rat mast cells is about 9.4 pmol/ 10^6 cells.⁹⁾ Calmodulin antagonists, including *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7), chlorpromazine and trifluoperazine, inhibited the mast cell histamine secretion induced by compound 48/80 and Ca^{2+} plus ionophore A-23187,^{10,11)} indicating that calmodulin participates in the secretory process. However, the existence of Ca^{2+} -binding proteins other than calmodulin remain unknown in mast cells, and the relation of these proteins to secretion has not been elucidated.

Permeabilized mast cells were utilized to clarify the role of CaBPs, including h-calcimedin, l-calcimedin, calmodulin, SMCaBP 11 and S100, on histamine release. In this paper, we describe that SMCaBP 11 shows an inhibitory effect on the histamine release induced by Ca^{2+} . We also examined the localization of actin and tubulin, indicating that SMCaBP 11 affects the behavior of actin in mast cells.

MATERIALS AND METHODS

Preparation of Proteins CaBPs, including h-calcimedin, l-calcimedin, calmodulin and SMCaBP 11, were prepared by the method of Mani and Kay with a slight modification.¹²⁾ Frozen chicken gizzard smooth muscle (100 g) was homogenized in 4 volumes of 40 mM Tris-HCl

buffer (pH 7.5) containing 80 mM NaCl, 2 mM EDTA, 1 mM α -phenylmethanesulfonyl fluoride and 0.05% NaN_3 using a Waring blender, followed by Polytron. The suspension was centrifuged at $10000 \times g$ for 75 min at 4 °C and the precipitate was discarded. Solid ammonium sulfate was added to give 90% saturation, and the resulting precipitate collected by centrifugation was dissolved in 40 mM Tris-HCl (pH 7.5) and 80 mM NaCl (buffer A). Calcium chloride was added to the 2 mM level. The sample was clarified by centrifugation and applied to a phenyl-Sepharose 4B column (2.5 $\times$ 20 cm) equilibrated with buffer A containing 2 mM CaCl_2 . The column was washed with the same buffer and the bound protein was eluted with buffer A containing 4 mM EGTA. The eluted CaBPs were collected and dialyzed overnight against 20 mM imidazole-HCl (pH 6.2) and 1 mM EDTA (buffer B). The dialysate was clarified by centrifugation and applied to a QMA column (Waters) equilibrated with buffer B. The column was washed with a linear gradient of NaCl, ranging from 0 to 600 mM. The 67 kDa-(h-calcimedin), 32 kDa-(l-calcimedin) and 11 kDa-(SMCaBP 11) protein fractions around 150–200 mM NaCl and calmodulin around 450 mM NaCl detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were collected and further purified by gel filtration on an Ultrogel AcA 54 column (1.5 $\times$ 60 cm) equilibrated with 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2 mM EDTA and 0.05% NaN_3 (buffer C). h-Calcimedin, l-calcimedin, SMCaBP 11 and calmodulin were collected and stored at –80 °C. S100 was purified from porcine brain according to the method of Isobe *et al.*¹³⁾ The purity of these proteins was checked by SDS-PAGE (Fig. 1). Actin was isolated from rabbit skeletal muscle according to the method of Spudich and Watt¹⁴⁾ and further purified by Sephadex G-150 column chromatography as previously described.¹⁵⁾

Preparation of Mast Cells Rat peritoneal mast cells were obtained from male Wistar rats. They were purified to at least 95% homogeneity by centrifugation through a 2 ml cushion of 24% metrizamide, and washed twice by

centrifugation at 4°C. Mast cells were suspended at approximately 10^6 ml^{-1} in mast cell medium (MCM) which was comprised of 144 mM NaCl, 2.7 mM KCl, 4.7 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 5.6 mM glucose, 10 U/ml heparin and 1 mg/ml bovine serum albumin (BSA), pH 7.

Permeabilization and Histamine Release Mast cell suspensions (2×10^4 /tube) containing CaBPs were pre-incubated for 5 min at 37°C and permeabilized by the addition of 15 $\mu\text{g/ml}$ β -escin for 15 min at 37°C. After the addition of 0.1 mM CaCl_2 , the cell suspension (0.2 ml) was further incubated for 10 min at 37°C. Reactions were terminated by adding 1.8 ml of ice-cold MCM buffer containing 1 mM EGTA. Mast cells were stimulated by compound 48/80 (0.4 $\mu\text{g/ml}$) or ionophore A23187 (0.4 $\mu\text{g/ml}$). IgE sensitized mast cells (0.18 ml) were prepared by incubation for 60 min at 37°C with rat IgE plus phosphatidyl serine (50 $\mu\text{g/ml}$) and then washed once by centrifugation, and 0.02 ml of antigen (ovalbumin, 50 $\mu\text{g/ml}$) was added to the sensitized cells for stimulation.

Fig. 1. SDS-PAGE of h-Calcimedin, l-Calcimedin, Calmodulin, SMCaBP 11 and S100

Electrophoresis was done on a 15% acrylamide gel. The arrows indicate the position of molecular markers: phosphorylase b (94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20000) and lysozyme (14400).

Incubation was carried out for 10 min at 37°C and reactions were terminated by adding 1.8 ml of ice-cold MCM buffer. After centrifugation, the histamine content of the supernatant (1 ml) was determined using o-phthalaldehyde as described by Shore *et al.*¹⁶⁾

Gel Electrophoresis and Immunoblotting SDS-PAGE was performed according to Laemmli,¹⁷⁾ using 0.8 mm-thick slabs stained with 0.1% Coomassie brilliant blue R-250. Proteins separated by SDS-PAGE were electrophoretically transferred to a nitrocellulose filter¹⁸⁾ and excess binding sites on the filters were blocked with 5% BSA and 1% calf serum in phosphate buffered saline (PBS) before incubation with the monoclonal antibodies against β -actin (Amersham) and α -tubulin. After incubation with anti-mouse IgG conjugated with peroxidase (Wako), the immunoblot was developed with the enhanced chemiluminescence immunodetection system (Amersham) using Fuji medical X-ray film. IgE and monoclonal antibody against α -tubulin, termed E₃B₈, were kind gifts from Dr. T. Arai (Science University of Tokyo).

Fluorescence Microscopy Mast cells on slides were fixed for 15 min with 3.7% formaldehyde in PBS, then permeabilized with 2% Triton X-100 in PBS for 5 min. Cells were incubated for 5 min with 10 U/ml rhodamine phalloidin (Molecular Probes) at 37°C. Following a rinse in PBS, the slides were examined and photographed with Fujicolor Super HG400 film using an Olympus BHS-FRK fluorescence microscope.

RESULTS

The effects of CaBPs from smooth muscle and brain, including h-calcimedin, l-calcimedin, calmodulin, SMCaBP 11 and S100, on histamine secretion induced by IgE, compound 48/80, ionophore A23187 and Ca^{2+} were examined using permeabilized or non-permeabilized rat peritoneal mast cells (Table I). SMCaBP 11 exhibited an inhibitory effect on Ca^{2+} -dependent secretion from β -escin-permeabilized cells. In contrast, no inhibition was found when non-permeabilized cells were stimulated by Ca^{2+} plus ionophore A23187, indicating that this protein can function after incorporation into mast cells. Calmodulin suppressed the histamine release induced by compound 48/80 only when Ca^{2+} was present in the medium. Since compound 48/80 has been reported to

TABLE I. Effects of CaBPs on Histamine Release from Rat Peritoneal Mast Cells

Proteins	Conc. ($\mu\text{g/ml}$)	Histamine release (%)				
		Permeabilized cells		Non-permeabilized cells		
		β -Escin + Ca^{2+}	Ionophore A23187	IgE	Compound 48/80 + Ca^{2+}	Compound 48/80 - Ca^{2+}
None	0	100	100	100	100	100
h-Calcimedin	10	98 $\pm$ 5	104 $\pm$ 3	96 $\pm$ 7	103 $\pm$ 2	106 $\pm$ 3
l-Calcimedin	2	100 $\pm$ 2	102 $\pm$ 1	98 $\pm$ 1	98 $\pm$ 6	100 $\pm$ 1
Calmodulin	6	96 $\pm$ 3	100 $\pm$ 1	102 $\pm$ 4	5 $\pm$ 1	102 $\pm$ 3
SMCaBP 11	1	61 $\pm$ 5	99 $\pm$ 2	99 $\pm$ 5	96 $\pm$ 6	100 $\pm$ 1
S100	5	98 $\pm$ 1	101 $\pm$ 1	99 $\pm$ 3	100 $\pm$ 5	100 $\pm$ 1

The histamine release over a 10 min period was expressed as a percentage of the amount of the histamine secretion by each stimulator.

interact with calmodulin in a Ca^{2+} -dependent manner,¹⁹⁾ the inhibition seems to be due to the direct interaction between compound 48/80 and calmodulin.

The inhibition of Ca^{2+} -dependent histamine release from β -escin-permeabilized mast cells with varying concentrations of SMCaBP 11 is illustrated in Fig. 2. The histamine release in the presence of SMCaBP 11 over 50 nM was decreased to about 60% of the original level in the absence of SMCaBP 11. The concentration of SMCaBP 11 required to obtain 50% inhibition was about 20 nM in the medium containing 2×10^4 mast cells. The number of mast cells remained almost unchanged during the procedure. Similar inhibitory action of SMCaBP 11 was also observed when digitonin was used instead of β -escin (data not shown).

Microfilaments and microtubules have been shown to

Fig. 2. Effects of SMCaBP 11 on Histamine Release from Permeabilized Mast Cells Induced by Ca^{2+}

Mast cells were preincubated with various concentrations of SMCaBP 11 as indicated for 15 min. The cells were permeabilized with 15 $\mu\text{g}/\text{ml}$ β -escin and then challenged with 100 μM CaCl_2 . Values are expressed as means $\pm$ S.D. for four determinations.

play important roles in the exocytosis of mast cells and calcium regulates the organization of cytoskeletal elements.^{2,3,6,20,21)} Using monoclonal antibodies against smooth muscle β -actin and α -tubulin, we examined the contents of actin and tubulin released from mast cells after they were treated with β -escin, Ca^{2+} and/or SMCaBP 11 (Fig. 3). When mast cells were treated with β -escin, a small amount of actin was released in the medium and the released amount was significantly increased with the additional exposure of Ca^{2+} . In contrast, tubulin was not released in the medium regardless of Ca^{2+} concentration. When SMCaBP 11 was preincubated with mast cells prior to the treatment of β -escin and Ca^{2+} , the leakage of actin was suppressed in a concentration-dependent manner, while the tubulin content was unchanged. These results suggest that SMCaBP 11 is associated with Ca^{2+} - and actin-dependent histamine release from the permeabilized mast cells. Koffer *et al.* showed that microfilaments are present in the cortical region of mast cells.³⁾ This was confirmed by our experiments (Fig. 4A). No significant change could be detected after β -escin treatment (Fig. 4B). The phalloidin-staining in Ca^{2+} -stimulated permeabilized cells preincubated with and without SMCaBP 11 exhibited a similar intense fluorescence in the periphery of the cells (Figs. 4C and 4D), though a considerable amount of actin was liberated in the medium (Fig. 3). This may be due to the rapid exchange between G- and F-actin, and also to the possibility that SMCaBP 11 may affect the state of actin filaments.

DISCUSSION

It is generally considered that calcium ions can initiate and modulate the processes of exocytosis and contractile response delivered from cytoskeletal elements, and Ca^{2+} -binding proteins act as transducers of an intracellular Ca^{2+} signal.^{1-3,21-23)} One of these proteins,

Fig. 3. Analysis of Actin and Tubulin of Mast Cells after Permeabilization by SDS-PAGE and Immunoblotting

Mast cells were incubated with SMCaBP 11, as described in Materials and Methods, and then stimulated with 0.1 mM CaCl_2 for 10 min at 37°C. At the end of the incubation, permeabilized and non-permeabilized mast cells (0.2 ml) were collected by centrifugation at $1000 \times g$ for 5 min and mixed with sample buffer (31 mM Tris-HCl, 1% SDS, 5% glycerol, 0.15% β -mercaptoethanol and 0.0005% pyronin Y, pH 6.8), then immediately heated to 95°C for 2 min. The supernatants (Sup) and pellets (Ppt) were analyzed by 12.5% SDS-PAGE and stained with Coomassie brilliant blue (A). After electrophoretic transfer to nitrocellulose, immunoblots were done using monoclonal anti- β -actin (B) and anti- α -tubulin (C) antibodies. a, Non-permeabilized mast cells; b, β -escin-permeabilized cells; c, β -escin-permeabilized cells stimulated by Ca^{2+} ; d, c + 20 nM SMCaBP 11; e, c + 48 nM SMCaBP 11.

Fig. 4. Localization of F-Actin in Mast Cells Treated with β -Escin, Ca^{2+} and SMCaBP 11

Rat mast cells from different experimental conditions were fixed and stained with rhodamine-phalloidin. A, non-permeabilized cells; B, permeabilized cells; C, permeabilized cells stimulated with Ca^{2+} ; D, Ca^{2+} -stimulated permeabilized cells preincubated with 48 nM SMCaBP 11. Bar, 20 μm .

having an apparent molecular weight of 11000 (SMCaBP 11) showed an inhibitory effect on Ca^{2+} -induced histamine release from permeabilized mast cells. When SMCaBP 11 was mixed with non-permeabilized mast cells, no influence on the histamine release induced by Ca^{2+} and ionophore A23187 was observed. SMCaBP 11 appears to function as a receptor of Ca^{2+} after incorporation into mast cells. The partial amino acid sequence of SMCaBP 11 has shown that this protein may be identical to calyculin, which belongs to the family of EF-hand type Ca^{2+} -binding proteins including calmodulin and troponin C²⁴) and has a high degree of sequence similarity to S100 α and β .^{25,26}) However, S100 had only a slight effect on histamine release in permeabilized and non-permeabilized mast cells (Table I). Using affinity chromatography and two-dimensional polyacrylamide gel electrophoresis, Filipek *et al.* have reported that calyculin can bind to annexin II and glyceraldehyde-3-phosphate dehydrogenase in a Ca^{2+} -dependent manner.²⁷) Several proteins from the annexin family could interact with F-actin only in the presence of Ca^{2+} , but their influence on the architecture of microfilaments is still unknown.^{28,29})

The release of histamine induced by Ca^{2+} was incompletely coupled with actin secretion, because histamine was still released in the permeabilized mast cells under conditions in which actin release was suppressed by SMCaBP 11 (Fig. 3). Morphological examination revealed that no significant change in the distribution of F-actin was found in Ca^{2+} -stimulated mast cells with or without SMCaBP 11, indicating that SMCaBP 11 may affect the

G-F state of actin. In addition, SMCaBP 11 did not co-sediment with skeletal muscle F-actin (data not shown). Recently, Koffer *et al.* suggested the presence of an actin severing- or depolymerizing-factor in rat mast cells.³) Thus, SMCaBP 11 seems to exert an influence on actin-regulatory proteins in mast cells and on the degranulation associated with microfilaments. None of the Ca^{2+} -binding proteins, other than calmodulin, have been detected in mast cells. The exogenous guanosine nucleotide analogue, guanosine 5'-[γ -thio]triphosphate (GTP[S]), has been reported to increase histamine release from permeabilized mast cells at μM levels of Ca^{2+} .^{4,30,31}) We have confirmed this increase and observed a similar inhibitory action of SMCaBP 11 between the presence and absence of GTP[S] (data not shown), indicating that the effects of SMCaBP 11 are not dependent on exogenous G-protein. It is generally believed that many kinds of CaBPs exist and participate in a variety of cellular functions. The inhibitory effects suggest that SMCaBP 11-like protein is a candidate as a critical receptor of Ca^{2+} in the secretory process of mast cells.

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