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A mastoparan analog without lytic effects and its stimulatory mechanisms in mast cells

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Abstract

Mastoparan, a tetradecapeptide isolated from wasp venom, is known to not only induce the secretion of histamine but also cause cell lysis in rat peritoneal mast cells. This lytic effect makes investigations concerning MP-induced signaling mechanisms difficult. Here, we report that a mastoparan derivative peptide, [Lys^{10}, Leu^{13}]mastoparan, also designated "mas 11", induces exocytosis with greater activity than mastoparan without the undesired lytic effect. The signaling mechanisms triggered by mas 11 were also investigated, and it was clearly demonstrated that mas 11 induced not only the non-lytic release of β-hexosaminidase but also an increase in the concentration of cytosolic free Ca^{2+} in the cells and these effects were mostly prevented by pertussis toxin, suggesting the involvement of G_i-type G protein in the signaling. Mas 11 is a promising stimulatory molecule with which to investigate the exocytotic mechanisms induced by not only mastoparan but also various amphiphilic peptides in the cells.

Keywords: Mastoparan; Mas 11; Exocytosis; Histamine release; Peritoneal mast cells; β-Hexosaminidase; Lactate dehydrogenase; Increase in the concentration of cytosolic free Ca^{2+}; G protein

Mastoparan (MP) is a toxic tetradecapeptide isolated from wasp venom as a histamine releaser in rat peritoneal mast cells [1]. MP has various biological activities; acting to stimulate the secretion of insulin from pancreatic β-cells [2], catecholamine from chromaffin cells [3,4], serotonin from platelets [5], and surfactant from pulmonary alveolar type 2 epithelial cells [6], to increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neutrophils [7], and to trigger proliferation in Swiss 3T3 cells [8]. MP also activates G_i-type and G_o-type GTP-binding regulatory proteins (G proteins) directly in reconstituted phospholipid vesicles [9–11], and is expected to be a useful stimulant for investigating the cellular signaling mechanisms in various cells, especially those whose endogenous stimulators are still to be identified. Rat peritoneal mast cells are known to secrete histamine when stimulated by not only IgE with multivalent antigens but also various amphiphilic compounds including MP [12–14], although it is controversial whether the stimulation of amphiphilic compounds has physiological and/or pathophysiological significance, and it is still not identified endogenous amphiphilic stimulators which act on the cells physiologically. We, therefore, tried to investigate the signaling mechanisms triggered by amphiphilic peptides in rat peritoneal mast cells utilizing MP. MP, however, not only induced exocytotic secretion at relatively low concentrations but caused lysis of the cells at higher concentrations, and it was impossible to clearly distinguish the cellular exocytotic events from the lytic effect. To overcome these difficulties, we sought MP-related peptides inducing exocytosis without undesired toxic effects. Here, we describe a MP analog peptide, [Lys^{10}, Leu^{13}]MP, also designated "mas 11" [10], as a promising stimulator of the cells,

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and its stimulatory mechanism which induces exocytosis and an increase in $[Ca^{2+}]_i$ but dose not cause cell lysis.

Materials and methods

Materials. Compound 48/80, lithium lactate, and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were purchased from Sigma (St. Louis, MO). NAD was obtained from Tokyo Kasei (Tokyo, Japan) and EGTA and Fura-2/acetoxymethyl ester (fura-2/AM) were from Dojin (Kumamoto, Japan). Percoll was obtained from GE Healthcare Life Science (Piscataway, NJ). Lipid-poor bovine serum albumin (BSA) and pertussis toxin (PTX) were from Calbiochem (La Jolla, CA, USA) and List Biological (Campbell, CA, USA), respectively.

MP and mas 11 were synthesized and purified as described previously [15,16]. The purity of peptides was proven to be over 98% by reverse-phase high-performance liquid chromatography on a column of C18 (5 μm, 4.5 × 150 mm, Wako Chemicals, Osaka, Japan). Amino acid analysis of the peptides confirmed the compositions. The identity of the peptides was monitored by fast atom bombardment mass spectrometry. Each synthetic peptide was dissolved in phosphate-buffered saline.

MP: Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2
Mas 11: Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Lys-Lys-Lys-Leu-Leu-NH_2

Animals. Male Wistar rats (400–600 g) were purchased from the Institute of Animal Reproduction (Ibaraki, Japan) and housed three per cage. The animals were maintained in a temperature (22–23 °C) and light-controlled environment on a 12-h light/dark cycle with free access to food and water. They were fasted from 12 h before experiments and sacrificed under anesthesia with ether. Experimental procedures in this study were approved by the Animal Care Committee of the University of Tsukuba.

Preparation of rat peritoneal mast cells. Rat peritoneal mast cells were collected and purified as described [17–19]. Mixed cells were collected from the peritoneal cavity in Hepes-buffered salt solution (HBS) (135 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.0 mM $CaCl_2$, 0.6 mM $MgSO_4$, 3.0 mM $NaHCO_3$, 5.6 mM glucose, 5.0 mM Hepes, 0.1% BSA, and 0.1% gelatin, pH 7.4) containing 10 U/ml of heparin. Mast cells were purified from these mixed cells using a Percoll discontinuous gradient as described [18]. The purity of the cells was over 95% according to toluidine blue staining, and the viability of the cells was about 98% according to Trypane blue exclusion. Purified mast cells were then washed three times with HBS and kept on ice prior to use. Some of the cells were incubated with or without 100 ng/ml of PTX at 37 °C for 2 h under bubbling with 95%O_2–5%CO_2, washed three times with HBS, then kept on ice until used.

Measurement of exocytotic secretion. The exocytotic secretion from rat peritoneal mast cells was assessed based on the amount of β-hexosaminidase (β-HA) released [17,18,20]. Mast cells (5×10^4 cells) in 90 μl of HBS (without $CaCl_2$ and with 0.1 mM EGTA to remove extracellular Ca^{2+} in some experiments) were stimulated with 10 μl of the stimulator solution for 10 min at 37 °C, following the preincubation of cells for 10 min at 37 °C. The stimulation was then terminated by the addition of 200 μl of ice-cold stop buffer I (25 mM Tris, 123 mM NaCl, and 2.7 mM KCl, pH 7.4). The released β-HA was separated from the cells by brief centrifugation at 4 °C, and 90 μl of cell-free supernatant was incubated in a 96-well plate with 60 μl of substrate solution for the β-HA reaction (10 mM of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 40 mM citric acid and 70 mM Na_2HPO_4, pH 4.5) at 37 °C for 2 h. Then, the enzyme reaction was terminated by adding 100 μl of 0.4 M glycine (pH 10.7), and absorbance at 415 nm was measured on a MTP-22 microplate reader (Corona Electric, Hitachi, Japan). The secretion of β-HA was estimated as a percentage of the amount of enzyme in the positive control, which was stimulated with 10 μg/ml of compound 48/80 [18] for 10 min at 37 °C in the presence of extracellular free Ca^{2+}. The chemicals investigated had no effect upon enzyme reactions.

Estimation of cell damage. The damage to rat peritoneal mast cells was assessed by measuring the leakage of lactate dehydrogenase (LDH) from the cells as described previously [18]. In brief, the cell-free supernatant (100 μl) obtained in the stimulation experiments described above was incubated with 400 μl of substrate solution II (125 mM 2-amino-2-metyl-1-propanol, 6.25 mM NAD, and 125 mM lithium lactate, pH 9.5) for 2.5 h at 37 °C in a glass tube, and the absorbance of each tube at 340 nm was then quickly measured using a UV/vis spectrophotometer (type 460, Japan Spectroscopy Inc., Tokyo, Japan). The leaked LDH was expressed as a percentage of the total cellular amount of LDH determined after solubllilizing the cells with 0.05 % Triton X-100.

Measurement of the change in the concentration of cytosolic free Ca^{2+}. The change in $[Ca^{2+}]_i$ in rat peritoneal mast cells induced by MP or mas 11 was measured using fura-2, a sensitive luminescent calcium chelator, as described previously [21,22] with modifications. Purified rat peritoneal mast cells were incubated with 4 μM of fura-2/AM for 1 h at 20 °C, and washed twice with HBS. The fura-2-loaded cells were resuspended at 1×10^6 cells/ml in HBS and placed in HBS at 20 °C prior to use. Then, 1 ml of the cell suspension (10^6 cells) was transferred to a cuvette, and stimulated by MP or mas 11 at 37 °C following the preincubation of cells at 37 °C for 2–3 min under continuous stirring. In some experiments, EGTA was added to the cell suspension before stimulation at a final concentration of 4 mM to chelate extracellular Ca^{2+}. The fluorescence of fura-2-loaded cells was recorded using a CAF-100 spectrofluorometer (Japan Spectroscopy Inc., Tokyo, Japan) with excitation at a wavelength of 340 and 380 nm, and emission at 500 nm, and then the $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. [23].

Statistical analysis. Each value in the text and figures is expressed as a mean ± SE. The values were compared using Student's *t*-test to determine statistical significance, and differences were considered significant at $p < 0.05$.

Results and discussion

Although MP was purified and identified from wasp venom as a histamine-releasing peptide in rat peritoneal mast cells [1] and is known to activate purified G_i- or G_o-types of G proteins by directly binding with them [9,10,24,25], little is known of the stimulatory or cellular signaling mechanisms by which it induces exocytosis. Therefore, we first investigated the mechanisms by which MP stimulates exocytosis in rat peritoneal mast cells.

The release of β-HA and LDH caused by MP in the presence or absence of extracellular free Ca^{2+}

To assess the stimulation of exocytosis by peptides in rat peritoneal mast cells, we measured the release of β-HA because this enzyme is found in histamine-containing secretory vesicles and is similarly secreted upon stimulation by not only IgE but also various amphiphilic compounds [17,18,20]. In addition, the secretion of β-HA is easier to quantify than the release of histamine.

We first investigated the time course of the secretion of β-HA stimulated by MP in the presence or absence of extracellular free Ca^{2+}. In the presence of extracellular free Ca^{2+}, the release of β-HA stimulated by 10 μM of MP was initiated within 3 s, and was completed in 5 min. In the absence of extracellular free Ca^{2+}, again the secretion was induced by 10 μM MP, but a slower time course was observed and the release took 10 min; the $t_{1/2}$ values of the secretion were less than 5 s in the presence of extracellular free Ca^{2+} and about 30 s

in its absence. Hence, we stimulated the cells with the peptides for 10 min when investigating the secretory mechanisms.

We then examined the concentration-dependency of MP to the secretion of β-HA. As shown in Fig. 1A, MP caused the secretion in a concentration-dependent manner with or without extracellular free Ca^{2+}, but the amounts of β-HA secreted were significantly higher in the absence of extracellular free Ca^{2+}. These effects, however, may be caused by the lysis of mast cells because amphiphilic peptides such as MP cause cell-lysis by perturbing plasma membranes. Thus, the leakage of LDH, which is a cytosolic enzyme and is not released by exocytosis, from the cells was measured for quantification of the lytic effect [18]. MP significantly caused the leakage of LDH at concentrations above 20 μM and these effects were more evident in the absence of extracellular free Ca^{2+} (Fig. 1B), suggesting that the difference in activity to secrete β-HA in the presence versus the absence of extracellular free Ca^{2+} may come from the difference in the lytic effects. These findings demonstrated that the exocytotic events induced by MP are indistinguishable from the peptide's lytic effect.

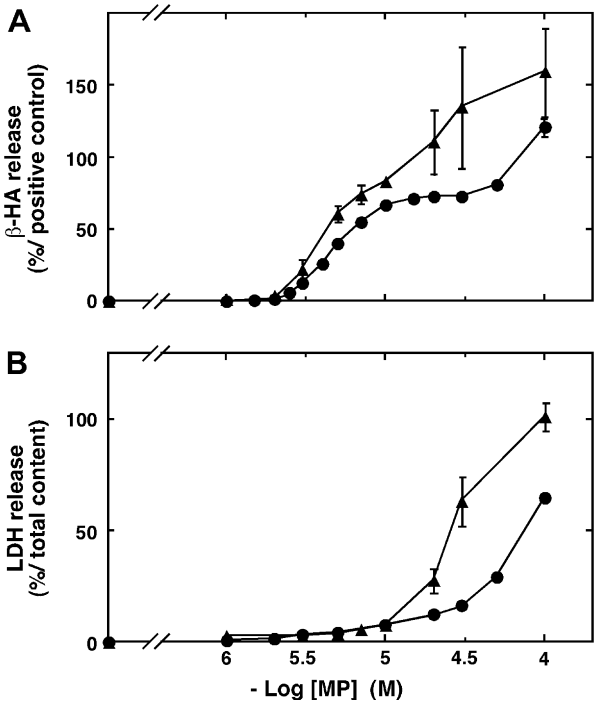

Fig. 1. Effects of MP on the release of β-HA (A) and LDH (B) in rat peritoneal mast cells. The mast cells were stimulated with various concentrations of MP in the presence (closed circles) or absence (closed triangles) of extracellular free Ca^{2+} for 10 min at 37 °C, and the amounts of β-HA (A) and LDH (B) in the cell-free supernatant were determined as described in Materials and methods. Each point represents the mean ± SE of separate experiments ($n = 6$). Abscissa; −log of the molar MP concentration. Ordinate; percentage of β-HA released by 10 μg/ml of compound 48/80 (A) or percentage of the total amount of LDH, which was determined after cell lysis by 0.05 % Triton X-100 (B).

A MP-related peptide which induces exocytosis without cell lysis

Since MP has short-comings as a mast cell stimulator, we sought MP-related peptides that induce exocytosis without undesired toxic effects by studying structure–activity relationships. Most of the MP-related peptides caused a loss of LDH from the cells. [Ala^{12}, Leu^{13}]MP, designated mas 7, was the most potent derivative to activate G_i or G_o proteins [10], however, it caused the release of not only β-HA but also LDH at the same concentration ranges, and was not observed to stimulate exocytosis without cell lysis. [Lys^{10}, Leu^{13}]MP, which was designated "mas 11" previously [10], induced the secretion of β-HA without cell lysis in a concentration-dependent manner. Namely, mas 11 induced the secretion of β-HA from the cells regardless of the presence of extracellular free Ca^{2+} but the effect was more potent in the absence of extracellular free Ca^{2+} at concentrations below 3 μM but weaker at concentrations above 10 μM (Fig. 2). The overall activity of mas 11 to induce the release of β-HA in the presence of extracellular free Ca^{2+} is considerably greater than that of MP (EC_{50} values: 3.1 ± 0.3 μM for mas 11 vs. 7.1 ± 0.2 μM for MP). A significant increase in the LDH concentration in the cell-free supernatant was not detected at up to 100 μM (Fig. 2).

The increase in $[Ca^{2+}]_i$ induced by MP or mas 11 in the presence or absence of extracellular free Ca^{2+}

Since mas 11 is expected to be a useful stimulator for rat peritoneal mast cells, we investigated its effects as well as those of MP on the increase in $[Ca^{2+}]_i$ in the presence or absence of extracellular free Ca^{2+}. As shown in Fig. 3.

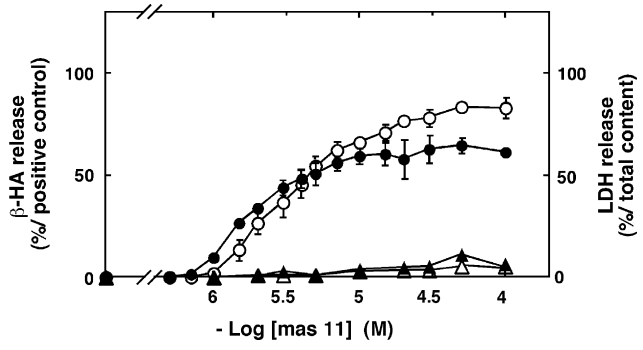

Fig. 2. Effects of [Lys^{10}, Leu^{13}]MP (mas 11) on the secretion of β-HA and LDH in rat peritoneal mast cells. The mast cells were stimulated with various concentrations of mas 11 in the presence (open symbols) or absence (closed symbols) of extracellular free Ca^{2+} for 10 min at 37 °C, and the amounts of β-HA (circles) and LDH (triangles) in the cell-free supernatant were determined as described in Materials and Methods. Each point represents the mean ± SE of separate experiments ($n = 8$). Abscissa; −log of the molar mas 11 concentration. Ordinate; percentage of β-HA released by 10 μg/ml of compound 48/80 or percentage of the total amount of LDH, which was determined after cell lysis by 0.05 % Triton X-100.

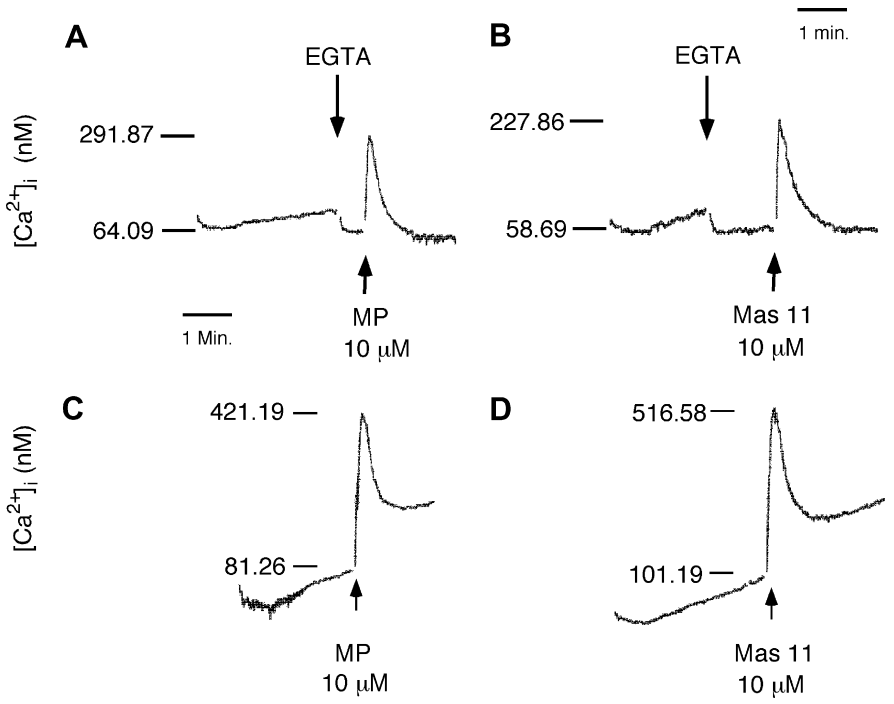

Fig. 3. Effects of MP (A,C) and [Lys[10], Leu[13]]MP (mas 11) (B,D) on the increase in $[Ca^{2+}]_i$ in the absence (A,B) or presence (C,D) of extracellular free Ca^{2+}. In panels A and B, 0.9 mM of extracellular free Ca^{2+} was chelated with 4 mM EGTA, and then the cells were stimulated with MP or mas 11. Each trace is a typical representation of six independent experiments.

Ten micromolar of MP and mas 11 caused an immediate but transient increase even in the absence of extracellular free Ca^{2+} chelated with 4 mM EGTA, though the increase was potentiated by the presence of free Ca^{2+}. These findings suggest that the increase in $[Ca^{2+}]_i$ is due to both the mobilization of intracellular Ca^{2+} stores and the influx of extracellular Ca^{2+}.

Exocytotic mechanisms induced by mas 11

Rat peritoneal mast cells secrete histamine in response to not only IgE with multivalent antigens but also various amphiphilic basic compounds such as MP, compound 48/80, and substance P, and G_i proteins have been indicated to be involved in the latter stimulation but not the former [12–14,24–31]. The physiological role and the mechanism of action of such basic amphiphilic compounds, however, remain unclear. PTX is known to specifically ADP-ribosylate G_i or G_o proteins and render them insensitive to regulation by receptors or G protein activators such as MP [9,28]. Therefore, to clarify whether mas 11, instead of MP, is a promising stimulator of rat peritoneal mast cells to induce exocytosis involving G proteins, we investigated the inhibitory effect of PTX on the exocytotic secretion and the $[Ca^{2+}]_i$ increase stimulated by mas 11. The effect of 10 μM of mas 11 on the release of β-HA was reduced about 85% by pretreatment of the cells with 100 ng/ml of PTX for 2 h, and the maximum $[Ca^{2+}]_i$ increase caused by 10 μM was also inhibited about 70% by PTX (Fig. 4). These findings indicate that both an immediate and transient increase in $[Ca^{2+}]_i$ and exocytosis induced by mas 11 are regulated by PTX-sensitive G_i proteins. Namely, the profiles of mas 11 and MP to induce the functions of mast cells were similar, and therefore [Lys[10], Leu[13]]MP, mas 11, is a promising stimulator of rat peritoneal mast cells to induce exocytosis without undesired effects. We are now not only investigating the cellular signaling mechanisms for exocytosis in mast cells utilizing mas 11, but also identifying the endogenous amphiphilic peptides that act on the cells by utilizing a recently developed methodology

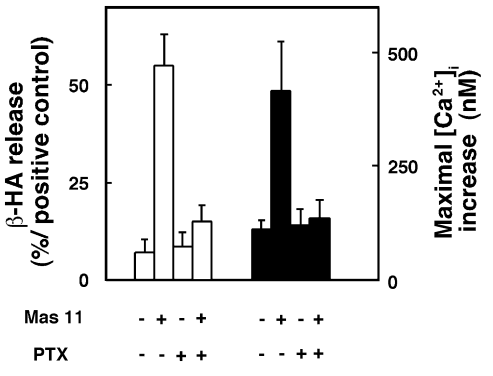

Fig. 4. Inhibitory effects of pretreatment of the cells with PTX on [Lys[10], Leu[13]]MP (mas 11)-induced secretion of β-HA (open columns) or the maximum transient increase in $[Ca^{2+}]_i$ (closed columns). The mast cells were cultured with (+) or without (−) 100 ng/ml of PTX for 2 h at 37 °C under humidified conditions with the bubbling of 95% O_2–5% CO_2, then stimulated with mas 11. Each value represents the mean ± SE of seperate experiments ($n = 6$).

for the bioinfomatic characterization of functional peptides [32].

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