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# Cryptide signaling: Amphiphilic peptide-induced exocytotic mechanisms in mast cells

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ABSTRACT

Amphiphilic peptides with positive charges such as substance P (SP) and mastoparan (MP) are known to induce exocytosis in rat peritoneal mast cells. To elucidate whether and how intracellular $Ca^{2+}$ signaling is involved in the peptide-induced exocytosis, here we investigated the relationships between an increase in intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and exocytosis caused by SP and MP. SP and MP induced exocytosis coinciding with an initial rapid and transient $[Ca^{2+}]_i$ increase, but not with a sustained increase. These stimulations were abolished by pertussis toxin, indicating the involvement of the $G_i$-family of G proteins in the peptide signaling. Moreover, the $[Ca^{2+}]_i$ increase was shown to accelerate and potentiate exocytosis, suggesting that the transient increase in $[Ca^{2+}]_i$ positively modified exocytotic secretion. However, it was indicated that the signal of $[Ca^{2+}]_i$ increase was not sufficient for the peptide-induced exocytosis, suggesting the participation of alternative mechanisms other than $Ca^{2+}$ signaling in the pathway.



Rat peritoneal mast cells are known to secrete histamine in response to not only IgE antibodies with multivalent antigens but also amphiphilic basic compounds [1–5]. Recently, the IgE-induced signaling mechanism in mast cells has been extensively investigated owing to its importance in the understanding of various allergic symptoms such as pollinosis rhinitis and anaphylaxis after food intake. The involvements of many signal molecules including Fc receptors and Fyn, Syk, and Lyn proteins as well as intracellular free $Ca^{2+}$ in signaling, and the regulation of signaling by various cytokines have been elucidated so far [2].

In the case of stimulation by basic compounds, many amphiphilic compounds including substance P (SP), mastoparan (MP), and bradykinin as well as compound 48/80 have been reported to induce histamine release from mast cells [1,3–6]. The presence of the $G_i$-family of G proteins ($G_i$ protein) in the cells has been demonstrated and the involvement of these proteins in basic compound-induced histamine release has been suggested [6–9]. The receptors of such amphiphilic basic compounds in the cells are not likely to be the usual G protein coupled receptors, but are proposed to be a $G_i$ protein since these compounds can directly regulate the $G_i$ protein in *in vitro* [10–15]. The signaling mechanisms induced by these compounds have also been investigated and the involvement of $Ca^{2+}$ signaling in the exocytotic pathway was proposed [6,11,15,16]. However, "the whole picture" of the regulatory mechanisms as controlled by amphiphilic compounds with positive charges remains unclear. In this regard, there seems to be a variety of mast cell-activating amphiphilic cryptides presumably derived from various cytosolic proteins [17,18]; nevertheless, it has remained unclear how these cryptides are concertedly involved in physiological and/or pathophysiological regulatory mechanisms. Although signaling mechanisms for inducing exocytosis in rat peritoneal mast cells have been biochemically and electrophysiologically investigated [6,11,15,16,19–22], the roles of $Ca^{2+}$ signaling in the exocytotic pathway induced by such amphiphilic peptides are still uncertain due to the difficulty in measuring changes in the concentration of intracellular free $Ca^{2+}$ ($[Ca^{2+}]_i$) in intact rat peritoneal mast cells.

Recently, we established a method for reproducibly measuring the increase in $[Ca^{2+}]_i$ in intact mast cells from rat peritoneum [22,23]. Here, we therefore investigated the relationships between $[Ca^{2+}]_i$ increase and exocytosis caused by SP and MP to elucidate

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the involvement of intracellular $Ca^{2+}$ signaling for the peptide-induced exocytosis.

## Materials and methods

*Peptides.* SP, neurokinin A (NKA), neurokinin B (NKB), and MP were synthesized by the conventional solid-phase method as described previously [24,25]. The purity of peptides given below was proven to be more than 98% by reverse-phase high-performance liquid chromatography on a C18 column (5 μm; 4.5 × 150 mm; Wako Chemicals, Osaka, Japan). The identity of the synthetic peptides was confirmed by amino acid analysis of the peptides after acid hydrolysis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Each synthetic peptide was dissolved in phosphate buffered saline.

- SP: H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$
- NKA: H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-$NH_2$
- NKB: H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-$NH_2$
- MP: H-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-$NH_2$

*Exocytosis assay.* Rat peritoneal mast cells were collected and purified as described previously [22,23]. In brief, Wister rats (400–500 g) were sacrificed under ether anesthesia, and mixed cells from the peritoneal cavity were collected 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% bovine serum albumin (BSA) and 0.1% gelatin (pH 7.4)) containing 10 U/ml heparin. Mixed cells were purified using discontinuous gradient of Percoll(GE Healthcare Life Science, Piscataway, NJ)-HBS, and then the cells were washed 3 times with HBS and stored on ice until use. Some cells were incubated with or without 100 ng/ml pertussis toxin (PTX; List Biological, Campbell, CA) for 2 h at 37 °C, washed 3 times with HBS, and then stored on ice until use. About $1 \times 10^6$ cells per rat were obtained at more than 95% purity (as determined by toluidine blue staining) and 98% viability (as assessed by trypan blue exclusion). Exocytotic secretion from cells was assessed based on β-hexisaminidase (β-HA) secretion [23]. Cells ($5 \times 10^4$ cells) in 90 μl of HBS (without $CaCl_2$ and with 0.1 mM EGTA to remove extracellular $Ca^{2+}$ in some experiments) were preincubated for 10 min (or otherwise indicated times (Fig. 3)) at 37 °C. β-HA secretion was induced by the addition of 10 μl of peptide solution, and then the cell suspension was incubated for the indicated times at 37 °C. Stimulation was terminated by the addition of 200 μl of ice-cold buffer I (25 mM Tris, 123 mM NaCl, and 2.7 mM KCl (pH 7.4)). The released enzyme was separated from the cells by brief centrifugation at 4 °C. Cell-free supernatant (90 μl) was incubated at 37 °C for 2 h with 60 μl of the substrate solution (10 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma, St. Louis, MO) in 40 mM citric acid and 70 mM $Na_2HPO_4$ (pH 4.5)) in the wells of 96-well immunoplates, and the enzyme reaction was terminated by adding 100 μl of 0.4 M glycine (pH 10.7). Then, absorbance at 415 nm was measured on a microplate reader. β-HA secretion was expressed as a percentage of the total contents of the enzyme estimated after a 10-min incubation period with 0.05% Triton X-100 at 37 °C (% of total content), or as a percentage of the positive control estimated after stimulation with compound 48/80 (3 μg/ml), a chemical stimulator of mast cells, at 37 °C for 10 min (% of positive control).

*Measurement of $[Ca^{2+}]_i$.* Changes in $[Ca^{2+}]_i$ were measured using fura-2, a sensitive luminescent calcium chelator described previously [26,27] with modifications [22]. Purified mast cells were washed twice with HBS, and incubated with 4 μM fura-2/acetoxymethyl ester (fura-2/AM; Dojin, Kumamoto, Japan) for 1 h at 20 °C to incorporate fura-2 into cells. After washing the cells twice, they were placed in HBS at 20 °C until use. About $1 \times 10^6$ fura-2-loaded cells/ml (1 ml) were transferred to a cuvette and stirred continuously. Then, the cells were stimulated by adding the peptides at 37 °C following preincubation at 37 °C for 2–3 min. In some experiments, 4 mM EGTA (final concentration) was added to the cell suspension 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 wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. [28].

## Results

*Effects of neurokinin peptides and MP on rat peritoneal mast cells*

We initially examined the effects of not only SP but also other neurokinin peptides, namely, NKA and NKB, in addition to MP on the stimulation of β-HA release and the increase in $[Ca^{2+}]_i$ in rat peritoneal mast cells. In the presence of extracellular free $Ca^{2+}$ (0.9 mM), SP and MP, but not NKA and NKB, promoted significant β-HA release at concentrations above 5 μM and 3 μM, respectively, in a concentration-dependent manner (Fig. 1A). Moreover, SP and MP, but not NKA and NKB, also caused concentration-dependent increases in $[Ca^{2+}]_i$ above 5 μM and 2 μM, respectively (Fig. 1B), with an immediate and transient increase in $[Ca^{2+}]_i$ (Fig. 1B-inset).

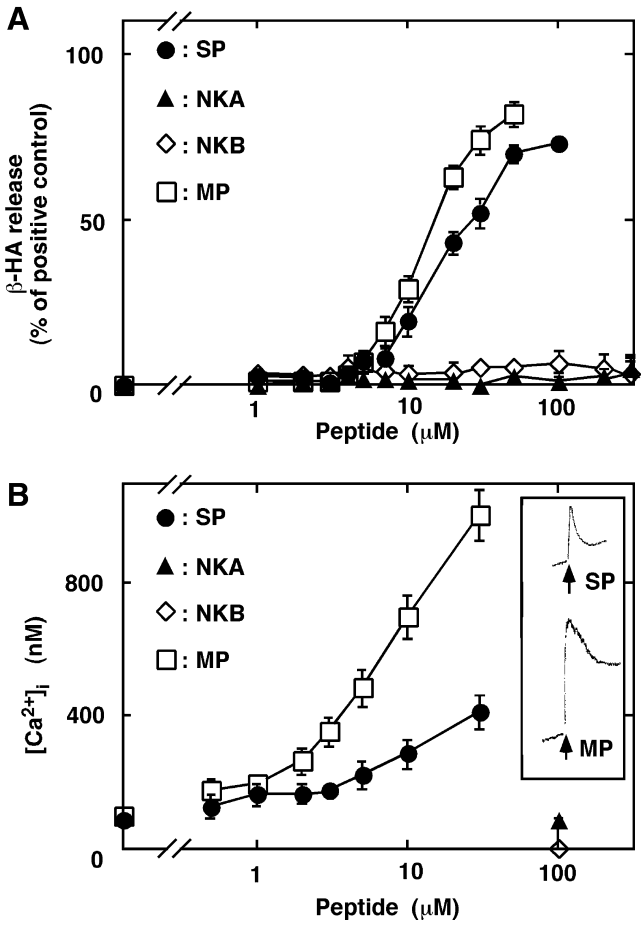


**Fig. 1.** Effects of SP (closed circles), NKA (closed triangles), NKB (open diamonds) and MP (open squares) on β-HA secretion (A) or increase in $[Ca^{2+}]_i$ (B). Each point represents the mean ± SE of 10–16 (A) or 3–5 (B) separate experiments. Traces of $[Ca^{2+}]_i$ changes caused by the addition of 30 μM SP and MP are shown in the inset of (B).

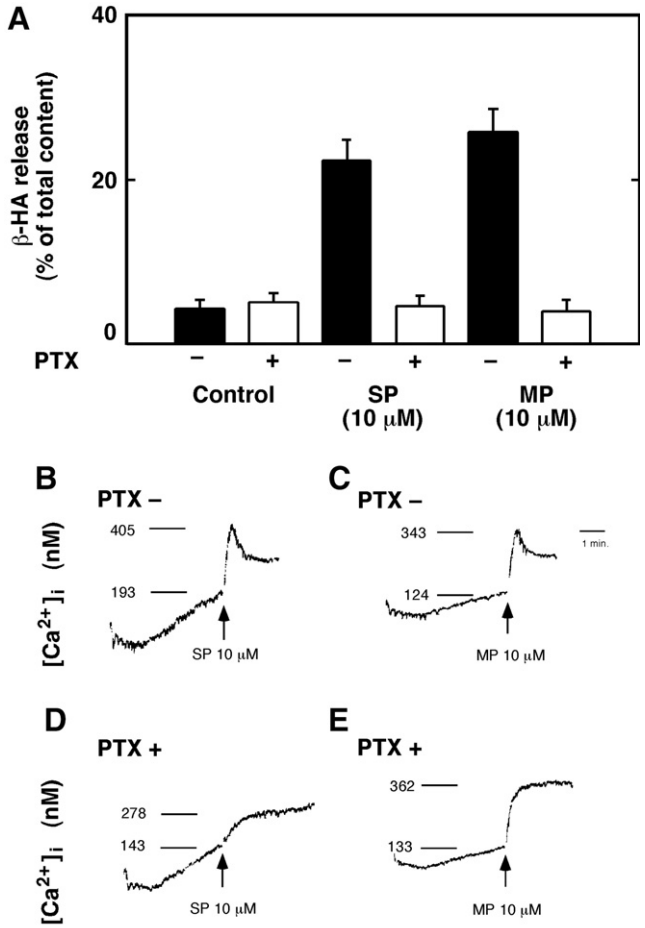


**Fig. 2.** Inhibitory effects of PTX on β-HA release (A) or $[Ca^{2+}]_i$ increase (B–E) stimulated by 10 μM of SP or MP. Purified mast cells were preincubated without (closed columns in panel A and traces in panels B and C) or with 100 ng /ml PTX for 2 h (open columns in (A) and traces in panels (D,E)) before the stimulation. (A) Each column represents the mean ± SE of four independent experiments. (B–E) Arrowheads indicate the addition of peptides. These results are typical of four individual experiments.

In addition to this transient increase, MP induced a slower and sustained increase; nevertheless, this increase was not evident in the case of the stimulation by SP (Fig. 1B-inset). These results demonstrate that SP and MP induced the increase in $[Ca^{2+}]_i$ and β-HA release at similar concentrations.

### *Involvement of $G_i$-family of G proteins in $[Ca^{2+}]_i$ increase and exocytosis*

PTX is known to specifically ADP-ribosylate the $G_i$- or $G_o$-family of G proteins and render them insensitive to stimulation [6,29]. As shown in Fig. 2A, stimulation of β-HA secretion by SP and MP at 10 μM was completely inhibited by the pretreatment of cells with 100 ng of PTX for 2 h. In the case of the induction of $[Ca^{2+}]_i$ increase by SP and MP, the immediate and transient increase in $[Ca^{2+}]_i$ was prevented by the pretreatment of cells, but not the sustained increase, which was more evident in the stimulation by MP (Fig. 2B–E; panels B and D, in the stimulation by SP; panels C and E, in the stimulation by MP; panels B and C, in the control cells; panels D and E, in the PTX-treated cells). These results indicate that not only exocytosis but also the immediate and transient increases in $[Ca^{2+}]_i$ induced by SP and MP, but not the slower and sustained $[Ca^{2+}]_i$ increase, are under the regulation of $G_i$ or $G_o$ proteins.

### *Involvement of $Ca^{2+}$ signaling in exocytotic pathway*

Figs. 1 and 2 show the correlation between $[Ca^{2+}]_i$ increase and β-HA secretion stimulated by SP and MP in the concentration range of stimulation, as well as the regulation of the secretion and the $[Ca^{2+}]_i$ increase by PTX-sensitive G proteins. The β-HA release induced by MP, however, is shown to be a consequence of not only exocytosis but also cell lysis, and it is impossible to discriminate between exocytotic secretion and β-HA leakage by cell lysis [22]. In contrast, SP caused no leakage of the cytosolic enzyme lactate dehydrogenase even at 100 μM (data not shown), indicating that the enzyme release caused by SP results from exocytotic secretion. Therefore, the involvement of $Ca^{2+}$ signaling in the exocytotic mechanisms induced by SP was further investigated.

First, the $[Ca^{2+}]_i$ increase induced by SP was examined either in the presence or absence of extracellular free $Ca^{2+}$ to clarify the roles of $Ca^{2+}$ influx in the pathway. To completely remove residual extracellular free $Ca^{2+}$, 4 mM EGTA was applied in addition to the removal of 0.9 mM extracellular free $Ca^{2+}$. SP at 30 μM, which promotes sub-maximal response in the stimulation of β-HA release

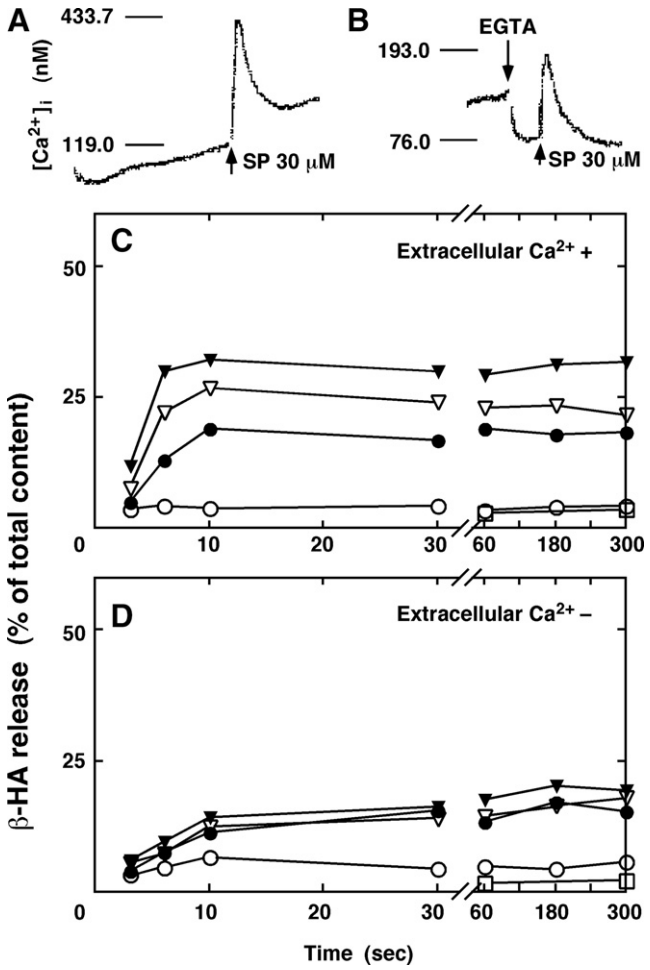


**Fig. 3.** Effects of presence of extracellular free $Ca^{2+}$ on $[Ca^{2+}]_i$ increase (A,B) or exocytotic secretion of β-HA (C,D) induced by SP. (A,B) Rat peritoneal mast cells were stimulated by adding 30 μM SP in the presence (A) or absence (B) of extracellular free Ca2+. Arrowheads indicate the addition of peptides. These results are typical of 5 individual experiments. (C,D) Purified mast cells were stimulated by adding the vehicle (open squares), and 1 μM (open circles), 7 μM (closed circle), 10 μM (open reverse triangles) or 30 μM (closed reverse triangles) of SP for the indicated intervals in the presence (C) or absence (D) of extracellular free Ca2+. Each point represents the average of duplicate experiments, and three independent experiments gave similar results.

(Fig. 1A), induced an immediate and transient $[Ca^{2+}]_i$ increase regardless of the presence or absence of extracellular free $Ca^{2+}$; however, the maximal increase was reduced to 37% by the removal of extracellular $Ca^{2+}$ (315 nM in the presence of extracellular $Ca^{2+}$, and 117 nM in the absence of extracellular $Ca^{2+}$; Fig. 3A and B). These results demonstrate that the mobilization of intracellular free $Ca^{2+}$ induced by SP is due to the influx of extracellular free $Ca^{2+}$ in addition to the mobilization from intracellular stores.

Next, we investigated the kinetics of β-HA release induced by SP in the presence or absence of extracellular free $Ca^{2+}$ to elucidate whether the entry of extracellular $Ca^{2+}$ and the $[Ca^{2+}]_i$ increase play any roles in the exocytotic pathways. As shown in panels C and D of Fig. 3, SP induced β-HA release despite the presence or absence of extracellular $Ca^{2+}$. Faster kinetics and a more efficacious effect were observed in the presence of extracellular $Ca^{2+}$, namely, the $t/2$ values of secretion were about 4 and 10 s after the stimulation with SP (30 μM) in the presence or absence of extracellular $Ca^{2+}$, respectively, although the exocytotic secretion was completed within 30 s regardless of the presence or absence of extracellular $Ca^{2+}$. The amounts of secreted β-HA in the stimulations by the addition of 7, 10 or 30 μM SP in the presence of extracellular $Ca^{2+}$ were higher than those in the absence of extracellular $Ca^{2+}$ (18.4%, 21.6%, and 31.7% of the total content in the presence of extracellular $Ca^{2+}$; 15.4%, 18.0%, and 19.3% of the total content in the absence of extracellular $Ca^{2+}$ for the 5-min stimulation by the addition of 7, 10 or 30 μM SP, respectively). These results demonstrate that the kinetics of exocytosis induced by SP coincides with transient increases in $[Ca^{2+}]_i$ immediately after stimulation. The present findings also demonstrate that the influx of extracellular $Ca^{2+}$ potentiates the rate and amount of β-HA secretion, suggesting that the increase in $[Ca^{2+}]_i$ positively modifies exocytosis.

## Discussion

In the present study, a mammalian tachykinin, SP, as well as a wasp venom toxin, MP, were shown to stimulate an increase in $[Ca^{2+}]_i$ and induce exocytosis in rat peritoneal mast cells at a similar concentration range. The stimulation by SP and MP to induce $[Ca^{2+}]_i$ increase and exocytosis was inhibited by PTX, which specifically ADP-ribosylates the $G_i$- or $G_o$-family of G proteins and renders them insensitive to stimulation, indicating the involvement of $G_i$ and/or $G_o$ proteins in the signaling pathway. It was firstly demonstrated that the increase in $[Ca^{2+}]_i$ as stimulated by SP affected the SP-induced exocytosis; the influx of extracellular free $Ca^{2+}$ induced by SP accelerated exocytosis and increased the amount of secretion. These results indicate the importance of $Ca^{2+}$ signaling in SP-induced exocytosis.

Exocytosis in rat peritoneal mast cells induced by SP and MP as well as compound 48/80 has been demonstrated to be inhibited by PTX [6,11,15,22]. These compounds-promoted $IP_3$ production by phospholipase C, which mobilized free $Ca^{2+}$ from intracellular $Ca^{2+}$ stores to cytosol, was also decreased by PTX pretreatment [6,11]. In rat peritoneal mast cells, the presence of a PTX-sensitive $G_{i3}$ protein and its involvement in the signaling pathway for exocytosis promoted by amphiphilic compounds have been reported [6–8,11]. The results of these previous studies propose that $Ca^{2+}$ signaling as a consequence of the activation of the $G_{i3}$ protein is involved in the exocytotic pathway. In fact, SP-induced $[Ca^{2+}]_i$ increase has already been reported in intact cells [21]; however, the mechanisms underlying such $[Ca^{2+}]_i$ increase remain to be clarified. In other words, it is still unknown whether the $[Ca^{2+}]_i$ increase promoted by SP is under the regulation of G proteins, owing to the difficulty in quantitatively and reproducibly measuring $[Ca^{2+}]_i$ changes in intact mast cells from the rat peritoneum. In fact, we tried to load fura-2 into the cells by incubating the cells with fura-2/AM at 37 °C for 1 h. However, only a slight increase in $[Ca^{2+}]_i$ triggered by SP and MP was observed (H. Mukai, unpublished data), possibly due to the leakage or translocation of the loaded fura-2. Moreover, we incubated the cells with fura-2/AM at various temperatures for several time periods and found that the $[Ca^{2+}]_i$ increase induced by SP and MP was maximized by the incubation of the cells with 4 μM fura-2/AM at 20 °C for 1 h. Using this condition, we were able to measure changes in $[Ca^{2+}]_i$ reproducibly; therefore, we further investigated the roles of $Ca^{2+}$ signaling in the exocytotic mechanisms.

In the present study, the immediate and transient increase in $[Ca^{2+}]_i$ induced by SP was inhibited by PTX pretreatment of cells (Fig. 2B and D). Moreover, the presence of extracellular $Ca^{2+}$ was shown to accelerate the rate of SP-induced exocytosis and to potentiate the amount of β-HA secretion (Fig. 3C and D). However, the secretion was not completely under the regulation of the $[Ca^{2+}]_i$ increase; different activities on β-HA secretion were observed comparable to the increase in $[Ca^{2+}]_i$ as stimulated by SP and MP (Fig. 1A vs. B). Taken together, including the fact that rat peritoneal mast cells express the $G_{i3}$ protein abundantly, the present results suggest that SP caused the immediate and transient increase in $[Ca^{2+}]_i$ as a consequence of the activation of the $G_{i3}$ protein, and that this increase positively modified the exocytotic events; however, independent mechanisms on $Ca^{2+}$ signaling, which are also regulated by the $G_{i3}$ protein, may also participate in the secretory pathway.

MP is known to induce not only an immediate and transient $[Ca^{2+}]_i$ increase, but also a slower and sustained increase (Fig. 1B-inset) [22]. SP also caused a slower and sustained increase although this was not evident in Figs. 1B-inset or 2B. These slower and sustained $[Ca^{2+}]_i$ increases were not inhibited by PTX (Fig. 2D and E), and such uninhibited increases may cause discrepant interpretations concerning the involvement of $[Ca^{2+}]_i$ increase in the exocytotic mechanisms induced by amphiphilic compounds [6,11,15,16,19,20,30].

Recently, we have attempted to purify peptides activating rat peritoneal mast cells from several mammalian tissues such as the heart and liver, and found the presence of various activating peptides presumably derived from cytosolic proteins, although their complete structures could not be determined due to the small limited amount of the purified peptides (H. Mukai, unpublished observation). We have also demonstrated the presence of many cryptides, namely, functional cryptic peptides hidden in protein structures, that possibly transduce inflammatory signals to neutrophils (H. Mukai et al., submitted for publication) [18], and found that some of them also activated mast cells. We are currently attempting to identify such mast cell-activating cryptides as well as neutrophil-activating cryptides utilizing the functional prediction of peptides [17,18]. The present investigation is expected to provide insights into the functional prediction of mast cell-activating peptides.

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