Human salivary histatins (Hsts), which belong to a salivary polypeptide family, have potent antifungal activity against *Candida albicans* and *Cryptococcus neoformans*, and are expected to be useful as therapeutic reagents against *Candida* species. However, little is known about the effect of Hsts on host immune systems. Thus we conducted a series of *in vitro* experiments with rat mast cells to determine whether histatin 5 (Hst 5) or histatin 8 (Hst 8) has a histamine-releasing effect on mast cells. Both Hst 5 and Hst 8 induced histamine release from rat peritoneal mast cells in a dose-dependent manner (10⁻⁴ to 10⁻² M). Hst 5 had a stronger releasing effect than Hst 8. The histamine release induced by Hst 5 (10⁻⁴ M) was increased by the presence of 0.5 mM Ca²⁺, but decreased by 2 mM Ca²⁺. Alternatively, the histamine release induced by Hst 8 (10⁻⁴ M) was inhibited by the presence of Ca²⁺ (0.5 to 2 mM). These results suggest that Hsts have limited usefulness as therapeutic agents due to induction of histamine release from mast cells.

**Key words** histatin 5; histatin 8; mast cell; histamine release; extracellular calcium

**MATERIALS AND METHODS**

**Animals and Drugs** Male Wistar rats, weighing 300—400 g (Japan SLC, Hamamatsu, Japan), were used in this study. The rats were housed under controlled environmental conditions (temperature 23 ± 1 °C and humidity 55 ± 5%) with commercial food and water freely available. All animal experiments were carried out according to the guidelines of Nagoya University School of Medicine for the care and use of laboratory animals. Human Hst 5 was purchased from the Peptide Institute (Osaka, Japan) and human Hst 8 was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were commercially available and were used at analytical grade without the need for further purification. Each peptide was dissolved in Ca²⁺- and Mg²⁺-free Tyrode's solution containing 0.1% (w/v) bovine serum albumin (BSA, Wako Pure Chemical Industries, Osaka, Japan) and 10 units/ml of sodium heparin. Peritoneal exuded cell (PEC) fluid in the peritoneal cavity was obtained after gentle massage of the abdomen for 3 min. The PECs were obtained by centrifuging the PEC fluid at 2 °C for 5 min. PECs were stained with 0.05% toluidine blue and adjusted to a concentration of 5 × 10⁶ mast cells/ml with Ca²⁺- and Mg²⁺-free Tyrode's solution containing 20 mM HEPES buffer (pH 7.0—7.4).

**Preparation of Crude Peritoneal Mast Cells** The preparation of crude peritoneal mast cells was performed as described elsewhere. Briefly, rats were killed under ether anesthesia. The peritoneum was exposed and the cavity was washed with 10 ml of Ca²⁺- and Mg²⁺-free Tyrode's solution containing 0.1% (w/v) bovine serum albumin (BSA, Wako Pure Chemical Industries, Osaka, Japan) and 10 units/ml of sodium heparin. Peritoneal exuded cell (PEC) fluid in the peritoneal cavity was obtained after gentle massage of the abdomen for 3 min. The PECs were obtained by centrifuging the PEC fluid at 2 °C for 5 min. PECs were stained with 0.05% toluidine blue and adjusted to a concentration of 5 × 10⁶ mast cells/ml with Ca²⁺- and Mg²⁺-free Tyrode's solution containing 20 mM HEPES buffer solution (pH 7.0—
7.4) (PEC fluid). This cell preparation showed a viability of greater than 95% and contained approximately 10% mast cells throughout the experiments.

**Cell Incubation** The PEC fluid (0.9 ml) in tubes was preincubated at 37°C for 10 min and 0.1 ml of each peptide solution was added to the fluid. In the first set of experiments, to investigate the time-dependent effect of peptides Hst 5 and Hst 8 on histamine release, the PEC fluid samples (0.9 ml each) were incubated for different times (0.5 to 15 min) in the presence or absence of each peptide (10^-8 M). In the second set of experiments, to investigate the concentration-dependent effect of Hst 5 and Hst 8 on histamine release from the PEC fluid, the PEC fluid samples (0.9 ml each) were incubated with different concentrations of peptide solution (10^-8, 10^-7, 10^-6, 10^-5 M) for 10 min. In the third set of experiments, to investigate the effect of extracellular calcium on the histamine release induced by Hst 5 and Hst 8, 10—40 μl of 5.0×10^-2 M CaCl2 (final concentrations of CaCl2 were 0.5—2 mM) were added to the assay buffers before preincubation. The PEC fluid samples were incubated with a 10^-6 M (Hst 5 and Hst 8) peptide solution for 10 min in the presence of different concentrations of CaCl2 at 0, 0.5, 1, and 2 mM. The concentrations of CaCl2 (0.5—2 mM) were selected to inhibit peptide-induced histamine release, as has been described elsewhere. After incubation, all test tubes were immediately placed in ice-cold water, and the supernatant solution was obtained by centrifugation at 2°C for 10 min.

**Measurement of Histamine** The concentration of histamine in the supernatant solution was determined by high-performance liquid chromatography (HPLC) using the method reported previously by May and colleagues. The apparatus for HPLC was a Shimadzu LC-6A system (Kyoto, Japan) equipped with a fluorescence detector (RF-535, Shimadzu) (emission: 460 nm, excitation: 360 nm) consisting of an LC-6A liquid pump. The conditions were as follows: column, a YMC Packed Column A-302 (Yamamura Chemical Laboratories, Kyoto, Japan); mobile phase, 40% methanol in water containing 42 mM acetate buffer (pH 4.0); and flow rate, 1 ml/min. The total histamine release in intact PEC fluid was determined after hydrolysis by boiling for 5 min. We also measured the concentrations of lactate dehydrogenase (LDH) in the solution before and after incubation.

**Statistical Analysis** Values are expressed as the mean±S.E. of the net percentage of total histamine release obtained in 3 to 4 different experiments. Statistical analysis was performed using Student’s t-test. The differences between means were considered to be significant when p values were less than 0.01.

**RESULTS**

Figure 1 shows the release–time profiles of histamine from mast cells after initiation of incubation with Hst 5 or Hst 8 at the concentration of 10^-6 M. Histamine release induced by both peptides was very rapid and reached the maximum concentration 0.5 min after initiation of incubation. Based on data from this experiment, we selected 10 min as the incubation period in the next experiments, because the maximum release of histamine occurred at 2 min. The concentration-dependent effects of Hst 5 or Hst 8 on histamine release are illustrated in Fig. 2. Both Hst 5 or Hst 8 released histamine from rat peritoneal mast cells in a dose-dependent manner. The histamine-releasing effect in the presence of Hst 5 was more potent than that of Hst 8, and the threshold concentration was around 10^-7 M and 10^-6 M, respectively. At the highest concentration tested (10^-5 M), histamine release induced

![Fig. 1. Time-Dependent Changes in the Histamine Release from Rat Peritoneal Mast Cells in the Presence of Hst 5 (1.0×10^-6 M, •) or Hst 8 (1.0×10^-8 M, □). Each point represents the mean±S.E. of 6 experiments. * Significantly different from the data at time zero of Hst 5 and Hst 8, respectively (p<0.01).](image1)

![Fig. 2. Dose-Dependent Effect of Hst 5 (•) and Hst 8 (□) on Histamine Release from Rat Peritoneal Mast Cells. Each point represents the mean±S.E. of 8 experiments. * Significantly different from the corresponding dose of Hst 8 (p<0.01).](image2)

![Fig. 3. Effect of Calcium on Histamine Release from Rat Peritoneal Mast Cells Induced by Hst 5 (1.0×10^-6 M, •) and Hst 8 (1.0×10^-8 M, □). Each point represents the mean±S.E. of 8—8 experiments. * Significantly different from the histamine release of Hst 5 and Hst 8 in the absence of CaCl2; respectively (p<0.01).](image3)
by Hst 5 and Hst 8 was 42.1 ± 3.8% and 33.3 ± 3.2%, respectively. Figure 3 shows the effects of extracellular Ca\(^{2+}\) on the histamine release induced by Hst 5 or Hst 8. That induced by Hst 8 was inhibited by the presence of Ca\(^{2+}\) at the concentrations studied (0.5 to 2 mM). In contrast, the presence of Ca\(^{2+}\) (0.5 mM) increased the histamine release induced by Hst 5, whereas it decreased in the presence of 2 mM of Ca\(^{2+}\).

Total histamine release from mast cells was approximately 1 µg/5 × 10⁴ mast cells (mean 1.16 ± 0.15 µg/5 × 10⁴ mast cells throughout the experiments), and spontaneous histamine release was less than 7% of total histamine release throughout the experiments, and spontaneous histamine release was less than 8% above background, data not shown), suggesting that histamine release from the mast cells induced by Hsts is not caused by cytotoxicity.

**DISCUSSION**

Murakami and colleagues suggested that the arginine residue in a histidine-rich peptide plays an important role in the inhibition of hemagglutination by *Porphyromonas gingivalis*. They also suggested that human salivary Hsts and lysozymes inhibit coagulation induced by *P. gingivalis* and *Streptococcus mitis*. Recent studies have suggested that *Cryptococcus neoformans*, which is a primary pathogen in immunocompromised patients, is the first manifestation of human immunodeficiency virus infection in 26—45% of patients. Tsai and coworkers have reported that Hsts possess potent antifungal activity against *C. neoformans* and *C. albicans* and that they are nontoxic because they are natural products in the human body. These properties of Hsts suggest that they are potential drugs for the treatment of fungal infections, especially those associated with AIDS. Oppenheim and colleagues have reported that the secretion of salivary Hsts is affected or modulated by candidal infection and that a feedback mechanism is of considerable importance for the nonimmune host defense functions. However, little is known about the effect of Hsts on host immune systems. Thus we attempted to determine the histamine-releasing effects of Hst 5 and Hst 8 from mast cells.

The present study demonstrated that both Hst 5 and Hst 8 dose-dependently increase the histamine release from rat peritoneal mast cells. The ability of Hsts to stimulate histamine release is almost equivalent to that of other bioactive polypeptides and of IgE. Considering the physiological concentrations of Hst 5 in saliva (15—30 µM), Hst 5 might be able to stimulate the release of histamine from mast cells in physiological conditions. On the contrary, the histamine-releasing effect of Hst 8 was weaker than that of Hst 5. Hst 8, the C-terminal fragment of Hst 5, is thought to be an important fragment to exert the antifungal activity, since Hst 8 and 16 amino acids of C-terminal fragment of Hst 5, called Hst M, revealed similar antifungal activity to that of Hst 5. Thus it has been suggested that the relative structural importance of the N-terminal region of Hst 5 is greater than its C-terminal region to regulate histamine release, which is different from their antifungal activity. Very recently, it has been reported that there is no structural relationship between the antifungal activity of Hsts and their cationic charges.

The differential histamine-releasing effects observed between Hst 5 and Hst 8, in spite of their common basic character, prompted us to examine whether the chemical structural characteristics of the two peptides are important in explaining the data observed in this study. Interestingly, it has been reported that these endogenous peptides are characterized by a net positive charge. It has also been suggested that a molecule with a greater overall positive charge increases the molecule’s effectiveness. For example, lysine and arginine are amino acids possessing one positive charge at physiological pH, and aspartic acid and glutamic acid are amino acids possessing a negative single charge. Hst 5, which consists of three arginine residues, four lysine residues, one aspartic acid residue, and one glutamic acid residue, has an overall positive charge of 5, whereas Hst 8 has an overall positive charge of only 2. The differential histamine-releasing effect of these compounds appears to be related to differences in the potency of positive charges in their molecule. Although the precise mechanism responsible for histamine release by Hsts is still unknown, we hypothesize that the number of overall positive charges in the molecule may play a partial role in induction of histamine release from mast cells.

Extracellular Ca\(^{2+}\) differently affected the histamine release from mast cells induced by Hst 5 and Hst 8. Hst 5-induced histamine release was activated by a low concentration (0.5 mM) of Ca\(^{2+}\), but decreased by a high concentration (2 mM), whereas Hst 8-induced histamine release was dose-dependently inhibited by extracellular Ca\(^{2+}\) (0.5—2 mM). Previous studies in our laboratories demonstrated that the histamine-releasing effect of several peptides including natriuretic peptides, nociceptin, and adrenomedullin was inhibited by extracellular Ca\(^{2+}\), suggesting the existence of a Ca\(^{2+}\)-sensitive pathway to regulate histamine release. Taken together, the results suggest that Hsts might share the same Ca\(^{2+}\)-sensitive pathway as these peptides to regulate histamine release. Further studies are necessary to understand how extracellular Ca\(^{2+}\) inhibits Hsts-induced histamine release although few reports have already proposed the possible action of extracellular Ca\(^{2+}\), i.e., the alteration of peptide binding to the cell surface and the direct inhibition of the activity of Ca\(^{2+}\)-inhibitable adenylyl cyclase and/or Ca\(^{2+}\)-sensing receptor.

The mechanism by which extracellular Ca\(^{2+}\) enhances on histamine release induced by Hst 5 remains unclear. Recently, Helmerhorst et al. have demonstrated that Hst 5 is internalized and subsequently located on mitochondria in *C. albicans*. Although the intracellular roles of Hst 5 are still unknown, this phenomenon might occur in host immune cells. Thus it would be of interest to elucidate further the underlying mechanisms of Hst 5-induced histamine release from mast cells.

In conclusion, the present study demonstrated that basic polypeptides Hsts have histamine-releasing activity in rat mast cells. Thus far, these findings indicate that Hsts have limited usefulness, except in saliva, as therapeutic agents due to their histamine-releasing effect from mast cells and will need to be the appropriate structural modification.

**Acknowledgments** We would like to thank Professor Tetsuo Hayakawa for his encouragement throughout this study and Drs. Kenju Hiramatsu and Hiroshi Yoshida for...
their helpful advice.

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