Inhibitory Effects of Emedastine Difumarate on Histamine Release

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ABSTRACT—The inhibitory effects of emedastine difumarate on histamine release were studied in rat peritoneal mast cells. Emedastine significantly inhibited substance P (SP)-induced histamine release at concentrations above 10⁻⁹ M in the presence of extracellular Ca²⁺ and at concentrations above 10⁻¹¹ M in its absence. At concentrations of 10⁻⁸ M or higher, emedastine significantly inhibited SP-induced Ca²⁺ release from intracellular Ca stores and SP-induced ⁴⁵Ca uptake into mast cells. Emedastine also inhibited passive peritoneal anaphylaxis in rats and guinea pigs. We conclude that the clinical antiallergic effects of emedastine involve the inhibition of histamine release and that this inhibition is mediated by the inhibition of Ca²⁺ release from intracellular Ca stores and the inhibition of Ca²⁺ influx into mast cells.

Keywords: Emedastine difumarate, Histamine release, Substance P, Mast cell, Intracellular Ca²⁺

Histamine release from mast cells is induced by various stimuli apart from IgE-mediated antigen-antibody reactions. In particular, histamine release induced by neuropeptides such as substance P (SP) has been discussed extensively in recent years (1–6). Intracutaneous injection of histamine produces circumscribed edema, wheals and flares; an axon reflex model has been suggested for the spread of flares. Some histamine-induced orthodromic impulses from sensory nerve endings spread as antidromic impulses through terminal arborizations. These antidromic impulses cause the release of SP which stimulates neighboring mast cells to release histamine (1). Emedastine difumarate is a new antiallergic drug which possesses an antihistaminic effect (7) and an inhibitory effect on IgE-mediated histamine release from rat peritoneal mast cells (8). In the present study, we examined the effects of emedastine on SP-induced histamine release from rat peritoneal mast cells. To elucidate the mechanism of histamine release inhibition induced by emedastine, we studied its effects on SP-induced Ca²⁺ release from intracellular Ca stores and ⁴⁵Ca influx into mast cells. In addition, we also studied the effects of emedastine on passive peritoneal anaphylaxis (PPA) in rats and guinea pigs to clarify the effectiveness of this drug in preventing in vivo anaphylaxis in relation to the inhibition of histamine release.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 210–420 g and male or female Hartley guinea pigs weighing 300–400 g were used. The animals were housed in a temperature- and humidity-controlled room with free access to food and water.

Drugs

Emedastine and ketotifen were synthesized by Kanebo, Ltd. Disodium cromoglycate (DSCG) was extracted from Intal (Fujisawa Pharmaceutical Co., Ltd., Osaka) by Kanebo, Ltd. Verapamil was purchased from Sigma Chemical Co. (St. Louis, MO, USA). To study the effects of the test compounds in the presence of extracellular Ca²⁺ in vitro, emedastine and ketotifen were dissolved in physiological buffered solution (PBS; 154 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl₂, 5.6 mM glucose, 0.1% bovine serum albumin, 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.4). Verapamil was dissolved in distilled water and diluted with PBS. To study the effects of test compounds in a Ca-free medium, Ca-free PBS containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) was prepared and substituted for PBS. For the in vivo studies, emedastine and ketotifen were dissolved in distilled water, and DSCG was dissolved in saline.
Histamine release from rat peritoneal mast cells

Rats were exsanguinated, 25 ml of glucose-free PBS was injected into the abdominal cavity, and the abdominal wall was gently massaged. Fluid from the abdominal cavity was collected and centrifuged at $80 \times g$ for 5 min at $4^\circ C$. The pellet was then washed with fresh glucose-free PBS and suspended in PBS. The cell suspensions ($5 \times 10^5$ cells/ml) and test compounds were preincubated at $37^\circ C$ for 10 min, after which 0.1 ml of cell suspension was added to 0.8 ml of each test compound solution or PBS as a control. After a 60-min incubation, 0.1 ml of SP was added, and incubation was continued for another 10 min. The reaction was terminated by the addition of 1 ml of ice-cold PBS. After centrifugation, histamine in the supernatant and residual histamine in the cell pellet were determined by spectrophotometric assay, according to the method of Shore et al. (9). In the study performed in the absence of extracellular Ca$^{2+}$, Ca-free PBS containing 0.1 mM EDTA was substituted for PBS. Histamine released in the supernatant was expressed as a percentage of the total histamine content in the specimen. The spontaneous histamine release was $4.3 \pm 0.8\%$ (n = 11) or $3.7 \pm 0.7\%$ in the presence or absence of extracellular Ca$^{2+}$, respectively. The percent inhibition was calculated as follows:

\[
\text{Inhibition (\%)} = \frac{(C - D)}{(C - S)} \times 100
\]

C: SP-induced histamine release without drugs
D: SP-induced histamine release with drugs
S: spontaneous histamine release

Fluorescence measurement of mast cells loaded with quin 2

Fluorescence measurement of mast cells loaded with quin 2 was carried out according to the method of Tasaka et al. (2). Rat peritoneal mast cells were harvested from the abdominal cavity and purified to a level higher than 87% by density gradient centrifugation with Percoll (Pharmacia Biotechnology AB, Uppsala, Sweden). The mast cells were suspended in PBS containing 10$^{-3}$ M of quin 2/AM (Dojindo Laboratories, Kumamoto) and incubated for 60 min at $37^\circ C$, followed by washing with Ca-free PBS. Mast cell suspension (0.4 ml) was then added to the microchamber, and test compounds were added; after 10 min of incubation, SP was added to the medium. Fluorescent changes derived from quin 2 chelated with intracellular Ca$^{2+}$ were measured as integrated values every 2 sec under a fluorescence microscope (XF, Nikon Corporation, Tokyo) connected to a video-intensified microscopy system (ARGUS-100, Hamamatsu Photonics, Hamamatsu). Results were expressed as a percentage of the fluorescence intensity determined before the addition of SP, and the maximum values detected in the control and drug-treated groups were compared. Fluorescence intensities were converted into pseudo-color from white to black according to pixel intensity.

$^{45}$Ca uptake into rat peritoneal mast cells

The measurement of $^{45}$Ca uptake into rat peritoneal mast cells was carried out according to a modification of the method of Spataro and Bosmann (10). Rat peritoneal mast cells purified with Percoll density gradient centrifugation were suspended in PBS. Mast cell suspension (0.35 ml, 2.86 $\times 10^5$ cells/ml) was preincubated at $37^\circ C$ for 10 min, after which 0.05 ml of $^{45}$CaCl$_2$ (1 MBq/ml) was added; the suspension was then incubated for 10 min prior to the addition of 0.05 ml of test compound solution or PBS as a control. After a 10-min incubation, 0.05 ml of SP was added, and the suspension was incubated for another 10 min. The reaction was terminated by the addition of 0.5 ml of ice-cold PBS. The cells, collected by centrifugation at $340 \times g$ for 5 min at $4^\circ C$, were washed twice with ice-cold PBS. After solubilization of the cell pellet in 0.2 ml of PBS containing 10% Triton X-100, 2 ml of liquid scintillator (Scintisol EX-H, Wako Pure Chemical Industries, Ltd., Osaka) was added to 0.15 ml of the specimen. The radioactivity was measured by a liquid scintillation counter (LS-1050, Aloka Co., Ltd., Mitaka). The percent inhibition was calculated as follows:

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S: spontaneous $^{45}$Ca uptake

Preparation of anti-egg albumin (EA) rat serum

Anti-EA rat serum was prepared according to a modification of the method of Scotton and Share (11). Rats were immunized by intracutaneous injection of 0.6 ml of saline containing 1 mg of EA, 2 mg of aluminum hydroxide gel, and 10$^{10}$ killed Bordetella pertussis (Tohama strain phase I, Chiba Serum Institute, Ichi-kawa) into the footpads. Five days after immunization, the animals were boosted with an intramuscular injection of 0.5 ml of 1 mg/ml EA. Sera were isolated from blood collected at 15 days after the first immunization. The IgE titer of the serum was examined by inducing 48-hour homologous passive cutaneous anaphylaxis, and the sera with levels of 1:256 or above were pooled and kept at $-80^\circ C$ until immediately before the experiment.

PPA in rats

Rats were passively sensitized by the intraperitoneal injection of 3 ml anti-EA rat serum (1/12 dilution). After 48 hours, 1 ml of 5 mg/ml EA was intravenously injected. Immediately after that, 5 ml of Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl$_2$, 1.0 mM MgCl$_2$, 8.1 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, 0.58 mM Na$_2$HPO$_4$) was injected into the abdominal cavity, and the abdominal wall was gently massaged. Fluid from the abdominal cavity was collected and centrifuged at $80 \times g$ for 5 min at $4^\circ C$. The pellet was then washed with fresh glucose-free PBS and suspended in PBS. The cell suspensions ($5 \times 10^5$ cells/ml) and test compounds were preincubated at $37^\circ C$ for 10 min, after which 0.1 ml of cell suspension was added to 0.8 ml of each test compound solution or PBS as a control. After a 60-min incubation, 0.1 ml of SP was added, and incubation was continued for another 10 min. The reaction was terminated by the addition of 1 ml of ice-cold PBS. After centrifugation, histamine in the supernatant and residual histamine in the cell pellet were determined by spectrophotometric assay, according to the method of Shore et al. (9). In the study performed in the absence of extracellular Ca$^{2+}$, Ca-free PBS containing 0.1 mM EDTA was substituted for PBS. Histamine released in the supernatant was expressed as a percentage of the total histamine content in the specimen. The spontaneous histamine release was $4.3 \pm 0.8\%$ (n = 11) or $3.7 \pm 0.7\%$ in the presence or absence of extracellular Ca$^{2+}$, respectively. The percent inhibition was calculated as follows:

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12.0 mM NaHCO₃, 0.4 mM NaH₂PO₄, and 5.6 mM glucose) was intraperitoneally injected. The animals were killed 5 min after the antigen injection, and the peritoneal fluid was collected. After centrifugation at 400×g for 5 min at 4°C, histamine in the supernatant was determined by a spectrofluorimetric assay according to the method of Shore et al. (9). The test compounds were administered orally 60 min before the antigen injection.

**Preparation of anti-benzylpenicilloyl bovine γ-globulin (BPO·BGG) guinea pig serum**

Anti-BPO·BGG guinea pig serum was prepared according to the method of Levine et al. (12). Female guinea pigs were immunized intraperitoneally by repeated injections (twice a month, a total of 15–21 times) of 1 ml of 0.1% aluminum hydroxide gel which contained 2 μg/ml of BPO·BGG as antigen. Sera were isolated from blood collected at 10 days after the last immunization. The IgE titer of the serum was examined by inducing 7-day homologous passive cutaneous anaphylaxis; sera with levels of 1:512 or above were pooled and kept at −80°C until immediately before the experiment.

**PPA in guinea pigs**

Male guinea pigs were passively sensitized with intraperitoneal injection of 3 ml anti-BPO·BGG serum (1/100 dilution). After 7 days, 0.3 mg/kg of pyrilamine was injected intravenously, followed 5 min later by the intravenous injection of 1 ml of BPO·BSA (protein concentration, 0.2 mg/ml). Immediately after that, 10 ml of Tyrode’s solution was intraperitoneally injected. The animals were killed 5 min after the antigen injection, and peritoneal fluid was collected. After centrifugation at 400×g for 5 min at 4°C, histamine in the supernatant was determined by spectrofluorimetric assay according to the method of Shore et al. (9). The test compounds were administered orally 60 min before the antigen injection.

**Statistics**

The results were expressed as the mean±S.E. A one-way analysis of variance with Dunnett’s test and a paired t-test were used to determine statistical significance.

**RESULTS**

**Effects on histamine release in the presence of extracellular Ca²⁺**

When rat peritoneal mast cells were exposed to 2×10⁻⁶ M of SP in the absence of extracellular Ca²⁺, histamine was released at 22.2±2.9% (n=9) of the total histamine content. Emedastine, at 10⁻¹¹ M or higher, significantly inhibited histamine release (Fig. 2), while ketotifen produced significant inhibition only at 10⁻⁹ M or higher.

![Fig. 1. Effects of emedastine and ketotifen on substance P-induced histamine release from rat peritoneal mast cells in the presence of extracellular Ca²⁺. Each point represents the mean of 5 or 6 experiments. Vertical bars indicate S.E. *P<0.05, **P<0.01, significantly different from the control. ○: emedastine, ●: ketotifen.](image)

![Fig. 2. Effects of emedastine and ketotifen on substance P-induced histamine release from rat peritoneal mast cells in the absence of extracellular Ca²⁺. Each point represents the mean of 4 or 5 experiments. Vertical bars indicate S.E. *P<0.05, **P<0.01, significantly different from the control. ○: emedastine, ●: ketotifen.](image)
Effects on increase of intracellular Ca\(^{2+}\) concentration

When mast cells loaded with quin 2 were exposed to \(2 \times 10^{-6}\) M of SP in the absence of extracellular Ca\(^{2+}\), the fluorescence intensity of these cells was increased transiently at 2–4 sec after SP stimulation and returned to the basal level by 10 sec after SP stimulation, indicating Ca\(^{2+}\) release from intracellular Ca stores. Emedastine, at \(10^{-8}\) M, significantly inhibited the fluorescence increase at 2–4 sec after SP stimulation (Figs. 3 and 4), while ketotifen did not show any significant effect at this concentration.

Effects on \(^{45}\)Ca uptake into rat peritoneal mast cells

When mast cells were exposed to \(3 \times 10^{-6}\) M of SP, \(^{45}\)Ca uptake into mast cells was increased in a time-dependent manner (Fig. 5). The increase of \(^{45}\)Ca uptake was continued for at least 30 min. In the following study, mast cells were incubated for 10 min after SP stimulation. SP (\(3 \times 10^{-6}\) M) increased \(^{45}\)Ca uptake by 1272 ± 236 cpm (n = 5) compared to the value of 139 ± 34 cpm (n = 5) in the control group. Emedastine inhibited the SP-induced increase of \(^{45}\)Ca uptake in a concentration-dependent manner at concentrations ranging from \(10^{-8}\) to \(10^{-3}\) M (Fig. 6), while ketotifen did not have any significant effect. Verapamil, a calcium channel blocker, significantly inhibited \(^{45}\)Ca uptake at concentrations of \(10^{-3}\) M or higher.
Effects on PPA in rats

When antigen was intravenously injected to passively sensitized rats, the histamine concentration in the abdominal fluid was increased to $1.17 \pm 0.25 \, \mu g/ml (n=10)$ compared to the concentration of $0.01 \pm 0.01 \, \mu g/ml (n=8)$ in the group that had not received antigen injections, indicating that histamine was released into the abdominal cavity from peritoneal mast cells. Emedastine and DSCG slightly inhibited PPA in rats at 30 mg/kg, p.o. and 10 mg/kg, i.v., respectively (Fig. 7).

Effects on PPA in guinea pigs

In a preliminary study, when antigen was intravenously injected into passively sensitized guinea pigs, all of the animals died of anaphylactic shock within 5 min. Therefore, in the present study, guinea pigs were pretreated with pyrilamine to avoid anaphylactic death and to obtain the control value for the evaluation of the emedastine effect. This treatment prevented anaphylactic death of the animals, and inhibited the increase of histamine concentration in the abdominal fluid; histamine concentrations of pyrilamine-nontreated group and pyrilamine-treated group were $92.0 \pm 16.3 \, ng/ml (n=12)$ and $60.5 \pm 12.6 \, ng/ml (n=12)$, respectively. However, this effect was not
significant. When antigen was intravenously injected into guinea pigs treated with pyrilamine, histamine concentration in the abdominal fluid was increased to 58.6±9.0 ng/ml (n=15) compared to the level of 4.3±0.6 ng/ml (n=15) in the group that had not received antigen injections. Emedastine produced significant inhibition of PPA at 0.03 mg/kg, p.o. (Fig. 8), while ketotifen did not inhibit it, even at 0.3 mg/kg, p.o.

DISCUSSION

Many factors, including allergic reactions, induce histamine release from mast cells. Although type I hypersensitive reactions mediated by IgE are thought to be the main reason for histamine release, proteins and peptides secreted by activated eosinophils and neutrophils during the anaphylactic reaction are also responsible for the histamine release from mast cells (13, 14). When the spreading of inflammation beyond the point of stimulation takes place via the axon reflex, neuropeptides, such as SP, secreted from nerve endings, induce histamine release from mast cells (1). In this study, we selected SP as a histamine releaser, and we examined the effects of emedastine on histamine release.

It has been reported that SP-induced histamine release was mediated by Ca\(^{2+}\) release from the intracellular Ca store in the absence of extracellular Ca\(^{2+}\) (2, 3). In fact, as shown in Figs. 2 and 3, SP elicits both histamine release and increase in cytoplasmic Ca\(^{2+}\) concentrations. On the other hand, in the presence of extracellular Ca\(^{2+}\), SP increased \(^{45}\)Ca uptake into mast cells. These findings were consistent with data showing that SP-induced histamine release was mediated not only by Ca\(^{2+}\) release from intracellular Ca stores, but also by Ca\(^{2+}\) influx into mast cells (3). SP-induced Ca\(^{2+}\) release from intracellular Ca stores occurred immediately after SP stimulation, and it was transient. On the other hand, the increase of SP-induced \(^{45}\)Ca uptake into mast cells continued for 30 min. These results suggest that the early phase of SP-induced histamine release from mast cells was mediated by Ca\(^{2+}\) release from intracellular Ca stores, and the late phase was mediated by Ca\(^{2+}\) influx of extracellular Ca\(^{2+}\) into mast cells. Emedastine inhibited the SP-induced histamine release and the increase of fluorescence intensity derived from quin 2 chelated with intracellular Ca\(^{2+}\) as well as SP-induced \(^{45}\)Ca uptake into mast cells. These results indicate that the inhibitory effects of emedastine on SP-induced histamine release are related to the inhibition of Ca\(^{2+}\) release from intracellular Ca stores as well as to the inhibition of Ca\(^{2+}\) influx into mast cells.

Emedastine inhibited SP-induced histamine release at concentrations as low as 10\(^{-9}\) M in the presence of extracellular Ca\(^{2+}\). The IC\(_{50}\) value of emedastine for antihistaminic activity is 6.1 x 10\(^{-9}\) M in vitro (7), and the plasma level of emedastine was about 10\(^{-9}\) g/ml (1.9 x 10\(^{-9}\) M) when 2 mg of emedastine was administered repeatedly in humans (15). This indicates that emedastine elicits the inhibition of histamine release and shows antihistaminic effects at clinical dose levels. Hence, we examined PPA models in rats and guinea pigs to determine whether the same results for histamine release obtained in vitro could be obtained in vivo. In rats, emedastine showed slight inhibition of PPA at 30 mg/kg, p.o. To prevent anaphylactic death, guinea pigs were pretreated with an antihistamine, pyrilamine, since pyrilamine, among many other antihistamines, is reported to have the least inhibitory effect on histamine release (16, 17). Pyrilamine at 0.3 mg/kg, i.v. prevented anaphylactic death in these animals due to its antihistaminic effects, and it gave the control value that is necessary for the evaluation of the emedastine effect. Pyrilamine was effective in inhibiting histamine release into the abdominal cavity. Emedastine exhibited an additive effect with pyrilamine in inhibiting the increase of histamine concentration, and this inhibitory effect of emedastine became significant at 0.03 mg/kg, p.o. This was equivalent to the dose at which other type I allergic reactions were inhibited in guinea pigs (8, 18). Therefore, it was concluded that orally administered emedastine was effective in preventing histamine release in vivo. The effective dose of emedastine in rats was different from that in guinea pigs. Sakai et al. (19) reported that there were great species differences in the Cmax/dose and AUC/dose of emedastine, and they were due to the species difference in the first pass effect between rats and guinea pigs. Therefore, it was considered that the species difference in bioavailabilities of emedastine was one of the reasons for the difference in effective dose between rats and guinea pigs.

From these results, we concluded that the clinical efficacy of emedastine, an antiallergic agent, can be attributed to its inhibition of histamine release in addition to its antihistaminic effect. The mechanisms through which it inhibits histamine release are related to its significant inhibition of both Ca\(^{2+}\) release from intracellular Ca stores and Ca\(^{2+}\) influx into mast cells.

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