Characteristics of Histamine Release from Rat Mast Cells Induced by a Bracken Toxin, Braxin A1

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Abstract—The effect of braxin A1, a new bracken glucoside, on histamine release from isolated rat peritoneal mast cells was studied. Braxin A1 caused the release of histamine in a dose-dependent manner; the release was slow and increased gradually with time, finally reaching a maximum release of 100%. The action of braxin A1 depended on the incubation temperature in the range from 4°C to 49°C, while it was almost abolished at 0°C. The action of braxin A1 was unaffected by removing calcium or any inorganic ions from the incubation medium and by the addition of 2,4-dinitrophenol or theophylline. The mast cells exposed to braxin A1 were vitally stained with trypan blue and swelled greatly. The cell swelling was characterized by the protrusion of swollen cytoplasmic granules. The present results for braxin A1 were similar to those for the ionophore X537A except for the extracellular inorganic ion dependency, but they were different from those observed with compound 48/80. These results suggest that braxin A1 releases histamine from mast cells without both exocytosis and membrane lysis, but with a cytotoxic action on cytoplasmic membranes by a different mode of action from that of X537A.

Bracken fern, Pteridium aquilinum (L) Kuhn, causes a serious poisoning characterized by edematous and hemorrhagic changes in domestic and experimental animals, so called "bracken poisoning" (1-4). Many studies have been done on bracken poisoning, but the causative substance has not yet been isolated. In view of the facts that plasma histamine (5) and heparin (5, 6) are increased in cattle with the acute toxic disease, we have used histamine releasing activity as an index for the fractionation of the active principles (7-10), and have isolated two novel glucosides, named braxin A1 and A2, from bracken rhizomes (11). It was suggested that braxin A2 changed from braxin A1 during the isolation procedures, and that both braxin A1 and A2 have different chemical natures from ptaquiloside (12, 13) isolated from bracken fronds as a carcinogen. Recently, it has been shown that braxin A1 induces bracken poisoning in guinea pigs, characterized by a marked edema and hemorrhage within 24 to 48 hours in the urinary bladder (14).

The aim of the present study was to elucidate the characteristics of braxin A1 in the effect on histamine release from isolated rat mast cells by comparing them with those of compound 48/80, a typical histamine liberator, and the monovalent cation ionophore X537A (X537A).

Materials and Methods
Preparation of mast cells: Peritoneal mast cells were collected from albino Wistar rats of either sex (250-350 g) by lavage with buffered salt solution containing 0.1% bovine serum albumin as described earlier (11). Mast cells were isolated by Ficoll density gradient centrifugation by the method of Uvnäs and Thon (15) with the minor modifications described earlier (11). The buffered salt solution (pH 7.1) had the following composition: 140 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl2, 1.2 mM MgCl2, 4.47 mM Na2HPO4, 2.23 mM KH2PO4 and 5.5 mM glucose; it was supplemented with 0.1% bovine serum albumin for the dissolution of Ficoll and for the wash-
ing of the cells. Mast cells were finally sus-
pended again in ice-cold buffered salt solu-
tion to give about 25×10^4 cells/ml. In some
experiments, the last suspension was done in
an appropriate incubation medium. In the
final preparation, the mast cells accounted for
more than 96% of all cells as judged by tolu-
idine blue staining, and their viability estimated
by trypan blue exclusion was more than 97%.

**Incubation procedures:** The cell suspen-
sions (about 5×10^4 cells/0.2 ml) in a poly-
carbonate tube were usually incubated first
for 10 min in a water bath at 37°C with gentle
mechanical agitation, and then the secretory
response was started by the addition of agents
dissolved in 0.2 ml of the appropriate isotonic
solution and stopped by the addition of 4 ml
of ice-cold solution of the same composition
as that used during incubation. The following
solutions were used for incubation: the buff-
ered salt solution, calcium-free buffered salt
solution in which CaCl_2 was replaced by
NaCl, and Tris-glucose solution that con-
tained 360 mM glucose buffered with 10 mM
Tris-HCl (pH 7.1). The final concentrations of
the agents are given in the legends to the
figures.

**Assay of histamine release:** After the re-
sponse was stopped, each tube was cen-
trifuged at 420×g for 5 min at 4°C. After the
supernatant was decanted, the cell pellet was
suspended in 1 ml of 0.4 N HClO_4 to release
residual histamine, and 3 ml of distilled water
was added. The tubes were centrifuged at
1600×g for 10 min, and 0.5 ml of the superna-
tant was used for the assay of histamine.
Histamine was measured by the fluorescence
method of Shore et al. (16), with the extrac-
tion steps omitted (17). Histamine release was
expressed as a percentage of the total his-
tamine content in the initial cell sample (per
5×10^4 cells, 1.23 μg±0.23 S.E., n=6). The
spontaneous release of histamine was sub-
tracted from the drug-induced values. The
results were expressed as mean±S.E. of
separate experiments. Statistical signficance
was evaluated by Student’s t-test.

**Vital staining:** Mast cells were incubated in
the buffered salt solution (20 μl, about 10^8
cells/ml) for various periods at 37°C on glass
coated with silicone. After a buffered salt
solution (12 μl) with 0.04% trypan blue was
added, the cells were observed in a micro-
scope within 4 min to assess vital staining.

**Preparation for scanning electron micro-
scopy (SEM):** Suspensions of mast cells
(about 2×10^4 cells/20 μl) were incubated
with an agent for 10 min at 37°C on a glass
slide coated with 2% gelatin. The cells were
fixed in 1% ice-cold glutaraldehyde buffered
with 0.1 M cacodylate (pH 7.2) for 30 min
and then fixed in steaming formalin for 10 min.
After being rinsed twice with the cacodylate-
buffed solution, the cells were stained in
2% tannic acid and postfixed in 1% OsO_4 buf-
fered with cacodylate for 30 min at room
temperature. After being rinsed twice, the
cells were dehydrated in a graded series of in-
creasing concentrations of ethanol, stained
with isoamyl acetate, dried at the critical point
and sputter-coated with gold. The specimens
were observed in a scanning electron micro-
analyzer (Hitachi X-650, Japan).

**Preparation of braxin A1:** Braxin A1 was
isolated from the methanol extract of bracken
rhizomes as described in the previous paper
(11). The isolated braxin A1 was freeze-dried
and stored at −20°C in a vacuum. It was dis-
olved in an appropriate incubation medium
immediately before use.

**Drugs and chemicals:** X537A was kindly
donated by Nippon Roche Co. (Tokyo,
Japan), and HCl-sinomenine was the gift of
Shionogi Co. (Osaka, Japan). Compound
48/80 was purchased from Sigma Chemical
Co. (St. Louis, U.S.A.); theophylline, 2,4-
dinitrophenol (DNP), o-phthalaldehyde
(OTP), bovine serum albumin, and tris(hy-
droxymethyl)aminomethane, from Wako Pure
Chemical Industries (Osaka, Japan); and
Ficoll, from AB Pharmacia (Uppsala, Swe-
den). All other reagents were of the highest
commercial grade available. OTP was re-
crystallized with ligroine. X537A was dis-
olved in dimethylsulfoxide (DMSO) to give
the final concentration of 10 mg/ml. Further
dilutions were made with incubation media.
DMSO in the incubation mixture did not af-
fect the histamine release.

**Results**

**Effect of braxin A1 on histamine release:**
The release of histamine with braxin A1 in-
creased in a dose-dependent manner at con-
Concentrations from 0.5 to 3 mg/ml, although it was less potent than the other releasers tested (Fig. 1). The spontaneous histamine release was 3.5±0.6% (mean±S.E., n=12) from mast cells incubated in buffered salt solution for 10 min at 37°C. The dose-response curves of braxin A1 and X537A were shifted to the left by prolonged incubation (60 min), but not those of compound 48/80 or sinomenine.

Since the results above showed that the action of braxin A1 at the lower concentrations was not completed within 10 min, the time course of the effect of braxin A1 was studied on histamine release from mast cells incubated in buffered salt solution at 37°C. The histamine release induced by braxin A1 at the concentrations of 1 and 3 mg/ml increased gradually with time, and finally reached 100%, like the release induced by X537A, while the response to compound 48/80 was completed in a short time (Fig. 2). The spontaneous release of histamine was slight even on prolonged incubation (60 min, 7.3±1.0%, n=6; Fig. 2A).

Effect of incubation temperature: The braxin A1-induced release of histamine depended on the incubation temperature from 4°C to 49°C, as with X537A, while the effect of compound 48/80 was depressed at temperatures above 44°C (Fig. 3). None of the agents tested released histamine at 0°C. The spontaneous releases of histamine at 4°C and 49°C were 1.2±0.4% (n=6) and 1.4±0.5% (n=6), respectively, which were less than that (3.3±0.5%, n=6) at 37°C (P<0.01). Braxin A1 and X537A still had their maximum effect on mast cells preincubated at 48°C for 10 min, while the effect of compound 48/80 was depressed with elongation of the preincubation time and was almost completely abolished after 10 min (Fig. 4). The spontaneous release of histamine by the heat-treatment for 10 min was 0.8±0.4% (n=6), which was lower than that (3.3±0.5%, n=6) at 37°C (P<0.01).

Effect of calcium-free medium and inorganic ion-free medium: Histamine release induced by braxin A1 was unaffected by the removal of calcium or of all inorganic ions from the incubating medium, while the release induced by X537A was much less in medium free of inorganic ions (Fig. 5). The compound 48/80-induced release was depressed in medium free of calcium or inorganic ions (Fig. 5). The spontaneous histamine releases in calcium-free and inorganic ion-free medium for 10 min at 37°C were 4.8±1.1% (n=6) and 6.3±0.9% (n=6), respectively, both of which

![Fig. 1. Concentration-response curves for the release of histamine from rat mast cells by braxin A1 (●), X537A (○), compound 48/80 (□) and sinomenine (△). The cells were incubated for 10 min at 37°C with the agent. Each value represents the mean±S.E. of 4–6 separate experiments.](image)
were slightly higher than those in ordinary buffered salt solution (P<0.05).

**Effects of some metabolic inhibitors:** To determine whether the action of braxin A1 in releasing histamine depends upon metabolic energy and upon the intracellular cyclic AMP level or not, the effects of DNP and theophyl-

![Figure 2](image-url)  
**Fig. 2.** Time course for the histamine release from rat mast cells induced by braxin A1 (●), X537A (○) and compound 48/80 (□) at low (A) and high (B) concentrations. A: braxin A1, 1 mg/ml; X537A, 6 μg/ml; compound 48/80, 1 μg/ml; ▲, spontaneous histamine release. B: braxin A1, 3 mg/ml; X537A, 60 μg/ml; compound 48/80, 10 μg/ml. Each value represents the mean±S.E. of 4–6 separate experiments.

![Figure 3](image-url)  
**Fig. 3.** Effect of incubation temperature on the histamine release from rat mast cells induced by braxin A1 (1.5 mg/ml, ●), X537A (15 μg/ml, ○) and compound 48/80 (10 μg/ml, □). The cells were incubated with the agent for 10 min at the indicated temperatures. Control experiments were done without the agent (▲). Each value represents the mean±S.E. of 4–6 separate experiments.

![Figure 4](image-url)  
**Fig. 4.** Effect of heat-treatment on the histamine release from rat mast cells induced by braxin A1 (3 mg/ml, hatched column), X537A (30 μg/ml, dotted column) and compound 48/80 (10 μg/ml, open column). The cells were incubated first at 48°C for a certain time, an agent added, and the cells were incubated again at 37°C for 5 min. ***P<0.001, compared with the control value. Each value represents the mean±S.E. of 3 separate experiments.
line on the braxin A1-induced histamine release were studied. Neither DNP nor theophylline affected the histamine release induced by braxin A1 (BA1, 3 mg/ml) and compound 48/80 (C48/80, 10 μg/ml). Controls (open column) were performed in a buffered salt solution. The cells were incubated first in a Ca-free buffered salt solution or Tris-glucose solution for 10 min at 37°C, and then incubated again with an agent added for 5 min at 37°C. ***P<0.001, compared with the control value. Each value represents the mean±S.E. of 4 separate experiments.

![Fig. 5. Effects of calcium-free (hatched column) and inorganic ion-free (dotted column) medium on the histamine release from rat mast cells induced by braxin A1 (BA1, 3 mg/ml), X537A (30 μg/ml) and compound 48/80 (C48/80, 10 μg/ml). Controls (open column) were performed in a buffered salt solution. The cells were incubated first in a Ca-free buffered salt solution or Tris-glucose solution for 10 min at 37°C, and then incubated again with an agent added for 5 min at 37°C. ***P<0.001, compared with the control value. Each value represents the mean±S.E. of 4 separate experiments.](image1)

Morphological changes: Unstimulated mast cells, which were about 8–15 μm in diameter, were not vitally stained with 0.015% trypan blue, although about 3% of the mast cells harvested were stained (Fig. 6A). Most of the mast cells exposed to braxin A1 were vitally stained with the dye during incubation for 10 min at 37°C (Fig. 6B); the stain was light blue in the cytoplasm and dark blue in the nucleus. These cells looked greatly swollen, without visible degranulation. Mast cells exposed to X537A were also vitally stained with the dye, but not those exposed to compound 48/80.

Under the scanning electron microscope, the unstimulated mast cells had large branching ruffles and cytoplasmic folds on the entire cell surface (Fig. 8A). Various morphological changes had occurred in mast cells exposed to braxin A1 (Fig. 8B). Common features were a marked swelling of the cells and protrusion of the cytoplasmic granules. The cells took on a so-called raspberry-like shape (18). In the cell membranes, tiny bridges had formed, but the branching ruffles and cytoplasmic folds were smaller and fewer. The granules, which had swollen to various sizes, seemed still to be covered with the cytoplasmic membrane. The morphological changes on the cell surface of mast cells exposed to X537A were similar to those exposed to braxin A1, although the swelling was less than with braxin A1. In agreement with the observations by Bytzer et al. (18), mast cells exposed to compound 48/80 had many holes and flaps in the cytoplasmic membrane resulting from the exocytotic process.

![Fig. 6. Effects of 2,4-dinitrophenol (2 mM; hatched column) and theophylline (20 mM, dotted column) on the histamine release from rat mast cells by braxin A1 (BA1, 3 mg/ml), X537A (30 μg/ml) and compound 48/80 (C48/80, 10 μg/ml). Controls (open column) were performed without inhibitors. The cells were exposed to an inhibitor for 10 min at 37°C and then incubated with a secretagogue added for 5 min at 37°C. ***P<0.001, compared with the control value. Each value represents the mean±S.E. of 4 separate experiments.](image2)
Fig. 7. Light micrographs of rat peritoneal mast cells incubated without (A) or with braxin A1 (3 mg/ml, B) for 10 min at 37°C. The cells were stained with 0.015% trypan blue. Note swelling and staining in mast cells exposed to braxin A1 (B). The horizontal bars represent 20 µm.

Fig. 8. Scanning electron micrographs of rat peritoneal mast cells unstimulated (A) or exposed (B) to braxin A1 (3 mg/ml) for 10 min at 37°C. The appearance of unstimulated cells showed large branching ruffles and cytoplasmic folds, and the cells exposed to braxin A1 swelled and acquired a raspberry-like shape upon protrusion of the swollen granules. The horizontal bars represent 2 µm.

Discussion

The present study confirmed the previous results (11) that braxin A1 has a dose-dependent histamine-releasing action on rat peritoneal mast cells. Mast cells respond to various agents and toxins by releasing histamine, heparin, and other chemical mediators with or without exocytosis. The secretory response requires a higher dose compared with the dose of secretagogues needed for secretory cells to release hormones or digestive enzymes. Among the histamine releasing agents, it is also known that many agents with adverse actions such as nicotine (19) or morphine (20) require higher doses compared with the doses of calcium- and energy-dependent agents such as compound 48/80 or the ionophore A23187, although a few exceptional agents such as dextran (21)
exist. The effective concentration of braxin A1 was the same order as that of these agents. Unlike with compound 48/80, the histamine release induced by braxin A1 was slow, and it did not depend on extracellular calcium or metabolic activity. It was also observed under the scanning electron microscope that most granules were still on the inside of the cells exposed to braxin A1, even when histamine release had reached 100%. These results suggest that the mechanism of histamine release induced by braxin A1 is independent of exocytotic processes, whereas the released induced by compound 48/80, is considered to involve calcium-mediated exocytosis that requires energy (22), in a similar manner to the ionophore A23187 (23), sinomenine (24) or antigen (25–28).

Diamant et al. (29, 30) have reported that the mechanism of histamine release induced by X537A could be distinguished from that by the releasing agents like compound 48/80 or A23187, although it was considered to be similar to that by the calcium- and energy-dependent releasing agents. The present results agree with findings that the secretory action of X537A affects heat-treated mast cells (31) and mast cell granules (30, 32), and that this action is not affected by metabolic inhibitors or the omission of extracellular calcium (31). In the present experiments, the histamine release evoked by braxin A1 resemble that by X537A in the following points: Both braxin A1 and X537A were effective in causing histamine release from rat mast cells incubated at 49°C or pre-incubated at 48°C. Mast cells exposed to braxin A1 and X537A were vitally stained with trypan blue and also swelled. In addition, they were highly active in releasing histamine from isolated histamine-retaining granules (T. Saito et al., unpublished data). These facts suggest that braxin A1, as well as X537A or chlorpromazine (33), has a cytotoxic effect on plasma and perigranular membranes to increase permeability and to release histamine. In addition, the action of braxin A1 could be distinguished from the simple lytic effect of decylamine, because braxin A1 did not release histamine at 0°C; decylamine causes histamine release at low temperatures (30, 34).

Some similarities were found in the histamine-releasing action of braxin A1 and X537A, as mentioned above, but the actions were quite different in their dependency on extracellular inorganic ions. Unlike X537A, the braxin A1-induced histamine release did not require any inorganic ions in the medium. X537A requires some monovalent cations in the medium to induce histamine release (31) and swelling (29), suggesting that both these effects of X537A may be the results of the ionophore-activity to carry monovalent cations across the plasma membrane with an influx of extracellular water as a direct consequence (29). Braxin A1 seemed not to have such an ionophore action, but to have a non-specific action on the plasma or perigranular membranes of mast cells to increase the permeability, resulting in the release of histamine.

In the experimental bracken poisoning in cattle, it was found that the blood levels of histamine (5), heparin (5, 6), or heparin-like substances (35) were increased with a blood coagulation disorder. The previous paper (14) showed that braxin A1 produced the bracken poisoning in guinea pigs, characterized by edematous and hemorrhagic changes in the urinary bladder. In addition, braxin A1 found to increase cutaneous vascular permeability in guinea pigs (T. Saito et al., unpublished data). Thus, the release of chemical mediators, histamine, heparin or others from mast cells by braxin A1 may be involved in the development of the clinical symptoms of bracken poisoning, which include edema, hemorrhage, and hemostatic disorders.

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