Magainin-2 Releases Histamine from Rat Mast Cells (42989)

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Abstract. **The magainins are basic 23 amino acid peptides with a broad spectrum of antimicrobial activity. Their bactericidal effect has been attributed to their capacity to interact with lipid bilayer membranes. We observed histamine release by magainin-2 amide from rat peritoneal mast cells (ED_{50} = 13 μg/ml) but not from human basophils. This histamine-releasing reaction from peritoneal mast cells was due to a secretory rather than cytolytic effect, i.e., release occurred without concomitant liberation of lactic dehydrogenase. Furthermore, the pretreatment of mast cells with magainin-2 amide did not desensitize cells against subsequent challenge with other secretagogues. Maximum histamine release occurred in less than a minute at 25 and 37°C. The addition of Ca^{2+} was not required for histamine release, although release was enhanced by the addition of 0.3–1 m*M* Ca^{2+}. The addition of 3 m*M* Ca^{2+} or Mg^{2+} was markedly inhibitory. The presence of Na^+ or Cl^- ions in the medium was not required for release. Therefore, histamine release is not due to the formation of anion-selective channels in the membrane of mast cells. The results indicated that the characteristics of histamine secretion induced by magainin-2 amide were unlike IgE-mediated release but were similar to the mechanism of release attributed to some other basic peptides and to compound 48/80.**
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A variety of biologically active peptides have been isolated from the skin of the clawed toad *Xenopus laevis* (1–3). These include two antibiotic peptides that have been named magainins. Magainin-1 and 2 each have 23 amino acids, differing by only 2 substitutions (1). They are water soluble, amphiphilic, nonhemolytic, and at low concentrations can inhibit the growth of many species of bacteria and fungi. Magainin-2 is the more active of the two peptides in bactericidal assays (2). We studied the histamine-releasing activity of magainin-2 amide after it was recognized that its structure is similar to other basic peptides that have mast cell-activating properties. Among these other secretagogues are the neuropeptides substance P and neurotensin (4), and the luteinizing hormone-releasing hormone (LHRH) as well as some of its structural analogs (5).

The release of histamine from mast cells and basophils serves as a model for secretory cell function. Histamine is stored in granules within the cell and release occurs by exocytosis. Stimuli for these cells include the cross-linking of IgE molecules by antigen, antibody to IgE, or by the addition of chemotactic peptides such as C5a (6) or formylated, methionine-containing di- and tri-peptides (7). In this report we describe the characteristics of histamine release from rat mast cells activated by magainin-2 amide and discuss the relationship of its structure to histamine-releasing activity.

Magainin-2, like several other basic peptides, releases histamine by a noncytolytic mechanism independent of added Ca^{2+}. Histamine is not released from human basophils. The present data demonstrate that magainin-2 amide, its nonbactericidal D-Lys, D-Phe-substituted analog Z-12, and the LHRH analog Nal-Arg share characteristics of their histamine-releasing activity that are similar to those of the nonpeptide polymer compound 48/80.

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Materials and Methods

Buffered Media. Pipes-buffered saline (Pipes AC) contains 119 m*M* NaCl, 5 m*M* KCl, 25 m*M* piperazine-*N*,*N*′-bis-2-ethanesulfonic acid (Pipes) NaOH to pH 7.3, 0.3 m*M* $CaCl_2$, 5.6 m*M* glucose, and 0.1% bovine serum albumin. For experiments with human basophils, the glucose was omitted and the bovine albumin replaced by 0.3% human albumin. For ion replacement experiments, the standard medium was modified to contain 10 m*M* Pipes adjusted with sufficient Tris base to achieve a pH of 7.3 at 25°C and salt-free (dialyzed)

bovine serum albumin (0.03%). Solid $CaSO_4$ was added to the solutions to achieve 0.1 m*M* Ca^{2+} as measured with a calcium ion electrode (Radiometer F220; The London Co., Cleveland, OH). The sugar or salt content of each medium was adjusted to 290 mOsmol/kg H_2O as measured with an Osmette Automatic Osmometer (Precision Systems, Inc., Sudbury, MA). Thus, there was an isosmotic substitution of the NaCl-KCl of the standard medium by other salts or sucrose. Twice (glass) distilled water was used for all media.

Mast Cells and Basophils. Male Sprague-Dawley rats (240–270 g) were purchased from Harlan (Madison, WI), sacrificed by carbon dioxide inhalation, and the peritoneal cavity lavaged with 35 ml of Pipes AC to collect mast cells. Following centrifugation at 200*g* for 8 min at 4°C, cells were washed again and resuspended to a concentration of 8–24 × 10^5 total leukocytes/ml in Pipes AC. This suspension contained 5–10% mast cells. Where specified, peritoneal cells were purified to approximately 95% mast cells by centrifugation through percoll (8). Mast cells were enumerated by staining with toluidine blue. Rat basophilic leukemia cells (RBL-2H3) were cultured for 2 days at 36°C in 5% CO_2 in Eagle's minimal essential medium supplemented with 15% fetal calf serum. Cells (approximately 10^5/well) were then transferred to 24-well plates which were then incubated 24 hr prior to the histamine release reaction (9). Human leukocytes were obtained from nonallergic healthy donors, washed, and resuspended in Pipes AC medium for the histamine release reaction (10).

Secretagogues. Magainin-2 amide as used in this study is (Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser) amide and Z-12 is its analog [D-Lys^{4,10,11,14}, D-Phe^{5,12,16}] magainin-2 amide. Both were supplied by Dr. M. Zasloff (Childrens Hospital, Philadelphia, PA) and had a purity of >95% as determined by high-performance liquid chromatography. The LHRH analog Nal-Arg([N-Ac-D-Nal(2)′,D-pF-Phe^2,D-Trp^3,D-Arg^6]LHRH) was obtained from Dr. J. Rivier (Salk Institute, La Jolla, CA). Compound 48/80 was purchased from Sigma (St. Louis, MO). Calcium ionophore A23187 was purchased from Calbiochem-Behring (San Diego, CA). Affinity purified antibody to human IgE (epsilon) was purchased from Kirkegaard and Perry Labs (Gaithersburg, MD). Peptides were dissolved in H_2O at a concentration of 2 mg/ml and stored at −20°C until used.

Histamine Release. Secretagogues were diluted (0.01–300 μg/ml) in Pipes AC medium, prewarmed to 37°C, and incubated in 12- × 75-mm polystyrene tubes with an equal volume (0.1 ml) of rat mast cells or basophils suspended in Pipes AC. After 15 min (45 min for basophils), 0.3 ml of ice-cold Pipes AC was added and the tubes were centrifuged to sediment cells. To RBL-2H3 cells on plates, 0.5 ml of the histamine-releasing agent was added and the release reaction carried out for 45 min at 36°C. Histamine in the supernatant was assayed by the automated fluorometric method (10). Spontaneous release in all experiments was <5% of the total histamine. Results are expressed as the percentage of maximum release obtained with leukocytes treated with 3% perchloric acid.

Desensitization Reaction for Mast Cells. Cells were first incubated in an ice water bath for 2 hr at 1°C with the indicated concentrations of secretagogue, washed once in Pipes AC at 1°C, the pellet resuspended to 0.1 ml in Pipes AC, and then challenged with 0.1-ml dilutions of magainin-2, Z-12, Nal-Arg, or compound 48/80 to release histamine by incubation for 15 min at 37°C (7). Supernatants from the first incubation were assayed to ascertain that there was no histamine release in the initial step.

Lactic Dehydrogenase (LDH) Assay. LDH release from purified mast cells was determined by the conversion of lactate to pyruvate using an automated procedure (11). The same samples tested for histamine were assayed for LDH with the exception that total LDH release was achieved by the addition of an equal volume of Brij detergent (Sigma Chemical Co.) diluted 1:100 in H_2O.

Results

Histamine Release. As shown in Table I, magainin-2 was active in releasing histamine from rat peritoneal mast cells (ED_{50} = 13 μg/ml) as was the nonbactericidal control peptide Z-12 (ED_{50} = 4.6 μg/ml). Both magainin-2 and Z-12 failed to release histamine from human basophils. Nal-Arg and compound 48/80 were highly active against peritoneal rat mast cells (ED_{50} of 0.19 and 0.45 μg/ml), yet both failed to release from human basophils. The amphiphilic peptide

Table I. Histamine Release from Rat Mast Cells and Human Basophils by Magainin-2 and Other Agents[a]

Agent	ED_{50}[b] (μg/ml)	
	Rat mast cells	Human basophils
Magainin-2	13 ± 3.2	>300[c]
Z-12	4.6 ± 1.7	>300
Melittin[d]	4.1 ± 4.3	32 ± 18
Nal-Arg	0.19 ± 0.12	>300
Compound 48/80	0.45 ± 0.17	>300
Goat anti-IgE_{hu}	NT[e]	0.53 ± 0.36

[a] Secretagogues at concentrations of 0.1–300 μg/ml were incubated in the presence of 0.3 m*M* Ca^{2+} with mast cells or basophils to release histamine.
[b] ED_{50}, concentration of agent giving 50% histamine release. Mean ± SD of 4–13 experiments done in duplicate. The concentration in micromoles required for 50% histamine release from rat mast cells: magainin-2, 5.3; Z-12, 1.9; melittin, 1.5; and Nal-Arg, 0.13.
[c] >300, less than 10% histamine release observed at a secretagogue concentration of 300 μg/ml or lower.
[d] Melittin is cytotoxic; e.g., the ED_{50} for LDH release from rat mast cells was 33 ± 5.0 μg/ml.
[e] NT, not tested.

melittin and the Ca^{2+} ionophore A23187 released from all types of cells tested. Goat anti-human IgE was used as a control for release from human basophils.

Magainin-2 is not cytotoxic for rat mast cells. Liberation of cytoplasmic LDH was measured to assess possible cell lysis (Fig. 1). Magainin-2, like peptides Z-12 and Nal-Arg, released histamine without causing release of LDH from purified mast cells. It is noteworthy that the 100 μg/ml concentration of secretagogues used here to test for LDH release was several fold higher than that required to achieve 50% histamine release (cf. Table I). Melittin was toxic for cells as evidenced by 100% release of LDH (Fig. 1). In other experiments (data not shown) magainin-2 released histamine from cultured RBL-2H3 cells (ED_{50} of 92 ± 28 μg/ml). However, this was accompanied by release of LDH and indicates toxicity toward these cells. In these experiments with RBL-2H3 cells, Z-12, Nal-Arg, and compound 48/80 did not release histamine or LDH.

Time and Temperature for Optimal Histamine Release. The release of histamine from rat mast cells by magainin-2 is very rapid; at 37°C the reaction reaches completion in less than 1 min (data not shown). This rapid time course was not appreciably influenced by changing the concentration of peptide from 3 to 100 μg/ml. Similar rapid release as obtained with Z-12, Nal-Arg, and compound 48/80. These agents were tested for release at temperatures between 1 and 37°C (Fig. 2). Maximum histamine release with these four secretagogues occurred at 25 and 37°C, with less occurring at 17°C and none at 1°C.

Effect of Divalent Cations on Histamine Release. Magainin-2 released histamine in the absence of added

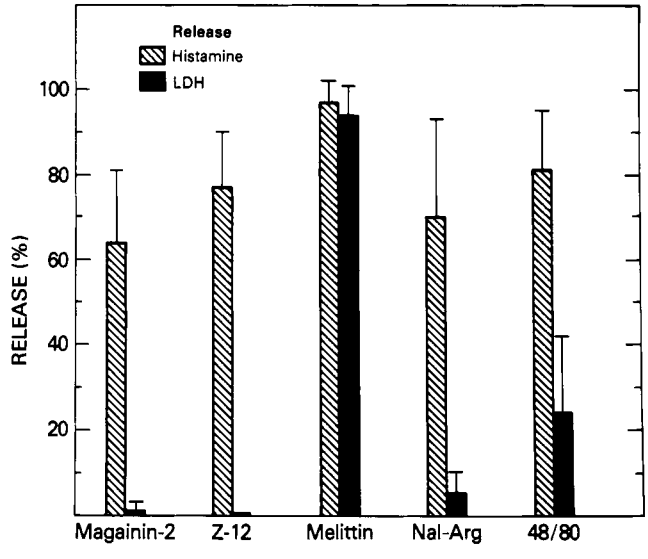

Figure 1. Histamine and lactic dehydrogenase release from rat mast cells with different secretagogues. Peritoneal cells were purified to contain 90–96% mast cells and incubated with secretagogues (100 μg/ml) at 37°C for 15 min. Supernatants were assayed for both histamine and lactic dehydrogenase release. The results were expressed as the mean and SD from four different experiments.

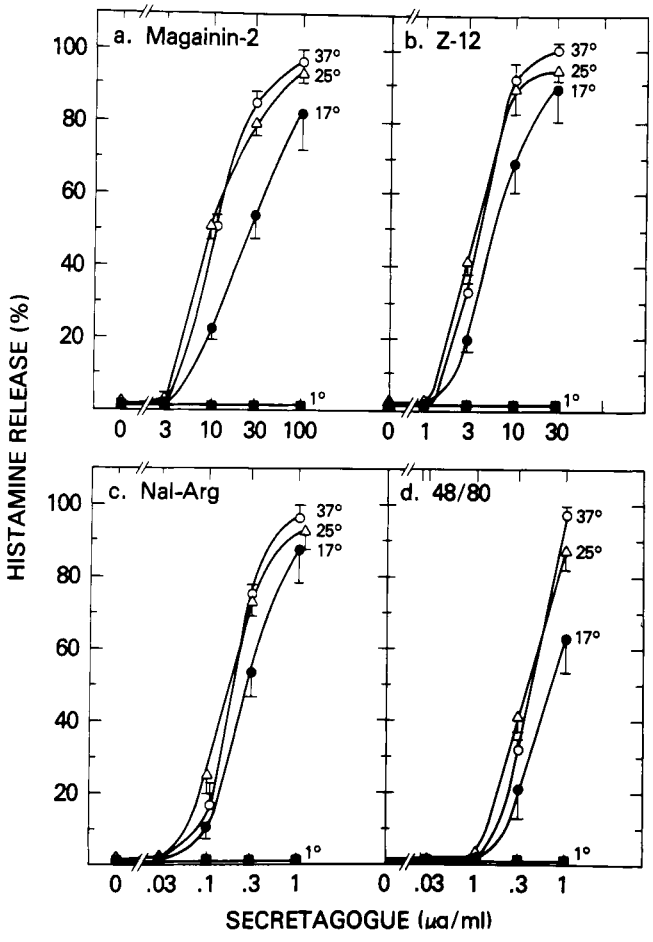

Figure 2. Temperature requirement for histamine release. Peritoneal rat mast cells and secretagogues were prewarmed separately in a water bath for 5 min at the indicated temperatures before combining and incubating for 15 min to release histamine. Results are the mean ± SD of duplicate samples from two different experiments.

Ca^{2+} or Mg^{2+}. In Figure 3a–d the abscissa gives the concentration of $CaCl_2$ added to the medium. Thus, there was little or no enhancement of release by magainin-2 when Ca^{2+} concentrations of 0.1–1 m*M* were added. However, with 3 m*M* of added Ca^{2+} there was a marked inhibition of release (Fig. 3a). The results were similar to the secretagogues Z-12, Nal-Arg, and compound 48/80 (Fig. 3b–d). In contrast, an IgE-mediated reaction using mast cells passively sensitized with antidinitrophenyl antibody and challenged with dinitrophenyl$_{44}$-human serum albumin required at least 3 m*M* added Ca^{2+} to achieve optimal histamine release (data not shown). It should be noted that the values on the abscissa represent calcium chloride added and that Pipes, like most buffering agents, chelates divalent cations. When an ion-specific electrode was used to measure the available calcium concentration, it was found that the ionized cation concentration was reduced by approximately two thirds. Thus, when 0.3 m*M* Ca^{2+} was added to the medium, only 0.1 m*M* was ionized. There was no requirement for Mg^{2+} with any of these

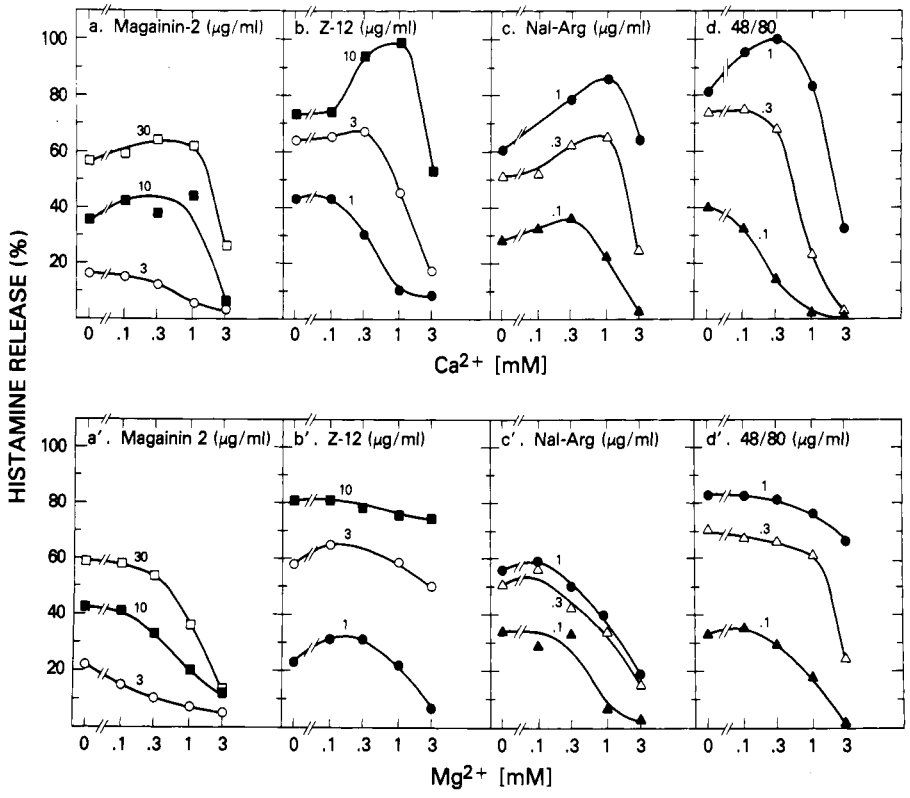

Figure 3. Influence of calcium and magnesium on the histamine release reaction. Rat mast cells and secretagogues were diluted in Pipes medium containing 0.04 m*M* EDTA and no added calcium or magnesium. $CaCl_2$ or $MgCl_2$ was then added to individual tubes to achieve the final concentrations listed on the abscissa. The ionized Ca^{2+} concentrations as measured by a specific ion electrode were 0, 0.03, 0.11, 0.43, and 1.1 m*M*, respectively. Tubes were incubated at 37°C for 15 min to release histamine. Results with the mast cells of one rat are shown and are representative of three experiments.

secretagogues. Mg^{2+} at a concentration of 1 m*M* or greater inhibited peptide-induced release (Fig. 3a'–d').

Effect of Ion Replacement on Histamine Release. The bactericidal effect of the magainins has been attributed to their capacity to form anion-selective channels. Therefore, we tested whether histamine release is due to the formation of anion channels by replacing the chloride in the medium with impermeant anions. Figure 4a shows the effect of replacing the NaCl-KCl of the standard medium with different potassium salts, both in the absence and presence of added Ca^{2+}. Without the addition of Ca^{2+}, the acetate, isethionate, and sulfate salts of potassium were effective in supporting histamine release. There was little release in media containing KBr or sucrose. On the other hand, when 0.3 m*M* Ca^{2+} was added (only 0.1 m*M* was available as ionized Ca^{2+}) all of the media supported release to an extent similar to the standard NaCl-KCl medium (Fig. 4b). Results with compound 48/80 were similar to those obtained with magainin-2 (Fig. 4c and d). In other experiments done at the same time, peptides Z-12 and Nal-Arg gave results similar to those achieved with magainin-2 and compound 48/80 (data not shown).

Magainin-2 does not Desensitize Mast Cells to Challenge. We previously reported that human leukocytes could be desensitized to C5a and f-Met-Phe-Met peptides by preincubation of cells with the homologous peptide under nonreleasing conditions (7). Therefore, experiments were done to test for cross-desensitization between magainin-2 and other mast cell secretagogues (Fig. 5). These agents were used at the indicated concentrations to pretreat cells before challenge. In Figure 5a the pretreated cells were challenged with magainin-2 and the control cells were pretreated with Pipes A medium only. No desensitization of cells to magainin-2 challenge occurred. The doses used for the desensitization step were approximately those amounts required to achieve 50% histamine release.

The inability to detect cell desensitization was also seen with peptides Z-12, Nal-Arg and compound 48/80 (Fig. 5). In some cases the dose-response curves shifted slightly to the left, indicating that a lower challenge dose was required to achieve 50% histamine release. This may have been due to residual peptide remaining after washing the cells. The inability to demonstrate a down-regulation of cells suggests that the release mechanism differs from that of basophils activated by f-Met peptides, C5a, or anti-IgE. Furthermore, it confirms that magainin-2, Z-12, Nal-Arg, and compound 48/80 were not toxic for mast cells because there was no inhibition of histamine release.

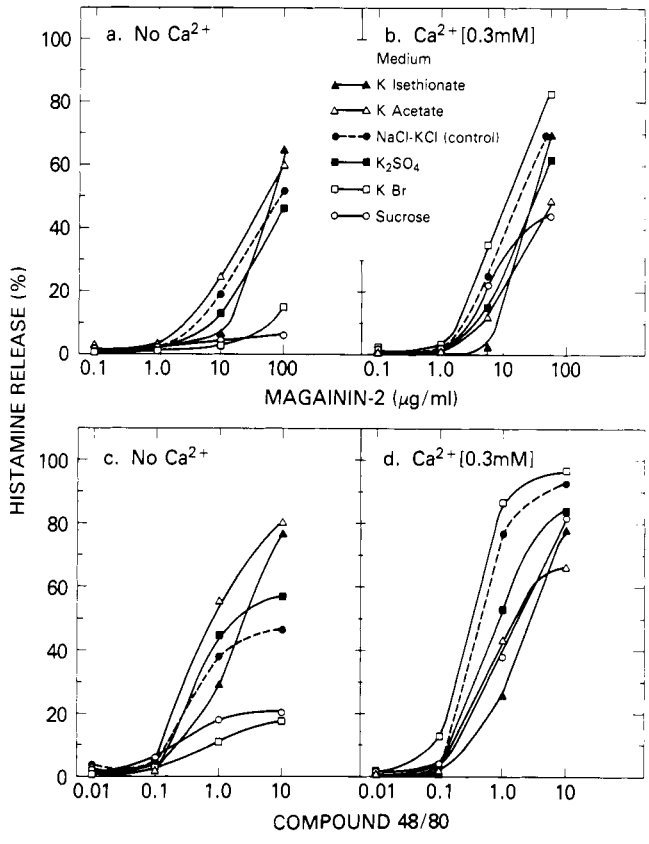

Figure 4. Effect of replacing the NaCl-KCl in the histamine release medium with other potassium salts or with sucrose. Isosmotic solutions of each medium buffered with Pipes-Tris to pH 7.3 were used to wash and suspend rat mast cells and to dilute magainin-2 or compound 48/80. Results are the mean values from four experiments.

Relationship between Magainin-2, Nal-Arg, and Compound 48/80-Induced Histamine Release. The capacity of mast cells from individual rats to release histamine with the aforementioned secretagogues was compared by calculating the coefficient of correlation. There was no significant correlation between the sensitivity of mast cells to release by magainin-2 and their response to Nal-Arg ($r = 0.20$) or to compound 48/80 ($r = 0.44$). On the other hand, there was a moderately strong relationship between cell sensitivity to Nal-Arg and compound 48/80 ($r = 0.85$).

Discussion

The magainins are a newly discovered family of peptides that may play a role in natural resistance to infection. Other antimicrobial peptides present in the glandular secretions of Xenopus species include the 21-residue peptide PGL[a] (3,12) and a 25-amino acid peptide derived from the xenopsin precursor XPF (14,15). These have a broad spectrum of bactericidal activity similar to the magainins (14).

Like certain other biologically active peptides, the magainin molecule may assume an α-helical formation after insertion into a lipid membrane, exhibiting on one face a hydrophobic surface and on the other a hydrophilic one. The magainins contain 23 amino acids, a size optimal for the formation of the transmembrane α helix. This helical structure may result from the contact of magainin with any hydrophobic environment (1,15). The D-lysine-substituted control peptide Z-12 is ineffective in antimicrobial assays (2); yet as shown here it is more active in releasing histamine from mast cells. Therefore, it may be concluded that an α helical configuration is not critical to achieve histamine release from mast cells.

Although the magainin structure suggests that it may be surface active, it does not cause lysis of human erythrocytes (1) and as shown here is not cytotoxic for mast cells. The antibacterial activity of the magainins may be related to their ability to form voltage-dependent anion (Cl^-) channels in cell membranes. There was good histamine release where the extracellular medium was free of any permeant anion (Fig. 4). Therefore, histamine release by the magainins does not appear to be dependent upon the formation of anion channels.

A variety of basic peptides, including the antibiotic

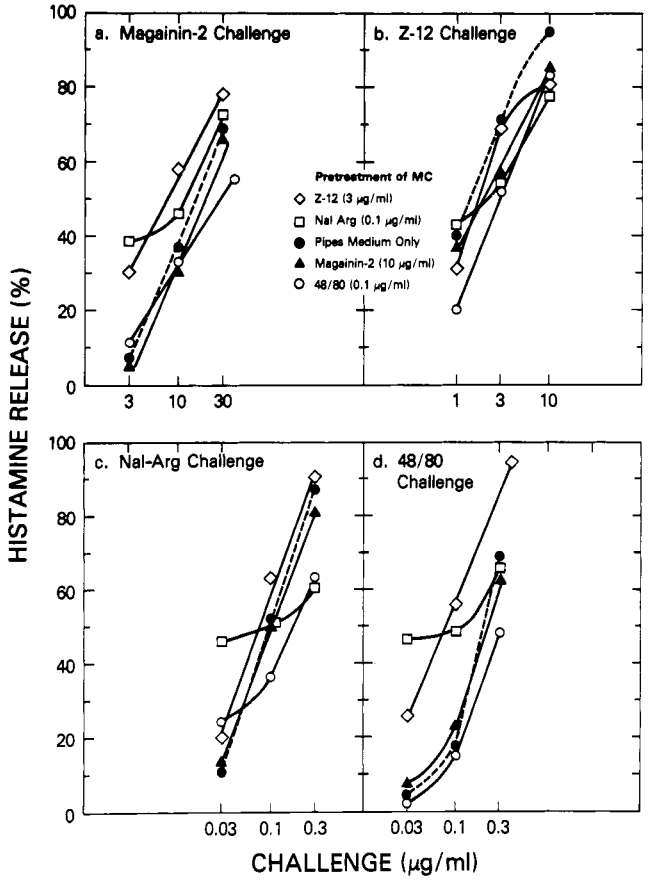

Figure 5. Magainin-2 does not desensitize mast cells against challenge by other secretagogues. Cells were incubated at 1°C for 2 hr in Pipes AC with peptides Z-12, Nal-Arg, magainin-2, or compound 48/80, followed by challenge with dilutions of the secretagogues at 37°C to release histamine. Results with the mast cells of one rat are given and are representative of three experiments.

polymyxin B, have been described as being capable of releasing histamine from rodent mast cells (16). In the case of the neuropeptide substance P, this characteristic has been attributed to the presence of two structural elements, a hydrophobic chain and a hydrophilic part with two positively charged amino acids (17).

We have extensively studied structural analogs of LHRH for their capacity to release histamine from rat mast cells (18). The structural characteristics of the most potent LHRH analogs in triggering histamine release *in vitro* involve a combination of a strongly basic D-amino acid side chain (Arg or Lys) at position 6 (in close proximity to Arg^8) and a cluster of hydrophobic aromatic amino acids at the N terminus. An example is the Nal-Arg used in these studies which has approximately 2000 times the histamine-releasing potency of LHRH. By themselves, two basic side chains in close proximity were insufficient to impart a high histamine-releasing potency to LHRH analogs (5,18). A limited number of LHRH analogs containing D-substituted amino acids have been studied for their histamine-releasing activity. In the case of Nal-Arg, a replacement of its D-Arg^6 with L-Arg^6 results in a 4-fold loss of histamine-releasing activity. Similarly, magainin-2 with all L-amino acids has about one third the histamine-releasing activity of the D-Lys, D-Phe-substituted analog Z-12. On the other hand, D-substituted analogs of magainin-2 retain their antimicrobial activity (15).

Neither Ca^{2+} nor Mg^{2+} ions were required to obtain near-maximum histamine release with magainin-2 (Fig. 3). This is in contrast to the IgE-mediated activation of mast cells which is dependent on the addition of 1 m*M* or more Ca^{2+} to achieve histamine release (16). The inhibition of the magainin-induced release by 3 m*M* Ca^{2+} or Mg^{2+} was unexpected. This inhibitory effect was also observed with 1 m*M* of added cation when the secretagogue concentration was less than the 50% histamine-releasing dose. On the other hand, at the higher concentrations of Z-12, Nal-Arg, or 48/80, 1 m*M* Ca^{2+} enhanced release. Therefore, the effect of cation concentration on histamine release is related to the amount of secretagogue used. The mechanism of this inhibition by divalent cations may involve an interaction with charged groups on the peptide.

The biologic significance of histamine release by magainin and the LHRH analogs is unclear. Because the magainin molecule contains a relatively large number of basic residues, it is not unexpected that it will cause histamine release from rat mast cells.

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