

Mechanisms of Nonimmunological Histamine and Tryptase Release from Human Cutaneous Mast Cells

Mette Veien, M.D.,* Fania Szlam, M.M.S.,† Jeannine T. Holden, M.D.,‡ Koji Yamaguchi, M.D.,*
 Donald D. Denson, Ph.D.,§ Jerrold H. Levy, M.D.||

Background: If mast cells are stimulated they release multiple mediators that delineate markers for immunologic and nonimmunologic reactions; histamine and tryptase are the two best known. Although histamine can be assayed in plasma, it is a nonspecific marker with a very short half-life. Tryptase has a longer half-life, but its release has not been proven to be specific for anaphylaxis. The authors investigated the mechanisms of nonimmunologic histamine release from human cutaneous mast cells to understand the mechanisms of mediator release and to determine whether tryptase was specific for allergic mediated activation.

Methods: Dispersed mast cell suspensions isolated from neonatal foreskins underwent challenge with vancomycin, calcium ionophore A23187, morphine, and atracurium, and histamine-tryptase release was measured. The effects of calcium and magnesium, along with phospholipase C and phospholipase A₂ inhibitors, also were investigated.

Results: Tryptase and histamine both were released by the known nonimmunologic stimuli (pharmacologic agents used in the current study; $r^2 = 0.6$). Furthermore, vancomycin- and atracurium-induced histamine release was calcium dependent. Phospholipase C and phospholipase A₂ inhibitors decreased vancomycin-induced histamine release, but not calcium ionophore A23187-induced release.

Conclusions: Tryptase is not a specific marker of mast cell

activation (*i.e.*, anaphylaxis), and signaling mechanisms for mast cell activation involve activation of phospholipase C and phospholipase A₂ pathways that are also involved in other cellular activation mechanisms. (Key words: Anaphylaxis; mediator release; phospholipases.)

ANAPHYLACTIC reactions represent complex acute inflammatory processes that are associated with significant morbidity and mortality rates. A key inflammatory cell in this clinical event is the mast cell. Mast cells can release histamine and tryptase through multiple mechanisms, including immunologic (by antigens) or nonimmunologic (by drugs or neurokinins) stimulation. Immunologic activation initiates the release of histamine and other mediators by a mechanism that takes 5 min to reach completion and needs influx of extracellular calcium.^{1,2} Although nonimmunologic mast cell activation is not well-understood, vancomycin,³ the calcium ionophore A23187,⁴ morphine,⁵ and atracurium^{6,7} consistently cause degranulation. A common feature of immunologic and nonimmunologic stimulation is the release of intracellular histamine. However, histamine is cleared within minutes from the circulation; in contrast, tryptase stays elevated for 1-2 h or more and appears to be released from mast cells with histamine. Although the physiologic role of tryptase as a mediator is not clear, measurement of tryptase concentrations potentially can provide a better diagnostic tool to evaluate suspected hypersensitivity reactions if sampling is delayed or as a more specific marker for mast cell activation.⁸ Schwartz *et al.*⁹ described that tryptase can be used *in vivo* as a diagnostic correlate of mast cell activation in systemic anaphylaxis and mastocytosis. However, there is some controversy about whether tryptase is released with histamine, especially if nonimmunologic degranulation occurs. Because tryptase is considered to be a better marker for immunologic activation, we investigated whether human cutaneous mast cells release tryptase with histamine if stimulated nonimmunologically with vancomycin, the calcium ionophore A23187, morphine, or atracurium.

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* Research Fellow.

† Research Associate.

‡ Assistant Professor of Pathology.

§ Associate Professor of Anesthesiology.

|| Professor of Anesthesiology.

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Address reprint requests to Dr. Levy: Department of Anesthesiology, Emory University Hospital, 1364 Clifton Road NE, Atlanta, Georgia 30322. Address electronic mail to: jerrold_levy@emory.org

MECHANISMS OF MEDIATOR RELEASE FROM HUMAN MAST CELLS

Little information is available about the mechanisms of nonimmunologic mast cell degranulation. An understanding of the nonimmunologic mechanisms of mast cell activation may provide therapeutic approaches to treating or preventing angioedema. Therefore, we investigated whether the mast cell degranulation was calcium dependent. In addition to histamine and tryptase, eicosanoids, such as prostaglandins and leukotrienes that arise from the metabolism of arachidonic acid, are also released after immunologic activation of cutaneous mast cells to produce inflammatory responses.^{1,5,10} Arachidonic acid production is thought to be associated with an activation of cytosolic phospholipase A₂ (PLA₂) in mast cells.¹¹ Because the calcium ionophore A23187 can activate the Ca²⁺-dependent cytosolic PLA₂, with a resultant increase in arachidonic acid, we evaluated the involvement of cytosolic PLA₂. We also evaluated the possibility that arachidonic acid arises *via* a phospholipase C (PLC) mechanism.

Materials and Methods

Isolation of Neonatal Mast Cells

Human foreskins were obtained after circumcision. The skin was immediately placed in Ca²⁺-Mg²⁺-free Hanks' balanced salt solution (CMF-HBSS; Sigma Chemical, St. Louis, MO) at 4°C and used within 24 h of circumcision.^{4,6,12-16} After removal of the subcutaneous fat the skin was weighed, chopped into 1- or 2-mm fragments, and washed twice with CMF-HBSS (10 ml/g) at room temperature. The tissue fragments were then incubated in CMF-HBSS with collagenase (20 mg/g), hyaluronidase (4 mg/g), and deoxyribonuclease (1,000 U/foreskin; Worthington Biochemical, Freehold, NJ) in a total volume of 5 ml/g wet weight of tissue for 3 h at 37°C, with constant stirring. After incubation the digested tissue was filtered through a 150- μ m pore-sized Nytex cloth (Tetko, Elmsford, NJ) and washed three times with 3.5% bovine serum albumin made in CMF-HBSS (Sigma Chemical).

Short-term Culture

Dispersed cells were resuspended in RPMI-1640 (Roswell Park Memorial Institute) culture medium (10 ml/g skin) with 25 mM HEPES (Sigma Chemical), 2 mM glutamine, 5% fetal calf serum (GIBCO, Grand Island, NY), 1,000 U/ml penicillin G, and 1% gentamicin (vol/vol; Sigma Chemical). The suspension was inoculated into a

Petri dish and incubated overnight at 37°C in humidified 95% air and 5% CO₂.

After overnight culture, the cells were harvested and washed three times with PAG buffer (5 ml/g), [Pipes, 25 mM piperazine-N,N'-bis(ethenesulfonic acid) (Sigma Chemical), 110 mM NaCl, 6 mM potassium chloride (KCL), and 0.1% dextrose, pH 7.4 at room temperature. They then were counted in a hemocytometer after staining with toluidine blue. Viability was assessed by trypan blue exclusion. If the assessed cell viability was less than 80%, the cells were discarded. A Wright-stained Cytospin preparation (Shandon Inc., Pittsburgh, PA) was used to determine the percentage of mast cells in each sample.

Histamine and Tryptase Release after Drug Challenge

One hundred-microliter aliquots of the cell suspension, containing between 2×10^4 and 5×10^4 mast cells/experimental tube were incubated in duplicate for 30 min at 37°C with the following drugs: 4.76×10^{-5} M calcium ionophore A23187 (Sigma Chemical); 3×10^{-3} M vancomycin (Vancocin HCl; Lilly, Indianapolis, IN); 10^{-3} M atracurium (Tracurium; Burroughs Wellcome, Triangle Park, NC); and 2.63×10^{-3} M morphine (Mallinckrodt, St. Louis, MO). The concentrations chosen were based on *in vitro* data³ and data applied to clinically relevant doses.¹⁷ To determine the effects of calcium on histamine release, cells were incubated either in buffer from which Ca²⁺ and Mg²⁺ were omitted or in buffer containing 2.8 mM Ca²⁺ and 1.0 mM Mg²⁺.

The release reaction was stopped by addition of 900 μ l ice-cold CMF-HBSS and centrifugation at 1,000g for 5 min at 4°C. Collected supernatants were split into two aliquots and frozen at -70°C until analysis time. The total-release tubes had 900 μ l of sterile ice water added instead. Total histamine-tryptase content of the skin mast cells was assessed by lysis of the cells by three cycles of freezing and thawing. The spontaneous release of histamine-tryptase was estimated from tubes to which buffer, instead of releasing agent, was added. Also, if the baseline release of histamine was greater than 15% of the total histamine release, the cells were deemed damaged during the preparation process and data were excluded. Histamine-tryptase release was calculated as percentage of total release. Net percentage of histamine-tryptase release is expressed as a percentage of total histamine-tryptase corrected for spontaneous release, according to the formula

$$\text{Net \%release} = (T - B)/T \cdot 100$$

where T is total histamine and B is spontaneous release.

Histamine concentrations were measured by the enzyme immunoassay method using commercially available kits (Immunotech, Westbrook, ME). Tryptase concentrations were measured using the UniCap 100 automated apparatus (Pharmacia and Upjohn, Kalamazoo, MI) and the UniCap 100 fluoroenzyme immunoassay.

Mechanisms for Nonimmunologic Degranulation

For elucidating the mechanisms of histamine and tryptase release, two different types of enzymatic inhibitors were studied using a stimulus-secretion paradigm, with histamine and tryptase release from the mast cells as the endpoint.

Phospholipase A₂ Inhibition by 3-(4-Octadecyl)-benzoylacrylic Acid. 3-(4-Octadecyl)-benzoylacrylic acid (OBAA; BIOMOL Research Laboratories Inc.; Plymouth Meeting, PA) is a potent inhibitor of PLA₂. Ten milligrams OBAA was dissolved in 9.34 ml absolute ethanol and split into 500- μ l aliquots that were stored at -70°C. Just before use, 40 μ l ethanol solution was further diluted with 1:1 solution (vol/vol) of ethanol-PAG to a final concentration of 24 μ M. For the inhibition studies, 5- μ l aliquots of the ethanol-PAG solution were added to 100 μ l cell suspensions (resulting in a final concentration of 1 μ M of the inhibitor). Tubes were preincubated for 30 min at 37°C before addition of vancomycin or calcium ionophore A23187, and the cell suspensions were incubated for an additional 30 min. Ethanol in PAG (2.08%) and OBAA without addition of drugs were used as controls.

Phospholipase C Inhibition by (1-(6-((17 β -3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U-73122). (1-(6-((17 β -3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U-73122; BIOMOL Research Laboratories Inc.) inhibits receptor-coupled generation of inositol 1,4,5-triphosphate and intracellular mobilization of Ca²⁺ (IC₅₀ [concentration of the inhibitor that causes 50% of inhibition] = 1.0–2.1 μ M) and exhibits little or no direct inhibition of PLA₂ and phospholipase D.

Five milligrams U-73122 was dissolved in 5 ml methylene chloride and split into 100- μ l aliquots, which were stored at -70°C. Just before use, methylene chloride was evaporated under nitrogen and U-73122 was redissolved in 150 μ l absolute ethanol. This was further diluted with 927 μ l PAG buffer, resulting in a 199.8- μ M U-73122 solution. For the inhibition studies, 5 μ l PAG-ethanol solution was added to 100 μ l cell suspension,

resulting in 8.36 μ M U-73122. Tubes were preincubated for 30 min at 37°C before addition of vancomycin or calcium ionophore A23187, and the suspensions were incubated for an additional 30 min. Ethanol in PAG (0.58%) and U-73122 without addition of mediators were used as controls.

Statistical Analysis

Results are presented as the mean and SD, and the number of experiments (n) is indicated. All two-group comparisons were performed using a paired or unpaired *t* test, with the level of statistical significance set at $P < 0.05$. Multigroup comparisons were made using one-way analysis of variance, followed by a Scheffé *post hoc* test for multiple comparisons, with $P < 0.05$ considered to be significant.

Results

Each foreskin yielded between 2×10^5 and 17×10^6 cells, with 4–8% being mast cells. In the presence of Ca²⁺ and Mg²⁺ in the incubating buffer, we found that vancomycin, calcium ionophore A23187, morphine, and atracurium resulted in significant histamine release (31 ± 5.1 , 60 ± 14.2 , 17.4 ± 10.2 , and $8.8 \pm 5.2\%$, respectively), with an average baseline release of $6.9 \pm 3.6\%$ (fig. 1). Tryptase release was 22.7 ± 6.8 , 18.1 ± 10.5 , 22.2 ± 13.0 , and 11.6 ± 6.3 , respectively, with an average baseline release of $10.8 \pm 5.9\%$ ($P < 0.005$; fig. 2). Also, there was a direct correlation between the net percentage releases of histamine and tryptase by vancomycin, morphine, and atracurium ($r^2 = 0.6$; fig. 3), but no correlation between net percentage releases of histamine and tryptase for calcium ionophore A23187.

If calcium and magnesium were omitted from the incubation buffer, histamine release from neonatal skin mast cells was decreased by 90% for vancomycin ($8.5 \pm 5.9\%$; baseline $6.0 \pm 4.0\%$) and virtually abolished for morphine ($5.7 \pm 2.5\%$; baseline $5.6 \pm 3.5\%$; $P < 0.05$). Conversely, calcium had no effect on release reaction of A23187 ($55.8 \pm 15.1\%$; baseline $6.5 \pm 3.7\%$; $P < 0.0001$) or atracurium ($5.4 \pm 2.5\%$; baseline $3.7 \pm 2.0\%$; $P = 0.0004$; fig. 4). Furthermore, there was a significant difference in net percentage histamine release between Ca-Mg(+) and Ca-Mg(-) groups if vancomycin ($P < 0.0001$) or morphine ($P = 0.0012$) was used as the mast cell activator, but no difference was seen with calcium ionophore A23187 ($P = 0.57$) or atracurium ($P = 0.85$; fig. 5).

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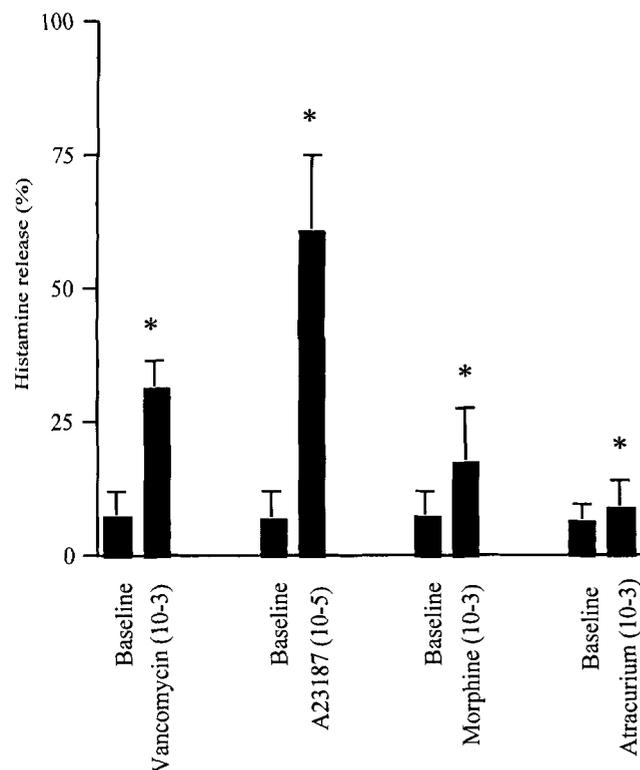


Fig. 1. Effects of vancomycin ($n = 7$), calcium ionophore A23187 ($n = 8$), morphine ($n = 8$), and atracurium ($n = 10$) on histamine release from human cutaneous mast cells in the presence of calcium and magnesium. The histamine release is calculated as (drug released histamine)/(total histamine content) \times 100%. The baseline for each group of drugs is presented together with the drug release. *Significant difference from baseline release ($P < 0.05$).

Preincubation of the dispersed mast cells with the secretory PLA₂ inhibitor OBAA attenuated vancomycin-induced histamine release from $19.4 \pm 8.5\%$ to $9.3 \pm 6.3\%$ (baseline: $6.0 \pm 2.7\%$; control: $5.8 \pm 2.3\%$; OBAA: $6.4 \pm 2.9\%$); whereas the calcium ionophore A23187-induced histamine release was not affected (77.0 ± 28.7 vs. $73.3 \pm 17.4\%$; baseline $7.3 \pm 2.0\%$; control: $7.1 \pm 1.1\%$; OBAA: $7.6 \pm 1.6\%$; fig. 6).

For the PLC inhibitor U-73122 we found a similar pattern as for the PLA₂ inhibitor: It abolished vancomycin-induced histamine release but not calcium ionophore A23187-induced histamine release. For the vancomycin group, data were as follows: baseline: $5.2 \pm 3.4\%$; control: $5.5 \pm 2.8\%$; U-73122: $8.9 \pm 3.8\%$; U-73122 with vancomycin: $9.3 \pm 3.8\%$; and vancomycin: $35.3 \pm 18.7\%$. For the calcium ionophore A23187 group, values were as follows: baseline: $7.3 \pm 3.0\%$; control: $5.7 \pm 2.3\%$; U-73122: $8.2 \pm 3.4\%$; U73122 and A23187: $80.4 \pm$

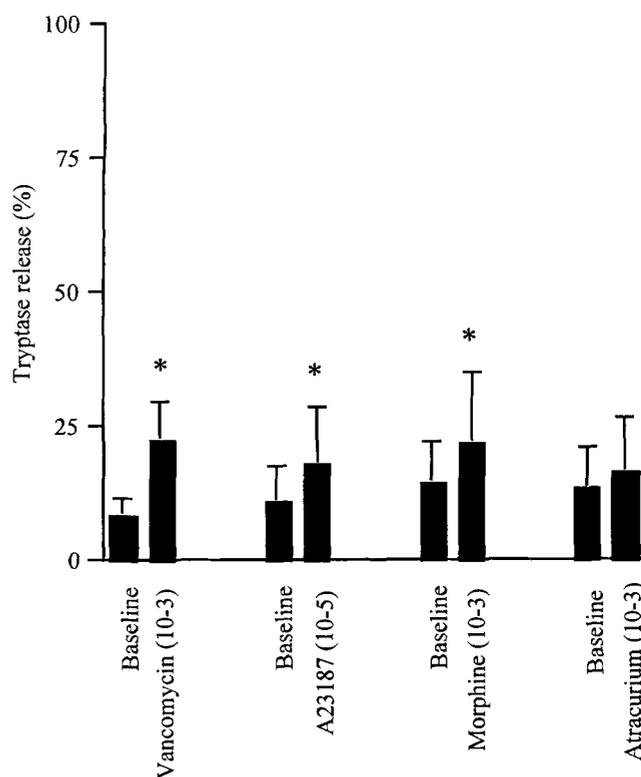


Fig. 2. Tryptase release for the experiments illustrated in figure 1. The tryptase release is calculated as (drug released tryptase)/(total tryptase content) \times 100%. The baseline for each group of drugs is presented together with the drug release. *Significant difference from baseline release ($P < 0.05$).

18.9% ; and A23187: $77.3 \pm 19.4\%$. The groups were compared using analysis of variance, and we found a significant release for vancomycin alone, for U73122 with A23187, and for A23187 ($P = 0.05$), compared with the baseline and the control (fig. 7).

Discussion

We found that tryptase is released in addition to histamine during nonimmunologic processes by known mast cell degranulators. Also, vancomycin, a prototypical mast cell degranulator, appears to be partially dependent on calcium and releases histamine and tryptase *via* PLC- and PLA₂-dependent mechanisms. This may be a result of either direct action on PLC and PLA₂ by vancomycin or stimulation of an unknown receptor mechanism. Our finding of significant tryptase release by vancomycin differs from the result described in a recent publication by Renz *et al.*¹⁸ Those authors looked at plasma levels of tryptase and histamine in patients receiving rapid vanco-

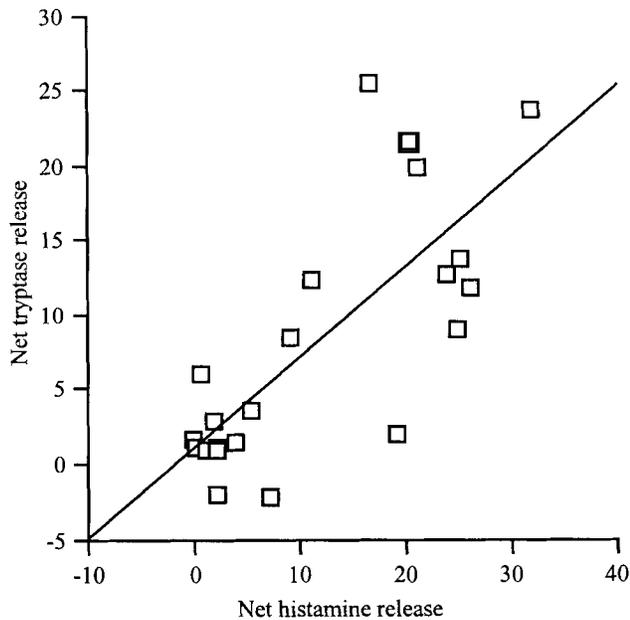


Fig. 3. Correlation between the histamine and tryptase release by vancomycin, morphine, and atracurium. The correlation is based on the calculated net histamine and tryptase release: (drug release-baseline release)/total mediator content \times 100%.

mycin infusion and saw increases in plasma histamine levels only. The difference in the results may be caused by either direct activation of mast cells *in vitro* or the fact that only *in vivo* basophil activation occurred. Conversely, mast cell degranulation induced by calcium ionophore A23187, which causes Ca^{2+} influx and releases Ca^{2+} from intracellular sites, such as the sarcoplasmic reticulum,^{19,20} was not inhibited by PLC and PLA_2 inhibitors or decreased in the absence of extracellular calcium. This extracellular calcium independence on the histamine release by A23187 is opposite to the response seen in the dispersed mast cells from human lungs. Church *et al.*²¹ reported that histamine release induced by the calcium ionophore A23187 was decreased by more than 90% if calcium was omitted from the incubation medium. Because mast cells are functionally heterogeneous and possess different characteristics,^{7,22} previously described pathways from animal experiments and nonconnective tissue mast cells may not be valid for human cutaneous infant mast cells, the cells used in this study.

Our mast cell degranulation results are in accordance with other studies. Levy *et al.*³ showed that vancomycin induced 22.9% histamine release. Benyon *et al.*²³ showed that calcium ionophore A23187 released 28.6% histamine. Stellato *et al.*⁵ showed a significant positive

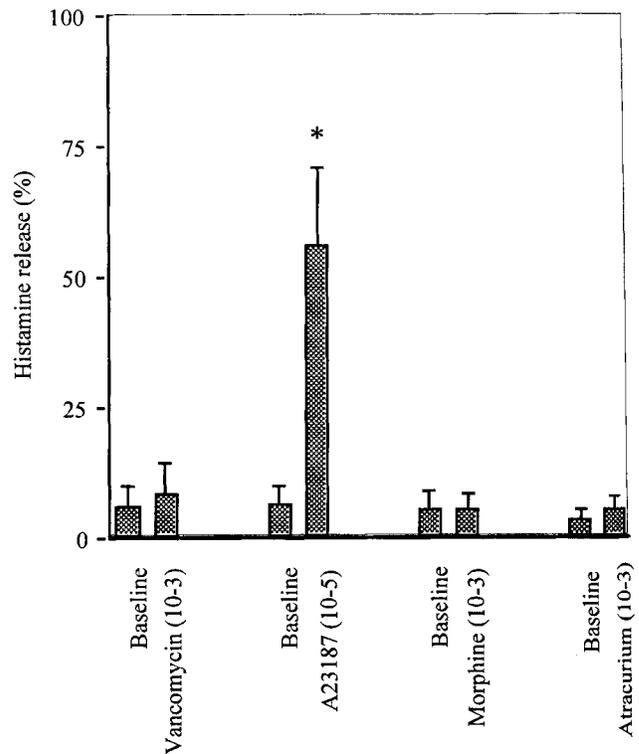


Fig. 4. Histamine release, if vancomycin ($n = 8$), calcium ionophore A23187 ($n = 11$), morphine ($n = 11$), or atracurium ($n = 9$) is added to human cutaneous mast cells without calcium and magnesium in the buffer. The histamine release is calculated as (drug released histamine)/(total histamine content) \times 100%. The baseline for each group of drugs is presented together with the drug release. *Significant difference from baseline release ($P < 0.05$).

correlation between the percentages of histamine and tryptase release induced by morphine from human skin mast cells, and the release was calcium dependent, and, as in our study, no release was observed if calcium was removed from the incubation buffer.¹³ Atracurium is a known histamine releaser, both *in vivo*²⁴ and *in vitro* (net histamine release 12% with 10^{-3} M),⁶ although another study reports that atracurium causes modest histamine release.²⁵ We also found only a modest release of histamine and tryptase after incubation with atracurium. Tryptase has been shown to be released with histamine from human heart and synovial mast cells, if stimulated immunologically *in vitro*,^{26,27} and others have shown that it is released with histamine after an immunoglobulin (Ig) E-mediated event *in vivo*.^{28,29} Stellato *et al.*⁵ showed a positive correlation between the percentage of histamine and tryptase release induced by buprenorphine or by anti-IgE from human lung mast cells. Watkins³⁰ previously defined the nonimmune mecha-

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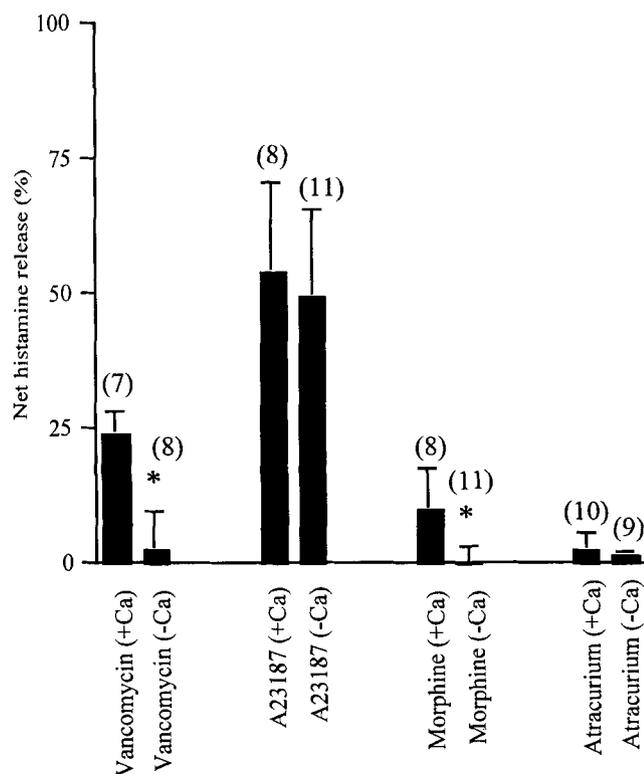


Fig. 5. Comparison of the histamine release with or without calcium and magnesium in the buffer. The comparison is based on the calculated net histamine release: (drug release - baseline release)/(total histamine content) \times 100%. The number above each bar graph represents the number of experiments for each drug. *Significant difference in the histamine release ($P < 0.05$).

nisms as absence of plasma tryptase but presence of increased urinary methylhistamine excretion. According to a study by Fisher and Baldo,³¹ increased concentrations of mast cell tryptase are not associated invariably with immunologic reactions. In a recent study, Edston and van Hage-Hamsten³² found elevated levels of tryptase in heroin addicts who died suddenly after injection, indicating a possible mast cell degranulation. There was no correlation between total IgE and tryptase levels, suggesting that heroin and morphine may have activated mast cells nonimmunologically.³² Data from our own *in vitro* study shows that tryptase also can be released, along with histamine, by nonimmunologic mast cell activation.

There was marked variability in histamine release from different foreskin preparations (all prepared using the same cell-dispersion protocol), suggesting that the ability to release influences the effect of the drugs on cutaneous mast cells. Furthermore, the number of mast cells iso-

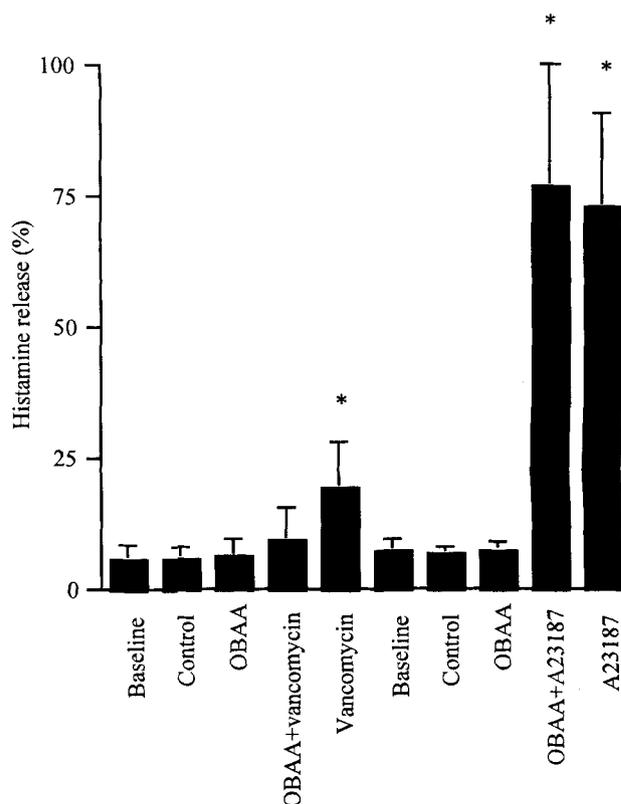


Fig. 6. Histamine release if the mast cells are preincubated with the phospholipase A₂ inhibitor 3-(4-octadecyl)-benzoylacrylic acid ($n = 6$). The histamine release is calculated as (drug-released histamine)/(total histamine content) \times 100%. *Significant difference from baseline or control release ($P < 0.05$). Comparison performed using analysis of variance.

lated per gram of skin varied markedly, which could be another factor that influences drug sensitivity or other nonimmunologic mast cell activators.

The mechanisms for nonimmunologic mast cell activation are not well-described but suggest a dose-dependent and reproducible noncytotoxic reaction.³³ Some authors describe the reaction *in vitro* to be independent of extracellular calcium¹; others describe it as partially calcium-independent^{23,34}; and still others find it to be dependent on extracellular calcium, with maximum release if incubated with 3-mM extracellular calcium.^{4,35-36,37} Our studies with neonatal, partially purified, mast cells showed that the release was partially dependent on extracellular calcium, except in the case of the calcium ionophore A23187, which can release calcium from intracellular stores.^{19,20}

Studies with peritoneal rat mast cells show that PLA₂ inhibitors can suppress histamine release but not prostaglandin release, if challenged with IgE or nonimmuno-

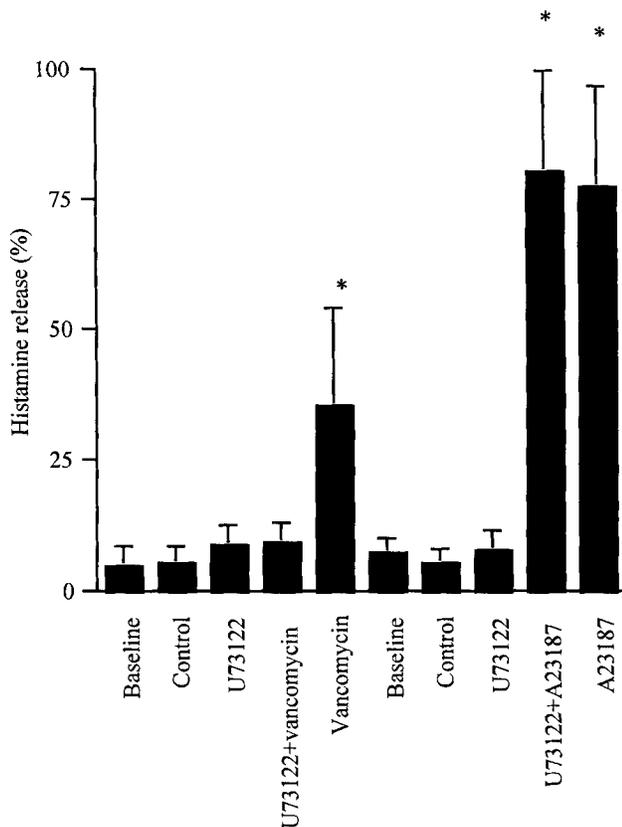


Fig. 7. Histamine release if the mast cells are preincubated with the phospholipase C inhibitor U-73122 ($n = 6$). The histamine release is calculated as (drug-released histamine)/(total histamine content) $\times 100\%$. *Significant difference from baseline or control release ($P < 0.05$). Comparison performed using analysis of variance.

chemical stimuli, such as calcium ionophore A23187, concanavalin A, compound 48/80, and substance P.³⁸ Murakami *et al.*³⁹ also showed that if rat mast cells are exposed to type II PLA₂, a significant release of histamine is observed. Our studies with the human cutaneous mast cell showed decreased histamine release if PLA₂ or PLC was inhibited (figs. 6 and 7). Elucidating pathways responsible for the histamine and tryptase release requires further studies to obtain a better understanding of *in vivo* and *in vitro* mast cell activation.

Laroche *et al.*⁴⁰ measured concentrations of plasma histamine and tryptase after exposure to radiocontrast media in 20 patients with mild to severe reactions. Increased plasma histamine levels developed in 14 patients and increased tryptase levels developed in 16.⁴⁰ Histamine and tryptase concentrations correlated with the severity of the reaction. The authors also reported the presence of IgE against the radiocontrast agents, al-

though radiocontrast reactions traditionally have been considered to be nonimmunologic anaphylactoid reactions. Tests to determine IgE antibodies against radiocontrast agents are not validated; the authors did not perform inhibition studies to further validate their assay; and radiocontrast reactions occurred without previous exposure, suggesting that the reactions they noted were anaphylactoid.

Based on our *in vitro* study, we conclude that tryptase is released with histamine during nonimmunologic mast cell activation and also can be an indicator for anaphylactoid reactions because it is only present in the cytoplasmic granules of mast cells.⁴¹ The mechanism of nonimmunologic histamine and tryptase release relies on membrane associated enzymes, such as PLA₂ and PLC. Although the physiologic role of tryptase is not clear, it is a neutral protease that can both activate and inactivate multiple inflammatory mediators. Further elucidation of direct molecular effects of mast cell activation may be important in understanding mechanisms of anaphylactoid reactions and other pathologic states of mast cell activation.

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