Stimulation of Histamine Release from Human Basophils by Human Platelet Factor 4

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ABSTRACT Human basophils were stimulated to release histamine noncytotoxically by purified human platelet factor 4 (PF4) and the synthetic substituent peptide PF4(59-70). Histamine release was augmented significantly by 10^{-7} M PF4 and 10^{-5} M PF4(59-70), increased in a concentration-dependent manner, and attained a maximum at 3×10^{-5} M PF4 and 3×10^{-4} M PF4(59-70) similar to that achieved by goat anti-human myeloma IgE. PF4 (1-60) failed to initiate the release of histamine, which confirmed that the critical determinant of activity is in the carboxy-terminal sequence. Histamine release from basophils by optimally effective concentrations of PF4 and PF4(59-70) reached a plateau by 1–3 min, as contrasted with 10 min or longer for anti-IgE. The elimination of calcium and magnesium from the buffer suppressed the release of histamine by anti-IgE by 79–83%, but had no effect on that elicited by PF4(59-70). The rate of uptake of [^{125}I]PF4 by purified basophils was similar on a molar basis to the rate of release of histamine by the same concentrations of PF4. The noncytotoxic release of histamine from human basophils by PF4 thus is temporally and biochemically distinct from that mediated by IgE and may be similar to that evoked by other polycationic stimuli.

INTRODUCTION

Platelet factor 4 (PF4)[1] is a 70-amino acid polypeptide that is stored in the α-granules of human platelets (1). Immunological stimuli, including immune complexes and phospholipid platelet-activating factors, and platelet agonists of other types evoke the release of PF4 in vitro and in vivo (2–4). PF4 not only appears in the circulation, in relation to acute activation of platelets by immunological and other stimuli, but also permeates the walls of blood vessels after endothelial cell injury (5) and thereby may accumulate at tissue sites of hypersensitivity reactions or inflammation. That some portions of the PF4 molecule are as basic as the small cationic peptides of polymorphonuclear (PMN) leukocyte granules that disrupt mast cells and basophils (6) suggested that PF4 also might have the capacity to activate basophils. The results of the present study indicate that PF4 stimulates human basophils to release histamine by a noncytotoxic and calcium-independent mechanism, which may represent the direct ionic displacement of histamine from the basophil granules by PF4.

METHODS

Heparin-Sepharose, Sephadex G-10, Ficoll-Hypaque, glass columns for chromatography (Pharmacia Fine Chemicals, Inc., Piscataway, NJ), Hypaque (Winthrop Laboratories, Inc., New York), recrystallized human serum albumin (Miles Laboratories, Inc., Elkhart, IN), histamine, protamine sulfate (salmon, grade X), reagents for the quantification of lactic acid dehydrogenase activity (Sigma Chemical Co., St. Louis, MO), ^{125}I-Bolton-Hunter reagent (New England Nuclear, Boston, MA), standard dansylated amino acids, carboxypeptidase Y (Pierce Chemical Co., Rockford, IL), *o*-phthalaldehyde (Dionex Co., Sunnyvale, CA), reagents for solid-phase peptide synthesis (Beckman Instruments, Inc., Palo Alto, CA), C18 Sep-Pak cartridges (Waters Associates, Bedford, MA), and solvents that had been redistilled from glass (Burdick and Jackson, Inc., Muskegon, MI) were obtained from the designated suppliers. Goat anti-human IgE serum was raised by immunizing a goat intradermally in multiple sites on the back with a total of 80 μg of partially purified Shackford myeloma IgE emulsified in 1 ml of 0.02 M Tris-HCl buffered 0.13 M NaCl (pH 7.6) and an equal volume of complete Freund's adjuvant (Gibco Laboratories, Grand Island, NY). 4 wk after a second immunization with an identical preparation of myeloma IgE by the same route, the goat was bled weekly and the sera tested for antibody content by basophil histamine releasing activity. At 8 wk after the second immunization, the dilution of serum re-

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[1] *Abbreviations used in this paper:* B-HSA, buffer with HSA; HSA, human serum albumin; LDH, lactic acid dehydrogenase; PF4, platelet factor 4; TFA, trifluoroacetic acid.

quired for maximum histamine release was 1/120. 200 ml of blood was then taken weekly for 4 wk and the sera were pooled, heated for 30 min at 56°C, sterilized by micropore filtration, and stored at −70°C in 2-ml aliquots. Each aliquot of serum was passed through a 10-ml column of Sepharose-human serum before use, which removed trace quantities of antibody to other immunoglobulins.

Purification of human basophils. Venous blood from normal subjects was collected in sodium citrate anticoagulant as described (7), layered in 20-ml portions on 10-ml cushions of Ficoll-Hypaque, and centrifuged at 400 *g* for 40 min at room temperature. The supernatant autologous plasma was removed and saved, and the basophil-containing mononuclear leukocytes at the plasma-cushion interface were transferred to clean test tubes, and washed twice and resuspended at 3×10^7/ml in 0.135 M NaCl-0.01 M Tris-HCl (pH 7.4) containing 0.002 M KCl, 0.001 M $CaCl_2$, 0.002 M $MgCl_2$, and 0.05 g of human serum albumin (HSA) per 100 ml (B-HSA). The basophil content of the mononuclear leukocyte suspensions was 2–9%, as assessed by differential counts of dried and methanol-fixed smears treated with Giemsa-Wright's stain. For some experiments, basophils were purified further by applying 10 ml of a mononuclear leukocyte suspension to a column of 5 ml of glass beads that was prewashed with 20 ml of distilled water and 10 ml of autologous plasma, which had been centrifuged at 5,000 *g* for 20 min at 4°C to remove platelets. After removing nonadherent cells by washing the column with 6 ml of autologous plasma, the basophils and other adherent cells were eluted in 10 ml of calcium- and magnesium-free B-HSA containing 0.1 g of disodium EDTA per 100 ml, and washed and resuspended in 10 ml of B-HSA. 1-ml portions of basophil-containing leukocyte suspension were layered on 4-ml linear gradients of 9.0–12.8 g of Hypaque per 100 ml of water with 0.1 g of HSA per 100 ml and centrifuged at 500 *g* for 10 min at room temperature. The basophils in the pellet were washed and resuspended in B-HSA; the purity ranged from 34–89% ($n = 8$).

Preparation of PF4 and substituent peptides. The source of PF4 was human platelets that were recovered from blood-bank outdated concentrates by centrifugation at 1,500 *g* for 20 min at 4°C. The platelets were washed twice in 0.154 M NaCl-3.8 *g* sodium citrate per 100 ml of water (9:1, vol/vol, pH 7.0), resuspended at a concentration of 7×10^9/ml in 0.05 M NaCl, and lysed by adjustment of the pH to 2.0 with 3 M HCl. After 20 min of stirring at 4°C, the lysate was centrifuged at 40,000 *g* for 20 min at 4°C, and the supernate was lyophilized and redissolved in 20 ml of 0.2 M Tris-HCl (pH 8.6). The PF4 was purified by adherence to a 30-ml column of heparin-Sepharose and elution in 4 ml of 2 M NaCl-0.2 M Tris-HCl (pH 8.6) as described (8). ^{125}I-PF4 was prepared by reacting PF4 with ^{125}I Bolton-Hunter reagent with a standard procedure (9) and removing the unreacted reagent by filtration of the ^{125}I-PF4 on a column of Sephadex G-10 in 2 M NaCl-0.2 M Tris-HCl (pH 8.6); the specific activity of the ^{125}I-PF4 was 33 Ci/mmol. Dr. Robert I. Handin (Brigham and Women's Hospital and Harvard Medical School, Boston, MA) generously supplied samples of PF4 and ^{125}I-PF4 for some studies.

PF4(59-70) and other substituent peptides were synthesized by standard solid-phase methods (10), purified by counter-current distribution and filtration on Sephadex G-10 in 0.1 M acetic acid, and assessed by amino acid analysis and determination of the amino acid sequence with standard Edman techniques (11).

PF4(1-60) was prepared by treating 70–100 μg of purified PF4 with carboxypeptidase Y (1:50, wt/wt) in 0.2 ml of 0.6 M NaCl-0.1 M sodium acetate (pH 5.0) for 1 h at 37°C. The mixture then was heated at 80°C for 15 min to inactivate the carboxypeptidase Y, before assessing the effects of PF4(1-60) on basophils. In initial efforts to establish the correct conditions, replicate samples of 7–10 μg of PF4 were treated with carboxypeptidase Y for different times and the amino acids released were dansylated and analyzed qualitatively by thin-layer chromatography as described (11). For each preparation obtained by the standardized method presented above, 10% of the solution of digested PF4 was diluted to 2 ml with 0.1% (vol/vol) trifluoroacetic acid (TFA) in water:methanol (70:30, vol/vol) and passed through a C18 Sep-Pak cartridge that had been activated with 20 ml of methanol and washed with 20 ml of 0.1% TFA in water and 10 ml of 0.1% TFA in water/methanol (90:10, vol/vol). The cartridge then was developed with 0.1% TFA in water/methanol (70:30) and 3 ml of effluent was collected, lyophilized, and subjected to amino acid analysis as described (11). Effluents contained 3.1–3.6 nmol lysine, 1.3–1.7 nmol isoleucine, 1.2–1.5 nmol leucine, 0.5–0.8 nmol glutamic acid, 0.2–0.6 nmol serine (range, $n = 2$), and <0.2 nmol of other amino acids.

Release of histamine from human basophils. Replicate suspensions of 1×10^7 basophil-containing mixed mononuclear leukocytes and of 2×10^5 purified basophils in 1 ml of B-HSA were incubated for 1–40 min at 37°C with buffer alone, PF4, PF4 substituent peptides, or goat anti-human IgE. The tubes were centrifuged at 100 *g* for 2 min at 4°C, the supernates transferred to clean test tubes, and the pellets resuspended in 1 ml of B-HSA and sonified at 4°C (200 W for 2 min; Branson Sonic Power, Inc., Danbury, CT). Histamine in the supernates and basophil pellet sonicates was quantified by a standard manual fluorometric assay (12), utilizing *o*-phthalaldehyde as the fluorophore. The percentage release of histamine was calculated from the ratio of the histamine in the supernate to the total of that in the pellet and the supernate, and net percentage histamine release was derived by subtracting the spontaneous release from unstimulated basophils in buffer alone. The release of lactic acid dehydrogenase (LDH) from replicate 1-ml suspensions of 2×10^5 basophils of 84 and 87% purity was assessed by quantifying the LDH activity in 100 μl of supernates and of sonicates of basophil pellets in 1 ml of buffer. The colorimetric assay for LDH (Sigma Chemical Co.) was based on the reaction of residual pyruvic acid with phenylhydrazone, the product of which absorbs at a maximum of 550 nm. The sonicates of basophil pellets gave a decrement in absorbancy of 0.092 at 550 nm, so that a release of 5% or more of the LDH would be detected reliably in the supernates.

RESULTS

PF4 and the substituent peptide PF4(59-70) stimulated the release of histamine from human basophils in a concentration-dependent manner (Fig. 1). The magnitude of histamine release was significantly greater than that in buffer alone at 10^{-7} M PF4 and 10^{-5} M PF4(59-70) and attained a similar maximal level at 3×10^{-5} M PF4 and 3×10^{-4} M PF4(59-70). The level of release of histamine by 10^{-3} M PF4(59-70) in two of the experiments was 41.8 and 38.5%, which indicates that the plateau had been reached at 3×10^{-4} M PF4(59-70). To examine whether the release of histamine by basophils was influenced by the mononu-

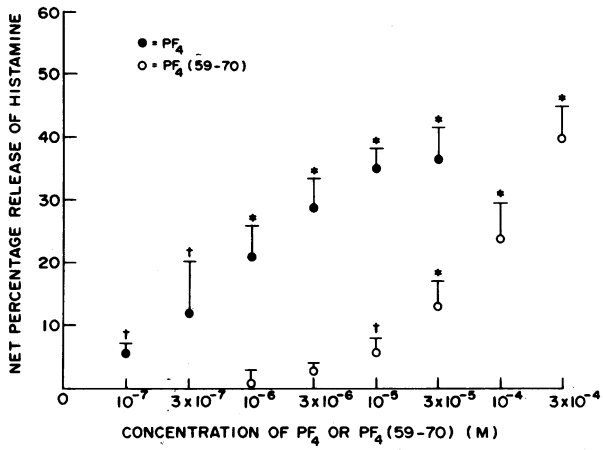

FIGURE 1 Relationship of the release of histamine from human basophils to the concentration of PF4 or PF4(59-70). Each point and bracket depicts the mean±SD of the results of four experiments with basophils from different subjects. The release of histamine from unstimulated basophils after 40 min at 37°C was 3.8±1.7% (mean±SD). The level of significance of the release of histamine by PF4 or PF4(59-70) relative to that in buffer alone was calculated by a standard t test and is represented by the symbols: † = $P < 0.05$ and * = $P < 0.01$.

clear leukocytes in the suspensions, basophils of 1.3, 6.5, and 51% purity, respectively, were obtained from the same donor by centrifugation of mixed leukocytes on Ficoll-Hypaque alone and by the sequential application of adherence to glass beads of leukocytes from Ficoll-Hypaque and centrifugation on Hypaque gradients of leukocytes eluted from glass beads. The density of basophils was adjusted to 2×10^5/ml for each suspension before incubation with PF4 and PF4(59-70). The three suspensions of basophils of different purities released a mean of 19.3, 24.7, and 22.0% of the histamine, respectively, with 10^{-6} M PF4; 33.6, 37.5, and 35.2% with 10^{-5} M PF4; and 24.3, 23.0, and 28.4% with 10^{-4} M PF4(59-70). Neither PF4 nor PF4(59-70) was cytotoxic for basophils, as indicated by the lack of release of LDH along with maximal release of histamine. The absence of cytotoxicity of PF4 and PF4(59-70) was further suggested by the ability of anti-IgE to release histamine from basophils exposed previously to the peptides. Replicate suspensions of basophil-containing mixed mononuclear leukocytes that had been incubated for 15 min at 37°C with 10^{-5} M PF4 or 10^{-4} M PF4(59-70), washed twice and resuspended in release buffer, and challenged with a 1/20 dilution of anti-IgE released 42.6±11.2% (mean±SD, $n = 3$) and 37.8±6.5% histamine, respectively, as compared with 43.7±9.9% for basophils preincubated in buffer alone.

With optimally effective concentrations of PF4 and PF4(59-70), the release of histamine reached a plateau by 1–3 min, whereas the achievement of a similar level of release of histamine by anti-human IgE required at least 10 min at 37°C (Fig. 2). The difference in the time-course of release of histamine by PF4 and anti-IgE was not a function of the magnitude of stimulation. When basophil-containing mononuclear leukocytes were challenged for 1, 3, 10, and 20 min, the mean release of histamine by 10^{-6} M PF4 was 18.6, 17.9, 21.3, and 19.8%, respectively, and by a 1/20 dilution of anti-IgE was 14.5, 22.1, 37.0, and 44.6%. That the release of histamine by PF4 and anti-IgE is additive was illustrated by the results of the effects of the two stimuli together. When replicate portions of basophils were challenged for 40 min at 37°C with 3×10^{-6}, 10^{-5}, and 10^{-4} M PF4(59-70), the mean release of histamine was 2.9, 6.5, and 23.6%, respectively, without anti-IgE. The concurrent introduction of a 1/40 dilution of anti-IgE, which alone released a mean of 19.6% of the histamine, raised the values for the respective concentrations of PF4(59-70) in a simple additive manner to 20.8, 24.3, and 41.2% histamine release.

The mechanisms of histamine release from human basophils by PF4 and anti-IgE were distinguished by a difference in the dependence on extracellular divalent cations (Fig. 3). The magnitude of histamine release by two concentrations of PF4(59-70) in calcium- and magnesium-free buffer containing 5 mM EDTA

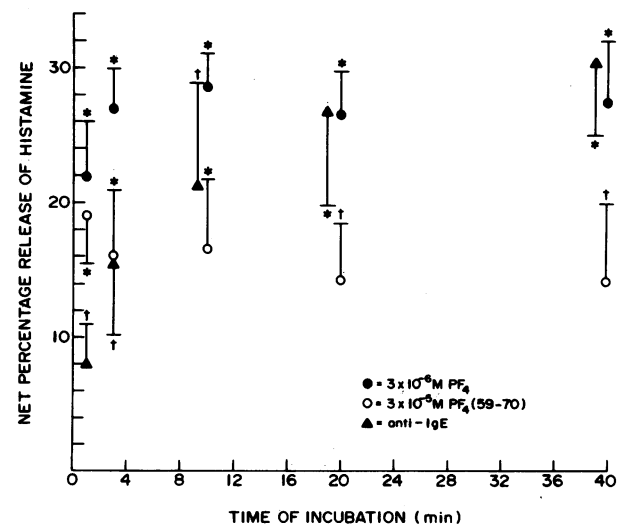

FIGURE 2 Time-course of the release of histamine from human basophils by PF4, PF4(59-70), or goat anti-human IgE. Each point and bracket depicts the mean±SD of the results of three experiments with basophils from different subjects. The concentrations of PF4 and PF4(59-70) were 3×10^{-6} and 3×10^{-5} M, respectively, and the dilution of anti-IgE was 1/30. The symbols denoting statistical significance are the same as in Fig. 1.

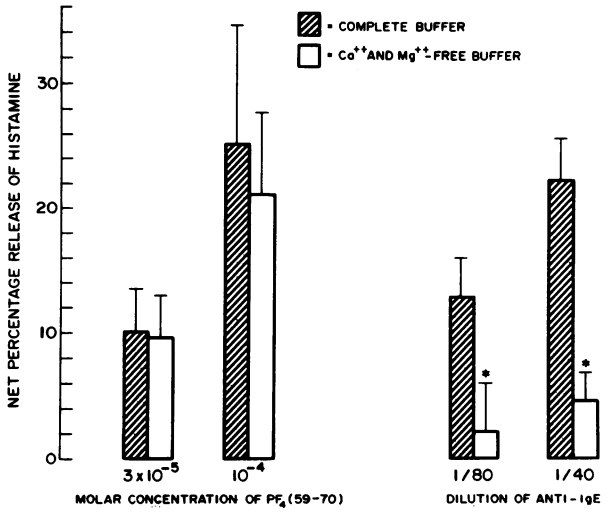

FIGURE 3 Relationship of the release of histamine from basophils to extracellular divalent cations. Each bar and bracket depicts the mean±SD of the results of three experiments with basophils from different subjects incubated in complete buffer or calcium- and magnesium-free buffer containing 5 mM EDTA. The symbols denoting statistical significance are the same as in Fig. 1.

was not significantly less than that observed in complete B-HSA. In contrast, histamine release by two concentrations of anti-IgE was suppressed by 79–83% in the absence of calcium and magnesium.

That a similar maximal release of histamine from human basophils was evoked by PF4 and PF4(59-70) suggested that the molecular determinants critical for basophil activation are located in the carboxy-terminal portion of PF4, although other sequences in PF4 may contribute to histamine-releasing potency. PF4(1-60), which lacks the carboxy-terminal 10 amino acids, exhibited one-fifth or less of the activity of native PF4 at concentrations of 10^{-6}–3×10^{-5} M, and failed to evoke a response equivalent to that observed with 3×10^{-7} M PF4 at a concentration as high as 10^{-4} M (Table I, Fig. 1). PF4(59-70) exhibited 1/100–1/30 the potency of PF4, while the carboxy-terminal pentapeptide PF4(66-70) retained only ~1/10 the potency of PF4(59-70).

To investigate the possibility that the effect of the strongly cationic PF4 on basophils was attributable to specific uptake into the granules and charge displacement of histamine from the matrix of the granules, the uptake of [^{125}I]PF4 by highly purified human basophils was examined at 2–30 min (Table II). Uptake of [^{125}I]PF4 by basophils was detectable at 10^{-8} M and increased in direct relation to the concentration of [^{125}I]PF4. After 2 min at 37°C, the extent of uptake of [^{125}I]PF4 by basophils reached a level that was 53–88% of that observed after 30 min of incubation with the same concentration of [^{125}I]PF4. The rapid apparent saturation of basophils with PF4 paralleled the time-course of release of histamine by PF4 and PF4(59-70) (Fig. 2).

DISCUSSION

The exposure of human basophils to purified PF4 initiates the release of histamine, which at PF4 concentrations of 10^{-5} M and higher is equivalent in magnitude to the maximal response elicited by anti-IgE (Fig. 1). That the histamine-releasing activity of PF4 is attributable predominantly to the cationic carboxy-terminal sequence of PF4 is suggested by the capacity of synthetic PF4(59-70) to exert a maximal effect similar to those of intact PF4 or anti-IgE (Fig. 1, Table I). The potency of PF4(59-70) is ~1–10% of that of PF4. In contrast, PF4(1-60) and PF4(66-70) exhibit only ~0.1% or less of the potency of PF4. The noncytotoxic release of histamine from basophils by PF4 and PF4(59-70) is distinguished from that achieved by anti-IgE, both in terms of the time-course and the

TABLE I
Peptide Structural Determinants of the Release of Histamine from Human Basophils by PF4

	Molar concentration of stimulus					
Peptide stimulus	10^{-6}	3×10^{-6}	10^{-5}	3×10^{-5}	10^{-4}	3×10^{-4}
PF4	21.0±5.2*	29.0±4.6	35.3±3.1	36.8±2.9	39.4±4.7	—
PF4(59-70)‡	0.9±1.7	2.7±1.3	5.8±2.1	13.3±3.8	24.1±5.6	40.2±4.8
PF4(66-70)§	0.2±0.6	1.5±0.8	2.0±1.4	4.6±2.9	7.7±3.4	12.3±3.2
PF4(1-60)	2.4±0.3	2.2±1.9	3.2±0.4	7.1±2.7	8.6±3.1	—

* Mean±SD (n = 3) of release of histamine from basophils of three different subjects.
‡ PF4(59-70) = Leu-Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-Leu-Glu-Ser.
§ PF4(66-70) = Lys-Leu-Leu-Glu-Ser.

TABLE II
*Uptake of [^{125}I]PF4 into Human Basophils**

Time	Molar concentration of [^{125}I]PF4			
	10^{-8}	10^{-7}	3×10^{-7}	10^{-6}
min				
2	1.0±0.4‡	7.8±3.1	33.3±4.5	68.6±27.4
10	1.3±0.5	9.3±3.2	—	69.5±22.5
30	1.9±0.7	10.2±3.7	—	78.2±20.2

* Replicate suspensions of 3×10^5 purified basophils in 1 ml of B-HSA were incubated with [^{125}I]PF4 at 37°C and washed twice before assessment of basophil-associated radioactivity. The purity of the two preparations of basophils was 84 and 87%.
‡ Picomoles of [^{125}I]PF4 taken up by 10^5 basophils; mean±range (n = 2).

dependence on the extracellular concentration of divalent cations. Maximum release of histamine from basophils by optimal concentrations of PF4 and PF4(59-70) is realized within 1–3 min of incubation, whereas the same effect of anti-IgE requires 10 min or longer (Fig. 2). When calcium and magnesium are eliminated from the extracellular fluid, the release of histamine from the basophils by anti-IgE is suppressed dramatically without influencing the level of release evoked by PF4(59-70) (Fig. 3) or PF4.

Some previously defined functions of PF4, including modulation of the activity of collagenase and elastase (13, 14) and stimulation of PMN leukocyte and monocyte chemotaxis (15), appear to result from one or more complex molecular and cellular events. Other functions of PF4, such as the binding and neutralization of heparin (16), are determined largely by charge interactions. The mechanism of activation of basophils by PF4 has not been elucidated definitively. However, it is of interest that the stimulation of histamine release from mast cells and basophils by some polycations is similarly independent of extracellular calcium and, in contrast to IgE-dependent release of histamine, is not suppressed by esterase inhibitors or elevation of intracellular cyclic AMP (17). The rapid attainment of maximal histamine release by PF4 in the absence of extracellular calcium and magnesium suggests that PF4 may be specifically and noncytotoxically taken up by basophil granules and displaces histamine from the proteoglycans of the granule matrix. The concentration-dependent uptake of [^{125}I]PF4 by basophils parallels temporally the release of histamine (Table II). It can be calculated that the uptake of 1 pmol of PF4 by basophils (Table II) is associated with the release of 1–3 pmol of histamine (Fig. 1), depending on the concentration of PF4. That the uptake of PF4 by the basophils may be specific was suggested by the finding in one experiment that 10^{-7}, 3×10^{-7}, and 10^{-6} M protamine sulfate failed to alter the uptake of 10^{-7} M [^{125}I]PF4. Studies of the uptake of PF4 by isolated basophil granules and of the concurrent release of histamine from the granules will be required to confirm the stoichiometry of the reaction in the absence of other cellular constituents that may bind PF4 and degrade histamine.

The release of histamine from human basophils by PF4 may represent an important positive feedback-mechanism in some immediate-hypersensitivity reactions. If IgE-dependent stimulation of basophils generates platelet-activating factors that recruit platelets and induce the liberation of PF4 (2), then the PF4 may be capable of augmenting and prolonging the hypersensitivity reaction by stimulating additional basophils, irrespective of their state of sensitization with IgE.

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