ACTIVATION AND DESENSITIZATION OF PLATELETS BY PLATELET-ACTIVATING FACTOR (PAF) DERIVED FROM IgE-SENSITIZED BASOPHILS

I. Characteristics of the Secretory Response*

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Release of histamine from sensitized rabbit blood cells was first clearly demonstrated in 1966 by Barbaro and Zvaifler (1). Later analysis of this phenomenon revealed the sequential interaction of antigen with blood basophils (2–4), which had been sensitized with IgE antibody (4), followed by release of histamine from blood platelets. Histamine is also liberated from the basophils, but in the blood of the rabbit, both histamine and serotonin are found primarily (5) in the dense granules of the platelets. We earlier showed that the effect on the platelets was mediated by a soluble factor released from the sensitized leukocytes (6, 7), a factor later termed platelet-activating factor or PAF[1] (4). PAF is a low molecular weight material which binds to albumin (4, 8) and has recently been shown to be phospholipid in nature (9, 10). In addition, we have also described a platelet-activating factor (PAF_L) which is liberated from sensitized chopped rabbit lung fragments upon incubation with specific antigen (11). This probably derives from the mast cells and was shown to be different (though it is similar in action) to PAF_B from circulating basophils. Demonstration of PAF release in man (4, 12) and rats (10) dispells the rabbits' possible uniqueness in possessing this material.

The in vivo pathologic significance of this system was indicated by its demonstrated involvement in the increased vascular permeability in acute experimental immune complex disease resulting in the deposition of the circulating complexes in vessel walls (13). The deposited complexes then initiate the injurious process (14). Involvement in other pathologic events may also be indicated or surmised, e.g. anaphylactic shock (15), or the Arthus reaction (16). The physiologic role of this process in amplification of an antigen-IgE-basophil interaction is as yet unclear and must await a clear understanding of the physiologic functions of the IgE system itself.

PAF has been shown to induce aggregation of platelets (4, 7) and secretion of

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[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; BSA, bovine serum albumin; PAF, platelet-activating factor; PBS, phosphate-buffered saline; ZC3, zymosan-C3.

their content of histamine and serotonin (7). The release of vasoactive amines is not cytotoxic (7), requires platelet energy metabolism and environmental calcium, and is an example of a secretory reaction similar to that observed in a wide variety of cells (17). The importance of the PAF-induced secretion as an in vivo mediation process, as a clearly demonstrated and studied example of cell cooperation, as an effect of a unique platelet activator, and as a means to study the secretory processes of platelets have prompted the studies reported herein. These comprise a characterization of the secretion induced by PAF_B and a demonstration of the unique features of this process, namely the essential interplay between activation and desensitization which serves to limit and control the secretory event.

Materials and Methods

Animals. New Zealand white rabbits of both sexes weighing from 2.5 to 4 kg were employed, both for supplying the platelets and the PAF. Blood for platelet preparation was not removed at intervals of less than 1 mo.

Immunization. Horse radish peroxidase (type II, Sigma Chemical Co., St. Louis, Mo.) was employed as antigen. The initial injection comprised 1.0 mg in saline subcutaneously into 2.5 kg rabbits. Thereafter, animals were reimmunized with 5 mg/injection at variable intervals (1-6 mo). The rabbits were bled for PAF production 10 days after each immunization.

Preparation of Platelets. Rabbit platelets were prepared as previously described (18). Blood was taken into acid citrate dextrose (ACD) (as for PAF production) and centrifuged at 550 *g* (1,600 rpm) for 20 min. The entire preparation procedure was carried out at room temperature and with plastic containers and pipettes. Platelets from different rabbits were usually pooled, a procedure that in our hands has not led to any behavioural differences from platelets prepared from a single animal. The platelet-rich plasma was removed and incubated at 37°C with 0.6 μCi [^{3}H]serotonin, binoxalate (4.3 Ci/mmol), New England Nuclear, Boston, Mass. for 15 min. The platelets were sedimented at 1,800 *g* (3,000 rpm) for 15 min and washed first in Tyrode's gelatin without Ca++ and with EGTA (see below) and then in Tyrode's gelatin without Ca++. The platelets were resuspended in the latter buffer and diluted to 4×10^9 or 2.5×10^9 depending on the experiments to be performed. The concentration was determined from a standard curve of absorbance at 530 nm. The preparation procedure is essentially that of Ardlie et al. (19) and preserves the platelet Mg++. Such platelets are readily aggregated by adenosine diphosphate (ADP) in μM concentrations and are predominantly discoid in shape. These characters are both indicative of relative normality. The platelets were maintained at room temperature and used within 3-4 h of preparation. While their reactivity to ADP declined somewhat over this time, that to PAF remained unaltered.

Buffers. (*a*) Tyrode's solution: NaCl, 8 g/liter; KCl, 0.195 g/liter; $NaHCO_3$, 1.02 g/liter; $MgCl_26H_2O$, 0.213 g/liter; $CaCl_2$ (anhydrous), 0.145 g/liter; glucose, 1 g/liter; pH 7.2. (*b*) Tris-Tyrode's solution: same as above with 0.01 M tris-(hydroxymethyl) amino-methane instead of $NaHCO_3$. (*c*) Tyrode's gelatin: same as (*a*), with 0.25% gelatin. (*d*) Tyrode's bovine serum albumin (BSA): same as (*a*) with 0.25% BSA. (*e*) Tyrode's gelatin without Ca++; basic Tyrode's gelatin with calcium omitted and a pH of 6.5. (*f*) Tyrode's gelatin without Ca++ and with EGTA; same as (*e*) but with 0.1 mM ethylene glycol tetraacetate. (*g*) Tris-Tyrode's without Ca++ or Mg++.

Preparation of PAF. Blood was obtained from the central ear artery into 1/6 vol of ACD (20). The procedure thereafter was similar to that previously described (4, 7). The blood was sedimented at 550 *g* (1,600 rpm) in an International PR6 centrifuge (International Equipment Co., Boston, Mass.) for 20 min at room temperature. The platelet-rich plasma was removed. The buffy coat layer on top of the erythrocytes was harvested with a siliconized (Siliclad, Clay-Adams, Inc. Parsippany, N. Y.) Pasteur pipette with tip bent at right angles. Once rabbits had been established as producing PAF (producing IgE antibody), buffy coat cells from all the positive animals were pooled. Erythrocytes, platelets, and lymphocytes were now the predominant contaminants of the basophil preparation. Addition of equal volumes of 2.5% gelatin in isotonic phosphate-buffered saline (PBS) was followed by incubation at 37°C for 30 min in an incubator to sediment most of the

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erythrocytes. The supernatant cell suspension was removed, centrifuged at 500 g (1,500 rpm) for 15 min at room temperature. The cell pellet was resuspended in a small volume of Tris-buffered Tyrode's solution with no Ca++ or Mg++ and with EGTA (see below). Remaining erythrocytes were removed by hypotonic lysis. For each 2 ml of cell suspension, 6 ml of distilled water was added and after 30 s, 2 ml of 3.5% saline was used to restore isotonicity. Platelets were removed by 4–5 washes, 8 min at 180 g (900 rpm) in 50-ml plastic tubes using Tris-buffered Tyrode's solution without Ca++ or Mg++. Cells were resuspended to 1×10^7 leukocytes in Tris-Tyrode's with 0.25% BSA and were contaminated with less than 10^{-4} per ml platelets. The cell concentration is important since if it is higher, the PAF apparently binds to the other cells present and the yield is lowered. Basophils comprise from 3 to 6% of the final leukocyte preparation. The albumin is essential and provides a carrier for the PAF that binds to it. Antigen (50 μg peroxidase for each 10^7 cells) was added and incubated for 20 min at 37°C. The cells were removed (500 g, 1,500 rpm for 10 min), and the supernatant fluid containing PAF-albumin was tested (see below), dialyzed against PBS or Tris-buffered saline overnight at 4°C, and frozen in aliquots at −70°C.

Extraction of PAF. PAF was extracted from the albumin by the method described by Benveniste (8). Ethanol was added to 80%, and after 15 min at room temperature the precipitated protein was removed by centrifugation. The extracted PAF was evaporated to dryness at room temperature and redissolved immediately in either ethanol or Tyrode's gelatin solution. In the latter instance the PAF was used immediately as it loses activity in aqueous solution (4, 8).

Platelet Stimuli. Antiplatelet antibody was prepared and absorbed as previously described (18), except that a goat rather than a sheep was immunized. A gamma globulin preparation (18) was used. Zymosan-C3 (ZC3) was prepared by incubating 17 mg zymosan (ICN Pharmaceuticals, Inc., Cleveland, Ohio) per ml one-third fresh rabbit serum in Tyrode's for 20 min at 30°C as previously described (21). The ZC3 was washed extensively and frozen in aliquots at −70°C. A preparation of zymosan incubated in serum with 10 mM EDTA (which prevents the binding of complement) was employed as control. Thrombin (bovine, Parke Davis & Co., Detroit, Mich.) was diluted in Tyrode's gelatin. Epinephrine (Parke Davis & Co.) was also diluted in Tyrode's gelatin, but only immediately before use. Collagen was prepared by homogenization and then sonication of bovine tendon collagen (Sigma Chemical Co.) in saline. Unlabeled serotonin (serotonin creatinine sulfate) was obtained from Sigma Chemical Co.

Platelet Secretion. The standard reaction mixture contained 0.1 ml 2.5×10^9 platelets, varying amounts of PAF, and Tyrode's gelatin in 1 ml. The final concentration of platelets was thus 2.5×10^8/ml. The platelets were generally added last, unless otherwise indicated. The reactions were performed in 12×75-mm polystyrene tubes (Lancer, Sherwood Medical Industries, Inc., St. Louis, Mo.) at 37°C for 10 min. Tubes were then cooled to 4°C in ice and centrifuged in the cold at 1800 g, 3,000 rpm for 15 min. An aliquot (0.1 ml) of the supernate was removed, and the radioactivity was counted in a mixture of equal parts toluene and Aquasol (New England Nuclear) in a Beckman liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). The secreted [^{3}H]serotonin was usually expressed as a percentage of the total taken up by the platelets, determined by lysis with Triton × 100. Generally, 0.1 ml of 2.5×10^8/ml platelets contained 18–25 $\times 10^3$ cpm. Background secretion from platelets incubated without stimulus was usually subtracted and seldom exceeded 2% of the total (see results section). Reactions were generally performed in duplicate. Kinetic experiments were performed by incubating 0.5 ml, 4×10^9/ml platelets with 0.5 ml of PAF dilution and 1.0 ml Tyrode's gelatin (i.e., 1×10^9/ml final concentration of platelets). At intervals 50-μl aliquots were removed and added to 0.45 ml of ice-cold Tyrode's gelatin containing 5 mM EGTA to completely and instantly stop the secretion. Tubes were then centrifuged and supernates counted.

Results

The Effect of PAF Concentration on Secretion. Increasing amounts of PAF complexed to albumin (4) were incubated with platelets (2.5×10^8/ml) for 10 min and the release of [^{3}H]serotonin was ascertained (Fig. 1). It should be noted that the use of [^{3}H]serotonin as a marker for endogenous serotonin (and histamine, 7 and 18) secretion has recently been validated (22). Since PAF has not yet been fully characterized, its absolute concentration is unknown. The data are ex-

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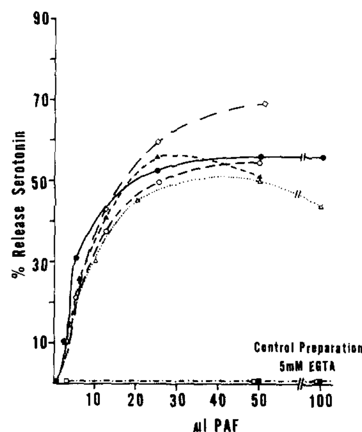

FIG. 1. Dose response of PAF-induced secretion of [^{3}H]serotonin from platelets. Each curve represents the mean release from duplicate incubations (10 min at 37°C in 1 ml) of different preparations of 2.5 × 10^8/ml platelets. Control (negative) PAF represents the supernatant fluid from sensitized basophils incubated with an unrelated antigen. Similar negative controls were seen with supernates from unsensitized basophils incubated with peroxidase. EGTA blocked the secretion.

pressed here as microliters of standard preparations (the supernatant from 1 × 10^7 sensitized leukocytes/ml, dialyzed, and containing 0.25% BSA). The data depicted in Fig. 1 results from five different platelet preparations and two different PAF preparations. The results (when expressed as percent secretion) are remarkably similar. The amount of serotonin released increases to a maximum value of between 45 and 60%, remains at that value as the PAF concentration increases, and then at high levels of PAF the amount of secretion declines. By concentrating the PAF-albumin complexes (ultrafiltration), PAF concentrations can be achieved which result in almost complete suppression of release (not shown). The peculiar nature of this dose response will be discussed below.

Control preparations, i.e. supernate from sensitized leukocytes (basophils) incubated with no antigen or with an unrelated antigen (e.g., BSA) or nonsensitized leukocytes reacted with peroxidase, gave no serotonin secretion (Fig. 1 and reference 7). Controls to confirm the secretory nature of the serotonin release have been reported earlier (7) and include demonstration of the absence of release of cytoplasmic constituents such as lactic dehydrogenase, amino acids, or $K+$. The complete inhibition of secretion with 5 mM EGTA is also shown, as this agent will be used to terminate the secretion in kinetic experiments.

Secretion Kinetics. Platelets were incubated with varying concentrations of PAF, and aliquots were removed at intervals into cold EGTA-containing buffer. Fig. 2 depicts the secretion rates. A number of points emerged from these experiments: (*a*) The secretion is extremely rapid, being generally complete within 1 min. This contrasts with other stimuli for rabbit platelet secretion (18, 21). Thus concentrations of PAF, thrombin, antiplatelet antibody, and collagen, which stimulate 50% secretion, yield initial secretion rates of 85, 28, 20, and 10% per min, respectively. However, high concentrations of thrombin (giving 100%

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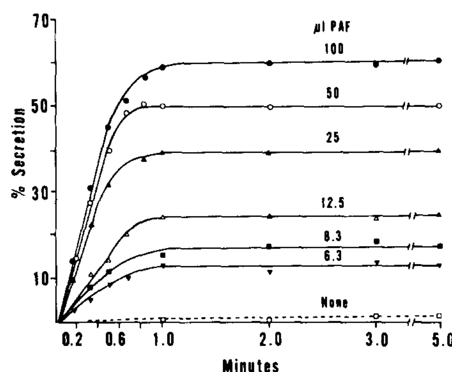

FIG. 2. Kinetics of secretion of [^{3}H]serotonin from platelets with different concentrations of PAF. PAF was incubated with 1×10^9/ml platelets, and aliquots were removed and diluted into ice-cold EGTA-containing buffer to halt the reaction.

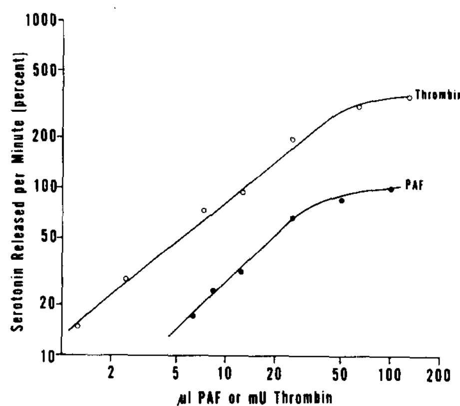

FIG. 3. The relationship of secretion rates to PAF concentration. Initial rates of secretion induced by PAF, or for comparison by thrombin, are expressed as percent released per minute. Concentrations of thrombin are expressed in mU (0.001 U). The incubation conditions were identical with those of Fig. 2.

secretion) result in maximum secretion rates three times greater than those of PAF (Fig. 3). (*b*) There is a distinct lag period before secretion starts, but this period is very short, about 0.1 min. Because of the expanded scale, this is more clearly shown in Fig. 4. Surprisingly, this lag is approximately the same over a wide range of PAF concentrations. (*c*) The rate and extent of secretion vary as the PAF concentration. Fig. 3 depicts this relationship, showing that the log of the initial secretion rate is proportional to PAF concentration over a considerable range, but approaches a maximum with high concentrations.

Platelet Concentration. To determine the effect of platelet concentration on PAF-induced secretion, the kinetic experiments were repeated, but with varying concentrations of platelets. It may be seen (Fig. 4 and 5) that secretion (rate and extent) was optimal at rather low platelet concentrations (2.5×10^8/ml), diminishing as the concentration increased or decreased. This contrasts with

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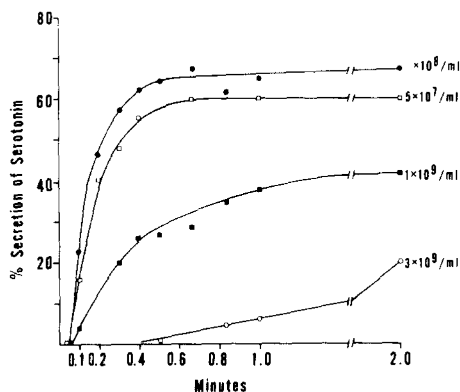

FIG. 4. Kinetics of secretion induced by PAF on different concentrations of platelets. PAF (100 μl/ml) was incubated with platelets at the indicated concentrations, and the reaction was terminated as described in Fig. 2.

secretion induced by thrombin that increases in rate with increased platelet concentration over all concentration tested (Fig. 5). Other stimuli (antiplatelet antibody and ZC3) also require much higher concentration of platelets for optimal secretion (data not shown). Accordingly, experiments were generally performed at a concentration of 2.5×10^8 platelets/ml.

Temperatures. The effect of temperature on the secretion induced by PAF is depicted in Fig. 6. Of interest, it may be noted that the extent of secretion was also markedly temperature dependent, indicating some mechanism for shutting off the secretory process (see below). This has been expressed as either initial rate of secretion or as extent of secretion (here as counts per minute of [^{3}H]serotonin released) in an Arrhenius plot in Fig. 7. It may be seen that secretion was maximal at 37°C and that a 'break' was observed in the Arrhenius plot at about 20°C. Subsequently, experiments were performed at 37°C.

pH. The effect of varying pH on platelet secretion is shown in Fig. 8. Optimal secretion was observed at about pH 7.7, and release was inhibited at pH 6.5. Higher pH's induced spontaneous release of serotonin, and backgrounds started increasing at pH 7.5. Consequently, experiments were performed at pH 7.4.

Are all the platelets affected by PAF? The inability to obtain more than 50–60% secretion of serotonin with PAF raises the possibility that only 50% of platelets respond to the stimulus. Alternatively, all the platelets respond, but by secreting only 50% of their contained vasoactive amines. This is an important question. Experiments were performed to determine whether the serotonin remaining after PAF-induced secretion could be secreted in response to another stimulus, in this case, ZC3. The data in Fig. 9 indicate that this residual serotonin is indeed releasable. However, this could still imply two populations of platelets.

The Effect of Ethanol-Extracted PAF. In Fig. 10 the dose response of PAF-albumin and of PAF extracted from this with ethanol is compared and provides an answer to the question of different platelet populations. It may be seen that the extracted PAF induces secretion of up to 90% of the serotonin. This clearly

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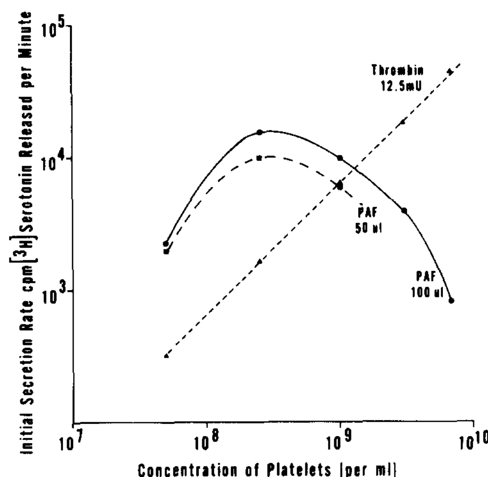

FIG. 5. The relationship of rate and extent of secretion to concentration of platelets. The conditions employed were those described in Fig. 2.

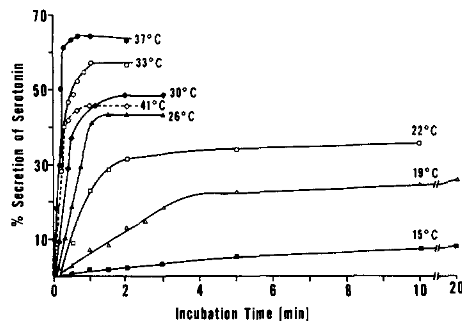

FIG. 6. Kinetics of secretion induced by PAF at different temperatures. Platelets (1×10^9/ml) were incubated with PAF (100 μl/ml) at different temperatures, and aliquots were diluted into cold EGTA-containing Tyrode's solution to halt the secretion.

rules out the possibility of two populations of platelets, demonstrates the likelihood that all the platelets interact with the PAF, and suggests some controlling process to limit the extent of the secretion. It also shows that extracted PAF behaves in this regard differently from albumin-bound PAF. However, secretion rates, desensitization, and the other parameters discussed are similar for both forms of PAF.

Specific Desensitization of Platelets by PAF. The data in Fig. 9 showed that platelets limited to 50% release with PAF were still capable of releasing serotonin with ZC3. This suggested the possibility that the platelets were desensitized to PAF but not to ZC3. To show this more clearly, platelets were incubated for 30 min at 37°C with PAF in the presence of 5 mM EGTA (i.e., in the absence of calcium) to inhibit secretion but still allow interaction of PAF and the induction of desensitization. Control platelets were incubated with EGTA alone. The platelets were then washed and tested for their ability to secrete serotonin in response to the different stimuli depicted in Table I. It may be seen that

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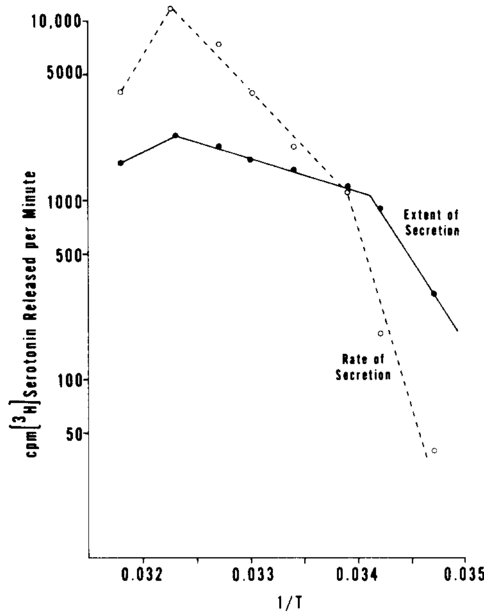

FIG. 7. Arrhenius plot of rate or extent of secretion (expressed as counts per minute released per minute or counts per minute released). The experimental conditions were those described in Fig. 6.

platelets were specifically desensitized to PAF if they had been pretreated with PAF, but still reacted perfectly well to all the other stimuli. In fact an enhanced response to the other stimuli was observed in platelets pretreated with PAF compared with those pretreated with EGTA alone. This enhancement is probably due to the lowering of the intracellular cyclic AMP by the PAF (even in the absence of Ca++) which enhances the secretory response (23). That this is the likely explanation is suggested by the effect of theophylline, which increases cyclic AMP by inhibiting phosphodiesterase, and which reduced the enhancement observed by pretreatment with PAF (Table II). It may also be observed that serotonin, which probably induces [^{3}H]serotonin release by some exchange process, not modulated by cyclic AMP (Henson unpublished observations) did not exhibit the enhancement phenomenon.

Rate of Desensitization. The desensitization of platelets to PAF might explain the limitation of the dose response (Fig. 1) to a maximum of about 50% secretion. Thus, as the desensitization increased, the secretion would be shut off. Fig. 11 depicts an example of an experiment to measure the rate of desensitization. Platelets were incubated with PAF and EGTA for varying times and then diluted in Tyrode's gelatin to noninhibitory concentrations of EGTA and to nonstimulating concentrations of PAF. Tubes were then incubated with or without additional PAF to determine the degree of desensitization. Control platelets (no PAF in the first incubation) exhibited no desensitization, whereas platelets pretreated with PAF showed increasing desensitization starting at about 1 min which, interestingly, is the point at which the secretion rate starts diminishing (also shown in Fig. 11).

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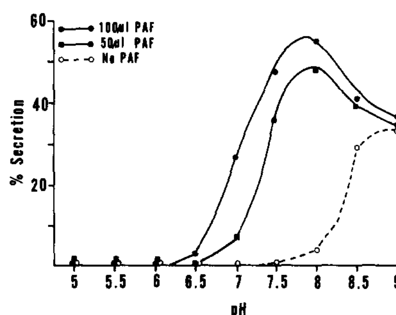

FIG. 8. Secretion induced by PAF at different pH's. Tyrode's solution was buffered with Tris and the platelets (2.5×10^8/ml) incubated with PAF for 10 min at 37°C.

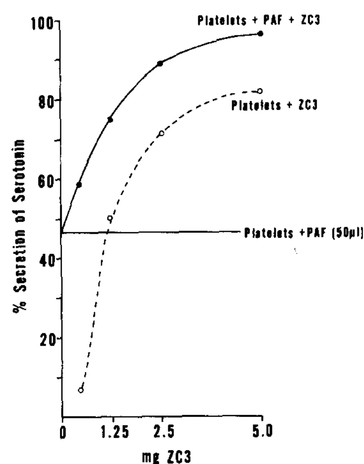

FIG. 9. The effect of ZC3 on platelets stimulated maximally with PAF. The ZC3 (50 mg/ml) was added simultaneously with the PAF and incubated with the platelets (2.5×10^8/ml) in 1 ml for 15 min at 37°C.

Desensitization with Ethanol-Extracted PAF. The PAF extracted from albumin with ethanol behaves in most respects like albumin-bound PAF. Experiments depicted (data not shown) that platelets can be desensitized to extracted PAF if incubated with it in nonreleasing conditions (i.e., in the presence of EGTA). Moreover, platelets made unresponsive to albumin-bound PAF remained unresponsive to the extracted material.

Discussion

The low molecular weight factor, PAF, which is released from IgE-sensitized basophils upon reaction with antigen has been shown to be a very potent stimulus for platelet secretion. It is our hypothesis (17) that secretion from mediator-containing cells such as platelets is an essentially similar process to secretion from a wide variety of cell types. Most of the characteristics of PAF-induced secretion, as described herein, do not dispute this point. Nevertheless, other factors, particularly the peculiar dose response relationship, appear to be unique to this particular platelet stimulus.

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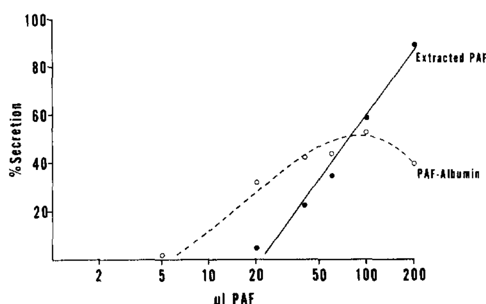

FIG. 10. Dose response of PAF extracted from albumin with 80% ethanol. The effect of the parent PAF-albumin preparation is also included.

TABLE I
Desensitization of Platelets to PAF

Stimulus	Percent release serotonin ± SEM	
	Platelets pretreated with EGTA	Platelets pretreated with PAF and EGTA
PAF (20 µl)	43.1 ± 1.4	9.0 ± 1.5
ZC3 (3 mg)	60.8 ± 4.0	77.6 ± 2.6
A-P Ab (100 µg immunoglobulin)	60.2 ± 1.5	74.0 ± 2.1
Collagen	23.7 ± 3.1	43.7 ± 5.3
Thrombin (0.016 U)	78.4 ± 2.5	92.3 ± 4.7
Epinephrine (4×10^{-4} M)	18.9 ± 4.3	31.0 ± 5.5
Serotonin (2×10^{-4} M)	38.3 ± 2.4	35.3 ± 2.1
None	2.8 ± 0.5	6.3 ± 1.2

Platelets (2.5×10^9) were pretreated for 30 min at 37°C with 5 mM EGTA in the presence or absence of 200 µl PAF. They were then washed, and aliquots (2.5×10^8) were incubated with the different stimuli for 15 min at 37°C.

The release of vasoactive amines from rabbit platelets by PAF is noncytotoxic (7) and was completely inhibited by calcium deprival. It is a true example of a secretory process (17) and requires platelet energy (7), and as described in the accompanying paper (23) is modulated by cyclic nucleotides and requires microtubule function and an activatable serine protease. This contrasts with the suggestion of Siraganian and Osler (24) that the effect of PAF is cytolytic. However, their conclusion was based on release of ^{51}Cr from chromium-labeled platelets, which may not be an adequate indication of cytotoxicity. The absence of release of K^+ (actually ^{86}Rb was used) or cytoplasmic enzymes, and the absence of morphologic evidence of lysis seems to refute this point (7). Moreover, as seen in Fig. 11, prolonged incubation of platelets after induction of secretion resulted in some uptake of the released serotonin, surely a contraindication of cytotoxicity.

The inability of PAF when bound to albumin to stimulate more than about 50% secretion of vasoactive amines may be explained by the concurrent desensitization induced in platelets by the PAF. The rate of induction of desensitization

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TABLE II
Inhibitory Effect of Theophylline on the Enhancement of Secretion Seen in PAF-Pretreated and Desensitized Platelets

Pretreatment (stimuli in second incubation)	Percent release serotonin		Percent enhancement due to PAF pretreatment
	Platelets pretreated with EGTA*	Platelets pretreated with EGTA and PAF	
Control pretreatment			
PAF	44.5	10.5	
ZC3	24.1	34.0	41
Anti-PAb	66.7	76.0	14
–	0.3	0.3	
Theophylline (10^{-3} M) present during pretreatment			
PAF	33.1	9.5	
ZC3	21.5	25.1	13
Anti-PAb	53.5	54.0	1
–	0.7	0.4	

* Pretreatment conditions were the same as those in Table I.

is certainly less than that of secretion, but before long becomes predominant, and secretion ceases. As the PAF concentration is increased, the secretion rate may also increase. However, a finite time is necessary for secretion to occur, and at a given point the desensitization is rapid or early enough that no further increment of secretion is seen. The desensitization results from interaction of PAF with the cell, a process that is also presumably related to PAF concentration. The relationship to each other of rates of 'activation', 'decay' and 'desensitization' are under investigation and will be reported elsewhere[2]. It is of interest that mast cells, whose secretory properties are very similar to platelets, also exhibit a limitation of secretion by desensitization (25) which may in part be a general phenomenon (17). A cobra venom activating agent acts on mast cells in a manner similar to that of PAF on platelets (26) to yield a maximum of only about 50% release of serotonin. This again appears to be due to the rapid desensitization.[2]

Another hypothesis to account for the maximum secretion of about 50%, namely that a unique population of platelets is involved, appears unfounded. The ability of extracted PAF to induce more than 90% secretion demonstrates that most of the platelets can respond. The unique effect of albumin-bound PAF may result from the density of PAF on the albumin, conferring a greater ability to induce desensitization, and this is under investigation. It should be noted that the binding to albumin is rapid, and the effects of PAF in vivo may involve totally or partially protein-bound material. Moreover, the extracted PAF is very

[2] Morrison, D. C., and P. M. Henson. Regulation of the rate and extent of secretion from mast cells and platelets by the nature of the stimulus-cell interaction. Submitted for publication.

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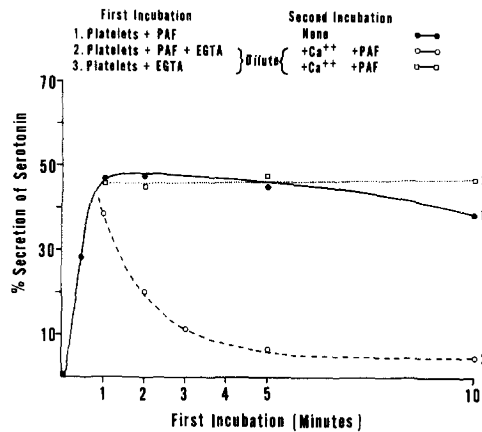

FIG. 11. Rate of desensitization of platelets to PAF. *(a)* Platelets (10^8/ml) were incubated with PAF (50 μl/ml) and the time-course of secretion determined as in Fig. 2. *(b)* Platelets (10^9/ml) were incubated with PAF (50 μl/ml) in the presence of 5 mM EGTA. At intervals aliquots were diluted one-tenth in Tyrode's gelatin containing Ca^{++} to which PAF (50 μl/ml) has been added and incubated for a further 10 min. *(c)* Platelets were incubated with EGTA, and at intervals aliquots were diluted in Tyrode's solution with PAF and incubated for a further 10 min.

unstable, and considerable activity is usually lost during the process. Despite this difference between the extracted and albumin-bound PAF, in other respects they appear identical. Thus, platelets can be desensitized by both forms of PAF (under conditions of calcium deprivation) and are thus unresponsive to both the bound and the extracted materials. Other evidence against unique populations of cells comes from data showing that in platelets exhibiting maximum release with PAF of 50%, this can be increased to over 70% in the presence of 1×10^{-5} M epinephrine (acting as an alpha adrenergic agent), again showing that more than 50% of the platelets can respond (see reference 23). The presence of an inhibitor, as well as an activator, in the PAF preparation might also explain the data, although the argument expressed above against unique populations of platelets would also serve to diminish the likelihood of this explanation.

The data also provides evidence to answer another important question, namely when 50% of the serotonin is released, do all the platelets respond by 50% of their capacity or do 50% respond fully? Inasmuch as total desensitization can be achieved with doses of PAF which induce only 50% secretion and yet there are not unique populations of platelets responsive to PAF, it appears that all the platelets respond to PAF. This is important since it implies that the platelets can be visualized and studied as a pure population of relatively uniform cells.

The rate of secretion induced by PAF is dependent upon the concentration of PAF and also, as expected, upon the concentration of platelets. Surprisingly, however, compared with other stimuli such as thrombin, antiplatelet antibody, or C3, the optimal platelet concentration (for rate and extent of secretion) was low, about 2.5×10^8/ml. It is perhaps significant, however, that this approximates the blood concentration of platelets in the rabbit. The mode of interaction

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of PAF with the platelets is not known. Preliminary data with BSA-PAF bound to Sepharose 4B suggests that platelet activation with insoluble PAF is possible, indicating that PAF probably, as do other platelet stimuli, acts at the surface membrane. The observation that increased platelet concentrations (above 2.5×10^8/ml) resulted in a decreased initial rate of secretion (in complete contrast to the enzyme thrombin), provides a number of clues about the PAF-platelet interaction. It would certainly suggest that the PAF did not dissociate from one platelet and then act on another. It thus implies a high binding affinity compared with the other stimuli (whose optimal cell concentration was above 5×10^9/ml) or an inactivation of the PAF once it has bound. Since the initial rate of secretion decreases with increased platelet concentration, this suggests that the rate of binding (expected to increase with increase of either platelet or PAF concentrations) is not the rate-limiting factor in the secretion.

The temperature and pH optima for the PAF-induced secretory process are as expected, those of normal physiologic conditions. Interestingly, when the temperature dependence is plotted as an Arrhenius plot, both rate and extent of secretion show a sharp break at about 20°C. This is similar to the temperature dependence curves described for the effect of polymyxin B (27) and of cobra venom activator (Morrison, D. C., personal communication) on mast cells as well as for other stimuli on rabbit platelets. This serves to further the ideas of similarities between secretion from different cells. It has been suggested that this 'break' corresponds to a phase change in the membrane lipids resulting in decreased receptor mobility (27) and has been used to argue for the role of receptor aggregation in cell stimulation. While this is an attractive hypothesis and indeed, receptor aggregation may well be a key point in cell activation, the temperature data is certainly open to other interpretations. All it really indicates is that from 37°C to 20°C, one temperature-sensitive step in the complicated secretion process is rate limiting and that below 20°C another predominates. Platelets undergo shape changes at 4°C (28) which if they also occur in part at higher temperature (15°C–20°C) may contribute to the secretion data. It is of interest with regard to PAF that the extent of secretion is also temperature sensitive. This implies that if indeed it is the desensitization that switches off the secretion, the desensitization process is not the temperature-sensitive, rate-limiting step.

The stimulus specificity of the desensitization was demonstrated, since all other stimuli examined induced greater secretion from PAF-desensitized than from control platelets. An early, stimulus-specific step in the secretion (activation) process is implied. Recent evidence (29, 30) has indicated that this may be a specific stimulus-activated platelet serine protease which is required for cell activation. The increased release seen with the other stimuli seems to result from the effect of PAF in reducing the levels of intracellular cyclic AMP in the platelets (23). This was shown by diminution of the increase in platelets treated with theophylline, an agent that increases cyclic AMP by inhibiting phosphodiesterase.

Since specific desensitization does result from interaction of PAF with platelets, this may also occur in vivo. It could then act as a physiologic controlling influence. It might also be detected experimentally and used to determine

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whether or not in vivo platelet secretion or temporary platelet sequestration (as in IgE anaphylactic shock, 15) had resulted from release of PAF. Preliminary observations with Dr. Neal Pinckard of the University of Arizona have indicated that PAF-desensitized platelets can indeed be detected in rabbits undergoing IgE anaphylaxis. This would demonstrate in IgE-mediated pathophysiologic events in the animal the involvement of the phenomena described herein in vitro.

Summary

The secretion of vasoactive amines from rabbit platelets induced by the platelet-activating factor (PAF) derived from IgE-sensitized rabbit basophils, was examined. The secretion required calcium has previously been shown to be noncytotoxic and was optimal in both rate and extent at 37°C and pH 7.2. Different temperature-sensitive steps were rate limiting for secretion above or below 20°C. The rate of secretion was dependent upon the concentration of PAF and also of platelets. Maximal rates were observed with relatively low concentrations of platelets (2.5×10^8/ml), sharply contrasting with other platelet stimuli such as C3 or thrombin. The extent of secretion was dependent upon PAF concentration until a maximum of 50 or 60% of the serotonin was released and then declined with increasing amounts of PAF. This was interpreted to result from the platelets becoming desensitized to the PAF, a process that shuts off the secretion. Such a desensitization was demonstrated and was shown to be stimulus specific, i.e., other stimuli could still induce secretion from PAF-desensitized platelets. PAF extracted with ethanol from the albumin to which it is usually bound during preparation, exhibited similar characteristics, except that secretion of up to 90% of the serotonin was induced. The extracted PAF thus seemed less able to induce the desensitization. Its use did provide important evidence that populations of rabbit platelets are relatively homogenous in their ability to respond to PAF.

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