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

II. The Role of Serine Proteases, Cyclic Nucleotides, and Contractile Elements in PAF-Induced Secretion*

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Platelet activating factor (PAF)[1] is a soluble molecule which is released from IgE-sensitized basophils upon stimulation with specific antigen or with anti-IgE antibody (1–5). It was shown (1) to be the active principle of a cooperative in vitro allergic reaction involving rabbit platelets and basophils first described by Barbaro and Zvaifler in 1966 (6). Recently, PAF has been described in man (2, 7, 8) and rats (9), and a material similar to PAF is released from antigen-stimulated, IgE-sensitized rabbit lungs (10). PAF appears to be a low molecular weight phospholipid (11) which binds avidly to albumin although retaining its biological activity in this form (2).

PAF stimulates platelets to secrete in a noncytotoxic manner their content of vasoactive amines (1). It also initiates platelet aggregation (1, 2), apparently by a direct and rather unique effect on the platelets rather than by a mechanism involving released adenosine diphosphate (ADP),[2] which is only poorly released in this system (1).

In the preceding paper (12), the nature of secretory reaction of rabbit platelets induced by PAF was characterized. The secretion was shown to be extremely rapid and its extent was apparently controlled by the concurrent induction in the platelets of desensitization, which was specific to PAF. The temperature, pH, and cell density optima were determined and were found to be those of the blood, i.e., 37°C, pH 7.4, and 2.5×10^8 platelets per ml. It was shown that all the platelets in a preparation responded to PAF.

Since the desensitization induced by PAF was shown to be specific for that stimulus (12), a unique secretion pathway was implicated. However, the simi-

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[1] *Abbreviations used in this paper:* AMP, adenosine 5′-monophosphate; cAMP, cyclic AMP; cGMP, cyclic guanosine 5′-monophosphate; DFP, diisopropylphosphofluoridate; OMTI, ovomucoid trypsin inhibitor; PAF, platelet-activating factor; PGE_1, prostaglandin E_1; PMSF, phenylmethyl sulfonyl; SBTI, soybean trypsin inhibitor; TAMe, tosyl-*l*-arginine methyl ester.

[2] Henson, P. M. Activation of platelets by platelet-activating factor (PAF) derived from IgE-sensitized basophils. III. Dissociation of the secretory and aggregating activities of PAF. Submitted for publication.

larities between the mechanisms of secretion induced in platelets by a variety of stimuli with the mechanisms of secretion in other cell types has been noted (13), and a general secretory pathway has been suggested. The experiments described herein were designated to examine this discrepancy and to further define some of the cell-dependent biochemical parameters of the secretory process. It has been determined that common elements do exist in the process of secretion, and that the unique characteristics of the PAF-induced platelet activation probably lie in the stimulus receptor interaction and in the nature of the serine protease which is activated.

Materials and Methods

PAF and platelets were prepared as described in the preceding paper (12). [^{3}H]serotonin was incorporated into the platelets before washing and its release used to indicate secretion (14).

Platelet Secretion. The standard reaction mixture contained [^{3}H]serotonin-labeled washed platelets (final concentration 2.5×10^8/ml), PAF, and where indicated, inhibitor, all in Tyrode's solution containing 0.25% gelatin in a final vol of 1 ml (12). The plastic tubes were incubated for 15 min at 37°C, centrifuged at 1,800 *g* in the cold, and a 0.1-ml aliquot of the supernate removed for assessment of released [^{3}H]serotonin. For many experiments inhibitors were incubated with the platelets for 10 min at 37°C or room temperature before addition of PAF. Inhibition experiments were performed with concentrations of PAF previously determined by yield submaximal secretion (see Fig. 1). All reactions were performed in duplicate, and the secretion is expressed as mean percent [^{3}H]serotonin released. Duplicates varied by less than 10%. The total [^{3}H]serotonin in the platelets was used for this calculation and was assessed by counting aliquots of platelets lysed with Triton 100. Radioactivity was determined in equal parts toluene and Aquasol (New England Nuclear, Boston, Mass.) in a Beckman scintillation counter (Beckman Instruments Inc., Fullerton, Calif.). Kinetic experiments were performed as described in the previous paper (12), the reaction being stopped by dilution into cold EGTA-containing buffer. Experiments were repeated at least three times.

Chemicals. Diisopropylphosphofluoridate (DFP) was purchased from Sigma Chemical Co., St. Louis, Mo. and dissolved in Tyrode's gelatin immediately before use. The trypsin inhibitors, soybean trypsin inhibitor (SBTI), (Sigma Chemical Co.), low molecular weight lima bean trypsin inhibitor (LBT) (Sigma Chemical Co.), and ovomucoid trypsin inhibitor (OMTI), (Worthington Biochemical Corp., Freehold, New Jersey), esters (Cyclo Sciences Inc., Palisades Park, N. J.), theophylline (Sigma Chemical Co.), cyclic 3′5′-adenosine monophosphate, cyclic guanosine monophosphate, 5′-adenosine monophosphate, (Sigma Chemical Co.), phentolamine, (Ciba Corp., Summit, N. J.), propranolol, (Ayerst Laboratories, New York), carbachol, acetyl choline, colchicine, (Sigma Chemical Co.), and epinephrine (Parke, Davis & Co., Detroit, Mich.) were also diluted or dissolved in Tyrode's gelatin, the epinephrine and colchicine immediately before use. Phenylmethyl sulfonyl fluoride (PMSF, Sigma Chemical Co.) was dissolved at 50 mM in propylene glycol, prostaglandin E (generously supplied by Dr. Pike, UpJohn Co., Kalamazoo, Mich.) was dissolved in ethanol at 30 mM. Cytochalasin B (ICI Research Laboratories, Alderley Park, Cheshire, England) was dissolved at 100 μg/ml in $^1/_{200}$ dimethyl sulfoxide (DMSO) Tyrode's mixture. It was ascertained that the solvents had neither an enhancing nor inhibiting action on PAF-induced secretion at the concentrations employed.

Expression and Calculation of Inhibition or Enhancement. Experiments on different days were compared by calculating the inhibition of the percent that was secreted without the inhibitor. In this way, differences between platelet preparations, content of [^{3}H]serotonin, etc. can be minimized. Generally, the percent inhibition or enhancement ± SEM of secretion by a particular inhibitor has been presented. For most inhibitors, the percent inhibition is independent of the amount of serotonin released in the control tubes (i.e., without inhibitors), and the validity of expressing the data this way is indicated by the reproducibility of the data. In some experiments the inhibition is expressed as the mean pI_{50} ± SEM. This is defined as the negative logarithm of the concentration of inhibitor required to give 50% inhibition and allows a direct comparison between inhibitors of a given class, e.g., small molecular weight esters. The pI_{50} was determined from plots of percent inhibition versus inhibitor concentration.

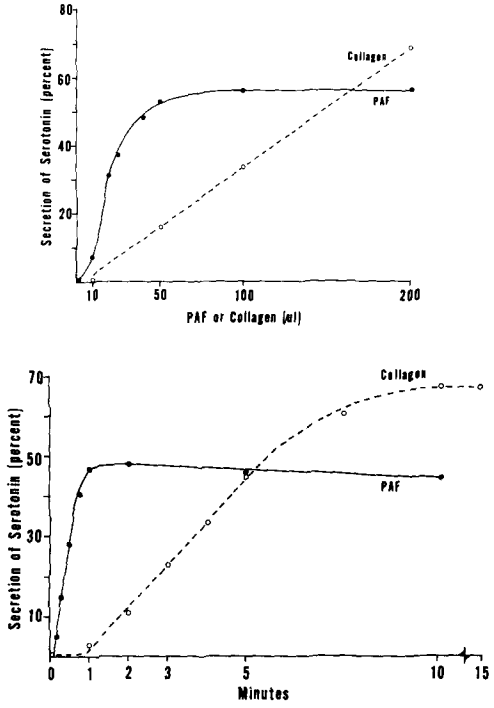

FIG. 1. Secretion of [^{3}H]serotonin from rabbit platelets induced by PAF or collagen. *(a)* Secretion from 2.5 × 10^8 platelets/ml incubated for 15 min at 37°C with increasing amounts of stimuli. The PAF preparation represents the dialyzed supernate from 1 × 10^7/ml sensitized rabbit leukocytes and contains 0.25% bovine serum albumin. The collagen concentration was 350 μg/ml. *(b)* Secretion expressed as a function of time of incubation. The reaction was stopped by dilution with cold EGTA-containing buffer.

Determinations of Cyclic AMP (cAMP). Platelets were reacted with PAF (5 × 10^8/ml with 200 μl PAF) for varying times at 37°C. They were stopped by extraction with 3 ml cold 5% trichloracetic acid (TCA). Excess TCA was removed by extraction with acidified ethyl ether. Subsequently, the aqueous portion was lyophilized and then resuspended in 0.05 M sodium acetate buffer, pH 6.2. The degree of recovery of cAMP through this procedure was ascertained by similarly extracting known, standard samples as controls. Alternatively, the secretion reaction was stopped by boiling for 20 min. The precipitated protein was removed by centrifugation, and the supernate was lyophylized and analyzed for cAMP. Concentrations of cAMP were measured according to the Schwarz/Mann radioimmunoassay procedure (assay kit, Schwarz/Mann, Orangeburg, N. Y.). A standard binding curve was prepared using known amounts of cAMP for each experiment. Measurements of cAMP were performed in duplicate on duplicate experimental samples.

Results

The Involvement of Serine Proteases in PAF-Induced Secretion

Experiments designed to examine the potential role of serine proteases and cyclic nucleotides in the secretory process rely heavily on the use of inhibitors. Before the initiation of such experiments, therefore, the dose response and time-course of PAF-induced secretion were determined. While these have been described at length in the preceding paper, they are also depicted in Fig. 1 for

direct reference. Inasmuch as some of the experiments involve a comparison of PAF-mediated and collagen-mediated secretion, these data are also included in Fig. 1. For subsequent experiments, concentrations of PAF to give less than maximal secretion were chosen (see Fig. 1 *a*), and an incubation time of 15 min was employed. Because secretion was normally complete in one minute, this proved sufficient to allow completion of secretion even when substantially inhibited.

We have suggested that activation of 'mediator' cells in general involves the activation of a precursor (zymogen) serine protease or serine esterase, which is then involved in the subsequent cell response (13). Experiments were initiated to extend this observation to PAF-induced secretion from platelets.

THE EFFECT OF DFP. DFP is an irreversible inhibitor of serine proteases (esterases). It will also inhibit the potent platelet activators thrombin and trypsin. As shown in Fig. 2, if DFP is present during the reaction of PAF with platelets, secretion is inhibited in a dose-dependent fashion. Since the inhibitor is irreversible, the possibility that the DFP inhibits preformed enzymes on the platelets or in the PAF preparations can be ascertained. Pretreatment of either platelets or PAF for 20 min with DFP (at different concentrations) followed by washing or dialysis, did not alter their ability to interact and initiate secretion (Fig. 2). These data suggest that the PAF directly or indirectly activates a precursor serine protease in the platelet that only then becomes inhibitable by DFP, and that this protease or esterase is required for the secretion process.

THE ACTION OF OTHER PROTEASE INHIBITORS. Table I indicates the inhibition achieved with PMSF, another general inhibitor of serine proteases. Again, this is dose dependent and does not occur if the platelets are pretreated. The inhibitor must be present during the secretory reaction, suggesting its action on the same serine esterase discussed above.

The trypsin inhibitors SBTI, limabean trypsin inhibitor (LBTI), and OMTI were also found to be inhibitory. The inhibition found with these agents was highly variable, perhaps reflecting their large size and a possible variable ability to gain access to the putative protease. Generally, 50% inhibition was achieved with concentrations varying from 10^{-5} to 10^{-4} M.

THE EFFECT OF LOW MOLECULAR WEIGHT ESTERS. The natural substrate for such an enzyme is unknown. However, the possibility that synthetic small molecular weight amino acid esters could inhibit the secretion was investigated, both to provide some information about a possible substrate and to add additional support to the hypothesis of an essential role for an activatable esterase. Fig. 3 depicts the inhibition of secretion achieved with seven esters. The data are expressed as the pI_{50}, the negative logarithm of the ester concentration to give 50% inhibition. A pI_{50} of 4, for example, represents 50% inhibition with 1×10^{-4} M ester. Of the esters examined, TAMe (tosyl-*l*-arginine methyl ester) was most inhibitory. LeuMe (leucine methyl ester) was not inhibitory at all and served as an important control to indicate that 5×10^{-2} M or more ester was not necessarily inhibitory to secretion. Esters with basic and/or aromatic amino acids seemed most inhibitory, and substitution of the amino group of lysine methyl ester with an acetyl group rendered it ineffective as an inhibitor. Also shown in Fig. 3 is the inhibition due to the amino acids, e.g. tosyl-*l*-arginine,

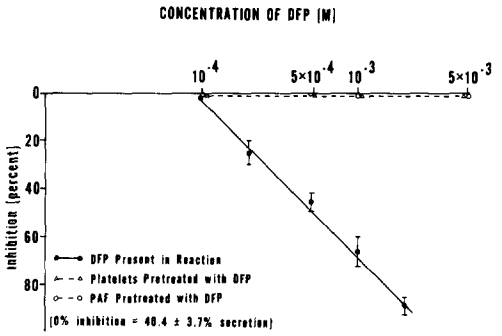

FIG. 2. Inhibition of secretion with DFP. Platelets were pretreated for 20 min at 37°C with buffer or DFP, washed, and incubated for 15 min at 37°C with PAF (25 μl) or PAF pretreated with DFP and dialyzed.

TABLE I
Effect of PMSF on PAF-Induced Release

	Percent release serotonin	Percent inhibition
Platelets + PAF	32.0 ± 2.3	
Platelets + PAF + PMSF 2.5×10^{-4} M	20.4 ± 3.0	36
5.0×10^{-4} M	14.2 ± 1.8	56
1.0×10^{-3} M	7.6 ± 1.1	76
Platelets pretreated with 10^{-3} M PMSF + PAF	39.5 ± 1.9	0
Platelets pretreated with propylene glycol + PAF	40.1 ± 2.3	0

Pretreatment was at 37°C for 20 min followed by washing. The PMSF was dissolved in propylene glycol at 5×10^{-2} M. Background secretion (PMSF, but no PAF) varied from 0.1 to 1.6%.

which is seen to be much less inhibitory than the esterified form. Methanol (the other product of hydrolysis of the methyl esters) in similar concentrations is not inhibitory. Taken together this indicates that it is the ester that is inhibitory and suggests substrate inhibition of a cell-bound esterase.

The Effect of Cyclic Nucleotides and Neurohormonal Stimulation on PAF-Induced Secretion

Secretion from a wide variety of cells has been shown to be under the control of intracellular levels of cyclic nucleotides. This was investigated.

THE EFFECT OF AGENTS THAT INCREASE INTRACELLULAR cAMP. To determine the role of cAMP in PAF-induced secretion, theophylline and prostagandin E_1 (PgE_1) were examined for their effect. Both agents induce increases in cAMP in platelets (see ref. 13), the former by inhibiting phosphodiesterase (15) and the latter by stimulating adenylate cyclase (16). PgE_1 was found to be a potent inhibitor (50% inhibition at about 10^{-7} M, Fig. 4). Theophylline was also inhibitory, although at much higher concentrations. Dibutyryl cAMP and 3'5'-

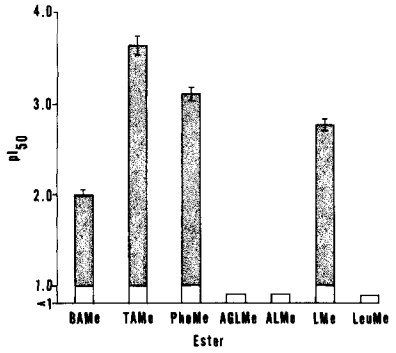

FIG. 3. Inhibition of secretion with amino acid esters. Esters were incubated with PAF (25–50 μl) and platelets, and the inhibition of secretion assessed. The shaded portion of the column represents inhibition with the ester, and the open area represents inhibition with the native amino acid. The data represent the mean ± SEM of at least three experiments. BAMe, benzoyl-*l*-arginine methyl ester; TAMe, tosyl-*l*-arginine methyl ester; PheMe, phenylalamine methyl ester; AGLMe, acetyl glycyl lysine methyl ester; ALMe, acetyl lysine methyl ester; LMe, lysine methyl ester; LeuMe, leucine methyl ester.

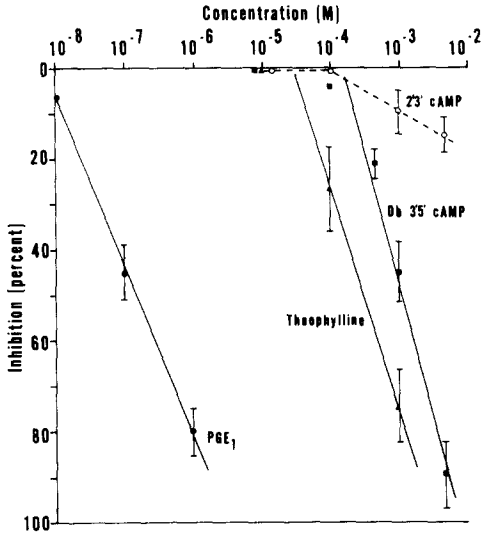

FIG. 4. Inhibition of secretion by agents that increase cAMP. The inhibitors were incubated with the platelet for 10 min at 37°C before addition of the PAF. Control secretion (no inhibitor) was 35.4 ± 4.3%.

cAMP itself were shown to be inhibitory, whereas control compounds 5′-AMP and 2′3′-cAMP were without effect. This is indirectly of additional interest, as AMP has been demonstrated to be an inhibitor of ADP-induced platelet aggregation (17).

If increased cAMP levels are indeed inhibitory to PAF-induced secretion, a synergistic effect between PgE_1 and theophylline would be expected (18). This was shown to be the case (Table II).

EFFECT OF ADRENERGIC AGONISTS. The cAMP system is usually modulated by adrenergic stimulation. Adrenergic agonists and antagonists were therefore

TABLE II
Synergistic Effect of PgE_1 and Theophylline on Inhibition of PAF-Induced Release

Treatment of platelets*	Percent release	Percent inhibition
No inhibitor	48.1 ± 2.3	
PgE_1 5×10^{-8} M	33.7 ± 5.1	30
Theophylline 10^{-4} M	39.1 ± 3.7	18
PgE_1 + theophylline	4.1 ± 1.0	92
Buffer	2.3 ± 0.5	

* Platelets incubated at 25°C with inhibitor for 10 min before addition of PAF.

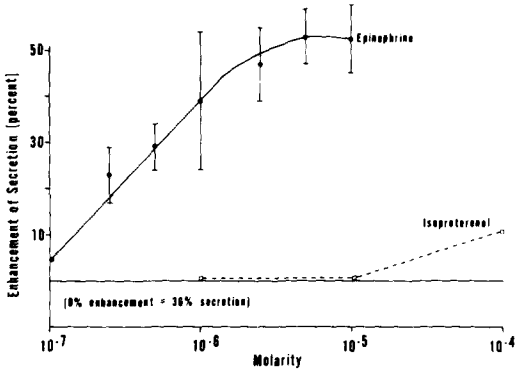

FIG. 5. Enhancement of secretion by epinephrine. Platelets were incubated with epinephrine or isoproterenol for 10 min at 37°C before addition of PAF. At concentrations below 10^{-4} M, epinephrine did not itself induce secretion.

examined for their effect on PAF-induced secretion. Fig. 5 depicts the effect of epinephrine. A potent enhancement is observed. That this observation was true enhancement was demonstrated by the failure of epinephrine alone in the same concentration to induce secretion. Fig. 6 shows that the enhancement is similar over the complete dose-response curve for PAF. Epinephrine usually exhibits both α- and β-adrenergic activity. Isoproterenol, predominantly β in action, produces little effect on PAF-induced secretion, a mild enhancement is observed at 10^{-4} M (Fig. 5). This suggests little effect of β-adrenergic stimulation (which usually stimulates adenylate cyclase and increases intracellular cAMP) in these platelets. The effect of epinephrine would therefore appear to be due to its α-adrenergic action in lowering the levels of cAMP and as a consequence, enhancing secretion. In our experiments, epinephrine was shown to reduce platelet levels of cAMP by more than 25%.

Fig. 7 depicts the results of experiments to demonstrate the adrenergic action of epinephrine, showing inhibition of its enhancing effect with phentolamine (an α-blocker) and virtually no alteration of its effect with propranolol (a β-blocker).

DECREASE IN INTRACELLULAR cAMP DURING SECRETION. The data described above suggested that increased levels of cAMP inhibited secretion, and that decreased levels induced by the α-adrenergic action of epinephrine, enhanced

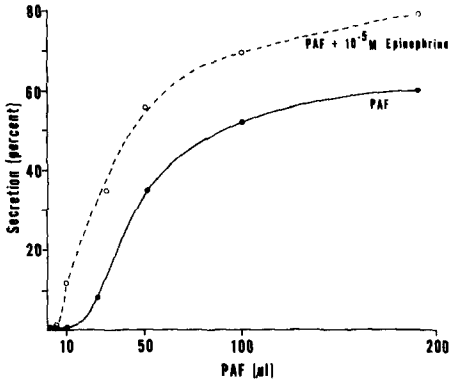

FIG. 6. The enhancing effect of epinephrine at different doses of PAF. The details are the same as those in Figs. 1 and 5.

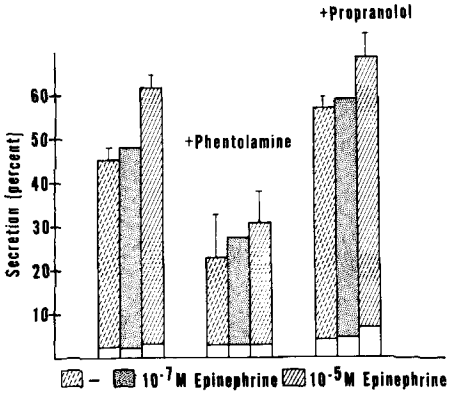

FIG. 7. The effect of propranolol or phentolamine on epinephrine enhancement of PAF-induced secretion. Incubation conditions are the same as those of Fig. 5. The open areas of the columns represent control secretion in the presence of the drugs but in the absence of stimulus. The first column (dashed hatching) represents the effect of PAF on platelets in the absence of adding epinephrine.

secretion. This would imply that if cAMP has any direct role in the PAF-induced secretion, its levels would be likely to decrease to permit the secretion to occur. To test this, we measured directly the levels of cAMP during PAF-induced secretion. As shown in Fig. 8, cAMP level did decrease in platelets stimulated with PAF. The rate of decrease was extremely rapid (less than 30 s), which is consistent with the rapid secretion induced by PAF (see Fig. 1). By contrast, collagen, which also decreases cAMP, does so more slowly (Fig. 8), which again correlates with its slower secretion rate (Fig. 1).

THE LACK OF EFFECT OF CYCLIC GMP (cGMP) AND CHOLINERGIC STIMULATION. To examine the possible role of cGMP (often found to antagonize the action of cAMP), cholinergic agents and cGMP were examined in a wide range of concentration for an effect on PAF-induced secretion (Table III). None was found. The secretory process, therefore, does not appear to be modulated by the antagonistic action of cGMP and cAMP, but by cAMP alone.

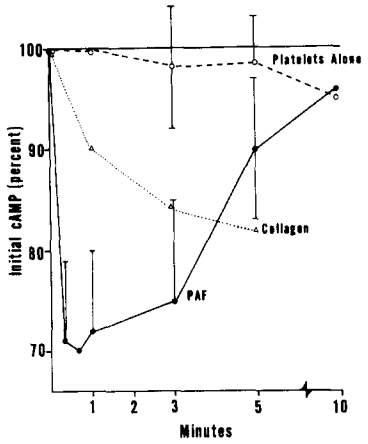

FIG. 8. Changes in the levels of cAMP during PAF-induced secretion. PAF induced a faster rate of cAMP decrease than did collagen which is consistent with a faster rate of secretion. See Fig. 1.

The Effect of Colchicine and Cytochalasin B. Another system suggested to be of importance in the secretory process is that of the contractile elements of the cell (see 13). Microtubules play an important cytoskeletal role in the platelet (14, 19), and their effect on PAF-induced secretion was accordingly examined using the inhibitor of microtubule assembly, colchicine. As shown in Fig. 9, colchicine is an effective inhibitor of PAF-initiated secretion, 50% inhibition being achieved with 10^{-4} M. These are concentrations similar to those required to "disrupt" microtubules in platelets (19, 20), suggesting that this is indeed the mechanism of action.

Microfilaments comprised of the contractile protein thrombosthenin can be observed in platelets underneath the plasma membrane (21). Cytochalasin B is thought to disrupt microfilaments (22), although it may have other actions as well. It was found to enhance the secretion from platelets stimulated with PAF (Fig. 8). In data not shown, the enhancement was seen over the entire dose-response range of the PAF and could be demonstrated only if the cytochalasin was present during the reaction and not if platelets were pretreated with cytochalasin B and washed. This is consistent with the known reversibility of the action of this drug (see 13).

Discussion

Secretion from platelets stimulated by PAF has many characteristics in common with the secretory responses initiated by a number of other stimuli on a variety of cells (13). The secretion is modulated by intracellular levels of cAMP, controlled in turn by neurohormonal stimuli or prostaglandins. Contractile and supporting elements within the cell are involved, and as shown previously (1, 12), there is a requirement for calcium and platelet energy metabolism, presumably supplying ATP. We have also shown a requirement for an activatable serine protease. PAF is then a unique activator of platelets, probably reacting with a unique cell surface receptor (12) and activating a unique serine protease

TABLE III
Lack of Effect of Cholinergic Agents and cGMP on PAF-Induced Secretion

Inhibitor		Percent release of serotonin	
		With PAF	Without PAF
None		35.9	2.2
Carbachol	10^{-4} M	34.7	1.3
	10^{-6} M	35.8	1.4
	10^{-8} M	35.1	1.5
	10^{-10} M	34.3	2.2
	10^{-12} M	33.9	1.1
Acetyl choline	10^{-4} M	33.5	1.7
	10^{-6} M	33.1	1.0
	10^{-8} M	32.9	2.4
	10^{-10} M	34.2	1.1
	10^{-12} M	35.5	1.5
cGMP	10^{-3} M	31.3	1.9
	10^{-5} M	32.4	1.7
	10^{-7} M	32.9	2.2
	10^{-9} M	31.8	1.6

This experiment was repeated three times with PAF concentrations giving secretion varying from 30 to 60%.

(23), which then acts to induce secretion through a common pathway within the platelet.

The Role of Serine Proteases in PAF-Induced Secretion. Secretion from a number of 'mediator' cells has now been shown to involve serine proteases, apparently activated during the stimulation process. These include mast cells, neutrophils, platelets (see 13), basophils (24), and lymphocytes (25). We have now obtained similar evidence for PAF-induced secretion from rabbit platelets in that DFP inhibits secretion, but only if present during the reaction. Since DFP is irreversible in its action and pretreatment of platelets, or PAF, is ineffective, activation of a precursor protease on or in the platelets, which only then becomes inhibitable with DFP, is suggested. The relative inability of DFP to inhibit serine proteases in their zymogen form is known (26). The high concentrations of DFP required in this system probably reflect the need for the inhibitor to gain access from the outside of the cell to an enzyme being activated in close proximity to its substrate, cleavage of which immediately progresses on to secretion.

Direct evidence for the involvement of such an activatable esterase will have to await its isolation and full characterization. Only with the neutrophil enzyme involved in chemotaxis has a substrate for one of these activatable proteases involved in cell activation been found (27). In the neutrophil we have shown, using [^{3}H]DFP (Becker, E. L. and P. M. Henson, to be published), that as few as 500–1,000 molecules of such an enzyme may be present per cell. The quantitative

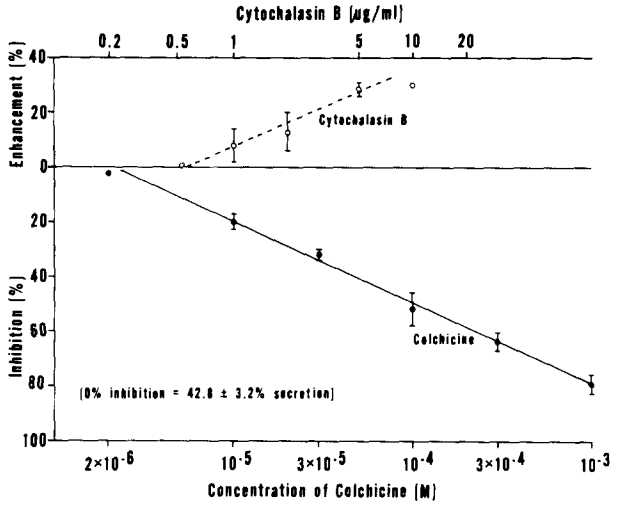

FIG. 9. Effect of colchicine and cytochalasin B on PAF-induced secretion. The drugs were incubated with platelets for 10 min at 37°C before addition of PAF.

problems in isolating the enzyme from neutrophils or platelets will be considerable. Nevertheless, additional evidence supporting the potential involvement of an activatable serine protease in PAF-induced platelet secretion has been obtained. Other inhibitors of such enzymes were also active against PAF-induced secretion, including PMSF. Low molecular weight amino acid esters were inhibitory, especially those with basic and aromatic groups. TAMe was most effective of the esters used, and leucine methyl ester was inactive. Substitution of the NH_2 of lysine methyl ester (inhibitory), with an acetyl group rendered it ineffective as an inhibitor. It is suggested that the esters are inhibiting the activated enzyme, effectively competing for it with the natural intracellular substrate, which is at present unknown. The serine proteases have esterolytic activity, and it is important to note that the small molecular weight esters are significantly more inhibitory than the native amino acids. The inhibition is reversible and is not due to hydrolysis products of the esters, since methanol itself is ineffective at these concentrations. While at this point it cannot be proven that the esters are inhibiting an activatable serine protease, the data are consistent with such a hypothesis.

The hypothesis that activation of cells such as platelets, mast cells, and neutrophils involves as a very early step the conversion of a precursor protease (esterase) to its active form is gaining credence as evidence mounts in its favor (13). We have recently obtained evidence, in addition, that different stimuli activate different platelet esterases (23).

It is possible that the cell receptor for a given stimulus and the precursor protease are one and the same (or at least are closely linked). If so, the analogy between the mechanisms of initiation of two plasma enzyme mediator systems (the complement and coagulation systems) becomes apparent. In both cases presumed conformational changes in precursor serine proteases induced by specific stimuli lead to generation of the active enzymes (C1 esterase or Hage-

man factor) and then to a sequence of amplifying and controlling steps until the complement action or coagulation is complete. We are suggesting that a similar process, i.e. a conformationally activated protease, may initiate secretion in platelets. Exogenous proteases which activate platelets, such as thrombin or trypsin, might either bypass the enzymes to act on their substrates or could themselves activate the putative enzymes. If a unique enzyme is involved in PAF-induced secretion, its decay or inactivation could account for the specific desensitization (see the previous paper, 12), as was suggested for the neutrophil chemotaxis system (27). If so, the small molecular weight esters may protect against the desensitization, as well as providing a possible way of determining substrates for the enzymes.

The Role of Cyclic Nucleotides in PAF-Induced Secretion. Increasing the intracellular levels of cAMP in rabbit platelets effectively inhibited the secretory process. This increase could be brought about by stimulating synthesis of cAMP with PgE_1 by inhibiting breakdown of the cAMP by the phosphodiesterase with theophylline, or by incubating the platelets with exogenously supplied cAMP. The effects of PgE_1 and theophylline in increasing cAMP levels in platelets have been described by others (see 13, 28) and were confirmed by our studies. It was also found, as expected of this system (18), that PgE_1 and theophylline acted together in a synergistic manner to prevent PAF-induced secretion.

Cyclic nucleotides, cAMP and cGMP, have been shown to act intracellularly to control or initiate secretion from a wide variety of cells (see 13). In many cases, an antagonistic (Yin Yang) relationship between cAMP and cGMP has been demonstrated (29). In cells secreting mediators of inflammatory processes, cAMP acts (as seen for PAF-induced secretion) to inhibit the secretory process which is somewhat different from its role in other cell types (13). cGMP, on the other hand, enhances secretion (30, 31) or cell movement (32). In the studies reported here, however, neither cGMP nor cholinergic agents such as carbachol or acetylcholine, which are known to increase levels within cells (29–32), had any effect on the secretion induced by PAF. We have also observed a lack of effect of the cGMP system on secretion from rabbit platelets induced by other stimuli (unpublished). The inference is that in this cell type, cAMP is the modulating nucleotide, and that cGMP and the so called "Yin Yang" effect is inoperative. However, increased cGMP has been reported in human platelets during collagen-induced secretion (33). Whether this difference from the data described herein is a species difference or whether it reflects a lack of action of exogenous cGMP or of cholinergic agents is at present unknown.

If increased levels of cAMP are inhibitory, the question may be raised as to how the levels of cAMP change during the secretory process itself. As expected, a measurable decrease in the cAMP level was detected. This decrease, although time dependent, was extremely rapid, which is consistent with a permissive effect of the lowered nucleotide level on the secretory process. By contrast, collagen, which also induces secretion, and in addition, induced lower cAMP levels, (as shown also by others, 34) stimulated both of these changes at a slower rate. A correlative effect between changes in cAMP levels and secretion is implied. It is suggested that the stimulus (PAF) induces a temporary decrease in

intracellular cAMP which permits the secretion to occur. When the cAMP levels return to normal, the secretion might then be terminated. In fact, as described in the previous manuscript (12), in the case of PAF as a stimulus, desensitization of platelets by the PAF probably serves to shut off the secretion before the cAMP can act. With other stimuli for platelet secretion, however, the cAMP effect may have more importance.

The PAF-induced desensitization of platelets itself (12) does not appear to result from changes in cAMP levels for a number of reasons: *(a)* Increased cAMP was not observed after PAF stimulation. *(b)* The desensitization to PAF is specific for that stimulus. *(c)* Platelets desensitized to PAF exhibit an enhanced secretion to other stimuli. This latter effect was shown to result from the decreased cAMP levels induced by the original PAF stimulation.

The observed decrease in cAMP levels is not extensive (about 30%), but is consistent with that seen for other stimuli on rabbit platelets (Henson, unpublished) or with human platelets (34). Presumably, the balance is very delicate, and it does not require much of a decrease to permit secretion to occur. It is also possible, though unlikely, that the cAMP is compartmentalized within the cell and that a much greater decrease is occurring within a particular pool.

The measurements of cAMP were made on the whole platelet population (which appears to be relatively homogenous in its response to PAF) including the surrounding fluid. A decrease thus represents increased destruction or decreased synthesis. The latter seems most likely since in human platelets a number of stimuli (including thrombin) are known to inhibit adenylate cyclase. It is suggested that the activated serine protease could act similarly on adenylate cyclase. In view of the possibility that thrombin might itself be contributing to the PAF effect, it is important to emphasize that hirudin, a potent thrombin inhibitor, did not alter the PAF-induced secretion (1, 10).

The Role of Microtubules and Microfilaments. The observation that colchicine inhibits PAF-induced secretion suggests a role for microtubules in the secretory process. Similar inhibition by colchicine of secretion induced by other stimuli has been reported or found (see 17, and Henson, unpublished observations). The concentrations of colchicine required are similar to those necessary to alter the platelet microtubules as observed by ultrastructural examination (19, 20), suggesting that the effect is indeed to 'disrupt' the microtubules or rather, to prevent their subunit aggregation.

Morphologic observations of the PAF-induced secretion demonstrate a migration of the marginal bundle of microtubules towards the center of the platelet as described for other stimuli by White (14). Whether this is essential for secretion is not known, but if it was, it could explain the effects of colchicine.

Cytochalasin B enhanced secretion induced by PAF. It also enhances secretion from human platelets induced by collagen (35). The explanation for this is unknown, although it is noteworthy that secretion from neutrophils (13) and basophils (36) is also enhanced by this drug. Cytochalasin is thought to disrupt the microfilaments (22), in this case comprised of the contractile protein thrombosthenin. However, this has been questioned (37). In the neutrophil, an increased mobility of granules is observed after cytochalasin, probably resulting in an increased likelihood of their interaction with the 'activated' plasma

membrane (38). It is unclear whether a similar phenomenon is operative in platelets. Cytochalasin B inhibits clot retraction (22), a phenomenon attributable to platelets and their content of thrombosthenin. The effect of cytochalasin B on secretion from rabbit platelets, however, has been variable in our hands with different stimuli. Thus, PAF-induced secretion is enhanced, but that induced by C3 bound to zymosan particles was inhibited (unpublished observations). The possibility that these differences might reflect the differences between a particulate or soluble stimulus is under investigation.

The results suggest some role for both microtubules and microfilaments in the secretory process initiated by PAF. However, while the exact mechanism by which a stimulus at the platelet membrane is translated to a discharge of granule contents remains unknown in physical, biochemical, or even morphologic terms, any suggestions as to the nature of the role played by these structural elements of the cell or their interaction with Ca^{++} and cAMP (39) remains highly speculative.

Conclusion. A consideration of all the data would suggest that PAF activates a precursor serine protease which then initiates the secretory process within the platelet. This latter is a programmed function of the cell and can be initiated by other stimuli including collagen, thrombin, antiplatelet antibody, or C3 (13). Secretion induced by any of these stimuli shows similar properties: is calcium and energy requiring, is modulated by cAMP, and in some way involves microtubules and microfilaments. A similar secretory process is observed in mast cells and basophils and, indeed, in neutrophils and macrophages as well (13). The uniqueness of the reaction lies in the stimulus, in this case PAF, its interaction with cell surface receptor, and its activation of an apparently specific serine protease (23).

Summary

Secretion of serotonin from platelets induced by platelet-activating factor (PAF) derived from antigen-stimulated, IgE-sensitized rabbit basophils was studied to further characterize the biochemical requirements. Inhibition of secretion with diisopropylphosphofluoridate (DFP) was observed if the DFP was present during the reaction, but not if platelets or PAF were pretreated with the inhibitor. This suggested a role for an activatable serine protease in the secretion. Supporting evidence came from the observation that other protease inhibitors and a variety of low molecular weight amino acid esters were also inhibitory. TAMe was most effective, and AGLMe and LeuMe were inactive, indicating a specificity for different esters. Secretion was reduced by agents that increased intracellular cyclic AMP (cAMP), but enhanced by α-adrenergic stimulation, which reduced the levels of cAMP. Concurrent with PAF-induced secretion, a reduction in cAMP levels was observed. No effect of cyclic GMP or cholinergic stimulation was found. Secretion was inhibited by colchicine and enhanced by cytochalasin B, suggesting a role for microfilaments and microtubules. The effects of these three systems on PAF-induced secretion indicate the basic uniformity of the secretory process in platelets (and other cells) whatever the stimulus. The uniqueness of the reaction apparently lies in the stimulus-receptor interaction and the nature of the serine protease which is activated.

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