ACTIVATION OF STIMULUS-SPECIFIC SERINE ESTERASES (PROTEASES) IN THE INITIATION OF PLATELET SECRETION

I. Demonstration With Organophosphorus Inhibitors*

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Platelets undergo a secretory reaction in response to a variety of noncytotoxic stimuli. We have earlier suggested that this secretion of the vasoactive amines, histamine and serotonin, from platelet granules, is another manifestation of a general secretory mechanism to be found in most cells capable of secreting granule-bound constituents (1). Particular similarities were noted among a number of 'mediator cells', e.g., mast cells, basophils, platelets, neutrophils, macrophages, and lymphocytes. A common feature of these cell types is that their physiologic response to particular stimuli can be inhibited by organophosphorus inhibitors of serine esterases. This raises the possibility that cell-associated serine esterases are involved in these responses. The cell responses include not only secretion, e.g. from platelets (see below), mast cells (2, 3), neutrophils (1, 4), and basophils (5), but also neutrophil chemotaxis (6) and phagocytosis (7) and lymphocyte cytotoxicity (8, 9) and movement (10, 11). The organophosphorus inhibitors used include diisopropylphosphonofluoridate (DFP) and a variety of phosphonate esters. They specifically and irreversibly inhibit serine esterases by phosphorylating the serine group in the active site of the enzyme (12).

Use of organophosphorus inhibitors to examine the role of esterases in cell activation can provide two additional pieces of information. First, the structure of the phosphonate inhibitors determines their relative inhibitory activity for different serine esterases. This presumably reflects the 'goodness of fit' of the inhibitor for the active center of the enzyme, since the inhibitors are substrate analogs which phosphorylate the serine group in the same manner as the substrate acylates it (12, 13). This property has been used to demonstrate different inhibition specificities (activity-structure profiles) for trypsin, chymotrypsin, C1s and acetylcholine esterase (13, 14). Phosphonates have been simi-
larly used to show that the esterase putatively involved in mast cell activation is different from the above four enzymes (2), and that there are two different esterases involved in the chemotactic response of neutrophils (6). Since a majority of the serine esterases have endopeptidase as well as esterolytic activity, and the proteolytic properties are more likely to be involved in the cell activation (see reference 1 and Discussion), we have generally employed the term serine proteases for cell-associated activities.

A second piece of information about the cell-associated serine proteases to be gained from use of the organophosphorus inhibitors relies on the observation that the inhibitors have relatively little activity, if any, on the precursor or zymogen form of the enzymes (15). This property has been used to show that some of the proteases involved in cell responses are in fact activated by the stimulus and only then became inhibitable by the organophosphorus agent. In this way, it was shown that of the two esterases involved in neutrophil chemotaxis, one was in an activated state and the other was in a precursor state and could be activated only by an interaction with the chemotactic stimuli (6). This has led to the concept of stimulus-activatable proteases as essential components of the initiation of a variety of cell responses (1).

In previous work, we have described and characterized three unique noncytotoxic stimuli which cause secretion from rabbit platelets. These include (a) platelet-activating factor (PAF) which is a low molecular weight material released from antigen-stimulated IgE-sensitized basophils (16, 17). It induces both aggregation of platelets and secretion of their content of vasoactive amines (17, footnote 2). (b) C3 bound to particles such as zymosan (ZC3) also induces secretion from platelets having a C3 receptor, e.g., those from the rabbit (18). (c) Antibody directed against the surface membrane of rabbit platelets which in the absence of complement, initiates noncytotoxic secretion of platelet constituents (19). The secretion induced by all three of these stimuli is inhibited by DFP (18–20). Additional stimuli examined were the potent platelet activators, collagen and thrombin. The latter is in itself a serine protease. This group of five stimuli is representative of platelet stimuli in that it includes two particulate stimuli (ZC3 and collagen), two soluble agents (PAF and anti-platelet antibody) of differing molecular weights, and a proteolytic enzyme (thrombin).

Experiments are described herein which were designed to determine whether the secretory response of rabbit platelets to five different stimuli involves activation of cell bound serine proteases. Since this appeared to be so for at least four of them, then we further attempted to determine whether each stimulus activates a different protease or whether one common enzyme is involved in the cell response. Since most of the pathway from stimulus to secretion is thought to be common for all the stimuli (1), this question also asks whether the protease is in the stimulus-specific or common portions of the pathway. The evidence suggests that specific serine proteases which are activated by each stimulus are required for platelet secretion.

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2 Henson, P. M., and Z. G. Oades. 1976. Activation of platelets by platelet-activating factor (PAF) derived from IgE-sensitized basophils. III. Characteristics of the aggregation and its dissociation from secretion. Submitted for publication.
Materials and Methods

Platelets. Blood was obtained from New Zealand white rabbits, and washed platelets were prepared by a modification of the technique of Ardlie et al. (21), as described earlier (18 and footnote 2). They were resuspended in Tyrode's solution containing 0.25% gelatin but lacking calcium to a concentration of \(2.5 \times 10^9/\text{ml}\). To examine the secretory reaction, exogenous \([^{3}\text{H}]\text{serotonin}\) was incorporated into the platelets before washing by incubating platelet-rich plasma with \(0.6 \mu\text{Ci/ml}\ \left[{^{3}\text{H}}\right]\text{serotonin binoxalate (4.3 Ci/mmol, New England Nuclear, Boston, Mass.) for 15 min at 37°C.}\)

Secretion. Platelets (0.1 ml containing \(2.5 \times 10^9\) platelets) were incubated with the stimulus (in 0.1 ml) and inhibitor (also generally in 0.1 ml) in a final vol of 1 ml of Tyrode's solution containing gelatin and calcium for 15 min with shaking at 37°C. At the end of the incubation the tubes (12 \times 75-mm polystyrene tubes, Lancer, Sherwood Medical Industries, St. Louis, Mo.) were chilled in ice and the platelets pelleted at 1,800 \(g\) for 15 min in the cold. An aliquot (0.1 ml) of the supernate was removed and the radioactivity (secretion of serotonin) determined in a mixture of equal parts Aquasol (New England Nuclear) and toluene in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The secretion was expressed as a percentage of the total incorporated \([^{3}\text{H}]\text{serotonin}\) which could be detected in 0.1 ml of the platelet suspension lysed with 0.1% Triton x 100. The total counts thus measured generally ranged from 18 to 95 \(\times 10^2\) cpm. Background secretion (without stimulus) was always measured. It was generally from 1-5% and in most cases was subtracted from the stimulus-induced secretion. High concentrations of a few phosphonates (e.g., the long chain derivatives of the p-nitrophenyl ethyl alkylphosphonate series when used above millimolar concentration) induced some secretion, and where this could not be avoided this control value was subtracted from the release found with stimulus and phosphonate together. All reactions were performed in duplicate or triplicate and the counts (which differed by less than 10%) averaged. The effect of the inhibitors was expressed as percent inhibition (see Results section).

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\text{1.0} - \frac{\text{percent serotonin secreted in the presence of inhibitor}}{\text{percent serotonin secreted by the stimulus alone}} \times 100
\]

To compare different inhibitors of the phosphonate series, the \(p_{10}\) was calculated. This is the negative logarithm (to the base 10) of the concentration of inhibitor which gives 50% inhibition of secretion. This figure was obtained from three or more separate experiments and expressed as the mean \(p_{10} \pm \text{SEM.}\)

Stimuli. PAF bound to bovine serum albumin was prepared as described previously (17). The activity is expressed as microliters of dialysed supernate from antigen-stimulated, sensitized leukocytes or as units of PAF, where 1 U is that amount which induces 20% secretion from \(2.5 \times 10^9\) platelets in 1 ml. The gamma globulin fraction of goat anti-rabbit platelet antibody (\(\alpha-PAb\)) was prepared as described earlier (19 and footnote 2), and ZC3 was prepared by incubating extensively washed zymosan (Nutritional Biochemicals Corp., Cleveland, Ohio) with fresh rabbit serum (17 mg/ml) for 20 min at 30°C (18). The ZC3 was thoroughly washed and frozen in aliquots. A control preparation of zymosan incubated with serum in the presence of 10 mM EDTA (to prevent binding of C3) did not stimulate secretion. Bovine thrombin was obtained from Parke, Davis & Co., Detroit, Mich. Collagen was prepared by homogenization and sonication in phosphate-buffered saline (PBS) of bovine tendon (Sigma Chemical Co., St. Louis, Mo.). All stimuli were resuspended or dissolved in PBS and where appropriate, protein determinations were performed by the Folin technique.

Organophosphorus Inhibitors. DFP was obtained from Sigma Chemical Co. It was dissolved directly in Tyrode's gelatin immediately before use. The \(p\)-nitrophenylethylphosphonate esters were described earlier (2, 6, 13, 14, 22). The cyclohexyl phosphonofluoridate esters were described in references 7 and 8. The phosphonates were dissolved in dry propylene glycol at a concentration of 100 mM and kept at \(-20°C\). Hydrolysis of the \(p\)-nitrophenyl compounds were determined by dissolving in phosphate buffer and immediately reading absorbation at 405 nM. Concentrations were corrected for the degree of hydrolysis. It was previously ascertained that propylene glycol was a suitable solvent and at the concentrations used did not cause release of serotonin from platelets. At high concentrations of propylene glycol (above 5%, representing approximately 5 mM...
concentrations of the dissolved phosphonates) some inhibition of secretion was observed, but most of the phosphonates were used at concentrations at which the solvent was without effect. The occasional use of slightly inhibitory concentrations of propylene glycol was accompanied by an appropriate positive control (i.e., solvent alone) so that accurate percent inhibition could be determined. Phosphonates and DFP were hydrolysed by treating solutions of the inhibitors with 0.1 M NaOH for 16 h and neutralizing with HCl.

Pretreatment of Stimuli and Platelets. Platelets were incubated for 15 or 30 min at 37°C with various concentrations of the inhibitors in Tyrode's gelatin without Ca++. They were then washed twice before testing the stimulus-induced secretion. Control platelets were similarly incubated with inhibitors and the DFP removed by overnight dialysis against PBS, or in the case of ZC3 by centrifugation and washing. Control preparations were incubated without inhibitors and then dialyzed or washed.

Results

The Secretory Response of Platelets to Five Stimuli. To examine most effectively the inhibitory action of the organophosphorus esterase (protease) inhibitors, concentrations of stimuli were determined, and the response was shown to be secretory in nature and not cytotoxic. Fig. 1 depicts the secretion induced by increasing concentration of thrombin, a-PAb, collagen, ZC3, and PAF. PAF produces an anomalous response in that the secretion is maximal at about 50-60%. This phenomenon has been described previously and probably results from the simultaneous induction of desensitization by this stimulus, which serves to limit the secretion (23). The cytoplasmic enzyme, lactic dehydrogenase (LDH), was not liberated by any of the stimuli, showing the noncytotoxic nature of these secretory reactions.

For the inhibition studies, concentrations of stimuli were employed to induce about 40% secretion of serotonin, i.e., on the linear portion of each dose response curve. Since exact percent release figures for a given experiment could not be predicted, it was important to show that the percent inhibition of secretion produced by the inhibitors was relatively independent of the amount of secretion. To do this different amounts of stimulus were incubated with platelets in the presence of a constant concentration of DFP. Data is shown in Table I for PAF and collagen and thrombin and was similar over the range of secretion values employed (on the linear portion of the curves) for the other stimuli.

It was also important to determine a suitable incubation time for the reactions. Although the rate of secretion induced by each stimulus varies, the secretion is complete in 15 min at 37°C. An inhibitor such as DFP reduced the rate of secretion (Fig. 2). However, the secretion was again complete by 15 min and no further effect was seen if a 30-min incubation period was employed. Accordingly, a standard 15-min incubation period was used throughout.

Inhibition of Secretion with DFP. To examine the potential role of serine proteases in platelet activation, DFP was examined for its ability to inhibit secretion by all five stimuli (Fig. 3). Concentration-dependent inhibition of secretion was observed for all stimuli. The range of concentrations at which inhibition was achieved, however, varied greatly with the stimulus. In fact, complete inhibition of collagen or anti-platelet antibody-induced secretion was not obtained. Nevertheless, the inhibition curves at lower degrees of inhibition were all parallel. pI50 values (negative log10 of concentration of DFP to yield 50% inhibition) for ZC3, thrombin, and PAF were 4.7, 3.9, and 2.8, respectively.
Table I

| Stimulus   | Percent secretion of serotonin |
|------------|-------------------------------|
|            | Without DFP | With DFP (2 x 10⁻³ M) | Percent inhibition |
| PAF        |              |                        |                    |
| 25 µl      | 68.4         | 37.4                   | 42                 |
| 10 µl      | 55.0         | 22.2                   | 59                 |
| 5 µl       | 27.8         | 11.9                   | 57                 |
| Collagen   |              |                        |                    |
| 100 µg     | 84.4         | 67.2                   | 20                 |
| 40 µg      | 48.1         | 34.5                   | 28                 |
| 20 µg      | 11.5         | 8.8                    | 23                 |
| Thrombin   |              |                        |                    |
| 5 mU       | 47.3         | 12.8                   | 72                 |
| 2.5 mU     | 20.3         | 6.3                    | 69                 |
| 1.25 mU    | 7.5          | 2.9                    | 62                 |

The Requirement for a Stimulus-Activatable Protease. To determine whether an active protease was present on the platelets which were being inhibited by the irreversible organophosphorus inhibitor, platelets were pretreated with concentrations of DFP and then washed. As shown in Fig. 3, no inhibition of secretion occurred under these circumstances. Another possible explanation for the effect of DFP is that the stimuli are themselves serine proteases responsive to the inhibitory action of DFP. That this is so for thrombin is depicted in Fig. 3. Pretreatment of thrombin with DFP followed by dialysis completely abrogated its serotonin-releasing capacity. However, pretreatment of the other stimuli with DFP did not prevent their subsequent action on platelets. For ZC3, over 1/500 concentration of DFP is required to inhibit if present during the reaction than if used to pretreat the stimulus on the platelets.
The time-course of PAF-induced secretion of serotonin in the presence or absence of DFP.

Fig. 3. Inhibition of secretion by DFP. Increasing concentrations of DFP were incubated in the reaction of stimulus and platelets (closed symbols) or used to pretreat the platelets (open symbols) or the stimuli (hatched symbols). The platelets (P) and stimuli were washed or dialyzed after the pretreatment. The figures represent the pl 50 for the respective stimuli.

These data suggest the involvement of a platelet serine protease which is activated by the stimulus and only then becomes inhibitable by DFP.

Inhibition of Secretion With Cyclohexyl Phenylalkylphosphonofluoridates. Experiments with the phosphonate inhibitors were initiated to (a) support the hypothesis of involvement of serine proteases in platelet secretion and (b) to attempt to determine whether the effect of the different stimuli required the action of one or more different enzymes.

The cyclohexyl phenylalkylphosphonofluoridates were found to be the most effective inhibitors of the platelet secretion. Fig. 4 depicts inhibition curves of these agents on the secretion induced by ZC3. The phenyl derivative was found to give 50% inhibition at $1.3 \times 10^{-6}$ M. It may be seen that all the curves are
Molar Concentration of inhibitor

FIG. 4. Inhibition of ZC3 induced secretion by cyclohexyl phenylalkylphosphonofluoridates. The numbers represent the number of carbon atoms in the phenyl alkyl side chain, i.e., 0 = phenyl, 1 = benzyl, 2 = phenyl ethyl, and 3 = phenylpropyl phosphonofluoridates. The arrows indicate 50% inhibition from which the pIso was calculated.

parallel, suggesting an action on the same enzyme and allowing a determination of the pIso. From experiments such as this, the data presented in Fig. 5 was obtained. ZC3 and thrombin were inhibited by very low concentrations of these inhibitors. However, a clear distinction between the activity-structure profiles of the phosphonofluoridates in their inhibition of all five stimuli was found. This strongly suggests that each stimulus, except possibly thrombin (see below), activates a unique serine protease, exhibiting its own activity structure profile. As with DFP, the phosphonofluoridates were found to be much less effective if used to pretreat the platelets than if added to the reaction of stimulus with cells. In this instance the inhibitors were from 10 to 100-fold more effective if included in the reaction.

Inhibition of Secretion with p-Nitrophenyl Ethyl Chloroalkylphosphonate Esters. Inclusion of these inhibitors in the reaction effectively prevented the secretion induced by all five stimuli (Fig. 6). This series of inhibitors was most effective against collagen, especially those with longer side chains. The chlorohexyl derivative caused 50% inhibition at $5 \times 10^{-5}$ M (pIso = 4.32). The inhibitors were least effective against anti-platelet antibody-induced secretion. Distinctive activity structure profiles were observed for all stimuli, except ZC3 and thrombin the action of which was similar and was maximally inhibited by the chloropentyl ($n = 5$) compound and minimally affected by the chloroheptyl ($n = 7$) derivative.

To determine whether the phosphonates were inhibiting a cell-dependent protease, i.e. an enzyme on the cell which was already active, rather than a stimulus-dependent protease, i.e. one which was activated by the stimulus, the platelets were pretreated with the inhibitors and then washed. Table II depicts the results with the chlorobutyl compound, although the other inhibitors gave similar results. Inclusion of the inhibitor in the reaction was much more effective than if the platelets were pretreated. Again, action of the phosphonates on a stimulus-activated esterase is suggested.

Two other series of phosphonate inhibitors were studied, the $p$-nitrophenyl ethyl phenylalkyl- and $p$-nitrophenyl acetoxyphenylalkylphosphonates. While these inhibitors were slightly less active than those shown in detail above, they
too were capable of distinguishing between the different stimuli and were more effective if included in the reaction mixture.

Effect of Nonphosphorylating Phosphonates and of Hydrolyzed Inhibitors. The possibility that the inhibition was not due to phosphorylation of the active center of the putative platelet proteases was examined. The data in Table III compare the effect of p-nitrophenyl ethyl pentylyphosphonate which is a good
phosphorylator with phenyl ethyl pentylyphosphonate which phosphorylates very poorly. The phenyl phosphonates are much less effective irreversible inhibitors of serine esterases than are the p-nitrophenyl derivatives (11). The phenyl phosphate was also much less effective in preventing serotonin secretion. Similarly, the nonphosphorylating diisopropyl methyl phosphate was compared with the phosphorylating compound, DFP, and found to be completely ineffective as an inhibitor of platelet secretion.

Another way of approaching the question of other actions of the phosphonates is to determine whether hydrolysis of the agents prevents their action (8) and whether some of the activity is due to the products of hydrolysis. Table IV shows the ineffectiveness of the hydrolyzed compounds (including DFP) and of products of the hydrolysis such as p-nitrophenol or fluoride. This supports the concept of the agents acting as irreversible serine esterase inhibitors.

**Phosphonate Inhibition of the Esterolytic Action of Thrombin.** The secretion from platelets which is induced by thrombin was also inhibited by these series of phosphonate and fluoridate inhibitors. The exogenous serine protease in this system could be acting either directly on some protease-sensitive substrate within the platelet or could itself activate a serine protease in or on the cell. In an attempt to distinguish between these possibilities we examined the activity-structure profiles of the cyclohexyl phenylalkyl phosphonofluoridates on the hydrolysis of benzoyl-\(\beta\)-arginine ethyl ester (BAEe) by thrombin was examined. If the profile were different from that for the action of thrombin on platelets the activation of another esterase within the platelet might be implicated. Fig. 7 depicts the hydrolysis of BAEe by 1 U of thrombin, measured by the increase in absorbance at 253 nm. Cyclohexyl phenylethylphosphonofluoridate effectively inhibited the enzyme in a time-dependent fashion as expected. Addition of the

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**Table II**

*Inhibition of Platelet Secretion by p-Nitrophenyl Ethyl Chlorobutyl-phosphonate Ester*

| Inhibitor present during the reaction | Platelets pretreated with the inhibitor and washed |
|--------------------------------------|-----------------------------------------------|
|                                      | \(p_{50}\)                                      |
| Collagen                             | 3.84 <2.0                                      |
| Anti-platelet antibody               | 3.3 <2.0                                       |
| PAF                                  | 3.84 <2.0                                      |
| ZC3                                  | 3.4  3.0                                       |
| Thrombin                             | 3.4  3.0                                       |

**Table III**

*Lack of Inhibition by Nonphosphorylating Compounds*

| Inhibitor | Percent inhibition of secretion |
|-----------|--------------------------------|
|           | ZC3   | Thrombin | Collagen |
| p-nitrophenylethyl pentylyphosphonate (2.5 x 10\(^{-4}\) M) (phosphorylating) | 58    | 54   | 38      |
| Phenylethyl pentylyphosphonate (2.5 x 10\(^{-4}\) M) (poorly phosphorylating) | 6     | 3    | 8       |
| Diisopropyl phosphofluoridate (DFP) (1 x 10\(^{-3}\) M) (phosphorylating)    | 100   | 100  | 25      |
| Diisopropyl methyl phosphate (DMP) (1 x 10\(^{-3}\) M) (nonphosphorylating)   | 0     | 0    | 0       |
ACTIVATION OF STIMULUS-SPECIFIC ESTERASES

TABLE IV
Lack of Inhibition by Hydrolyzed Compounds

| Inhibition                                      | Percent inhibition of secretion |
|------------------------------------------------|---------------------------------|
|                                                 | ZC3    | Thrombin | Collagen |
| DFP (2 × 10⁻³ M)                                | 100    | 100      | 39       |
| DFP hydrolyzed* (2 × 10⁻³ M)                    | 0      | 0        | 0        |
| Cyclohexyl phenylpropyl (1 × 10⁻³ M)            | 100    | 100      | 48       |
| Cyclohexyl phenylpropyl hydrolyzed* (1 × 10⁻³ M)| 13     | 0        | 0        |
| Propylene glycol                                | 4      | 0        | 0        |
| Propylene glycol hydrolyzed*                    | 4      | 0        | 0        |
| p-nitrophenol (5 × 10⁻⁴ M)                      | 0      | 0        | 0        |
| Sodium fluoride (10⁻³ M)                         | 3      | 0        | 1        |

* Treated with 0.1 M NaOH and then neutralized.

Fig. 7. Inhibition of the esterolytic activity of thrombin. 1 U of thrombin was added to 1.5 ml 1 mM BAEe in 0.15 M NaCl buffered to pH 7.6 with 0.01 M Tris at 37°C and the changes in OD at 253 nm determined on a strip recorder. Cyclohexyl phenylethylphosphonofluoridate was added at the start of the incubation or was preincubated with the enzyme for 10 min before addition of substrate.

inhibitor concurrently with the enzyme and substrate was much less effective than preincubation of the thrombin with the inhibitor. This may explain the relatively high concentration of the inhibitors required to inhibit the stimulus-activated proteases in the platelets, since this experimental circumstance is essentially the same as that of concurrent addition of inhibitor, enzyme, and substrate.

The activity structure profiles of the phosphonofluoridates in inhibiting thrombin-induced serotonin secretion from platelets and thrombin-induced hydrolysis of BAEe are compared in Fig. 8. They were found to be identical. The absolute values of the pIₐ's cannot be compared because of the 10-min preincubation of inhibitor and thrombin. In the platelet experiments the phosphonates were apparently acting on the thrombin itself and not detectable on a thrombin-activated esterase.
Fig. 8. Activity structure profiles of the cyclohexyl phenylalkylphosphonofluoridates in inhibiting the action of thrombin on platelets (secretion) or on BAEe (esterolysis).

Discussion

Induction of the secretory response in rabbit platelets by five different stimuli was prevented by organophosphorus inhibitors of serine proteases (esterases). The involvement of such enzymes in the platelet release reaction was thereby suggested. In addition, the data support the concept of the enzymes being stimulus-activatable proteases, since inhibition was only achieved by concurrent addition of the inhibitor and stimulus to the platelets and nonphosphorylating or poorly phosphorylating analogues were inactive. Finally, the unique activity structure profiles found for the phosphonate inhibition of secretion induced by the different stimuli were highly suggestive of the requirement for a unique activatable platelet protease for each of the stimuli.

The Requirement for an Activatable Serine Protease. Stimulation of secretion from rabbit platelets by collagen, PAF, anti-platelet antibody, particle bound C3, and thrombin was in each case inhibitable by concurrent addition of organophosphorus inhibitors. DFP was effective against all five stimuli, but in most cases only at high concentrations. The \( p \)-nitrophenyl ethyl chloroalkylphosphonates and cyclohexyl phenylalkylphosphonofluoridates, however, were highly active in inhibiting the secretory response. Since the inhibition of serine esterases by these compounds is both time and temperature dependent, the relatively high concentrations required in most cases for inhibition is readily explained. As depicted in Fig. 7, concurrent addition of inhibitor to an active enzyme (thrombin) in the presence of its substrate is an inefficient way to prevent the cleavage of the substrate. And yet this is precisely the situation in the cell, where the protease is presumably activated in some way by the stimulus (see below) and then finds itself in the presence of both substrate (in this case an unknown substrate in the cell) and the inhibitor. The ability of, for example, cyclohexyl phenylphosphonofluoridate to inhibit ZC3-induced secretion with a \( p_{1/2} \) of about 6.0 therefore indicates great potency and considerable specificity.
Before the existence of cell-associated proteases could be inferred, it was most important to eliminate the possible action of the inhibitors on the stimuli themselves. Pretreatment of ZC3, collagen, PAF, or α-PAb had no effect on their subsequent action on platelets. As expected, however, the serine protease, thrombin, was completely inhibited. In view of the great sensitivity of ZC3-induced secretion to inhibition by organophosphorus agents, the similarities of the inhibition profiles to those of thrombin, using the p-nitrophenyl ethyl chloroalkylphosphonates, the possible peptidase activity of bound C3 (25), and the method of preparation of ZC3 (i.e., incubation of zymosan with fresh serum, from which a number of proteases could have been absorbed) indicated the special need for this control. However, the action of ZC3 was not prevented by pre-treatment with DFP or phosphonates and is not inhibited by the thrombin inhibitor hirudin (18). Moreover, the inhibition profiles with the phosphonofluoridates for ZC3 and thrombin were found to be significantly different.

It is also important to examine the possibility that the organophosphorus inhibitors are acting on the platelets in some way other than as serine esterase inhibitors. A number of lines of evidence serve to render this possibility highly improbable. The most likely action would be as detergents. However, the compounds with the larger hydrophilic side chain would be expected to be more effective, and this was not found to be the case. In addition, the different inhibitory activities of the various phosphonates or fluoridates in a series against the different stimuli argue against this possibility as does the effectiveness of DFP itself. Further support for the involvement of the specific phosphorylating action of the inhibitors came from examination of the nonphosphorylating, diisopropyl methyl phosphate and the poorly phosphorylating phenyl ethyl pentylyphosphonate. These were found to be inactive as inhibitors when compared with their phosphorylating counterparts, DFP and p-nitrophenyl ethyl pentylyphosphonate. Hydrolysis of the organophosphorus compounds prevented their inhibitory action, indicating that the complete and active molecule is required. It was also shown that the inhibition was not produced by the hydrolysis products p-nitrophenol or fluoride.

If the protease or esterase that is being inhibited is in or on the platelet, the question now arises (see reference 1) as to whether it is already activated (cell dependent) or is activated by the effect of the stimulus (stimulus dependent). Pretreatment of the platelets with the inhibitors followed by washing did not prevent the subsequent activation of the cells by the stimuli. Since these are irreversible inhibitors of serine proteases (12–14), the data suggest that the enzymes involved in the secretory response of platelets are stimulus dependent. The presumption that in the instances studied the organophosphorus compounds are acting as irreversible inhibitors is strongly supported by the demonstration that the nonphosphorylating or poorly phosphorylating analogues are inactive. The differences in pI50 for phosphonates, fluoridates, and DFP added concurrently with those when the cells were pretreated were found to vary with the stimuli and the inhibitor. In some cases, the difference was more than 500-fold, in others less than 10-fold. The latter circumstance was particularly evident when poorly inhibited stimuli were employed (e.g., anti-platelet antibody) or when inhibitors with long side chains were examined. It is believed that
the effect is in part due to absorption of the inhibitors, particularly if they have long hydrophobic side chains, by the platelet membranes so that they are not effectively washed out after pretreatment. Activation studies with \[^\text{3H}\]DFP (Becker and Henson, unpublished observations) would support this contention. It is concluded, therefore, that the data overestimate the inhibitory effect of pretreatment of the platelets and that with all four stimuli studied the involvement of stimulus-activated proteases is strongly indicated.

This probable involvement of stimulus-activated proteases or esterases in platelet activation provides another example to support the hypothesis that such stimulus-activated proteases represent a general phenomenon, at least in the triggering of 'mediator cells' such as mast cells, basophils, neutrophils, and lymphocytes (1). Mast cells have been shown by similar techniques to have stimulus-activated esterases which are required for secretion (2, 3). The anti-Ig-induced movement of B lymphocytes have also been demonstrated to require a stimulus-activated esterase (11), and there is suggestive evidence that this is true of human T lymphocyte-mediated cytotoxicity (9). Neutrophil chemotaxis was found to involve both a stimulus-dependent and a cell-dependent esterase (6). In other cell processes, e.g. neutrophil phagocytosis (7) or mouse T-lymphocyte cytotoxicity (8), cell-dependent esterases have been demonstrated, but conclusive evidence for stimulus-dependent enzymes has not yet been presented. While secretion from rabbit platelets has been shown to involve predominantly stimulus-activated esterases, the concurrent requirements for a cell-dependent (i.e., previously activated) enzyme of this type, which is relatively resistant to inhibition by the phosphonates, cannot be completely excluded.

**Stimulus Specificity of the Activatable Proteases.** The data presented using three series of p-nitrophenyl ethyl phosphonate inhibitors and one of phosphonofluoridates indicate that the effect of each stimulus was uniquely inhibited by these compounds. Activity-structure profiles for the series of inhibitors against the five stimuli were both characteristic and different for each stimulus. This was particularly evident for the cyclohexyl phenyalkylphosphonofluoridates which were the most inhibitory group of compounds tested. However, it was also seen with the p-nitrophenyl ethyl chloroalkylphosphonates as also with a group of p-nitrophenyl ethyl alkylphosphonates and of p-nitrophenyl ethyl phenyalkylphosphonates (for which the data were not presented). These results represent strong evidence in support of the conclusion that each stimulus activates a unique serine protease which is then required for the secretory process. Other explanations for the effect of different inhibitors, for example a differing solubility in the platelet membrane, would not account for the unique inhibition profiles for each stimulus.

Thrombin is itself a serine protease and exhibits its own unique profile of inhibition. The action of thrombin on the platelets might therefore be either to activate a platelet protease or perhaps to act on the intracellular substrate(s) of these putative proteases, i.e., to act on the next stage in the sequence. The inhibition profile (phosphonofluoridates) for the esterolytic action of thrombin was identical with that for its secretion-inducing action. At this time, therefore, these two possibilities cannot be distinguished. The unique profile does suggest that prothrombin on the platelets is not being activated by the other stimuli.
However, the similarities in the profile for thrombin and ZC3 exhibited by most of the phosphonate series raised the question of ZC3 activation of a prothrombin-like enzyme. Two lines of evidence suggest that this is not so. (a) The inhibition profile of the cyclohexyl phenylalkylphosphonofluoridates for ZC3 and thrombin is different. (b) Repeated attempts to prevent ZC3-induced release by hirudin have been unsuccessful (see also reference 18).

The substrate(s) for these proteases in the platelet are not known. Other areas still to be investigated include the site of the enzymes and their position in the sequence of events leading from stimulus to secretion (stimulus-secretion coupling). Preliminary data (24, 26, and, to be published) suggest that the DFP-inhibitable steps in the secretion sequence are the earliest that can be detected. This would be compatible with the idea of an early stimulus-specific step or steps which then initiate a common pathway resulting in secretion (1). The ability to induce stimulus-specific desensitization of platelets (23) also supports this concept and raises the possibility that the desensitization involves decay or inhibition of the specific activated esterase. A similar phenomenon has been described in neutrophils where the desensitization was prevented by low molecular weight amino acid esters (27).

The earliest step in the sequence is presumably the binding of the stimulus to its receptor. The question arises as to how the stimulus-receptor interaction could activate the protease. The simplest hypothesis equates the receptor with the precursor protease since any other mechanism involves two highly specific steps, i.e., stimulus-receptor interaction and the interaction of specific receptor with specific protease. This possibility will only be proven by isolation of the receptor and the protease as a single entity. Experiments are proceeding in this direction. Indirect support for the hypothesis, however, comes from the demonstrated mechanism of activation of two plasma mediation systems. The complement and intrinsic coagulation systems are initiated by activation of the precursor serine proteases C1 and Hageman factor, respectively. The activation may involve a conformational change in the enzyme to expose an active site. This can then be inhibited by DFP or phosphonate esters (2, 28). By limited proteolysis the enzymes then cleave and activate the next components in the system, C4 and Factor XI, respectively. It is proposed that a similar system may exist in the platelets (and other cells). The stimulus, which is usually at least divalent, could bind to the receptor (which is close to or identical with the precursor serine protease) and then conformationally alter the protease to expose its active center. A possible role for cross-linking of receptors (aggregation of receptor) could also be explained in this manner. The enzyme could then initiate the secretory process within the platelet.

Direct proof of this hypothesis will require isolation of the esterases and desensitization of their activation mechanism and their effect in the cell. However, indirect evidence is accumulating. Thus stimulus-dependent binding of [3H]DFP to C5a-stimulated neutrophils has been found (Becker and Henson, unpublished observations). A minimum of 1,000 molecules of protease per cell was calculated. Moreover, the platelet secretion can be inhibited by low molecular weight amino acid esters (Henson, Landes, and Becker, manuscript in preparation). As with the phosphonates, different esters optimally inhibit differ-
ent stimuli. Taken together these experiments strongly suggest a role for unique stimulus-specific activatable proteases in platelet secretion.

Summary

The effect of organophosphorus inhibitors of serine esterases (proteases) on secretion from washed rabbit platelets was examined. Five noncytotoxic stimuli were employed: collagen, thrombin, heterologous anti-platelet antibody (in the absence of complement), rabbit C3 bound to zymosan, and platelet activating factor derived from antigen-stimulated, IgE-sensitized rabbit basophils.

Diisopropyl phosphofluoridate, three series of p-nitrophenyl ethyl phosphonates, and a series of cyclohexyl phenylalkylphosphonofluoridates were all found to be inhibitory to the platelet secretion. These are irreversible inhibitors of serine proteases but in this system were only inhibitory if added to the platelets concurrently with the stimuli. Pretreatment of either the platelets or the stimuli with the inhibitors followed by washing, was without effect on the subsequent reaction. This suggested the involvement of stimulus-activatable serine proteases in the secretory process. The concept was supported by finding that nonphosphorylating phosphonates or hydrolyzed phosphonates or phosphonofluoridates were without inhibitory action.

The effect of a series of phosphonates or phosphonofluoridates in inhibiting each stimulus exhibited a unique activity-structure profile. The demonstration of such unique profiles with four series of inhibitors for each of the five stimuli was interpreted as demonstrating that a specific activatable serine protease was involved in the platelet secretory response to each stimulus.

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