# Thrombin-Induced Platelet Secretion

## FURTHER EVIDENCE FOR A SPECIFIC PATHWAY

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ABSTRACT We have studied the interaction between thrombin and washed, human platelets using prostacyclin, a reversible inhibitor of platelet secretion. The effect of thrombin is limited to those reactions that are not inhibited by an increased concentration of platelet cyclic adenosine 3',5'-monophosphate, because prostacyclin is a potent inducer of the latter. Prostacyclin-treated platelets were briefly (15–30 s) exposed to low concentrations of human thrombin (0.01–0.2 U/ml). After removal of the prostacyclin and thrombin, the platelets were incubated with fresh thrombin. Although they had not undergone the release reaction after the first thrombin incubation, these platelets had a diminished capacity to secrete [³H]serotonin when exposed to thrombin the second time. Refractoriness was concentration dependent: the higher the initial thrombin concentration, the greater the degree of inhibition of serotonin secretion on subsequent thrombin exposure. Inhibition was closely related to the ability of thrombin to induce platelet secretion and not to its esterase or fibrinogen clotting activity. Diisopropyl fluorophosphate-inactive thrombin did not induce refractoriness. Refractoriness to thrombin did not increase when the time of the initial incubation with thrombin was lengthened, nor was it reversible.

Inhibition was thrombin specific: serotonin secretion induced by collagen, wheat germ agglutinin, and the ionophore A23187 was minimally affected. For an equivalent amount of thrombin bound, a decrease was observed in serotonin secretion by thrombin-pretreated platelets compared to control platelets. Thus, there is at least one step in the secretory pathway between thrombin binding and regulation of adenylate cyclase. This step appears to transmit the signal that leads to extrusion of intracellular granular contents.

## INTRODUCTION

Thrombin, a blood coagulation factor with proteinase activity, is also capable of initiating platelet secretion (1). Because it stimulates platelet secretion at concentrations that are generated physiologically (1–2 nM), it is likely to be important in the initiation of the platelet thrombus formed in response to vascular injury (2, 3). The mechanism by which thrombin stimulates platelet secretion is unknown. Recent evidence suggests that thrombin initiates the platelet release reaction[1] through a different pathway from that of other mediators of secretion (4, 5). Most agents known to induce secretion require formation of platelet prostaglandin endoperoxides and release of adenosine diphosphate. Thrombin, however, does not.

Equilibrium binding of thrombin to the platelet surface appears to be the first step in this pathway (6–10). Thrombin binding is specific, saturable, and reversible (9). The precise correlation that has been demonstrated between the amount of thrombin bound to the platelet and the extent of secretion suggests that binding may be of physiological importance (11). However, although it may be the necessary first step in the secretory pathway, binding in itself is insufficient to induce the release reaction. This is indicated by the fact that thrombin inactivated at its serine center and native thrombin bind identically to platelets, whereas only the native form induces secretion (7, 12).

The specific reactions that follow thrombin binding have not been characterized. Although thrombin stimulates a variety of metabolic changes in platelets (13–16), these reactions occur in response to most agonists that stimulate the release reaction. One reason for the paucity of information on the sequence of events that takes place after thrombin binding is the rapidity with which secretion occurs. Because in human platelets secretion takes place in <1 min (17), it has been dif-

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[1] The term "release reaction" refers to platelet secretion and the terms are used interchangeably.

ficult to separate the unique effects of thrombin on platelets from the general changes associated with secretion. By incubating platelets with prostacyclin (PGI₂),[2] a reversible inhibitor of secretion, we have been able to study the presecretory effects of thrombin on platelets. In this report, we describe the nature of some of these effects and how they relate to subsequent platelet secretion.

## METHODS

*Preparation of platelets, thrombin, and other materials.* Platelets were prepared from freshly drawn blood according to the method of Baenziger and Majerus (18).

Human alpha and gamma thrombin were prepared as described previously (11, 19, 20). The specific activities were 2,400–4,000 and 1.88 U/mg, respectively.

Thrombin (0.022 mg) was incubated for 1–2 min at 20°C with 0.038 U lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.), 0.062 U glucose oxidase (Type V, Sigma Chemical Co.), 0.18% β-d glucose, 0.75 mCi [$^{125}$I]sodium iodide (New England Nuclear, Boston, Mass.), 0.01 M benzamidine, and buffer (0.2 M NaCl–0.05 M sodium phosphate, pH 7.0) in a total volume of 0.5 ml. [$^{125}$I]Thrombin was separated from [$^{125}$I]sodium iodide by gel filtration.

Thrombin was incubated for 30 min at 20°C in 0.75 M NaCl–0.025 M imidiazole, pH 7.3, with diisopropyl fluorophosphate (iPr₂ PF) diluted 1:100 to a final concentration of 0.01 M to prepare diisopropylphosphoryl thrombin (DIP-thrombin). The iPr₂ PF was removed by chromatography over a column of Sephadex G-25-80 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.). The DIP-thrombin had no demonstrable clotting activity.

Both the [$^{125}$I]thrombin and DIP-thrombin were stored at −70°C. The [$^{125}$I]thrombin was used the same day of preparation and the DIP-thrombin over a period of 3 wk. Acid soluble collagen was a gift from Mr. Heinz Scheuenstuhl. It was prepared from bovine achilles tendon, Type 1, purchased from Sigma Chemical Co. Wheat germ lectin was prepared by affinity chromatography using ovamucoid-Sepharose (Pharmacia Fine Chemicals Inc.) as described by LeVine et al. (21). PGI₂ was synthesized by K. C. Nicolau of the University of Pennsylvania (22), and kindly supplied by Dr. J. Bryan Smith of Thomas Jefferson University, Philadelphia, Pa. Rabbit antihuman thrombin antibody and F(ab')₂ fragments were prepared as described previously (8).

*Preincubation of platelets with thrombin.* Platelets (2–4 × 10⁹/ml) prelabeled with [$^3$H]serotonin (7), were suspended in 0.5 ml of buffer containing 0.14 M NaCl, 0.015 M Na₂HPO₄/NaH₂PO₄, 0.005 M glucose, 0.5% bovine serum albumin (fraction 1, Sigma Chemical Co.), 0.002 M EDTA, and 2 μM imipramine (Sigma Chemical Co.), pH 7.4. PGI₂(20 nM final concentration) was added in a 1:100 dilution for 1 min before the addition of thrombin (0.01–0.2 U/ml). Platelets and thrombin were incubated at room temperature for 15–30 s, then anti-thrombin F(ab')₂ fragments were added in a concentration that completely inactivated the thrombin within 30 s. Platelets were then centrifuged at 12,000 rpm for 2 min in a Beckman Microfuge B (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The supernate was collected and the radioactivity counted to determine the extent of secretion of [$^3$H]serotonin.

The platelet pellet was resuspended in isotonic buffer (0.14 M NaCl, 0.015 M Na₂HPO₄/NaH₂PO₄, 0.005 M glucose, pH 7.4). The platelet concentration was then determined with a hematocytometer (23).

Samples of platelets (5 × 10⁷), previously exposed to alpha thrombin (or its derivatives, DIP- or gamma thrombin), were suspended in 0.5 ml of the isotonic phosphate buffer, pH 7.4. Controls were treated identically except for preincubation with thrombin. A second release inducing agent was added (thrombin, collagen, wheat germ lectin, or the ionophore A23187). Platelets were collected by membrane filtration and the extent of secretion determined by the amount of [$^3$H]serotonin retained by the platelets (7). The time from removal of PGI₂ to addition of the second agent varied 15–30 min.

*Correlation of binding of [$^{125}$I]thrombin to platelets with the release of [$^3$H]serotonin.* [$^{125}$I]Thrombin, 0.013–1.025 U, was added to 1 × 10⁸ platelets prelabeled with [$^3$H]serotonin in a total volume of 0.65 ml of buffer: 0.14 M sodium chloride, 0.015 M Tris-HCl, 0.0055 M glucose, and 0.5% fat-free albumin (Sigma Chemical Co.), pH 7.4. After incubation for 5 min at 22°C, aliquots were removed and thrombin binding or serotonin release measured simultaneously.

*Thrombin binding.* Platelets (0.4 ml) were centrifuged through a mixture of *n*-butyl phthalate and type A Apiezon oil (James G. Biddle Co., Plymouth Meeting, Pa.), and the radioactivity contained in the platelet pellet determined in a gamma scintillation spectrometer (24). Net thrombin bound and nonspecific binding were determined as described previously (7).

*Platelet secretion.* Platelets (0.2 ml) were membrane filtered and the retained [$^3$H]serotonin was measured in a liquid scintillation counter. Gross counts per minute were corrected for $^{125}$I using $^{125}$I standards which were counted by beta and gamma scintillation spectrometry (11).

## RESULTS

*Inhibition of thrombin-induced platelet secretion of [$^3$H]serotonin by PGI₂.* To determine the effects of thrombin on platelets apart from those changes associated with the release reaction, it was necessary to develop a technique by which platelets could be incubated with thrombin without subsequent secretion. Furthermore, it was required that the inhibition be reversible so that platelet responsiveness could be studied after the removal of thrombin. PGI₂, an inhibitor of platelet aggregation (25), appeared to be a likely agent because its effects are rapidly reversible (26, 27).

Platelets incubated for 1 min with 20 nM PGI₂ did not secrete [$^3$H]serotonin when exposed to thrombin concentrations that would normally induce 10–60% secretion (Fig. 1). In addition, we determined that once PGI₂-induced inhibition was reversed, the platelets responded normally to thrombin (data not shown).

*Effect of preincubating platelets with thrombin on their secretory response to subsequent thrombin stimulation.* As shown in Fig. 2, when PGI₂-treated platelets were briefly exposed to low concentrations of thrombin, centrifuged to remove both the PGI₂ and thrombin, then resuspended in buffer, there was inhibition of [$^3$H]serotonin secretion upon reexposure of the platelets to thrombin. The extent of inhibition was
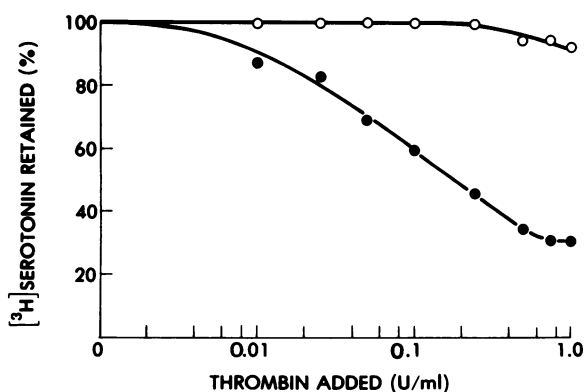
FIGURE 1 Inhibition of thrombin-induced platelet secretion of [³H]serotonin by PGI₂. Washed platelets (5 × 10⁷) prelabeled with [³H]serotonin were suspended in 0.5 ml of buffer containing 0.14 M sodium chloride, 0.015 M sodium phosphate, 0.005 M glucose, 0.5% bovine serum albumin, 0.002 M EDTA, and 2 μM imipramine, pH 7.4. Either PGI₂ diluted in ethanol (final concentration, 20 nM) or an equivalent volume of ethanol (control) was added 1 min before the addition of thrombin. After incubation for 15–30 s at room temperature, the extent of secretion of [³H]serotonin was determined by membrane filtration (7). Secretion is expressed as the percentage of serotonin retained compared to control incubations without thrombin. O, prostacyclin-treated platelets; ●, control platelets.

dependent on the initial thrombin concentration (Fig. 2). Complete inhibition of release of [³H]serotonin was observed when PGI₂-treated platelets were preincubated with 0.1 U/ml of thrombin, a concentration which would otherwise induce ≅50% [³H]serotonin secretion.

That this effect was not due to centrifugation of the platelets or to the PGI₂ was indicated by the fact that control platelets were treated identically except for the initial exposure to thrombin. Similarly, inhibition did not result from platelet secretion after the first thrombin incubation, because <1% of the [³H]serotonin appeared in the supernate after centrifugation. Imipramine was included in the initial incubation mixture to block reuptake of [³H]serotonin (28). Anti-thrombin F(ab')₂ fragments were added to the platelets 15 s after the thrombin to limit the duration of its effect. When thrombin was preincubated with anti-thrombin F(ab')₂ fragments for 30 s before its addition to platelets, a 90% decrease in its inhibitory effect on subsequent platelet secretion was seen.

It is interesting that platelets preincubated with PGI₂ before thrombin exposure did not secrete [³H]serotonin once the effect of PGI₂ had been reversed and the thrombin removed. It should be noted also that the incubation period with thrombin (15–30 s) was limited to the time necessary for thrombin-induced platelet secretion (17). Thus, although thrombin binding was normal in the presence of PGI₂ (data not shown), after binding,

the secretory stimulus did not progress to a point that resulted in the release reaction once PGI₂ inhibition was reversed.

*Effect of modification of native thrombin on its reaction with PGI₂-treated platelets.* Next, we used modified thrombins to determine the relationship between their ability to induce platelet secretion and their effect on PGI₂-treated platelets. Platelets were preincubated with PGI₂, then with thrombin inactivated at its serine-active site with iPr₂PF(DIP-thrombin). Thrombin modified in this way binds to platelets identically to native thrombin but does not cause the release reaction (7). As shown in Fig. 3, there was no difference in the subsequent secretion of [³H]serotonin induced by native thrombin compared to platelets not preincubated with DIP-thrombin. On the other hand, there was complete inhibition of serotonin secretion in platelets preincubated with an equivalent molar concentration of native thromin (Fig. 3). These results suggest that to induce refractoriness, thrombin must have the capability to initiate secretion.

Because DIP-thrombin and native thrombin bind to the same site on the platelet, we examined the possibility that an excess of DIP-thrombin would prevent the inhibition induced by native thrombin. Platelets were incubated for 10 min first with DIP-thrombin then for 15 s with ¹⁄₁₀ as much native thrombin. As shown in
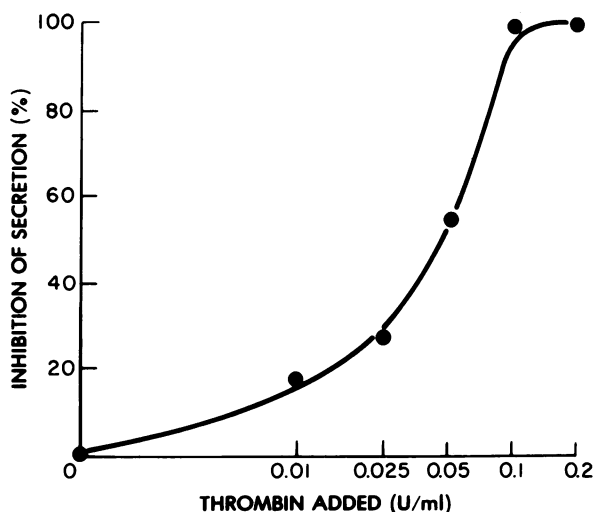


FIGURE 2 Effect of preincubating platelets with thrombin on their secretory response to subsequent thrombin stimulation. Thrombin (0.01–0.2 U/ml) or buffer (control) was added to platelets which were then centrifuged and resuspended in fresh buffer as described in Methods. Aliquots of 5 × 10⁷ platelets were removed, incubated with 0.2 U/ml of thrombin, and secretion of [³H]serotonin was determined by membrane filtration. Inhibition of secretion was calculated from the formula: ([% [³H]serotonin secreted, control platelets − % [³H]serotonin secreted, thrombin-pretreated platelets]/ [% [³H]serotonin secreted, control platelets]) × 100.
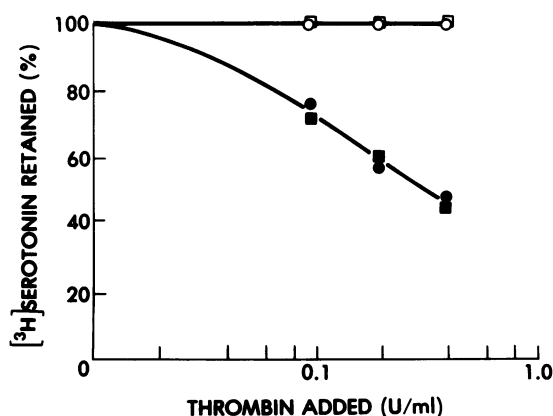
FIGURE 3 Effect of preincubating platelets with DIP-thrombin on their subsequent response to native thrombin. PGI$_2$-treated platelets were preincubated with either 0.196 U/ml DIP thrombin (■); 0.196 U/ml native thrombin (○); 1.96 U/ml DIP-thrombin for 10 min; then 0.196 U/ml native thrombin (□); or buffer (●); for 15–30 s; then centrifuged and resuspended as described in Fig. 2. Thrombin (0.1–0.4 U/ml) was added to aliquots of 5 × 10$^7$ platelets for 30 s and secretion of [³H]serotonin determined by membrane filtration.

Fig. 3, DIP-thrombin did not alter the subsequent inhibition of platelet secretion by native thrombin. The explanation for this is unclear, but it should be noted that DIP-thrombin does not block thrombin-induced platelet secretion either (7).

Gamma thrombin, a product of limited tryptic proteolysis of alpha thrombin, has ≅0.1% of the fibrinogen clotting activity of native thrombin, 1% of the platelet release-inducing activity, and full esterase activity (5, 20). When either alpha or gamma thrombin was prein-cubated with PGI$_2$-treated platelets at equivalent concentrations based on the ability to induce platelet secretion, there was complete inhibition of secretion on reexposure to thrombin (Table I). The platelets were preincubated with a concentration of alpha or gamma thrombin sufficient to give 50% secretion of [³H]serotonin in the absence of PGI$_2$. On a molar basis the concentration of gamma thrombin was 75-fold higher than the alpha thrombin and had only 2% of the clotting activity. Apparently the inhibition of secretion that resulted from thrombin preincubation was specifically related to its ability to induce secretion and not to its esterase activity. Nor does it appear to be a nonspecific proteolytic effect, because an equivalent inhibitory effect was seen at a 50-fold lower concentration based on the ability to cleave fibrinogen.

*Effect of increasing the thrombin concentration on platelet secretion inhibited by prior incubation with thrombin.* Next, we determined whether the platelet refractoriness to thrombin could be overcome by increasing the thrombin concentration with which the platelets were stimulated the second time. As shown in Fig. 4, a fourfold higher concentration of thrombin was required to achieve the same extent of serotonin secretion (50%) as for platelets not previously exposed to thrombin. When the time of preincubation of platelets with thrombin was increased from 15–30 to 300 s, no additional increase in the concentration of thrombin was necessary to achieve secretion (Fig. 4). The same result was obtained when a concentration of thrombin that resulted in 50% inhibition of secretion was used. There was no increase in the percentage of inhibition when the preincubation was lengthened to 300 s.

TABLE I
Effect of Preincubation of Platelets with Alpha or Gamma Thrombin on
Subsequent Thrombin-Induced Platelet Secretion

| | Thrombin concentration used in preincubation | Clotting activity | Release of platelet [³H]serotonin control platelets* | Inhibition of platelet secretion |
|---|---|---|---|---|
| | μg/ml | U/ml | % | |
| Alpha thrombin | | | | |
| Experiment 1 | 0.042 | 0.192 | 50 | 100 |
| Experiment 2 | 0.042 | 0.192 | 57 | 100 |
| Gamma thrombin | | | | |
| Experiment 1 | 1.0 | 0.039 | 50 | 100 |
| Experiment 2 | 0.5 | 0.020 | 21 | 64 |

Platelets prelabeled with [³H]serotonin were incubated first with PGI$_2$ (25 nM), then either alpha or gamma thrombin, or buffer (control) for 15–30 s. After centrifugation and resuspension in fresh buffer, 0.192 U/ml of alpha thrombin was added to the platelets and the extent of [³H]serotonin secretion determined. Calculation of inhibition of platelet secretion of [³H]serotonin was determined as described in Fig. 2.
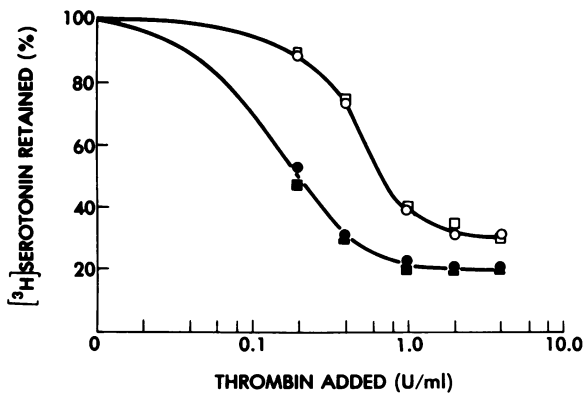* The release-inducing activity of thrombin was determined in a parallel experiment with platelets not pretreated with PGI$_2$.

FIGURE 4  Effect of increasing thrombin concentrations on platelet refractoriness induced by prior incubation with thrombin. $PGI_2$-treated platelets were preincubated with thrombin (0.196 U/ml) or buffer for 15–30 or 300 s, then centrifuged and resuspended in fresh buffer. Thrombin (0.196–4.0 U/ml) was added to aliquots of $5 \times 10^7$ platelets for 15–30 s and secretion of [$^3$H]serotonin was determined by membrane filtration. ●, control platelets, 15–30 s; ○, platelets preincubated for 15–30 s with thrombin; ■, control platelets, 300 s; □, platelets preincubated for 300 s with thrombin.

*Specificity of inhibition of platelet secretion induced by thrombin preincubation.*  The effect of thrombin pretreatment on the release reaction induced by other agonists was studied also. When collagen, wheat germ agglutinin, or the calcium ionophore A23187 was used to stimulate platelet secretion, there was little difference in the extent of [$^3$H]serotonin release whether or not the platelets were first preincubated with thrombin (Table II). The concentration of thrombin used, 0.19 U/ml, however, resulted in 90% inhibition of thrombin-induced secretion. A small, but consistent, inhibition of A23187-induced secretion was seen on several occa-

sions. The significance of this is unknown. This marked refractoriness to thrombin but not other platelet stimuli suggests that the effect of thrombin preincubation is specific.

*Binding of [$^{125}$I]thrombin to platelets previously exposed to thrombin.*  To determine whether the refactoriness of platelets to thrombin was because of an effect on thrombin binding, the following experiment was performed: Platelets were preincubated with a concentration of thrombin (0.2 U/ml) which resulted in 93% inhibition of serotonin secretion (platelets not pretreated with thrombin showed 59% secretion; thrombin-pretreated platelets, 7% secretion). The thrombin was removed by centrifugation and both binding of [$^{125}$I]thrombin to the resuspended platelets and extent of serotonin secretion were measured. As shown in Fig. 5, for the same amount of thrombin bound, a marked decrease was seen in serotonin secretion in those platelets preexposed to thrombin compared to control platelets. Approximately four times as much bound thrombin was required for 40% serotonin secretion in thrombin-pretreated platelets. Thus, the inhibitory effect on platelet secretion that followed thrombin pretreatment was because of alteration of a step subsequent to thrombin binding to platelets.

*Duration of inhibition of platelet secretion induced by thrombin preincubation.*  In many tissues, reversible desensitization to agonist stimulation has been observed (29). We examined the possibility that the inhibition of platelet secretion we had observed was related to a reversible desensitization of the platelets to thrombin. Platelets were preincubated with thrombin in the presence of $PGI_2$. Then, thrombin was inactivated with anti-thrombin F(ab')$_2$ fragments, and both the thrombin and $PGI_2$ were removed after centrifugation of the platelets. The resuspended plate-

TABLE II
*Effect of Thrombin Pretreatment of Platelets on Subsequent Secretory Response*

| Stimulus | [$^3$H]Serotonin secreted | |
|---|---|---|
| | Control | Thrombin pretreated |
| | % | |
| Thrombin (0.2 U/ml) | 53 | 5 |
| Collagen (16 μg/ml) | 17 | 21 |
| Collagen (40 μg/ml) | 34 | 28 |
| Wheat germ lectin (300 μg/ml) | 17 | 13 |
| A23187 (4.0 μg/ml) | 59 | 48 |

$PGI_2$-treated platelets were preincubated with 0.19 U/ml of thrombin or buffer for 15–30 s, then sedimented by centrifugation. After resuspension in fresh buffer, secretion of [$^3$H]serotonin in response to the indicated agonist was determined. The results depicted are from one of four experiments, each of which gave similar results.
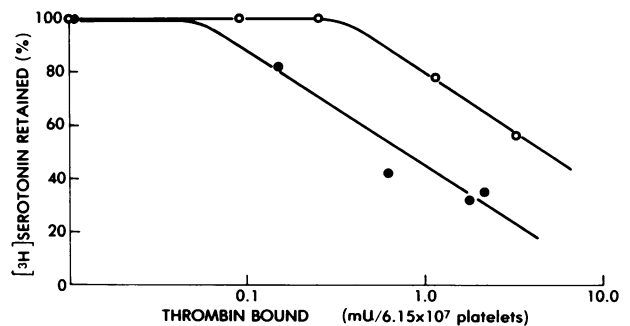


FIGURE 5  Effect of thrombin pretreatment of platelets on the relationship between thrombin binding and serotonin secretion. Platelets ($1 \times 10^8$) previously treated with 0.2 U/ml of thrombin were incubated with 0.02–1.5 U/ml of [$^{125}$I]thrombin. Then both the amount of [$^{125}$I]thrombin bound to the platelets and retained [$^3$H]serotonin were determined on separate aliquots simultaneously. ●, platelets preincubated with buffer; ○, platelets preincubated with thrombin.

TABLE III

*Effect of Time on the Reversibility of Platelet Refractoriness to Thrombin*

| Time, *min* | [³H]Serotonin secreted | | | |
|---|---|---|---|---|
| | 45 | 100 | 150 | 210 |
| | | % | | |
| Control platelets | 59 | 38 | 40 | 50 |
| Thrombin-pretreated platelets | 5 | 4 | 8 | 0 |

PGI$_2$-treated platelets were preincubated with 0.192 U/ml of thrombin or buffer (control) for 15–30 s, sedimented by centrifugation, and resuspended in fresh buffer (0.14 M NaCl, 0.0154 M Na/PO$_4$, 0.005 M glucose, 0.0026 M KCl, 0.001 M MgCl$_2$, 0.5% fat-free albumin, and 0.001 M EDTA, pH 7.4) as described in Methods. Aliquots were removed at the intervals indicated and 0.192 U/ml of thrombin added. Secretion of [³H]serotonin was determined by membrane filtration.

lets were then tested for thrombin-induced secretion of [³H]serotonin over a period of several hours. As indicated in Table III, inhibition was not diminished over a period of 3.5 h. It would appear, therefore, that in the time period studied, the effect of thrombin preincubation on the platelets was irreversible.

## DISCUSSION

Investigators have shown that once platelets have secreted in response to thrombin, their capacity to respond to thrombin a second time is diminished (30, 31). However, the step in the secretory pathway at which the platelets become modified has not been established. We have studied the interaction between PGI$_2$-treated platelets and thrombin to determine the effects of thrombin on platelets before secretion. These platelets compared to those not prestimulated with thrombin exhibit a marked decrease in serotonin secretion. That refractoriness to thrombin stimulation is related to an alteration of a step in the secretory pathway rather than a nonphysiological effect is indicated by the following observations. First, the amount of thrombin used to induce refractoriness was not in excess of that necessary to induce secretion. Second, this effect was not due to prolonged exposure of the platelets to thrombin, because the incubation was limited to the time necessary to induce the release reaction. Third, the secretory and inhibitory effects of thrombin on platelets are both dose dependent. The higher the thrombin concentration, the greater the degree of secretion or inhibition. Fourth, the capacity of thrombin to render the platelets refractory correlates closely with its ability to induce secretion. This is indirect evidence that the inhibitory effect on the platelet is not secondary to a nonspecific esterolytic or proteolytic ac-

tion of thrombin. Similarly, the marked discrepancy between the platelet-secretory and fibrinogen-clotting activity of the gamma thrombin suggests that inhibition is not the result of an alteration of platelet fibrinogen. Fifth, the effect was specific for thrombin: platelet secretion induced by wheat germ agglutinin, the ionophore A23187, and collagen was relatively unaffected by previous exposure of the platelets to thrombin. In addition, preincubation of PGI$_2$-treated platelets with collagen has no effect on either thrombin- or collagen-induced platelet secretion.[3]

We have demonstrated that platelet refractoriness to repeated thrombin stimulation is not a consequence of the release reaction or of subsequent events. Refractoriness does not appear to result from an alteration of thrombin binding, the first step in the secretory pathway, but instead from a decrease in its effectiveness in initiating secretion once it has bound to platelets. The observation that DIP-thrombin does not inhibit subsequent secretion induced by thrombin suggests that the inhibitory effect of native thrombin may be a consequence of limited proteolysis. Electrophoretic analysis of the membranes from platelets that have been exposed to thrombin in the presence of PGI$_2$ should resolve this issue.

The diminished responsiveness of platelets to thrombin does not resemble the desensitization that has been observed in a variety of tissues in response to cholinergic or adrenergic agents, and drugs (29). The effect was seen after exposure of the platelets to thrombin for only 15–30 s. Although desensitization of parotid acinar cells to epinephrine after a 2-min exposure has been described (32), a longer incubation period is generally required. Also, desensitization generally increases with time: the longer the exposure of target cells to the agonist, the greater the degree of refractoriness to subsequent stimulation. This is not the case with thrombin and platelets. As much inhibition was seen after 15 s of exposure to thrombin as after 300 s. Also, inhibition was not reversed after the removal of thrombin and PGI$_2$. Another difference between our findings and those of studies demonstrating desensitization is that frequently a correlation has been noted between desensitization and resensitization with a change in the number of receptors on the target cell. On the contrary, we observed that for the same amount of thrombin bound, there was less secretion in thrombin-pretreated platelets than control platelets. Thus, if refractoriness to thrombin is a result of desensitization, it differs in several respects from previously described examples.

Considerable evidence suggests that regulation of adenylate cyclase is an essential step in the platelet secretory pathway (33, 34). Most platelet agonists, in-

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[3] Botney, M. B., A. L. Swanson, and M. A. Shuman. Manuscript in preparation.

cluding thrombin, inhibit stimulation of adenylate cyclase (33–35). Conversely, agents such as $PGI_2$, which cause an increase in cyclic adenosine 3',5' monophosphate (cyclic AMP), inhibit platelet secretion (33, 34). Reactions known to be inhibited by these secretory antagonists include hydrolysis of arachadonic acid from platelet phospholipid, and phosphorylation of platelet proteins in response to thrombin (36, 37). It is unlikely, therefore, that the refractoriness that results from the exposure of $PGI_2$-treated platelets to thrombin is a result of an effect on either of these reactions or on events that occur subsequently.

Friedman and Detwiler have shown that cyclic AMP inhibits thrombin-induced platelet secretion in a complex manner (38). On the basis of kinetic studies of ATP secretion, they concluded that both the rate of the initial steps in secretion and the total amount of secretion were inhibited by drugs that elevate cyclic AMP. At low concentrations of prostaglandin $E_1$ ($PGE_1$), there was both a delay in the onset of secretion and a prolongation in the time required for secretion, once initiated. At high $PGE_1$ concentrations, complete inhibition of secretion occurred. Our observations suggest the presence of at least one step in the secretory pathway between thrombin binding and regulation of adenylate cyclase. This step appears to be specific for thrombin; it may also be an essential mechanism for transmitting the signal that leads to the extrusion of intracellular granular contents.

## ACKNOWLEDGMENTS

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