Kinetics of the Thrombin-Induced Release of Calcium(II) by Platelets†

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ABSTRACT: The kinetics of the thrombin-induced release of Ca^{2+} from human blood platelets have been followed using the calcium-complexing dye murexide with a dual-wavelength spectrophotometer. The observed reaction shows three phases, a lag followed by an exponential phase and a final slow zero-order change. From the dependence of the kinetic parameters on concentration, at least four steps must be proposed for the reaction: (i) a rapid formation of a thrombin–platelet complex, (ii) a first-order transformation of the complex, (iii) a slower first-order release of Ca^{2+}, and (iv) slow turnover of thrombin leading to release of additional Ca^{2+}. The first order release of Ca^{2+} from the transformed platelet has rate constant $k_2 = 0.038$ sec^{-1} (SD 0.008). The yield of Ca^{2+}, as well as the kinetic parameters, is dependent on thrombin, saturating at high concentrations. Several models are considered for the reaction and it is concluded that thrombin sites on platelets are not independent, but that cooperative interactions are required to explain the kinetics and yield. From the saturation of the various parameters, as well as from binding studies at high thrombin, the number of thrombin sites is estimated at 2×10^4/platelet.

Platelets are anucleate blood cells that play crucial roles in hemostasis and thrombosis by forming compact, adhesive aggregates (for reviews, see Johnson, 1971, Marcus, 1969, and Mustard and Packham, 1970). A variety of physiological agents, such as thrombin, collagen, and ADP, induce the profound morphological and biochemical changes in platelets that lead ultimately to aggregation and contraction of the aggregate. These changes include pseudopod formation, movement of granules, increased metabolism, and the specific release of certain platelet constituents such as adenine nucleotides, calcium, 5-hydroxytryptamine, and certain enzymes (Holmsen *et al.*, 1969). Release of calcium (Murer, 1969) is of particular interest since comparisons with other secretory cells (Douglas, 1968; Stormorken, 1969) and limited experimental evidence (Grette, 1962; Sneddon, 1972) suggest that Ca^{2+} may be the agent that mediates other changes.

The most potent physiological stimulator of platelets is thrombin, the enzyme that catalyzes conversion of fibrinogen to fibrin as well as other reactions in the overall process of blood coagulation. Thrombin is one of a class of proteolytic enzymes that are distinguishable by an active serine believed to function as a nucleophilic catalyst in the hydrolysis of peptides (for review, see Magnussion, 1971). It is similar to trypsin, with specificity for arginyl and lysyl peptide and ester bonds. However, thrombin has a much higher degree of specificity than trypsin. For example, thrombin will hydrolyze only 4 out of 100 or so trypsin-sensitive bonds in fibrinogen. Like other serine proteases, thrombin is inhibited by DFP, a reagent that forms a covalent, inactive diisopropylphosphoryl derivative (DIP-enzyme)[1] by reacting with the active serine.

The mechanism by which thrombin modifies platelets is not known. The fact that the reaction is observed with trypsin but not with DIP-thrombin (Davey and Luscher, 1967) suggests that a proteolytic mechanism is involved. Several investigators have reported the existence of thrombin-labile proteins in, or on the surface of, platelets (Ganguly, 1971; Baenziger *et al.*, 1972; Salmon and Bounameaux, 1958; Nachman, 1965; Cohen *et al.*, 1969) but a description of the primary reaction and how it leads to the observed morphological and biochemical changes has not yet been achieved.

In an attempt to better understand the reaction of thrombin with platelets and the role of Ca^{2+} in platelet function, we have studied the kinetics of the thrombin-induced release of Ca^{2+} using the metallochromic indicator murexide to follow the reaction spectrophotometrically. From analysis of kinetic data obtained by independently varying concentrations of thrombin and platelets, we have developed a simple model of the reaction. The model includes at least four steps, a rapid binding of thrombin to platelets, a change in the thrombin–platelet complex, a thrombin-independent first-order release of Ca^{2+} from platelets, and a slow turnover of thrombin. The number of thrombin binding sites has been estimated to be approximately 2×10^4/platelet.

Methods

Preparation of Platelets. Human platelets were isolated by differential centrifugation from blood obtained from healthy donors. All equipment used for collection of blood and isolation of platelets was either silicone coated or plastic and temperature was maintained at 0–4° during the entire procedure. Blood was collected by venipuncture into 0.15 volume of 120 mM disodium citrate containing enough EDTA to give a final concentration of 1 mM. The small amount of EDTA was used because it seemed to facilitate resuspension of the platelets while a larger amount would interfere with the subsequent calcium assay. Red cells and white cells were sedimented by centrifugation in a swinging-bucket centrifuge at 300*g* for 15 min. Platelets were obtained from the supernatant fluid by centrifugation at 1500*g* for 20 min. The platelets were washed twice by gently suspending them in 10 ml of a solution that was 136 mM NaCl, 25 mM Tris-HCl (pH 7.4), 5 mM glucose, and 0.6 mM disodium citrate (wash solution) and

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[1] DIP, diisopropylphosphoryl derivative.

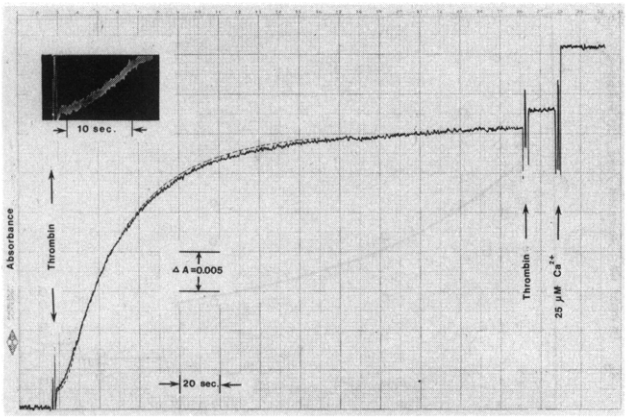


FIGURE 1: Thrombin-induced release of Ca^{2+} from platelets. The experiment was carried out as described under Methods with 2.4×10^8 platelets/ml and 0.6 unit of thrombin/ml. The vertical axis is the difference between absorbance at 540 and 507 nm. The theoretical curve (broken line) was generated by analog computer for a series first-order mechanism with $k_1 = 0.20$ sec^{-1} and $k_2 = 0.036$ sec^{-1}.

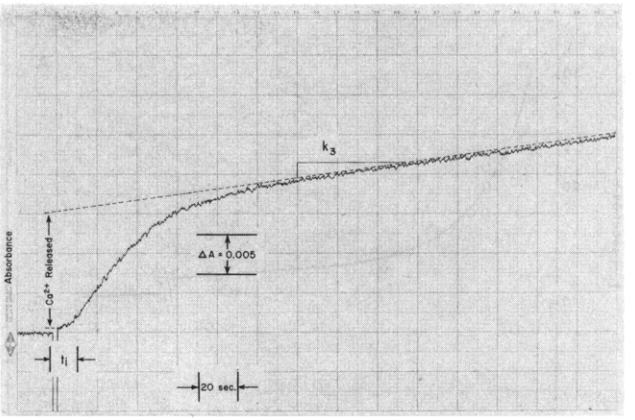


FIGURE 2: Thrombin-induced release of Ca^{2+} from platelets showing parameters that have been measured. The experiment was carried out as for Figure 1 with 2.4×10^8 platelets/ml and 0.15 unit of thrombin/ml. The theoretical curve is the extrapolated zero-order line added to the integrated rate equation for a series first-order mechanism with $k_1 = 0.08$ sec^{-1} and $k_2 = 0.043$ sec^{-1}.

sedimenting by centrifugation. The washed platelets were suspended in the wash solution to give a concentration of 2–4 $\times 10^9$ platelet/ml. Washed platelets suspended in a simple medium were used in order to maintain an inherently complex reaction mixture in as well defined a state as possible.

Observation of the Thrombin-Induced Release Reaction. Spectrophotometric measurement of released calcium was done with an Aminco-Chance dual-wavelength spectrophotometer using the method described by Ohnishi and Ebashi (1963). This method is based on measurement of the spectral shift of murexide when it forms a complex with Ca^{2+}. It is necessary to have a Ca^{2+} chelator present in platelet suspensions to prevent spontaneous aggregation of platelets, but Ca^{2+} chelators in the assay medium reduce the sensitivity of the assay by competing with murexide for Ca^{2+}. To obtain maximum sensitivity, it was therefore necessary to use a rather high concentration of murexide and a minimum of citrate. Satisfactory results were obtained with 0.3 mM murexide and a final concentration of citrate, added with the platelet suspension, less than 0.12 mM. This gave a linear response to Ca^{2+} to about 0.3 mM, whereas most experiments involved changes of less than 0.15 mM Ca^{2+}. In experiments where thrombin-induced release of ATP was studied (T. C. Detwiler and R. D. Feinman, in preparation), it was found that 0.3 mM murexide had no effect on the kinetics of that related process, suggesting that this concentration of murexide does not modify platelets with respect to their reaction with thrombin or the release reaction.

The calcium release experiments were done at 22° in cuvets with a 10-mm light path and with a reaction mixture that contained 0.05 ml of 6 mM murexide plus incubation solution (136 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 5 mM glucose) and platelet suspension to give a final volume of 1.0 ml. The method for observing the calcium release reaction by recording changes in absorbance of murexide is illustrated in Figure 1. After establishing a base line, the reaction was started by the rapid addition of thrombin (5–20 μl) using a flattened, plastic mixing rod ("plumper," Calbiochem, Los Angeles, Calif.). At completion of the reaction, the addition of thrombin was repeated to establish that the instantaneous absorbance change accompanying the first addition was due to the thrombin solution itself. This was followed by addition of a standard calcium solution for calibration. There was no visible platelet aggregation, presumably because of chelation of Ca^{2+} by the murexide.

To permit more detailed analysis of the important initial phase of the thrombin–platelet reaction, the change was also observed on a storage oscilloscope with a faster time base (Figure 1, inset). The signal to the oscilloscope does not go through a log converter so the vertical axis, which is set at 2 mV/division, represents per cent transmission. Changes on both the oscilloscope and recorder are displayed with increasing Ca^{2+} corresponding to upward deflection.

Reagents. Thrombin was purified from Parke-Davis Topical thrombin by the method of Glover and Shaw (1971). Specific activities ranged from 1300 to 1950 NIH units per mg. Thrombin activity was assayed by measuring the time required to clot a fibrinogen solution under the conditions described by Baughman (1970), using NIH standard thrombin as primary standard. Fibrinogen was purified by the method of Blombäck and Blombäck (1956). For each release experiment fresh dilutions of a stock thrombin were made with the incubation solution.

All other reagents were prepared with water that had been treated with Chelex-100 resin (Bio-Rad, Richmond, Calif.) to remove traces of Ca^{2+}. Murexide was obtained from Sigma Chemical Co., St. Louis, Mo. The calcium standard was prepared from dry $CaCO_3$ dissolved in a minimum of HCl before diluting to volume with water. Hirudin was a gift from Dr. Erik Murer (Institute for Thrombosis Research, Oslo). Its activity was determined by its ability to inhibit standardized thrombin in the clotting assay.

Results

The kinetics of the thrombin-induced release of Ca^{2+} show three distinct phases (see Figure 1): (i) an initial lag; (ii) an apparently exponential phase; and (iii) a final slow, nearly linear phase, which is more obvious with experiments at low concentrations of thrombin (Figure 2). The rate of approach to the zero-order phase, *i.e.*, the second phase of the reaction, is first order over most of the time of reaction as indicated by linearity of log plots (time *vs.* the logarithm of the difference

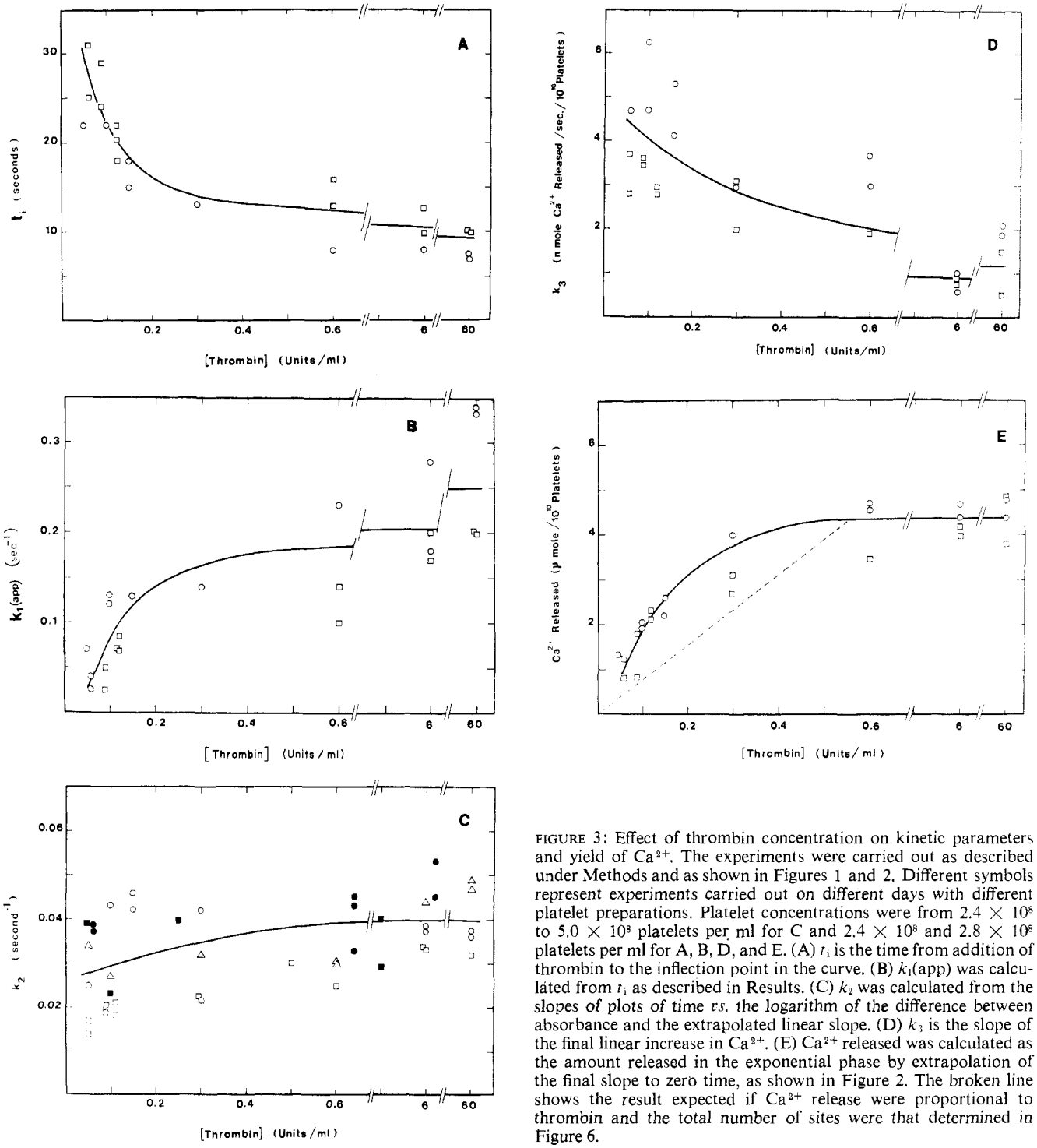


FIGURE 3: Effect of thrombin concentration on kinetic parameters and yield of Ca^{2+}. The experiments were carried out as described under Methods and as shown in Figures 1 and 2. Different symbols represent experiments carried out on different days with different platelet preparations. Platelet concentrations were from 2.4×10^8 to 5.0×10^8 platelets per ml for C and 2.4×10^8 and 2.8×10^8 platelets per ml for A, B, D, and E. (A) t_i is the time from addition of thrombin to the inflection point in the curve. (B) k_1(app) was calculated from t_i as described in Results. (C) k_2 was calculated from the slopes of plots of time *vs.* the logarithm of the difference between absorbance and the extrapolated linear slope. (D) k_3 is the slope of the final linear increase in Ca^{2+}. (E) Ca^{2+} released was calculated as the amount released in the exponential phase by extrapolation of the final slope to zero time, as shown in Figure 2. The broken line shows the result expected if Ca^{2+} release were proportional to thrombin and the total number of sites were that determined in Figure 6.

between the curve and the extrapolated slope). The first-order rate constant determined from the slope of these log plots is referred to as k_2 and is shown below to be independent of concentrations of thrombin and platelets with a value of 0.038 (SD = 0.008) sec^{-1}.

The kinetic curves at the higher thrombin concentrations (Figure 1) have the characteristics of appearance of product in a series first-order reaction

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

The theoretical line in Figure 1 is an analog computer simulation based on this mechanism. At lower thrombin (Figure 2) the curves appear to represent the same mechanism followed by a slower zero-order change. If the extrapolated zero-order line is subtracted from the curve, the resulting points can be fitted with the integrated rate equation

$$\frac{[C] - [A_0]}{[A_0]} = \left(\frac{k_2}{k_2 - k_1}\right) e^{-k_1 t} - \left(\frac{k_1}{k_2 - k_1}\right) e^{-k_2 t} \quad (1)$$

(Frost and Pearson, 1961), where $[A_0]$ is assumed proportional to the final amount of Ca^{2+} released. We thus consider the reaction to be represented by

$$P \xrightarrow{k_1(app)} P^* \xrightarrow{k_2} P' + Ca^{2+} \quad (2)$$

where P = unreacted platelets, P* = thrombin-activated platelets, P′ = platelets after release of Ca^{2+}, and k_1(app) is the apparent first-order rate constant for the first step.

We have analyzed the experimental curves as shown in Figure 2. Values for k_1(app) were calculated from the time to the inflection point in the curve (t_i). The relation between t_i and the rate constants for a series first-order reaction is $t_i = \ln (k_1/k_2)/(k_1 - k_2)$, which is derived from eq 1. The values of k_1(app) are thrombin dependent with saturation values of about 0.25 sec^{-1} (Figure 3B). The final zero-order rate was measured and its rate constant is referred to as k_3. The amount of calcium released in the exponential (k_2) phase was calculated from the difference between the starting absorbance and the absorbance extrapolated from the final slope to zero time.

Variation of Thrombin with Constant Platelets. Experiments were carried out with different concentrations of thrombin (from 0.05 to 60 U per ml) and constant concentrations of platelets and the curves were analyzed as just described. The results are shown in Figure 3. As thrombin was increased, the lag, quantitated as t_i, was shortened (Figure 3A) so that k_1(app), the calculated rate constant for the first step, was correspondingly increased (Figure 3B). The rate constant for the second step, k_2, was independent of thrombin concentration[2] (Figure 3C), while k_3 decreased as thrombin increased (Figure 3D). Figure 3E shows that not only the rate constants but the actual yield of calcium is dependent on thrombin concentration, increasing with higher thrombin. Thus, as thrombin was lowered: (i) the rate constant of the first step(s), k_1(app), decreased, (ii) the amount of calcium released during the exponential phase decreased, and (iii) the final slope increased, but (iv) the rate constant of the second step did not change.

Variation of Platelets with Constant Thrombin. Similar measurements were made over a range of platelet concentrations at two thrombin concentrations, one just less than required for maximum release and one near the lower limit for accurate measurement. Neither the lag (t_i) nor the rate of calcium release (k_2) was dependent on platelet concentration. The amount of calcium released in the k_2 phase (Figure 4A) was proportional to the concentration of platelets, reaching a maximum above which it decreased slightly. The value of k_3 (Figure 4B) also increased with increasing platelet concentration. Comparisons of Figures 3D,E and 4A,B indicate that k_3 is a function of the ratio thrombin: platelet. When thrombin is in excess, all releasable calcium appears in the initial exponential phase of the reaction and there is essentially no k_3 phase; when thrombin is less than saturating, release continues at a slow rate beyond the initial reaction. We interpret this to mean that thrombin becomes tightly bound in the course of the reaction and that the slow rate is an indication of the slow turnover of thrombin. It should be noted that the units of k_3 include platelet^{-1}, so that the actual slope of the final part of the calcium release curve increases as a function of platelet concentration more than is obvious in Figure 4B.

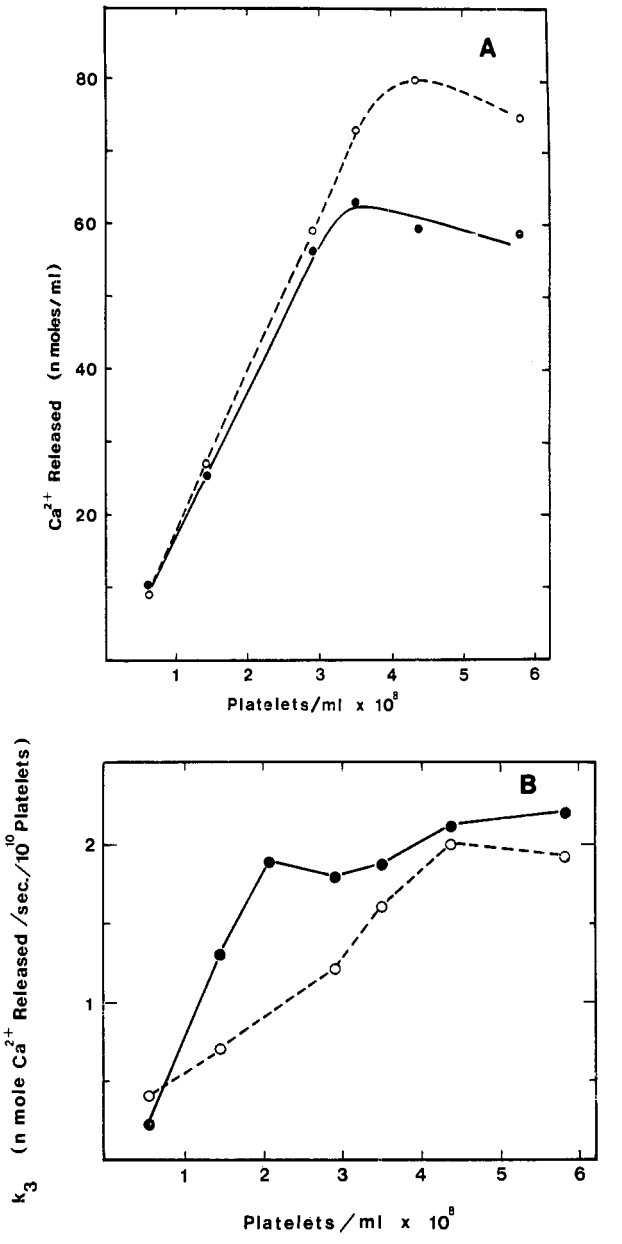


FIGURE 4: Effect of platelet concentration on kinetic parameters and yield of Ca^{2+}. Experiments were carried out as for Figure 3. Open symbols with broken lines, 0.4 unit of thrombin/ml; closed symbols with solid lines, 0.16 unit of thrombin/ml.

Inhibition of Thrombin with Hirudin. Hirudin, a protein found in salivary glands of leaches, is a specific and potent inhibitor of thrombin (Markwardt, 1970). A fivefold excess of hirudin, added before thrombin, completely inhibited the release of Ca^{2+} (upper and lower curves of Figure 5). Addition of hirudin 5 sec after thrombin (center curve of Figure 5) caused remarkably little inhibition of the further course of the reaction, although the total yield seems to have been reduced. This is consistent with the observation that k_2 is thrombin independent, whereas k_1(app) depends on thrombin. When a lower concentration of thrombin (0.1 unit/ml) and a higher concentration of platelets (5.4×10^8/ml) were used to give a greater rate for k_3 (ΔA = 0.0042 /min), hirudin added at the beginning of the k_3 phase completely inhibited this phase of the reaction, consistent with the hypothesis that this step is due to the appearance of free thrombin.

[2] The slight decrease in k_2 at low thrombin is due to the fact that at this concentration k_1(app) approaches the value of k_2, and thus influences the k_2 phase from which a log plot is made. We have studied the series first-order reaction with analog computer simulations (R. D. Feinman and T. C. Detwiler, unpublished observations) and when k_1 is much larger than k_2, a plot of log ($c_\infty - c$) *vs.* time is linear with the slope giving a true value of k_2. However, when k_1 is approximately equal to k_2, a log plot still gives a straight line, but the slope gives a value less than the actual k_2. The average and standard deviation of k_2 were determined from values where thrombin was at least 0.30 unit/ml, when $k_1/k_2 = 5$.

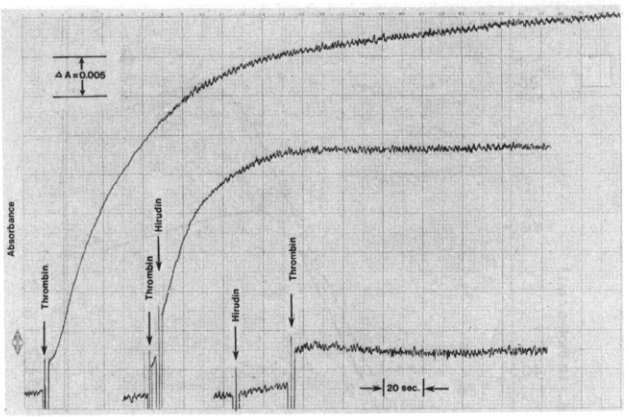


FIGURE 5: Effect of hirudin on the thrombin-induced release of Ca^{2+}. The experiments were carried out as described for Figure 1 under Methods with 1 unit/ml of thrombin, 5 unit/ml hirudin, and 3.6×10^8 platelets/ml.

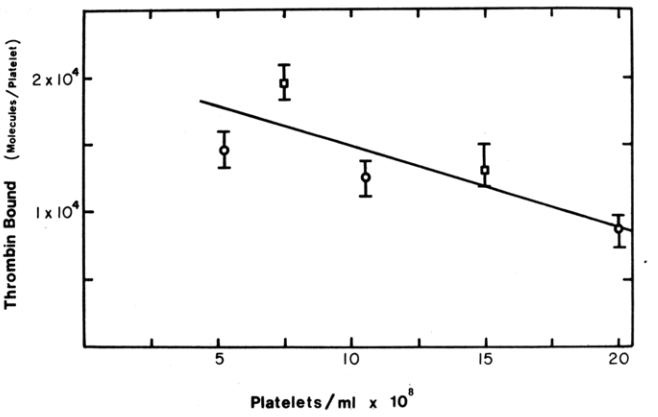
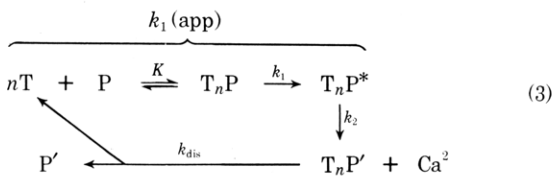


FIGURE 6: Binding of thrombin to platelets. Platelets were suspended in the same solution used for measuring Ca^{2+} release. Thrombin was added to the suspensions and the tubes were immediately centrifuged at 10,000*g* for 4 min at room temperature. The supernatant solutions were removed and assayed for thrombin by the clotting assay standardized against standard thrombin in identical solutions. Each point is the average of either duplicate or triplicate experiments with the ranges indicated by brackets. The different symbols represent experiments carried out on different days with different platelet and thrombin preparations. (□) 4 units of thrombin/ml in control tubes (no platelets); (○) 3 units of thrombin/ml in control tubes.

Qualitative Consideration of Data: a Simple Kinetic Model. The data have been analyzed with the goal of a simple kinetic model for the thrombin-induced platelet release reaction. A kinetic model of this reaction must be consistent with the following qualitative observations. (1) The amount of Ca^{2+} released is a function of thrombin concentration (Figure 3E). It is also a function of platelet concentration, with a maximum depending on thrombin concentration (Figure 4A). (2) The rate constant for Ca^{2+} release, k_2, is independent of thrombin (Figures 3C and 5) or platelet concentrations. (3) The rate constant for the first step(s) of the reaction, k_1(app), is a function of thrombin concentration and saturates at high thrombin (Figure 3B), but is not dependent on platelet concentration.

To develop a satisfactory kinetic model, it is first necessary to define the involvement of thrombin. k_1(app) is a function of thrombin concentration and hence must be a pseudo-first-order rate constant containing terms in enzyme concentration. From the fact that k_1(app) is not linear with thrombin, saturating at high values (Figure 3B), we exclude second-order as the true order.[3] The simplest model, and one consistent with classical enzymology, includes the formation of a platelet–thrombin complex, as shown in reaction 3, where T represents



(3)

thrombin, k_{dis} is the constant for dissociation of thrombin from P′, and the other symbols are the same as in reaction 2. It should be pointed out that from the determination of number of sites on platelets, as discussed below, most of these experiments were done at approximately equal concentrations of thrombin and sites. In reaction 3 the equilibrium is followed by an irreversible step and the amount of Ca^{2+} released is proportional to the amount of T_nP^* formed, which in turn is a function of the concentration of platelets or thrombin, whichever is limiting.

As indicated above, we consider the observed rate constant k_3 to reflect the turnover of thrombin, that is, the dissociation of thrombin from the activated platelet to react with unreacted platelets. The observed k_3 would thus include the constants K, k_1, and k_2, the concentrations of all dissociating species of thrombin–platelet complexes, the rate constant of dissociation of these complexes, k_{dis}, and the concentration of unreacted platelets. The rate of dissociation of the thrombin–platelet complex is probably the rate-limiting step and very slow, because a rapid turnover of thrombin would not lead to the observed dependence of amount of Ca^{2+} released on thrombin concentration. For simplicity in reaction 3, k_{dis} is shown to involve only T_nP', but it could also involve T_nP^*, and it may actually be an equilibrium constant.

Estimation of the Number of Thrombin Sites on Platelets. The model depicted in reaction 3 suggests that the release of Ca^{2+} in the k_2 phase represents the titration of thrombin sites on platelets, so that the saturating amount of thrombin, or the ratio thrombin:platelets for maximum release in the k_2 phase, should correspond to a minimum value for the number of thrombin binding sites per platelet. Using the value of 10^{13} molecules of thrombin per unit of activity,[4] the saturating ratio for Figure 3E is about 2×10^4 thrombin/platelet.

A direct experimental test of this estimate of the number of thrombin binding sites was made by measuring the extent to which platelets removed thrombin, assayed by the fibrinogen clotting test, from solution (Figure 6). The conditions for this experiment were somewhat different from those for the kinetic experiments because the low sensitivity of the clotting assay necessitated higher concentrations of both platelets and

[3] For example, if the true order were second, $-d[P]/dt = k_1([P_0] - [TP^*])([T_0] - [TP^*])$ and if thrombin were in excess, $-d[P]/dt = k_1[T_0]([P_0] - [TP^*])$ and $k_1(app) = k_1[T_0]$. While the measured rate constant k_1(app) may not actually be pseudo first order, the approach here is to assume pseudo first order and see if the predictions made from this assumption lead to a consistent model (see footnote 5).

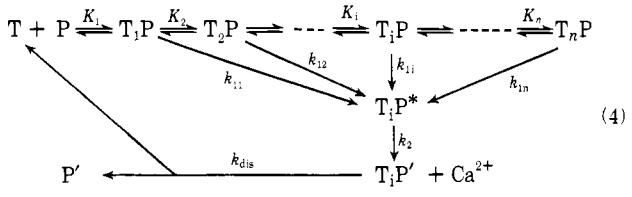
[4] Based on an activity for pure thrombin of 2000 units/mg (Seegers *et al.*, 1958; Magnusson, 1965; Baughman and Waugh, 1967) and a molecular weight of 30,000.

thrombin. However, the experiment confirms that about 2×10^4 thrombin molecules bind per platelet. The fact that the number of thrombins bound per platelet decreases as platelet concentration increases may indicate an equilibrium adjusting to the decreased concentration of free thrombin at high platelet concentrations; at the lowest concentration of platelets, free thrombin is about 2.4 units/ml and at the highest concentration it is 1 unit/ml. This equilibrium would presumably involve either P* or P′ (see reaction 3) and not P, since P would be rapidly converted to P* under the conditions of this experiment. To establish that the removal of clotting activity by platelets was actually due to binding of thrombin by platelets and not to release of a thrombin inhibitor, an experiment similar to that described in Figure 6 was carried out with 0.5 unit/ml of thrombin and an incubation of 5 min before centrifugation. The supernatant then had no detectable clotting activity but also did not inhibit clotting by a standard thrombin solution.

Calculation of the ratio thrombin:platelet for maximal release per platelet in experiments where platelets were varied would be expected to give the same estimation of the number of sites. We therefore replotted the data in Figure 4A as calcium per platelet *vs.* thrombin per platelet. From this plot (Figure 7), the thrombin:platelet ratio for maximal release per platelet can be calculated as 0.6×10^4 and 1.2×10^4 for 0.16 and 0.4 unit of thrombin per ml. These values are not only different from that calculated above, but, surprisingly, depend on thrombin concentration. Unfortunately, these experiments could not be carried out with a wider range of thrombin concentration; at lower thrombin, the amount of Ca^{2+} released is too small for accurate measurement at low platelet concentrations, and at the higher platelet concentrations required for saturation with higher thrombin, turbidity is too great. This is an unusual aspect of the reaction and is considered in the discussion. We believe, however, that the maximum number of thrombin sites is about 2×10^4.

Discussion

The kinetics of the release of calcium from platelets has the characteristics of the appearance of product in a series first-order reaction, and the results have been analyzed on this basis. From the dependence of the first step on thrombin we have further postulated a rapid association between enzyme and platelets. The most general mechanism that is consistent with our data is shown in reaction 4.



(4)

In the absence of information about the number and kinds of subcellular structures which release calcium and the nature of the thrombin binding step, it is not possible to propose a precise mechanism for the reaction. A number of special cases can be considered, however, and at least one specific case can be excluded.

Rejection of Models Based on Equivalent and Independent Sites. The simplest model for the reaction is one in which all K_i's are equal and all k_{1i}'s are equal, *i.e.*, sites are equivalent and noninteracting. This can be written as a model in which

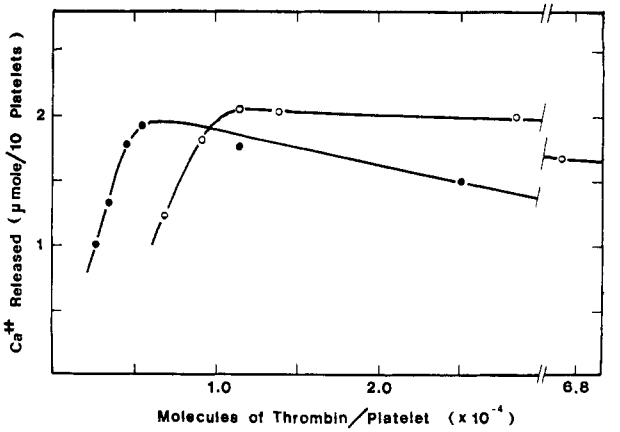


FIGURE 7: Ca^{2+} released as a function of mean number of thrombin per platelet. The data is replotted from Figure 4A.

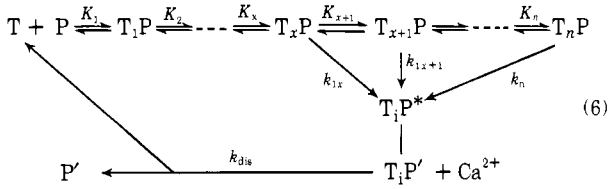
one thrombin reacts with one site, leading to release of a certain number of moles of Ca^{2+}, as shown in reaction 5, where

$$T + P \overset{K}{\rightleftharpoons} Tp \xrightarrow{k_1} Tp^* \xrightarrow{k_2} Tp' + Ca^{2+} \qquad (5)$$

lower case p is a site on platelets. This model can be rejected for three reasons. (i) If the sites are independent, each one must lead directly to release of Ca^{2+} and the yield of Ca^{2+} should be proportional to the amount of thrombin added until the point of saturation, but experimentally a nonlinear relationship is observed (Figure 3E). (ii) The ratio thrombin:platelet for maximal release depends on thrombin concentration (see Figures 3E, 4A, and 7). (iii) Reaction 5 predicts that, in the approximately stoichiometric range of concentrations used in these experiments, k_1(app) depends on concentrations of both platelets and thrombin, since k_1(app) would not be a pseudo-first-order rate constant.[5] While k_1(app) is thrombin dependent, no platelet dependence was observed.

We therefore exclude all models that involve equivalent and independent sites and we will only consider models that can account for the unusual features described above.

Possible Models with Cooperativity between Sites. The thrombin dependence of Ca^{2+} release is consistent with a model in which there is a critical number, x, of thrombins per platelet required for release to occur, *i.e.*, in reaction 4, all k_{1i}'s for $i < x$ are zero and P would be transformed to P* only after x thrombins were bound. This is shown in reaction 6.



(6)

With the addition of less than saturating amounts of thrombin, the number of platelets binding x or more thrombins would

[5] The rate of transformation of TP to TP* is $d[TP]/dt = k_1[TP]$. The equilibrium expression for TP is $[TP] = ([P_0] - [TP])([T_0] - [TP])/K$. If T is in excess, then solving for TP and substituting, $d[TP]/dt = k_1(app)\cdot[P]$, where $k_1(app) = k_1[T_0]/([T_0] + K)$, and the reaction is first order in platelets. Where P and T are of similar concentration, k_1(app) as measured here is not a pseudo-first-order rate constant and depends on both platelets and thrombin.

be simply a function of the probability distribution of thrombin on platelets. The number of platelets binding x or more thrombin will be determined, for a given mean value of thrombins/platelet, by a statistical distribution. The value of x is estimated as the ratio thrombin:platelet giving half-maximal release and is 0.5×10^4 thrombins/platelet.

The amount of calcium released would be proportional to the area under a distribution curve between x and infinity. Such curves are tabulated (Korn and Korn, 1968) and show sigmoid characteristics, although without independent evidence for the nature of the dispersion it would be impossible to choose any particular curve to fit Figure 3E. This model also predicts that increasing the number of platelets above the point of maximum release would actually decrease the number with x or more thrombins by simple dilution, the result actually observed in Figure 4A. The model in reaction 5 thus accounts for the dependence of Ca^{2+} released on thrombin and on platelets, but it does not explain the dependence of the ratio thrombin:platelet for maximal release on thrombin concentration (Figure 7). This model can therefore hold only in conjunction with some additional mechanism.

The problem appears to be that the number of thrombin binding sites increases as the concentration of thrombin increases. A number of possible mechanisms are applicable including the classic case of cooperative interactions in enzymes. (Here the substrate exhibits cooperativity.) Both the induced-fit model (Koshland, 1963) and the allosteric model of Monod (Monod *et al.*, 1965) are consistent with our data. For example, binding of thrombin may cause a change in the binding constant at other sites. Alternatively, there may be some inactive, or unproductive, thrombin binding sites. If the active and inactive sites existed in an equilibrium that was influenced by thrombin, the number of active sites would thus be controlled by thrombin. Mechanisms of this type could not only account for the observed dependence of the stoichiometry on thrombin concentration, but might also explain the lack of dependence of k_1(app) on platelet concentration, since the initial *effective* concentration of thrombin sites might be enough less than total sites to make k_1(app) pseudo first order in platelets (*i.e.*, thrombin in excess of sites). Other mechanisms, such as progressive "unmasking" of sites, are also possible. Our results do not permit evaluation of these proposals, but they do demand some form of cooperativity between the thrombin sites.

Interpretation of the Rate Constants. In the models considered, k_1(app) contains the true first-order rate constant k_1 for the transformation of a TP complex as well as the equilibrium expression for the complex formation, terms containing platelet and thrombin concentrations and the equilibrium constants. Because no platelet dependence was observed for this apparent first order constant we suggested a cooperative model in which the number of active platelet sites was low at low thrombin, leading to pseudo-first-order behavior. An alternative model to explain the k_1(app) step is to consider that the value of k_1 is a function of the number of thrombins bound. That is, in reaction 6, $k_{1i} < k_{1j}$ for $i < j$. Thus k_1(app) would increase with increasing thrombin as the mean number of thrombins per platelet increased and would saturate at k_{1n}. This model predicts that k_1(app) would actually increase as platelet concentration decreased, since the average number of thrombins per platelet would increase. Since no dependence of k_1(app) on platelet concentration was observed, neither of these explanations alone is satisfactory. Further understanding of k_1(app) will depend in large part on direct studies of the initial binding of thrombin to platelets.

The first-order rate constant k_2 apparently represents the actual release of Ca^{2+} from the platelet and depends on neither thrombin nor platelet concentrations. Whether the release process takes place by a pump or by simple diffusion is not known.

We have interpreted the rate constant k_3 as a complex function for the rate at which thrombin dissociates from thrombin activated platelets to react with unreacted platelets. This interpretation is based primarily on the observation that (i) an appreciable k_3 is observed only when the ratio thrombin:platelet is low enough to leave a large proportion of platelets unreacted in the initial reaction, and (ii) hirudin inhibits the k_3 step, suggesting that the mechanism involves free thrombin. Since the turnover of thrombin must be very slow compared to the rate of the initial reaction to account for the observed dependence of the amount of Ca^{2+} released on thrombin concentration, the rate-limiting step in k_3 is probably the dissociation of thrombin, referred to as k_{dis}. Whether this step is an equilibrium or an irreversible step is not known.

Chemistry of the Thrombin–Platelet Interaction. One of the unusual features of these studies is that all of the releasable calcium does not appear in the k_2 phase, but is limited by the amount of thrombin (even though more calcium can be released later at a slow rate). This means that the thrombin does not turn over rapidly. The experiments in Figure 6 and the hirudin studies confirm that thrombin is tightly bound during the reaction, and that the turnover of enzyme is slow. If the reaction is proteolytic, such a long-lived enzyme–product complex is unusual. It is also unlikely that this is the covalent acyl-enzyme which is formed by serine proteases between the carboxyl group of the cleaved bond and the active serine hydroxyl; for peptide substrates the formation of this intermediate is usually rate determining although this is not proven for thrombin (Magnusson, 1971). More likely is that the tight binding does not involve the active site of the enzyme and/or that transformation to the P* state causes an increased affinity for thrombin in the platelets.

The inhibition of the release reaction by hirudin sheds some light on the thrombin–platelet association. The work on this inhibitor (Markwardt, 1970, and references therein) strongly suggests that the site of inhibition is not the active site, but rather part of the fibrinogen binding site. Evidence for this idea comes from the following observations: hirudin binds to DIP-thrombin; acetylated thrombin (presumed to be acetylated at amino groups on the surface of the enzyme) does not bind hirudin; esterase activity is markedly less inhibited than clotting activity. It appears, then, that the thrombin binding site on platelets recognizes that part of the molecule that binds fibrinogen. Although binding on the surface of the platelet seems the most likely form of binding, we cannot exclude binding within the membrane or transport of the thrombin to the inside of the platelet.

Physiological Significance of This Work. Platelets contain a large amount of calcium, most of which is not exchangeable with extracellular Ca^{2+} (Wallach *et al.*, 1958; Murer and Holme, 1970). Murer (1969) and Murer and Holme (1970) observed that about 75% of the platelet's calcium was released on addition of thrombin, but the role of this phenomenon is not known. It seems unlikely that the release serves as a source of extracellular Ca^{2+}, for it would lead to an increase in the normal concentration of blood Ca^{2+} of less than 10%. One intriguing possibility is that release to the extracellular phase is associated with an increase in intracellular Ca^{2+} due to release of bound calcium and that intracellular Ca^{2+}

regulates metabolism and function. Such a mechanism is suggested by the work of Stormorken (1969), who compared the platelet-release reaction with release by secretory cells, in which Ca^{2+} mediation is well known (Douglas, 1968; Rubin, 1970); by the observation of Grette (1962) that the platelet release reaction could be separated into two steps, a proteolytic step followed by a Ca^{2+} mediated step; and by the reports of Grette (1963) and of Statland *et al.* (1969) that platelets contain an ATP-dependent Ca^{2+} binding protein, in some respects similar to the sarcoplasmic reticulum of skeletal muscle. We made several attempts to get murexide into platelets so that changes in intracellular Ca^{2+} concentration could be observed, as has been reported for frog muscle (Jobsis and O'Connor, 1966), but without success. Thus, while the data presented in this paper indicate that release of Ca^{2+} can be observed soon enough after addition of thrombin for it to serve as a triggering agent for the other thrombin-induced phenomena, the essential evidence in support of such a role is not available.

There is little known about the location of releasable Ca^{2+} in platelets, or the mechanism of the actual release. Other released constituents, such as adenine nucleotides and 5-hydroxytryptamine, are stored in granules which disappear during release, so that it may be assumed that release occurs at least from one entire granule, and most likely from all granules in a platelet at once. In other words, for any platelet release is probably all or none. Our data offer no evidence about whether release of Ca^{2+} is also all or none.

The results of this work suggest two significant facts about the physiological actions of thrombin and platelets. First, the only models capable of explaining our results involve cooperative interactions, either by virtue of a critical number of thrombins before release or a system where bound thrombin increases the further binding of thrombin. This means that small amounts of thrombin could be in circulation without danger of reacting with platelets. Second, there is very little turnover of thrombin, the enzyme becoming tightly bound throughout the reaction. Thus, the platelets exhibit appreciable anti-thrombin activity by binding up to 2×10^4 thrombins/platelet. This would correspond to approximately 1 unit of thrombin/ml of blood, and is consistent with the self-limiting nature of the overall process of blood coagulation.

The methods described here for observation of the kinetics of the thrombin-induced release of Ca^{2+} should prove valuable for studies of the effect of drugs on platelets or of the nature of the lesions in pathological platelets.

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