Kinetics of the Thrombin-Induced Release of Adenosine Triphosphate by Platelets. Comparison with Release of Calcium†

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ABSTRACT: The kinetics of the release of ATP from human platelets induced by thrombin has been studied and compared to the previously reported kinetics of Ca^{2+} release. The release of ATP was observed by recording the luminescence resulting from the reaction of released ATP with firefly extract. The reaction was characterized by a delay of several seconds followed by a first-order release. ATP-release curves were analyzed by measuring the length of the delay, the first-order rate constant, and the amount of ATP released. The delay varied inversely with thrombin, while the first-order rate constant was a direct function of thrombin, saturating at about 2 units of thrombin/ml with a value of 0.03 sec^{-1}. Both the delay and the first-order rate constant were independent of platelet concentration. The amount of ATP released was a function of both platelet and thrombin concentrations, indicating a very slow turnover of thrombin. Although much of the data for ATP release is consistent with a previously proposed model for the reaction of thrombin with platelets, ATP is released by a mechanism that is kinetically distinct from release of Ca^{2+}. Direct comparisons of release of ATP and Ca^{2+} indicate that release of Ca^{2+} preceeds release of ATP. The results are discussed in terms of a model in which thrombin causes intracellular release of bound calcium, which in turn mediates other thrombin-induced processes, including the release reaction.

The platelet release reaction is a specific process by which certain platelet constituents are secreted to the extracellular phase. It is an essential step in the sequence of events leading to formation of the dense, adhesive aggregates of platelets observed in hemostasis and thrombosis (for reviews, see Mustard and Packham, 1970, and Holmsen *et al.*, 1969). This secretory process can be induced by a variety of agents (proteolytic enzymes, collagen, polystyrene particles, epinephrine, and 5-hydroxytryptamine), the most potent being thrombin, a trypsin-like protease that functions in several steps in blood coagulation. The substances released include ADP, ATP, 5-hydroxytryptamine, and certain enzymes. Holmsen *et al.* (1969) reported that those compounds released are located in the granule fraction of platelets, while those constituents not located in the granule fraction are not released, leading to the conclusion that the release reaction involves the specific secretion, perhaps by exocytosis, of the contents of the granules.

The actual mechanism of release, or the mechanism by which the initial stimulus triggers the release, is not known. It is known that in most secretory cells Ca^{2+} mediates the stimulus–secretion process (Douglas, 1968; Stormorken, 1969; Rubin, 1970) and that under certain conditions, extracellular Ca^{2+} appears to be required for the platelet release reaction (Grette, 1962; Murer, 1971; Sneddon, 1972). The report by Murer and Holme (1970) that Ca^{2+} was also released by platelets was therefore especially interesting. One problem in evaluating the role of release of Ca^{2+} is that the location of the stored calcium is not known. It has been suggested that the releasable Ca^{2+} is located in the granules where it serves as a counterion for released substances (Murer and Holme, 1970). On the other hand, a membranous fraction capable of an ATP-dependent binding of calcium has been isolated from platelets (Statland *et al.*, 1969), suggesting that release of Ca^{2+} might be associated with an intracellular release of Ca^{2+} from storage membranes or vesicles, in analogy with release of Ca^{2+} by sarcoplasmic reticulum with electrical stimulation of muscle. Ca^{2+} release could then be a reflection of a major regulatory mechanism in platelets.

We previously described the kinetics of the thrombin-induced release of Ca^{2+} from platelets (Detwiler and Feinman, 1973). To extend the analysis of the kinetics of the platelet release reaction and to further evaluate the role of Ca^{2+} release we have now compared the kinetics of release of constituents known to be secreted from platelet granules with the previously described kinetics of release of Ca^{2+}. The major purpose of this work was to test the hypotheses (i) that Ca^{2+} is secreted from the granules, perhaps as a counterion for other released substances, and (ii) that the release reaction is mediated by Ca^{2+}. As an indication of secretion from granules, we measured released ATP by the firefly luminescence assay. The experiments described in this paper indicate that the kinetics of the thrombin-induced release of Ca^{2+} and ATP are different, primarily because release of ATP begins only after a delay of at least 4 sec. This is inconsistent with the first hypothesis above and consistent with the second.

Methods

Preparation of Platelets. Suspensions of washed human platelets were prepared as previously described (Detwiler and Feinman, 1973). For most experiments, platelets from 50 ml of blood were suspended in 1.0 ml to give concentrations of about 4×10^9/ml.

Measurement of ATP. ATP was measured by the luminescence method of Strehler and Totter (1952) (for review, see Strehler, 1968) using firefly lantern extract as a source of luci-

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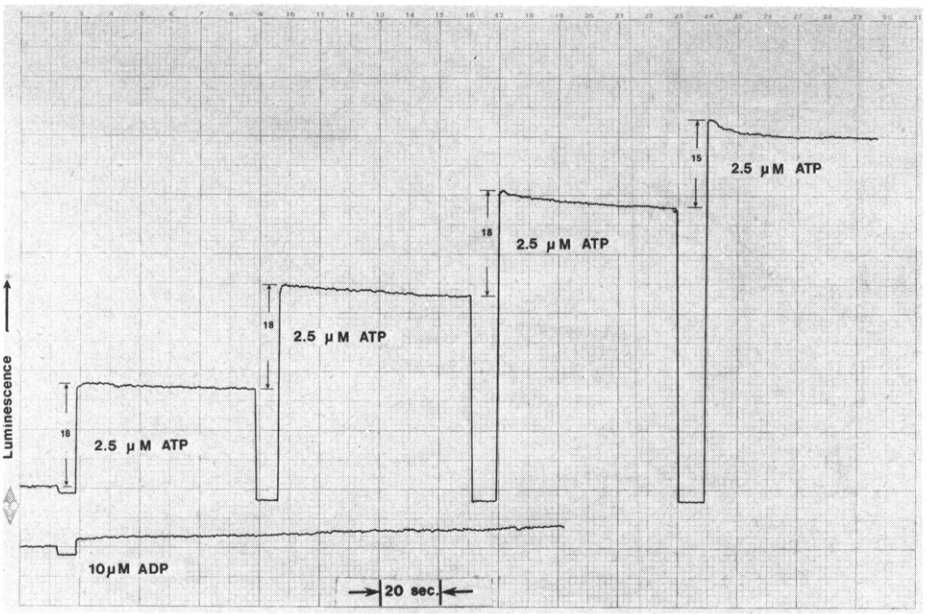

FIGURE 1: Luminescence caused by ATP and ADP. Luminescence was measured as described in the text with platelets omitted.

ferin and luciferase. Luminescence was measured with an Aminco-Chance dual-wavelength spectrophotometer with the lamp turned off and the phototube high-voltage set to 1.25 kV. The signal current from the phototube was converted to voltage and amplified using Philbrick Model EP-85AU operational amplifiers. The circuitry also included a variable zero supression voltage. The amplified signal was recorded with a storage oscilloscope and a recorder.

Thrombin was purified and assayed as described previously (Detwiler and Feinman, 1973). Firefly lantern extracts were prepared by homogenizing 30 mg of desiccated firefly tails (Sigma Chemical Co., St. Louis, Mo.) in 3 ml of 50 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 2000*g* for 20 min and the supernatant was used for the ATP assay. Stock solutions of ATP and ADP, neutralized with KOH, were standardized using enzyme spectrophotometric assays (Lamprecht and Trautschold, 1965; Adam, 1965).

In order to use the firefly luminescence assay to follow the kinetics of release of ATP, it was necessary to establish conditions under which luminescence is proportional to ATP concentration. In Tris-HCl buffer, as well as in most other dilute buffers, *initial* luminescence is proportional to added ATP, but the luminescence decays rapidly, so that a very difficult correction would be required for time–progress curves of ATP release. However, it is known (Strehler, 1968) that phosphate inhibits both the initial luminescence and the subsequent decay. By using 45 mM phosphate (Figure 1) we found that the initial response to ATP was linear to about 7.5 μM ATP and that the decay was appreciable only above 5 μM ATP. For comparison, most experiments involved changes of less than 2 μM ATP. Therefore kinetic experiments were performed with 45 mM phosphate (pH 7.4) unless otherwise stated.

It is also known (Strehler, 1968) that firefly lantern extracts contain adenylate kinase, which can convert ADP to ATP. Since platelets release about as much ADP as ATP, a slow conversion of ADP to ATP would greatly complicate analysis of kinetics of the release reaction. However, Figure 1 (lower trace) shows that under the conditions of these experiments, addition of 10 μM ADP, which is about five times the amount that would be released under most conditions studied, caused only a very slight increase in luminescence.

The method of measuring thrombin-induced release of ATP is shown in Figure 2. To a cuvet 10-mm wide with a 5-mm light path, the following were added: 0.1 ml of firefly extract, 0.05 ml of 154 mM $MgSO_4$, 0.4 ml of 112 mM sodium phosphate (pH 7.4), platelet suspension, and suspending solution to bring the total volume to 1.0 ml. The suspending solution was 25 mM Tris-HCl (pH 7.4), 136 mM NaCl, 5 mM glucose, and 0.6 mM disodium citrate. The trace in Figure 2 began with the cuvet in the cell compartment and the shutter open, recording a slight background luminescence. When the shutter was closed, the signal dropped to dark current level. Thrombin (5–20 μl) was quickly added with a flattened plastic mixing rod exactly as the oscilloscope sweep was triggered. The cell compartment cover was replaced and the shutter was opened, causing the signal to return to the original level before ATP release began. Calibration was with standard ATP solutions.

Results

Progress curves of the thrombin-induced release of ATP (Figure 2) are qualitatively similar to curves of release of Ca^{++} studied previously (Figure 3). The approach to the final, nearly linear, slope is exponential as indicated by straight lines on log plots. The curves of ATP and Ca^{2+} release appear to differ, however, in the early lag phase, where a distinct delay is observed before any ATP is released, especially obvious in the oscilloscope trace (Figure 2, inset). This difference seems especially significant because the ATP experiment was done with a higher thrombin concentration, which shortens the lag phase.

The curves were analyzed in a manner similar to that described previously for Ca^{2+} release. The lag phase was quantitated as the *delay* before any observed reaction, measured as the time from addition of thrombin to the point where the signal started its upward deflection, and as the time from addition of thrombin to the inflection point, t_i. A first-order rate

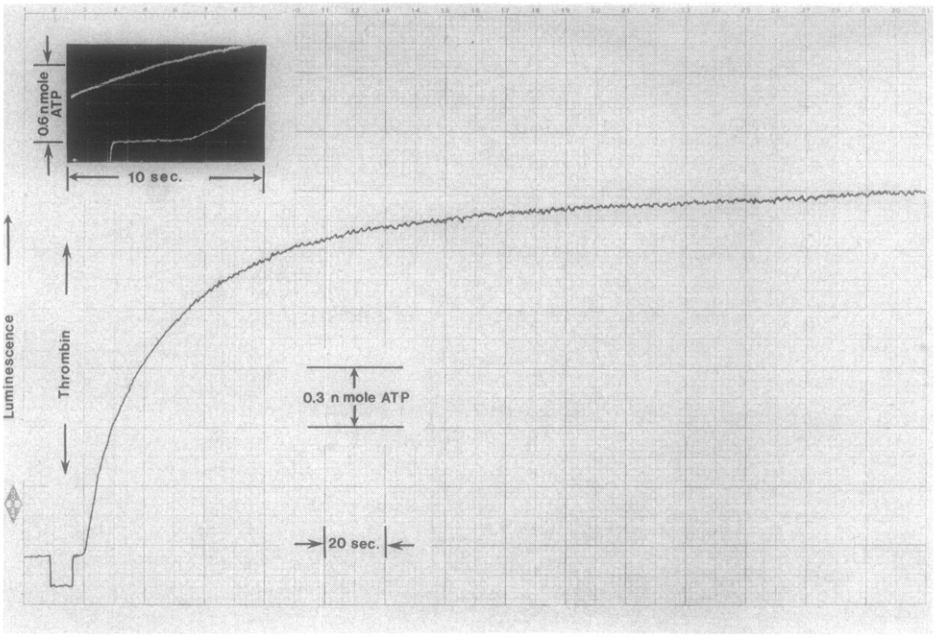

FIGURE 2: Progress curve of the thrombin-induced release of ATP. The procedure is described under Methods. The reaction mixture contained 7.8×10^7 platelets/ml and 8 units of thrombin/ml.

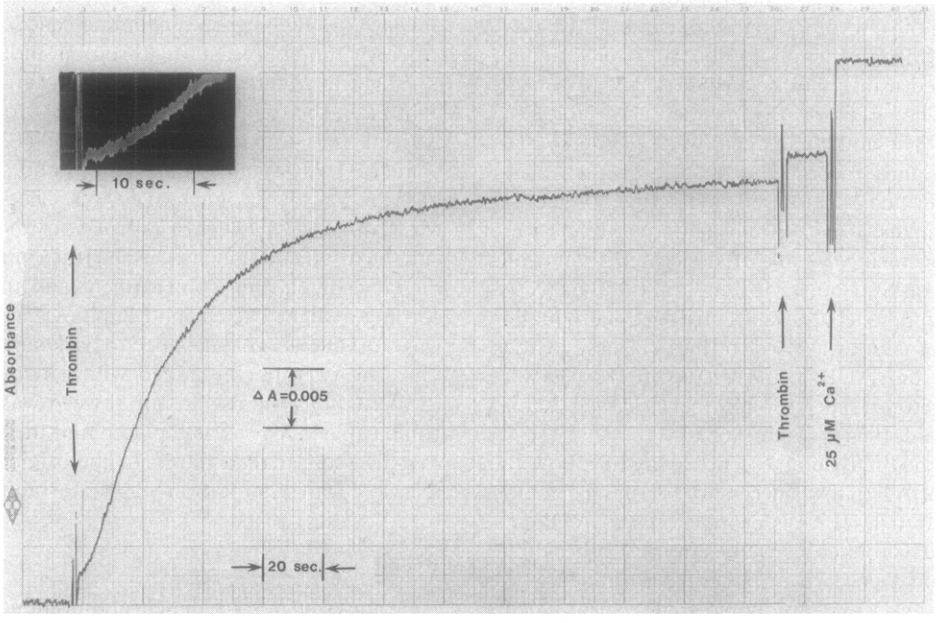

FIGURE 3: Progress curve of thrombin-induced release of Ca^{2+} from platelets. Release was measured by spectrophotometrically recording the formation of a calcium–murexide complex. The reaction contained 2.4×10^8 platelets/ml and 0.6 unit of thrombin/ml; from Detwiler and Feinman (1973).

constant, referred to as k_2 to be consistent with terminology used for Ca^{2+} release, was calculated from log plots (time *vs.* the logarithm of the difference between the curve and the extrapolated linear phase). At lower thrombin concentrations, release of ATP was incomplete, and a slow, nearly linear, increase in luminescence followed the exponential phase, similar to the k_3 phase observed with Ca^{2+} release. This linear increase was not measured in this work because of the error that could result from the slow decay of luminescence and the slow increase due to reactions of released ADP (see Methods).

Experiments were carried out with constant concentrations of platelets while thrombin concentration was varied from 0.15 to 40 units per ml (Figure 4). Both the delay and t_i (Figure 4A) were an inverse function of thrombin, essentially reaching limiting values at about 2 units of thrombin/ml. The value of k_2 was constant above 2 units of thrombin/ml with an average of about 0.03 sec^{-1}, but decreased to about 0.02 at very low thrombin (Figure 4B). As with Ca^{2+} release, not only the kinetic parameters but also the yield of ATP was a function of thrombin concentration, also saturating at about 2 units/ml (Figure 4C).

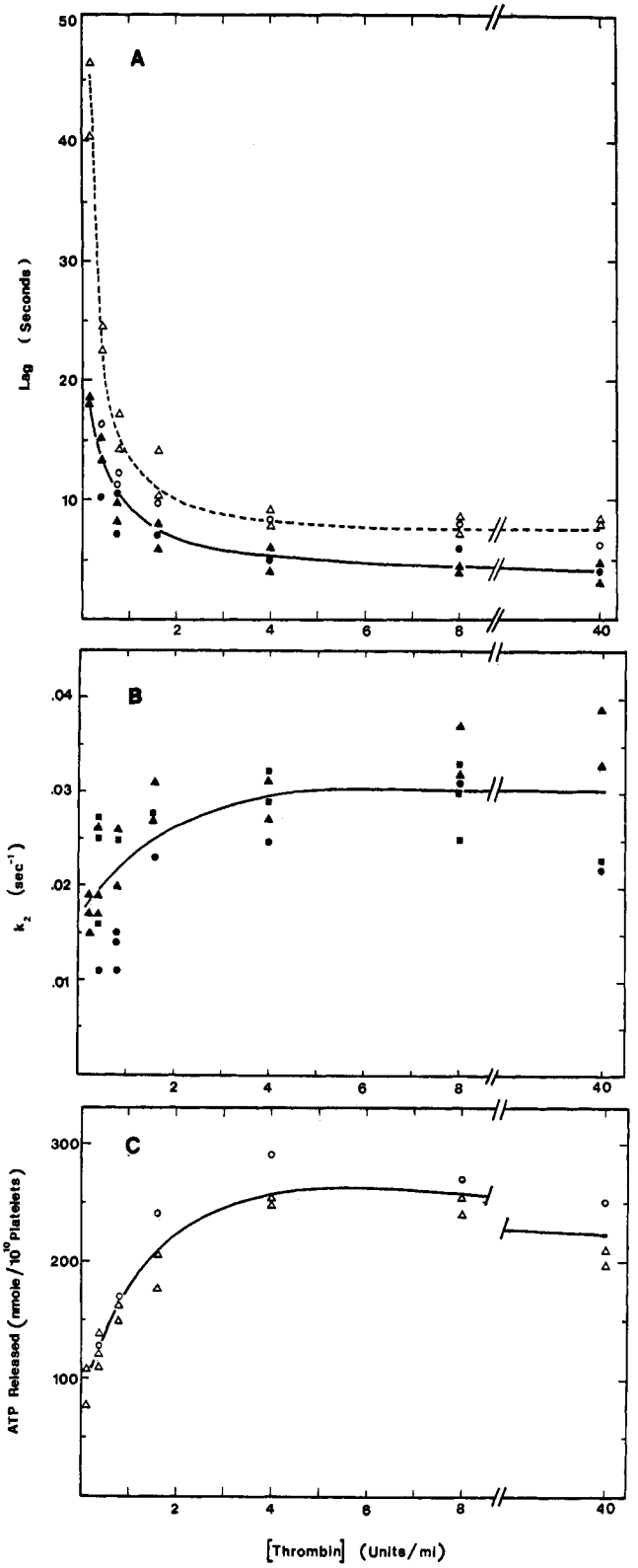

FIGURE 4: Effect of different thrombin concentrations on kinetic parameters and amount of ATP released. The experiments were performed as shown in Figure 2. Platelet concentrations were 3.9×10^7 to 2.2×10^8 per ml for B and 7.8×10^7 to 2.0×10^8 for A and C. (A) The lag phase is shown as *delay*, the time from addition of thrombin to the beginning of the reaction (solid symbols, continuous line) and as t_i, the time from addition of thrombin to the inflection point of the curve (open symbols, broken line). (B) k_2 is calculated from the slope of a plot of time *vs.* the logarithm of the difference between the curve and the extrapolated final line. (C) ATP released is calculated from the difference between the starting luminescence and the final line extrapolated to zero using standardized ATP solutions for calibration.

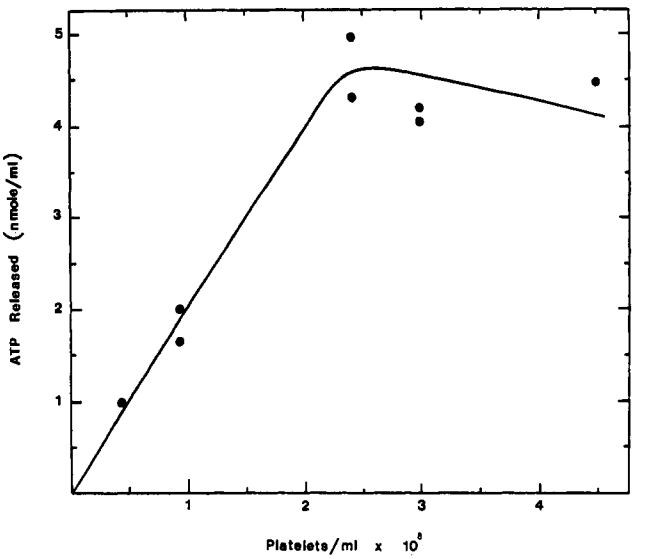

FIGURE 5: Effect of different platelet concentrations on the amount of ATP released. The experiment was carried out as described for Figure 2. Thrombin concentration was 0.4 unit/ml.

The concentration of platelets was also varied at constant thrombin concentration (0.4 unit/ml). Neither the lag phase nor k_2 was affected by a 20-fold change in platelet concentration, but the amount of ATP released was proportional to platelet concentration, reaching a maximum above which it appeared to decrease slightly (Figure 5), a pattern similar to that observed for release of Ca^{2+}.

To determine whether the thrombin-induced release of Ca^{2+} and ATP follow the same time course, it is necessary to determine whether observed differences can be due to differences in the experimental conditions. A major experimental difference is that for measurement of Ca^{2+} release the medium contained a rather high concentration of the calcium-complexing dye, murexide, which could conceivably modify the reaction either by complexing Ca^{2+} or by some direct effect on the platelets. To test this, an experiment was performed with 0.3 mM murexide in the assay mixture. This decreased the response, due to absorbance of the emitted light, but by increasing amplification 2.5-fold, a trace that was essentially superimpossable on the trace without murexide was obtained. Addition of 0.1 mM EGTA to chelate Ca^{2+} was also without effect on the reaction. The other major difference between the systems for measuring ATP release and Ca^{2+} release is the high concentration of phosphate in the measurement of ATP release. It is not possible to measure Ca^{2+} release in high phosphate because of precipitation of calcium phosphate and it is not possible to measure release of ATP without high phosphate because of the rapid decay of luminescence. However, the most important difference observed is the delay before any ATP release, and the problem of decay would not interfere with measurement of the lag phase. Thus, experiments were carried out to compare the lag phases of release of Ca^{2+} and release of ATP under conditions that were essentially identical (Figure 6). The distinct delay before release of ATP was still observed with conditions of recording that resulted in traces with identical slopes, eliminating any error in interpretation due to differences in sensitivity. The observed differences between lag phases for release of Ca^{2+} and ATP are therefore not due to differences in the methods of observation but apparently indicate different mechanisms.

Discussion

In our previous studies of the kinetics of the platelet release reaction, we found that observation of the release of Ca^{2+} was very satisfactory for fundamental investigations of the interaction of thrombin and platelets. The kinetics of the thrombin-induced release of ATP are less satisfactory for such detailed analysis for several reasons. First, the complications of a slight decay in luminescence and the slow reaction with ADP make it difficult to accurately establish the final, linear reaction. This not only prevents measurement of a rate constant for this phase of the reaction, it also may cause some error in the values of k_2 and the amount of ATP released in the exponential phase since both of these require extrapolation of the final slope to zero time. Second, the ATP-release reaction cannot be fit to such a simple mechanism as the Ca^{2+}-release reaction, making the observed rate constants more difficult to interpret, and finally, the method of recording the release of ATP is more complicated and requires conditions that are farther from physiological than the method for measuring Ca^{2+} release. On the other hand, measurement of ATP has the major advantage of greater sensitivity than measurement of Ca^{2+}.

The purpose of the work described here was to compare the release of ATP and Ca^{2+}, with a specific goal to test the hypothesis that regulation of Ca^{2+} concentration is an essential aspect of regulation of platelet function, including the release reaction. For this, the above disadvantages of the ATP-release reaction for studying the interaction of thrombin and platelets are not critical. Experimental release curves were analyzed using parameters similar to those used for Ca^{2+} release, although it is recognized that the meaning of these parameters may not be exactly the same.

In general the kinetics of the thrombin-induced release of ATP are consistent with our previous conclusions about the interaction of thrombin and platelets based on measurement of Ca^{2+} release. The major *similarities* between release of Ca^{2+} and ATP are: (i) both reactions are characterized by a lag phase followed by a first-order release, (ii) the lag phases are inversely a function of thrombin concentration and independent of platelet concentration, and (iii) the amount released in the exponential phase is a function of both thrombin and platelets. The most striking *differences* between the kinetics of ATP release and Ca^{2+} release are the following: (i) ATP release is observed only after a distinct delay, whereas Ca^{2+} release appears to begin within the period of mixing; (ii) release of Ca^{2+} follows the kinetics of appearance of product in a simple series first order reaction, but release of ATP does not approximate such a mechanism; and (iii) while the kinetic parameters and yield as a function of thrombin concentration for the two reactions are qualitatively similar, there are appreciable quantitative differences.

For the thrombin-induced release of calcium, we were able to describe the reaction as series first order

$$A \xrightarrow{k_{1,app}} B \xrightarrow{k_2} C$$

The first rate constant, $k_{1,app}$, was actually a complex constant containing binding constants (K) and a first-order rate constant (k_1)

$$T + P \overset{K}{\rightleftharpoons} TP \xrightarrow{k_1} TP^* \xrightarrow{k_2} TP' + Ca^{2+} \qquad (1)$$

where T is thrombin, P is unreacted platelet, P* is thrombin activated platelet, and P′ is platelet after release. Several aspects of the data for ATP release are consistent with a mechanism of this type. For example, eq 1 predicts that the lag phase would be an inverse function of thrombin while k_2 would be independent of thrombin concentration. It can also account for the dependence on thrombin concentration of the amount of calcium released or, in these experiments, ATP released, since the tight binding of thrombin makes the amount of P*, the releasing unit, a function of both T and P. The thrombin-induced release of ATP cannot, however, be completely described by eq 1, since the delay before any reaction begins excludes this simple series first-order mechanism (see Appendix). It thus appears that the delay observed with ATP release represents a kinetic step in addition to those shown in eq 1.

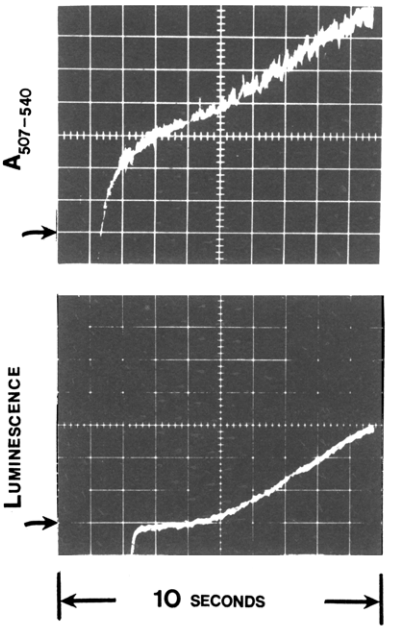

FIGURE 6: Comparison of the lag phases for release of Ca^{2+} and ATP. Ca^{2+} release (upper trace) was measured as described previously (Detwiler and Feinman, 1972). Each cuvet contained 0.05 ml of 6 mM murexide; 0.05 ml of 154 mM $MgSO_4$; 0.8 ml of a solution that was 25 mM Tris (pH 7.4), 136 mM NaCl, and 5 mM glucose; and 0.1 ml of a platelet suspension to give a final concentration of 2.1×10^8/ml. In addition, the ATP release cuvet contained 0.05 ml of firefly extract, the only difference between the two experiments. The oscilloscope sweeps were started as 4 units of thrombin was added. Arrows indicate position of traces before addition of thrombin.

Quantitative comparisons also suggest that release of Ca^{2+} and ATP are similar but not identical. The value of k_2 for Ca^{2+} release was 0.038 (SD 0.008) and for ATP it is 0.031 (SD 0.006), calculated from the values obtained with 8 and 40 units of thrombin/ml. Considering the differences in conditions of assays, these values are remarkably similar and suggest a similar mechanism for the actual release. In contrast, the saturation of the measured parameters (t_i, k_2, and yield) is observed at higher thrombin with ATP release (2–4 units/ml) than with Ca^{2+} release (1 unit/ml). This could be due in part to a different rate-limiting step or to different binding constants (K in eq 1) with the different buffers used.

It can thus be concluded that the thrombin-induced release of Ca^{2+} and ATP are by kinetically distinct mechanisms and the hypothesis that Ca^{2+} is secreted from the granules as a counterion for adenine nucleotides can be rejected for the major part of released Ca^{2+}. An alternative hypothesis, that the release reaction is mediated by Ca^{2+}, is consistent with, but not proved, by the data.

As a reasonable working hypothesis that is consistent with our kinetic data and other platelet research, we propose that platelets contain a calcium storage organelle, either a membrane or a vesicle (Statland *et al.*, 1969), that tightly binds calcium. Thrombin reacts with the platelet membrane to cause discharge of Ca^{2+} from the storage site. The intracellular Ca^{2+} is free to diffuse out of the cell but it also activates any Ca^{2+}-sensitive process, including secretion of the contents of granules. There are several aspects of this model that should be especially noted. (1) It implies that platelets are similar to other secretory cells in that secretion is mediated by Ca^{2+}, but differ in that the source of Ca^{2+} would normally be intracellular for platelets. (2) A requirement for Ca^{2+} for the release reaction could be demonstrated only if the intracellular release of stored calcium were blocked or if calcium chelators got inside. This might account for some of the conflicting reports about the requirement of Ca^{2+} for the platelet-release reaction. (3) The mechanism by which the stimulus, thrombin, modifies the membrane and how this signal is translated to release of Ca^{2+} are not known but the calcium-storage organelle may be continuous with the cell membrane, perhaps as part of the canalicular system (White, 1971).

The intracellular release of stored calcium could also mediate other thrombin-induced processes, of which the most obvious are: (1) activation of phosphorylase. The quickest metabolic change yet reported to occur after addition of thrombin to platelets is activation of glycogen phosphorylase, observed as an increase in the level of glucose 1-phosphate within a few seconds (Detwiler, 1972). It is known that phosphorylase can be activated by a Ca^{2+}-activated phosphorylase kinase in muscle (Heilmeyer *et al.*, 1971; Brostrom *et al.*, 1971), and it is possible that a similar mechanism exists in platelets. (2) Regulation of contraction. Platelets contain a contractile protein, in many ways similar to contractile proteins from muscle (Luscher and Bettex-Galland, 1971). Although the exact functions of these proteins are not known they probably participate in at least some of the thrombin-induced changes in platelets. Regulation of platelet actomyosin ATPase by Ca^{2+} has been reported (Cohen and Cohen, 1972; Hanson *et al.*, 1972) and Abramowitz *et al.* (1972) have reported that CaATP specifically affects the state of polymerization of platelet contractile protein. It is also known that troponin from muscle actomyosin is phosphorylated by the Ca^{2+}-dependent phosphorylase kinase, (Stull *et al.*, 1972) a possible regulatory mechanism in platelets. (3) Modification of microtubules. Platelets contain microtubules that undergo changes induced by thrombin (White, 1971), and microtubule assembly *in vitro* has been shown to be Ca^{2+}-dependent (Weisenberg, 1972).

Acknowledgments

We are indebted to Mr. Kao-Chih Hsu of the Department of Computer Science, Downstate Medical Center, for the computer program for Figure 7.

Appendix

Series first-order kinetic curves can not be linearized in any simple way and it is, therefore, frequently difficult to decide whether this mechanism is applicable to a particular experiment. This is especially true where the concentrations cannot be determined due to rapid reactions or, as in this case, the actual concentration of the reacting species is not known. We have attempted to define parameters which can be used to determine if observed kinetics fit this mechanism.

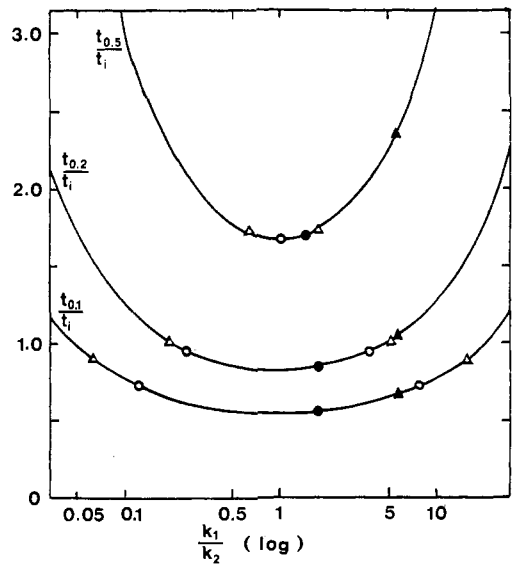

FIGURE 7: Time ratio curves for product appearance in a series first-order reaction. Lines were calculated by computer for the ratio of time for 10, 20, or 50% reaction to the time to the inflection point in the curve (t_i). The points are from experimental progress curves, (○,△) from ATP-release experiment (two possible solutions for each set of ratios are shown) and (●,▲) from Ca^{2+}-release experiments (only one set of points for each reaction are shown since the absolute values of the rate constants are known (Detwiler and Feinman, 1973)). (○) 0.4 unit of thrombin and 4.4×10^8 platelets/ml; (△) 5 units of thrombin and 2.2×10^8 platelets per ml; (●) 0.15 unit of thrombin and 2.4×10^8 platelets per ml (from Figure 2 of Detwiler and Feinman (1973)); (▲) 0.6 unit of thrombin and 2.4×10^8 platelets per ml (from Figure 3 of this paper).

For the kinetic scheme

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

the rate of appearance of C shows a characteristic lag phase which becomes more pronounced as the ratio k_1/k_2 becomes smaller. However, when this ratio is less than unity, the shape of the curve changes as well, reflecting the greater contribution of the first step which is becoming rate determining. Thus, although the lag (quantitated here as t_i, the time to the inflection point), continues to increase, its fraction of the total reaction time might be expected to reach a maximum when $k_1 = k_2$. We have compared t_i to the overall reaction course in attempting to find diagnostic parameters for this reaction.

As measures of the extent of reaction we chose the times to 10, 20, and 50% reactions, $t_{0.1}$, $t_{0.2}$, and $t_{0.5}$. Ratios of these times to t_i are plotted as a function of k_1/k_2 for theoretical reactions in Figure 7. For Figure 7, there are two criteria that must be met by an experimental reaction if it is series first order. The three measured ratios must all fall on the curves at the same ratio k_1/k_2 and no ratio may be less than the theoretical minimum.

This approach is similar to the time ratio method of Swain (1944), although more sensitive to the case at hand. Although this method has general utility for qualitative judgments, the actual values of the rate constants will not be more accurate than those determined directly from t_i (Detwiler and Feinman, 1973; Frost and Pearson, 1961).

When data from representative curves of Ca^{2+} release, previously shown by computer simulation to closely approximate series first-order kinetics, are entered in Figure 7, the three parameters give nearly the same value for k_1/k_2. In contrast,

data for ATP release show ratios of k_1/k_2 that differ by a factor of 10. In addition, several of the curves of ATP release that were tested had time ratios less than the theoretical minimum of Figure 7, excluding series first order as a possible mechanism.

References

Abramowitz, J., Stracher, A., and Detwiler, T. C. (1972), *Biochem. Biophys. Res. Commun. 49*, 958.

Adam, H. (1965), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic Press, p 573.

Brostrom, C. O., Hunkeler, F. L., and Krebs, E. G. (1971), *J. Biol. Chem. 246*, 1961.

Cohen, I., and Cohen, C. (1972), *J. Mol. Biol. 68*, 383.

Detwiler, T. C. (1972), *Biochim. Biophys. Acta 256*, 163.

Detwiler, T. C., and Feinman, R. D. (1973), *Biochemistry 12*, 282.

Douglas, W. W. (1968), *Brit. J. Pharmacol. 34*, 451.

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N. Y., Wiley, pp 166–169.

Grette, K. (1962), *Acta Physiol. Scand. 56*, Suppl. 195.

Hanson, J. P., Repke, D. I., Katz, A. M., and Aledort, L. M. (1972), *3rd Congr. Int. Soc. Thromb. Haemost.*, 200.

Heilmeyer, L. M. G., Jr., Meyer, F., Haschke, R. H., and Fischer, E. H. (1971), *J. Biol. Chem. 245*, 6649.

Holmsen, H., Day, H. J., and Stormorken, H. (1969), *Scand. J. Haematol.*, Suppl. 2.

Lamprecht, W., and Trautschold, I. (1965), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic Press, p 543.

Luscher, E. F., and Bettex-Galland, M. (1971), *in* The Circulating Platelets, Johnson, S. A., Ed., New York, N. Y., Academic Press, p 225.

Murer, E. (1971), *Biochim. Biophys. Acta 261*, 435.

Murer, E. H., and Holme, R. (1970), *Biochim. Biophys. Acta 222*, 197.

Mustard, J. F., and Packham, M. A. (1970), *Pharmacol. Rev. 22*, 97.

Rubin, R. P. (1970), *Pharmacol. Rev. 22*, 389.

Sneddon, J. M. (1972), *Nature (London) 236*, 103.

Statland, B. E., Heagen, B. M., and White, J. G. (1969), *Nature (London) 223*, 521.

Stormorken, H. (1969), *Scand. J. Haematol.*, Suppl. 9.

Strehler, B. L. (1968), *Methods Biochem. Anal. 16*, 99.

Strehler, B. L., and Totter, J. R. (1952), *Arch. Biochem. Biophys. 40*, 28.

Stull, J. T., Brostrom, C. O., and Krebs, E. G. (1972), *J. Biol. Chem. 247*, 5272.

Swain, C. G. (1944), *J. Amer. Chem. Soc. 66*, 1696.

Weisenberg, R. C. (1972), *Science 177*, 1104.

White, J. G. (1971), *in* The Circulating Platelet, Johnson, S. A., Ed., New York, N. Y., Academic Press, p 46.

Membrane D-Lactate Dehydrogenase from *Escherichia coli*. Purification and Properties†

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ABSTRACT: D-Lactate dehydrogenase has been solubilized from the membrane of *Escherichia coli* and purified to a practically homogeneous state using conventional procedures. The enzyme had a pH optimum of 8–9 and an approximate molecular weight of 72,000 (acrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate) or 71,000 (sucrose density gradient). It contains flavine, which was found to be flavine adenine dinucleotide by the D-amino acid oxidase test and the characteristic fluorescence change as a function of pH. Fluorescence of the flavine in the purified enzyme, which is quenched at neutral pH, was increased by boiling, guanidine-HCl, or acidification. The apparent K_m for D-lactate was 6.0×10^{-4} M using an assay based on measuring the reduction of tetrazolium dye mediated by phenazine methosulfate.

One of the membrane-bound primary dehydrogenases in the respiratory chain of *Escherichia coli* (Cox *et al.*, 1970) is D-lactate dehydrogenase. Recently this enzyme has been implicated in the electron transfer reactions coupled to active transport of various amino acids and sugars into bacterial membrane vesicles (Barnes and Kaback, 1971). It is therefore of interest to characterize the enzyme and to determine its localization in the cytoplasmic membrane. In this study, D-lactate dehydrogenase has been solublized by deoxycholate from membranes of *E. coli* and purified about 400-fold to a practically homogeneous state, mainly by ammonium sulfate fractionation and DEAE-Sephadex column chromatography in the presence of Triton X-100. The purified enzyme is a flavoprotein containing FAD^+, and some of its properties are described. This enzyme has been independently purified by Kohn and Kaback (unpublished), but referred to in a review of Kaback (1972).

Materials and Methods

Growth of the Bacteria and Preparation of Membranes. *E. coli* ML 308-225 ($i^- z^- y^+ a^+$) was grown with vigorous aera-

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