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CONTROL OF ENERGY METABOLISM IN PLATELETS

THE EFFECTS OF THROMBIN AND CYANIDE ON GLYCOLYSIS

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SUMMARY

1. The effects of thrombin on platelet glycolysis were studied by measuring levels of glycolytic intermediates. Comparisons were made with the effects of inhibition of respiration by cyanide.

2. Glycogen phosphorylase and phosphofructokinase were identified as major regulatory enzymes with both cyanide and thrombin.

3. Activation of both enzymes occurred at the same time with cyanide. With thrombin, activation of phosphorylase was observed sooner (2.5 sec after addition of thrombin) than activation of phosphofructokinase (10 sec after addition of thrombin).

4. The results are interpreted as indicating that thrombin activates phosphofructokinase allosterically by changing the energy charge of the adenine nucleotide pool as a result of increased utilization of ATP, while activation of phosphorylase is by conversion of phosphorylase *b* to *a*, probably by a Ca^{2+}-activated phosphorylase kinase.

INTRODUCTION

Addition of thrombin to platelet suspensions causes profound changes in the platelets[1,2]. These changes are believed to be analogous to the *in vivo* processes associated with the phenomena of thrombosis and platelet plug formation. There is an initial "release", or active secretion, of certain platelet constituents that are involved in the hemostatic process. This is followed, in the presence of divalent cations, by aggregation of the platelets and then by contraction of the aggregated platelet mass. These processes all require energy and have been shown to depend on active platelet metabolism[3–7]. There have been numerous reports that thrombin also causes a stimulation of platelet glycolysis[4,8–11], but no confirmed reports of an increased rate of respiration[4,12–14]. It has been suggested that the thrombin stimulation of platelet glycolysis could be due to specific effects on certain glycolytic enzymes or to an increased permeability to glucose[9]. It seemed more likely that the effect of thrombin on glycolysis was not direct, but was the effect of an increased energy expenditure on the basic glycolytic regulatory mechanisms.

Regulation of metabolism can be studied by comparing the steady-state levels of intermediates in a pathway during different metabolic fluxes (for reviews see refs. 15–17). This type of analysis depends on the identification of rate-limiting (and therefore potentially regulatory) enzymes as those catalyzing non-equilibrium reactions, or reactions for which the mass action ratio (calculated as an equilibrium constant under steady-state conditions) is much less than the equilibrium constant. If these rate-limiting enzymes are actually regulatory, higher metabolic fluxes will be caused by their activation and will therefore be accompanied by a shift of the mass action ratio toward the equilibrium constant. The shifts are usually detected on "crossover" plots in which levels of intermediates at one flux are plotted as percent of levels at a control flux.

Using these methods to study the regulation of glycolysis in platelets, glycogen phosphorylase and phosphofructokinase were identified as major regulatory steps[18]. By analogy with other tissues their regulation is presumed to depend on the relative amounts of ATP, ADP, and AMP, with metabolism poised to maintain a constant "energy charge"[15–17, 19, 20]. Thus if there is an increase in the rate of utilization of ATP or a decrease in its rate of production (for example by inhibition of oxidative phosphorylation) the change in the energy charge would lead to stimulation of glycolysis, primarily by activation of phosphofructokinase. If the thrombin stimulation of glycolysis were a response to a greater utilization of ATP, as opposed to a more direct effect on enzymes or membranes, it should cause a pattern of changes in glycolytic intermediates similar to those observed with any agent that stimulates glycolysis by changing the "energy charge".

This paper describes a study of the stimulation of glycolysis by thrombin using the techniques described above. Comparisons are made with the effects of cyanide, which stimulates glycolysis by inhibiting respiration.

METHODS

Platelets were isolated from rat blood and washed twice as described previously[18]. They were suspended in an incubation solution to give a concentration of $3 \cdot 10^9$–$6 \cdot 10^9$ platelets per ml. The basic incubation solution was essentially a Krebs–Ringer phosphate solution[21] without Ca^{2+} or Mg^{2+} and with 4 mM glucose and 0.2 mM EDTA. Where indicated, glucose and EDTA were omitted. Platelet suspensions were incubated at 37° in silicone-coated glass chambers under a gentle stream of oxygen as previously described[18].

Methods of extraction with $HClO_4$ and measurement of metabolic intermediates have been described[22]. Samples of platelet suspensions were taken with Eppendorf Push-Button automatic pipets. To obtain a uniform sample of aggregated platelets and to permit more rapid sampling, the disposable pipet tips were cut off to form an opening approx. 1 mm in diameter.

Bovine thrombin was obtained from the Upjohn Company, Kalamazoo, Mich., U.S.A.

RESULTS

Evaluation of control mechanisms is facilitated if the changes in metabolic flux are known. The rate of lactate production was used as a measure of the rate of

glycolysis, since it has been shown that under the conditions used glycolysis is predominately of the anaerobic type[18]. The effects of cyanide or thrombin on the rate of lactate production, either with or without glucose and EDTA, are shown in Table I. In the absence of EDTA, platelet aggregation was observed about 1.5 min after addition of thrombin*. There was about a 2-fold increase in glycolysis caused by either cyanide or thrombin under all of the conditions used. These rates are calculated from changes during the first 5 min after addition of cyanide or thrombin and are based on the assumption of an immediate change to the new and constant rate of lactate production. If this assumption is not valid, the actual changes could be somewhat greater, but not less, than shown in Table I.

TABLE I

EFFECTS OF CYANIDE AND THROMBIN ON LACTATE PRODUCTION BY WASHED RAT PLATELETS

Platelet suspensions were incubated as described under METHODS. The rates of lactate production are calculated from samples taken 5 min before the addition, at the time of the addition, and 5 min after the addition of either cyanide or thrombin. NaCN was added at a final concentration of $1 \cdot 10^{-4}$ M and thrombin at 1 unit/ml. The numbers in parentheses are the number of experiments. Units are nmoles/10^{10} platelets per min.

Conditions of incubation		*Addition*	*Lactate production*		
4 mM glucose	*0.2 mM EDTA*		*Before addition*	*After addition*	$\frac{\text{After}}{\text{Before}} \times 100$
+	−	None (5)	195	184	94
+	+	Cyanide (5)	206	433	210
+	+	Thrombin (4)	186	321	173
+	−	Thrombin (5)	201	422	210
−	+	Cyanide (6)	83	191	230
−	+	Thrombin (6)	88	215	245
−	−	Thrombin (4)	104	191	184

In each experiment in Table I, the levels of those glycolytic intermediates useful for studying regulation at phosphorylase and phosphofructokinase and the levels of ATP and ADP were measured in extracts made 0.5, 1.5, and 5 min after addition of cyanide or thrombin and were compared with levels measured in extracts made immediately before the addition**. Levels of AMP, which would be more useful than levels of ADP and ATP for evaluating allosteric regulation by adenine nucleotides, were not measured because the very low levels in platelets require the use of more extract than was available in these experiments.

The data for the experiments where cyanide was added to platelets incubated with glucose are shown in Fig. 1A with a conventional "crossover" plot. The decrease in levels of metabolites before, and the increase in levels of metabolites after, the

* To avoid spontaneous aggregation before thrombin was added, Ca^{2+} and Mg^{2+} were not included in the incubation, but there apparently was enough Ca^{2+} released from the platelets, on addition of thrombin, to promote aggregation.

** The experiments were restricted to the first 5 min because previous investigations indicated that some glycolytic intermediates were slowly released from platelets following addition of thrombin[23]. During the first 5 min, the amounts released were small compared to the intracellular amount, so that measurements of intermediates in the total suspensions gave a good estimate of the amounts actually in the platelets. After 5 min, a difficult correction would be required.

reaction catalyzed by phosphofructokinase clearly indicate that the increased rate of glycolysis was associated with activation of this enzyme. The measurements of glucose 1-phosphate, which is present in low levels in platelets incubated with glucose, were not satisfactory in these experiments and no data are shown for this metabolite. Subsequent experiments (Fig. 5) revealed an increase in glucose 1-phosphate, indicating that phosphorylase also is activated by cyanide under the conditions of these experiments. The changes occur mostly within the first 0.5 min, after which there is little additional change. Fig. 1 B shows that with no addition there is no change during the 5-min period of incubation.

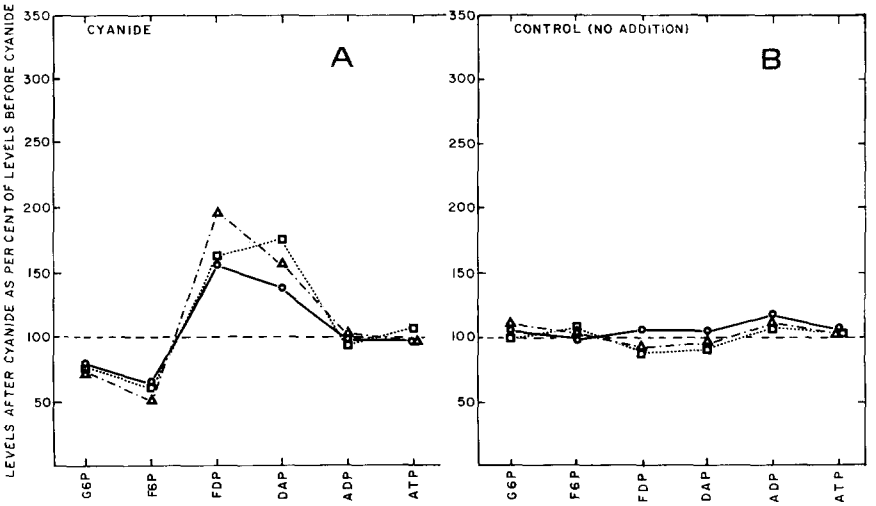

Fig. 1. Effect of cyanide on levels of metabolites in platelets incubated with 4 mM glucose. Data were obtained from extracts made in experiments described in Table I. Non-standard abbreviations are: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-di-phosphate; DAP, dihydroxyacetone phosphate. ○ — ○, 0.5 min after addition; △ – · – △, 1.5 min after addition; □ · · · · · □, 5 min after addition.

When thrombin was added with aggregation inhibited by EDTA (Fig. 2A), the changes in levels of glycolytic intermediates were similar to those found with cyanide, indicating that stimulation of glycolysis by thrombin is also through activation of phosphofructokinase. The initial increase in glucose 1-phosphate indicates activation of glycogen phosphorylase also. When EDTA was omitted to permit platelet aggregation (Fig. 2B), the changes in levels of metabolites before aggregation were similar to those found with EDTA, but aggregation was associated with drastic changes, the most obvious being the very large increases in glucose 6-phosphate and fructose 6-phosphate.

One possible explanation for the observed changes in metabolites after aggregation was an accelerated production of gluocse 6-phosphate from glucose, either through activation of hexokinase or increased penetration of glucose into the platelet. To investigate this possibility, the experiments were repeated without added glucose. Fig. 3 shows that the effects of cyanide on intermediates in platelets without glucose was similar to its effects with glucose (Fig. 1). Addition of thrombin to platelets incubated without glucose (Fig. 4) had effects similar to those observed with glucose,

but with evidence of a more pronounced and prolonged activation of phosphorylase, as would be expected when glycogen is the only source of substrate. Following aggregation (Fig. 4B), glucose 6-phosphate and fructose 6-phosphate increased in the absence of glucose. Although the increase was not as much as when glucose was present, the fact that it increased at all in the absence of glucose eliminates the possibility that the increase after aggregation was due to an increased uptake and phosphorylation of glucose.

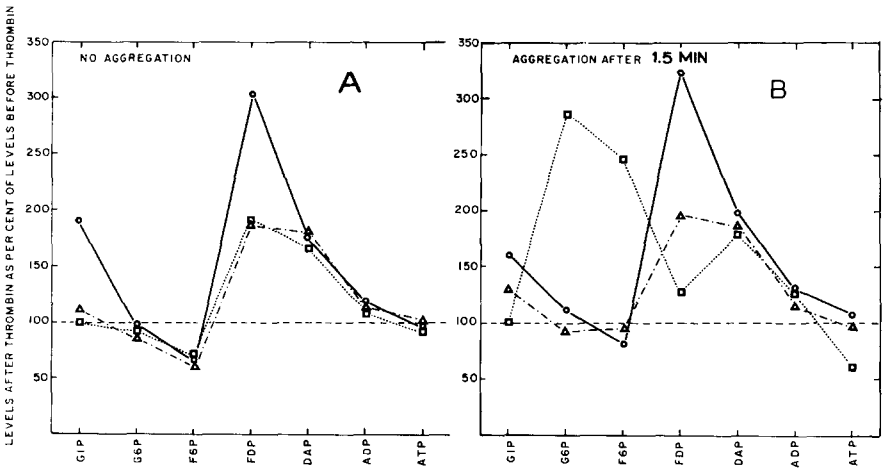

Fig. 2. Effect of thrombin on levels of metabolites in platelets incubated with 4 mM glucose. Data were obtained from extract made in experiments described in Table I. GIP is glucose 1-phosphate. Other abbreviations and symbols are the same as in Fig. 1. A. Incubation solution contained 0.2 mM EDTA. B. Incubation solution contained no EDTA.

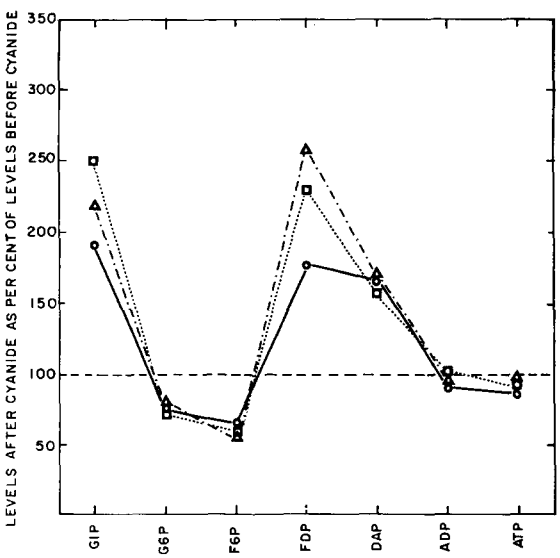

Fig. 3. Effect of cyanide on levels of metabolites in platelets incubated without glucose. The data are from experiments in Table I. Abbreviations and symbols are the same as for Figs. 1 and 2.

Another possible explanation for the changes in levels of metabolites after aggregation was a modification of the intracellular compartmentation of glycolysis so that the measured amount of glucose 6-phosphate would include that in the glycolytic compartment *plus* glucose 6-phosphate that is outside, or unavailable to the glycolytic enzymes. This seemed possible because of the known thrombin-induced release of platelet constituents. In a previous study of the release to the suspending

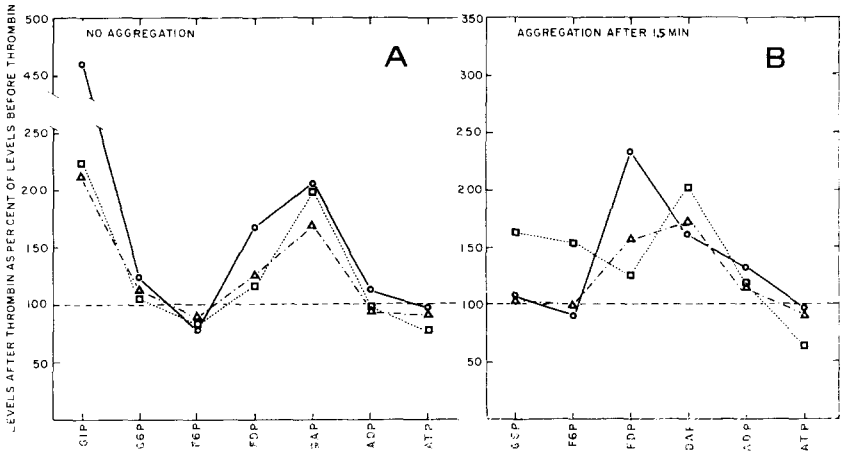

Fig. 4. Effect of thrombin on levels of metabolites in platelets incubated without glucose. The data are from experiments in Table I. Abbreviations and symbols are the same as for Figs. 1 and 2. A. Incubation solution contained 0.2 mM EDTA. B. Incubation solution contained no EDTA.

medium of some platelet glycolytic intermediates[23], slow rates or release were established with platelet suspensions containing EDTA to inhibit aggregation and there was some suggestion of a faster release with aggregation, but the conditions of the experiments did not permit accurate estimation of rates of release without EDTA. The changes in levels of intermediates following aggregation observed here also suggested that the release of these intermediates might be much faster in the absence of EDTA. This was tested by measuring the levels of intermediates in the extracellular phase just before and after aggregation. In Table II, the amounts in the extra-

TABLE II

LEVELS OF METABOLITES IN EXTRACELLULAR PHASE AFTER ADDITION OF THROMBIN WITHOUT EDTA

Platelets were incubated with 4 mM glucose without EDTA as described under METHODS. Thrombin was added to give a final concentration of 1 unit/ml. At the time indicated, samples were transferred to heavy glass tubes in a NaCl–ice–water bath at −7°, mixed, and quickly centrifuged. Perchloric acid extracts were made with the supernatant solutions.

Metabolite	*Extracellular phase*			*Total suspension* *
	Before thrombin	*1 min after thrombin*	*5 min after thrombin*	*5 min after thrombin*
Glucose 6-phosphate	0.1	1.7	19.9	26.6
Fructose 6-phosphate	0	0.7	2.6	6.0
Fructose 1,6-diphosphate	0.4	0.3	0.4	5.6
Dihydroxyacetone phosphate	0.4	0	21.2	10.1
ADP	0	38	58	101
ATP	14	68	66	217

* Data from experiments in Fig. 4A.

cellular phase are compared with the total amounts in the suspension 5 min after addition of thrombin. The release of the glycolytic intermediates measured is slight until after aggregation, when extracellular glucose 6-phosphate, fructose 6-phosphate and dihydroxyacetone phosphate become a major portion of the total in the suspension. The larger amount of dihydroxyacetone phosphate in the supernatant than in the total suspension is due to the fact that these two values represent different experiments and the levels of dihydroxyacetone phosphate vary considerably. In contrast to the glycolytic intermediates, the release of ADP and ATP is almost immediate, as expected[23,24], and is not dependent on aggregation. These data clearly indicate that the large changes in levels of glycolytic intermediates accompanying aggregation are due to rapid leakage into the extracellular phase.

The data in Figs. 2 and 4 indicate that the stimulation of platelet glycolysis by either cyanide or thrombin occurs within the first 30 sec. Therefore, similar experiments were carried out using shorter time periods. Fig. 5 shows the changes

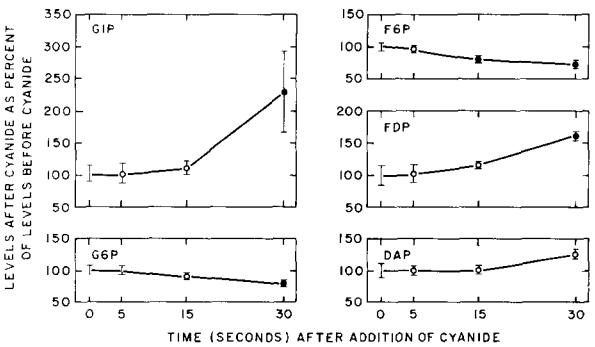

Fig. 5. Effects of cyanide on levels of metabolites in platelets. Experiments were carried out as described under METHODS and in Table I with 4 mM glucose and 0.2 mM EDTA. ○, means of 5–7 experiments with standard errors of means indicated by bars; ●, values significantly different ($P < 0.05$) from values before addition of cyanide. Abbreviations are defined in Figs. 1 and 2.

in levels of metabolites during the first 30 sec after addition of cyanide to platelet suspensions. The changes indicative of activation of phosphorylase (increase in glucose 1-phosphate) and of phosphofructokinase (decrease in glucose 6-phosphate and fructose 6-phosphate and increase in fructose 1,6-diphosphate and dihydroxyacetone phosphate) occur between 15 and 30 sec after addition of cyanide. In contrast, after addition of thrombin (Fig. 6), activation of phosphorylase is apparent within 2.5 sec while activation of phosphofructokinase occurs within 10–15 sec. The times of activation can be seen more clearly by plotting ratios of product to substrate for key reactions (Fig. 7). The ratio glucose 6-phosphate/glucose 1-phosphate, the mass action ratio for phosphoglucomutase, is near the equilibrium constant of 18 in normal platelet metabolism[18,22]. With activation of phosphorylase, the metabolic flux exceeds the capacity of phosphoglucomutase to maintain an equilibrium condition and the ratio glucose 6-phosphate/glucose 1-phosphate decreases[18]. Thus, this ratio is an indication of the flux through the phosphoglucomutase step, a low ratio corresponding to a high flux. The ratio fructose 1,6-diphosphate/fructose 6-phosphate is proportional to the mass action ratio for phosphofructokinase as long as there are

no changes in the ratio ATP/ADP*. Since the mass action ratio of the rate-limiting phosphofructokinase is much less than the equilibrium constant[18], it will increase

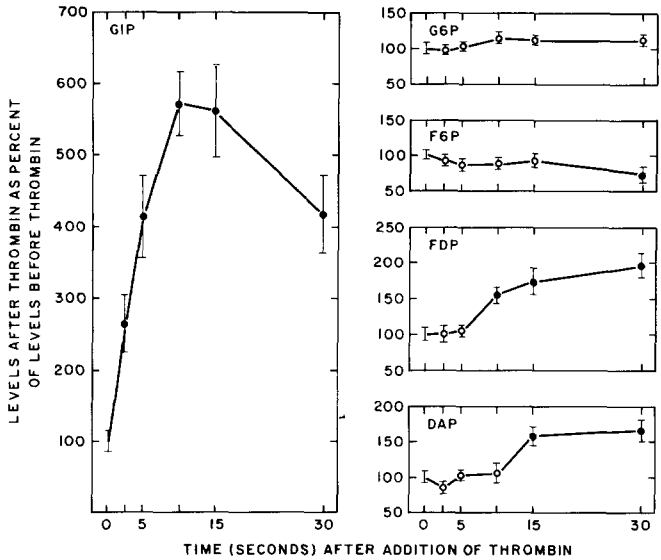

Fig. 6. Effect of thrombin on levels of metabolites in platelets. Experiments were carried out as described under METHODS and in Table I with 4 mM glucose and 0.2 mM EDTA. Symbols are explained in Fig. 5 and abbreviations in Figs. 1 and 2.

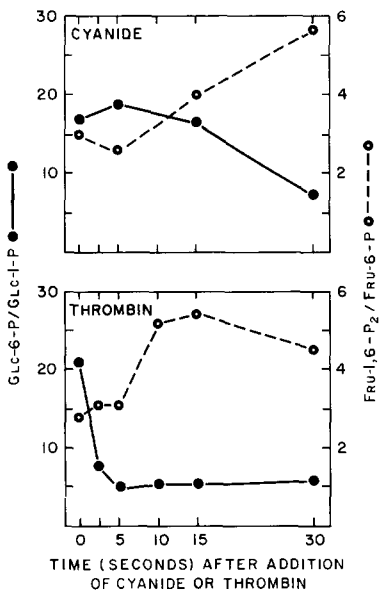

Fig. 7. Ratios of product over substrate for reactions catalyzed by phosphoglucomutase and phosphofructokinase after addition of cyanide or thrombin. The data for these graphs are from experiments described in Figs. 5 and 6.

* The mass action ratio for phosphofructokinase is equal to [fructose 1,6-diphosphate] [ADP]/[fructose 6-phosphate] [ATP].

as the enzyme is activated. There are only slight changes in either ratio during the first 15 sec after addition of cyanide. But with addition of thrombin, the ratio glucose 6-phosphate/glucose 1-phosphate decreases within 2.5 sec and the ratio fructose 1,6-diphosphate/fructose 6-phosphate increases to a maximum by 10 sec. It is thus clear that with thrombin-induced activation of glycolysis, activation of phosphorylase precedes activation of phosphofructokinase, indicating different mechanisms of activation, whereas cyanide-induced activation of these two enzymes occurs simultaneously. The shortest time studied, 2.5 sec, may actually represent even a shorter time period, since it is the time required to transfer a sample from the incubation chamber to $HClO_4$. Thus, thrombin was added to the platelet suspension and a sample taken immediately without time for complete mixing.

DISCUSSION

The data presented here confirm the previous observation[18] that phosphofructokinase is a major regultory enzyme of platelet glycolysis, which is consistent with observations in other tissues[15, 16, 19, 20]. The data also indicate that the effect of thrombin on platelet glycolysis is through activation of phosphofructokinase. Although kinetic data on phosphofructokinase from platelets are not available, it is reasonable to assume that it is regulated by allosteric mechanisms similar to those found for phosphofructokinases from other sources. Thus it is probable that platelet phosphofructokinase activity is regulated by the relative levels of adenine nucleotides, with ATP inhibiting while ADP and AMP activate. The rate of glycolysis is thereby poised by the "energy charge", or the extent to which the adenine nucleotide pool is charged with high energy phosphate. Inhibition of oxidative phosphorylation by cyanide would lead to activation of phosphofructokinase by blocking a major mechanism for producing high energy phosphates, lowering the energy charge.

The observation that thrombin also stimulates glycolysis by activating phosphofructokinase suggests a related mechanism. Thrombin is known to trigger a sequence of platelet processes that utilize ATP. This energy utilization presumably lowers the energy charge, activating phosphofructokinase. The action of thrombin on the glycolytic pathway is therefore not direct but is the result of a change in energy utilization.

Glycogen phosphorylase is also indicated as a primary regulatory enzyme of platelet glycolysis, especially when glycolysis is stimulated by thrombin. The activation of phosphorylase after addition of thrombin is observed within 2.5 sec, thus preceding the observed functional or morphological changes caused by thrombin. It is therefore, very significant for understanding the mechanism of the reaction of thrombin with platelets.

From studies on other tissues, (see refs. 20 and 25 for reviews) as well as on platelets[26–28], it is known that phosphorylase activity can be controlled in two ways: (i) allosteric regulation of phosphorylase b and (ii) conversion of phosphorylase b to phosphorylase a*. Phosphorylase b, normally the predominant form in cells, is

* KARPATKIN AND LANGER[27] reported evidence that platelets may have an additional mechanism for activation of phosphorylase. They ovserved a MgATP-dependent conversion of an inactive monomer to phosphorylase *a*. KARPATKIN *et al.*[42] observed a particulate phosphorylase from platelets that was completely dependent on divalent cations. The physiological significance of these observations is not yet known.

activated by AMP and inhibited by ATP and glucose 6-phosphate .This aspect of its control is therefore similar to the control of phosphofructokinase, consistent with the parallel activation of phosphorylase and phosphofructokinase when glycolysis was stimulated by cyanide. Conversely, the lack of parallel activation of these two enzymes when glycolysis was stimulated by thrombin makes this type of allosteric regulation of phosphorylase *b* unlikely and suggests activation by conversion to phosphorylase *a*, the activity of which is independent of AMP.

The phosphorylase *b* to *a* conversion is a phosphorylation catalyzed by phosphorylase kinase[25]. In muscle, phosphorylase kinase is activated by a cyclic AMP-dependent protein kinase and by Ca^{2+} (refs. 29 and 30).

There are several reasons why release of Ca^{2+} is a more plausible mechanism than production of cyclic AMP for the thrombin-induced activation of phosphorylase. (1) Cyclic AMP is known to be associated with effects on platelets opposite those of thrombin[31–34]. (2) Some of the effects of thrombin on platelets are analogous to processes in other tissues in which Ca^{2+} has been the mediating factor, for example release and contraction (for reviews see refs. 35 and 36). (3) Platelets contain large amounts of calcium, much of which is not exchangeable and therefore probably tightly bound[7,37], and Ca^{2+} binding components have been reported[38,39]. Thrombin is known to cause extracellular release of Ca^{2+} (ref. 7), so that the mechanism for regulation by release of Ca^{2+} seems to be present. (4) The time of thrombin-induced activation of phosphorylase in platelets is more consistent with Ca^{2+} mediated electrical activation of muscle phosphorylase, which occurs within a few seconds, than with cyclic AMP-mediated epinephrine-induced activation of muscle phosphorylase, which requires minutes[40].

GRETTE[2] demonstrated that Ca^{2+} was required for the thrombin-induced platelet release reaction, and separated the process into two steps, a proteolytic step followed by a Ca^{2+} mediated step. The results presented here are consistent with and support such a mechanism. However, it remains to be demonstrated that intracellular Ca^{2+} actually increases after treatment with thrombin and before other metabolic and morphological changes are observed and that Ca^{2+} actually regulates platelet contractile protein and phosphorylase kinase.

Two aspects of the role of thrombin in regulation of platelet energy metabolism have been demonstrated. The first is activation of phosphofructokinase, apparently through stimulation of energy utilizing processes and allosteric control. The second is activation of phosphorylase, possibly through a Ca^{2+}-mediated mechanism. It is unlikely that the effects are restricted to these two enzymes since other enzymes are also subject to allosteric control[15,16,19,20]. Control of hexokinase may also be expected. In experiments with deoxyglucose, it was shown that the capacity of hexokinase in platelets exceeds the rate of glycolysis[41], indicating that it is normally under some form of control, and the rate of glucose consumption by platelets has been shown to increase after addition of thrombin[11]. Changing the "energy charge" would also be expected to change the rate of oxidative phosphorylation, a major source of ATP production in platelets[18], but there have as yet been no confirmed reports of a thrombin-induced stimulation of platelet respiration[13,14].

The apparently specific release to the extracellular medium of some, but not all, glycolytic intermediates continues to be an observation that is perplexing as to both its function and mechanism. The data presented here permit some perspective

regarding its physiological significance. The leakage of large amounts of vital intermediates would seem to be disastrous to a cell. However, if the amount lost is compared not to the steadystate level in the cell but to the metabolic flux, the loss appears to be less. For example, Table II indicates that in 5 min, the loss of glucose 6-phosphate equals about 3 times the amount in the platelet. But from Table I, the flux is 211 nmoles glucose 6-phosphate per min. (422 nmoles lactate per min) or 1055 nmoles glucose 6-phosphate per 5 min, so that the 5-min loss represents less than 2 % of the flux. Therefore in terms of metabolic efficiency, the loss of glycolytic intermediates may be insignificant. The smaller loss in the absence of glucose (compare Figs. 2A and 4A) is probably because the levels of intermediates are less without glucose[18].

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