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REGULATION OF PLATELET PHOSPHORYLASE

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Summary

A sensitive fluorimetric enzyme assay was developed for study of activation of glycogen phosphorylase (EC 2.4.1.1) in intact platelets and in platelet extracts. Activity was calculated as AMP independent (activity in the absence of AMP), total (activity in the presence of 1 mM AMP), and AMP dependent (difference between AMP independent and total).

The following observations were made with intact rat platelets. (1) Stimulation of platelets with thrombin caused a 7-fold increase in total activity, with increases in both AMP-dependent and AMP-independent activities. Maximum activation was obtained within 10 s after addition of thrombin. (2) The divalent cation ionophore A23187 caused a similar, though less pronounced, activation of phosphorylase. (3) Acceleration of glycogenolysis by inhibition of respiration with cyanide caused similar changes in phosphorylase activity but with the maximum effect observed only after 45 s. (4) Dibutyryl cyclic AMP had two effects; it partially activated phosphorylase and blocked further activation by thrombin, but not A23187.

Similar effects were observed with human platelets, but low resting levels of phosphorylase activity could not be maintained so that changes were not as large as with rat platelets.

Experiments with extracts of rat platelets gave the following results. (1) Phosphorylase activity in many extracts of non-stimulated platelets could be increased by incubation with Mg^{2+}-ATP and Ca^{2+}; ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA) partially inhibited. (2) In some extracts there was essentially no activation by incubation with Mg^{2+}-ATP and Ca^{2+}, but addition of cyclic AMP gave partial activation while addition of rabbit muscle phosphorylase kinase gave full activation. (3) Incubation of extracts of thrombin-stimulated platelets caused conversion of AMP-dependent to AMP-independent activity.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

It is concluded that platelet phosphorylase exists in an inactive and two active forms. Conversion of the inactive to the active forms and of the AMP-dependent to the AMP-independent form is catalyzed by a kinase(s) that requires Ca^{2+} for full activity and is activated through a cyclic AMP-mediated process. The major change following physiological stimulation is an increase in both active forms, with little change in their ratio.

Introduction

The response of platelets to various physiological stimuli is the essence of their crucial roles in hemostasis and thrombosis (for reviews see Marcus [1—3] and Mustard and Packham [4]). The stimuli include such diverse agents as thrombin, collagen and ADP; the response involves major changes in morphology, function and metabolism and includes all aspects of platelet physiology and biochemistry. While the responses are known, there is little known about the mechanism of stimulation or of the coupling of the initial stimulus to the observed metabolic and functional changes. One of the quickest metabolic responses observed is the thrombin-induced increase in the level of glucose 1-phosphate (Detwiler [5]) presumably due to activation of glycogen phosphorylase (EC 2.4.1.1). Thus, phosphorylase activation appears to be directly coupled to stimulation, and knowledge of the regulation of platelet phosphorylase would be a substantial step toward establishing the mechanism of stimulus-response coupling.

There is much known about control of phosphorylase in other tissues (for reviews see Fischer et al. [6] and Ryman and Whelan [7]); the regulation involves both allosteric and covalent modulation of enzyme activity. Muscle phosphorylase exists in two forms, a non-phosphorylated species (phosphorylase *b*) that is active only in the presence of AMP and a phosphorylated species (phosphorylase *a*) with activity independent of AMP. Liver phosphorylase is similar except that the non-phosphorylated, *b*, form is only slightly activated by AMP. There are conflicting reports about whether platelet phosphorylase is predominantly of the muscle type (Yunis and Arimura [8]) or the liver type (Proux and Dreyfus [9]) based on electrophoretic mobility. Karpatkin and co-workers [10—12] reported that platelet phosphorylase existed in the AMP-dependent and AMP-independent forms found in muscle, but that it also existed as an inactive species.

Although stimulation of platelets clearly results in increased glycogenolysis (Bettex-Galland and Luscher [13], Corn [12], Warshaw et al. [15], Karpatkin [16], Karpatkin and Langer [17], Detwiler [5]), the expected conversion of phosphorylase *b* to *a* has not been observed (Karpatkin and Langer [18], Deisseroth et al. [19]). This paper describes studies of the activation of phosphorylase by stimulation of platelets with thrombin or the divalent cation ionophore A23187, and by increasing glycolysis with a respiratory inhibitor. A sensitive fluorimetric assay was used to measure AMP-dependent and AMP-independent phosphorylase activity in platelet extracts. The major change after

stimulation was an increase in total activity, a change that could also be observed by incubation of extracts with Mg^{2+}-ATP.

Materials and Methods

Preparation and incubations of platelets. Experiments were with rat and human platelets. Rat platelets were used because they have frequently been more satisfactory for metabolic studies and because they were used in the metabolic studies (Detwiler [5]) that led to the present work. Comparisons were made with human platelets because of the more extensive literature on human platelets. Rat platelets were isolated as previously described (Detwiler and Zivkovic [20]) except that the blood was diluted with 0.5 volumes of 154 mM NaCl to improve yield. The platelets were washed twice in the incubation solution (see below) and were suspended in the incubation solution to give a final concentration of $7 \cdot 10^9$—$10 \cdot 10^9$ platelets/ml. Washed human platelets were prepared as previously described (Detwiler and Feinman [21]) and were suspended in the incubation solution to give a final concentration of $3 \cdot 10^9$—$5 \cdot 10^9$ platelets/ml. The incubation solution was 124 mM NaCl, 5 mM KCl, 16 mM sodium phosphate, and 4 mM glucose with a final pH of 7.4. The suspension was placed in a water-jacketed flask equipped with a stirring bar and maintained at 37°C; a gentle stream of O_2 was blown over the suspension. After equilibration, usually for 3—5 min, a sample was taken for extraction of phosphorylase.

Extraction of phosphorylase. Samples were diluted with three volumes of a cold solution containing 60% glycerol, 5 mM EDTA, 20 mM NaF, 10 mM mercaptoethanol, and 40 mM sodium glycerophosphate (pH 6.8). This diluent was quickly expressed into a tube containing the platelet suspension while mixing on a vortex mixer and the tube was immediately immersed in an ethanol/solid CO_2 bath (—50°C). The mixture was sonicated five times for 15 s in a salt ice bath (—7°C). The microtip of the sonifier was cooled to dry ice temperature before each sonication period. Each sample was then centrifuged at 15 000 × *g* for 10 min and the supernatant solution was recentrifuged after addition of 40 mg/ml of acid washed activated charcoal. The supernatant solution was assayed for phosphorylase the same day. Low temperatures and use of a kinase inhibitor (EDTA) and a phosphatase inhibitor (NaF) were to prevent interconversions of the phosphorylase forms during extraction (Danforth et al. [22]).

As an alternative method for membrane disruption (Doery et al. [23]), a final concentration of 0.5% Triton X-100 was added plus 5 µl octanol to prevent foaming. The suspension was placed in a salt ice bath (—7°C) and was briefly homogenized three times at 10-min intervals with a motor-driven teflon pestle homogenizer. Results of these experiments are not shown here but were similar to those obtained with sonication.

Phosphorylase assay. The assay for phosphorylase was in the direction of glycogen degradation with product measured by enzymatic coupling through glucose 1-phosphate and glucose 6-phosphate to the reduction of NADP by glucose-6-phosphate dehydrogenase. Activity was monitored in the absence and in the presence of 1 mM AMP. This is essentially the assay used by Karpatkin

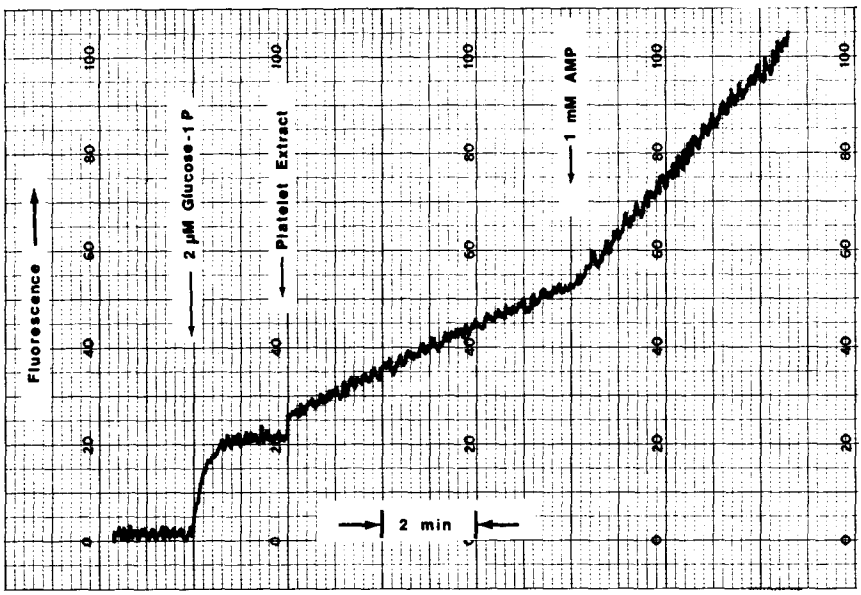


Fig. 1. Fluorimetric assay of phosphorylase. The assay is based on the reaction glycogen → glucose 1-phosphate → glucose 6-phosphate → 6-phosphogluconate + NADPH, with NADPH formation followed fluorimetrically using an Eppendorf fluorimeter with a calibrated suppression attachment (Brinckman Instrument Co., Westbury, N.Y.). Exciting light was the 313 and 366 nm bands of a mercury lamp and emitted light above 420 nm was measured. Reactions were in 1.0 ml cuvettes with a 5-mm light path. The reaction mixture was (final concentrations) 20 mM sodium glycerophosphate (pH 6.8), 3 mM $MgCl_2$, 2 mM potassium phosphate, 16.3 mM glycogen (glucose equivalents), 0.46 mM NADP, 5 μg phosphoglucomutase, 30 μM glucose 1,6-diphosphate, and 5 μg glucose-6-phosphate dehydrogenase. Glucose 1-phosphate was added to calibrate the fluorescence change prior to beginning the assay by addition of the platelet extract, usually 0.1 ml. Activity was calculated as AMP-independent and total activity (after addition of AMP) and, by difference, AMP-dependent activity. Full scale on this chart would correspond to a change in A_{340nm} of about 0.0016. Sensitivity can be further increased, but with some increase in instrument noise.

and Langer [17], but in order to increase sensitivity we measured NADP reduction fluorimetrically instead of spectrophotometrically. Details of the procedure and a typical assay are shown in Fig. 1. For each sample, blank assays were run in the absence of glycogen and phosphate; if there was any reaction, phosphorylase activity was corrected. Phosphorylase activity was calculated as "AMP-independent" activity (from the slope before addition of AMP), as "total" activity (from the slope after addition of AMP), and as "AMP-dependent" activity (the difference between total and AMP-independent). The unit of activity is μmol glucose 1-phosphate/min at 30°C. However, activities are usually expressed as a percent of maximum total activity, which we take as the total activity observed 15 s after addition of thrombin to intact platelets at 37°C (cf. Fig. 2). This method of expressing activity was chosen to eliminate variation in level of activity between platelet preparations; the level of phosphorylase in resting platelets could not be used as a reference since it was very low, sometimes essentially zero, and any error in these low activities would cause a large variation in calculated percent activation.

Materials. Bovine thrombin was purified from Parke-Davis topical throm-

bin by the method of Glover and Shaw [24]. Prostaglandin E_1 was given to us by Dr J. Pike, Upjohn Co., and A23187 was a gift of Dr R. Hosley, Lilly Co. NADP, glucose 1,6-diphosphate, phosphoglucomutase and glucose-6-phosphate dehydrogenase were obtained from the Boehringer Mannheim Co. Cyclic AMP and dibutyryl cyclic AMP were purchased from Sigma. Purified rabbit skeletal muscle phosphorylase kinase was a generous gift from Dr Hubert Blum, Department of Biochemistry, University of Washington.

Results

Rat platelets incubated for 5 min at 37 °C as described in Materials and Methods had a very low level of phosphorylase activity, but this could be substantially increased above the resting level by various treatments of the intact platelets or of the extracted phosphorylase as described below.

Activation in intact platelets

Thrombin. Thrombin is a potent physiological platelet stimulus that induces both functional and metabolic changes. Addition of thrombin to suspensions of washed rat platelets caused a 4—20-fold increase in total phosphorylase activity within 10 s (Fig. 2). From these and other experiments, the average maximum total activity in thrombin-treated platelets was 0.05 unit (±0.01, $n = 18$)/10^{10} platelets.

The increase in both AMP-dependent and AMP-independent activities, giving rise to the increase in total activity, suggests that this system is different from those of muscle (where activation is observed primarily as conversion of AMP-dependent to AMP-independent activity with little change in total activity) or liver (where activation causes an increase in total activity but little AMP-dependent activity is observed under conditions of assay used here.

A23187. The divalent cation ionophore A23187 has been reported to induce secretion, an important functional response of platelets to stimuli, by a mechanism that bypasses several early steps in the overall thrombin-response coupling mechanism (Feinman and Detwiler [25], Friedman and Detwiler [26]). It also causes activation of phosphorylase (Fig. 3), with a pattern and time course similar to those for activation by thrombin.

Cyanide. Platelet glycolysis and glycogenolysis are increased after inhibition of respiration with cyanide (Detwiler [5]), but the pattern of activation is distinct from that due to thrombin (the quick increase in glycogen degradation is not observed with cyanide). The mechanism is presumed to be through adjustment to a change in energy charge. Addition of 10^{-4} M cyanide to suspensions of rat platelets caused increases in both AMP-dependent and AMP-independent phosphorylase activity, with total activity reaching 80% of the thrombin-stimulated activity. However, the change was much slower, with no change for 10 s and full activity only after 40 s.

Cyclic AMP. In many tissues cyclic AMP causes activation of phosphorylase through activation of protein kinase and phosphorylase kinase, but this is a questionable mechanism in platelets since cyclic AMP is known to inhibit many platelet functions (Salzman [27], Mills [28]). To ascertain its effects on phosphorylase activity in platelets, intracellular cyclic AMP was elevated by addi-

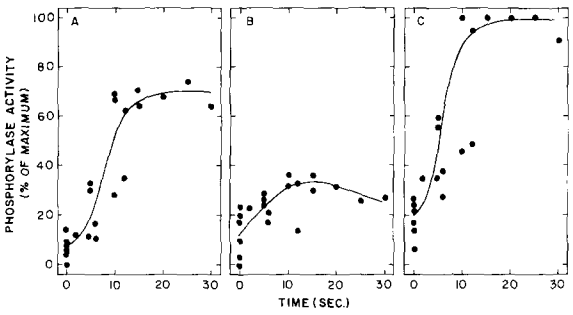


Fig. 2. Thrombin-induced activation of phosphorylase in intact platelets. Suspensions of rat platelets were incubated as described in Materials and Methods with aliquots taken for extraction of phosphorylase before (t = 0) and at various times after addition of from 3 to 6 units thrombin/ml. All activities are expressed as percent of maximum total activity for each experiment. (A) Shows AMP-independent activity. (C) Total activity (with 1 mM AMP), (B) AMP-dependent activity (the difference between C and A).

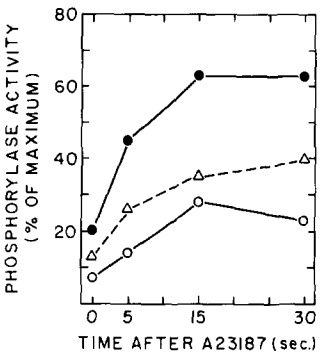


Fig. 3. A23187-induced activation of phosphorylase in intact platelets. The experiment was as described for Fig. 2 but with stimulation by A23187 instead of thrombin. A solution of A23187 in the incubation buffer was prepared by dilution of a stock solution of A23187 in ethanol just prior to use. This was necessary because ethanol was found to interfere. The final concentration of A23187 in the suspension was calculated to be 1 μM, but because of its low solubility in water, the actual concentration may have been substantially less. All activities are expressed as percent of maximum total activity, defined as the total activity in a 15-s thrombin sample. ●———●, total activity (with 1 mM AMP); △- - - - -△, AMP-independent activity; ○———○, AMP-dependent activity (the difference between total and AMP independent).

tion of dibutyryl cyclic AMP or prostaglandin E_1 (an adenyl cyclase activator) either alone or together with theophylline (a phosphodiesterase inhibitor). The effects of dibutyryl cyclic AMP on phosphorylase activity in intact platelets are shown in Table I. Two effects are observed; phosphorylase is partially activated (compare "control" values in Table I) but activation by thrombin is inhibited (compare "thrombin" with "control" for each experimental conditions). Cyclic AMP does not inhibit stimulation by A23187 but actually enhances its effect. Similar results were obtained with prostaglandin E_1 or prostaglandin E_1 plus theophylline and with human platelets.

Activation in platelet extracts

Phosphorylase activity in extracts of rat platelets could be increased by incubation with Mg^{2+}-ATP and Ca^{2+}, but the extent of activation varied greatly, from essentially no activation to nearly maximum activity. Examples of each of these cases are shown in Table II. Activation always required incubation with Mg^{2+}-ATP and was partially, but never completely, blocked by ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) (Table II, Expt. 1). In extracts in which little activation by incubation with Mg^{2+}-ATP and Ca^{2+} was observed (e.g. Table II, Expt. 2), complete activation could be obtained by addition of purified rabbit muscle phosphorylase kinase or partial activation by including cyclic AMP. It thus appears that the required kinase was extracted, but in an inactive form that could be activated by cyclic AMP. Incubation of extracts of thrombin-treated platelets, which already have maximum total activity, caused a conversion of AMP-dependent to AMP-independent activity

TABLE I

THE EFFECT OF DIBUTYRYL CYCLIC AMP ON PLATELET PHOSPHORYLASE ACTIVITY

Suspensions of rat platelets were incubated for 5 min as described in Materials and Methods with 5 mM dibutyryl cyclic AMP and 250 μM theophylline added as indicated. Samples were transferred to tubes containing 0.025 volume thrombin or A23187 in the incubation solution or incubation solution alone (control), and 15 s later phosphorylase was extracted. Final concentrations were 4 units/ml thrombin and 2 μM A23187.

Additions	Stimulus	Phosphorylase activity (percent of maximum)	
		AMP independent	Total
None	Control	5	13
	Thrombin	66	100
	A23187	26	60
Dibutyryl cyclic AMP	Control	5	16
	Thrombin	24	68
	A23187	39	76
Dibutyryl cyclic AMP + theophylline	Control	17	56
	Thrombin	20	58
	A23187	59	115

(Table II, Exp. 3). With added phosphorylase kinase, essentially all activity is AMP independent. Thus, the AMP-independent activity must be a manifestation of a distinct form of phosphorylase that is not appreciably stimulated by AMP; conversely, the AMP-dependent activity must represent another form of phosphorylase. The major implications of Table II are that (i) platelet phosphorylase can be activated by muscle phosphorylase kinase, (ii) platelets have a phosphorylase kinase that is dependent on Ca^{2+} for maximum activity, (iii) the platelet enzyme system that activates phosphorylase can be activated by a cyclic AMP-dependent process, and (iv) essentially all of the total phosphorylase activity can be converted to an AMP-independent form.

Human platelets

Experiments were done with human platelets exactly as with rat platelets. After 5 min incubation at 37°C followed by 15 s thrombin treatment, the mean total activity from 20 separate experiments was 0.09 unit (±0.02)/10^{10} platelets; 53% (±12) of the activity was AMP independent. Because of the larger size of human platelets, this is approximately the same activity per gram as in rat platelets. In control platelets (before thrombin), the total activity was 74% (±10) and the AMP-independent activity was 20% (±7) of this maximum total activity. It thus appeared that the activation was similar to that in rat platelets, but with a very high control, or resting, activity. Many efforts were therefore made to obtain lower resting levels of phosphorylase activity; platelets were isolated without cooling and/or without washing; they were kept in plasma; and they were incubated at different temperatures for various times with or without oxygen. The only condition that appreciably changed any activity was extraction without prior incubation. In two experiments one with platelets in buffer and one with unwashed platelets in plasma, prior to any incubation phosphorylase activities (as percent of maximum total activity)

TABLE II

ACTIVATION OF PHOSPHORYLASE IN PLATELET EXTRACTS

Extracts from either control or 15 s thrombin-treated platelets were prepared as described in Materials and Methods. The extracts were incubated with various additions at 37° C for 30 min (except as indicated) before assaying phosphorylase activity. Concentrations added were: 10 mM, Mg^{2+}-ATP; 1.5 mM, $CaCl_2$; 2 mM, EGTA; and 0.1 mM, cyclic AMP. Maximum activity is total activity in a 15-s thrombin extract.

Additions to extract	Phosphorylase activity (percent of maximum)		
	AMP independent	Total	AMP independent/Total
Expt 1, control extract			
None (no incubation)	5	19	0.26
None	3	12	0.25
Mg^{2+}-ATP, Ca^{2+} (no incubation)	5	19	0.26
Mg^{2+}-ATP, Ca^{2+}	33	54	0.61
Mg^{2+}-ATP, EGTA	16	35	0.46
Expt 2, control extract			
None (no incubation)	6	24	0.25
Mg^{2+}-ATP, Ca^{2+}	3	26	0.12
Mg^{2+}-ATP, Ca^{2+}, phosphorylase kinase	77	105	0.73
Mg^{2+}-ATP, Ca^{2+}, cyclic AMP	14	49	0.29
Mg^{2+}-ATP, Ca^{2+}, cyclic AMP (no incubation)	5	21	0.23
Expt 3, "thrombin" extract			
None (no incubation)	51	100	0.51
Mg^{2+}-ATP, Ca^{2+}	62	89	0.70
Mg^{2+}-ATP, Ca^{2+}, phosphorylase kinase	76	85	0.89

were 3 and 15% AMP independent and 55 and 52% total. After 3 min incubation, AMP-independent activities had increased to 12 and 30% and total activity to 88 and 83%. It is thus clear that the conditions of incubation were not satisfactory for maintenance of a resting state in human platelets, but that the pattern of activation was the same as with rat platelets, with increases in both AMP-dependent and AMP-independent activities.

The effects of thrombin, A23187 and dibutyryl cyclic AMP were essentially the same for human platelets as those described for rat platelets.

Discussion

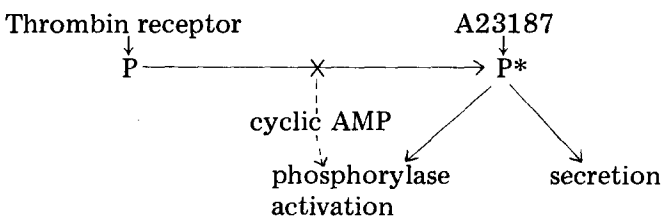
The activation of phosphorylase associated with stimulation of rat platelets is characterized by increases in both AMP-dependent and AMP-independent activities. The data suggests that platelet phosphorylase exists in three forms, an inactive form and two active forms, one AMP dependent and one AMP independent*. The evidence for the inactive form is the very low activity in

* The inactive and AMP-independent species might be considered analogous to liver type phosphorylases *b* and *a*, and AMP-dependent activity is a characteristic of muscle type phosphorylase *b*. We, therefore, considered the possibility that platelet phosphorylase is simply a mixture of liver and muscle types, but this would not explain our data since there is no combination of these two well-known phosphorylases that on activation could show such a large increase in AMP-dependent activity and yet have the potential to exist completely in an AMP-independent form.

unstimulated platelets; the evidence for two separate active forms is the observation of essentially all activity as either AMP dependent or AMP independent (Table II)*. Cowgill and Cori [29] observed a similar system in lobster muscle and Karpatkin and Langer [10] previously reported the presence in platelet extracts of an inactive phosphorylase that could be converted to either an AMP-dependent or an AMP-independent form. From 18 different preparations of rat platelets, the mean ratio of these three forms for resting (non-stimulated) platelets was (inactive:dependent:independent) 84 : 12 : 4 (range, 75 : 17 : 8 to 94 : 5 : 1) while 15 s after thrombin it was 0 : 42 : 58 (range, 0 : 63 : 37 to 0 : 26 : 74) Thus stimulation caused a 15-fold increase in the independent activity, a 3.5-fold increase in the dependent activity and a 6-fold increase in total activity. However, activity of the independent form as a percent of total activity, the parameter usually reported, only increased from 25% in resting platelets to 58% in stimulated platelets.

The activation of platelet phosphorylase is apparently catalyzed by a kinase(s). This was demonstrated by incubation of platelet extracts with Mg^{2+}-ATP and Ca^{2+} (Table II), resulting in conversion of the inactive to the active forms (Expt. 1) or of the dependent to independent form (Expt. 3), and by incubation with purified phosphorylase kinase. The platelet kinase(s) that catalyzes the conversions requires Ca^{2+} for maximum activity and can be extracted in either an active (Table II, Expts 1 and 3) or an inactive (Table II, Expt. 2), form. Partial activation by cyclic AMP was observed in either intact cells (Table I) or extracts (Table II, Expt. 2).

One major goal of this work was to compare the stimulus-response coupling mechanism for a metabolic response with that for a functional response. The experimental approach and interpretation are best understood in the context of a previous scheme for stimulus-secretion coupling (Friedman and Detwiler [26]). This involves an intermediate "activated" state, P*, that preceeds secretion; it can be obtained by thrombin through a cyclic AMP-blocked process or by A23187 through a mechanism that bypasses the thrombin, cyclic AMP-inhibited steps (reaction 1). This same intermediate state is apparently involved in phosphorylase activation, which also follows a cyclic AMP-inhibited thrombin process or a cyclic AMP-independent A23187 process. Thus, as shown in reaction 1, stimulus-response coupling involves a common intermediate "activated" state for either a functional response (secretion) or a meta-



* It is known that phosphorylases may exist in different forms with different AMP dependence. For example: muscle phosphorylase *a* is AMP dependent when assayed with very low substrate (Lowry et al. [33]), 0.1 M $NaClO_4$ (Sealock and Graves [34]) or 3.0 M NaCl (Wang and Graves [35]); and liver phosphorylase *b* is normally inactive, but in 0.7 M Na_2SO_4 it is converted to a form that is partially activated by AMP (Appleman et al. [36]). But the crucial point here is the difference in AMP dependence observed under identical assay conditions.

bolic response (phosphorylase activation). In the context of reaction 1, there need be no contradiction in the observation that cyclic AMP blocks thrombin activation of phosphorylase while itself causing activation. It has been suggested, but not proved, that the activated state represents an intracellular Ca^{2+} flux (for discussion see Detwiler et al. [30]), and it is possible, but not necessary, that direct activation of phosphorylase kinase by Ca^{2+} is the mechanism for activation of phosphorylase. Partial inhibition of the kinase by EGTA was, in fact, observed (Table II) and Gear and Schneider [31] have recently reported that platelet phosphorylase kinase is stimulated by micromolar concentrations of Ca^{2+}.

The previous failure to observe activation of platelet phosphorylase under conditions where glycogenolysis is known to be accelerated (Karpatkin and Langer [18], Deisseroth et al. [19]) has been a perplexing inconsistency in knowledge of platelet energy metabolism. Karpatkin and Langer [11] suggested that this might be because of predominantly allosteric instead of covalent regulation of platelet phosphorylase. The results here demonstrate that there is substantial covalent regulation and suggest two reasons why this has not been previously observed. First, activation of platelet phosphorylase is in large part observed as an increase in total activity, a change that would be missed if only the fraction of total activity that is AMP independent is recorded. Second, it is very difficult to maintain a low resting level of phosphorylase activity in human platelets, the most widely studied species. In this regard, our results are consistent with those of Scott [32], who found that both AMP-independent and total activity in human platelets increased with any manipulations, and of Gear and Schneider [31], who found it necessary to suspend human platelets in a medium containing imidazole (to activate phosphodiesterase) to obtain low AMP-independent activity.

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