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ALTERATIONS IN ^{32}P-LABELLED INTERMEDIATES DURING FLUX ACTIVATION OF HUMAN PLATELET GLYCOLYSIS

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Using a newly developed isotopic tracer technique for the measurement of ^{32}P-labelled intermediates in glycolysis and nucleotide metabolism in platelets, we studied the variations in ^{32}P-labelled intermediates during activation of the glycolytic flux by cyanide and platelet-activating agents. The major variations occurred in [^{32}P]Fru-1,6-P_2, dihydroxy acetone phosphate, ATP and P_i. There was a quantitative covariance between the increase in lactate production and the rise in [^{32}P]Fru-1,6-P_2 induced by different platelet-activating agents. In contrast, cyanide induced weaker activation of the flux and greater accumulation of [^{32}P]Fru-1,6-P_2. Variations in ^{32}P-labelled intermediates were apparent 5 s after flux activation, but the major changes in [^{32}P]Fru-1,6-P_2 occurred much later and fell in periods in which a constant lactate formation was maintained. The cyanide-induced changes in ^{32}P-labelled intermediates depended on the extracellular level of glucose, showing a predominant ATP → P_i conversion in glucose-depleted medium that shifted to an ATP → Fru-1,6-P_2 conversion at excess glucose. At about 50 μM glucose, flux activation occurred without major changes in [^{32}P]Fru-1,6-P_2, dihydroxy acetone phosphate and P_i, with only a small fall in [^{32}P]ATP. The data provide evidence for a role of the aldolase reaction in flux control and demonstrate rapid changes in Fru-1,6-P_2 and ATP during flux activation with an additional role for Fru-1,6-P_2 as an energy buffer during post-activation periods.

Introduction

Stimulation of platelets by a variety of physiological agents causes changes in the shape of the cells, liberation of arachidonate from membrane phospholipids, secretion of granule-stored components and aggregation of the platelets [1–3]. Each of these processes is dependent on metabolic energy, and the increase in aerobic and anaerobic ATP regeneration seen during platelet stimulation, is generally considered as a compensation for an increased demand for ATP [2].

So far, it has been difficult to establish the mechanism by which ATP production is accelerated. Secretion of granular constituents from platelets is paralleled by a fall in the cytoplasmic (i.e., metabolic) ATP level which, by analogy with the situation in many other cells, could increase the activity of phosphofructokinase, a major glycolytic control enzyme. Purified platelet phosphofructokinase is indeed strongly inhibited by ATP, and the levels of its reactants in the intact platelet indicate that this enzyme catalyzes a non-equilibrium reaction [4–6], which is often seen in a control step. Stimulation of ATP-supplying processes may also result from an increase of the cytosolic Ca^{2+} concentration, which is thought to

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mediate aggregation and secretion [7]. Ca^{2+} could activate glycogen phosphorylase and thereby accelerate glycogenolytic flux, a suggestion consistent with the increase in the Glc-1-*P* concentration seen after platelet stimulation [8].

Recently we have employed the acidification by platelets of a poorly buffered suspension medium as a means for continuous registration of lactate formation [9]. Those studies showed that flux activation by platelet activating agents occurred in a matter of seconds and was followed by periods of at least 20 min with a constant lactate production. These observations called for a reappraisal of earlier attempts to elucidate flux controlling mechanisms from variations in the concentrations of glycolytic intermediates [4,6]. Those studies were based on spectro-fluorimetric techniques, which lacked sufficient sensitivity to be applied to the initial phase of flux activation.

In the present study we have investigated the role of glycolytic intermediates during flux activation using a recently developed isotopic tracer technique [10], which, apart from a high sensitivity, has the additional advantage of detecting exchange of energy-rich phosphate groups between phosphorylated carbohydrates and adenine nucleotides. This technique therefore better takes into account the balance between ATP production and utilization, which appears of crucial importance for the variations in glycolytic intermediates during flux activation.

Materials and Methods

Preparation of [^{32}P]P_i-labelled platelets

Platelet-rich plasma prepared from citrate-containing human blood [11] was incubated with 0.5 mCi $H_3{}^{32}PO_4$ (New England Nuclear, carrier free) per ml for 2 h at 22°C. The platelets were then transferred by gel filtration into Ca^{2+}-free Tyrode's solution containing 0.2% bovine serum albumin (Sigma) without added P_i and with or without 5 mM glucose (referred to as 'glucose-rich' and 'glucose-free' medium, respectively). In the Tyrode's solution, $MgCl_2$ was replaced by $SrCl_2$ to enhance the yield of the gel filtration step, but immediately after collection of the gel-filtered platelets, $MgCl_2$ was added to a final concentration of 2 mM. Final platelet concentrations were in the range (1.5–3.0) $\cdot 10^8$ cells/ml.

Incubation studies

The platelet suspensions were stirred at 900 r.p.m. in capped polystyrene tubes at 37°C after allowing the suspensions to warm up for 3 min. At different times, samples were collected and immediately mixed with equal volumes of freshly prepared 7.7 mM EDTA in 82% ethanol (0°C). These extracts were used for measurement of the radioactivity of ethanol-soluble [^{32}P]P_i-labelled metabolites with a newly developed technique described elsewhere [10]. In short, [^{32}P]P_i-labelled metabolites were separated by two-dimensional paper chromatography. The spots were detected by radioautography, and the radioactivity of each spot was counted according to standard procedures. Spot identification was based on cochromatography of known phosphate esters and nucleotides as well as by enzymatic identification [10]. The [^{32}P]Fru-1,6-P_2 spot, in particular, could be degraded almost completely by incubating platelet extract with aldolase, indicating that it was not contaminated with [^{32}P]Fru-2,6-P_2, which does not react with aldolase [12]. Measurement of the specific radioactivity of the various spots confirmed that the ^{32}P measurement was a true reflection of the quantities of the various fractions [10]. Spectrophotometric analysis of Fru-2,6-P_2 was carried out according to the method of van Schaftingen et al. [13] in alkaline extracts of gel-filtered platelets as well as in extracts of concentrated platelet suspensions obtained by repeated centrifugation-resuspension in EDTA-containing Ringer solution [14].

Lactate was measured as previously described [15]; the coefficient of variation for single determinations was 4% ($n = 10$). Since in platelets only about 3% of the glucose and glycogen derivatives enter the citric acid cycle, lactate formation is a good estimation of the overall glycolytic flux [16].

Variations in glycolytic flux were induced with 1 mM CN^- or by activating the platelet with bovine thrombin (topical, Parke-Davis), collagen [17], epinephrine (Sigma), ADP (Boehringer) or the divalent cationophore A23187 (Eli Lilly). When ADP or epinephrine was used, 0.1 volume of au-

tologous platelet-poor plasma was added 30 s prior to addition of the platelet-activating agent as a source of fibrinogen, a cofactor for these types of aggregation. Control studies performed at 37°C in an aggregometer (Payton) revealed normal aggregation patterns of ^{32}P-labelled platelets upon addition of these agents.

Expressions

Since the total ^{32}P radioactivity per volume of platelet extract was constant throughout each experiment, the radioactivity of each intermediate was expressed as a percentage of total ^{32}P radioactivity. Between suspensions, the total ^{32}P radioactivity varied between 50 and $150 \cdot 10^3$ cpm/chromatogram.

Results

Activation of glycolytic flux by platelet-activating agents and CN^-

Platelets suspended in a glucose-rich medium had a basal glycolytic flux, measured as the rate of lactate formation, of 2.59 ± 0.08 μmol/min per 10^{11} cells (mean ± S.E., $n = 11$). In the presence of platelet-activating agents, the flux increased but the degree of stimulation differed with the type of activator added, with thrombin (5 U/ml) showing the strongest and ADP the weakest effect (Table I). The flux was also activated by CN^-, an inhibitor of mitochondrial ATP-resynthesis, which induced only a modest acceleration in lactate production (Pasteur effect). In the absence of extracellular glucose, a much lower flux was maintained by unstimulated platelets (1.01 ± 0.01 μmol lactate/min per 10^{11} cells ($n = 11$). Again, the flux was increased by the platelet-activating agents and by CN^-, and although the degree of stimulation was slightly higher than in the presence of glucose, the relative efficiency of each activator seemed to be the same. Under all conditions, the increase in lactate production was complete within the first 4 min after flux activation and was followed by periods of at least 26 min in which a constant lactate production was maintained (data not shown).

TABLE I

ACTIVATION OF LACTATE PRODUCTION BY CN^- AND PLATELET-ACTIVATING AGENTS

The velocity of lactate formation in platelet suspensions was measured in the presence and absence of 5 mM glucose. Data are expressed as percentage of controls (no stimulators added) and are calculated from duplicate lactate measurements at 4-min intervals over a 30-min incubation period at 37°C (means of five experiments ± S.E.). Control rates: 2.59 and 1.01 μmol/min per 10^{11} cells with and without glucose, respectively.

	5 mM glucose	No glucose
Thrombin (5 U/ml)	225 ± 20	305 ± 25
A23187 (4 μM)	185 ± 20	220 ± 20
Collagen (100 μg/ml)	170 ± 15	215 ± 15
Adrenalin (5 μM)	155 ± 15	215 ± 15
ADP (10 μM)	150 ± 10	190 ± 20
CN^- (1 mM)	140 ± 5	170 ± 15

The effect of platelet-activating agents on the levels of ^{32}P-labelled intermediates

Platelet activation also affected the levels of ^{32}P-labelled intermediates. The main variations were found in [^{32}P]Fru-1,6-P_2, dihydroxyacetone phosphate, ATP and P_i. [^{32}P]UTP + GTP accounted for about 10% of total radioactivity and decreased with not more than 3% in the presence of platelet-activating agents. [^{32}P]Glc-6-*P*, Fru-6-*P* and ADP did not change. In platelets suspended in glucose-rich medium, maximal variations were found between 1 and 3 min after stimulation with 5 U/ml thrombin (see below). An incubation time of 2 min was therefore chosen in order to compare the early events produced by the various platelet-activating agents (Table II). All activators tested induced an accumulation of [^{32}P]Fru-1,6-P_2, although to different extents. Thrombin (5 U/ml) induced the greatest accumulation, whereas adrenalin induced only a minor increase. This pattern was not parallelled by the variations induced in dihydroxyacetone [^{32}P]phosphate, where the levels decreased upon addition of platelet-activating agents. All activators except adrenalin induced a considerable [^{32}P]ATP → [^{32}P]P_i conversion, with the divalent cationophore A23187 showing the greatest effect.

In glucose-free medium, unstimulated platelets showed a steady decline in [^{32}P]Fru-1,6-P_2 and dihydroxy acetone phosphate and a pronounced hydrolysis of [^{32}P]ATP. Addition of platelet activators did not result in aggregation and secre-

TABLE II

VARIATIONS IN [^{32}P]-LABELLED Fru-1,6-P_2, DIHYDROXY ACETONE PHOSPHATE, ATP AND P_i INDUCED BY PLATELET-ACTIVATING AGENTS

^{32}P-labelled intermediates (expressed as percentage of total ^{32}P radioactivity) at 2 min after stimulation with 5 U/ml thrombin, 100 μg/ml collagen, 4 μM ionophore A23187, 10 μM ADP and 5 μM adrenalin (means of three experiments ± SE). The platelet suspension contained 5 mM glucose. The sum of the radioactivity in the four intermediatees varied between 74 and 78% of the total ^{32}P radioactivity. DHAP, dihydroxyacetone phosphate.

Addition	Fru-1,6-P_2	DHA-P	ATP	P_i
None	1.8 ± 0.3	1.7 ± 0.3	47.5 ± 1.5	26.0 ± 0.5
Thrombin	6.4 ± 1.1	0.9 ± 0.3	35.5 ± 2.0	32.5 ± 3.0
Collagen	3.9 ± 0.3	1.5 ± 0.2	30.5 ± 0.5	38.5 ± 1.5
A 23187	3.5 ± 0.2	0.8 ± 0.1	26.0 ± 1.0	45.0 ± 2.0
ADP	2.6 ± 0.2	0.8 ± 0.2	34.5 ± 1.0	38.5 ± 1.0
Adrenalin	2.1 ± 0.2	1.0 ± 0.1	49.0 ± 1.0	27.0 ± 2.0

tion, which accords with earlier findings [18]. In these platelets, the levels of ^{32}P-labelled intermediates were similar as those found in unstimulated cells (data not shown).

Time course of flux activation by thrombin and CN^- in the presence of glucose

The time course of flux activation induced by 5 U/ml thrombin, a strong activator of lactate production, was compared with the effect of 1 mM CN^-, which only weakly increased the flux (Table I). Both agents induced a transient fall in [^{32}P]Fru-1,6-P_2 during the first 10 s, which was followed by a rapid increase to more than twice the levels found in resting platelets (Fig. 1). Within 5 min after the addition of thrombin, [^{32}P]Fru-1,6-P_2 had decreased to control values, which contrasted with the effect of CN^-, where this intermediate remained high throughout this incubation period. Following a slight increase, dihydroxyacetone [^{32}P]phosphate decreased upon addition of thrombin. It returned to normal values after about 5 min. This contrasted with the variations induced by CN^-, where dihydroxyacetone [^{32}P]phosphate increased rapidly and remained high during the 5 min incubation period. Thrombin and CN^- did not differ in their effect on [^{32}P]ATP and P_i, where both induced a rapid but limited ATP → P_i conversion during the first 30 s.

Variations in ^{32}P-labelled intermediates induced by CN^-

Fig. 1 had shown that the CN^--induced variations in ^{32}P-intermediates lasted much longer than those induced by thrombin. A long-term study of the effect of cyanide in glucose-rich medium showed that it took about 30 min before the level of [^{32}P]Fru-1,6-P_2 had normalized (Fig. 2). This pattern was not paralleled by dihydroxyacetone [^{32}P]phosphate, which accumulated throughout the 30 min of incubation. When glucose was omitted

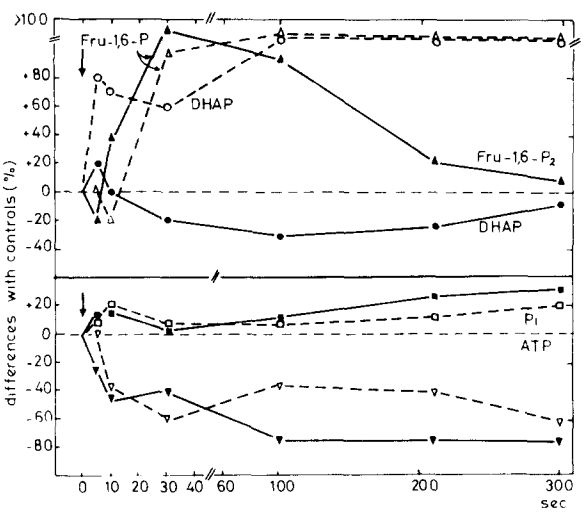

Fig. 1. Variations in ^{32}P-labelled intermediates induced by thrombin and CN^-. ^{32}P-labelled intermediates (differences with unstimulated platelets) induced by 5 U/ml thrombin (closed symbols) and 1 mM CN^- (open symbols). The symbols refer to Fru-1,6-P_2 (▲, △), dihydroxyacetone phosphate (●, ○), ATP (▼, ▽ and P_i (■, □) in platelets in the presence of 5 mM glucose. The addition of thrombin and CN^- is indicated by the arrow. Representative example of three similar experiments.

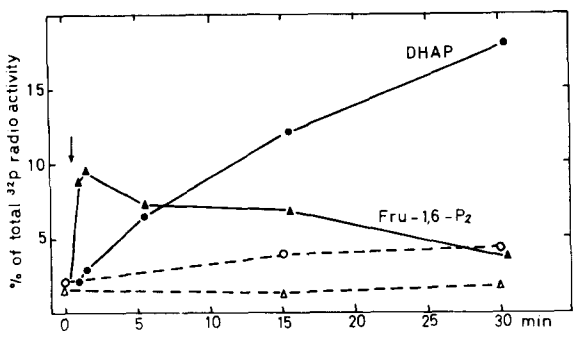

Fig. 2. Variations in ^{32}P-labelled intermediates induced by CN^- in the presence of glucose. ^{32}P-labelled Fru-1,6-P_2 (▲, △) and dihydroxyacetone phosphate (●, ○) expressed as percentage of total ^{32}P radioactivity in the presence (closed symbols) and absence (open symbols) of 1 mM CN^-. The platelets were incubated in the presence of 5 mM glucose, and CN^- was added as indicated by the arrow. Representative example of four similar experiments.

from the medium, CN^- still induced a 1.5-fold increase in lactate production (Table I), but the changes in ^{32}P-intermediates were quite different (Fig. 3). After a slight increase [^{32}P]Fru-1,6-P_2 fell to almost zero. Dihydroxyacetone [^{32}P]phosphate increased again, although to much lower ranges than with glucose present and stabilized after about 5 min.

Influence of extracellular glucose on CN^--induced variations in ^{32}P-labelled intermediates

The observations with and without added glucose made it clear that flux activation by CN^- was

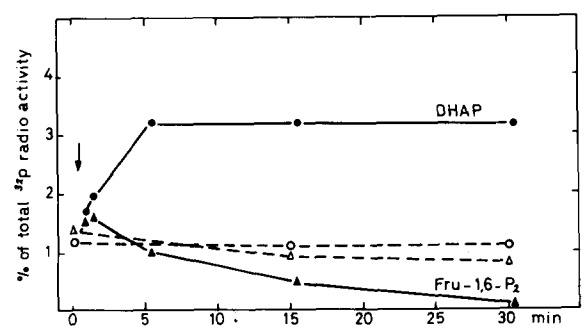

Fig. 3. Variations in ^{32}P-labelled intermediates induced by CN^- in the absence of glucose. Further details as in Fig. 2.

accompanied by changes in ^{32}P-intermediates that depended on the concentration of glucose in the medium, which is consistent with the very low level of intracellular glucose in platelets [16]. A detailed study (Fig. 4) showed that within 3 min after CN^- addition [^{32}P]Fru-1,6-P_2 decreased at low and increased at high glucose concentration. Dihydroxyacetone [^{32}P]phosphate increased with and without glucose but its greatest accumulation was found in the presence of 250–1000 μM glucose. [^{32}P]ATP decreased strongly in glucose-free medium, but fell only slightly in the presence of

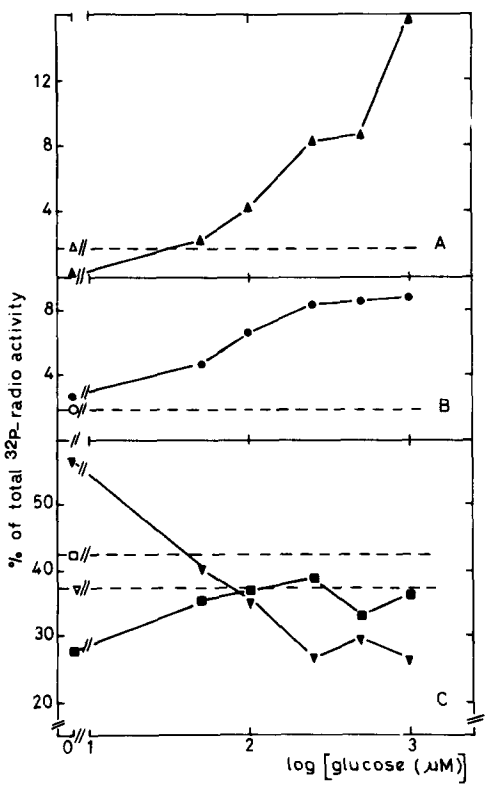

Fig. 4. Influence of extracellular glucose on CN^--induced variations in ^{32}P-labelled intermediates. ^{32}P-labelled Fru-1,6-P_2 (▲, △), dihydroxyacetone phosphate (●, ○), P_i (▼, ▽) and ATP (■, □), expressed as percentage of total radioactivity. Platelets were incubated in glucose-free medium. The intermediates were analyzed (open symbols) and 3 s later, different amounts of glucose were added. CN^- (1 mM) was added 30 s after glucose, and its effect on ^{32}P-labelled intermediates was measured 3 min later (closed symbols). Glucose addition alone did not change these ^{32}P-labelled intermediates during the short period of incubation. Representative example of five similar experiments.

250 μM glucose or more. [^{32}P]P_i accumulated at low but decreased at high levels of extracellular glucose. At all glucose concentrations tested, CN^- did not change the ^{32}P radioactivity in ADP, Glc-6-*P* and Fru-6-*P*, whereas the changes in [^{32}P]UTP + GTP paralleled [^{32}P]ATP but to a much smaller extent. It follows from Fig. 4 that flux activation by CN^- at about 40–100 μM glucose is not accompanied by significant changes in [^{32}P]Fru-1,6-P_2, dihydroxy acetone phosphate and P_i with only a minor decrease in [^{32}P]ATP.

Fructose 2,6-diphosphate present in human platelets?

In order to investigate whether a newly identified glycolytic intermediate, Fru-2,6-P_2, would play a role in flux activation, attempts were made to measure this sugar phosphate in extracts of human platelets. Gel-filtered platelets were incubated in glucose-free and in glucose-rich medium and before and at several intervals after addition of CN^- (1 mM) samples were withdrawn for analysis of Fru-2,6-P_2. None of these samples contained detectable amounts of this intermediate. Similar results were obtained in suspensions concentrated by centrifugation to a cell count of $9 \cdot 10^9$ cells/ml and incubated under the same conditions. Hence, human platelets do not contain detectable amounts of Fru-2,6-P_2, indicating that if it is present its concentration is far below 1 nM.

Discussion

Platelets stimulated with platelet activating agents show a quantitative correlation between the rate of the glycolytic flux and the accumulation of [^{32}P]Fru-1,6-P_2. Thrombin, which is a strong stimulator and initiates rapid aggregation and complete secretion, induced the greatest increase in lactate formation and [^{32}P]Fru-1,6-P_2. Weak activators such as adrenalin, which triggers slow aggregation and partial secretion, weakly increased the flux and the level of [^{32}P]Fru-1,6-P_2. These data accord with the concept that platelet functions require concurrent energy support, which for a major part is furnished by anaerobic degradation of glucose [9,18,19]. A similar covariance between Fru-1,6-P_2 and glycolytic flux has been observed in *Escherichia coli*, which proved to be part of a coordinate control mechanism that couples ATP production to ATP utilization [20]. A time course of the effect of thrombin revealed the first changes in [^{32}P]Fru-1,6-P_2 after 5 s. Earlier studies based on pH measurements showed an increase in lactate production within 5 s after addition of thrombin or ADP, followed by periods of at least 20 min in which lactate production was kept constant [9]. Our present lactate measurements are in line with this observation. The first changes in [^{32}P]Fru-1,6-P_2 therefore coincide with flux activation, which further strengthens a causal relationship between flux activation and variations in the level of Fru-1,6-P_2.

Compared to platelet-activating agents, CN^- induced quite different effects. Although CN^- was a weak activator of the glycolytic flux, it increased the concentration of [^{32}P]Fru-1,6-P_2 much more than thrombin. This contrasts with earlier findings by Detwiler [8], who found that thrombin and CN^- had the same effect on the total concentrations of glycolytic intermediates. A time course of cyanide's effect showed similar changes in [^{32}P]Fru-1,6-P_2 during the first 30 s compared with thrombin, but the CN^--induced increase lasted for about 30 min, whereas the thrombin-induced changes had disappeared after 5 min. A possible explanation lies in a recently discovered glycolytic intermediate, Fru-2,6-P_2, which is a powerful activator of the glycolytic flux in hepatocytes under aerobic conditions in glucose-rich medium [21]. By contrast, under anaerobic conditions this intermediate is not formed in sufficient amounts to be involved in flux control [22,23]. However, attempts to measure this intermediate in platelets were negative, both under aerobic and anaerobic conditions and with or without excess of extracellular glucose.

Our studies reveal a great discrepancy between variations in [^{32}P]Fru-1,6-P_2 and in dihydroxyacetone [^{32}P]phosphate. In the presence of glucose, both sugar phosphates increased upon CN^- addition, but not in parallel. In glucose-free medium [^{32}P]Fru-1,6-P_2 fell after a transient increase, whereas dihydroxyacetone [^{32}P]phosphate remained above control values. This difference was even greater with platelet-activating agents, which all increased [^{32}P]Fru-1,6-P_2 and decreased dihydroxyacetone [^{32}P]phosphate. The aldolase reaction is generally considered to be near equilibrium

[24–26]. It is difficult to confirm this by calculating the mass/action ratio because of the involvement of the triosephosphate isomerase reaction and the uncertainty about the concentration of glyceraldehyde-3-phosphate, which in most tissues is below detection limits [24]. An additional problem is the strong compartmention of aldolase, which in many tissues is bound to membranes and a part of multienzyme complexes bound to F-actin and F-actin-tropomyosin [27–29]. A number of observations do favour a regulatory function, such as the relatively low V_{max} as compared to the flux, a property of most regulatory enzymes [24], its complex kinetics [24], the effect of P_i on the aldolase reaction in situ [30,31] and the fact that the changes in Fru-1,6-P_2 and dihydroxyacetone phosphate often are not completely parallel [6,8]. Our present results are in favour of an important regulatory role of aldolase and support the concept that also near-equilibrium reactions take part in flux control and contribute to an extent that depends on the control coefficient of the particular enzymes [32].

The present study raises the question which role the variations in Fru-1,6-P_2, ATP and P_i play in flux control. Fru-1,6-P_2 is a potent stimulator of pyruvate kinase [33] and a rise in Fru-1,6-P_2 would activate mechanisms that prevent further accumulation of this intermediate. However, it is difficult to understand why changes in [^{32}P]Fru-1,6-P_2 last for 5 min (thrombin) to 30 min (CN^-), while the acceleration of the flux is a matter of seconds. This suggests an additional role for this sugar phosphate. Fru-1,6-P_2 contains two energy-rich phosphate groups and represents a considerable amount of metabolic energy. Its accumulation, during flux activation by CN^- is proportional to the extracellular concentration of glucose. This accords with the fact that platelets do not contain detectable amounts of glucose [16], which offers a tool to modulate the flux by changing the glucose content of the medium [34]. Accumulation of Fru-1,6-P_2 might therefore be the result of a transient overproduction of metabolic energy and reflect an energy buffer that stabilizes the level of metabolic ATP. This would be in agreement with the results from Rapoport et al. [25], who in a computer model of erythrocyte glycolysis demonstrated that Fru-1,6-P_2 stabilizes the ATP level.

Activation of energy-generating sequences is thought to be initiated by a transient imbalance between ATP-producing and -utilizing systems and a concomitant ATP → P_i conversion. Such a conversion is apparent during flux activation by platelet-activating agents and by CN^- in the absence of extracellular glucose. In the presence of glucose, however, CN^- increases the flux without a concomitant ATP → P_i conversion, and most of the decrease in [^{32}P]ATP is accounted for by an increase in [^{32}P]Fru-1,6-P_2. Surprisingly, human platelet glycolysis can be activated without an apparent change in P_i, which contrasts with the crucial role of the ATP/P_i ratio in the regulation of glycolysis in many types of cells [35].

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