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Mechanism of thrombin-induced rise in platelet fructose 2,6-bisphosphate content

Studies using phorbol myristate acetate, dioctanoylglycerol and ionophore A23187

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The mechanism by which thrombin increases platelet fructose 2,6-bisphosphate content was investigated. The action of thrombin was mimicked by phorbol 12-myristate 13-acetate and 1,2-dioctanoylglycerol. Ca^{2+} with A23187 potentiated the action of both these compounds. The action of thrombin required mobilization of intracellular and extracellular Ca^{2+} and was not decreased by indomethacin. This study suggests that protein kinase C activation and Ca^{2+} mobilization are both involved in the activation of glycolysis by thrombin.

INTRODUCTION

Fructose 2,6-bisphosphate (Fru-2,6-P_2) has been demonstrated to be the most potent activator of 6-phosphofructose-1-kinase, the rate-limiting enzyme of the glycolytic pathway (Hers & Van Schaftingen, 1982). The role of this compound in the basal and hormone-stimulated glycolytic flux was first established in isolated hepatocytes (Bartrons *et al.*, 1983). After this observation, the importance of Fru-2,6-P_2 was evaluated in other carbohydrate-utilizing tissues. Evidence has been presented for its involvement in the control of glycolysis in different cell systems, such as human fibroblasts (Bruni *et al.* 1983, 1986), chick-embryo fibroblasts (Bosca *et al.*, 1985, 1986), pancreatic islets (Sener *et al.*, 1984) and adipocytes (Sobrino & Gualberto, 1985), whereas in skeletal muscle (Minatogawa & Hue, 1984), mammary gland (Ward & Kuhn, 1985) and spermatozoa (Philippe *et al.*, 1986) Fru-2,6-P_2 concentrations are not strictly related to the rate of glycolysis.

We have previously demonstrated the important role of Fru-2,6-P_2 in the control of basal and thrombin-stimulated glycolysis in human platelets (Farnararo *et al.*, 1986). Thrombin elicits a marked increase in Fru-2,6-P_2, and this is responsible for a sustained activation of 6-phosphofructo-1-kinase, which may account for the observed enhancement of glycolytic flux. The particular importance of this effect, which enables the glycolytic flux to meet the energy requirements of the stimulated platelets, led us to investigate the mechanism by which thrombin affects Fru-2,6-P_2 concentrations.

The interaction between thrombin and its platelet receptor induces a rapid Ca^{2+} mobilization and protein kinase C activation that appear to mediate physiological cellular responses such as aggregation and secretion (Rasmussen, 1986; Kaibuchi *et al.*, 1983).

Therefore we have investigated whether these two early receptor-linked events are involved in the observed increase in Fru-2,6-P_2. This has been performed by two approaches: first, by studying the effect of selective protein kinase C activators such as phorbol 12-myristate 13-acetate (PMA) (Castagna *et al.*, 1982) and 1,2-dioctanoylglycerol (diC_8) (Lapetina *et al.*, 1985; Bishop & Bell, 1986), and secondly by studying the effect of different Ca^{2+} availability on the action of thrombin. Moreover, as it has been reported that platelet response to thrombin is amplified by the agonist-induced arachidonic acid cascade (Siess *et al.*, 1983), the importance of this pathway on the observed enhancement of Fru-2,6-P_2 has also been investigated.

MATERIALS AND METHODS

Biochemicals and auxiliary enzymes for the assay of Fru-2,6-*P* were purchased from Boehringer (Mannheim, Germany). Fru-2,6-P_2, PMA and diC_8 were from Sigma (St. Louis, MO, U.S.A.); topical bovine thrombin was from Roche (Milan, Italy); D-[U-^{14}C]glucose (358 mCi/mmol) was from New England Nuclear; prostacyclin was from Wellcome (London, U.K.).

Freshly drawn venous blood (50 ml portions) was collected from healthy young volunteers and anti-coagulated with 10 ml of ACD buffer (85 mM-trisodium citrate, 111 mM-dextrose, 71 mM-citric acid). Platelet-rich plasma was obtained by centrifugation at 200 *g* for 20 min. Prostacyclin was added (0.3 μg/ml), and the platelet-rich plasma centrifuged at 2000 *g* for 15 min. The resulting pellet was resuspended in modified Tyrode–Hepes buffer containing no glucose (134 mM-NaCl, 12 mM-$NaHCO_3$, 2.9 mM-KCl, 0.36 mM-NaH_2PO_4, 1 mM-$MgCl_2$, 5 mM-Hepes, pH 7.4). Further prostacyclin was added and the washing procedure repeated once. The washed platelets were resuspended in the above-mentioned buffer and the suspension was briefly centrifuged in a Microfuge to eliminate other contaminating cells. The suspension was diluted to give $(4–5) \times 10^8$ platelets/ml and, depending on the experimental purposes, 1 mM-$CaCl_2$ was added or not. The platelet suspension was kept in capped polystyrene tubes at 37 °C in a water bath under agitation for 90 or 180 min before starting the experiments.

Abbreviations used: Fru-2,6-P_2, fructose 2,6-bisphosphate; PMA, phorbol 12-myristate 13-acetate; diC_8, dioctanoylglycerol.

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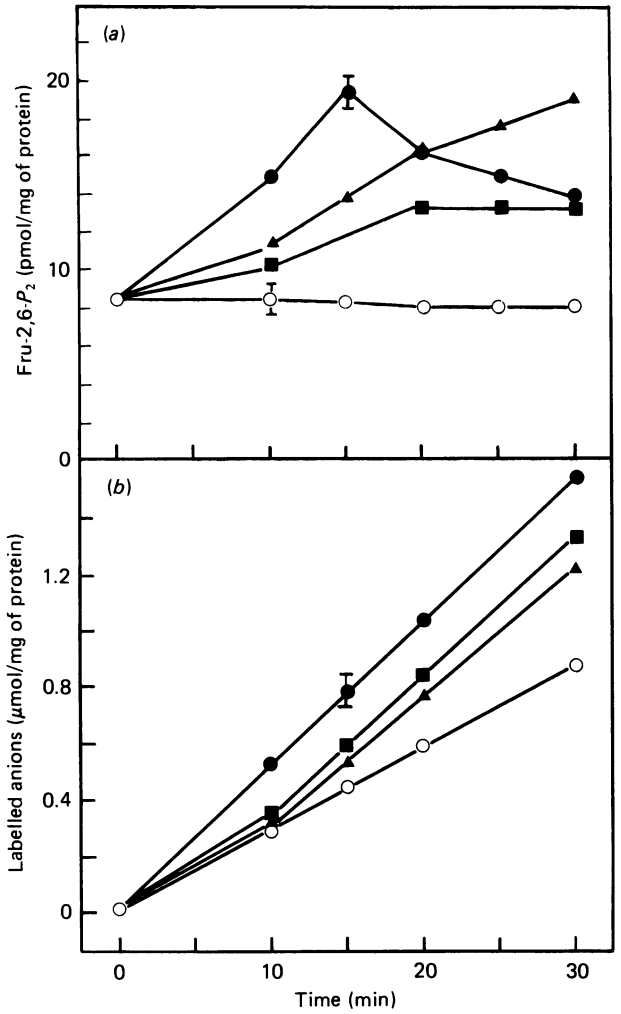

Fig. 1. Time course of the effects of thrombin, PMA and diC_8 on platelet Fru-2,6-P_2 contents (*a*) and glycolytic flux (*b*)

Platelet suspension was preincubated for 90 min at 37 °C with agitation in Tyrode–Hepes buffer with 1 mM-$CaCl_2$ without glucose; 5 min before the beginning of the experiment, 5 mM-glucose (*a*) or 5 mM-D-[U-^{14}C]glucose (1 μCi/ml) (*b*) was added. After this preincubation, to samples of the platelet suspension were added 4 units of thrombin/ml (●), 100 ng of PMA/ml (■) or 20 μg of diC_8/ml (▲). All the substances were dissolved in dimethyl sulphoxide, and its final concentration in the experiment was 0.1%. The controls (○) received the same amount of dimethyl sulphoxide. At various time intervals the incubations were terminated by addition of NaOH (final concn. 50 mM) (*a*) or by addition of $HClO_4$ (final concn. 0.5 M) (*b*). The samples were then used for Fru-2,6-P_2 (*a*) and glycolytic-flux (*b*) determinations as described in the Materials and methods section. Results are means ± S.E.M. of triplicate samples from one single representative experiment performed four times.

For the experiments, 0.2 ml portions of platelet suspension were preincubated for 2–5 min in the presence of 5 mM-glucose, and thereafter the substances to be tested were added.

For Fru-2,6-P_2 determinations, the incubations were terminated by addition of NaOH (final concn. 50 mM). The samples were heated at 80 °C for 10 min and then assayed for Fru-2,6-P_2 content by the method of Van Schaftingen *et al.* (1982).

Table 1. Effect of Ca^{2+} addition in the presence of 0.1 μM-A23187 on the increase in Fru-2,6-P_2 elicited by PMA and diC_8

Platelets were preincubated in the absence of Ca^{2+} as described in Fig. 1; 5 min before the beginning of the experiment, 5 mM-glucose was added with or without 1 mM-$CaCl_2$ and 0.1 μM-A23187. After this preincubation, 0.1% dimethyl sulphoxide, 150 ng of PMA/ml or 20 μg of diC_8/ml was added to 0.2 ml portions of platelet suspension, which were incubated for 15 min with agitation in a water bath. The incubation was stopped by addition of NaOH (final concn. 50 mM), and the samples were used for Fru-2,6-P_2 determination as described in the Materials and methods section. Values are means ± S.E.M. from triplicate samples of one representative experiment repeated four times. Significant differences between values, by unpaired Student's *t* test, are: *b* versus *a*, $P < 0.02$; *c* versus *a*, $P < 0.01$; *d* versus *a*, *e* versus *b* or *d*, *f* versus *c* or *d*, $P < 0.001$.

	Fru-2,6-P_2 content (pmol/mg of protein)	
Addition	No addition	−1 mM-$CaCl_2$ + 0.1 μM-A23187
DMSO	(*a*) 4.0 ± 0.3	(*d*) 6.8 ± 0.3
PMA	(*b*) 5.8 ± 0.2	(*e*) 12.8 ± 0.6
diC_8	(*c*) 6.7 ± 0.4	(*f*) 17.5 ± 0.4

For the estimation of glycolytic flux, analogous incubations of platelet suspension were carried out in the presence of D-[U-^{14}C]glucose (1 μCi/ml). The incubations were terminated by addition of ice-cold $HClO_4$ (final concn. 0.5 M). After centrifugation (2000 *g* for 10 min) and neutralization with K_2CO_3, labelled anions were separated from glucose by ion-exchange chromatography (Mojena *et al.*, 1985). Portions of samples were applied on Dowex AG1X8 (Cl^- form; 200–400 mesh; 0.5 cm × 4 cm column), [^{14}C]glucose was eluted with 6 ml of water, and successively the ^{14}C-labelled anions were eluted with 4 ml of 1 M-NaCl. The radioactivity of both fractions was measured.

Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Fig. 1 shows the time course of the effect of thrombin, PMA and diC_8 on platelet Fru-2,6-P_2 concentrations (*a*) and on the glycolytic flux (*b*), estimated as the production of labelled anions (primarily lactate and pyruvate) from [^{14}C]glucose, All the substances examined are able to increase in parallel Fru-2,6-P_2 concentrations and glycolytic flux, although they display different patterns. Thrombin is the most potent activator of the glycolytic flux, and is also responsible for a rapid increase in Fru-2,6-P_2 that reaches a maximum at 15 min. PMA induces a smaller and slower increase in Fru-2,6-P_2 that attains a plateau at 20 min, whereas diC_8 is responsible for a more marked increase in Fru-2,6-P_2, with a maximal effect after 30 min, decreased by 30% after 60 min (results not shown). PMA and diC_8 activate

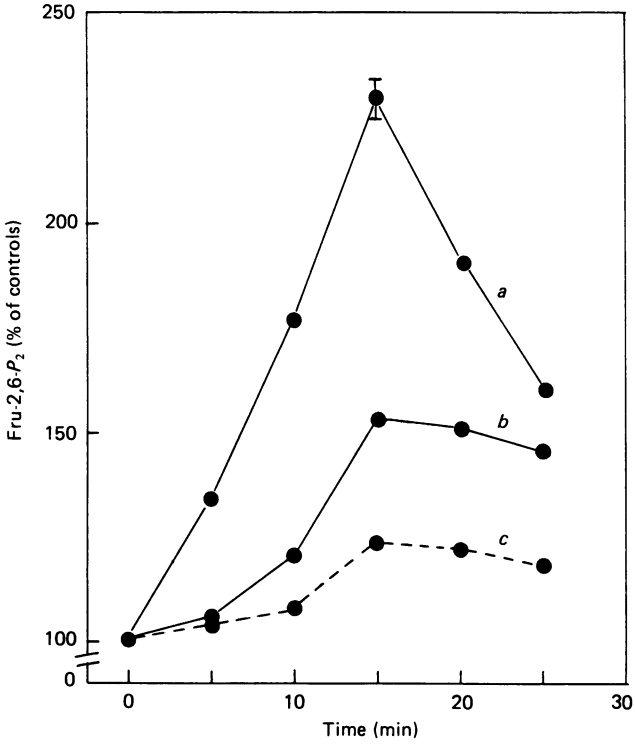

Fig. 2. Effect of different Ca^{2+} availability on the action of thrombin on platelet Fru-2,6-P_2 contents

Platelets from the same pool were preincubated for 180 min at 37 °C with agitation in Tyrode–Hepes buffer without glucose, in the presence (——) or absence (----) of Ca^{2+}; 2 min before the beginning of the experiment, 5 mM-glucose alone (*a*) or with 2 mM-EGTA (*b*, *c*) was added. The experiment was then initiated by adding bovine thrombin (4 units/ml) to 0.2 ml portions of the platelet suspensions. The incubations were terminated at different time intervals by addition of NaOH (final concn. 50 mM). The samples were used for Fru-2,6-P_2 determinations as described in the Materials and methods section. Results are means ± S.E.M. of triplicate samples from one representative experiment performed seven times.

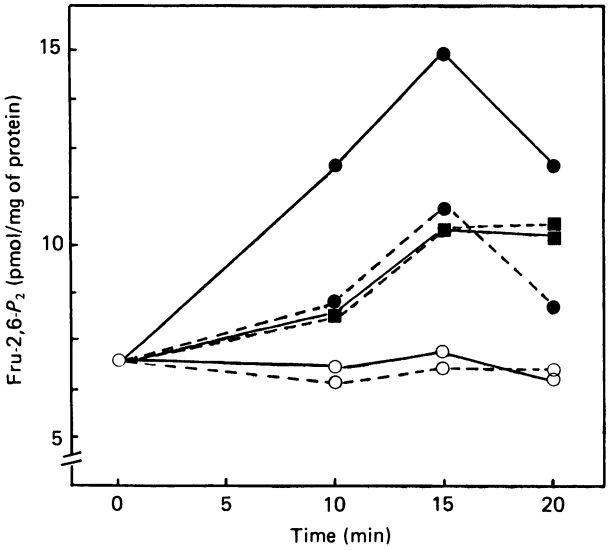

Fig. 3. Comparison of the effect of thrombin and PMA on platelet Fru-2,6-P_2 contents in the presence or absence of EGTA

Platelets were preincubated for 180 min at 37 °C in Tyrode–Hepes buffer without glucose in the presence of Ca^{2+}; 2 min before the beginning of the experiment, 5 mM-glucose alone (——) or with 2 mM-EGTA (----) was added. The experiment was then initiated by adding 4 units of bovine thrombin/ml (●) or 100 ng of PMA/ml (■) to 0.2 ml portions of the platelet suspension. Results are means ± S.E.M. of triplicate samples from one representative experiment performed three times.

glycolytic flux to a similar extent, with a delay in comparison with thrombin.

Table 1 reports the effect of Ca^{2+} addition, in the presence of 0.1 μM-A23187 [concentration unable to activate protein kinase C (Kaibuchi *et al.*, 1983)], on the increase in Fru-2,6-P_2 elicited by PMA and diC_8 in platelets preincubated for 90 min at 37 °C in a Ca^{2+}-free medium. Ca^{2+} addition increases basal concentrations of Fru-2,6-P_2 and is synergistically effective with PMA and diC_8 in causing an increase in the metabolite.

The importance of Ca^{2+} mobilization in the action of thrombin has been further investigated by preincubating platelets for 3 h in Tyrode–Hepes buffer containing 1 mM-$CaCl_2$ or not, and evaluating the effect of thrombin on Fru-2,6-P_2 concentrations in the presence or absence of 2 mM-EGTA. In view of the observed variable extent of increase in Fru-2,6-P_2 induced by thrombin in different platelet preparations, these experiments were performed with the same pool of platelets preincubated in parallel with or without Ca^{2+}. Fig. 2 shows that, in platelets preincubated with Ca^{2+}, thrombin elicits a less rapid and smaller increase in Fru-2,6-P_2 in the presence of EGTA than that observed when extracellular Ca^{2+} is not chelated. The effectiveness of thrombin is further decreased in platelets preincubated in the absence of Ca^{2+} and thus at least partially depleted of intracellular Ca^{2+}. In platelets preincubated with Ca^{2+} and stimulated in the presence of EGTA, thrombin exhibits the same potency as PMA in increasing Fru-2,6-P_2 (Fig. 3). Under these experimental conditions, the time course of the effect of diC_8 is also superimposable on that of thrombin (results not shown).

In Table 2 the concentrations of Fru-2,6-P_2 in platelets stimulated with thrombin in the presence of 1 μM-indomethacin are reported. The cyclo-oxygenase inhibitor does not affect the action of thrombin; analogous results were obtained with 10 μM-indomethacin.

DISCUSSION

We have previously reported that in human platelets thrombin induces a sustained increase in Fru-2,6-P_2 which may account for the well-known stimulatory effect on glycolytic flux elicited by the agonist (Farnararo *et al.*, 1986).

In the present work we have investigated the role of early molecular events triggered by the thrombin–receptor interaction in mediating the observed increase in platelet Fru-2,6-P_2. Protein kinase C activation has been demonstrated to occur after platelet stimulation with thrombin (Kaibuchi *et al.*, 1983). The stimulation of the same kinase activity has been shown to be involved in the activation of oxidative metabolism in polymorpho-

Table 2. Fru-2,6-P_2 contents in platelets stimulated with thrombin in the presence of 1 μM-indomethacin

The experiment was performed as described in Fig. 1, except that platelets were preincubated for 2 min in the absence or presence of 1 μM-indomethacin. The incubations were terminated at various time intervals by adding NaOH (final concn. 50 mM), and the samples were used for Fru-2,6-P_2 determinations. Values are means ± S.E.M. for triplicate samples from one representative experiment performed three times: $^*P < 0.001$ compared with respective controls.

			Fru-2,6-P_2 content (pmol/mg of protein)	
	Indomethacin	Time of incubation...	10 min	20 min
Control	−		7.5±0.5	8.0±0.1
	+		6.6±0.2	8.2±0.2
Thrombin (4 units/ml)	−		13.2±0.4*	15.1±0.2*
	+		14.8±0.7*	16.0±0.3*

nuclear leucocytes (Christiansen *et al.*, 1986) and adipocytes (Skoglund *et al.*, 1985); in addition, in chick-embryo fibroblasts the increase in glycolytic flux induced by PMA, a known activator of protein kinase C, has been ascribed to the increase in Fru-2,6-P_2 (Bosca *et al.*, 1985). Therefore we have examined PMA and diC_8, another selective activator of protein kinase C, for their ability to mimic the action of thrombin on Fru-2,6-P_2 concentrations. Interestingly, both compounds were found to be effective in rising the concentrations of the metabolite and the glycolytic rate, the temporal relationship of these two events confirming the relevant role of Fru-2,6-P_2 in human platelets (Fig. 1). This is, to our knowledge, the first evidence for the stimulation of an oxidative process brought about by PMA and diC_8 in platelets. These results indicate that protein kinase C activation may lead to an increase in Fru-2,6-P_2, and suggest that the same process may mediate the action of thrombin on the metabolite. However, the more rapid effect elicited by thrombin, compared with that observed with PMA and diC_8, suggests that the mechanism of action of the agonist may require more complicated processes than the simple activation of protein kinase C.

It is well known that thrombin, in addition to activating protein kinase C, can increase platelet intracellular Ca^{2+}, causing its release from intracellular stores and activating the rate of the ion influx (Rasmussen, 1986). In this connection, the importance of Ca^{2+} mobilization for the enhancement of Fru-2,6-P_2 concentrations was investigated in a series of experiments. Initially Ca^{2+}, in combination with the bivalent-ion ionophore A23187, was added to human platelets previously incubated for 90 min in the absence of Ca^{2+}. In these conditions the addition of Ca^{2+} to platelets elicited an increase in Fru-2,6-P_2, demonstrating that the metabolite values may also be affected by an increase in Ca^{2+}, besides protein kinase C activation. Moreover, Ca^{2+} was able to potentiate the effect of PMA and diC_8 (Table 1). This is the first evidence that protein kinase C activation and Ca^{2+} mobilization act synergistically to elicit a specific platelet metabolic response, the increase in Fru-2,6-P_2, as well as acting similarly on a complex cellular event such as the release of 5-hydroxytryptamine (Kaibuchi *et al.*, 1983). Successively we have investigated whether Ca^{2+} mobilization plays a role in the effect of thrombin on Fru-2,6-P_2 concentrations. The increase in Fru-2,6-P_2 induced by thrombin in Ca^{2+}-depleted platelets was always lower than that observed in platelets not Ca^{2+}-depleted where the influx was prevented by the presence of EGTA (Fig. 2). These results indicate that the availability of intracellular Ca^{2+} represents a limiting factor for the action of thrombin on Fru-2,6-P_2 concentrations. On the other hand, thrombin-stimulated Ca^{2+} influx must also be taken into account to evaluate the mechanism by which thrombin affects Fru-2,6-P_2 concentrations; in fact, from the same experiment it appears that the effectiveness of thrombin in non-Ca^{2+}-depleted platelets is strongly decreased by the presence of EGTA (Fig. 2). Therefore the data on the whole indicate that both intracellular and extracellular Ca^{2+} play an important role for the full metabolic response to thrombin. Interestingly, in the presence of EGTA the time course of the Fru-2,6-P_2 increase induced by thrombin closely resembles those elicited by PMA (Fig. 3). and diC_8, suggesting once more that the activation of protein kinase C represents a relevant step in the action of thrombin on the metabolite. The more rapid and sustained effect of thrombin, compared with that induced by PMA and diC_8, when extracellular Ca^{2+} was not chelated is conceivably due to the concomitant mobilization of Ca^{2+} elicited by the agonist.

The activation of the arachidonic acid cascade induced by thrombin, known to amplify the cellular response to the agonist, appears not to be involved in the increase in Fru-2,6-P_2, since indomethacin did not decrease the effect of thrombin (Table 2).

The present studies suggest that in human platelets protein kinase C activation and Ca^{2+} mobilization mediate the enhancement of Fru-2,6-P_2 induced by thrombin, which is responsible for the activation of glycolytic flux.

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