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FRUCTOSE-1,6-DIPHOSPHATE INHIBITS PLATELET ACTIVATION

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Abstract—Fructose-1,6-diphosphate (FDP) is a physiological product which exhibits pharmacological properties. This study shows that FDP (1–3 mM) inhibits platelet aggregation induced by the agonists thrombin, vasopressin, platelet activating factor, ADP, adrenaline, arachidonate and the stable thromboxane analogue U 44069. Thrombin-promoted ATP secretion and cytosolic Ca^{2+} rise are also drastically inhibited by FDP, which decreases, although to a lesser extent, the protein kinase C-dependent phosphorylation of the 47 kDa protein. The inhibition on thrombin-induced aggregation is shared, albeit less efficiently, by glucose-1,6-diphosphate and fructose-2,6-diphosphate but not by other phosphorylated monosaccharides (fructose-1:2 cyclic,6-diphosphate, glucose-1- and glucose-6-phosphate, fructose-1- and fructose-6-phosphate, mannose-6-phosphate and 5-phosphoryl ribose-1-pyrophosphate). FDP does not affect platelet activation induced by the protein kinase C activators dioctanoylglycerol or phorbol 12-myristate 13-acetate. No increase of cAMP concentration is observed in FDP-treated platelets. Altogether, these results indicate that FDP inhibits platelet activation at a level preceding phospholipase C. The data are consistent with a general inhibitory action of FDP on signal transmission.

Stimulation of surface membrane receptors of platelets, as of many other cell types, is accompanied by a membrane phospholipase C activation producing the second messengers diacylglycerols and inositol trisphosphate. These, in turn, bring about a cytosolic Ca^{2+} increase, shape change, protein kinase C (PKC†)-dependent phosphorylation, aggregation and secretion of the dense granule content (see review [1]). The cyclic nucleotides cAMP and cGMP have been shown to inhibit the agonist-induced platelet response by interacting at the intracellular level [2–5]. In the present study, we present evidence that fructose-1,6-diphosphate (FDP) inhibits agonist-promoted platelet activation possibly by interacting with the signal transmission. FDP is a physiological product that has been used as a pharmacological compound showing positive effects on the haemodynamics during hypoxemia [6], alcohol-induced damages [7, 8], ischemic–hypoxic brain and anoxic heart injury [9, 10], as well as on doxorubicin cardiotoxicity [11].

MATERIALS AND METHODS

Materials. Thrombin, prostacyclin, apyrase, 1,2-dioctanoylglycerol, phorbol 12-myristate 13-acetate (PMA), fructose-1,6-diphosphate, fructose-2,6-diphosphate, fructose-1:2 cyclic, 6-diphosphate, fructose-1- and fructose-6-phosphate, glucose-1- and glucose-6-phosphate, glucose-1,6-diphosphate, mannose-6-phosphate, 5-phosphorylribose-1-pyrophosphate, arachidonic acid, 9,11-dideoxy-9α,11α-epoxymethanoprostaglandin, L-α-phosphatidylcholine, β-acetyl-γ-*o*-alkyl and [Arg^8]-vasopressin (vasopressin) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). $^{32}P_i$ was from Amersham (Buckinghamshire, U.K.), fura acid, fura 2/AM and BCECF/AM from Calbiochem (La Jolla, CA, U.S.A.). All other reagents were of analytical grade.

Platelet preparation. Platelet-rich plasma and washed platelets were prepared as reported previously [4] from fresh blood drawn from healthy volunteers and mixed with acid citrate–dextrose anticoagulant supplemented with apyrase (20 μg/mL) and prostacyclin (0.2 μg/mL).

Measurement of platelet aggregation, ATP secretion, cytosolic Ca^{2+} rise and protein phosphorylation was performed in washed platelets as reported in Ref. 5, essentially as follows.

Protein phosphorylation in intact platelets. Platelets were gently resuspended at a final count of 2×10^9/mL of prewarmed medium consisting of 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 20 mM Hepes, pH 7.4 (buffer A), supplemented with 3 mg/mL bovine serum albumin, 50 ng/mL prostacyclin, 20 μg/mL apyrase, and incubated with carrier-free [^{32}P]orthophosphate (0.4 mCi/mL) for 90 min at 37°. The cellular suspension was then centrifuged at 800 *g* for 20 min and the supernatant discarded. The platelets were resuspended in buffer A at a count of 1×10^8 cells/mL and kept at room temperature until use. Prior to experimental

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† Abbreviations: FDP, fructose-1,6-diphosphate; DiC_8, dioctanoylglycerol; PMA, phorbol 12-myristate 13-acetate; PGI_2, prostacyclin; U44069, 9,11-dideoxy-9α, 11α-epoxymethano-prostaglandin $F_{2\alpha}$; PAF, L-α-phosphatidylcholine,β-acetyl-γ-*o*-alkyl; vasopressin, [Arg^8]-vasopressin; PKC, protein kinase C; fura 2/AM, fura 2 acetoxy-methyl-ester; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein.

procedures aliquots of cellular suspension were preincubated for 10 min at 37° with 1 mM $CaCl_2$. Incubations (1 mL) were carried out at 37° for 30 sec with thrombin. When present, FDP was added 1 min before thrombin. The reactions were terminated by the addition of 0.1 mL of 3 M perchloric acid and centrifuged for 7 min at 12,000 *g*. The precipitated proteins were dissolved in 0.1 mL of buffer containing 0.6 M Tris–HCl (pH 6.8), 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 2% (w/v) SDS, boiled for 5 min and subjected to 12% SDS–PAGE on vertical slabs according to Laemmli [12]. The gels were stained with Coomassie brilliant blue, dried, autoradiographed and scanned (1 hr/lane) for the measurement of $^{32}P_i$-incorporated radioactivity with a Bioscan Imaging Scanner System 200-IBM. The apparent molecular mass of the proteins was calculated by calibration with the following marker proteins: bovine serum albumin (67 kDa), glyceraldehyde phosphate dehydrogenase (36 kDa), trypsinogen (25 kDa) and ribonuclease (13.7 kDa). The areas corresponding to the 47 and 20 kDa bands were integrated by the NSCAN ver.2.247 MS-Dos program, and the values, subtracted of the radioactivity measured in unstimulated platelets, were utilized for the calculation of the percent of inhibition by FDP.

Determination of cytosolic free Ca^{2+} *concentration.* The intracellular Ca^{2+} concentration was determined with fura 2 essentially according to Pollock and Rink [13]. Briefly, platelet-rich plasma was incubated with 3 μM fura 2/AM for 30 min at 37°, centrifuged for 20 min at 800 *g*, gently resuspended at about 2×10^8 cells/mL of buffer A supplemented with 100 μM sulfinpyrazone to prevent the cellular efflux of fura 2 [14], and used within 1 hr. Fluorescence was measured at 37° in a thermostated, magnetically stirred cuvette, in a Shimadzu RL-5000 spectrofluorimeter with excitation and emission wavelengths of 340 and 505 nm, respectively.

Platelet aggregation. Platelet aggregation was evaluated with an Elvi Logos aggregometer as reported elsewhere [5].

ATP secretion. ATP secretion was measured with the luciferin–luciferase reagent (LKB) [5].

Intracellular pH. The intracellular pH of platelets was monitored according to the method of Rink *et al.* [15].

cAMP concentration. cAMP concentration was measured with the [^{3}H]cAMP kit of Amersham in samples deproteinized with 10% (v/v) perchloric acid containing 5 mM EGTA. After neutralization of the supernatant with $KHCO_3$ and removal of excess K-perchlorate by centrifugation, the samples were lyophilized, dissolved in 120 μL of 50 mM Tris–HCl, pH 7.4, containing 4 mM EGTA and used for the cAMP determination. Internal standards of cAMP (3–30 pmol), added to 1 mL of the control platelet suspensions (2×10^8 cells/mL), were almost completely recovered (> 95%).

RESULTS

FDP inhibited both platelet aggregation and secretion induced by thrombin in a concentration-dependent manner (in the range of 0.5 to 2 mM) (Fig. 1A and B); unlike aggregation shape change was not significantly affected.

The effects of a number of other phosphorylated sugars on platelet aggregation were also checked. The results reported in Table 1 show that only fructose-2,6-diphosphate and glucose-1,6-diphosphate, albeit less efficiently than FDP, inhibited the thrombin-induced platelet aggregation. Maximum

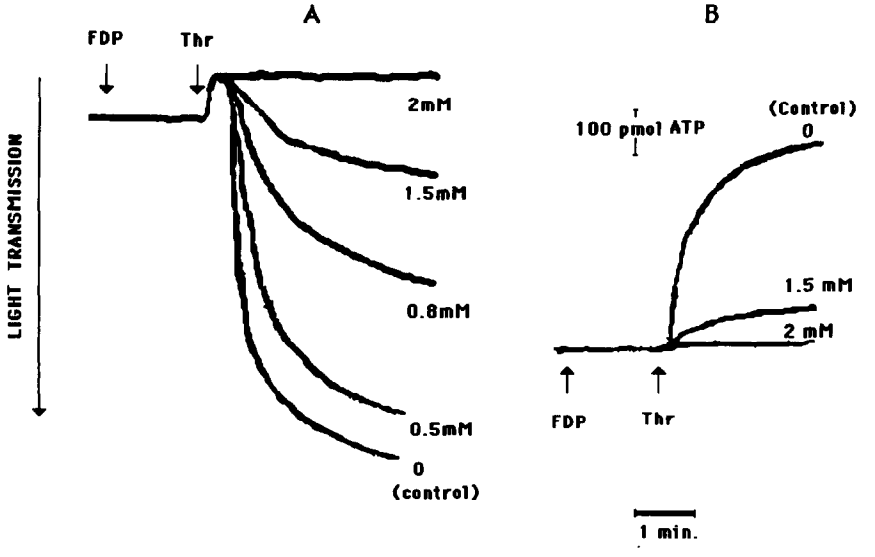

Fig. 1. Platelet aggregation (A) and ATP-secretion (B) induced by thrombin in the absence or presence of FDP. At the arrows, 0.03 U/mL of thrombin (Thr) and the indicated amounts of FDP were added to 1 mM $CaCl_2$ containing platelet suspension. Tracings are representative of at least five different experiments. Other experimental details are given in Materials and Methods.

Table 1. Inhibition of thrombin-evoked aggregation by different phosphorylated monosaccharides

Additions	Inhibition (%)
FDP	100
Fructose-2,6-diphosphate	70
Glucose-1,6-diphosphate	70
Glucose-6-phosphate	20
Glucose-1-phosphate	0
Fructose-6-phosphate	0
Fructose-1-phosphate	0
Mannose-6-phosphate	0
Fructose-1:2c,6-diphosphate	0
5-Phosphorylribose-1-pyrophosphate	0

All phosphorylated monosaccharides (2 mM) were added to the platelet suspension 1 min before 0.03 U/mL thrombin. The inhibition (% average of at least three different determinations) is calculated by comparison with control aggregation obtained with thrombin alone in a 1 mM Ca^{2+}-containing suspension.

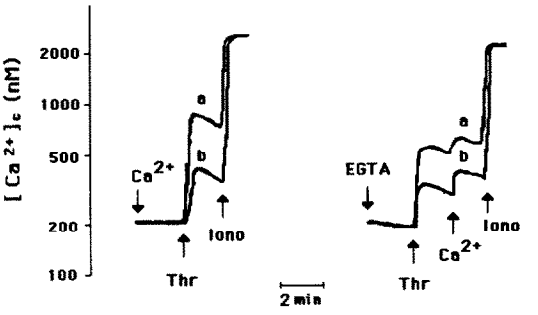

Fig. 2. Inhibition of FDP on thrombin-induced cytosolic Ca^{2+} increase in the presence or absence of external calcium. Where indicated, 1 mM $CaCl_2$, 0.3 mM EGTA, 0.03 U/mL thrombin (Thr) or 0.35 μM ionomycin (Iono) were added. FDP (2 mM) was added 1 min prior to thrombin (trace b).

Table 2. Effect of FDP on platelet aggregation induced by different agonists

Agonist	FDP concentration (mM)	Inhibition (%)
Thrombin (0.03 U/mL)	1.5 ± 0.5 (6)	100
ADP (20 μM)	2.5 ± 0.5 (4)	100
PAF (160 nM)*	3.5 ± 0.5 (3)	100
Vasopressin (300 nM)*	3.5 ± 0.5 (3)	100
Arachidonate (2 μM)	3.5 ± 0.5 (3)	100
U 44069 (2 μM)*	3.5 ± 0.5 (4)	50
DiC_8 (30 μM)	3.5 (3)	0
PMA (20 nM)	3.5 (3)	0

The concentrations of FDP needed to obtain the indicated inhibitions are expressed as means ± SD, for the number of experiments reported in parentheses. In all cases, FDP was added 1 min prior to the agonist, and the incubation lasted for at least 5 min. Added Ca^{2+} was 1 mM.

* In these experiments the agonist was added 1 min after 4 μM adrenaline.

inhibition was observed with 1.5–2 mM FDP and 2.5–3 mM fructose-2,6-diphosphate or glucose-1,6-diphosphate, respectively. A slight inhibition was also shown by glucose-6-phosphate, whereas mannose-6-phosphate, fructose-1-phosphate, fructose-6-phosphate, fructose-1:2 cyclic,6-diphosphate, glucose-1-phosphate and 5-phosphorylribose-1-pyrophosphate added at concentrations of 2–3 mM did not show significant inhibitory effects.

FDP inhibited, although to different extents, the aggregation evoked by PAF, vasopressin, ADP, arachidonic acid and U 44069, but not that promoted by dioctanoylglycerol (DiC_8) or PMA (Table 2). In all cases, the platelet shape change was not affected.

Agonist-binding to membrane receptor triggers a cascade process involving G protein-mediated phospholipase C activation, increase of the cytosolic Ca^{2+} and protein kinase C-catalysed protein phosphorylation [1]. In order to understand better the mode of FDP inhibition we have therefore studied its effect on thrombin-induced cytosolic Ca^{2+} increase. Figure 2 shows that FDP (2 mM) strongly inhibited the thrombin-induced Ca^{2+} rise both in the presence or absence of extracellular Ca^{2+}. In the latter case, the thrombin-induced cytosolic Ca^{2+} increase was lower, and the per cent inhibition by FDP (about 50%) was also less than that observed in the presence of external calcium (about 75%). Under our experimental conditions FDP showed only a negligible Ca^{2+} chelating effect, as demonstrated by the Ca^{2+} concentration fluorimetric measurement with fura 2-free acid (not shown). Cytosolic Ca^{2+} rise was similarly inhibited by FDP in platelets challenged with PAF, vasopressin and ADP (not shown).

Phosphorylation of the major substrate of platelet protein kinase C, i.e. a 47 kDa protein, was also inhibited by FDP as shown in Fig. 3, where the patterns of phosphorylated proteins induced by thrombin in the presence or absence of FDP are reported. A lower inhibition was found on the phosphorylation of the 20 kDa protein which, on the one hand, occurs later than that of the 47 kDa protein [16] and, on the other hand, is substrate for both protein kinase C and myosin light chain kinase [1]. Under these conditions, FDP alone did not induce any appreciable increase of phosphorylation of platelet proteins including the substrates of cAMP- and cGMP-dependent protein kinases [2, 3, 17].

In contrast with the effect on thrombin-induced phosphorylation, no inhibition by FDP was observed on the 47 kDa protein phosphorylation if platelets were stimulated with the direct activators of PKC, PMA or DiC8 (not shown).

The experiments of Fig. 4 show that FDP inhibited also the adrenaline-induced aggregation both when added before and at different times after adrenaline, even if, as expected, the inhibitory effect decreased by delaying the FDP addition. These experiments seem to rule out cAMP as a mediator of the FDP-

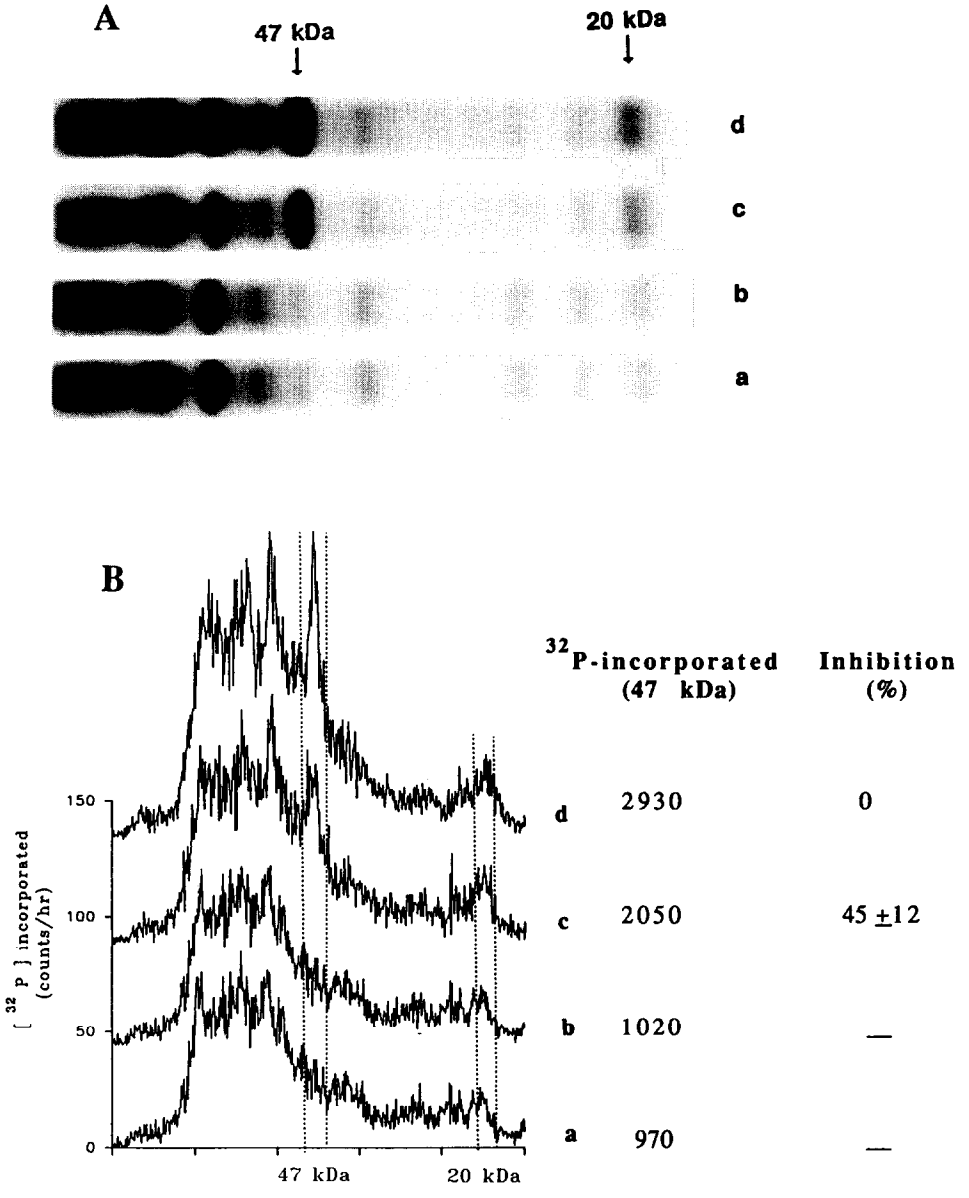

Fig. 3. Effect of FDP on thrombin-induced protein phosphorylation. Representative autoradiographies (A) and radioactive profiles (B) of the electrophoretic lanes of $^{32}P_i$-loaded platelets treated with: none for 2 min (lane **a**), 2 mM FDP for 2 min (lane **b**), 2 mM FDP for 1 min before 0.03 U/mL thrombin (30 sec) (lane **c**), none for 1 min prior to 0.03 U/mL thrombin (30 sec) (lane **d**). Quantitation of the phosphorylation of the 47 kDa protein, the major substrate of PKC, is reported as counts/hr/10^8 cells. The % of inhibition (means ± SD of four different experiments) refers to the values obtained with thrombin alone, subtracted from the values of unstimulated platelets.

induced inhibition, since adrenaline is a powerful inhibitor of platelet adenylate cyclase [18, 19].

Accordingly, the direct measurement of cAMP proved that FDP was without effect on the level of this nucleotide (Table 3). For comparison, the treatment with prostacyclin (PGI_2) was also performed which, as expected, induced a 10-fold increase of cAMP.

FDP addition did not appreciably modify either the extracellular or intracellular pH of platelets, the latter measured by means of the pH fluorescent probe BCECF (not shown).

DISCUSSION

Experiments reported in this paper show that thrombin-induced aggregation was inhibited by glucose-1,6-diphosphate, fructose-2,6-diphosphate and FDP. Since the latter exhibited the highest inhibitory effect, it was chosen to elucidate the

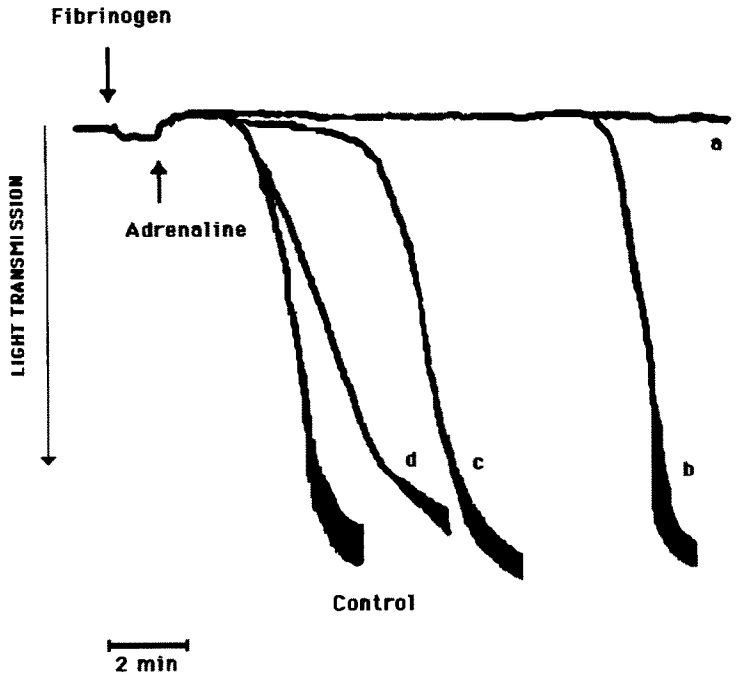

Fig. 4. Inhibition by FDP on adrenaline-induced aggregation. FDP (3 mM) was added 1 min before (**a**); immediately after (**b**), 30 sec after (**c**) and 2 min after (**d**) adrenaline (80 μM). The medium was also supplemented with 1 mM Ca^{2+} and 0.2 mg/mL fibrinogen.

Table 3. cAMP levels in FDP-treated platelets

Additions	Incubation time (min) 0	1	6
None	1.47 ± 0.22	1.55 ± 0.25	1.84 ± 0.30
FDP (2 mM)	1.40 ± 0.19	1.34 ± 0.30	1.68 ± 0.31
PGI_2 (40 nM)	1.56 ± 0.42	13.86 ± 1.45	16.10 ± 1.52

Values (means ± SD of at least three different experiments) are expressed as pmol/1 × 10^8 platelets.

mechanism underlying the antiaggregatory property of these hexose diphosphates. Thrombin-induced ATP secretion was inhibited by FDP, which also decreased the platelet aggregation induced by a variety of other receptor-mediated agonists such as ADP, PAF, vasopressin, the thromboxane analogue U 44069 and adrenaline, thus excluding a specific receptorial hinderance. On the other hand, FDP did not affect platelet aggregation promoted by DiC_8 and PMA, direct activators of PKC. This evidence prompted us to investigate the effect of FDP on agonist-evoked Ca^{2+} increase. Since also this process was deeply reduced by FDP, it was concluded that the phosphorylated monosaccharide probably acts at a step preceding the phospholipase C activation. Accordingly the thrombin-induced PKC-dependent protein phosphorylation was also inhibited, although to a lesser extent, by FDP.

Even if we have no clear explanation for the less evident inhibition on thrombin-promoted protein phosphorylation, the finding that FDP did not affect the phosphorylation elicited by the direct activators of PKC, namely PMA or DiC_8, supports the conclusion that FDP acts at a level preceding the formation of the PKC activators diacylglycerols and inositol trisphosphate.

It is well established that the cyclic nucleotides cAMP and cGMP are potent inhibitors of the platelet activation [1–5, 20, 21]. In order to verify whether the effect of FDP occurred through an increase of cyclic nucleotides, the level of cAMP in FDP-treated cells was assayed and no significant changes in comparison to untreated cells were found.

Although we have not performed direct measurements of cGMP concentration, we believe that this was not significantly affected by FDP. In fact, (1) an increase of cGMP by FDP should also have induced some increase of cAMP, cGMP being an

inhibitor of cAMP phosphodiesterase [22–24], and (2) unlike cGMP [20, 21], FDP did not inhibit the platelet activation promoted by diacylglycerol or phorbol esters.

It is generally believed that phosphorylated sugars do not penetrate the cellular membrane, therefore it is conceivable that the antiaggregatory action of FDP occurs at the cellular surface most probably by interfering with the transmission of signal at the level of the receptor–G protein–phospholipase C system.

It should be noted finally that the concentrations of FDP effective on the modulation of platelet activation, as determined in this paper, are much higher than those present in the plasma under physiological conditions [25], but we think that these concentrations are most likely reached in the plasma during i.v. pharmacological treatment with FDP at the dosages recommended.

It is tempting to speculate that some pharmacological action of FDP, such as the improvement of the ischemic–hypoxic brain injury upon reperfusion [9] may be related to the inhibition of platelet activation as described in this paper.

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