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Interaction of fructose-1,6-diphosphate with human red cell membrane

Interazione del fruttosio-1,6-difosfato con la membrana dell'eritrocita

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Summary: *Fructose-1,6-diphosphate is an activator of the Ca/Mg-ATPase of human red cell "in vitro" and under the same conditions it is able to bind to the isolated human red cell membrane. Fructose-1,6-diphosphate also influences ionic equilibria through the red cell membrane when incubated with intact red cells by stimulating potassium ion uptake and increasing internal pH. These effects are in turn responsible of the activation of glycolysis observed when fructose-1,6-diphosphate is added to the suspension medium.*

Riassunto: *Il fruttosio-1,6-difosfato è un attivatore della Ca/mg-ATPasi dei globuli rossi umani "in vitro" e, nelle stesse condizioni, è in grado di legarsi alla membrana isolata. Incubato con globuli rossi integri, il fruttosio-1,6-difosfato influenza gli equilibri ionici di membrana stimolando l'assunzione di potassio e aumentando il pH endocellulare. Questi effetti, a loro volta, sono responsabili dell'attivazione della glicolisi che si osserva quando il fruttosio-1,6-difosfato viene aggiunto al mezzo di sospensione.*

Introduction

Fructose-1,6-diphosphate (FDP) is a key intermediate of glycolysis and its administration in large amounts proved to be clinically useful in the treatment of adynamic ileus[10] and in the management of polytransfused patients[5].

The effect of FDP seems related to its ability to interact with different types of animal cell membranes[11] and the FDP-membrane interaction seems, in turn, responsible for a number of intracellular changes[9] although, by itself, FDP should not be able to cross the membrane barrier[3].

The present study deals with the effects of FDP on H^+ and K^+ equilibria through the human erythrocyte membrane and with two other possibly related phenomena, such as FDP binding to the membrane and activation of Ca/Mg-ATPase.

Materials and Methods

Potassium ion was measured by atomic absorption (Perkin-Elmer 305 B spectrophotometer Newark, Conn. USA) and pH with a glass electrode connected to a Beckman Expandomatic recording pH-meter (Geneva, CH) as described previously[12]. Human red cells prepared according to Scutari et al. [14] were used, protein was measured by the Lowry et al. procedure[8] and hemoglobin (Hb) according to Beutler [1].

K^+ level and pH were measured inside red blood cells after 30 min incubation at 25°C with 140 mM KCl, 10 mM NaCl, 5 mM Tris-HCl pH 7.4. A final volume of 10 ml contained 20 mg Hb and 5 mM FDP (Biomedica Foscama, Roma) or equimolecular fructose + phosphate (F + P).

1 ml samples, taken at time 0 and 60 min were centrifuged 10 min at 500 x g, the pellet washed twice with saline and lysed with 2 ml of b.d. water for pH measurement. K^+ was measured in the supernatant obtained after deproteinization with 0.5 ml of 14% (w:v) perchloric acid and 10 min centrifugation at 500 x g.

Red cell membranes obtained as described by Scutari et al. [14] showed 60% of inside-out vesicles by the test of accessibility to acetylcholinesterase (EC 3.1.1.7; ACC). The membranes were prepared by diluting 1:5 the red cells with 30 mM Na-Hepes buffer pH 7.4. The suspension was centrifuged 30 min at 45.000 x g, resuspended and centrifuged again until a white pellet was obtained.

The direct interaction of FDP with the prepared membranes was measured by incubating increasing amounts of ^{14}C-FDP (The Radiochemical Centre, Amersham UK) and unlabelled FDP with 10 mg of membrane protein at 25°C for 3 min in 2.5 ml of a medium with 240 mM KCl, 80 mM Na-Hepes, 0.38 mmol/L $CaCl_2$, 9.3 mM $MgCl_2$ and 1 mM ouabain [6]. The mixture was centrifuged 1 min at 25° C in an Eppendorf Microfuge, (Hamburg, FRG) the pellet washed twice and resuspended in 0.5 ml of the medium. The pellet from the last suspension was dissolved in 10 ml of Instagel and the residual radioactivity measured with a Beckman liquid scintillator (Geneva, CH).

Ca/Mg-ATPase activity was assayed enzymatically as

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Tab. I - *Effect of FDP on internal level of K^+ and pH in the red cells (Mean ± SE of 6 experiments)*

	K^+ (μmol/h/mg Hb)	(Δ pH/h/mg Hb) x 10^{-2}
Control	8.30 ± 2.31	0.33 ± 0.05
+ FDP	12.50 ± 3.20	0.75 ± 0.10
+ F + P	8.50 ± 1.20	0.36 ± 0.03

a rate of adenosindiphosphate (ADP) formation in the above described medium with 2 mg/ml protein. ADP formation was measured on the basis of reduced nicotinadenindinucleotide ($NADH_2$) disappearance by a coupled assay with pyruvate kinase (EC 2.7.1.40; PYK) and lactic dehydrogenase (EC 1.1.1.27; LAD) as described [14].

The medium, at pH 7.4, contained 1.9 mmol ATP. Pyruvate kinase was 0.15 mmol, phosphoenol pyruvate 3.06 mmol, lactate dehydrogenase 0.3 mmol and NADH 3.8 mmol. All sugars were 7.66 mmol. Protein was 10 mg.

All reagents and enzymes used throughout were Boehringer (Mannheim, FRG) products.

Results

The effect of FDP on potassium movements through the red cell membrane and internal pH is summarized in Table I. The intraerythrocytic level of K^+ and the internal pH are increased in the presence of FDP in agreement with our previous findings on intact rat red blood cells [12] showing a decrease of K^+ and an ejection of H^+ in the incubation medium. None of the other phosphorylated sugars i.e. fructose-1-phosphate (F1P) and fructose-6-phosphate (F6P) was able to imitate the effects of FDP (results not shown).

The effect of FDP and other phosphorylated sugars on Ca/Mg-ATPase of the red cell membrane is shown in Table II. FDP activates the enzyme in a concentration range between 6.2 and 7.6 mM and the effect is most evident at ATP concentrations higher than 1.5 mM. The effect of FDP seems rather specific since no effect is exerted by the other sugars.

FDP also protects Ca/Mg-ATPase from heat inactivation at 60°C. In fact while a decrease of activity of 16% is observed after 2 min heating in the control, a decrease of only 6% is observed in the presence of 7.66 mmol/L FDP and the difference is statistically significant ($p < 0.01$).

Tab. II - *Effect of different phosphorylated sugars on red cell Ca/Mg-ATPase (Mean ± SE of 6 experiments)*

Compound	Specific activity (nmol ADP/min/mg protein)
None	0.62 ± 0.05
F1P	0.60 ± 0.04
F6P	0.59 ± 0.04
F + P	0.61 ± 0.06
FDP	0.85 ± 0.03

Tab. III - *Interaction of ^{14}C-FDP and ^{14}C-F6P with red cell membranes in the presence and absence of Ca/Mg/ou (0.38 mM Ca, 9.3 mM Mg and 1 mM ouabain). Stock solutions of 7 x 10^6 dpm/ml of ^{14}C-FDP and 0.6 mM FDP were used. (Mean ± SE of 4 experiments)*

FDP_{free}(μM)	^{14}C-FDP_{free}(dpm)	^{14}C-FDP bound(μmol/g) Ca/Mg/ou	
		absence	presence
12	28,000	11.5 ± 1.1	27.5 ± 5.1
24	56,000	18.7 ± 4.0	45.3 ± 11.5
36	84,000	26.5 ± 6.0	54.0 ± 10.2
48	112,000	39.4 ± 8.7	84.0 ± 23.5
60	140,000	32.1 ± 8.4	108.3 ± 28.5

$F6P_{free}$(μM)	^{14}C-$F6P_{free}$(dpm)	^{14}C-F6P bound(μmol/g) Ca/Mg/ou	
		absence	presence
12	26,000	0.6 ± 0.02	3.3 ± 0.4
24	50,000	1.2 ± 0.4	4.6 ± 0.7
36	72,000	2.6 ± 0.2	5.1 ± 0.8
48	100,000	7.4 ± 0.7	5.7 ± 0.9
60	120,000	8.2 ± 1.2	7.8 ± 1.1

dpm = disintegration per minute

The direct interaction of ^{14}C-FDP with the red cell membrane is shown in Table III. FDP is bound to the membrane with an apparent Ks of 22 μM and the binding of ^{14}C-fructose-6-phosphate, under the same conditions, is much lower. The binding is approximately three times more effective in the presence of Ca, Mg and ouabain i.e. under conditions of inactivity of the Na/K-ATPase [6].

Discussion and Conclusions

Ca/Mg-ATPase is a red blood cell membrane enzyme utilizing ATP as a specific substrate. It is insensitive to ouabain, stimulated by Ca, regulated by calmodulin and its physiological function is possibly related to Ca efflux and K permeability processes [13]. The enzyme has two sites of binding of Ca, exists in two conformational states and its activity depends on a phosphorylated intermediate [2]. ATP interacts with the enzyme at a catalytic site and is capable of stimulating the hydrolysis of a phosphorylated intermediate by interacting with a second non-catalytic site [4].

FDP acts as a positive effector of Ca/Mg-ATPase and is bound by a red cell membrane preparation with an apparent K_s which is intermediate between the two K_s values of ATP for Ca/Mg-ATPase [4]. These possibly related phenomena may be associated to a third effect, reported elsewhere [11] on different intact cells and rat erythrocytes [12], and here on human red cell consisting of a FDP-dependent stimulation of K^+ uptake and acidification of medium. The present results also support the previously put forward contention of a specific binding site for FDP in the red cell membrane [11]. The increased uptake of K^+ supports the previously observed hyperpolarizing effect of FDP [10] and the increase of internal pH accounts for the ability of FDP to act as an activa-

tor of glycolysis[7-9]. In conclusion, FDP in able to interact with the human red cell membrane and such an interaction triggers a chain of a endocellular events which result in its eumetabolic effects.

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