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Effect of exogenously added acylphosphatases on inositol lipid metabolism in human platelets

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In this paper we demonstrate that human platelets contain an acylphosphatase isoenzyme. We then investigated the effect of exogenously added human muscle and erythrocyte acylphosphatases on inositol lipid content in human platelets permeabilized with saponin. Alterations in the level of the polyphosphoinositides were observed: in particular, the levels of phosphatidylinositol 4,5-bisphosphate, and of phosphatidylinositol 4-monophosphate were decreased, whereas the level of phosphatidylinositol was increased. These results suggest that acylphosphatases promote polyphosphoinositide dephosphorylation, possibly through intracellular Ca^{2+} mobilization.

Inositol lipid; Platelet; Acylphosphatase

1. INTRODUCTION

It is well known that muscle acylphosphatase (EC 3.6.1.7) catalyzes the hydrolysis of acylphosphates in solution and bound to membrane proteins in several vertebrate species; examples of this activity include the Na^+,K^+ and Ca^{2+},Mg^{2+}-dependent ATPases [1,2]. Recently, a novel acylphosphatase has been purified and sequenced from human erythrocytes. Although similar in molecular mass, this enzyme exhibits greater than 50% variability in amino acid residues when compared to the muscle isoenzyme; however, the kinetic parameters are very similar [3]. The two acylphosphatases are differently distributed in organs and tissues in different species: in horse, the muscular form is abundant in skeletal muscle, heart, and brain, although lower amounts were found in liver, kidney and erythrocytes [4]; on the other hand, in human the erythrocyte form is more abundant in brain, erythrocytes and blood platelets. The content of the muscular form in platelets has not been determined as yet.

Permeabilization of human platelets with saponin has recently been used to investigate the effect of various compounds on inositol lipid metabolism and protein phosphorylation [5–10]. In the present study we describe the effect of exogenously added acylphosphatases on inositol lipid metabolism and protein phosphorylation in intact and permeabilized platelets.

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Abbreviations: DTT, dithiothreitol; Hepes, 2-(4-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis

2. EXPERIMENTAL

Acylphosphatases were purified from human muscle (-SH form) and erythrocytes as previously reported [3,11–13]. Acylphosphatase activity was determined by continuous optical test at 238 nm using benzoylphosphate as substrate, according to Ramponi et al. [14]. Saponin was purchased from Merck; carrier-free ^{32}P-orthophosphate was from Amersham; precoated silica gel plates were from Whatman; phosphatidylinositol 4,5-bisphosphate (PIP_2) and thrombin were

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from Sigma; [^{3}H]PIP_2 was from New England Nuclear. All other reagents were analytical grade, or the best commercially available.

Preparation of washed platelets from healthy volunteers, and pre-labelling with ^{32}P-orthophosphate were performed as described [6]. Samples (0.5 ml) of $^{32}P_i$-labelled platelets were then placed in aggregometer tubes at 37°C, while stirring, in a Elvi aggregometer, in the presence of 1 mM ATP, 0.1 mM DTT, and 80 units of erythrocyte, or skeletal muscle acylphosphatase (-SH form). After 1 min, saponin was added, and incubation was carried out for 2 min. Extraction and separation of inositol phospholipids on thin-layer chromatography (TLC) plates were performed as described [6,7]. In some experiments, samples of $^{32}P_i$-labelled platelets were analyzed by polyacrylamide gel electrophoresis, performed according to Laemmli [15], using a 11% polyacrylamide continuous gel. Radioactivity of the bands was evidenced by autoradiography. In another set of experiments, designed to study the effect of acylphosphatase on PIP_2 in vitro, 37.5 μg of PIP_2, or 37.5 μg of PIP_2 plus 0.375 μCi of [^{3}H]PIP_2 were dissolved in chloroform and the solvent was evaporated with a stream of N_2. The residue was dissolved in 0.5 ml of 50 mM Hepes, pH 7.4, containing 1 mM $MgCl_2$, or in 50 mM acetate buffer, pH 5.3, containing 1 mM $MgCl_2$. The incubation of the sonicated solutions with human skeletal muscle acylphosphatase (100 units) was carried out at room temperature for 15 min, and stopped by addition of 2 ml of a solution of chloroform/methanol/HCl (100:200:2, v/v). The polyphosphoinositides were then separated and identified as previously described [7].

3. RESULTS AND DISCUSSION

We have determined acylphosphatase content in human platelets by a non-competitive enzyme-linked immunoadsorbent assay (ELISA) carried out with polyclonal anti-(erythrocyte acylphosphatase) antibodies [12]. Acylphosphatase content determination by this method resulted in 23.15 ± 0.74 ng/mg protein (mean ± SE, n = 3).

In order to investigate the role of the enzyme on platelet inositol lipid metabolism, we have added exogenous acylphosphatases to intact and saponin-permeabilized human platelets. Table 1 shows that addition of acylphosphatases to permeabilized human platelets caused a modification of polyphosphoinositides level. Both human skeletal muscle and human erythrocyte isoenzyme produced a loss of PIP_2 and phosphatidylinositol 4-monophosphate (PIP), and an increase of phosphatidylinositol (PI). The decreased levels of PIP_2 and PIP with a concomitant increase of PI indicate phosphomonoesteratic cleavage of the phosphates in position 4 and 5 of the inositol moiety of the polyphosphoinositides. It should be noted

Table 1

Effect of acylphosphatases and calcium on inositol lipid levels in permeabilized human platelets

Treatment	PIP_2	PIP	PI
None	5226 ± 38	10450 ± 741	6257 ± 225
HSM-AP	4668 ± 90*	6555 ± 348*	7239 ± 164*
E-AP	4969 ± 28*	8495 ± 176*	6707 ± 97
Ca^{2+}	1201 ± 107*	2612 ± 128*	7408 ± 112*

Washed human platelets, pre-labelled with $^{32}P_i$, were treated with 20 μg/ml of saponin, and with human skeletal muscle acylphosphatase (HSM-AP), erythrocyte acylphosphatase (E-AP), or Ca^{2+} (500 μM) for 2 min. Phosphoinositides were extracted and separated on thin-layer chromatography plates. PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol. Results, expressed as cpm, are means ± SE of six replicate samples in a single experiment, one out of six that gave almost identical results. Statistical significance was assessed by Student's t-test. * $P < 0.02$ versus control (no addition). Control experiments have shown that addition of saponin alone does not affect the metabolism of inositol lipids, in agreement with previous reports [6,7]

that the effect of the muscular isoenzyme seemed more pronounced. Addition of acylphosphatases to intact platelets did not induce any change in platelet aggregation or inositol lipid metabolism (not shown). Addition of 500 μM Ca^{2+} to platelets pre-treated with saponin, produced a marked dephosphorylation of PIP_2 and PIP with a resultant accumulation of PI. The smaller increase of ^{32}P radioactivity in PI compared with the loss in PIP_2 and PIP could be explained by the observation that [^{32}P]PI has a specific activity 15 and 30 times lower than that of PIP_2 and PIP, respectively [16].

Alterations in inositol lipid metabolite levels were not accompanied by any modification of the pattern of platelet protein phosphorylation, as determined by SDS-PAGE analysis of samples incubated in the presence of $^{32}P_i$ and treated with saponin, or with saponin and the muscular or the erythrocyte acylphosphatase isoenzymes (fig.1). The effects reported in table 1 were not due to a direct hydrolytic action of acylphosphatases on PIP_2. Indeed, the [^{3}H]PIP_2 content in samples containing this inositol lipid, incubated with the two acylphosphatase isoenzymes, at pH 7.4 and 5.3, was the same as that of controls, as monitored by autoradiography of TLC separation of the polyphosphoinositides (table 2).

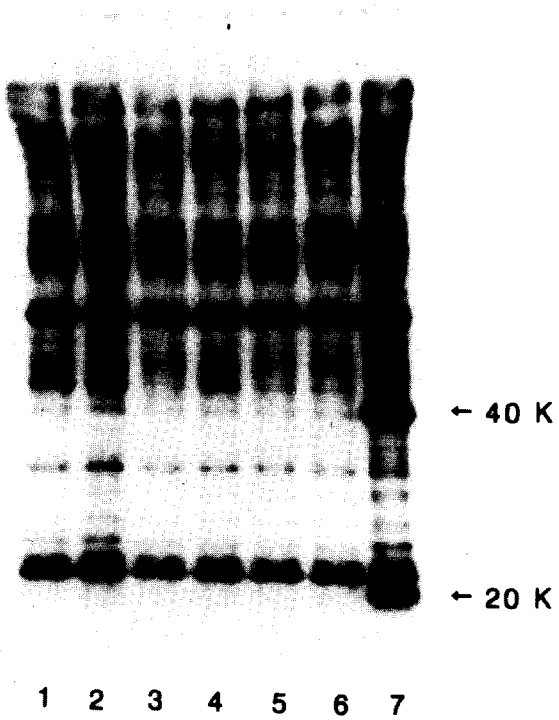

Fig.1. Effect of acylphosphatase and thrombin on protein phosphorylation in human platelets prelabelled with $^{32}P_i$. Washed human platelets prelabelled with $^{32}P_i$ were incubated in the aggregometer tubes as in table 1. Autoradiography shows the ^{32}P-labelled protein separated on an 11% SDS-polyacrylamide gel. Each sample is equivalent to 0.015 ml of the original 0.5 ml platelet suspension. Lanes: 1,2, control (no addition); 3,4, saponin; 5,6, human skeletal muscle acylphosphatase plus saponin; 7, thrombin (0.5 units/ml). It is worth noting that acylphosphatase does not induce any apparent modification in the pattern of protein phosphorylation in permeabilized platelets. Thrombin induces the phoshorylation of the 20 and 40 kDa proteins which are, respectively, the substrates for myosin light chain kinase and protein kinase C.

The results described above indicate that: (i) human platelets contain an acylphosphatase isoenzyme as determined by ELISA; (ii) added acylphosphatases are able to modify the pattern of inositol lipid content in human platelets; (iii) this effect is not accompanied by modifications of the protein phosphorylation pattern; (iv) this effect is not caused by direct hydrolytic action of the two isoenzymes on PIP_2.

Previous studies [1,2] have demonstrated that acylphosphatase is able to hydrolyze the phosphorylated intermediate formed during the activity of the Na^+,K^+- and Ca^{2+},Mg^{2+}-dependent ATPases, the latter in sarcoplasmic reticulum vesicles. It is well known that the Ca^{2+},Mg^{2+}-dependent ATPase is also present in the dense tubular system [17], that represents one of the major Ca^{2+} stores in platelets; consequently, it is conceivable that acylphosphatase might catalyze the hydrolysis of the phosphorylated intermediate which is formed during Ca^{2+} transport in the dense tubular system. Indeed, functional similarity between the Ca^{2+},Mg^{2+} ATPase from sarcoplasmic reticulum vesicles and that from membrane of the dense tubular system, has been demonstrated. Thus, acylphosphatase action on the phosphorylated intermediate might raise the level of free Ca^{2+}, promoting the phosphomonoesteratic cleavage of polyphosphoinositides [6,18]. In this regard, it is important to note that the protocol for purification of the enzymes, described in detail in [3,12,13], excludes the possibility of contamination by Ca^{2+}.

Results shown in table 1 indicate that Ca^{2+} promotes a more drastic dephosphorylation of PIP_2 and PIP in comparison to acylphosphatases. However, it should be noted that the amount of Ca^{2+} used in those experiments (500 μM) is presumably much higher than that possibly raised by the action of acylphosphatases on the Ca^{2+},Mg^{2+}-dependent ATPase.

The amount of acylphosphatases used in the experiments reported above is higher than that physiologically present in human platelets; however, it should be considered that, in a permeabilized system, one is forced to administer large quantities of a compound in order to observe an effect. This holds true, as an example, for the intracellular Ca^{2+}-mobilizer, inositol (1,4,5)-trisphosphate, that has to be administered in the 15–45 μM range in order to induce platelet activation [8].

In conclusion, this study demonstrates that human platelets do contain an acylphosphatase isoenzyme and that acylphosphatase might play a role in the metabolism of inositol lipids; the exact nature and extent of this involvement require further study.

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Table 2

Effect of human skeletal muscle acylphosphatase on phosphatidylinositol 4,5-bisphosphate in vitro

	pH 5.3		pH 7.4	
	Control	HSM-AP	Control	HSM-AP
[^{3}H]PIP_2	78265 ± 692	78423 ± 897	70817 ± 971	71690 ± 1073

37.5 μg of PIP_2, or 37.5 μg of PIP_2 plus 0.375 μCi of [^{3}H]PIP_2 were dissolved in chloroform and the solvent was evaporated with a stream of N_2. The residue was dissolved in 0.5 ml of 50 mM acetate buffer, pH 5.3, containing 1 mM $MgCl_2$, or in 50 mM Hepes, pH 7.4, containing 1 mM $MgCl_2$. Incubation of the sonicated solutions with human skeletal muscle acylphosphatase (HSM-AP, 100 units) was carried out at room temperature for 2 h, and stopped by addition of chloroform/methanol/HCl (100:200:2, v/v). Inositol phospholipids were separated by thin-layer chromatography as described [7]. Results expressed as cpm, are means ± SE (n = 3). It should be mentioned that 99.7 ± 0.02% of the total recovered radioactivity was found associated with PIP_2

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