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Association of phosphatidylinositol kinase and phosphatidylinositol 4-phosphate kinase activities with the cytoskeleton in human platelets

Nabeel Nahas*, Monique Plantavid, Gérard Mauco and Hugues Chap

INSERM Unité 101, Biochimie des Lipides, Hôpital Purpan, 31059 Toulouse, France and **Birzeit University, Birzeit, Occupied West Bank*

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The inositol lipid kinases were investigated in the cytoskeletons of human platelets. In the absence of added lipids the kinases were only barely detectable in the Triton-soluble fractions and undetectable in cytoskeletons of resting cells. However at least 30% of the total phosphatidylinositol kinase was present in the cytoskeleton as revealed by saturation of the enzyme. Phosphatidylinositol 4-phosphate kinase was also found in significant amounts in the cytoskeletons. On the other hand, both enzymes being only recovered in the particulate fraction of the cells, we suggest that inositol lipid kinases may be present near the anchoring points of the cytoskeletons at the membranes.

Cytoskeleton; Lipid kinase; Phosphatidylinositol; Phosphatidylinositol 4-phosphate; Phosphatidylinositol 4,5-bisphosphate; (Platelet)

1. INTRODUCTION

The role of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) in platelet activation may be viewed from several aspects. Firstly, there have been numerous studies describing its hydrolysis by phospholipase C as one of the earliest events following the binding of a variety of agonists to their membrane receptors [1–7]. This contributes to an increase of intracytoplasmic Ca^{2+} [8,9] and to activation of calmodulin-dependent protein kinase and protein kinase C [10]. Secondly, the relationship between PtdIns(4,5)P_2 metabolism and the dramatic morphological changes also occurring immediately after agonist binding begins to be explored. Reorganization of cytoskeleton is accompanied by a series of protein phosphorylations including those of myosin light chain and actin-binding protein [11–13] and so may be understood as a consequence of PtdIns(4,5)P_2 breakdown. On the other hand, Lassing and Lindberg [14] have shown that PtdIns(4,5)P_2 can bind to profilin, cause a rapid and efficient dissociation of profilactin and thus liberate G-actin for polymerisation. In this case, the generation of PtdIns(4,5)P_2 could control the first morphological changes following F-actin formation [15]. Sustaining this hypothesis, we have previously described an increase in the platelet content of phosphatidylinositol 4-phosphate (PtdIns(4)P) and PtdIns(4,5)P_2 upon thrombin treatment [16,17].

Here, we report on further evidence for a possible close relationship between polyphosphoinositides and cytoskeletal proteins, since the present study demonstrates the association of phosphatidylinositol kinase (PtdIns kinase) and PtdIns(4)P kinase with the cytoskeleton in human platelets.

Correspondence address: G. Mauco, INSERM Unité 101, Biochimie des Lipides, Hôpital Purpan, 31059 Toulouse, France

Abbreviations: PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate

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2. EXPERIMENTAL

2.1. *Isolation of cytoskeleton*

Washed human platelets ($2-3 \times 10^9$ ml^{-1}) were suspended in Tyrode's solution (pH 7.35) containing 2.5 mM Ca^{2+}, 1 mM Mg^{2+} and 0.35% (w/v) bovine serum albumin [18]. Aliquots (0.5 ml) were triggered by thrombin (1 IU·ml^{-1}) for 1 min at 20°C in the presence (stimulated platelets) or absence (aggregated platelets) of 10 mM EGTA. Cytoskeletons were obtained by addition of 1 vol. of a lysis buffer containing 2% Triton X-100, 10 mM EGTA, 2 mM PMSF, 0.2 mM leupeptin and 50 mM Tris-HCl (pH 7.4). After 5 min at room temperature followed by 10 min on ice, cytoskeletons were pelleted by centrifugation ($4000 \times g$/20 min/4°C), as described elsewhere [19]. The pelleted cytoskeletons were further washed with lysis buffer diluted twice with 50 mM Tris-HCl, pH 7.4, and finally resuspended in 0.5 ml of 50 mM Tris-HCl, pH 7.4, by brief sonication (3×10 s, MSE sonifier, maximum output). Supernatants (0.1 ml) i.e. Triton-soluble material, were diluted with 4 vol. of 50 mM Tris-HCl (pH 7.4) when assayed for PtdIns and PtdIns(4)P kinase activities. Protein content was measured as in [20].

2.2. *Assay of PtdIns and PtdIns(4)P kinases*

The assay mixture (0.52 ml) was made of 80 μM [γ-^{32}P]ATP (10 Ci/mmol), 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4). When noted, sonicated PtdIns and/or PtdIns(4)P were added at the concentrations indicated in the legends. After 5 min at 37°C in a shaking water bath, the reaction was stopped by adding 0.025 ml of 200 mM EDTA (pH 7.4) and lipids were immediately extracted [21,22]. Inositol lipids were separated by thin-layer chromatography [22]. After autoradiography (MP films, Amersham) radioactive spots were scraped off and counted in Instagel (Packard).

2.3. *Electrophoresis*

5 vols of washed cytoskeletons were dissolved in 1 vol. of 6.6% SDS, 0.6 M β-mercaptoethanol, 15 mM EDTA, 60% glycerol and heated in boiling water for 2 min. SDS-polyacrylamide gel electrophoresis was then carried out as in [23] using a 7.5–12% acrylamide gradient for the resolving gel. Gels were stained with Coomassie brilliant blue.

2.4. *Primary fractionation of platelets*

Platelet suspension in 50 mM Tris-HCl, pH 7.4 (0.5 ml), was sonicated on ice for 3×10 s (MSE sonifier, maximum output) and then centrifuged for 90 min, $105000 \times g$, at 4°C. Particulate and cytosoluble fractions were then assayed for PtdIns and PtdIns(4)P kinases as described above (150–250 μg proteins per assay).

2.5. *Materials*

PtdIns, PtdIns(4)P, PtdIns(4,5)P_2 were from Sigma (St. Louis, MO); [γ-^{32}P]ATP was from Amersham plc (Amersham, England).

3. RESULTS

3.1. *Association of PtdIns and PtdIns(4)P kinases with the particulate fraction*

As previously described in other cells [24–26], we have noted an almost exclusive localisation of PtdIns and PtdIns(4)P kinases in the particulate fraction of platelets (table 1). Addition of exogenous PtdIns revealed little PtdIns and PtdIns(4)P kinase activities in the cytosol and a non-significant enhancement of particulate activities. The same kind of effects were observed upon addition of PtdIns(4)P in both cytosol and particulate fractions. In conclusion, it appeared that most if not all of the activities were associated with organelles.

3.2. *Association of PtdIns and PtdIns(4)P kinases with the cytoskeleton*

When platelets were extracted with 1% Triton X-100, we obtained a so-called cytoskeleton as shown in fig.1. Stimulation by thrombin (1 IU·ml^{-1}) in the absence or presence of EGTA led to the appearance of aggregated or only stimulated platelets, respectively, allowing one to

Table 1

Inositol lipid kinases in cytosol and particulate fractions from platelets

		PtdIns kinase			PtdIns4P kinase		
Addition		None	PtdIns	PtdIns4P	None	PtdIns	PtdIns4P
Cytosol	SA	0.7 ± 0.3	3.5 ± 1.6	0.8 ± 0.2	0.1 ± 0.02	1.0 ± 0.5	2.7 ± 2.1
	%	2.6 ± 1.1	11.8 ± 6.4	0.9 ± 0.4	1.6 ± 0.2	9.8 ± 5.7	13.1 ± 4.9
Membranes	SA	47.7 ± 7.0	63.1 ± 20.6	68.7 ± 22.4	10.2 ± 2.4	11.9 ± 2.5	12.0 ± 4.1
	%	97.4 ± 1.1	88.2 ± 6.4	99.1 ± 0.4	98.4 ± 0.2	90.2 ± 5.7	86.9 ± 4.9

Results are mean values ± SE (n = 4) and are expressed as specific activities (SA): pmol of phosphorylated lipid·min^{-1}·mg protein^{-1} or as percentages of the total activities (%)

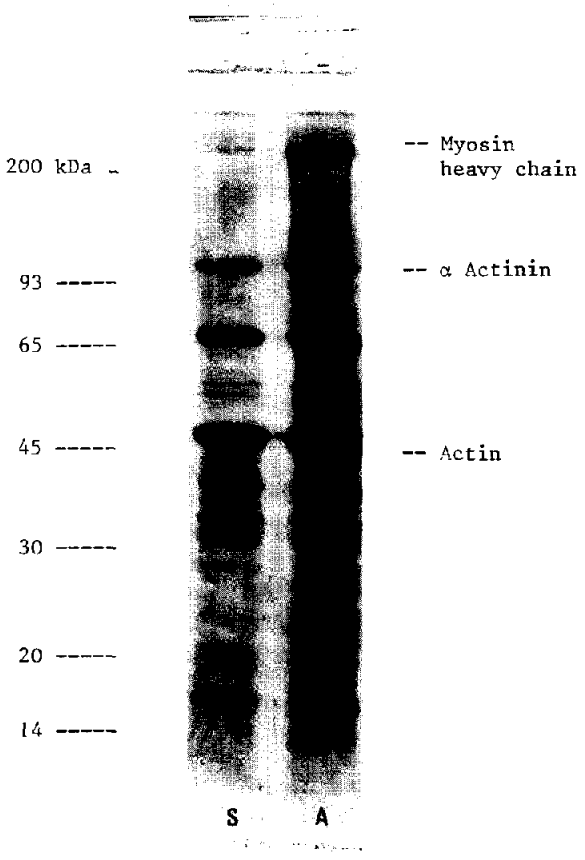

Fig.1. Electrophoretic patterns of Triton insoluble material from platelets. SDS-polyacrylamide gel electrophoresis on 7.5–12% acrylamide of Triton-insoluble cytoskeletons prepared from thrombin-activated platelets (S) and thrombin-aggregated platelets (A).

obtain electrophoresis patterns similar to those previously described by others [19,27].

We could detect both PtdIns and PtdIns(4)P kinases in these cytoskeletons, in the presence of added inositol phospholipids (figs 2,3). Such an activity could also be found in the supernatants obtained after removal of Triton X-100-insoluble material (i.e. cytoskeletons) and diluted in order to obtain a final Triton X-100 concentration of 0.2% for PtdIns and PtdIns(4)P kinase assays. This dilution also allowed us to adjust the protein concentration of Triton-soluble proteins to that obtained with cytoskeletons. Furthermore, it avoided inhibition of kinase activities observed with high Triton

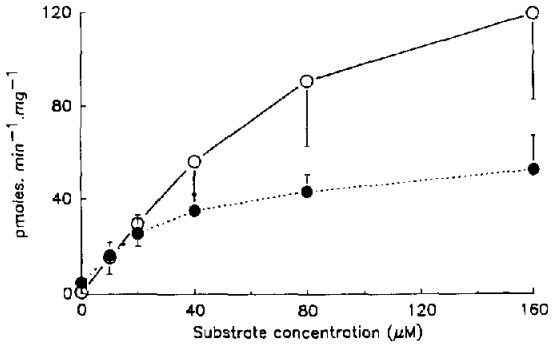

Fig.2. Substrate dependency of PtdIns kinase. Triton X-100-soluble and -insoluble fractions from stimulated platelets were incubated as indicated in section 2. Results are mean values ± SE from 5 to 8 independent experiments for cytoskeleton (open circles) and from 4 experiments for the Triton-soluble supernatant (closed circles). Protein concentrations were 285 ± 38 μg per assay for cytoskeleton and 185 ± 22 μg per assay for the Triton-soluble fraction.

concentrations (not shown). It was verified that 0.2% Triton X-100 did not significantly modify the kinase activities in the cytoskeleton (95.0 ± 5.8% of control values, $n = 4$).

Figs 2 and 3 depict the saturation curves of PtdIns and PtdIns(4)P kinases obtained for both the cytoskeleton and Triton-soluble fraction of stimulated platelets. It was clear that addition of PtdIns and PtdIns(4)P to either preparation increased inositol lipid kinase activities.

As seen in fig.2, the mean specific activity of PtdIns kinase was 120 ± 37 pmol PtdIns(4)P produced·min^{-1}·mg protein^{-1} ($n = 5$) in the cytoskeleton and 53 ± 15 pmol PtdIns(4)P pro-

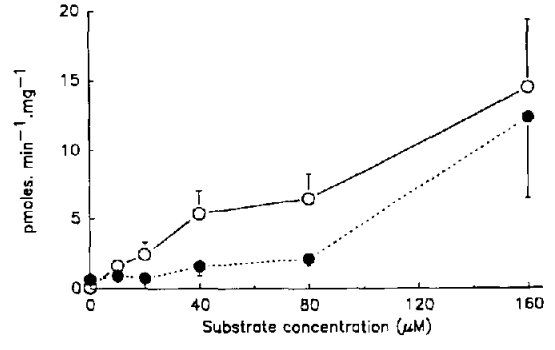

Fig.3. Substrate dependency of PtdIns(4)P kinase. For legend see fig.2.

Table 2

Comparison of the properties of cytoskeletons from stimulated and aggregated platelets

	Added lipids (μM)	Stimulated	Aggregated
% of total proteins		15.4 ± 0.2	16.5 ± 1.1
Specific activities	0	0.6 ± 0.2	3.7 ± 1.0
	160	120.0 ± 37.0	93.5 ± 2.7
Total amounts	0	0.2 ± 0.1	1.4 ± 0.4
	160	32.3 ± 10.4	30.0 ± 3.2

Results are mean values ± SE (n = 5) and are expressed as pmol of phosphorylated lipids · mg protein^{-1} · min^{-1} for specific activities and as pmol of phosphorylated lipids · min^{-1} for total activity in cytoskeletons

duced · min^{-1} · mg protein^{-1} (n = 4) in the Triton-soluble fraction when phosphoinositides were adjusted to 160 μM. Since 15.4 ± 0.2% (n = 4) of the total proteins were recovered in the cytoskeleton of stimulated platelets, at least 30% of PtdIns kinase were associated with the cytoskeleton. On the other hand, due to the atypical behaviour of the PtdIns(4)P kinase (fig.3), we could not obtain quantitative comparisons of activities in the two fractions.

Spontaneous activities of both PtdIns and PtdIns(4)P kinases (i.e. without any substrate addition) were only detectable in Triton-soluble fractions while cytoskeleton failed to incorporate significant amounts of ^{32}P into lipids. However, the comparison of the data obtained in either stimulated or aggregated platelet cytoskeletons even in the presence of 160 μM PtdIns plus PtdIns(4)P did not reveal any significant variation in their total amounts of PtdIns kinase.

4. DISCUSSION

These data show an association of PtdIns and PtdIns(4)P kinases with the Triton-insoluble fraction of platelets, i.e. with the so-called cytoskeleton. A similar finding has been reported for PtdIns kinase in erythrocytes [28]. On the other hand, we have confirmed the association of the kinases with the particulate fraction of platelets. It appears therefore that PtdIns and PtdIns(4)P kinases might be present at anchoring points of the cytoskeleton in the plasma membrane (and possibly other organelles). Such an association of the cytoskeleton with membrane enzymes has already been described even for intrinsic proteins such as glycoproteins IIb/IIIa, Ib or Ia [29–32].

We have also previously reported the association of some membrane lipids with the cytoskeleton [33] and more importantly, Verhallen et al. [34] have shown the role of intact cytoskeleton in maintaining membrane asymmetry which is essential for platelet function. We report here a new type of interaction between cytoskeleton and lipids: since PtdIns(4,5)P_2 availability is a key process in the agonist-activated production of diglyceride and inositol 1,4,5-trisphosphate, it appears that an important part of the enzyme activities responsible for the stability of PtdIns(4,5)P_2 level is associated with cytoskeleton. This opens a new way for understanding the regulation of this process. Actually, an interesting point is the possible regulation of PtdIns kinase by tyrosine kinases [35–37] and it is interesting to note that two major tyrosine kinases, pp60^{src}, which is present in significant amounts in platelets [38,39], and epidermal growth factor receptor, have been described associated with cytoskeletal structures [40,41].

The existence of cytoskeletal interactions with phospholipids can also be viewed in the opposite way, the phospholipids regulating the status of the cytoskeleton. For instance, PtdIns(4,5)P_2 causes a rapid dissociation of profilactin which is accompanied by polymerisation of G-actin [14]. We have shown that PtdIns(4,5)P_2 are not only degraded upon platelet stimulation but, after a rapid decrease, are synthesized in excess compared to the basal state [16,17]. Therefore, such an increase can be suggested as a means of regulating actin polymerization, an essential step in platelet activation.

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