Biochimica et Biophysica Acta, 1069 (1991) 27–36

ADONIS 0005273691003605

BBAMEM 75372

Asymmetric distribution of phosphoinositides and phosphatidic acid in the human erythrocyte membrane

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(Received 25 March 1991)

Key words: Phosphoinositide; Phosphatidic acid; Antibody to polyphosphoinositide; Phospholipase A_2; Erythrocyte membrane

The distribution of phosphoinositides and phosphatidic acid (PA) between the outer and inner layers of the human erythrocyte membrane was investigated by using two complementary methodologies: hydrolysis by phospholipase A_2 (PLA_2) and immunofluorescence detection with monoclonal antibodies against polyphosphoinositides. The contents of phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-phosphate (PIP) and PA were decreased by 15–20% after 60 min incubation with PLA_2, while that of phosphatidylinositol (PI) was increased. Studies with ^{32}P-labelled cells revealed that PLA_2 treatment led to indirect effects on the metabolism of these phospholipids. Therefore, the asymmetric distribution of phosphoinositides and PA was inferred from the data obtained in ATP-depleted erythrocytes. In these cells with arrested phosphoinositide metabolism, the asymmetric distribution of the major phospholipids was maintained: PLA_2 hydrolyzed approx. 20% of PI, PIP_2 and PA (but no PIP) indicating their localization in the outer layer of the membrane. This finding was confirmed by immunofluorescence studies with antibodies specific to each phosphoinositide. External addition of anti-PIP_2 but not anti-PIP gave a positive reaction both in control and in ATP-depleted erythrocytes. A pretreatment of cells with PLA_2 led to a decrease in the intensity of anti-PIP_2 staining. These results demonstrate that significant fractions of PIP_2, PI and PA are localized on the outer surface of the erythrocyte membrane.

Introduction

Phospholipid asymmetry is clearly established in erythrocyte membranes. The aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), are concentrated in the inner leaflet while the choline-containing phospholipids, sphingomyelin (SM) and phosphatidylcholine (PC), are mainly located in the external leaflet [1]. Specific lipid asymmetry may be a transient feature of many membranes to serve some define functions. For instance, platelet activation results in a reorientation of PS from the inner to the outer layer promoting the activation of the blood coagulation system [2]. An asymmetric distribution of phosphatidylinositol (PI) has been also reported with 20–40% located in the outer layer of the membrane of various mammalian erythrocytes [3–5]. A separate class of externally located and glycosylated PI molecules are responsible for anchoring cell surface proteins [6]. In contrast, although physiologically relevant, polyphosphoinositides, phosphatidylinositol 4-monophosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidic acid (PA) have never been localized. These phospholipids, especially PIP_2 play a major role as precursor of second messengers in many cells [7]. They also regulate a number of membrane properties such as controlling calcium pumps [8–10], protein–protein interactions [11] or transfer within the membrane [12], the anchoring of polar proteins by covalent linkage [13] or the exocytosis process [14,15]. This functional diversity may be associated with structural variation of the fatty acid pattern and/or with asymmetrical distribution across the membrane bilayer.

Abbreviations: PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; BSA, bovine serum albumin; PLA_2, phospholipase A_2; PIC, phosphoinositidase C; FITC, fluorescein isothiocynate; PBS, phosphate-buffered saline.

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In the present study, we investigated the transmembrane distribution of phosphoinositides and PA in human erythrocytes using two different methods: hydrolysis by phospholipase A_2 (PLA_2) and immunofluorescence detection with monoclonal antibodies raised against polyphosphoinositides [16]. Experiments with PLA_2 were performed both in control and in ATP-depleted erythrocytes. However, only the data obtained under these latter conditions, under which the rapid metabolism of phosphoinositides and PA is arrested while their asymmetric distribution is unaffected as already shown for the other phospholipids [17–19], were used to estimate their transbilayer distribution. A small fraction (15–25%) of PIP_2, PI and PA but no PIP was found to be located in the outer layer of the membrane. The immunofluorescent studies confirmed directly that a significant fraction of PIP_2 was localized within the outer layer, whereas all PIP was contained within the inner layer of the erythrocyte membrane. A preliminary report of this work has been published elsewhere [20].

Materials and Methods

Chemicals

$[^{32}P]P_i$ (sodium salt) was purchased from International CIS (Saclay, France). PLA_2 (bee venom P-9279 and *naja naja* P-6139), sphingomyelinase C from *S. aureus* (S-8633), lysoPC (L-4129), sodium oleate (O-7501), arachidonic acid (A-6382), iodoacetamide (I-6125), bovine serum albumin (BSA), fraction V fatty acid free (A-6003), FITC-avidin (A-2901), ovalbumin (A-5378) and standards for thin-layer chromatography were obtained from Sigma (St. Louis, MO, U.S.A.). Cobalt chloride hexahydrate was from Merck (Darmstadt, F.R.G.) and inosine from Boehringer-Mannheim (F.R.G.). All other reagents used were of analytical grade. Silica gel 60-precoated plates were from Schleicher and Schüll (Dassel, F.R.G.). Monoclonal antibody (ascite fluid) to PIP_2 (IgG2b) and PIP (IgG3) were produced as reported previously [16]. Anti-spectrin antibody (IgG) produced from rabbit was a gift of Dr. D. Dhermy (U.160 INSERM, Paris, France). Anti-mouse IgG biotinylated antibody was from Amersham (France). Citifluor was from Citifluor Ltd. (London, U.K.).

Preparation of erythrocytes

Freshly drawn and heparinized human blood was centrifuged (1300 × *g*, 10 min, 4°C) to remove plasma and buffy coat. Packed erythrocytes were washed three times in buffer A (154 mM NaCl, 1.5 mM Hepes-NaOH (pH 7.4), 10 mM glucose). After each run, the upper layer of the cell pellet (approx. 10% of total cells) was carefully discarded. With the exception of the incubations, all steps were performed at 4°C.

Incubation and treatments of erythrocytes

Cell suspensions were prewarmed (15 min, 37°C) in buffer A (hematocrit 40%) and were incubated for 2 h at 37°C with (or without) $[^{32}P]P_i$ (0.45–0.90 MBq/ml of cells). The incubation was stopped by the addition of ice-cold buffer A. Cells were washed twice in the same buffer and resuspended at an hematocrit of 25% before further treatments.

Phospholipase treatments. Cell suspensions were incubated for various times (15 to 90 min) at 37°C with or without bee venom PLA_2, alone or combined with *naja naja* PLA_2 (20 to 100 units/ml of cells) in the presence of $CaCl_2$ (250 μM to 1 mM, final concentration). The reaction was stopped by the addition of 4 vol. of ice-cold buffer A containing 1.2 mM EGTA (buffer B). When required, sphingomyelinase C (2 units/ml of cells) was added after 30 min of incubation with bee venom PLA_2. ATP depletion was performed prior PLA_2 treatment by an incubation (60 min, 37°C) in a buffer containing 154 mM NaCl, 10 mM Hepes-NaOH (pH 7.4), 6 mM iodoacetamide and 10 mM inosine. The ATP content measured as previously described [21] was decreased by more than 95% after this treatment (results not shown).

LysoPC or fatty acid treatments. Cell suspensions were incubated for 60 min at 37°C with or without lysoPC, sodium oleate or arachidonic acid (this latter dissolved in ethanol). All these drugs were used at 0.5 μmol/ml of cells. The incubation was stopped by the addition of 4 vol. of ice-cold buffer B.

Cobalt treatment. $CoCl_2$ (1 mM, final concentration) was added to cell suspensions just before the addition of $CaCl_2$ (250 μM, final concentration) and bee venom PLA_2 (20 units/ml of cells). The incubation was carried out for 60 min at 37°C and was stopped by the addition of 4 vol. of ice-cold buffer B.

The extent of red cell hemolysis was determined from the release of hemoglobin in the suspension medium at the end of the incubation, by photometric absorption at 540 nm.

Membrane preparation, lipid extraction and thin-layer chromatography of lipids

Packed erythrocytes were lysed in 25 vol. of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (buffer C) and centrifuged (25000 × *g*, 10 min). The resulting membrane pellet was incubated for 5 min at 4°C with 25 vol. of buffer C containing 1% (w/v) of fatty acid-free BSA and centrifuged to remove lysophospholipids and free fatty acids. Membranes were washed twice more in buffer C. Membrane protein concentration was determined by the method of Bradford [22], using BSA as a standard. Acidic lipid extraction, thin-layer chromatography (TLC) separations, and quantification of phosphoinositide and PA contents and radioactivities were carried out as previously described [23,24]. TLC of

major phospholipids (SM, PC, PS and PE) was performed using chloroform/methanol/acetic acid/water (77:49:10:6, v/v). Although the washing procedure with BSA is known to extract quantitatively lysoPC [25], we have checked that BSA was also able to extract lysoPE, lysoPA and lysoPIP$_2$. Under these conditions, there was no possible cross-contamination between lysophospholipids and the phospholipids in the chromatographic systems. When the presence of [^{32}P]lysoPIP$_2$ or [^{32}P]lysoPA was investigated, membranes were not washed with BSA and phospholipids were separated by TLC as described in Ref. 24. In that system, the radioactive spots of lysoPIP$_2$ and lysoPA were clearly separated from those of PIP$_2$, PIP and PA. LysoPIP$_2$ migrated between the origin and PIP$_2$, and lysoPA between PIP and PA as checked with standards prepared from ^{32}P-labelled membranes incubated with PLA$_2$. Phospholipid contents were measured by phosphorus assays after perchloric acid digestion of silica gel spots revealed by I_2 vapour or by autoradiography (for polyphosphoinositides and PA of ^{32}P-labelled cells). Radioactivity was measured in the same samples by Cerenkov counting after correction for quenching (dpm) as described by Gascard et al. [26].

Preparation of erythrocytes and thin sections for immunofluorescence detection

Washed erythrocytes were fixed for 2 h in freshly prepared periodate/lysine/paraformaldehyde fixative [27] and stored at 4°C in 2% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) until use. Intact erythrocytes were settled on polylysine-coated slides. Semi-thin sections (0.5 μm) of gelatin-embedded cells or ghosts obtained by cryoultramicrotomy were used for immunological detection of PIP, PIP$_2$ and spectrin. Cells and cell sections were washed in PBS containing 50 mM NH_4Cl to saturate the free aldehyde groups due to fixation and then incubated 30 min at 20°C with 1% ovalbumin to block non-specific reactions. Cells were then incubated overnight at 4°C in appropriate dilutions of antibodies. After rinsing in PBS, cells were incubated with 1:100 dilution of biotinylated anti-mouse IgG antibody for 1 h at room temperature. After an additional rinsing, cells were treated for 30 min with FITC-avidin diluted to 1:100, mounted in citifluor and observed with an epifluorescence microscope equipped with FITC emission filters (Axiovert 35 Zeiss).

Results and Discussion

Effect of PLA$_2$ on phospholipids in control erythrocytes

Preliminary experiments were undertaken to investigate whether polyphosphoinositides and PA were substrates for PLA$_2$. Artificial vesicles prepared by sonication of PC, PC/PS, PC/PIP$_2$, PC/PIP or PC/PA

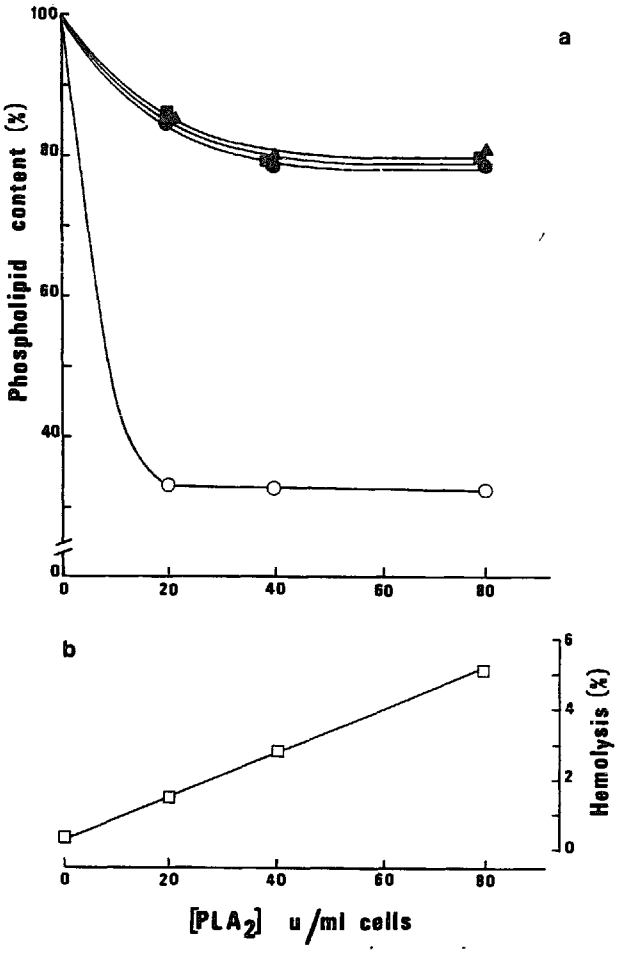


Fig. 1. Changes in the content of phospholipids (a) and extent of hemolysis (b) in erythrocytes upon treatment with increasing doses of PLA$_2$. Erythrocytes were preincubated for 2 h in buffer A before treatment for 60 min with different doses of bee venom PLA$_2$ in a medium containing 250 μM $CaCl_2$. Aliquots of the suspensions were centrifuged and cells were washed and hemolysed. The resulting membranes were washed once in the presence of BSA and twice without BSA. Phospholipids were extracted, separated by TLC and their content was measured (see Materials and Methods). Results shown are the means of three experiments. S.E. values (1–3%) were too small to be included in the gragh. PIP$_2$ (●); PIP (▲); PA (■); PC (○); cell hemolysis (□).

were incubated for 60 min at 37°C with bee venom PLA$_2$ (10–20 units/μmol phospholipid) and 250 μM $CaCl_2$. Under these conditions, 70–80% of each phospholipid were hydrolysed. These figures are consistent with the fraction of phospholipids present in the outer leaflet of single unilamellar vesicles [28].

The experimental conditions required for optimal PLA$_2$ action in erythrocytes (concentration dependence, origin of PLA$_2$ and time-course of action) were investigated in the presence of 250 μM $CaCl_2$. Fig. 1 shows the dose-response curves for the action of the bee venom PLA$_2$ on the breakdown of phospholipids and hemolysis of red cells incubated for 60 min. PC hydrolysis was maximal (66%) at a PLA$_2$ concentration

of 20 units/ml of cells (Fig. 1a). The PLA_2 treatment also promoted a decrease in the total content of polyphosphoinositides and PA. Higher enzyme concentrations, from 40 to 80 units/ml of cells, or longer periods of incubations, were inefficient in inducing further hydrolysis. A similar observation was made using the *naja naja* enzyme (data not shown). Fig. 1b shows that cell hemolysis was 1.5% at 20 units/ml of cells and increased to 5% with 80 units/ml of cells. To minimize direct access of the enzyme to the inner leaflet of hemolysed cells, the concentration of 20 units/ml of cells of the bee venom enzyme was used in all further experiments. It has been reported that PLA_2 accessibility to PC is increased when SM, the other major phospholipid of the outer half of the membrane, is hydrolysed by sphingomyelinase C [29]. This treatment indeed resulted in the hydrolysis of about 80% of SM and in an increase in the accessibility of PLA_2 to PC (from 62 to 74%) but did not cause any effect on PIP_2, PIP and PA, as compared to PLA_2 alone (results not shown).

The decrease of approx. 15% in the content of PIP_2, PIP and PA was associated with an increase of 18% in that of PI after 60 min incubation with PLA_2 (Table I). Thus, the diminution in PIP_2 and PIP contents was almost totally balanced by an increase in PI so that the total content in phosphoinositides remained nearly constant (Table I). To investigate whether these changes were due to metabolic effects resulting from PLA_2 treatment, erythrocytes were prelabelled with [^{32}P]Pi for 2 h prior to the addition of the enzyme. The specific radioactivity of PIP_2 did not change after PLA_2 treatment, whereas that of PIP decreased significantly and that of PA greatly increased (Table I). TLC analysis of the lipid extracts from PLA_2-treated samples (from membranes not washed with BSA) revealed the presence of a labelled compound comigrating with an authentic standard of lysophosphatidic acid. This clearly indicates that a fraction of PA was accessible to the enzyme in intact cells (results not shown). LysoPC was also observed under these conditions. However, no $lysoPIP_2$, lysoPIP or lysoPI were ever detected, partly because approximately half of these amphiphilic compounds is lost in the aqueous phase during the extraction procedure (results not shown) and partly for metabolic reasons. The hydrolysis of PIP_2 by PLA_2 should lead to decreasing, and not to maintaining constant, the total phosphoinositide content. Since all the relevant enzymes are functional in the red cell, a plausible explanation of these data is that $lysoPIP_2$ generated from PIP_2 was hydrolysed by phosphatases in lysoPI, itself reacylated in PI.

To assess whether the two split-products of PC hydrolysis were able to affect phosphoinositide and PA metabolism, erythrocytes were incubated for 60 min in the presence of either lysoPC, fatty acids (oleic or arachidonic) or both, at the concentrations estimated from the amount of PC hydrolysed by the PLA_2 (0.5 μmol of each/ml of cells). Hemolysis was between 8 and 12% with lysoPC, 2 and 3% with fatty acids and 6 and 7% with both drugs. The large extent of hemolysis as compared to that induced by PLA_2, indicated that these treatments were more drastic to the membrane integrity than the simple replacement of the molecules of hydrolysed PC by their split-products. LysoPC induced a decrease in the specific radioactivity of PIP (23%). Oleic (or arachidonic) acid caused a decrease in the content of PIP_2 (12%) and an increase in the specific radioactivity of PA (37%). In ATP-depleted cells, lysoPC had no effect. Only the fatty acids decreased the content of PIP_2 (12%). Thus, lysoPC and fatty acids likely inhibit PI kinase and stimulate diacylglycerol kinase, respectively. Some of the effects of these amphipilic molecules on the activity of membrane-bound enzymes involved in the metabolism of phosphoinositides and PA have been previously observed in cells other than erythrocytes [30–32].

These data indicate that the effects of PLA_2 on phosphoinositides and PA do not result only from the breakdown of molecules localized in the outer half membrane, but also from an alteration in the equilibrium between the kinases and phosphatases involved in their turnover. Another possibility is that the decrease in the content of polyphosphoinositides, together with the increase in the specific radioactivity of PA, could result from an activation of phosphoinositidase C (PIC).

TABLE I

Effect of PLA_2 on the content and ^{32}P specific activity (S.A.) of phospholipids in control erythrocytes

Cells were labelled for 2 h with [^{32}P]P_i, washed and reincubated for 1 h with or without PLA_2 (20 units/ml of cells) as described in Legend to Fig. 1. The values are the means ± S.E. of duplicate determinations in five experiments. Sum is the total content of phosphoinositides.

	% of controls						
	PIP_2	PIP	PI	Sum	PA	PC	PS
Content	83.9±3.2 *	88.7±2.7 *	118±6.3 *	95.9±3.0	84.3±1.1 *	36.0±2.3 *	103±1.5
S.A.	100.6±1.7	85.4±3.8 *			152 ±8.9 *		

* Significantly different from matched controls ($P < 0.05$).

TABLE II

Effect of PLA_2 on the content of phospholipids in erythrocytes incubated under different conditions

Incubation was conducted for 1 h with or without PLA_2 (20 units/ml of cells) in media containing the indicated concentrations of $CaCl_2$ and $CoCl_2$. The values are the means ± S.E. of duplicate determinations in three to five experiments. Changes in contents induced by PLA_2 are expressed as percentages of paired controls incubated under the same conditions. When compared to matched controls, PLA_2 had a statistically significant effect ($P < 0.05$) on all phospholipids in the three media. No significant difference was found between the three media.

	% of controls			
	PIP_2	PIP	PA	PC
Ca^{2+} (0.25 mM)	82.5 ± 2.5	90.8 ± 3.6	80.0 ± 1.3	33.8 ± 1.2
Ca^{2+} (1 mM)	87.5 ± 5.5	90.8 ± 3.3	84.3 ± 1.0	35.0 ± 2.0
Ca^{2+} (0.25 mM) + Co^{2+} (1 mM)	86.3 ± 2.8	87.8 ± 6.7	82.5 ± 3.9	37.4 ± 2.5

Action of PLA_2 does not lead to an activation of PIC

The possibility of a Ca^{2+}-dependent activation of PIC was investigated because lysoPC (one of the hydrolysis product of PC by PLA_2) has been reported to stimulate Ca^{2+} influx and to increase intracellular Ca^{2+} concentration in pigeon erythrocytes [33]. The effect of PLA_2 on phosphoinositides and PA content and specific radioactivity was measured in the presence of increasing concentrations of Ca^{2+} from 250 μM to 1 mM (Table II). The decreases in polyphosphoinositide and PA contents were maximal at 250 μM Ca^{2+} and not affected by a further rise to 1 mM. In addition, the increase in PA specific radioactivity was not affected either (results not shown). Addition of 1 mM $CoCl_2$, which is known to substitute for Ca^{2+} and thus to block Ca^{2+} influx [34] did not suppress the effects of PLA_2 on polyphosphoinositides and PA (Table II). These results support the view that the effect of PLA_2 on polyphosphoinositide and PA are unlikely due to a Ca^{2+}-dependent PIC activation. In addition, PIC can be activated directly by arachidonic acid in some cells [35–37] but not in others [32]. In erythrocytes, PIC activation causes a 60% decrease in PIP_2 content and a 80% decrease in that of PIP [26]. Addition of fatty acids did not activate PIC since it only led to a slight decrease in PIP_2 content (12%) without any change on that of PIP.

Effect of PLA_2 on phospholipids in ATP-depleted erythrocytes

To further investigate whether PLA_2 was able to hydrolyse polyphosphoinositides and PA without indirect effect on their metabolism, the action of the lipase was determined in ATP-depleted erythrocytes. ATP depletion resulted in a diminution in the content of PIP_2 and in an augmentation of that of PIP and PI as previously reported [26,38,39] (Table III). These changes were initiated by inactivation of the kinases which led to depletion of the phosphatase-sensitive PIP_2 and, to some extent, PIP pools. The new steady-state reached after 1 h of ATP depletion was main-

TABLE III

Effect of ATP depletion on the content of phospholipids in erythrocytes

Cells were incubated for 1 h either in the normal medium (control) or in the presence of iodoacetamide and inosine (ATP-depleted). The values are the means ± S.E. of duplicate determinations in five experiments. Sum is the total content of phosphoinositides.

	nmol/mg of membrane proteins						
	PIP_2	PIP	PI	Sum	PA	PC	PS
Control	4.73 ± 0.39	1.49 ± 0.08	3.90 ± 0.32	10.1 ± 0.7	10.3 ± 0.8	146 ± 7.9	82.4 ± 2.5
ATP-depleted	2.76 ± 0.37 *	2.73 ± 0.18 *	4.20 ± 0.27	9.70 ± 0.60	9.10 ± 0.70	139 ± 7.9	78.0 ± 7.5

* Significantly different from matched controls ($P < 0.05$).

TABLE IV

Effect of PLA_2 on the content and ^{32}P specific activity (S.A.) of phospholipids in ATP-depleted erythrocytes

Cells were labelled for 2 h with [^{32}P]P_i, washed and reincubated for 1 h in the presence of iodoacetamide and inosine. They were washed and transferred to a medium containing or not PLA_2 (20 units/ml of cells) for 1 h as described in Legend to Fig. 1. The values are the means ± S.E. of duplicate determinations in five experiments. Sum is the total content of phosphoinositides.

	% of controls						
	PIP_2	PIP	PI	Sum	PA	PC	PS
Content	75.8 ± 3.9 *	99.1 ± 1.1	86.5 ± 4.2 *	86.5 ± 1.7 *	74.1 ± 3.3 *	32.7 ± 2.4 *	97.7 ± 4.5
S.A.	94.5 ± 1.8	95.4 ± 1.7			87.2 ± 4.8		

* Significantly different from matched controls ($P < 0.05$).

tained at least for a further hour, the period during which the effect of PLA_2 was investigated (data not shown). Even if there was an ATP-dependent translocation of phosphoinositides and PA as in the case of aminophospholipids [40,41], this process could create an asymmetrical distribution but not alter a pre-existing distribution of these phospholipids [17–19]. Indeed, the extent of PC hydrolysis induced by PLA_2 was the same in control and in ATP-depleted cells and PS remained unaccessible to PLA_2 in depleted cells (Table IV).

PLA_2 induced a decrease in the content of PIP_2 (24%) similar to that in control cells (16%) and no significant change in its specific radioactivity (5%). The effect of PLA_2 in decreasing the content and the specific activity of PIP was abolished in ATP-depleted erythrocytes confirming the inhibition of PI kinase in control cells and arguing against the presence of PIP in the outer half-membrane. In addition, PI content, instead of being increased after PLA_2 treatment as in control cells, was decreased by 14% after the same treatment in ATP-depleted cells. This increase in PI content in the former case can be attributed to PI kinase inhibition together with recycling of lysoPIP$_2$ into PI (see above). The decrease in the content of PA induced by PLA_2 in ATP-depleted cells (26%) was slightly larger than in control cells (16%) but PA specific radioactivity, instead of being increased, was not significantly changed. These data again confirmed the activation of diacylglycerol kinase in control cells. The total content in phosphoinositides was decreased by 14% and this decrease was significantly different from that in control cells (4%) suggesting a PLA_2-induced hydrolysis of PIP_2 and PI in depleted cells. The changes in the content of phosphoinositides induced by ATP depletion are expected to affect the molecules of the inner leaflet, where the kinases are located [42–44]. As a result, the percentage of externally located molecules will be overestimated (PIP_2) or underestimated (PIP and PI) when measured in ATP-depleted cells. In fact the decrease in the content of PIP_2 induced by PLA_2 in depleted cells ($24 \pm 4\%$) although not significantly different from that in fed cells ($16 \pm 3\%$) was slightly higher. The external location of about 20% of PIP_2 in the membrane was thus confirmed. The content in PI was only increased by less than 10% and that of PA was not changed after ATP depletion. The 14% decrease in PI content and the 26% decrease in that of PA induced by PLA_2 in ATP-depleted cells can be considered to represent the fraction of externally located molecules in control erythrocytes. The presence of lysoPA in the extracts of PLA_2-treated cells (either control or ATP-depleted) is also consistent with a direct attack of this phospholipid by the lipase and thus with its external localization. The data concerning PI are in agreement with previous ones reporting that approx. 20–25% of this phospholipid was located in the outer layer of the erythrocytes of sheep, ox and pig [3], and of human erythrocytes [5]. In contrast, 42% of PI has been reported to be located in that layer of the membrane of mouse erythrocytes [4]. However, this value results from an extrapolated estimation and not from a direct determination since only the content in anionic phospholipids was measured.

Immunological detection of PIP and PIP_2

Interestingly, the localization of PIP_2 on the external erythrocyte surface was confirmed by immunofluorescence staining of intact erythrocytes with a monoclonal antibody to PIP_2. The very high specificity of anti-PIP and anti-PIP_2 monoclonal antibodies first demonstrated by Fukami et al. [16] was confirmed by dot-blotting and TLC immunostaining. Each antibody only recognized its antigen even when erythrocyte lipid extracts were used (data not shown). Chemically-fixed intact cells incubated with anti-PIP_2 antibody displayed a positive reaction (Fig. 2a). The staining was observed in all cells but unevenly distributed on the cell surface suggesting an heterogeneous distribution of PIP_2. Control experiments performed on the same cells revealed no staining with the antibody raised against spectrin (Fig. 2b). Since spectrin molecules are exclusively localised on the inner leaflet of the erythrocyte membrane, these data indicate that cells remained impermeable to antibodies during the period required for immunofluorescence study and that the positive reaction for PIP_2 could reasonably be ascribed to an external localization of part of the phospholipid in the cell membrane. As it may be expected from an internal localisation of the other part of PIP_2 and of spectrin, erythrocyte sections treated under the same expertimental conditions showed a positive reaction with both anti-PIP_2 and anti-spectrin (Fig. 2a′ and b′). The specificity of the anti-PIP_2 antibody was confirmed by the fact that the reaction was abolished by a preincubation with an excess of PIP_2 (Fig. 2c) but not by excess of PC (Fig. 2d), PI, PIP or inositol 1,4,5-trisphosphate (data not shown). It is noteworthy that anti-PIP antibody was unable to stain intact erythrocytes (Fig. 2e), though it markedly stained ghost sections (Fig. 2f), validating the biochemichal data of its exclusive localisation in the inner half-membrane.

Further experiments were undertaken to confirm that the PLA_2-mediated decrease in PIP_2 content found in control and ATP-depleted cells resulted from a direct breakdown of the phospholipid located on external surface of the plasma membrane. Control and ATP-depleted erythrocytes were preincubated with or without PLA_2, then washed, fixed and treated with anti-PIP_2. The unincubated controls (Fig. 3a) and the cells untreated with the enzyme (Fig. 3b) exhibited the usual positive staining in almost all the cells examined.

In contrast, the staining was perceptibly lowered, but not completely suppressed, after PLA_2 treatment (Fig. 3c). It is likely that lysoPIP$_2$ can react with the antibody. This possibility has not been tested. LysoPIP$_2$ could be partially lost under our experimental conditions (cell washing following incubation with PLA_2 and

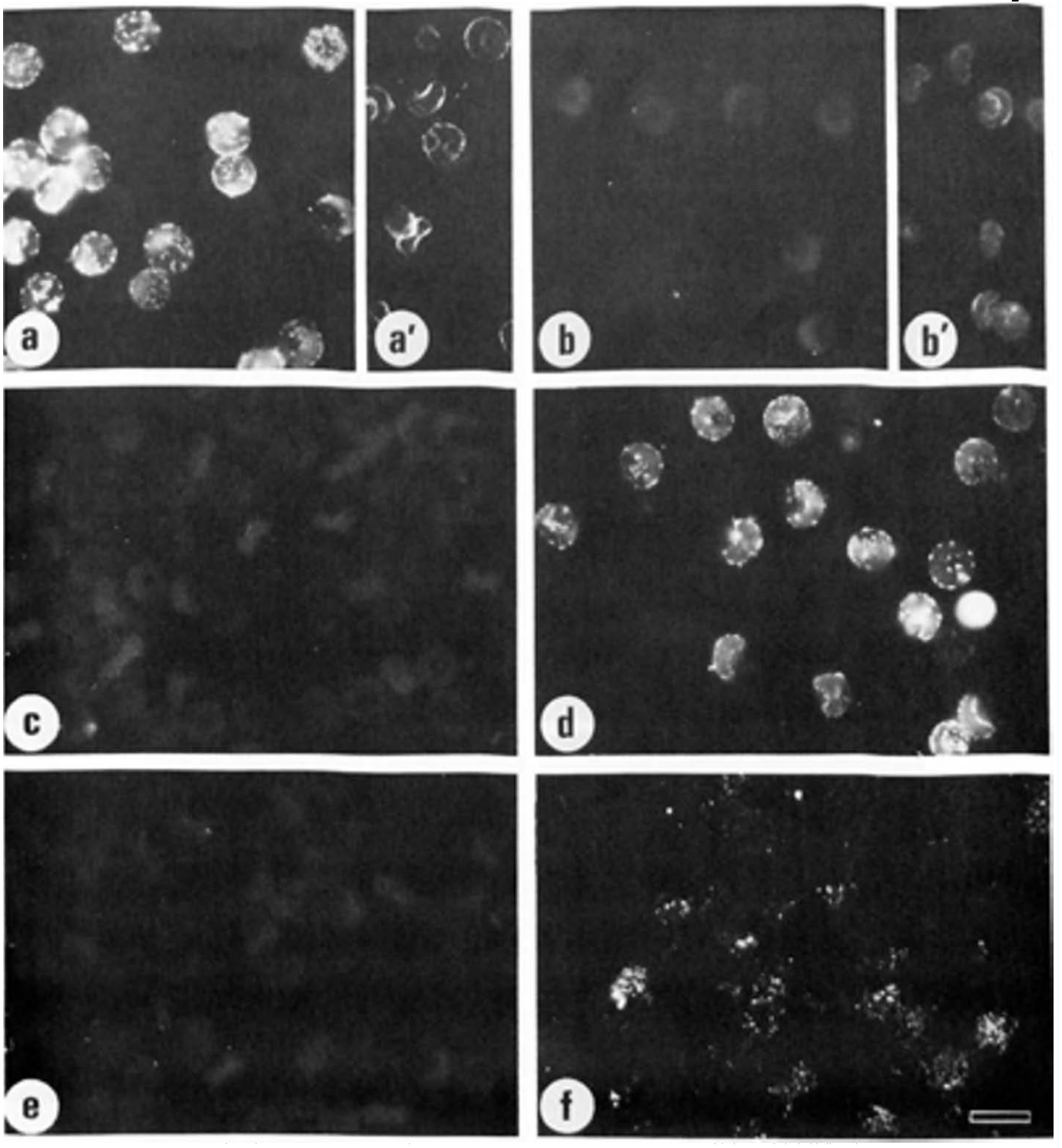
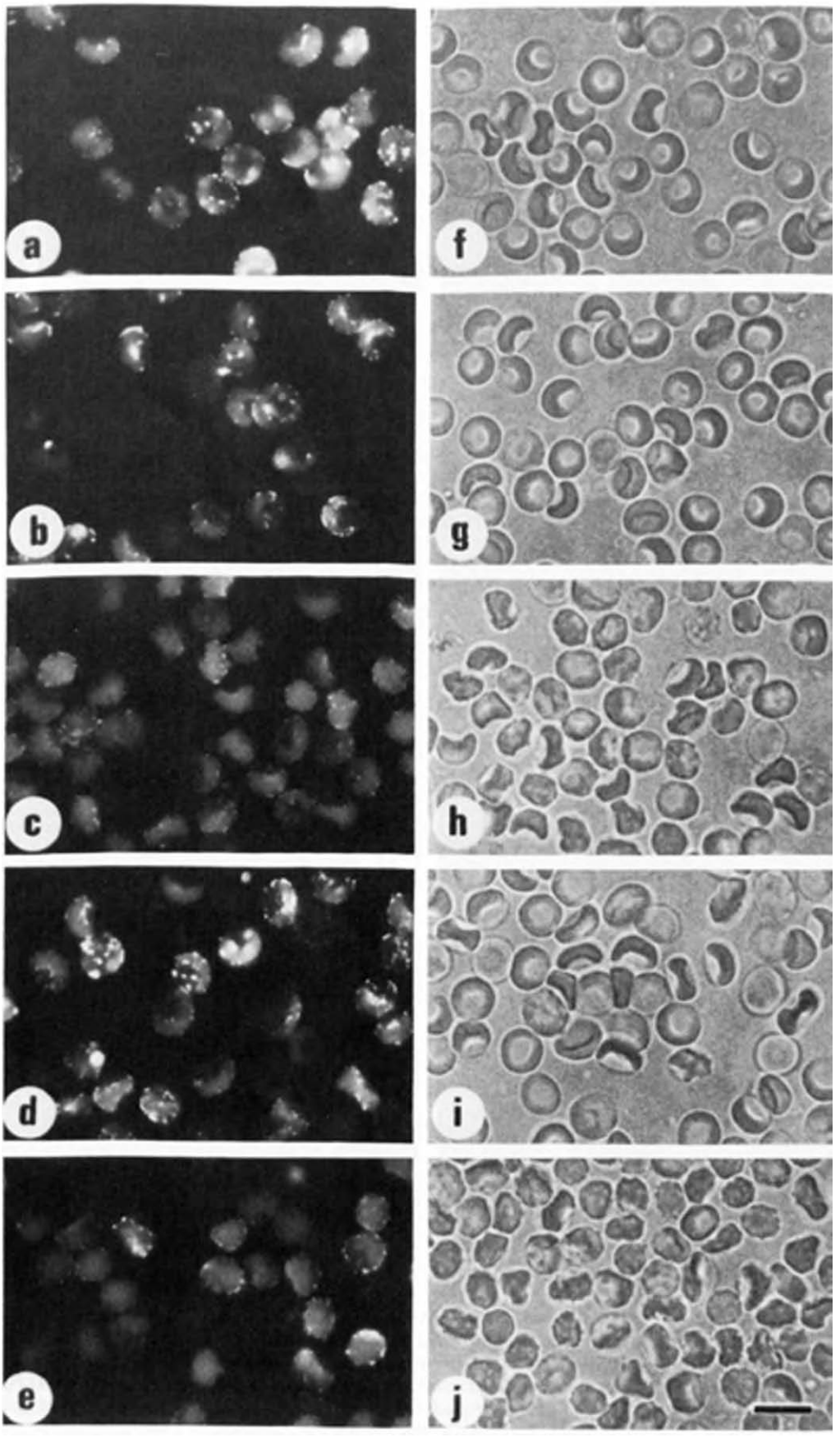


Fig. 2. FITC-avidin-biotin immunofluorescence localization of PIP$_2$ and PIP in human erythrocytes. (a) Intact erythrocytes stained by anti-PIP$_2$ antibody (1:16000). The positive reaction appeared unevenly distributed on the cell surface. (a') 0.5 μm cryosections of erythrocytes performed under the same conditions as in a. The staining delineated almost continously the cell membrane. (b) Controls of a showing that intact cells remained impermeable to antibodies during immunostaining. Erythrocytes were incubated with anti-spectrin antibody (1:1600) under the same conditions as in a. No staining was observed. (b') Erythrocyte sections treated in the same way as in b displayed a positive reaction for anti-spectrin. (c and d) Specificity of anti-PIP$_2$ antibody. The positive reaction for PIP$_2$ observed in a was abolished by a preincubation of the antibody with an excess of PIP$_2$ (c) but not with an excess of PC (d). (e) Intact erythrocytes incubated with anti-PIP antibody (1:1600). No staining was observed. (f) Ghost cryosections stained with anti-PIP antibody (1:1600). The positive reaction appeared as bright points. Horizontal bar, 10 μm.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j

treatment of the fixed samples with ovalbumin). The conditions of albumin treatment used (ovalbumin not fatty acid-free, concentration, time and temperature of action) may not allow a total extraction of this lyso derivative and thus could explain the partial disappearance of the staining. The ATP-depleted cells displayed the same positive labelling as controls (Fig. 3d) confirming the lack of effect of ATP depletion on the transbilayer distribution of this phospholipid. When ATP-depleted erythrocytes were treated with PLA_2 and exposed to anti-PIP_2, staining was also decreased as compared to the relevant controls (Fig. 3e).

In the right part of the panel, phase-contrast micrographs of the cell suspensions are presented. Fig. 3f and g show that control and PLA_2-untreated cells were normal discocytes. Thus, the punctate pattern of staining observed with anti-PIP_2 did not correspond to a PIP_2 distribution in spiculated areas of echinocytes but rather to special domains of the normal discocyte membrane. Echinocytic conversion of erythrocytes during PLA_2 treatment, attributed to the accumulation of lysoPC in the external leaflet of the membrane [45,46] has not been always observed [47]. In our study, only some echinocytic shapes were present (Fig. 3h). These discrepancies may be due to slight differences in the methodology used for sample treatment before microscopic examination. ATP-depleted erythrocytes exhibited nearly normal discocytic shapes (Fig. 3i) as already described when ATP depletion is induced by enzymatic inhibition [48,49]. The combination of ATP depletion and PLA_2 treatment gave rise to a more drastic shape change with almost all cells converted to echinocytes (Fig. 3j).

Functional significance

In summary, our data demonstrate that approx. 20% of PIP_2, PI, PA and no PIP can be hydrolysed by PLA_2 suggesting their external localisation on the erythrocyte membrane. The use of very specific monoclonal antibodies strongly supported the view that a significant fraction of PIP_2 but no PIP is localized in the outer surface. This is an unexpected finding since most of the functions of PIP_2, described up to now, take place in the cytoplasmic membrane surface: as a precursor of the intracellular second messengers inositol 1,4,5-trisphosphate and diacylglycerol in nucleated cells [7], as a promotor of the interactions between membrane and skeleton proteins glycophorin-protein 4.1 in erythrocytes [11], as an activator of membrane-bound enzymes such as Ca^{2+}-ATPase [8–10]. Externally located PIP_2 could be a site of covalent linkage with polar cell surface proteins as such covalent binding has been shown to occur with myelin basic protein [13]. Another possible function for this external PIP_2 could be linked to its role as a precursor of second messengers and the need for its rapid replacement following its breakdown upon cell stimulation by hormones, neurotransmitters or growth factors. Resynthesis, resulting from phosphoinositide kinase activation is the mechanism classically involved. Fast transbilayer inward transport (flip) of this external PIP_2 could be an alternative mechanism providing an even more rapid replacement of the hydrolysed PIP_2. The half-time for the energy-dependent inward transport of PS and PE by the aminophospholipid translocase in erythrocytes [40] is, at 37°C, of approx. 3 and 34 min, respectively [50]. It is not known whether this ATP-dependent translocase or another one, is able to transport PIP_2. In any case, it seems very likely that the transfer of such a highly polar phospholipid is energy-dependent and protein-mediated rather than occurring through spontaneous diffusion.

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Fig. 3. Immunofluorescence localization of PIP_2 on control or ATP-depleted erythrocytes with or without PLA_2 treatment (FITC-avidin-biotin method, anti-PIP_2 antibody diluted to 1:16000). (a) Unincubated controls. A positive reaction for PIP_2 was observed. (b) Incubated controls. The positive reaction was comparable to that seen in a. (c) PLA_2-treated cells. The positive reaction seen in b was clearly reduced. (d) ATP-depleted cells. The positive reaction was similar to that seen in a and b. (e) ATP-depleted cells treated with PLA_2. The reaction for PIP_2 was very weak (compared to d). (f–j) Phase contrast micrographs showing the cell morphology corresponding, respectively, to a–e. Unincubated (f) and incubated (g) controls showed a normal discocyte shape. PLA_2-treated cells showed an echinocytic shape (h). ATP-depleted cells (i) were nearly normal discocytes. Combination of ATP-depletion and PLA_2-treatment led to a marked transformation to echinocytic shape (j). Horizontal bar, 10 μm.

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