Articles

Characterization of Structural and Functional Phosphoinositide Domains in Human Erythrocyte Membranes†

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Received January 28, 1993; Revised Manuscript Received March 31, 1993

ABSTRACT: In the erythrocyte membrane, only a fraction (50–60%) of phosphatidylinositol 4,5-bisphosphate (PIP_2) and of phosphatidylinositol 4-phosphate (PIP) is rapidly turned over by specific kinases and phosphatases and accessible to hydrolysis by the polyphosphoinositide (PPI)-specific phospholipase C (PLC). To investigate whether the metabolic segregation of PPI resulted from preferential interactions with proteins, we have measured the accessibility of PPI to bee venom phospholipase A_2 (PLA_2) in native erythrocyte membranes, or after treatments designed to remove peripheral proteins and cytoplasmic domains of integral proteins. In native membranes, PPI, as well as the other major phospholipids, behaved as two distinct fractions (R_1 and R_2) differing by their sensitivity to PLA_2. Such a behavior was not observed in PIP and PIP_2 containing artificial vesicles. Evidence was provided that the highly sensitive fraction of PIP and PIP_2 (R_1) may be identical to the PLC-sensitive and rapidly metabolized pool. Removal of peripheral proteins, followed by proteolysis of the cytoplasmic domain of integral proteins, mainly glycophorins and band 3, led to a reduction of the R_1 fraction of PIP and of PIP_2. It is proposed that the rapidly metabolized pool of PIP_2 and PIP, involved in the regulation of major cellular functions, would be maintained in its functional state through interactions with integral proteins.

Despite the dynamic nature of lipids, different membranes within a cell have different lipid composition. A membrane may have an asymmetric distribution of phospholipids across the bilayer, and lipids within a membrane may be organized laterally into discrete regions or domains.

Recent observations by fluorescence digital imaging microscopy of intact erythrocytes or ghosts labeled with fluorophore-labeled phosphatidylcholine (PC)[1] and phosphatidylserine (PS) have revealed an uneven distribution of the phospholipids in the membranes, thereby demonstrating the presence of membrane domains (Rodgers & Glaser, 1991). Previous data obtained with two phospholipases A_2 (PLA_2), acidic or basic from snake (*Agkistrodon halys blomhofii*) venom, also demonstrated that, in erythrocyte membranes, there are different domains enriched in PC recognized by the differential action of the two lipases (Shukla & Hanahan, 1982).

Polyphosphoinositides (PPI), especially phosphatidylinositol 4,5-bisphosphate (PIP_2), play important roles in many cellular functions. It is involved in signal transduction triggered by a number of hormones, growth factors, and neurotransmitters, as a precursor of two second intracellular messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) (Berridge, 1984), in the modulation of membrane-bound enzymes (Missiaen et al., 1989; Brockman & Anderson, 1991) and in the regulation of glycophorin–protein 4.1 interaction in erythrocytes (Anderson & Marchesi, 1985; Anderson, 1989). Recent reports also indicate that PIP_2 could interact with cytoskeletal proteins (profilin, gelsolin, α-actinin) regulating actin polymerization or the bundling of actin filaments (Lassing & Lindberg, 1988; Yin et al., 1988; Fukami et al., 1992). In addition, there is good evidence of discrete pools of inositol lipids that turn over at distinct rates and may serve different functions. A number of studies indicated that, in the erythrocyte membrane, both PIP_2 and phosphatidylinositol 4-phosphate (PIP) were compartimentalized with only a fraction (50–60%) being rapidly turned over by specific kinases and phosphatases and accessible to hydrolysis by the PPI-specific phospholipase C (PLC) (Muller et al., 1986; King et al., 1987; Gascard et al.,1989). The origin of this metabolic compartimentalization is not known. One possible mechanism is that skeletal or integral membrane proteins (Devaux & Seigneuret, 1985; Haverstick & Glaser, 1989) could bind or sequester lipids, maintaining them in separate domains.

In the present work, we have investigated whether the metabolic segregation of PPI resulted from a nonrandom distribution maintained by preferential interactions with proteins. To this end, we have measured the accessibility of PPI to the bee venom PLA_2 in native erythrocyte membranes, or after treatments designed to remove peripheral proteins and cytoplasmic domains of integral proteins. In native membranes, PPI, as well as the other major phospholipids, behave as two distinct fractions characterized by their sensitivity to PLA_2. Our results indicate that the fractions of PPI highly sensitive to PLA_2 represented the rapidly turned over and PLC-sensitive metabolic pools. Removal of the cytoplasmic fragments of transmembrane proteins resulted

† This work was supported by INSERM Grant CRE 910410.

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[1] Abbreviations: BSA, bovine serum albumin; IOV, inside-out vesicle(s); IP_3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, polyphosphoinositide-specific phospholipase C; PPI, polyphosphoinositide(s); PMA, phorbol myristate acetate; PMSF, phenylmethanesulfonyl fluoride; sIOV, stripped inside-out vesicle(s); SUV, single unilamellar vesicle(s).

0006-2960/93/0432-5941$04.00/0

in a drastic reduction in the fractions of PPI highly sensitive to PLA_2 (not observed for the other phospholipids), suggesting that the metabolic segregation of PPI in native membranes resulted from interactions with proteins.

MATERIALS AND METHODS

Materials. PC (P 5763), phosphatidylethanolamine (PE) (P 6386), PIP sodium salt (P 9638), PIP_2 sodium salt (P 9763), PLA_2 from bee venom (P 9279), bovine serum albumin fatty acid free (BSA) (A 6003), calcium ionophore A23187 (C 7522), TPCK-treated trypsin (T 8642), TLCK-treated α-chymotrypsin (C 3142), phenylmethanesulfonyl fluoride (PMSF) (P 7626), phorbol myristate acetate (PMA) (P 8139), and reagents for gel electrophoresis were obtained from Sigma. Aprotinin (trypsin inhibitor, pancreas type) was purchased from Boehringer Mannheim. Silica gel G 1500 precoated plates for thin-layer chromatography (TLC) were from Schleicher & Schuell, Germany. All other reagents were of analytical grade.

Preparation of Erythrocyte Ghost Membranes and Treatment with PLA_2. Human blood, withdrawn from healthy volunteers in heparinized tubes, was centrifuged at 1500*g*, at 4 °C, for 10 min. Plasma and buffy coat were removed by aspiration, and packed cells were washed twice in 150 mM NaCl/5 mM sodium phosphate (pH 7.6). Membranes were prepared either by hypotonic lysis in 5 mM sodium phosphate (pH 7.6), 1 mM EDTA (medium A), and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) or by isotonic lysis in the presence of saponin [100 mM KCl, 50 mM Tris-HCl (pH 7.2), 0.5 mM EDTA, and 0.3 mg/mL saponin]. This second method allows inactivation of PLC (Hegewald et al., 1987). These membranes, referred to in the text as respectively "hypotonic" and "saponin" membranes, were incubated (2 mg of protein/mL) in medium B (150 mM NaCl/1.5 mM HEPES–NaOH, pH 7.4) at 37 °C, with 1 mM EGTA or 1 mM $CaCl_2$ and PLA_2 at the concentrations indicated in each experiment. The reaction was stopped by the addition of 1 volume of medium B containing 1.2 mM EGTA. Membranes were then washed 3 times in 10 mM Tris-HCl (pH 7.4)/1 mM EDTA. The first wash contained 1% BSA (fatty acid free) in order to remove lysophospholipids (Mohandas et al., 1982) produced by PLA_2 treatment which could have interfered with the phospholipids during TLC separation. Membranes were extracted with acidic $CHCl_3/CH_3OH$, and neutral phospholipids and PPI were separated by TLC and assayed as described previously (Wallace et al., 1983; Giraud et al. 1984; Gascard et al., 1991).

Preparation of Single Unilamellar Vesicles (SUV) and Treatment with PLA_2. SUV were prepared from pure phospholipids (PC, PC/PE, PC/PIP, or PC/PIP_2). CH_3Cl/CH_3OH solutions of PC and PE were evaporated under a stream of nitrogen at 4 °C. After addition of medium B and, when required, PIP or PIP_2 (sodium salt) in aqueous solution, the mixture was vortexed for 2 min and subjected to probe sonication (4 cycles of 7 min, 50 W at 4 °C). The sonicates were centrifuged (30000*g*, 60 min, 4 °C) to remove undispersed lipids and multilamellar liposomes. SUV, recovered in the supernatants, were incubated for 5 min, at 25 °C, in medium B containing 500 μM $CaCl_2$ (2–4 μmol of phospholipids/mL) with or without different concentrations of PLA_2. The reaction was stopped by the addition of 1 volume of medium B containing 1 mM EGTA. Phospholipid extraction, separation, and assay were carried out as above.

PLC Activation in Intact Erythrocytes. In some experiments, the PLC-sensitive pools of PIP and PIP_2 were depleted in intact erythrocytes, prior to treatment of membranes with PLA_2. Erythrocytes, suspended at a hematocrit of 10% in 75 mM KCl, 75 mM NaCl, 0.15 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES–NaOH (pH 7.4), were incubated for 20 min, at 37 °C, with the calcium ionophore A23187 (5 μM) dissolved in dimethyl sulfoxide as a 2 mM stock solution. In control experiments, erythrocytes were incubated under the same conditions, but with 1 mM EGTA instead of 1 mM $CaCl_2$. The incubation was terminated by the addition of 1.5 volumes of medium B containing 1.2 mM EGTA. Samples were centrifuged, and cell pellets were lysed as described above.

IP_3 Assay. Erythrocytes, suspended at a hematocrit of 40% in 150 mM NaCl, 10 mM glucose, and 1.5 mM HEPES–NaOH (pH 7.4), were incubated for 3 h with [^{32}P]P_i (1.85 MBq/mL of cells). The cells were washed 3 times in isotonic phosphate medium and lysed in medium A as described above. Membranes were incubated in medium B with 1 mM EGTA or 1 mM $CaCl_2$ and various concentrations of PLA_2. The reaction was stopped by the addition of 1 volume of medium B containing 1.2 mM EGTA. Samples were centrifuged, and IP_3, present in the supernatants, was assayed by ion-pair high-performance liquid chromatography as described previously (Rhoda et al., 1988; Sulpice et al., 1989).

Protein Kinase C (PKC) Activation in Intact Erythrocytes. In some experiments, erythrocytes, suspended at a hematocrit of 20% in medium B containing 10 mM glucose, were incubated, at 37 °C, for 60 min with 500 nM PMA dissolved in dimethyl sulfoxide as a 1 mM stock solution. The incubation was terminated by the addition of 15 volumes of ice-cold medium B containing 1 mM EGTA. Erythrocytes were washed once in this medium and lysed.

Preparation of Stripped Inside-Out Vesicles (sIOV) and Proteolyzed sIOV. Membranes were prepared as described above by hypotonic lysis in medium A containing 0.1 mM PMSF. Skeletal protein extraction (spectrin and actin) was achieved essentially according to Tyler et al. (1979). Membranes were incubated in 30 volumes of 0.3 mM sodium phosphate (pH 7.6), 0.2 mM EDTA, and 0.2 mM PMSF, at 37 °C for 30 min, resulting in the formation of inside-out vesicles (IOV). These IOV were washed in medium A and depleted of remaining peripheral proteins (bands 2.1, 4.1, and 6) by incubation, at 37 °C for 30 min, in medium A containing 1.5 M KCl, 2 mM PMSF, and 17 μg of aprotinin/mL. The resulting sIOV were washed in medium A. During these preparations, no attempt was made to obtain sealed IOV or sIOV. Only 10–15% maximum of these vesicles can be assessed to be sealed when the accessibility of membrane acetylcholinesterase was measured (Kant & Steck, 1973). Some of these sIOV were proteolyzed by incubation, at 20 °C for 30 min, at a concentration of 1 mg of vesicle protein/mL, in medium A containing either 10 μg/mL TPCK-treated trypsin alone or together with 10 μg/mL TLCK-treated α-chymotrypsin. The reaction was stopped by the addition of 5.4 μg/mL aprotinin when only trypsin was present or of 11 μg/mL aprotinin when both trypsin and α-chymotrypsin were present. After 15 min, sIOV were diluted in medium A and centrifuged for 15 min, at 25000*g* at 4 °C. Membranes, sIOV, and proteolyzed sIOV were washed once in medium B just before incubation, at 37 °C, with 1 mM EGTA or 1 mM $CaCl_2$ and various concentrations of PLA_2. The reaction was stopped by the addition of 1 volume of medium B containing 1.2 mM EGTA. Samples were centrifuged and washed once in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1% BSA and twice in the same buffer without BSA. Lipid extraction, phospholipid separation, and assay were carried out as above.

Other Procedures. Gels were run according to Laemmli (1970) in 10% acrylamide and stained either with Coomassie

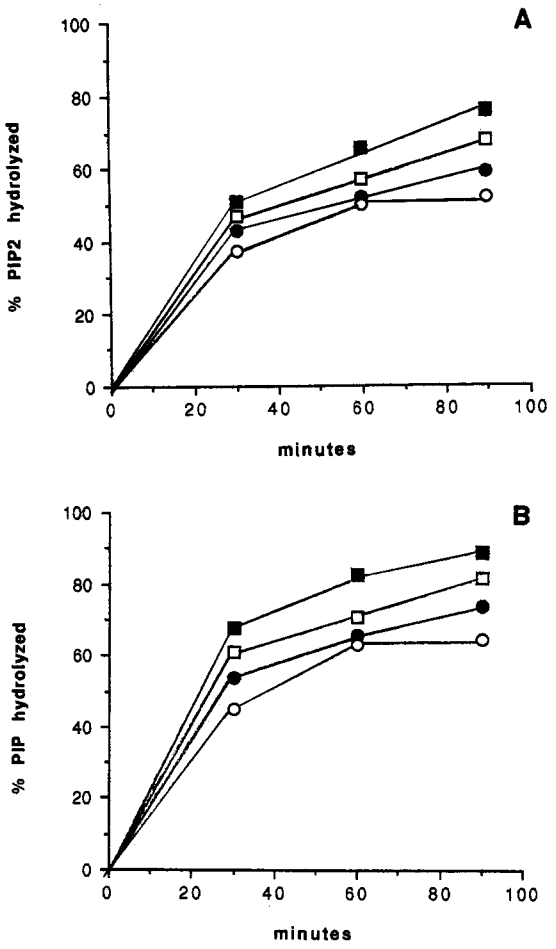

FIGURE 1: Kinetics of PLA_2-induced hydrolysis of PIP_2 (A) and PIP (B). Hypotonic membranes were incubated, at 37 °C, in medium B containing 1 mM Ca (○) and various concentrations of PLA_2 (IU/μmol of phospholipids): 0.6 (●), 2.5 (□), or 6.5 (■). The reaction was stopped by the addition of 1 volume of medium B containing 1.2 mM EGTA. The packed membranes were extracted, and phospholipids were separated by TLC and assayed. Values shown are from one experiment. Two others gave similar data.

blue or with periodic acid Schiff reagent. Protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

RESULTS

Characteristics of the Hydrolytic Activity of PLA_2 on Phospholipids in Erythrocyte Ghost Membranes. Erythrocyte membranes, prepared by hypotonic lysis, were incubated for various times at 37 °C in the presence of 1 mM $CaCl_2$ and different concentrations of PLA_2. The kinetics of PIP_2 and PIP hydrolysis are shown in panels A and B, respectively, of Figure 1. The extent of hydrolysis increased with the time of incubation and with the enzyme concentration. In the absence of PLA_2, Ca^{2+} activated the endogenous PLC, resulting in a maximal breakdown of 50% of PIP_2 and 65% of PIP, reached by 60 min of incubation. The effect of various concentrations of PLA_2 (from 0.25 to 24 IU/μmol of phospholipids) on phospholipid hydrolysis was studied after 60 min of incubation, a condition allowing, in principle, discrimination of the effect of PLC, already completed, from that of PLA_2. However, from these kinetic data, it cannot be concluded whether endogenous PLC was still active in the presence of exogenous PLA_2. This question will be addressed below. Figure 2A shows that 85–95% of PA, PC, and PE was hydrolyzed at a PLA_2 concentration of 4 IU/μmol of phospholipids. In contrast, PIP_2 and to a smaller extent PIP

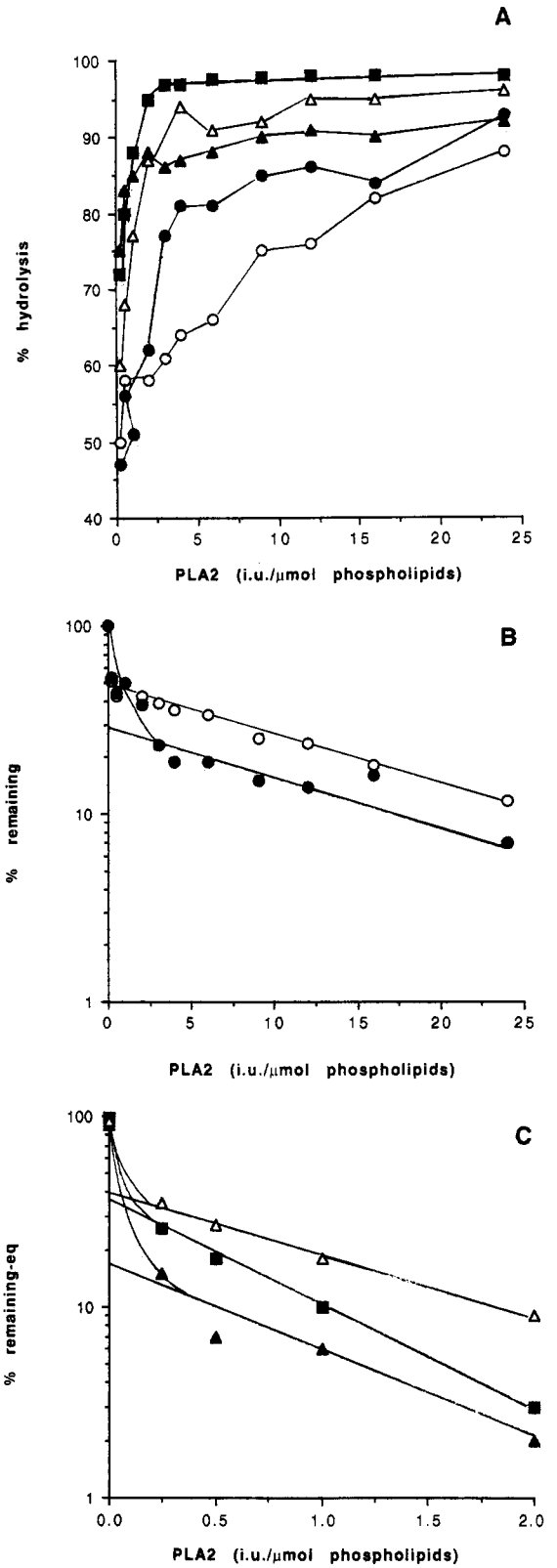

FIGURE 2: Effect of PLA_2 on the hydrolysis of various phospholipids in erythrocyte membranes prepared by hypotonic lysis. Hypotonic membranes were incubated with various concentrations of PLA_2 for 1 h, at 37 °C, in medium B containing 1 mM $CaCl_2$. The reaction was stopped as described in the legend of Figure 1. Membranes were centrifuged and washed once in hypotonic medium containing 1% BSA and twice in the same medium without BSA. The packed membranes were extracted, and phospholipids were separated by TLC and assayed. The percentage of hydrolysis of each phospholipid (A) and the percentage of phospholipids remaining (B and C) are presented. Values shown are means of two (PA, PC, PE) or three experiments (PIP, PIP_2). eq: Percent of PA, PE, and PC remaining at the maximal dose of PLA_2; PIP_2 (○); PIP (●); PE (▲); PC (△); PA (■).

Table I: PLA_2 Sensitivity of Phospholipids in Native Erythrocyte Membranes, in Stripped Vesicles (sIOV), and in Proteolyzed sIOV[a]

	R_1 (%)				
	PIP_2	PIP	PA	PC	PE
membranes	52 ± 4.5	71 ± 5.5	66 ± 4.5	60	84
sIOV	39 ± 5.4[b]	72 ± 5.5	73 ± 4.1	69	88
proteolyzed sIOV	18 ± 4.5[b]	48 ± 3.9[b]	67 ± 3.9	52	86

[a] Membranes, sIOV, and proteolyzed sIOV were incubated in the presence of increasing concentrations of PLA_2. The PLA_2-sensitive fraction of each phospholipid (R_1) was determined from plots of percent phospholipids remaining vs the concentration of PLA_2 for PIP_2, PIP, and PA (see Figure 6) and for PC and PE (not shown). Values are means ± SE of three experiments or means of two experiments. [b] Significantly different from corresponding control membranes.

were more resistant to the hydrolytic action of the enzyme, requiring between 16 and 24 IU/μmol of phospholipids to reach the same level of hydrolysis. The semi-logarithmic plots of Figure 2B,C (percent nonhydrolyzed phospholipids versus concentration of PLA_2) were biphasic, revealing the presence of two populations of phospholipids differing by their sensitivity to PLA_2: a highly sensitive pool (R_1) estimated from extrapolation of the regression lines at the concentration of PLA_2 equal to 0 and a relatively resistant one (R_2). The values of R_1 for different phospholipids are shown in Table I (first row).

Characteristics of the Hydrolytic Activity of PLA_2 on Phospholipids in Artificial Vesicles. It was important to know whether the biphasic curves of phospholipid hydrolysis toward increasing concentrations of PLA_2 in natural membranes reflected a heterogeneous organization of phospholipids or only a self-inhibition of the enzyme resulting from an increase in lateral surface pressure in the membrane due to accumulation of the products generated by PLA_2 action (Zwaal et al., 1975). This question was addressed by looking at the effect of increasing concentrations of PLA_2 on SUV prepared from different phospholipid mixtures. Incubations were carried out at 25 °C for 5 min in the presence of 500 μM Ca^{2+}, conditions under which the bilayer of SUV remained impermeable to PLA_2 (Wilshut et al., 1979). Figure 3A shows the dose–response curves of the hydrolysis either of PC in PC SUV or of PC and PE in PC/PE (1/1.2) SUV. In both types of SUV, the maximal level of phospholipid hydrolysis was about 60–70%, in agreement with previous observations of the complete degradation of the outer half of vesicle bilayers by PLA_2 (Wilschut et al., 1979; Jain & Berg, 1989) and of the outer/inner ratio (about 1.8) of the phospholipid molecules in SUV (Berden et al., 1975). The semilogarithmic plots of the percent of nonhydrolyzed phospholipid (minus percent of phospholipids remaining at the maximal concentration of PLA_2) versus the concentration of PLA_2 showed that the hydrolysis of PC and of PC and PE was a monotonic function of the concentration of PLA_2 (Figure 3B). In PC/PIP (1/0.3) SUV, maximal hydrolysis of PIP reached 80% while that of PC was only 40% (Figure 3C). These figures were respectively higher and lower than the 65% observed for PC in PC SUV or PC and PE in PC/PE SUV. By contrast, Figure 2C shows that in PC/PIP_2 (1/0.2) SUV, maximal hydrolysis of PIP_2 reached only 30%, while that of PC (70%) was in the range observed for PC in PC SUV or PC and PE in PC/PE SUV. Nevertheless, the hydrolysis of PC and PIP (in PC/PIP SUV) and of PC and PIP_2 (in PC/PIP_2 SUV) was a monotonic function of the concentration of PLA_2 (Figure 3D). Consequently, the biphasic plots of Figure 2B,C possibly reflected an heterogeneous organization of the phospholipids within the natural membranes.

Identification of the Pools of PPI Highly Sensitive to PLA_2 as the PLC-Sensitive Pools. We then tried to characterize these highly sensitive and relatively resistant fractions of phospholipids to PLA_2 in the erythrocyte membrane. Two pools of PIP and PIP_2 differing by their accessibility to the specific kinases, phosphatases, and PLC have been described previously (Gascard et al., 1989). After 60 min of activation of PLC by 1 mM Ca^{2+} in hypotonic membranes, 54% and 66% of PIP_2 and PIP, respectively, were hydrolyzed (Table II, see also Figure 1). These figures were similar to the values of R_1 (Table I) and to those hydrolyzed by a low dose of PLA_2 (0.6 IU/μmol of phospholipids) (Table II and Figure 1). IP_3 production, which results from cleavage of the polar head of PIP_2 by PLC, was the same either in the presence or in the absence of a low dose of PLA_2 (data not shown). This indicated that PLC was entirely responsible for PIP_2 hydrolysis at this concentration of PLA_2. To determine the effect of PLA_2 without interference with PLC, PPI hydrolysis was measured in saponin membranes which lack PLC activity (Hegewald et al., 1987). The electrophoretic protein pattern, the phospholipid composition, and the cholesterol/phospholipid ratio were similar in the saponin membranes and in the hypotonic membranes (data not shown). Incubation of the saponin membranes with 1 mM Ca^{2+} had no effect on PIP_2 or PIP hydrolysis (Table II). In the presence of PLA_2 (0.6 IU/μmol of phospholipids), PIP_2 hydrolysis was equal to that induced by PLC activation in the hypotonic membranes. These data suggest that PLC and PLA_2, at this concentration, attack the same pool of PIP_2 molecules. The effect of PLA_2 on PIP, in saponin membranes, cannot be determined because their incubation at 37 °C in the isotonic medium used in these experiments, with or without Ca^{2+}, resulted in the activation of a PIP phosphatase, a membrane-bound enzyme (Hegewald et al., 1987), which reduced the PIP content by about 75% and interfered with the measurement of PIP hydrolysis by PLA_2 (data not shown).

The effect of increasing concentrations of PLA_2 on the hydrolysis of PIP_2 was investigated in the saponin membranes. The semi-logarithmic plot (Figure 4) was biphasic, with a value of 55% for R_1, consistent with its identification to the PLC-sensitive pool. To gain further confirmation of this point, PLC was first activated in intact erythrocytes by incubation in a medium containing 1 mM Ca^{2+} and the ionophore A23187. This treatment resulted in hydrolysis of the PLC-sensitive pools (53% of PIP_2 and 66% of PIP), in agreement with previous observations (Gascard et al., 1989). Membranes were then prepared from these erythrocytes by hypotonic lysis and incubated with increasing concentrations of PLA_2 (Figure 5, triangles). The semi-logarithmic plots exhibited a monoexponential decrease for both PIP_2 (Figure 5A) and PIP (Figure 5B). The data for control (Figure 5, circles) were obtained in membranes prepared from erythrocytes incubated with A23187 and 1 mM EGTA. It is clear that, when the PLC-sensitive pools of both PIP_2 and PIP have been previously hydrolyzed, the PLC-resistant pools exhibited exactly the same sensitivity to PLA_2 as did the PLA_2-resistant pools in membranes in which PLC has not been activated. The hypothesis that the pools highly sensitive to PLA_2 and the PLC-sensitive pools represent the same fractions of PIP_2 and PIP is consistent with these data.

Effect of Protein Phosphorylation, Skeletal Protein Stripping, and Proteolysis of the Cytoplasmic Domains of Integral Proteins on the Distribution of Phospholipid Pools. The state of protein phosphorylation is known to affect protein–protein interactions [reviewed in Gascard and Cohen, (1992)]. To investigate whether the organization of the phospholipids in

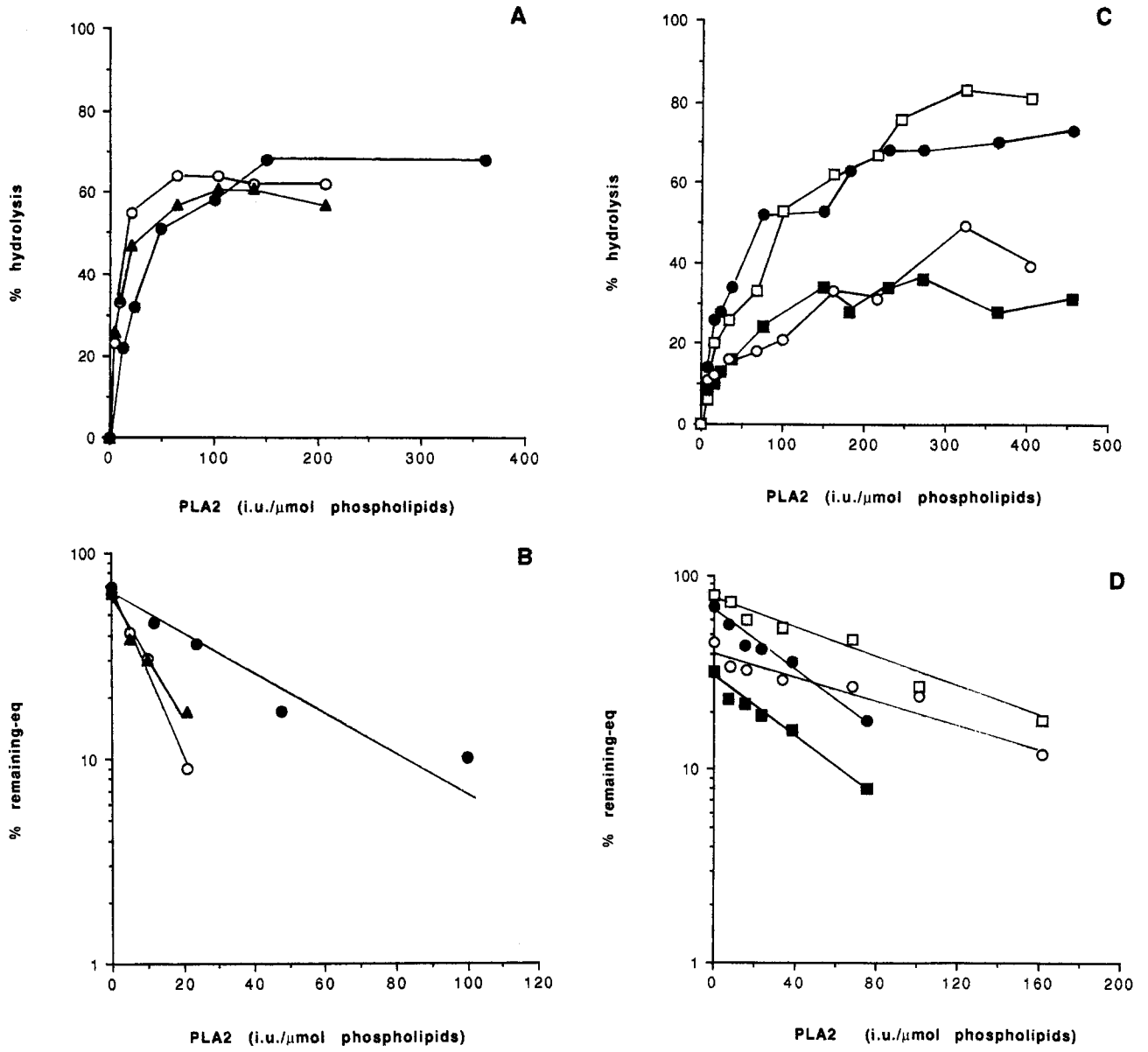

FIGURE 3: Effect of PLA_2 on the hydrolysis of various phospholipids in SUV. SUV, prepared in medium B by sonication and centrifugation, were incubated with various concentrations of PLA_2 for 5 min, at 25 °C, in medium B containing 500 μM $CaCl_2$. The reaction was stopped by the addition of 1 volume of medium B containing 1 mM EGTA. The vesicle suspensions were extracted, and phospholipids were separated by TLC and assayed. Values shown are means of three experiments. (A and B) PC SUV, PC (●); PC/PE SUV, PC (○), PE (▲). (C and D) PC/PIP SUV, PC (○), PIP (□); PC/PIP_2 SUV, PC (●), PIP_2 (■). eq: Percent of phospholipids remaining at the maximal dose of PLA_2.

Table II: Effect of Ca^{2+} Alone or with PLA_2 (0.6 IU/μmol of Phospholipids) in Erythrocyte Membranes Prepared by Hypotonic or Saponin Lysis[a]

	% hydrolysis			
	Ca^{2+}		Ca^{2+} + PLA_2	
conditions	PIP_2	PIP	PIP_2	PIP
hypotonic membranes	54.0 ± 2.9	65.5 ± 1.7	59.0 ± 5.1	68.0 ± 3.6
saponin membranes	1.5 ± 2.7	6.2 ± 6.1	59.0 ± 4.2	*b*

[a] Hypotonic or saponin membranes were incubated for 60 min, at 37 °C, in the presence of 1 mM $CaCl_2$ alone or with PLA_2 (0.6 IU/μmol of phospholipids) and treated as described in the legend of Figure 2. Values are means ± SE of three experiments. [b] The effect of PLA_2 on PIP hydrolysis in saponin membranes is not shown since it cannot be correctly estimated (see text).

two pools depended on the state of membrane protein phosphorylation, we have first measured the effect of PLA_2 on phospholipid hydrolysis in membranes prepared from erythrocytes preincubated in the presence of PMA, a treatment known to activate PKC. The increase in the phosphorylation of membrane proteins (mainly adducin and proteins 4.1 and 4.9), substrates of this protein kinase (Horne et al., 1985; Palfrey & Waseem, 1985; Cohen & Foley, 1986; Giraud et al., 1988), did not result in any change in the dose–response curve of phospholipid hydrolysis by PLA_2 (data not shown).

To investigate whether the difference in the sensitivity to PLA_2 could arise from interactions with proteins, the pool distribution of phospholipids was probed with PLA_2, under different conditions leading to depletion of skeletal proteins or proteolysis of extracellular domains of integral proteins. The efficiency of the following treatments: spectrin and actin extraction (IOV), stripping of bands 2.1, 4.1, and 6 on IOV (sIOV), and proteolysis of the cytoplasmic domains of transmembrane proteins (mainly band 3 and glycophorins) on sIOV, was monitored by SDS–PAGE electrophoresis (data not shown). None of these treatments had any effect on the content in phospholipids and particularly on the concentrations of PIP_2 and PIP expressed as a percentage of total phospholipids. However, the concentration of phospholipids, expressed per protein mass, was increased proportionally to the expected decrease in proteins (minus 33% and minus 60%, respectively,

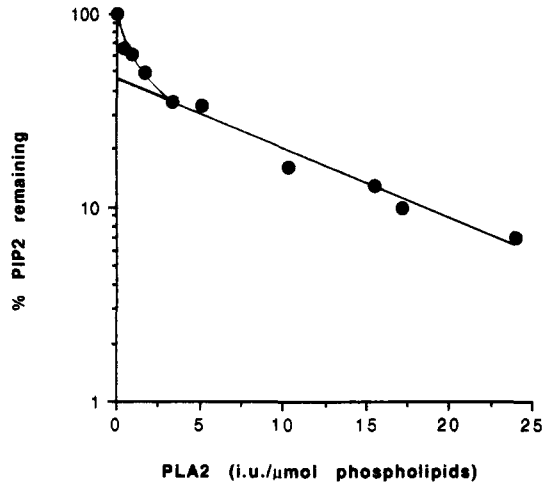

FIGURE 4: Effect of PLA_2 on PIP_2 hydrolysis in erythrocyte membranes prepared by saponin lysis. Saponin membranes were incubated with various concentrations of PLA_2 for 1 h, at 37 °C, in medium B containing 1 mM $CaCl_2$ and treated as described in the legend of Figure 2. Values shown are taken from three independent experiments, carried out at different concentrations of PLA_2.

in sIOV and proteolyzed sIOV, relative to native membranes) (data not shown).

Although it was observed that PLC activity was lost after spectrin and actin extraction, this treatment had no effect on the PPI pool distribution as probed by PLA_2, when compared to that in ghost membranes (data not shown). The two other treatments (stripping of the peripheral proteins or proteolysis of the cytoplasmic fragments of the integral proteins) produced a small decrease in the maximal percentage of hydrolysis of all the phospholipids. For instance, in the case of PA, this percentage was 98% in intact membranes, 93% in sIOV, and 90% after proteolysis of sIOV. The same decrease was observed also for PC and PE. Hydrolysis of PIP_2 and PIP did not seem to reach a plateau, at least in the range of PLA_2 concentrations used. Stripping of bands 2.1, 4.1, and 6 led to a significant decrease in the size of the highly sensitive pool (R_1) of PIP_2 (Figure 6A). R_1 for all the other phospholipids analyzed, PIP (Figure 6B) and PA (Figure 6C), PC, and PE, was not modified by the stripping procedure (Table I). Proteolysis of the cytoplasmic domains of mainly glycophorins and band 3 induced more drastic effects: R_1 decreased from 52% in ghost membranes to 18% in protelyzed sIOV for PIP_2 and and from 71% to 48% for PIP (Figure 6A,B and Table I). These modifications in pool sizes were specific for PPI: R_1 for PA, PC, and PE remained unchanged (Table I).

DISCUSSION

In contrast to intact erythrocytes, in which PLA_2 only hydrolyzes the phospholipids of the external membrane leaflet (Zwaal et al., 1975), the extent of phospholipid hydrolysis in white ghost membranes, due to their leakiness, was found to be almost 100%, as already reported (Kahlenberg et al., 1974; Van Meer et al., 1980). The PLA_2 dose dependence curves of hydrolysis of all the phospholipids examined exhibited a biexponential shape, characteristic of each phospholipid, providing evidence for a highly sensitive and a relatively resistant pool of phospholipids to PLA_2. This suggests that phospholipids could be arranged in, at least, two domains differing by their sensitivity to the enzyme. This conclusion is in agreement with direct observations, by fluorescence digital imaging microscopy, of PC and PS domains in human erythrocyte membranes (Rodgers & Glaser, 1991) and of PC clustering into domains as probed by an acidic and a basic snake venom PLA_2 (Shuckla & Hanahan, 1982).

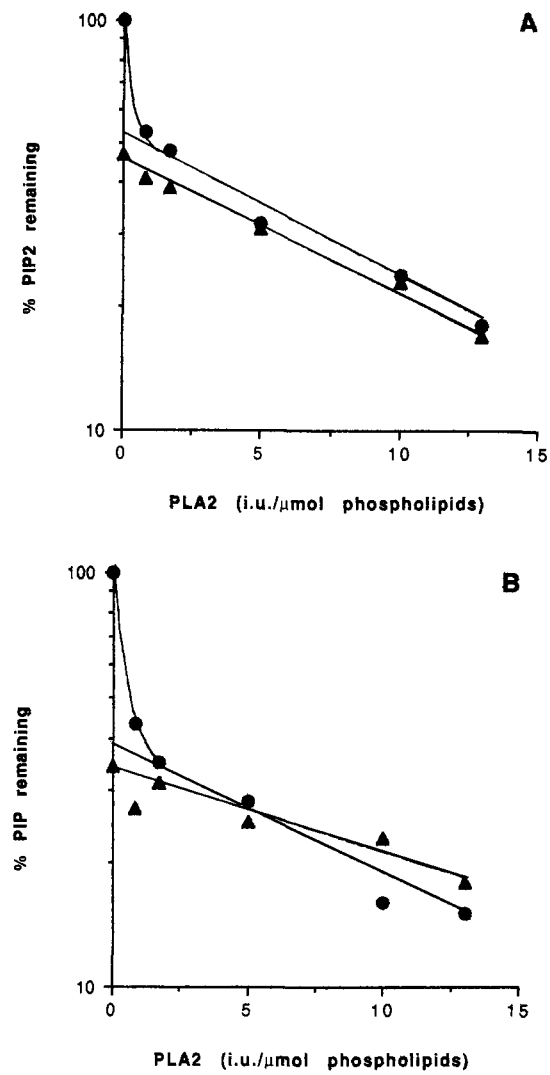

FIGURE 5: Effect of PLA_2 on PIP_2 (A) and PIP (B) before and after depletion of the PLC-sensitive pools. Hypotonic membranes, prepared from erythrocytes pretreated with the ionophore A23187 and either 1 mM $CaCl_2$ (▲) or 1 mM EGTA (●), were incubated with various concentrations of PLA_2 for 1 h, at 37 °C, in medium B containing 1 mM $CaCl_2$ and treated as described in the legend of Figure 2. Values shown are from one experiment. Another one gave similar data.

To validate the use of PLA_2 as a suitable tool to probe the heterogeneous distribution of phospholipids in natural membranes, it was necessary to show that, when pure phospholipids in SUV were used as substrates, this heterogeneity was not observed. In such vesicles, PLA_2 only attacks the phospholipids of the outer layer (Wilschut et al., 1979) which contains about 60–65% of the total molecules (Litman, 1973; Berden et al., 1975), due to the small diameter of the vesicles (20–25 nm) (Huang, 1969). In agreement with these data, we found that the maximal hydrolysis of PC and PE, either in PC or in PC/PE SUV, was about 60–70%. The maximal hydrolysis of the phosphoinositides in PC/PIP and in PC/PIP_2 SUV was different (respectively 80 and 35%), indicating that PIP was preferentially located in the outer and PIP_2 in the inner layer of the bilayer. However, the extent of their hydrolysis was a monoexponential function of the concentration of PLA_2. Thus, the biphasic plots of phospholipid hydrolysis, observed in natural membranes, were not an artifact linked to the use of PLA_2 since they were not observed in SUV.

The major goal of this study was to determine the origin of the nonrandom distribution of phospholipids in the erythrocyte membrane. The metabolic compartmentation of PPI

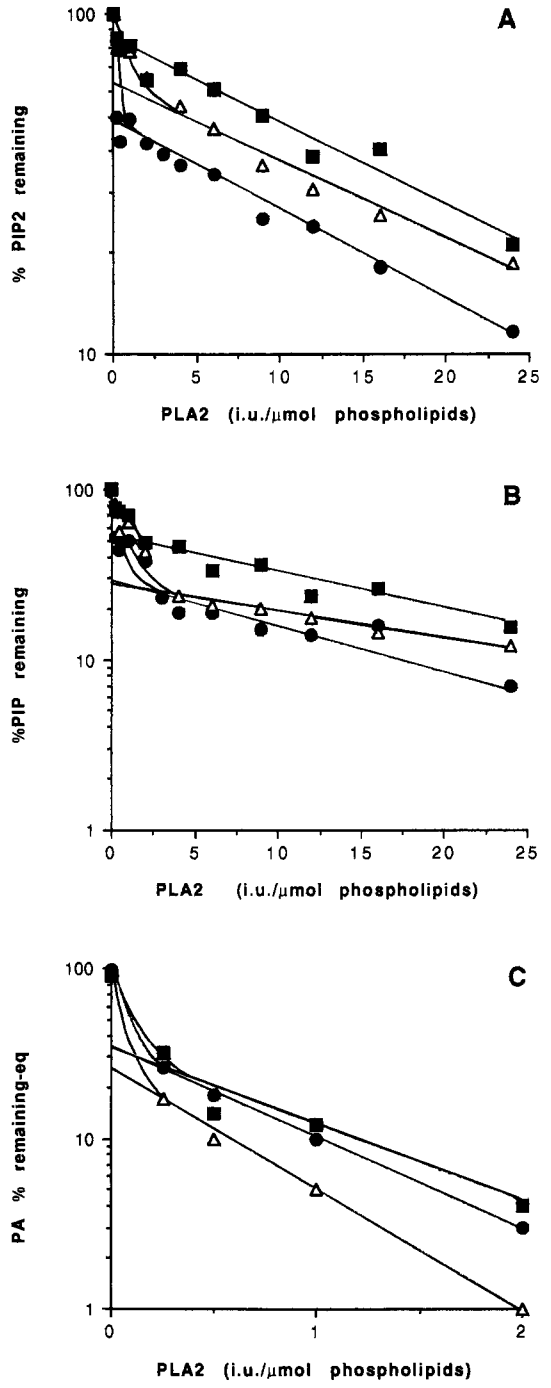

FIGURE 6: Effect of skeletal and anchoring protein depletion from erythrocyte membranes (sIOV) and of proteolysis of sIOV on the PLA_2-induced hydrolysis of various phospholipids. Membranes, prepared by hypotonic lysis, were first depleted of spectrin and actin in hypotonic medium, and then of proteins 2.1 and 4.1 and band 6 in hypertonic medium (sIOV). Proteolysis of the cytoplasmic domains of mainly band 3 and glycophorins was achieved by incubation with trypsin and α-chymotrypsin. Membranes (●), sIOV (△), and proteolyzed sIOV (■) were washed and incubated in medium B, for 1 h at 37 °C, in the presence of 1 mM $CaCl_2$ and various concentrations of PLA_2 and treated as indicated in the legend of Figure 2. Values shown are means of two (PA) or three experiments (PIP, PIP_2). (A) PIP_2; (B) PIP; (C) PA remaining at each concentration of PLA_2 minus PA remaining at the maximal dose (eq).

is well documented (Müller et al., 1986; King et al., 1987; Gascard et al., 1989). We have shown previously that the turnover of the phosphate monoester groups on both PIP and PIP_2 is heterogeneous with a rapidly turned over pool directly accessible to the specific kinases and phosphatases and to the PLC (when activated by intracellular Ca^{2+}) and a slowly turned over pool only exchangeable with the rapidly turned over one (Gascard et al., 1989). Different observations argue in favor of the identification of the highly sensitive fractions of PIP and PIP_2 to PLA_2 as the PLC-sensitive pools: (1) they have equal sizes; (2) in saponin membranes, devoid of PLC activity, the pool of PIP_2 highly sensitive to PLA_2 was equal to the PLC-sensitive pool characterized in hypotonic membranes; (3) after depletion of the PLC-sensitive pools of PIP and PIP_2 in intact erythrocytes, the resulting membranes only displayed the PLA_2 more-resistant pools. These data suggest that the two kinetic pools of PPI, PLC-sensitive and -insensitive, may be identical to the PLA_2-sensitive and PLA_2 more-resistant fractions.

We have investigated whether the difference in sensitivity to PLA_2 between the R_1 and R_2 fractions of phospholipids resulted from the association of one of these fractions with skeletal or integral membrane proteins. PLA_2-induced phospholipid hydrolysis was measured on erythrocyte membranes after various treatments leading to skeletal and anchoring protein depletion and to proteolysis of the cytoplasmic domains of integral proteins. Extraction of peripheral proteins leading to inside-out vesiculation occurred without any change in the phospholipid composition, in agreement with the data of Kahlenberg et al. (1974). As about 90% of the IOV and sIOV were unsealed (see Materials and Methods), both sides of the membrane were accessible to PLA_2. Limited depletion of skeletal proteins (spectrin and actin), although accompanied by a loss of PLC activity, had no effect on the sensitivity of PPI or of the other phospholipids to PLA_2. Membrane stripping of ankyrin, bands 4.1 and 6, significantly reduced the fraction of PIP_2 highly sensitive to PLA_2 (R_1, in Table I). Among the skeletal proteins, protein band 4.1 was the most likely to interact with PIP_2 since PIP_2 has been shown to regulate the glycophorin–band 4.1 association (Anderson & Marchesi, 1985; Gascard et al., 1993). However, a direct interaction of band 4.1 with PIP_2 was ruled out since band 4.1 failed to bind to liposomes reconstituted with PIP_2 in the absence of glycophorins (Anderson & Marchesi, 1985). Our study also revealed that the state of phosphorylation of band 4.1, adducin, and band 4.9, increased by PKC activation in intact cells, did not affect PPI distribution.

When the removal of peripheral proteins was followed by proteolysis (mostly of cytoplasmic domains of glycophorins and band 3), the R_1 fraction of PIP was also reduced and that of PIP_2 even more. The redistribution of PPI in favor of the R_2 fraction, induced by protein cleavage, was specific for these phospholipids. Such a change was not observed for either PC, PE, or PA, indicating that the decreased accessibility of PPI to PLA_2 could not be explained by a nonspecific effect related, for instance, to an increase in lateral pressure in proteolyzed sIOV as compared to native membranes. These data could support the hypothesis that the hydrophilic tails of integral proteins contribute to maintain PPI in domains where they are easily accessible to PLA_2 and to the endogenous PLC. In contrast, the segregation of PC, PE, and PA in domains differing by their sensitivity to PLA_2 does not seem to depend on such interactions. Both band 3 (Lux et al., 1989) and glycophorin A (Tomita et al., 1978) cytoplasmic segments have a cluster of cationic amino acid residues close to the bilayer interface which could interact with the negatively charged phosphate groups of PPI. In red cells, the lipids which copurify with band 3 do not contain arachidonic acid (Maneri & Low, 1989), a fatty acid very abundant in red cell PPI (Allan & Cockcroft, 1983), whereas isolated glycophorin retains phosphoinositides (Armitage et al., 1977; Yeagle & Kelsey, 1989). It has been shown recently that only the PLC-sensitive pool of PIP_2 was involved in the regulation of binding

of protein 4.1 to glycophorins in sIOV (Gascard et al., 1993). This is consistent with the proposal that this pool, the R_1 fraction, belongs to protein-rich lipid domains, including those surrounding the glycophorins. Conversely, the R_2 fraction would be free of interactions with proteins and could correspond to the diffusible PPI molecules which were recovered in the exocytic microvesicles released from erythrocytes upon various treatments (Hagelberg & Allan, 1990).

In conclusion, we present evidence that the two domains of PPI, that differ in their sensitivity to PLA_2, may be identical to the two metabolic pools previously characterized. Our data suggest that the rapidly metabolized pool, involved in the regulation of major cellular functions (signal transduction in nucleated cells, protein–protein interaction, ionic transport), may be maintained in its functional state through interactions with integral proteins.

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