Antagonist Effects of Ca^{2+} and Spermine on Phosphatidylinositol 4,5-Bisphosphate-Mediated Transmembrane Redistribution of Phospholipids in Large Unilamellar Vesicles and in Erythrocytes†

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ABSTRACT: We have previously suggested the involvement of a Ca^{2+}−phosphatidylinositol 4,5-bisphosphate (PIP_2) complex in the phospholipid transmembrane redistribution triggered by cytosolic Ca^{2+} in erythrocytes. Indeed, the lipid scrambling was induced by extracellular Ca^{2+} in erythrocytes loaded with PIP_2 and was abolished in inside-out vesicles prepared from PIP_2-depleted erythrocytes (Sulpice, J. C., Zachowski, A., Devaux, P. F., & Giraud, F. (1994) *J. Biol. Chem. 269*, 6347−6354). Here, we show that Ca^{2+} triggers a partial redistribution of spin-labeled phospholipids in protein-free large unilamellar vesicles (LUVs), only when they contain PIP_2. Spermine, a polyamine known to interact with PIP_2 and reported to inhibit lipid scrambling in resealed ghosts, was found to inhibit also the Ca^{2+}-induced scrambling in LUVs and in PIP_2-loaded erythrocytes, presumably by interacting with PIP_2 and preventing the formation of Ca^{2+}-PIP_2 complexes. A similar mechanism can account for spermine inhibition in natural membranes, confirming the role of PIP_2 in the scrambling process without excluding the participation of proteins. In erythrocytes, activation of the phosphoinositide phospholipase C (PLC) or a 20 h ATP depletion, which both led to a reduction in the PIP_2 content by 40−60%, did not affect Ca^{2+}-induced phospholipid scrambling. In contrast, longer ATP depletion, resulting in a 80% reduction in the PIP_2 content, did induce a significant decrease in lipid scrambling, suggesting that only the PIP_2 pool resistant to the PLC was involved. Spermine was able to inhibit hydrolysis of this pool by an exogenous PLA_2. It is thus likely that spermine antagonized the Ca^{2+}-induced scrambling in resealed ghosts by interacting with the PLC-resistant pool of PIP_2.

It is well established that phospholipids are asymmetrically distributed across the plasma membrane of erythrocytes (Bretscher, 1972; Op den Kamp, 1979), resting platelets (Chap et al., 1977), other circulating blood cells (Devaux, 1992), and various animal cells (Zachowski, 1993). The asymmetry is maintained in part by the activity of the aminophospholipid translocase (APLT)[1] or of a protein complex which catalyzes the transport of phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the outer to the inner leaflet of the membrane (Devaux, 1991; Schroit & Zwaal, 1991). Loss of this asymmetry has been reported in a number of circumstances including cellular activation (Zwaal et al., 1989), apoptosis (Fadok et al., 1992; Martin et al., 1995; Sambrano & Steinberg, 1995; Verhoven et al., 1995), erythrocyte aging (Shukla & Hanahan, 1982; Connor et al., 1994), and sickle cell anemia (Franck et al., 1985). There is increasing evidence that Ca^{2+} is the triggering factor of this loss of asymmetry (known as phospholipid scrambling), as it can be induced in erythrocytes or resealed ghosts by an increase in intracellular Ca^{2+} upon treatment with a divalent cation ionophore (Williamson et al., 1985; Chandra et al., 1987; Comfurius et al., 1990; Williamson et al., 1992). Although Ca^{2+} strongly inhibits the APLT (Bitbol et al., 1987), the phospholipid redistribution cannot be solely attributed to inactivation of the translocase (Comfurius et al., 1990; Verhoven et al., 1992). Scrambling is independent of other Ca^{2+}-dependent proteins or enzymes, such as transglutaminase, calpain, calmodulin, phosphoinositide-specific phospholipase C (PLC), and phospholipase A_2 (Chandra et al., 1987; Comfurius et al., 1990; Verhoven et al., 1992; Henseleit et al., 1990; Connor et al., 1990).

It has been proposed that scrambling would result from interactions between Ca^{2+} and anionic phospholipids (Lin et al., 1994; Bratton, 1994) and/or between Ca^{2+}, proteins, and anionic phospholipids (Bratton, 1994). Others have suggested the implication of phosphorylated protein(s) (Martin & Jesty, 1995) or of a specific protein deficient in Scott syndrome erythrocytes (Zwaal et al., 1993). We have recently used PIP_2-loaded erythrocytes and erythrocyte inside-out vesicles (IOVs) to show that PIP_2 was required in the Ca^{2+}-induced phospholipid scrambling (Sulpice et al., 1994). External Ca^{2+}, which has no effect on phospholipid redistribution in normal erythrocytes, induced in the PIP_2-loaded cells a rapid randomization of the four major phospholipids, without PIP_2 hydrolysis, when APLT was

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[1] Abbreviations: APLT, aminophospholipid translocase; BSA, bovine serum albumin; ESR, electron spin resonance; IOVs, inside-out vesicles; LUVs, large unilamellar vesicles; PC, phosphatidylcholine; PC*, spin-labeled PC; PE, phosphatidylethanolamine; PE*, spin-labeled PE; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLA_2, phospholipase A_2; PLC, phosphoinositide-specific phospholipase C; PS, phosphatidylserine; PS*, spin-labeled PS; SM, sphingomyelin; SM*, spin-labeled SM.

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inhibited. Ca^{2+}-induced scrambling was abolished in IOVs prepared from PIP_2-depleted erythrocytes and enhanced in PIP_2-enriched IOVs. In erythrocyte membranes, PIP_2 is metabolically heterogeneous: a first pool is rapidly turned over, being accessible to the specific kinase and phosphatase and to the PLC, whereas a second pool is only slowly turned over by exchanges with the former one (Gascard et al., 1989). There is some evidence to suggest that the second pool of PIP_2 would be involved in scrambling (Sulpice et al., 1994). PIP_2 is known to regulate the interactions between glycophorin and protein 4.1 (Anderson & Marchesi, 1985; Gascard et al., 1993a) and to interact with proteins containing plekstrin homology (PH) domains (Harlan et al., 1994) and with actin-binding proteins (Janmey, 1994, 1995). The existence of such PIP_2−protein interactions raises the possibility that the Ca^{2+}-induced pathway leading to phospholipid scrambling involves both PIP_2 and a protein. Spermine, a naturally occurring polyamine, has been shown to bind tightly to polyphosphoinositides, especially to PIP_2 in artificial membranes (Tadolini & Varani, 1986; Toner et al., 1988; Meers et al., 1986). Spermine is the most positively charged polyamine, when compared to spermidine and putrescine, which makes it a potential candidate to inhibit Ca^{2+} binding to PIP_2 (Schuber, 1989). On the other hand, Bratton (1994) has reported that polyamines inhibit phospholipid scrambling in resealed erythrocyte ghosts with a characteristic potency of spermine > spermidine > putrescine. It is thus likely that this inhibition could result from polyamine−PIP_2 interactions which would prevent Ca^{2+} accessibility to PIP_2.

The purpose of the present study is to confirm this hypothesis using conditions under which PIP_2 is unambiguously responsible for the Ca^{2+}-induced phospholipid scrambling. The results show that Ca^{2+} induced phosphatidylcholine (PC) scrambling in large unilamellar vesicles (LUVs) as long as they contained PIP_2 and that spermine inhibited this movement as well as in PIP_2-loaded erythrocytes. Similar inhibition of PC scrambling by spermine was observed in resealed ghosts. Furthermore, we found that spermine preferentially blocked the PLA_2-mediated hydrolysis of one of the two PIP_2 metabolic pools. Therefore, the polyamine provided a valuable tool for investigating whether PIP_2 was involved in the Ca^{2+}-induced lipid scrambling in erythrocyte membranes and which of the PIP_2 pools was concerned. In parallel, the same question was addressed by manipulating the PIP_2 content of each pool. The data strongly suggest that the slowly turned over PIP_2 pool, inaccessible to endogenous PLC, would be one of the Ca^{2+} targets responsible for phospholipid scrambling.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA) (fatty acid free) (A 7511), bovine brain PIP_2 (sodium salt) (P 9763), bovine brain phosphatidylinositol 4-monophosphate (PIP) (sodium salt) (P 9638), spermine (S 4264), calcium ionophore A23187 (C 7522), bee venom phospholipase A_2 (PLA_2) (P 9279), and egg yolk phosphatidylcholine (PC) (P 3556) were obtained from Sigma (France). Spin-labeled phospholipids with a short (C5) β-chain bearing a nitroxide probe, phosphatidylcholine (PC*), sphingomyelin (SM*), phosphatidylethanolamine (PE*), and phosphatidylserine (PS*), were synthesized as previously described (Fellmann et al., 1994). [^{32}P]P_i was purchased from ORIS (France).

Transbilayer Redistribution of Spin-Labeled Phospholipids in LUVs. PC, solubilized in chloroform, was dried under vacuum and suspended by vigorous vortexing in the incubation medium (145 mM NaCl, 0.1 mM EGTA, and 10 mM HEPES (pH 7.4)) at a final phospholipid concentration of 5 mM to obtain multilayered vesicles. When present, PIP_2 or PIP (sodium salt) was solubilized in the medium and added to the dried lipid film at a PIP_2/PC molar ratio of 5/95. This ratio was chosen knowing that in small PIP_2/PC unilamellar vesicles the yield of incorporation of PIP_2 was about 50% (unpublished data). As PIP_2 represents about 1% of the total membrane phospholipids in the erythrocyte membrane and is mainly concentrated within the inner leaflet, its concentration within this leaflet is around 2% of the phospholipids (Gascard et al., 1991). Under our experimental conditions, the initial 5/95 mixture would end with LUVs at a final 2−2.5% PIP_2 concentration, close to its physiological level. Multilayered vesicles were frozen and thawed three times, and large unilamellar vesicles (LUVs) were produced by extruding the lipid dispersion through filters of decreasing diameter (0.4, 0.2, and 0.1 μm) (Hope et al., 1985; Meers et al., 1986).

To assay inward movement of phospholipids, the probes (PC*, SM*, PE*, or PS*) dispersed in the medium as monomers and micelles were added to the LUV suspension, resulting to their incorporation in the outer leaflet, at a final concentration of 1% of the total lipids. LUVs were incubated in the presence or absence of $CaCl_2$. At different time intervals, aliquots were diluted with fatty acid-free BSA (2% final concentration) and kept 1 min on ice. The spin-labeled phospholipids, originally embedded in the outer leaflet and extracted by BSA, were rapidly reduced by addition of sodium ascorbate (65 mM final concentration). The residual intensity of the low field electron spin resonance (ESR) line, attributed to the spin-labeled phospholipids translocated to the inner leaflet, was expressed as a percentage of that obtained without addition of BSA and sodium ascorbate. In each sample, the signal of the probe inaccessible to sodium ascorbate remained stable over several minutes, showing that the lipid bilayer was poorly permeable to sodium ascorbate.

Unilamellarity of the vesicles was tested on LUVs with a symmetrical distribution of the PC* probe, prepared from a mixture of phospholipids containing the probe. The percentage of the inner probe, after BSA extraction and reduction by ascorbate of the probe present in the outer leaflet, was 53.5%, instead of 50% expected for unilamellar vesicles. Assuming that the population contains some bilamellar vesicles, with 75% of unextractable and nonreduced probe, it can be calculated that 86% of the vesicles were unilamellar. The presence of some vesicles of higher multilamellarity would even increase the proportion of unilamellar vesicles.

To study phospholipid outward movement, LUVs were prepared with a symmetrical distribution of the probe (see above). The probe in the outer leaflet was reduced by ascorbate (65 mM final concentration), resulting in an ESR signal corresponding to the probe located in the intravesicular leaflet. The amount of probe remaining in the inner leaflet was measured as a function of the incubation time in the presence or absence of Ca^{2+}.

PIP_2 Loading and Depletion of Erythrocytes. Blood was withdrawn from healthy volunteers, heparinized, and centrifuged (1300*g*, 10 min, 4 °C). The packed erythrocytes were washed three times in medium A (145 mM NaCl, 2 mM KCl, 10 mM glucose, 10 mM phosphate buffer (pH 7.4)).

Erythrocytes, suspended at 2.5% hematocrit in medium A supplemented with 1 mM EGTA and 1 mM EDTA, were incubated for 10 min at 37 °C in the presence of 50 μM PIP_2. These conditions led to an incorporation of PIP_2 which

amounted to about 1% of the total membrane phospholipids, equal to the endogenous PIP_2 content (Sulpice et al., 1994). After PIP_2 loading, cells were washed once in K medium (55 mM NaCl, 90 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.4) and resuspended at 30% hematocrit in the same medium containing either 0.1 mM EGTA or 1 mM $CaCl_2$ for measurements of phospholipid redistribution by ESR (see below) or at 2.5% hematocrit for morphological observations. Hemoglobin release was assayed during the incubations by measuring the absorbance of the supernatant at 540 nm.

ATP depletion has been previously shown to induce a dramatic decrease in membrane PIP_2 content resulting from an inhibition of phosphoinositide kinases (Gascard et al., 1989; Ferrell & Huestis, 1984; Bütikofer et al., 1989; Thompson et al., 1987). Erythrocytes were ATP-depleted with a 20−72 h incubation at 37 °C under nitrogen in 145 mM NaCl, 1 mM EDTA, 1 mM $MgSO_4$, 10 mM Tris-HCl (pH 7.4), and penicillin (100 μg/mL).

To determine the PIP_2 content, erythrocytes were pelleted and lysed in 30 volumes of ice cold 10 mM Tris-HCl and 1 mM EDTA (pH 7.4). The membranes were centrifuged (25000*g*, 10 min) and washed in the same buffer at 4 °C. Membrane lipids were extracted for 60 min at room temperature by addition of 3.75 volumes of $CHCl_3$/MeOH/13 M HCl (20/40/1, v/v). Phase partitioning was obtained by addition of 1.25 volumes of $CHCl_3$ and 1.25 volumes of H_2O. The lower organic phase was removed and dried under vacuum. Phospholipid separation and phosphorus assay were carried out as described before (Gascard et al., 1991, 1993b).

Hydrolysis of PIP_2 by PLC or Phospholipase A_2 (PLA_2) in Sealed and Unsealed Ghosts. Erythrocytes, suspended at 40% hematocrit in Na medium (145 mM NaCl, 10 mM glucose, and 10 mM HEPES, pH 7.4), were first incubated for 4 h at 37 °C with [^{32}P]P_i (3.7 MBq/mL of cells) to label the polyphosphoinositides. Under these conditions, the specific radioactivity of both [^{32}P]PIP_2 pools (PLC-sensitive and PLC-resistant) was identical, although equilibration with that of the γ-^{32}PATP pool was not yet reached (Gascard et al., 1989). The cells were washed three times in medium B (137 mM KCl, 2.7 mM NaCl, 10 mM K_2HPO_4, 8.5 mM KH_2PO_4 (pH 7.4)) and sealed ghosts were prepared as described by Bratton (1994), except using K^+ instead of Na^+ in the hypotonic lysis buffer. The cells were resuspended in 20 volumes of medium B diluted to 1:5 and supplemented with 1 mM $MgCl_2$ and 0.1 mM EGTA, and incubated for 1 h at 4 °C. These conditions allow ghosts to retain the native asymmetric transbilayer distribution of phospholipids (Bratton et al., 1991). When required, spermine was added to the lysis buffer and the pH was readjusted to 7.4. After 60 min, isotonicity was restored with 5-fold concentrated medium B, and ghosts were resealed by warming to 37 °C for 1 h. Resealed ghosts were isolated by centrifugation (15 min at 1300*g*) and washed twice in Na medium (without glucose). Endogenous PLC was activated upon ghost incubation in the presence of Ca^{2+} and ionophore A23187.

Unsealed ghosts were prepared from erythrocytes, prelabeled with [^{32}P]P_i as indicated above, by hypotonic lysis at 4 °C in 30 volumes of 5 mM sodium phosphate (pH 7.6) and 1 mM EDTA and centrifugation (15 min at 15000*g*). Pellets were washed twice at 4 °C and incubated at 37 °C for 60 min at about 2 mg of membrane protein/mL of Na medium (without glucose) containing various concentrations of Ca^{2+} and PLA_2 (100 IU/μmol of phospholipids). The reaction was stopped by the addition of 1 volume of the incubation medium containing 1.2 mM EGTA. [^{32}P]PIP_2 radioactivity in sealed and unsealed ghost membranes was measured after direct acidic extraction and separation of phospholipids as previously described (Gascard et al., 1993b). The Ca^{2+}-and/or PLA_2-induced decrease in [^{32}P]PIP_2 radioactivity was used as a measure of the extent of PIP_2 hydrolysis.

Transbilayer Redistribution of PC in Erythrocytes and Resealed Ghosts*. Erythrocytes (control, PIP_2-loaded, or PIP_2-depleted) or sealed ghosts were resuspended either in Na medium or in K medium without glucose at 30% hematocrit (corresponding to a membrane phospholipid concentration of 1.2 mM). When required, ionophore A23187 or spermine (in the case of PIP_2-loaded cells) were added to the suspension at room temperature, and 1 min later spin-labeled PC (PC*) was added in an amount corresponding to 0.8% of total phospholipid content of the suspension. After addition of $CaCl_2$, $BaCl_2$, or EGTA, the suspension was transferred to 37 °C (time 0 of the kinetics). Treatment of cells or ghosts with Ca^{2+} and ionophore in Na medium may induce shedding of microvesicles. Therefore, inward PC* translocation was measured from the signal remaining in the remnant cells after removal of the vesicles and of BSA-extractable PC* by centrifugation. At given time intervals, aliquots (70 μL) were withdrawn and mixed at 4 °C with 50 μL of either BSA (4%) or incubation medium. After 1 min, 700 μL of cold medium was added and the suspension was centrifuged (30 s, 8000*g*). The cell pellets were lysed with 30μL of water, and the amount of associated PC* was assayed by ESR spectroscopy after reoxidation by ferricyanide (Williamson et al., 1992; Fellmann et al., 1994). The intensity of the central peak of the signal measured in the BSA-treated pellets (PC* in the inner leaflet), compared to that in the corresponding untreated pellets (total PC*, inner plus outer leaflets), gave the percentage of spin-labeled on the inner monolayer. In other experiments (PIP_2 load or PIP_2 depletion), cells were incubated without or with ionophore, in K medium to avoid microvesicle formation (Allan & Thomas, 1981), and the BSA-extractable PC* corresponding to the PC* present in the outer leaflet was directly measured in the supernatant.

RESULTS

PIP_2 Requirement for Ca^{2+}-Induced Phospholipid Scrambling in LUVs. Inward movement of PC* was measured in LUVs by BSA extraction of the probe contained in the external leaflet and reduction of the nitroxide by ascorbate. In LUVs containing exclusively PC, PC* redistribution was hardly detectable, either in the absence or in the presence of Ca^{2+} (Figure 1A), suggesting that ascorbate or Ca^{2+} did not induce alterations in the vesicle structure which could affect the measurements. Likewise, in PC/PIP_2 (5/95) LUVs no PC* movement was detectable in the absence of Ca^{2+} even in the presence of spermine (Figure 1B). Remarkably, a rapid internalization of about 15% of PC* was observed by 2 min incubation in the presence of Ca^{2+} (Figure 1A), already significant at 0.3 mM Ca^{2+}, maximal at 2 mM Ca^{2+}, and unaffected by an increase in the incubation temperature from 20 to 37 °C (Figure 1B). A smaller Ca^{2+}-induced PC* redistribution (6−8%) was also observed in PIP2/PC (2/98) LUVs (data not shown). The effect of Ca^{2+} was specific for PIP_2: in LUVs prepared from a mixture of PIP and PC (5/95), Ca^{2+} did not trigger any redistribution of PC* (Figure 1B). The transmembrane redistribution was not restricted to PC*, since PS*, PE*, and SM* were also randomized (data not shown). The outward movement of the probe in PIP_2-containing LUVs exhibited the same characteristics as those of the inward movement. The addition of Ca^{2+} resulted in

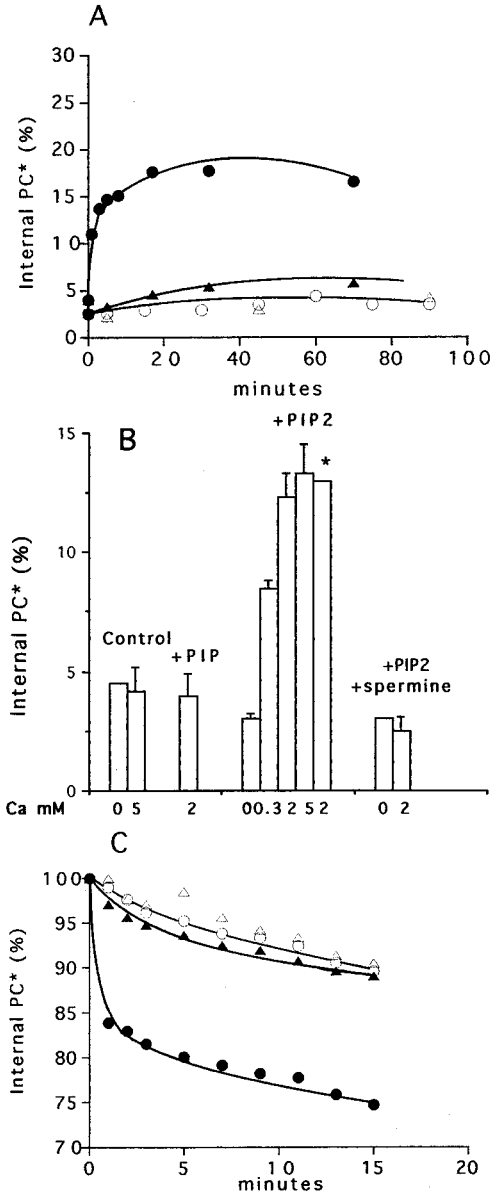

FIGURE 1: The effect of Ca^{2+} on inward (A and B) and outward (C) transbilayer movement of PC* in LUVs. (A and B) LUVs were prepared from PC, PIP/PC (5/95 mol/mol), or PIP_2/PC (5/95 mol/mol) mixtures. After incorporation of the PC* probe, LUVs were incubated at 20 or 37 °C (*), with or without $CaCl_2$ (2 mM in panel A and the indicated concentrations in panel B). In panel B, spermine concentration was 2 mM in the absence of $CaCl_2$ and 0.5 mM in presence of 2 mM $CaCl_2$. At various times, aliquots were extracted with BSA and treated with ascorbate to reduce the extractable PC*. The remaining ESR signal intensity represented the fraction of PC* on the inner leaflet. The time course in (A) is from one experiment representative of 7 experiments. Data shown in (B) are plateau values at 20 or 30 min and are means of 2 or means ± SE of 3−9 experiments. (C) LUVs were prepared from PC or PIP_2/PC (5/95 mol/mol) mixture containing the PC* probe, and the outwardly exposed probe was reduced by ascorbate prior to the incubation at 20 °C without or with 4 mM $CaCl_2$. The ESR signal was measured at different times. Data shown are from one experiment representative of 2 experiments. Symbols in panels A and C: PC LUVs, no Ca^{2+} (△); PC LUVs, $+Ca^{2+}$ (▲); PIP_2/PC LUVs, no Ca^{2+} (○), PIP_2/PC LUVs, $+Ca^{2+}$ (●).

the externalization of about 15% of probe by 1 min incubation, whereas Ca^{2+} had no effect on PC* redistribution in LUVs without PIP_2 (Figure 1C).

Inhibition of Ca^{2+}-Induced Phospholipid Scrambling by Spermine in LUVs and Erythrocytes. The effect of spermine on Ca^{2+}-induced phospholipid scrambling was investigated under two conditions in which this process was unambiguously dependent on PIP_2. In the PIP_2-containing LUVs, the inward redistribution of PC* induced by 2 mM Ca^{2+} was totally inhibited by a preincubation with 0.5 mM spermine, whereas up to 2 mM spermine had no effect on PC* redistribution in the absence of Ca^{2+} (Figure 1B). In erythrocytes loaded on the external leaflet with exogenous PIP_2, phospholipid scrambling could be induced upon addition of Ca^{2+} in the external medium (Sulpice et al., 1994). Figure 2A shows that the inward diffusion of PC* was low in PIP_2-loaded erythrocytes incubated in a Ca^{2+}-free medium, whereas addition of 1 mM Ca^{2+} induced a rapid transbilayer randomization of the probe, which was nearly maximal after 30 min. Phospholipid redistribution proceeded in conjunction with the formation of stomatocytes as reported before (Sulpice et al., 1994). Furthermore, a significant release of hemoglobin was observed, whose time course was delayed when compared to the rate of PC* scrambling (Figure 2B), indicating that PC* scrambling could not result from hemoglobin leak, in contrast with recent conclusions from Bevers et al. (1995). The mechanism responsible for the loss of hemoglobin may be related to the late spherostomatocytosis (Haest et al., 1981). Indirect evidence suggests that membrane permeability to Ca^{2+} was preserved during the time of the experiments. Indeed, PIP_2 loading did not result in APLT inhibition (Sulpice et al., 1994), an effect observed upon an increase in the cytosolic Ca^{2+} concentration above 1 μM (Bitbol et al., 1987). Addition of 2 mM spermine totally inhibited Ca^{2+}-induced PC* redistribution (Figure 2A) and prevented stomatocytosis (data not shown) and hemoglobin release (Figure 2B), consistent with the idea that shape changes (Sulpice et al., 1994) and subsequent cell lysis resulted from phospholipid scrambling. To determine the effect of various amounts of spermine on the extent of scrambling, PC* redistribution was calculated as the difference between the percentage of PC* in the inner leaflet after 50 min incubation with and without 1 mM Ca^{2+}. Half-maximal inhibition occurred at about 0.8 mM spermine (Figure 2C).

We have also confirmed that spermine inhibits Ca^{2+}-induced phospholipid scrambling in resealed ghosts (Bratton, 1994). Ca^{2+} at concentrations as low as 20 or 50 μM induced a randomization of PC * which was drastically reduced by 0.3 mM spermine and entirely prevented by 2 mM spermine (Figure 3). In the absence of Ca^{2+}, spermine had no effect on PC* movement (data not shown). In conclusion, spermine inhibited Ca^{2+}-induced phospholipid scrambling in resealed erythrocyte ghosts and in both conditions under which PIP_2 was a necessary requirement.

PIP_2 Pool Involved in Ca^{2+}-Induced Phospholipid Redistribution in Erythrocytes. To determine whether spermine inhibited the scrambling in erythrocyte membrane by interacting with a given PIP_2 pool, its effect on the Ca^{2+} accessibility to each PIP_2 pool was investigated by measuring PIP_2 hydrolysis by Ca^{2+}-dependent phospholipases, either endogenous PLC or exogenous PLA_2. Figure 4 shows that hydrolysis of the PLC-sensitive pool of PIP_2, induced in sealed ghosts by Ca^{2+} (20 or 50 μM) in the presence of the ionophore A23187, was not significantly reduced when the ghosts were resealed in a medium containing 2 mM spermine. In unsealed white ghosts, 20 or 50 μM Ca^{2+} was unable to induce PIP_2 hydrolysis (Figure 4), in agreement with previous data (Allan & Thomas, 1981). Addition of exogenous PLA_2 in the presence of Ca^{2+} caused a drastic hydrolysis of all glycerophospholipids (Gascard et al., 1993b). The PLA_2-induced PIP_2 hydrolysis was about 90%, affecting both PIP_2 pools. It was partially inhibited (30%,

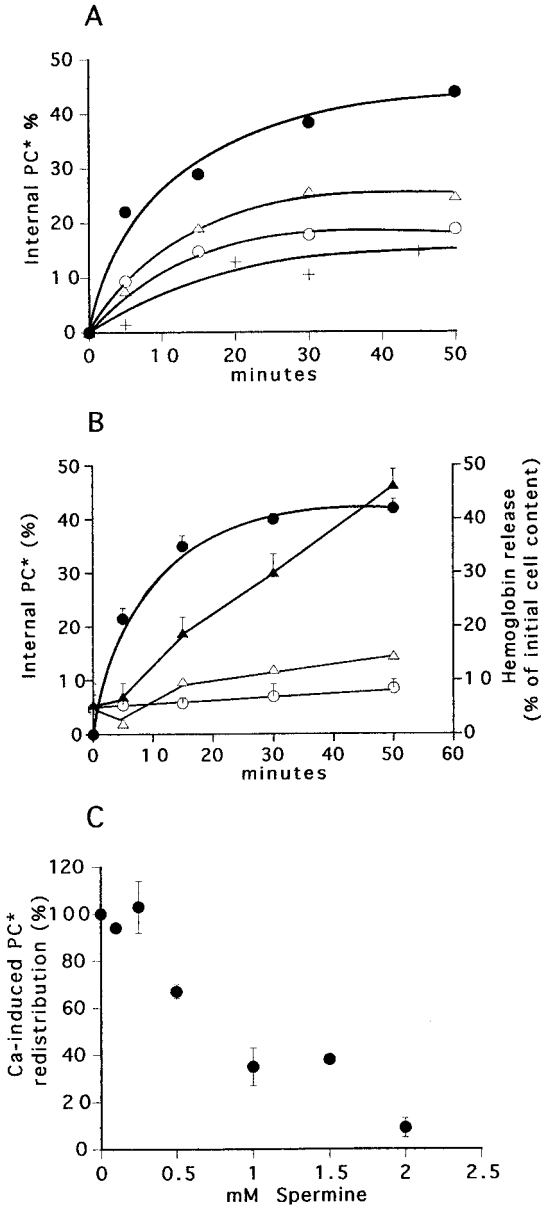

FIGURE 2: Inhibition by spermine of Ca^{2+}-induced inward transbilayer movement of PC* and of hemoglobin release in PIP_2-loaded erythrocytes. Erythrocytes were loaded with PIP_2, washed, and resuspended in K medium, with or without spermine. The PC* probe was incorporated 1 min prior to addition of either 100 μM EGTA or 1 mM $CaCl_2$ and transferred to 37 °C (time 0). At different intervals of times, aliquots were mixed with BSA and centrifuged. The percentage of hemoglobin release was measured in the supernatant. The percentage of the probe in the outer leaflet was determined by measuring the ESR signal in BSA supernatants. The percentage of the probe in the inner leaflet (internal PC*) was calculated from the difference to 100%. (A) Inward movement of PC: no Ca^{2+} (○), 1 mM Ca^{2+} (●), 1 mM Ca^{2+} + 2 mM spermine (△), 1 mM spermine (+). (B) Inward movement of PC: 1 mM Ca^{2+} (●) (means ± SE of 6 experiments) and hemoglobin release: no Ca^{2+} (○), 1 mM Ca^{2+} (▲), 1 mM Ca^{2+} + 2 mM spermine (△) (means ± SE of 5 experiments). (C) Effect of spermine concentration on Ca^{2+}-induced PC redistribution after 50 min incubation with or without 1 mM Ca^{2+} (means of 2 experiments or means ± SE of 3−7 experiments). Ca^{2+}-induced PC* redistribution was calculated from the difference between the percentage of PC* present in the inner leaflet with and without Ca^{2+}.

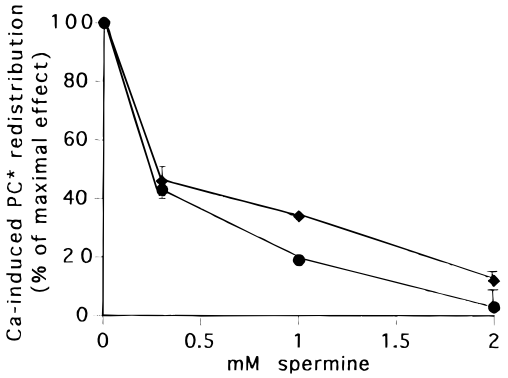

FIGURE 3: The effect of spermine on Ca^{2+}-induced inward redistribution of PC* in sealed ghosts. Ghosts were prepared by hypotonic lysis with various concentrations of spermine, sealed, and resuspended in Na medium. They were loaded with the PC* probe and incubated for 90 min at 37 °C, with 8 μM A23187 and either 100 μM EGTA or 20 (●) or 50 μM (◆) $CaCl_2$. Aliquots were extracted with BSA, diluted with cold medium, and centrifuged. Internal PC* was estimated by measuring the ESR signal in the pellets and is expressed as a percentage of the signal measured at time 0 in the total suspension without BSA. Ca^{2+}-induced PC* redistribution was calculated as explained in the legend of the Figure 2C (means ± SE of 3−5 experiments).

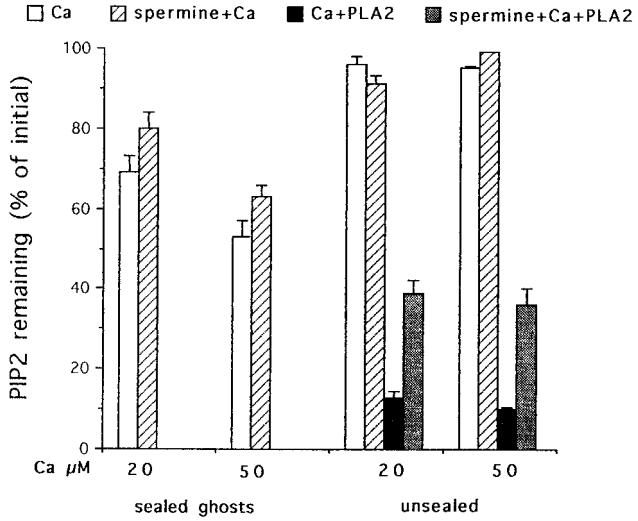

FIGURE 4: The effect of spermine on PIP_2 hydrolysis by Ca^{2+}-dependent endogenous PLC in sealed ghosts or exogenous PLA_2 in unsealed ghosts. Ghosts were prepared by hypotonic lysis with or without 2 mM spermine, sealed, resuspended in Na medium, and incubated for 60 min at 37 °C with 8 μM A23187 and $CaCl_2$. Unsealed ghosts, in Na medium, were preincubated for 5 min with or without 2 mM spermine and then incubated for 60 min at 37 °C with $CaCl_2$, in the presence or absence of PLA_2. PIP_2 content or radioactivity (nmol or cpm/nmol of membrane phospholipids) was measured before and after the incubations. Data are means ± SE of 3 or 4 experiments.

p <0.001) by a pretreatment of the ghosts with 2 mM spermine (Figure 4). In contrast, PLA_2-induced hydrolysis of PS, as well as of PC, PIP, and phosphatidic acid, was not inhibited by spermine (data not shown), demonstrating the specificity of the interaction of the polyamine with PIP_2. As spermine had no effect on the hydrolysis of the PLC-sensitive pool of PIP_2, the simplest hypothesis to explain these data is that spermine inhibited almost exclusively the PLA_2-induced hydrolysis of the PLC-resistant PIP_2 pool, presumably by interacting preferentially with this pool. It is thus likely that the inhibition of scrambling by spermine in resealed ghosts resulted from an interaction of the polyamine with the PLC-resistant PIP_2 pool.

The PIP_2 pool involved in the Ca^{2+}-induced phospholipid scrambling was also investigated by depleting either the PLC-sensitive pool or both pools. Treatment of erythrocytes with the ionophore A23187 and 1 mM Ca^{2+} to activate PLC resulted in the hydrolysis of 60% of PIP_2 corresponding to the PLC-sensitive pool (Gascard et al., 1989) (Figure 5a).

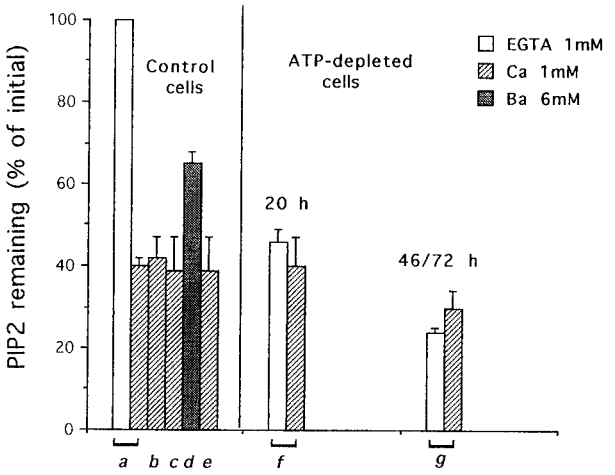

FIGURE 5: The effect of intracellular divalent cations on PIP_2 hydrolysis in control or ATP-depleted erythrocytes. Control erythrocytes, suspended in Na medium, were incubated at 37 °C (final hematocrit 30%) in the presence of A23187 (8 μM with EGTA or $CaCl_2$ or 15 μM with $BaCl_2$): (a) for 30 min with $CaCl_2$; (b) as in (a), then washed in Na medium containing 3 mM EGTA and reincubated for 60 min with $CaCl_2$; (c) as in (b) except the second incubation was for 120 min; (d) for 60 min with $BaCl_2$; (e) as in (d), then washed in Na medium containing 10 mM EGTA and reincubated for 60 min with $CaCl_2$; (*f* and *g*) following ATP depletion for 20−72 h (see Materials and Methods), cells were washed twice, resuspended in K medium, and incubated for 60 min in the presence of 8 μM A23187 and either EGTA or $CaCl_2$. PIP_2 content (nmol/μmol of total phospholipids) was measured at the end of the incubations with the ionophore and is expressed as percent of the initial content (means ± SE of 3−7 experiments).

In agreement with previous studies (Williamson et al., 1992; Smeets et al., 1994), this treatment randomized the four major phospholipids as illustrated by PC* behavior (Figure 6A). In another batch of cells treated in the same way but without PC* added, Ca^{2+} was chelated with EGTA after 30 min incubation. Cells were washed once, loaded with the PC* probe, and reincubated for 60 min either without or with Ca^{2+}. In the former case no phospholipid scrambling was detected, whereas in the latter case Ca^{2+} was still able to induce a scrambling of PC* (Figure 6B), indicating that the presence of the PLC-sensitive PIP_2 pool was not necessary to allow PC* redistribution. The second addition of Ca^{2+}, for 60 or 120 min, did not produce any significant additional PIP_2 hydrolysis (Figure 5b,c). Ba^{2+} (6 mM), added for 60 min instead of Ca^{2+} in the first incubation, resulted in a 35% PIP_2 breakdown (Figure 5d) but without any PC* scrambling (Figure 6A). After removal of Ba^{2+}, addition of 1 mM Ca^{2+} again induced PC* scrambling (Figure 6B), together with the hydrolysis of the remaining PLC-sensitive PIP_2 (Figure 5e). Thus the breakdown of PIP_2, presumably resulting from PLC activation by Ba^{2+}, did not by itself trigger the PC* redistribution, and again the unhydrolyzed pool of PIP_2 seemed to fulfil this role, as far as Ca^{2+} was present.

The PIP_2 content of erythrocyte membranes can be reduced without increasing intracellular Ca^{2+}, by inhibiting the phosphoinositide kinases by metabolic ATP depletion in a Ca^{2+}-free medium (Gascard et al., 1989; Ferrell & Huestis, 1984; Bütikofer et al., 1989; Thomson et al., 1987). After 20 h of glucose starvation, PIP_2 content was reduced by 54%, corresponding to the depletion of the PLC-sensitive pool (Figure 5f and Gascard et al., 1989). Figure 6C shows that the Ca^{2+}-induced redistribution of PC* was slightly reduced in these conditions. When the incubation without glucose was prolonged for 46 or 72 h, the PIP_2 content was further decreased, leading to a significant reduction of the PLC-

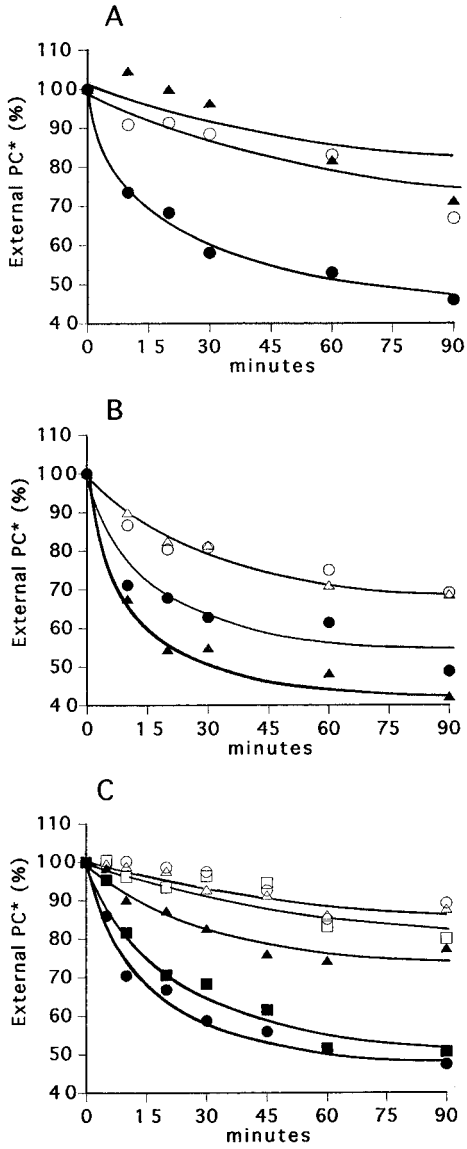

FIGURE 6: The effect of intracellular divalent cations on inward transbilayer movement of PC in control (A and B) or ATP-depleted erythrocytes (C). (A) Erythrocytes, suspended in Na medium, were loaded with the PC* probe and incubated at 37 °C (final hematocrit 30%), with 8 μM A23187 and either 1 mM EGTA (○) or 1 mM $CaCl_2$ (●), or with 15 μM A23187 and 6 mM $BaCl_2$ (▲). (B) Other samples were incubated in the absence of the probe with the ionophore and Ca^{2+} for 30 min (○, ●) or Ba^{2+} for 60 min (△, ▲), washed in Na medium containing EGTA (3 mM after Ca^{2+} treatment and 10 mM after Ba^{2+} treatment), loaded with the probe, and reincubated with either 1 mM EGTA (○, △) or 1 mM $CaCl_2$ (●, ▲). At different time intervals, aliquots were extracted with BSA, diluted with cold Na medium, and centrifuged. The percentage of the probe in the inner leaflet was determined from the ESR signal measured in cell pellets. The percentage of the probe in the outer leaflet (external PC*) was calculated from the difference to 100%. Data shown are from one experiment representative of 3 experiments for Ca^{2+} treatment and of 2 experiments for Ba^{2+} treatment. (C) Erythrocytes were either kept at 4 °C in medium A (○, ●) or ATP-depleted for 20 h (□, ■) or 72 h (△, ▲), washed twice to eliminate microvesicles and lysed cells, resuspended in K medium, and incubated at 37 °C (final hematocrit 30%) with 8 μM A23187 and either 1 mM EGTA (○, □, △) or 1 mM $CaCl_2$ (●, ■, ▲). The percentage of the probe in the outer leaflet was determined from the ESR signal measured in the supernatants after centrifugation of BSA-extracted cells. The presence of membranes (vesicles or ghosts) released in the supernatants from control or ATP-depleted cells was checked at the end of the incubation by phospholipid assay. In all conditions, this release never exceeded 6%, attesting that cell lysis was low. Data shown are means of two determinations in one experiment and are representative of 3 experiments.

resistant pool (Figure 5g and Ferrell & Huestis, 1984; Thomson et al., 1987) and, in parallel, to a drastic inhibition of PC* redistribution (Figure 6C). Regardless of the extent of the PIP_2 depletion, the remaining pool was not altered by a subsequent treatment with the ionophore and Ca^{2+}, consistent with its identification as the PLC-resistant pool (Figure 5f,g). These data suggest that the Ca^{2+}-induced PC* scrambling required the presence of the PLC-resistant pool of PIP_2.

DISCUSSION

In this study, PC* scrambling can be considered to represent the scrambling of all the phospholipids. Indeed, in erythrocytes and resealed ghosts, Ca^{2+}-induced scrambling of PC as well as of PE, PS, and SM were observed simultaneously (Williamson et al., 1992; Smeets et al., 1994; Sulpice et al., 1994; Bratton, 1994).

Ca^{2+} triggered partial transmembrane phospholipid redistribution in pure lipid membranes (PC LUVs), when they were prepared with 5% PIP_2. The inward and outward phospholipid movements induced by Ca^{2+} were symmetric and independent of the polar head group of the phospholipid. They concerned only 10−15% of the spin-labeled probe in each membrane leaflet. As in the PIP_2-loaded erythrocytes (Sulpice et al., 1994), Ca^{2+}-induced phospholipid translocation was specific for PIP_2 over PIP, another acidic phospholipid. Bevers et al. (1995) did not detect any Ca^{2+}-induced translocation of a fluorescent PC probe incorporated in PIP_2/PC/PS/cholesterol LUVs. However, under the conditions used in their study, low initial percentage of PIP_2 (1%) and inefficient solubilization of PIP_2 sodium salt in organic solvents (Toner et al., 1988), the amount of PIP_2 incorporated in LUVs was likely to be less than 1% (see Materials and Methods). A striking point is that the extent of redistribution observed in LUV membranes was limited and never led to lipid randomization of the monolayers. On the contrary, in erythrocyte membranes, Ca^{2+} could induce a maximal scrambling, even when exogenous PIP_2 was incorporated into the external leaflet. This difference could reflect intrinsic properties of the structure of each membrane (curvature radius, packing of the lipids) or could suggest that in biological membranes PIP_2 acts in synergy with another component to promote the lipid randomization.

At physiological pH, PIP_2 is negatively charged, providing potential binding sites for divalent cations, particularly Ca^{2+}. Such a mechanism is thought to be responsible for inducing lateral movements of PIP_2 and segregation of Ca^{2+}-PIP_2 complexes (Gadella et al., 1990), resulting in membrane destabilization and providing phospholipid flip sites as we have proposed before (Sulpice et al., 1994).

Spermine inhibited the Ca^{2+}-induced phospholipid scrambling in LUVs containing PIP_2 and in PIP_2-loaded erythrocytes. This polyamine bearing positive charges binds tightly to phosphoinositides, especially to PIP_2 in artificial membranes even in the presence of physiological concentrations of Mg^{2+} (Tadolini & Varani, 1986; Toner et al., 1988; Meers et al., 1986; Schuber, 1989). The distribution of the charges at fixed lengths along the molecule of spermine (Chung et al., 1985) would allow specific bridging between PIP_2 polar heads, preventing Ca^{2+} binding, lateral redistribution of PIP_2 molecules, and thus phospholipid scrambling. This mechanism could explain the inhibitory effect of spermine on Ca^{2+}-induced phospholipid redistribution at least in the two systems, such as PIP_2-containing LUVs or PIP_2-loaded erythrocytes, where PIP_2 is the obvious Ca^{2+} target.

In resealed ghosts, spermine also totally suppressed the redistribution of PC* induced by low Ca^{2+} concentrations (our results and Bratton, 1994). It has been proposed that Ca^{2+}-induced scrambling could involve interactions between either Ca^{2+} and anionic phospholipids (Lin et al., 1994; Bratton, 1994) or proteins and Ca^{2+} and anionic phospholipids and that such interactions would be inhibited by spermine (Bratton, 1994). PIP_2 which binds divalent cations (see above) and is likely to interact with proteins of the red cell membrane (Sheetz et al., 1982; Hagelberg & Allan, 1990; Gascard et al., 1993a; Hanicak et al., 1994) could play a pivotal role in the scrambling process. Spermine could inhibit the scrambling by preventing the formation of Ca^{2+}-PIP_2 complexes at least in LUVs and/or by inhibiting PIP_2−protein interactions in natural membranes. The proteins interacting with PIP_2 could be a putative scramblase, as proposed by Zwaal et al. (1993), or a phosphorylated protein, as suggested by Martin and Jesty (1995). In Scott red cells, elevation of intracellular Ca^{2+} was unable to induce phospholipid scrambling (Bevers et al., 1992), even though the PIP_2 content and metabolic pools were normal (Bevers et al., 1995), indicating that Ca^{2+}-PIP_2 complex was not the only participant in the induction of scrambling. Furthermore, PIP_2 loading of these cells did not result in Ca^{2+}-induced scrambling (Bevers et al., 1995) as it did in the normal erythrocytes (Sulpice et al., 1994). This suggests that PIP_2 might act in concert with a membrane component (protein) required for scrambling and deficient in pathological cells, thus preventing the membrane rearrangement. Alternatively, Scott cells could contain an altered or overexpressed protein inhibiting the scrambling process (a spermine-like protein).

As Ca^{2+} was still able to cause PC* redistribution after removal of the PLC-sensitive pool of PIP_2, either by metabolic depletion or by Ca^{2+}-induced hydrolysis, it can be concluded that this pool is not required in the scrambling process and that protein dephosphorylation resulting from 20 h ATP depletion as well as Ca^{2+}-induced proteolysis or enzyme activation do not prevent scrambling induction. Additionally, a possible role in phospholipid scrambling of diacylglycerol and/or phosphatidic acid resulting from PIP_2 hydrolysis can be ruled out, as their presence in remnant cells after a first Ca^{2+} burst was unable to maintain or induce scrambling in the absence of Ca^{2+}, in agreement with previous data (Lin et al., 1995). In contrast, PC* scrambling was inhibited either after removal of the PLC-resistant pool by long-term ATP depletion or by incorporation of intracellular spermine, which presumably prevented Ca^{2+} access to this PIP_2 pool, as deduced from the PLA_2 hydrolysis data.

The present data support a role of a Ca^{2+}-PIP_2 complex, but do not exclude the participation of proteins. Further investigations would be necessary to identify the putative protein and its characteristics of interaction with PIP_2. The PIP_2 pool responsible for Ca^{2+}-induced phospholipid scrambling would be the PLC-resistant and slowly turned over one. The PLC-sensitive PIP_2 pool has been reported to be involved in the interactions between protein 4.1 and glycophorin (Gascard et al., 1993a). Thus the two PIP_2 pools could play specific roles in cell membrane organization, such as Ca^{2+}-induced phospholipid transmembrane redistribution and cytoskeleton−membrane interactions. These conclusions are consistent with recent reports suggesting that PIP_2 is involved in cellular functions, such as exocytosis and membrane trafficking in various nonerythroid cells (Eberhard et al., 1990; Del Castillo et al., 1992; Cooper & Holz, 1993; Hay & Martin, 1993; Hay et al., 1995; Ohashi et al., 1995; Yamamoto et al., 1995).

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