Phosphatidylinositol 4,5-Bisphosphate Domain Inducers Promote Phospholipid Transverse Redistribution in Biological Membranes†

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ABSTRACT: Transmembrane phospholipid redistribution (scrambling), leading to exposure of phosphatidylserine on the cell surface, plays a physiological role to induce platelet procoagulant activity and clearance of injured or apoptotic cells. Scrambling is generally attributed to an increase in intracellular Ca^{2+} and would be mediated by a protein (scramblase), whose activity could be modulated by cofactors. We reported previously that phosphatidylinositol 4,5-bisphosphate (PIP_2) is a positive regulator of Ca^{2+}-induced scrambling. We show here, using inside-out vesicles from erythrocyte membranes, that a pleckstrin homology (PH) domain, which interacts with high affinity with PIP_2, inhibited Ca^{2+}-induced scrambling, confirming the role of PIP_2. As Ca^{2+} is known to interact with PIP_2 and to promote the formation of lateral domains of acidic phospholipids in membranes, we investigated whether PIP_2 domain formation could be involved in scrambling. Spermine, polylysine, and MARCKS (151−175) peptide caused scrambling in parallel to their reported ability to form domains of acidic phospholipids, including PIP_2. Similarly, neomycine, another PIP_2-interacting polycation, induced scrambling. A PIP_2 antibody was also found to induce scrambling, presumably by a similar mechanism, since phospholipid antibodies are known to promote phospholipid capping. In conclusion, Ca^{2+} is not the sole inducer of scrambling, and formation of PIP_2 domains could play a critical role in this process.

In resting cells, phospholipids are asymmetrically distributed across the plasma membrane, with phosphatidylcholine (PC)[1] and sphingomyelin primarily located in the outer leaflet, whereas the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) are maintained in the inner leaflet, mainly by the activity of a Mg^{2+}- and ATP-dependent aminophospholipid translocase (*1*−*3*). Loss of the asymmetric distribution of phospholipid scrambling has been reported in a number of circumstances generally involving an increase in the concentration of intracellular Ca^{2+}, due to cell activation, cell injury, or apoptosis. External PS exposure is implicated in the coagulation cascade and in the process of clearance of apoptotic or injured cells by the reticuloendothelial system (*4*−*6*).

The mechanisms by which Ca^{2+} induces scrambling are not fully understood. A protein fraction, partially purified from erythrocyte or platelet membranes and reconstituted in large unilamellar vesicles (LUVs), was shown to permit a limited phospholipid scrambling in the presence of Ca^{2+} (*7, 8*). A 37 kDa protein, exhibiting scrambling activity, was recently identified and cloned (*9*). There is some evidence that its activity would be regulated by other factors (*9*−*12*). Scrambling can be induced artificially with the Ca^{2+} ionophore A23187 in platelets, erythrocytes, or erythrocyte ghosts (*11, 13*−*16*), or by addition of Ca^{2+} to erythrocyte inside-out vesicles (IOVs) (*10, 17*). The proposal that scrambling could result from the formation of microvesicles (*18*), a concomitant phenomenon induced by Ca^{2+}, has been experimentally invalidated (*12, 19, 20*). A role for a direct interaction of Ca^{2+} with anionic phospholipids has been suggested (*21*), and we have reported that phosphatidylinositol 4,5-bisphosphate (PIP_2) is a good candidate for such an interaction. Indeed, scrambling can be induced by external Ca^{2+} in erythrocytes or in LUVs loaded with physiological concentrations of PIP_2 (*11, 17*). However, in erythrocytes, the effect of PIP_2 could be indirect, as it is associated with a redistribution of intracellular Ca^{2+} (*22*). Further confirmation of a role for PIP_2 in scrambling was obtained: (1) by manipulating the level of PIP_2 in IOVs (*17*); (2) from the use of vinblastine or chlorpromazine, which induce scrambling in erythrocytes and in LUVs, only when they contain PIP_2 (*23*); and (3) from the inhibition of Ca^{2+}-induced scrambling in sealed ghosts by high concentrations (>1 mM)

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; LUVs, large unilamellar vesicles; IOVs, inside-out vesicles; PIP_2, phosphatidylinositol 4,5-bisphosphate; MARCKS, myristoylated alanine-rich C kinase substrate; PH domain, pleckstrin homology domain; BSA, bovine serum albumin; C6-NBD, 1-oleoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl.

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of spermine (*11*, *12*, *16*), a natural polyamine interacting with PIP_2 (*24*, *25*) and preventing Ca^{2+} binding to PIP_2 (*11*, *12*).

Bivalent cations, such as Ca^{2+}, induce the formation of PS or phosphatidic acid domains in LUVs (*26*), and Ca^{2+} is able to cause a lateral redistribution of PIP_2 in LUVs (*27*) or PIP_2 aggregates in water (*28*). Polycations, such as polylysine and natural polyamines (spermine) or a myristoylated alanine-rich C kinase substrate (MARCKS) (151−175) peptide, which contains five contiguous lysine residues, induce the formation of acidic phospholipid (PS and PIP_2) domains in artificial membranes (*29*, *30*). Bivalent ligands, such as antibodies, promote the capping of antigenic analogues of PE in cell membranes (*31*). An interesting possibility would be that induction of lateral domains of PIP_2 contributes to phospholipid tranversal redistribution. In the present study, we show that polylysine, spermine at a low concentration, neomycine, MARCKS peptide, and anti-PIP_2 antibody induce scrambling in IOVs. In addition, a PH domain of phosphoinositide-specific phospholipase C-δ_1 (PLC-δ1), highly selective in its interaction with PIP_2 (*32*), was able to inhibit scrambling induced by Ca^{2+} or polylysine. The present data confirm the role of PIP_2 in phospholipid scrambling and demonstrate that various cationic effectors, which promote the formation of PIP_2 domains, can induce scrambling in natural membranes.

MATERIALS AND METHODS

Materials. Fatty acid free BSA (A7511), spermine (S4264), polylysine (P8954) (MW approximately 1000, containing about 10 lysine residues), neomycin (N1876), diamide (D3648), and nonspecific anti-mouse IgG (I5381) were obtained from Sigma. MARCKS peptide (151−175) (162004M001) was from Alexis. PH domains of PLC-δ (PLC-δ1, 1−175 and 1−290) were a generous gift from Dr. M. Katan, and the monoclonal (IgG2b) antibody Kt3g (ascite fluid) to PIP_2 was obtained from Dr. K. Fukami; IgG2b was purified by affinity chromatography with protein A−Sepharose (Pharmacia). 1-Oleoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl (C6-NBD) analogues of PC, sphingomyelin (SM), and PS were synthesized as previously described (*33*) or purchased from Avanti Polar Lipids.

Preparation of IOVs. Blood was withdrawn from healthy volunteers, heparinized, and centrifuged (1300*g*, 10 min, 4 °C). The packed erythrocytes were washed 3 times in a medium containing 140 mM NaCl, 5 mM phosphate buffer (pH 8), 0.1 mM EGTA. IOVs were prepared according to Sarkadi et al. (1980) (*34*) with some modifications. The lysing solution contained 5 mM phosphate buffer (pH 8). The hemoglobin-free membranes were diluted in 40 volumes of 0.1 mM EGTA, 0.5 mM phosphate buffer (pH 8−8.2) and incubated for 18 h at 4 °C to extract skeletal proteins. After centrifugation of the suspension, the pellet was washed once in the same medium before homogenization with a 27 gauge needle. The vesicles were further purified through a Dextran barrier ($d = 1.03$) by centrifugation (120 min, 100000*g*). The vesicles remaining above the barrier were collected, washed once in the hypotonic medium (0.1 mM EGTA, 10 mM Tris-HCl, pH 7.4), and resuspended in the same medium or in the isotonic medium (140 mM KCl, 0.1 mM EGTA, 10 mM Tris-HCl, pH 7.4). The preparations of IOVs were used within a day for the measurement of phospholipid movements. The membrane sidedness and the percentage of sealed vesicles were determined as described previously (*17*) by measuring, in the hypotonic medium, the latent activities of glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase (*35*), enzymes localized on the cytoplasmic and exofacial leaflet of the erythrocyte membrane, respectively. The accessibility of both enzymes demonstrated that, in the present preparation, about 80−90% of the vesicles had inside-out orientation, and were impermeable to small molecules (such as acetylthiocholine). The PIP_2 content of IOVs determined previously (*17*) (1% of total phospholipids) was the same as that of the erythrocyte membranes. Interestingly, in contrast to erythrocyte membranes, addition of Ca^{2+} to IOVs had no effect on PIP_2 content, suggesting that the Ca^{2+}-activated specific phospholipase C was lost during IOV preparation (*17*).

Transbilayer Redistribution of NBD-PC, NBD-PS, or NBD-SM in IOVs. Transbilayer movement of NBD-labeled phospholipids was measured by the BSA back-exchange method (*36*). IOVs were resuspended in the hypotonic, or isotonic, incubation medium, at a final phospholipid concentration of 3 mM. The NBD-labeled PC, SM, or PS analogues (1% of the endogenous phospholipids) were introduced in the external side of the IOVs (cytoplasmic leaflet of the original membranes) by a 5 min incubation at 4 °C. The loaded IOVs were washed once with the incubation medium and incubated at 37 °C for up to 30 min, either in the absence or in the presence of Ca^{2+}, polycations, peptides, or antibodies. To measure the redistribution of the probes in the internal side (exoplasmic leaflet), aliquots of 30 μL of IOV suspensions were taken at times 0, 15, and 30 min, rapidly mixed with 1 mL of medium containing or not containing 2% (w/v) BSA, and incubated for 5 min at 25 °C. After centrifugation (15 min, 3500*g*), supernatants were removed, and pellets were solubilized in 1 mL of medium containing 1% (w/v) Triton X-100. The NBD fluorescence of dissolved pellets was measured (I_{ex} 472 nm, I_{em} 534 nm) on a spectrofluorometer (JOBIN-YVON, France). The NBD signal remaining in the pellets after BSA extraction was considered to represent the internalized fraction of NBD analogues and is calculated as the percentage of the signal measured in the pellets without BSA treatment.

RESULTS

Ca^{2+} and Acidic pH Induce Redistribution of NBD-Phospholipids. IOVs, suspended in hypotonic or isotonic medium and loaded with NBD-PC or -PS analogues, were incubated in the presence of either 0.1 mM EGTA or 0.1 mM EGTA and 0.2 mM $CaCl_2$ (resulting in a free Ca^{2+} concentration of about 0.1 mM), or adjusted, by HCl addition, to different pH values (from 7.4 to 3). The extent of PC, SM, and PS redistribution in the inner leaflet of IOVs was measured after bovine serum albumin (BSA) extraction. In the hypotonic medium, at pH 7.4, incubation for 30 min without Ca^{2+} resulted in the internalization of 10−15% of the analogues, corresponding to the passive movement of the phospholipids. In the presence of Ca^{2+}, PC, PS (Figure 1 A), or SM (data not shown) were similarly and rapidly redistributed to about 30−35% (scrambling). Increasing the Ca^{2+} concentration from 1 to 5, 10, or 20 mM only slightly increased the extent of PC redistribution, leading to a full randomization (data not shown). Since the interactions of

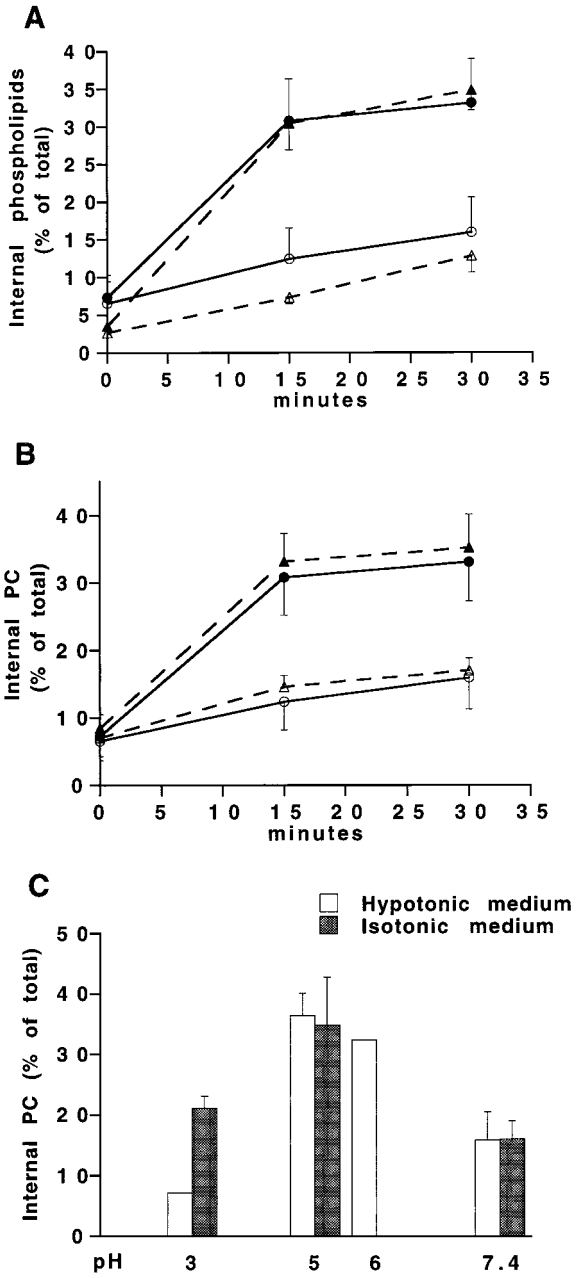

FIGURE 1: Ca^{2+} or acidification induces redistribution of NBD-PC and PS. (A) IOVs were loaded with fluorescent NBD-PC (circles) or NBD-PS (triangles) (1% of total phospholipids), resuspended in the hypotonic medium, and incubated without (open symbols) or with 0.2 mM Ca^{2+} (closed symbols); (B) IOVs loaded with NBD-PC were incubated either in the hypotonic (circles) or in the isotonic (triangles) medium without (open symbols) or with 0.2 mM Ca^{2+} (closed symbols); (C) IOVs, loaded with NBD-PC, were incubated either in the hypotonic or in the isotonic medium, adjusted to different pHs. At the indicated times, aliquots were taken and mixed with washing solution without or with BSA. After centrifugation, supernatants were removed and pellets solubilized in 1 mL of medium containing 1% (w/v) Triton X-100. The internalized fraction of the probes was calculated as a percentage of the fluorescence signal remaining in the pellets after extraction with BSA compared to that measured in the pellets without BSA treatment. Data shown are means of 2 experiments or means ± SE of 3−5 independent experiments.

Ca^{2+} with acidic lipids or proteins are dependent on the ionic strength (*37*), we have also measured the Ca^{2+}-induced redistribution of NBD-PC in an isotonic medium (140 mM KCl). Figure 1B shows that, under these conditions, the extent and the kinetics of PC internalization were not significantly modified.

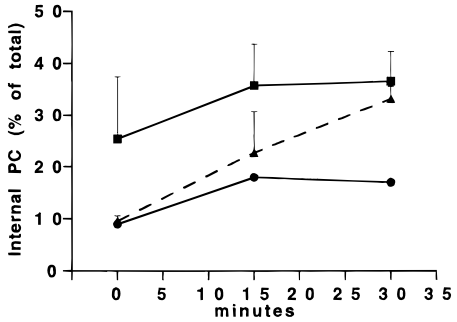

FIGURE 2: Anti-PIP_2 antibody induces redistribution of NBD-PC. IOVs were loaded with NBD-PC, resuspended in the hypotonic medium, and incubated in the presence of anti-PIP_2 antibody at a 2/1 stoichiometric ratio toward endogenous PIP_2 either in hypotonic (squares) or in isotonic medium (triangles), or at a 0.2/1 ratio in hypotonic medium (circles). The fraction of internalized NBD-PC was determined as described in the legend of Figure 1. Data shown are means of 2 experiments or means ± SE of 3−5 independent experiments.

In IOVs and in proteoliposomes containing a protein fraction with a scramblase activity, the redistribution of NBD-PS was reported to be induced in the absence of Ca^{2+} by lowering the pH in an isotonic medium (*10*). As shown in Figure 1C, in IOVs, redistribution of PC could be also triggered by acidic pH in both hypotonic and isotonic media. The effect was biphasic, with a maximum around pH 5, as already reported for PS (*10*).

Anti-PIP_2 Antibody and Polycationic Phospholipid Domain Inducers Promote NBD-PC Redistribution. In the hypotonic Ca^{2+}-free medium, Kt3g, an antibody specific for PIP_2 (*38*), in a 2/1 stoichiometric ratio versus endogenous PIP_2, induced a very rapid redistribution of NBD-PC (noticeable but variable at time 0), reaching a plateau value by 15 min, equal to that induced by 0.2 mM Ca^{2+} (30−35%) (Figure 2). A 10-fold dilution of anti-PIP_2 antibody totally suppressed the PC internalization. A nonspecific mouse IgG, or the anti-PIP_2 antibody preincubated with PIP_2, had no effect on PC redistribution (data not shown), demonstrating that the effect of the antibody was specific for PIP_2. In the isotonic medium, the antibody (2/1) had the same effect on the extent of PC redistribution, although it developed more slowly than in the hypotonic medium (Figure 2).

The effects of various polycationic compounds, known to interact with acidic phospholipids and particularly with PIP_2 on PC redistribution, are shown in Figure 3. Some of these compounds were also reported to induce the formation of phospholipid domains (*29*, *30*). The IOVs were incubated in the Ca^{2+}-free (0.1 mM EGTA) hypotonic medium at pH 7.4 in the presence of various concentrations of polycations. Neomycin (1 mM) or polylysine (1 mM) had the same effect as that induced by Ca^{2+} or acid pH, e.g., a 30−40% redistribution of NBD-PC after 30 min incubation (Figure 3A). Polylysine (1 mM) induced a similar redistribution of NBD-PS and NBD-SM (data not shown). In addition, spermine (100 μM), as well as MARCKS (151−175) peptide (10 μM), induced a 25−30% internalization of PC. The effect of these low concentrations of spermine and MARCKS required that the concentration of EGTA was reduced to 10 μM, to allow chelation of contaminating Ca^{2+} and to avoid

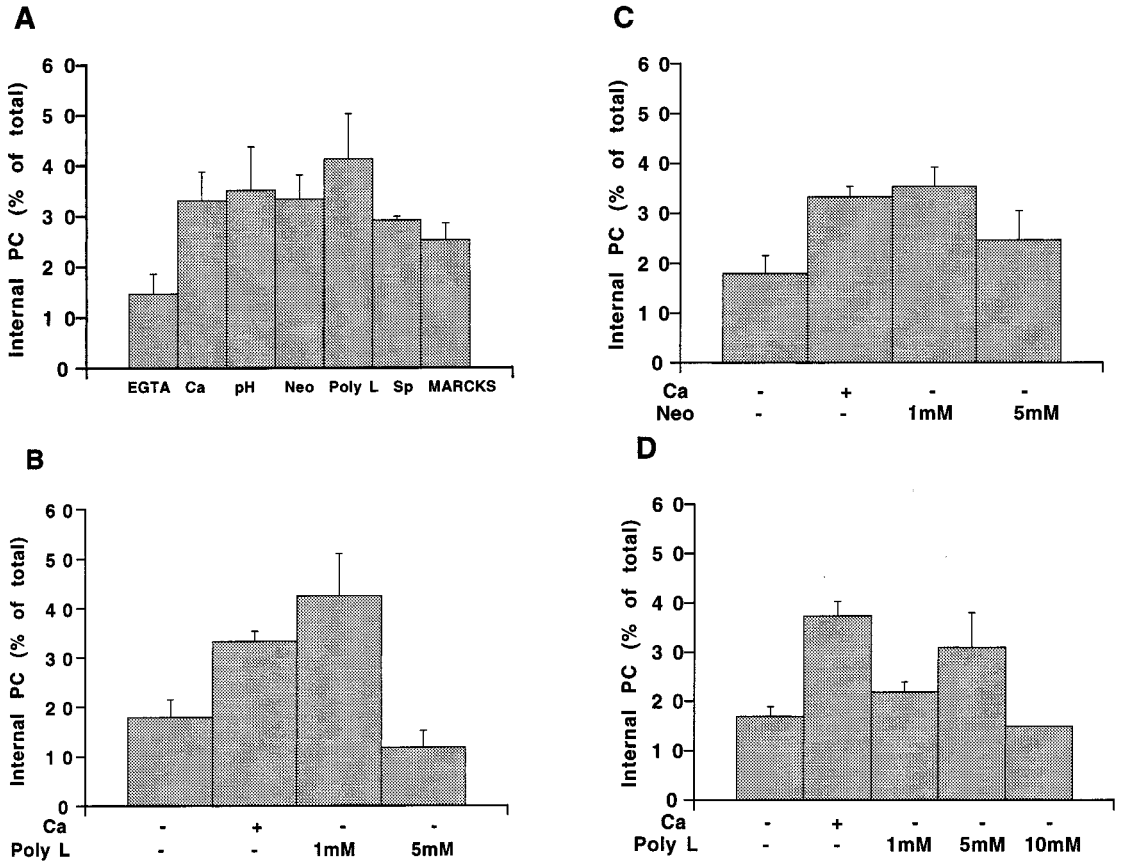

FIGURE 3: Various inducers of phospholipid domains cause redistribution of NBD-PC. (A−C) IOVs were loaded with NBD-PC, resuspended in the hypotonic medium, and incubated with 0.2 mM Ca^{2+}, neomycin, or polylysine (1 mM or the indicated concentrations). Alternatively, NBD-PC-loaded IOVs were resuspended in the hypotonic medium containing only 10 μM EGTA and incubated with spermine (0.1 mM) or MARCKS (10 μM). (D) IOVs loaded with NBD-PC were incubated in the isotonic medium with 0.2 mM Ca^{2+} or the indicated concentrations of polylysine. The fraction of internalized NBD-PC, after 30 min incubation at 37 °C, was determined as described in the legend of the Figure 1. Data shown are means ± SE of 3−5 independent experiments.

a possible interaction between EGTA and spermine or MARCKS.

Surprisingly, increasing the concentration of polylysine, or neomycin, to 5 mM failed to activate redistribution of PC (Figure 3B,C). In the same way, spermine (1 mM) was without effect on PC redistribution and polylysine (5 mM) on PS and SM redistribution (data not shown).

As the interaction of polycations with acidic phospholipids could depend on the ionic strength of the incubation medium, the effect of various concentrations of polylysine was tested in the isotonic medium (Figure 3D). Under these conditions, a biphasic activation of PC redistribution was still observed, but the maximum was shifted toward higher concentrations of polylysine (5 mM instead of 1 mM in the hypotonic medium; see Figure 3B). In contrast with the polycations, the effect of MARCKS on PC scrambling was independent of its concentration or of the ionic strength of the medium (data not shown).

PH Domain Peptides and High Concentrations of Polycations Inhibit the Ca^{2+}-Induced NBD-PC Redistribution. We have tested the effect of two recombinant peptides (1−175 and 1−290), derived from PLC-δ1 PH domain, which bind PIP_2 with high affinity (*32, 39*), on the Ca^{2+}-induced redistribution of NBD-PC in hypotonic or isotonic medium. The media did not contain EGTA, to avoid possible neutralization of positive charges of the peptides. Addition of PH domains, in an amount equivalent to 4 times the concentration of PIP_2 in IOVs, had no effect on PC redistribution. However, preincubation of the IOVs with the same concentration of PH domains (1−175) inhibited by about 90% the effect of Ca^{2+}, either in hypotonic or in isotonic medium (Figure 4 A). The PH domain (1−290) also inhibited by 83% the effect of Ca^{2+} and interestingly by 51% that of 300 μM polylysine in the hypotonic medium (data not shown).

As reported above, in the hypotonic medium, polylysine or neomycin (5 mM) failed to activate PC redistribution (Figure 3B,C). However, preincubation of the IOVs with the polycations, at this concentration, drastically inhibited the Ca^{2+}-induced redistribution (Figure 4B). In the same way, in the isotonic medium, spermine (100 μM) failed to induce PC redistribution, whereas the effect of Ca^{2+} was inhibited by 1 mM spermine (data not shown).

IOV Aggregation. Polycations are known to induce aggregation of lipid vesicles, when they contain acidic phospholipids, but never fusion in the absence of Ca^{2+} (*24, 25*). In our experiments, the relative extent of IOV aggregation was estimated from the turbidity change of the suspension, measured by the absorbance at 440 nm. It was dependent on the concentration of each polycation, occurring at a threshold of 10 μM for polylysine and of 1 mM for neomycin and spermine. Aggregation of IOVs could prevent quantitative extraction of noninternalized fluorescent phospholipid analogues, leading to an overestimation of the internalized

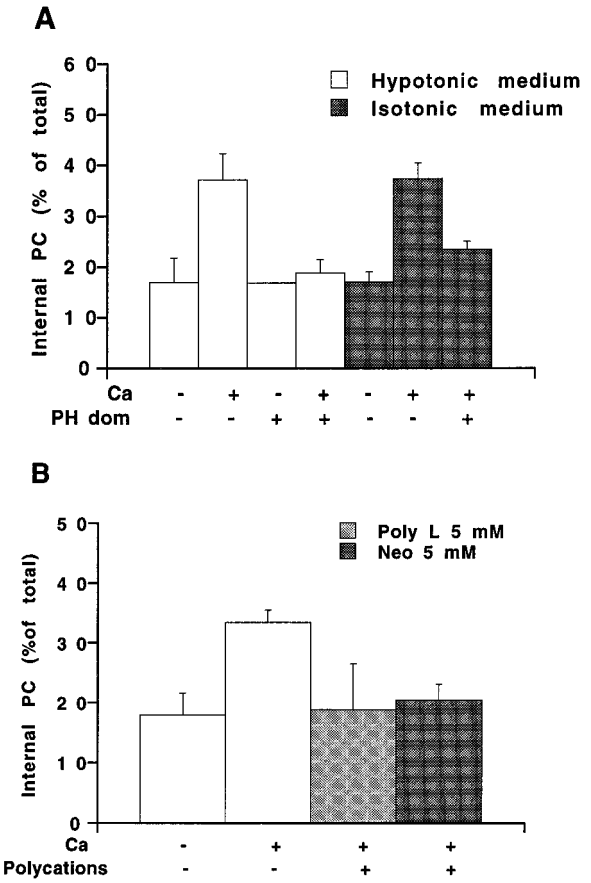

FIGURE 4: Ca^{2+}-induced NBD-PC redistribution is inhibited by PH domains of PLC-δ1 or by high concentrations of polycations. IOVs were loaded with NBD-PC and resuspended in the hypotonic or isotonic medium without (A) or with 0.1 mM EGTA (B) and preincubated for 5 min at 37 °C in the presence of either (A) PH domain (1−175), at a concentration equivalent to 4 times that of PIP_2 in IOVs, or (B) neomycin or polylysine (5 mM). Incubation was started by the addition of 0.1 mM EGTA or 0.2 mM Ca^{2+}. The fraction of internalized NBD-PC, after 30 min incubation at 37 °C, was determined as described in the legend of the Figure 1. Data shown are means ± SE of 3−5 independent experiments.

fraction. However, the following data argue against this possibility: (1) in the hypotonic medium, under conditions of maximal aggregation (5 mM neomycin or polylysine, or pH 3), no internalized NBD-PC was detected; (2) polylysine-induced PC redistribution, but not aggregation, was drastically inhibited by the PH domain; (3) at the beginning of the kinetics, aggregation induced by 1 mM polylysine or neomycine was already elevated, whereas scrambling was not detectable.

DISCUSSION

In the present work, we have used IOVs as a model of biological membranes to further explore the mechanisms by which PIP_2 could participate in phospholipid redistribution. These experimental conditions have been previously and successfully used (*10*, *17*). The advantage of IOVs is to provide a direct accessibility of the effectors to the cytosolic side of the membrane where PIP_2 is primarily located (*40*). NBD analogues of phospholipids are good reporters of natural phospholipids to measure transversal movements (*33*, *36*). In the present study, we have measured NBD-PC movements, as a test of scrambling, which affects unspecifically all the major phospholipids in the erythrocyte membranes (*14*, *36*). However, to prove global scrambling, redistribution of NBD-PS and SM was evaluated under some conditions (Ca^{2+} and polylysine).

Ca^{2+} which interacts with acidic phospholipids, PS or PIP_2, forms domains of PS or phosphatidic acid (*26*). Moreover, Ca^{2+} modifies the lateral mobility of PIP_2 in LUVs (*27*) and induces, at physiological concentrations, the formation of aggregates of PIP_2 (*28*). Similar changes in the PIP_2 physical state could be expected upon protonation of phosphate groups of PIP_2 by acidification (*41*). Therefore, induction of scrambling by Ca^{2+} and acidification in IOVs could also result from the formation of PIP_2 domains.

Lateral domains of acidic phospholipids are induced in LUVs by pentalysine, with two important characteristics (*30*). First, as its concentration increases up to 2 mM, domains form that are enriched in acidic lipids, whereas above that concentration domains break up. Second, as the ionic strength of the medium increases, domains no longer form. The ability of polylysine (decalysine) to induce PC scrambling was lost as the polylysine concentration was increased above 1 mM in the hypotonic medium (Figure 3B) and above 5 mM in the isotonic medium (Figure 3D). Therefore, there is an excellent correlation between the effects of polylysine on formation of phospholipid domains and on phospholipid scrambling. In addition, MARCKS (151−175) peptide, another inducer of lateral phospholipid (PS or PIP_2) domains (*29*), also caused phospholipid scrambling at a concentration of 10 μM. Interestingly, the ability of MARCKS peptide to sequester PIP_2 was maintained over large range of concentrations and independently of the ionic strength of the medium (*30*), as was its effect on scrambling. This property could be attributed to a dual interaction of MARCKS peptide with PIP_2: electrostatic, by its pentalysine segment; and lipophilic, by the aliphatic side chains of amino acids interacting with the fatty acid chains of PIP_2 (*30*). Neomycin and spermine, other polycations known to interact with PIP_2 (*25*, *42*), induced PC scrambling in the hypotonic medium also with biphasic concentration dependence. It is thus possible that neomycin at 1 mM induces the formation of PIP_2 domains, leading to phospholipid scrambling, whereas at 5 mM lateral domain formation and thus scrambling would no longer occur. In contrast to the biphasic effect of polycations (polylysine and neomycin) on PC redistribution, the effect of Ca^{2+} was not diminished by increasing its concentration from 0.2 to 20 mM. Consistent with this observation, the effect of Ca^{2+} on acidic phospholipid domain formation in LUVs, erythrocytes, or LUVs prepared from erythrocyte membrane phospholipids was not biphasic, but "of all or none" type and detectable up to a concentration of 10 mM (*26*). In the same way, the lateral mobility of PIP_2 in LUVs could be induced from 0.1 to 10 mM Ca^{2+} and remained constant up to 100 mM (*27*). Therefore, there is also a good correlation between the Ca^{2+} concentration dependence of scrambling and of domain formation.

Domain formation by polycations is not quite selective for PIP_2 over PS. However, all the polycationic effectors cause PC as well as PS redistribution, suggesting that, in our conditions, the interaction of the polycations with PS would be weak enough not to modify its own transversal movements. On the other hand, there are several lines of evidence in favor of a predominant role of PIP_2, over PS, in the scrambling mediated by domain formation: (1) in IOVs,

polylysine-induced scrambling was inhibited by PH domains which do not interact with PS (*32*); (2) in PIP_2-depleted IOVs, though PS is present, Ca^{2+} was unable to induce scrambling (*17*); (3) in PC-containing LUVs, Ca^{2+}-induced scrambling absolutely required the presence of PIP_2, PS being inefficient (*11*). In addition, anti-PIP_2 antibody (Kt3g), a more specific PIP_2 ligand, induces phospholipid scrambling. Kt3g could sequester PIP_2 in lateral domains, as reported for a divalent anti-PE antibody which caused capping of haptenized PE (*31*). Such a clustering effect would be consistent with the punctated lateral distribution of PIP_2 in the erythrocyte membrane, that we observed previously in immunolocalization studies with Kt3g (*40*).

The drastic inhibition of Ca^{2+}- and polylysine-induced phospholipid redistribution in IOVs, by a PH domain, confirms the role of PIP_2 domains. Indeed, in contrast to polycations which induce phospholipid domains (*30*), the PH domain of PLC-δ1 used in that study forms high-affinity 1/1 complexes of known structure with PIP_2 (*32*, *39*) and could competitively displace the interaction between Ca^{2+} and PIP_2, or polylysine and PIP_2, inhibiting their capacity to induce domain formation. In the same way, polylysine or neomycin, at the concentrations unable to promote scrambling (5 mM in hypotonic medium), inhibited the scrambling induced by Ca^{2+}. A similar inhibitory effect has been already reported for spermine in resealed ghosts (*11, 16*) and in LUVs containing PIP_2, and was shown to result from a competition with Ca^{2+} for binding to PIP_2 (*11*). In the IOVs (present work), spermine (1 mM) also inhibited scrambling induced by Ca^{2+}. Accordingly, the inhibitory effect of high concentrations of polylysine and neomycin could also result from a competition with Ca^{2+} for the interaction with PIP_2.

PIP_2 can directly interact with numerous proteins containing various peptidic sequences, the nature and the arrangement of basic amino acids being important for the interaction with the polar head of PIP_2 (*43*−*47*). The surface density of PIP_2, in the membrane, plays an important role in modulating its binding properties with cations and proteins (*28*−*30*), suggesting that PIP_2 domains are required to optimize the interactions of PIP_2 with proteins. A possible mechanism to explain the role of PIP_2, after domain formation, in regulating the function of some proteins could involve an interaction with their cytoplasmic part, resulting in their oligomerization, as shown for syndecan-4 (*48*). On the other hand, oligomerization of the scramblase by Ca^{2+} has been suggested as a mechanism leading to its activation (*49*). Although no classical PIP_2-binding sites have been identified in the scramblase sequence, PIP_2 could interact with small clusters of positively charged amino acids, present in the cytosolic part of the protein. Alternatively, PIP_2 could interact with a putative cofactor of the scramblase, previously proposed to explain the defect in scrambling activity in Scott cells (*10*).

The above data confirm the role of PIP_2 in scrambling and suggest that PIP_2 domain formation would be involved in this process. Furthermore, although Ca^{2+} is generally considered as the trigger for phospholipid scrambling, other candidates include pH, polycations, and proteins with PIP_2-binding domains. Their potential role in PS redistribution would deserve to be investigated in platelet activation or apoptosis. Recent data, indeed, indicate that polyamines could positively regulate the PS exposure in apoptotic cells (*50*).

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