The Complex of Phosphatidylinositol 4,5-Bisphosphate and Calcium Ions Is Not Responsible for Ca\(^{2+}\)-Induced Loss of Phospholipid Asymmetry in the Human Erythrocyte: A Study in Scott Syndrome, a Disorder of Calcium-Induced Phospholipid Scrambling

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Elevation of cytoplasmic Ca\(^{2+}\) levels in human erythrocytes induces a progressive loss of membrane phospholipid asymmetry, a process that is impaired in erythrocytes from a patient with Scott syndrome. We show here that porcine erythrocytes are similarly incapable of Ca\(^{2+}\)-induced redistribution of membrane phospholipids. Because a complex of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and Ca\(^{2+}\) has been proposed as the mediator of enhanced transbilayer movement of lipids (J Biol Chem 269:8347, 1994), these cell systems offer a unique opportunity for testing this mechanism. Analysis of both total PIP\(_2\) content and the metabolic-resistant pool of PIP\(_2\) that remains after incubation with Ca\(^{2+}\) ionophore showed no appreciable differences between normal and Scott erythrocytes. Moreover, porcine erythrocytes were found to have slightly higher levels of both total and metabolic-resistant PIP\(_2\) in comparison with normal human erythrocytes. Although loading of normal erythrocytes with exogenously added PIP\(_2\) gave rise to a Ca\(^{2+}\)-induced increase in prothrombinase activity and apparent transbilayer movement of nitrobenzoxadiazolyl (NBD)-phospholipids, these PIP\(_2\)-loaded cells were also found to undergo progressive Ca\(^{2+}\)-dependent cell lysis, which seriously hampers interpretation of these data. Moreover, loading Scott cells with PIP\(_2\) did not abolish their impaired lipid scrambling, even in the presence of a Ca\(^{2+}\)-ionophore. Finally, artificial lipid vesicles containing no PIP\(_2\) or 1 mole percent of PIP\(_2\) were indistinguishable with respect to transbilayer movement of NBD-phosphatidylethanolamine in the presence of Ca\(^{2+}\). Our findings suggest that Ca\(^{2+}\)-induced redistribution of membrane phospholipids cannot simply be attributed to the steady-state concentration of PIP\(_2\), and imply that such lipid movement is regulated by other cellular processes.

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inability to rearrange phospholipids in response to elevation of cytoplasmic Ca\(^{2+}\). We have used both Scott and porcine erythrocytes to determine whether a deficiency in Ca\(^{2+}\)-induced lipid rearrangements results from a deficiency in the PIP\(_2\) content of the membrane. We have also investigated whether exogenously added PIP\(_2\) can correct this scrambling defect and whether introduction of PIP\(_2\) into a protein-free reconstituted lipid vesicle induces a Ca\(^{2+}\)-dependent enhancement of transbilayer phospholipid movement.

MATERIALS AND METHODS

**Materials.** Bovine serum albumin (BSA; globulin and fatty acid free), ionomycin, and PIP\(_2\) (sodium salt) were obtained from Sigma Chemical Co (St Louis, MO). PIP\(_2\) was dissolved in chloroform:methanol:water (7:2:0.15) to a stock concentration of 0.666 mmol/mL. Coagulation factors Xa, Va, and prothrombin were prepared as described by Roseng et al\(^{12}\); thrombin-specific chromogenic substrate subst 2238 was from AB Kabi Diagnostica (Stockholm, Sweden). 1-Oleoyl-2-(6-7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)-caproyl-sn-glycero-3-phosphocholine (NBD-PC) and 1-Oleoyl-2-(6-7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)caproyl-sn-glycerol-3-phospho- serine (NBD-PS) were obtained from Avanti Polar Lipids (Alabaster, AL). 1,2-Diacyl-sn-glycerol-3-phosphocholine from egg (egg-PC) and 1,2-Diacyl-sn-glycerol-3-phosphoserine from bovine brain (brain-PS) were from Sigma. Cholesterol was from Baker (Deventer, The Netherlands). Sodium orthovanadate was from Janssen Chimica (Geel, Belgium). All other reagents used were of analytical grade.

**Preparation and PIP\(_2\) loading of erythrocytes.** Blood, collected in acid-citrate-dextrose, was obtained from healthy volunteers and from patient M.S., and shipped at 4°C by air express to both Milwaukee and Maastricht. Immediately upon receipt, erythrocytes were washed and loaded with PIP\(_2\), essentially according to Sulpice et al\(^4\): cells were washed three times in buffer A (145 mmol/L NaCI, 2 mmol/L KCI, 10 mmol/L glucose, 10 mmol/L phosphate buffer, pH 7.4). After evaporation of solvent, PIP\(_2\) was dispersed to a final concentration of 20 and 50 pmol/L in buffer A, containing 1 mmol/L EGTA and 1 mmol/L EDTA. Erythrocytes were added to a final hematocrit of 2.5% and were allowed to incorporate PIP\(_2\) for 10 minutes at 37°C. Subsequently, cells were washed once in buffer A (containing EGTA and EDTA) and twice in HEPES buffer (20 mmoK sodium citrate, 10 mmol/L D-glucose, 25 mmol/L phenylmethylsulfonyl fluoride, 3% Triton X-100, 1 mmol/L MgCl\(_2\) plus 2 mmoVL CaCl\(_2\)) and 1 pmol/L NBD-PS by incubation at 37°C for 2 minutes. Incubations at 37°C were initiated by addition of 2 mmol/L sodium o-vanadate was added before addition of NBD-PS, to block aminophospholipid translocase activity.

**Outward movement of NBD phospholipids by flow cytometry.** Flow-cytometric monitoring of transbilayer movement of NBD-PS and NBD-PC in PIP\(_2\)-loaded erythrocytes was monitored by modification of methods previously described.\(^{13}\) To monitor outward movement of NBD-PS, erythrocytes suspended to 10% were washed and loaded with 50 pmol/L PIP\(_2\), as described above and suspended in HEPES buffer to 10^7/mL. The samples were divided in half and at time zero received either 0.1 mmol/L EDTA plus 0.1 mmol/L EDTA, or 2 mmol/L CaCl\(_2\). At the times indicated in figures, 5-µL aliquots were diluted into 5 µL of HEPES buffer in the presence or absence of 2% BSA. After 2 minutes of incubation time to extract NBD-PS from the outer membrane leaflet, samples were immediately analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The light scatter and fluorescence channels were set at logarithmic gain. Five thousand events from each sample were analyzed for forward and right angle light scatter and for fluorescence caused by NBD-PS using a 530/30 bandpass filter in the FL1 channel. A similar protocol was used to monitor outward movement of NBD-PC, except that erythrocytes were first loaded with 1 µmol/L NBD-PC by overnight incubation at 37°C. In each case, the amount of NBD lipid distributed in the inner leaflet of the plasma membrane at each time point was derived from the erythrocyte- (or erythrocyte microparticle-) associated NBD fluorescence, measured for samples extracted with albumin, and expressed as a percentage of the total membrane-associated NBD derived from the NBD fluorescence of matched samples incubated in the absence of albumin.

**Assay of phosphatidylinositol-4-kinase and phosphatidylinositol 4-phosphate 5-kinase.** The activity of phosphatidylinositol-4-kinase and phosphatidylinositol 4-phosphate 5-kinase in lysates of Scott and normal erythrocytes was determined from the measured rates of incorporation of ATP-(y-{\(^{32}\)P}) into phosphatidylinositol-4-phosphate (PIP) and PIP\(_2\). Washed erythrocytes were suspended to 2.5 × 10^7/mL in an ice-cold solution containing 100 mmol/L KCl, 20 mmol/L sodium citrate, 10 mmol/L D-glucose, 25 mmol/L HEPES, 10 mmol/L MgCl\(_2\), 1 mmol/L EGTA, 50 µmol/L dihydrothreitol, 20 µL leupeptin, 5 µg/mL antipain, 100 µmol/L o-vanadate, 1 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride, 3% vol/vol glycerol (pH 7.4) and sonicated (10 seconds) on ice. Aliquots (100 µL) of these lysates were distributed into microtuge tubes at 37°C, and kinase reactions started by addition of 50 µL of 0.5 mmol/L L-α-phosphatidylinositol, 0.5 mmol/L L-α-phosphatidyserine, 2.5 mmol/L Mg\(^{2+}\)-ATP [containing 2 µCi ATP-(y-{\(^{32}\)P}); New England Nuclear (NEN), Boston, MA]. At various times, the reactions were stopped by addition of 400 µL 1 N HCl. The phospholipids were then extracted with 800 µL volumes of 50:50 (vol/vol) chloroform:methanol, the organic phase collected, and back extracted with 400 µL volumes of 50:50 (vol/vol) methanol:1 N HCl. To quantitate incorporation of {\(^{32}\)P} into PIP and PIP\(_2\), 5 µL of each organic phase was spotted on CDTA-treated 0.2-mm 20-cm × 20-cm silica 60 (Alttech Assoc Inc, Deerfield, IL) thin-layer chromatography plates, and resolved in a solvent phase containing 65% n-propanol, 35% 2 N acetic acid. Unlabeled phosphatidylinositol (PI), PIP, and PIP\(_2\) (Sigma; 10 µg each) served as standards. Radioactivity in PIP and

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PIP_{2} fractions were quantitated by photon counting (AMBIS from the same incubation used to measure NBD-phospholipid movement were diluted to a final concentration of 1 \times 10^{9} \text{mL} in TRIS-buffered saline (TBS; 50 mmol/L TRIS; 120 mmol/L NaCl, pH 7.4), containing 0.5 mg/mL of BSA and incubated at 37°C with 3 mmol/L factor Xa and 6 mmol/L factor Va for 2 minutes in the presence of 3 mmol/L CaCl_{2}. Thrombin formation was initiated by addition of 4 \mu mol/L prothrombin and additional CaCl_{2} (final concentration, 3 mmol/L). Exactly 1 minute after addition of prothrombin, an aliquot of the suspension was transferred to a cuvet containing 50 mmol/L TRIS-HCl, 100 mmol/L NaCl, 2 mmol/L EDTA, pH 7.9. Thrombin activity was determined using chromogenic substrate S2238 at 150 \mu mol/L. The rate of thrombin formation was calculated from the time-based change in absorbance at 405 nm, using a calibration curve generated with active-site titrated thrombin. It should be noted that in experiments with NBD-PS, this lipid probe did not affect the prothrombinase activity (probably because upon dilution of the cells in BSA-containing TBS buffer, the probe is rapidly sequestered by the protein).

**Lipid extraction and determination of PIP_{2} content.** Five milliliters of packed erythrocytes was lysed in 300 mL of 10-fold diluted TBS containing 0.5 mmol/L EGTA. Erythrocyte membranes were pelleted by centrifugation for 15 minutes at 30,000g and washed twice to remove hemoglobin. Lipids were extracted after the procedure described by Sulpice et al: 5 mL of packed membrane material was extracted with 18.75 mL of chloroform/methanol/conc HCl (20:40:1 vol/vol) at room temperature for 30 minutes with continuous stirring. Phase separation was obtained by addition of 6.25 mL of chloroform and an equal volume of water and stirring for 15 minutes at room temperature, after which the mixture was centrifuged for 15 minutes at 800g. The lower organic phase was collected and dried under vacuum using a rotavapor. Thin-layer chromatography was performed using a modification of the procedure described by Mallinger et al. Prefabricated thin-layer plates (20 × 20 cm; Merck, Darmstadt, Germany) were impregnated with 1% potassium oxalate dissolved in methanolic HCl (3:2), containing 2 mmol/L EDTA and dried for 1 hour at 120°C. Lipid extracts from 2 to 5 mL of packed membrane material were applied as a 3-cm-wide spot, and plates were developed in one dimension using a solvent system of chloroform/methanol /4.3 mmol/L NH_{4}OH (90:65:20 vol/vol) that allowed good separation of PIP_{2} from the other phospholipids. After detection by iodine vapor, spots were scraped from the plate and lipid phosphorus was determined using the method of Pries et al. PIP_{2} content was expressed as mole percent of total phospholipid.

**Preparation of lipid vesicles with and without PIP_{2} and assay for inward movement of NBD-PC.** Aliquots of egg-PC, brain-PS, and cholesterol, dissolved in chloroform, were mixed at a molar ratio of 18:2.5 and subsequently dried under a stream of nitrogen at room temperature. Lipids were dispersed at 37°C in a buffer composed of 50 mmol/L KCl, 50 mmol/L TRIS-HCl, 0.5 mmol/L EGTA, pH 8.0, to obtain a final phospholipid concentration of 10 mmol/L; in some preparations, PIP_{2} was included at a final concentration of 1 mole percent (of phospholipid). The dispersed lipids were extruded 10 times through a filter with a pore size of 0.1 \mu m (Millipore Corp, Bedford, MA), resulting in the formation of unilamellar vesicles.

To test for leakiness, 0.2 mole percent (of phospholipid) of NBD-PC was included in the mixture before dispersion in buffer to incorporate label on both sides of the membrane. These vesicles were diluted 400-fold in buffer in the presence or absence of 1 mmol/L CaCl_{2}. Distribution of the probe between the two leaflets of the membrane was determined by comparing fluorescence intensity (\lambda_{m}, 472 nm, \lambda_{e}, 534 nm) before and after addition of dithionite (final concentration, 10 mmol/L) according to McIntyre and Sleight. To assay for inward movement, 0.2 mole percent NBD-PC was added to the extruded vesicles from a stock solution in DMSO (to a final DMSO concentration of 1%) and the vesicles were placed at 37°C in the presence and absence of 1 mmol/L CaCl_{2}. At different time intervals, aliquots were diluted 400-fold in buffer, and residual fluorescence intensity was read after addition of dithionite.

**RESULTS**

Defective Ca^{2+}-induced lipid scrambling in erythrocytes and blood platelets from Scott syndrome was originally observed as an impaired surface exposure of endogenous PS, reflected by the absence of development of prothrombinase activity and factor Xa-Va binding sites. The inability of Scott erythrocytes to develop prothrombinase activity has been confirmed (Fig 1A). As another illustration of defective scrambling, the outer leaflet of erythrocytes was labeled by exogenously added fluorescent-labeled phospholipid analogs, and Ca^{2+}-induced transbilayer movement of the probes measured (Fig 1B). When labeled normal erythrocytes were incubated with ionophore in the presence of 1 mmol/L Ca^{2+}, 50% of NBD-PS moved to the inner leaflet. However, uptake of this lipid analog in Scott erythrocytes was less than 10%. Identical results were obtained with NBD-PC (data not shown). The absence of inward movement of NBD-PS in Scott erythrocytes upon elevation of intracellular Ca^{2+} not only reflects an impaired scrambling of lipids, but also clearly shows that Ca^{2+} inhibits the aminophospholipid translocase activity in these cells, as was previously found for normal erythrocytes.

Because porcine erythrocytes resemble Scott erythrocytes in their inability to produce microvesicles upon treatment with Ca^{2+} ionophore, lipid scrambling was also examined in porcine erythrocytes and found to be greatly impaired (Fig 1A and B). By contrast, transport of PS from outer to inner leaflet of the plasma membrane was normal in untreated Scott and porcine erythrocytes (data not shown), indicating no defect in the aminophospholipid translocase in these cells.

The proposal that a complex of Ca^{2+} and PIP_{2} is responsible for inducing lipid scrambling was based on increased Ca^{2+}-induced redistribution of spin-labeled probes in erythrocytes and erythrocyte-derived inside-out vesicles supplemented with PIP_{2}. Because an increase in intracellular Ca^{2+} induces phospholipase C-catalyzed hydrolysis of PIP_{2}, the investigators further postulated that only the metabolic-resistant pool of PIP_{2} can be responsible for scrambling. Accordingly, we investigated the PIP_{2} content of Scott and porcine erythrocytes before and after treatment with Ca^{2+} ionophore to establish whether the abnormal behavior of these cells can be explained by reduced levels of this particular phospholipid species. As shown in Table 1, the total PIP_{2} content in Scott erythrocytes is virtually identical to that of normal erythrocytes. Treatment of the cells with Ca^{2+} ionophore caused a 60% reduction in PIP_{2} content, but no significant difference was found between Scott and normal erythrocytes. In addition, the total PIP_{2} content of porcine erythrocytes and the residual PIP_{2} content after incubation with Ca^{2+} ionophore were found to be slightly higher than the corre-
phosphate 5-kinase in broken cell preparations of erythrocytes
phosphatidylinositol 4- and phosphatidylinositol 4-phosphate
incorporation of PIP2,
the rate of incorporation of Scott
not shown).

Total PIP2

Table 1. Total and Metabolic-Resistant PIP2 Content of Human and Porcine Erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>Human (n = 12)</th>
<th>Scott Syndrome (n = 4)*</th>
<th>Porcine (n = 5)</th>
</tr>
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<tbody>
<tr>
<td>Total PIP2</td>
<td>0.66 ± 0.29</td>
<td>0.61 ± 0.18</td>
<td>0.88 ± 0.35</td>
</tr>
<tr>
<td>Metabolic-resistant PIP2†</td>
<td>0.25 ± 0.06</td>
<td>0.24 ± 0.07</td>
<td>0.36 ± 0.04</td>
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Data are expressed as mole percent of total phospholipid.

* Values represent the average of PIP2 analysis performed on four different erythrocyte samples from patient MS. The average values for normal human and porcine erythrocytes are derived from analysis on samples from different individuals.

† The metabolic-resistant pool of PIP2 was determined in ionophore-treated erythrocytes: erythrocytes at 2.5% hematocrit were incubated with 5 μmol/L ionomycin in TBS containing 1 mmol/L CaCl2. Lipid extraction and PIP2 analysis were subsequently performed as described in Materials and Methods.

In erythrocytes, measurement of the enzymatic activities of phosphatidylinositol 4- and phosphatidylinositol 4-phosphate 5-kinase in broken cell preparations of erythrocytes showed no differences between control and Scott cells (data not shown).

To determine whether loading Scott erythrocytes with PIP2 could correct their defect in Ca2+-induced lipid scrambling, cells were loaded with 50 μmol/L PIP2 for 10 minutes. Using change in cell morphology as a qualitative index of incorporation of PIP2, the rate of incorporation of Scott erythrocytes was not appreciably different from that of normal erythrocytes, although the final extent of deformation was slightly less for Scott cells, likely because these cells were less deformed than the normal controls at the beginning of the incubation (data not shown). Inward movement of NBD-PC and NBD-PS was measured as the disappearance of BSA-extractable probe, and outward movement of endogenous PS was measured as an increase in procoagulant activity of the cells. To prevent inward transport of PS by the aminophospholipid translocase, cells were pretreated with vanadate. Figure 2, A and B show that addition of Ca2+ (extracellular; no ionophore) to normal erythrocytes loaded with PIP2 caused an increase in the apparent inward movement of NBD-PC and NBD-PS: ≈35% of NBD-PS and 45% of the NBD-PC were resistant to extraction by BSA after 45 minutes of incubation in the presence of Ca2+. In contrast, addition of Ca2+ to PIP2-loaded Scott erythrocytes resulted in uptake of only 15% of NBD-PC, whereas NBD-PS uptake leveled off at less than 8%. In the absence of extracellular Ca2+, no appreciable movement of either probe in either type of cell was observed. Similarly, incubation of PIP2-loaded normal erythrocytes with Ca2+ for 45 minutes resulted in prothrombinase activity of almost 500 nmol thrombin·L⁻¹·min⁻¹, whereas this activity in Scott erythrocytes was found to be much lower (≈125 nmol thrombin·L⁻¹·min⁻¹, Fig 2C). However, it was consistently found that apparent movement of the fluorescent lipid probes and expression of prothrombinase activity were closely correlated with loss of membrane integrity, as judged from the release of hemoglobin in the cell supernatant (Fig 2D). Loss of membrane integrity was only observed after addition of Ca2+, was time- and temperature-dependent, and was independent of cell hematocrit, presence of NBD-probes, and different PIP2 preparations. Although reduced cell lysis was observed at lower concentrations of added PIP2, a corresponding reduction in apparent movement was always observed using either assay.
No conditions were found that resulted in NBD-phospholipid scrambling or development of prothrombinase activity in the absence of hemolysis.

When Ca\(^{2+}\) was added to PIP\(_2\)-loaded erythrocytes in the presence of ionophore, a significant development of prothrombinase activity (Fig 3A) and inward transport of NBD-phosphatidylcholine (Fig 3B) was observed, but this was also accompanied by extensive lysis of the cells (80% hemolysis in 45 minutes; Fig 3C). In comparison, Scott erythrocytes were considerably less able to develop procoagulant activity, and less NBD-phosphatidylcholine migrated to the inner leaflet of the membrane. However, the action of ionophore on these PIP\(_2\)-loaded cells also resulted in considerable membrane damage, with 40% hemolysis occurring in 45 minutes. This extensive cell lysis hampers reliable interpretation of the data.

To gain further insight into the role that induced hemolysis might play in the apparent scrambling of membrane phospholipids by the Ca\(^{2+}\)-PIP\(_2\) complex, flow cytometry of PIP\(_2\)-treated erythrocytes was used to monitor the transmembrane movement of NBD-PS in both the intact erythrocytes and lysed ghost membranes. In these experiments, NBD-PS was loaded into the inner membrane leaflet of erythrocytes. After addition of vanadate to prevent inward transport of PS during subsequent steps, erythrocytes were loaded with 50 \(\mu\)mol/L PIP\(_2\) for 10 minutes at 37°C. As shown in Fig 4, addition of Ca\(^{2+}\) to PIP\(_2\)-loaded erythrocytes initiated a distinct change in particle scatter arising from the erythrocytes (events falling within the gate of forward and side scatter shown in Fig 4). Additionally, this treatment resulted in the appearance of new particles (erythrocyte membrane-derived microparticles) with distinctly reduced forward and side angle scatter, falling outside the erythrocyte gate shown in Fig 4. Comparison of PIP\(_2\)-loaded erythrocytes from Scott syndrome and normal controls showed that the Scott cells were distinctly less sensitive to this effect of the Ca\(^{2+}\)-PIP\(_2\) complex. In all experiments performed under the conditions of Fig 4, the appearance of erythrocyte membrane-derived particles was accompanied by the release of hemoglobin into the supernatant of the Ca\(^{2+}\)-PIP\(_2\)-treated erythrocytes (not shown) sug-
Fig 3. Time course of development of prothrombinase activity (A), inward movement of NBD-PC (B), and cell lysis (C) of PIP₂-loaded normal erythrocytes (■) and erythrocytes from patient MS with Scott syndrome (○) incubated with ionophore and Ca²⁺. Cells (2 × 10⁸/mL) were loaded 50 μmol/L PIP₂ for 10 minutes, washed, and incubated with 1 μmol/L NBD-PC for 2 minutes. Subsequently, ionophore and CaCl₂ were added to a final concentration of 5 μmol/L and 1 mmol/L, respectively.

Fig 4. Effect of PIP₂ plus Ca²⁺ on the particle light scatter properties of erythrocytes. Scott and normal control erythrocytes (2 × 10⁸/mL) were loaded with 1 μmol/L NBD-PS for 1 hour at 37°C, washed in the presence of 2 mmol/L vanadate and subsequently loaded with 50 μmol/L PIP₂ for 10 minutes at 37°C. At time 0, CaCl₂ and MgCl₂ (final concentration, 2 mmol/L) were added, and samples were diluted and analyzed by flow cytometry at the indicated times as described in Materials and Methods. Dot plots denote forward (abscissa) versus side angle scatter (ordinate) for control (A and C) and Scott erythrocytes (B and D) at time 0 (A, B) and 1 hour (C, D). Polygons denote gates used to discriminate intact from damaged erythrocytes.
suggesting that these particles represent ghosts and membrane vesicles derived from lysed cells. Consistent with this interpretation, we found that the change in light scatter depicted in Fig 4 mimicked that observed when erythrocytes were osmotically lysed by suspension in hypotonic medium (data not shown). No PIP2-induced changes in particle light scatter were observed in the absence of Ca²⁺. To determine whether the apparent movement of NBD-PS from inner to outer leaflets initiated by interaction of Ca²⁺ with PIP2 (Fig 2) reflected scrambling of lipids across the membranes of intact erythrocytes or reflected loss of phospholipid asymmetry in lysed ghost membranes, extractable inner leaflet NBD-PS was monitored in each population by gating selectively on the intact erythrocytes and lysed ghost membranes using the forward and side angle light scatter gates depicted in Fig 4. As illustrated in Fig 5, this analysis showed that addition of Ca²⁺ to PIP2-loaded erythrocytes caused little to no scrambling of NBD-PS in the intact erythrocytes, whereas virtually all of the extractable NBD-PS could be accounted for by a loss of phospholipid asymmetry in the lysed membrane fraction. Similar results were obtained when the outward movement of NBD-PC was monitored under these conditions (not shown). As shown in Figs 4 and 5, in PIP2-loaded Scott cells virtually all Ca²⁺-induced lipid scrambling was attributable to the lysed ghost population. Inspection of these data also confirms that the reduced phospholipid scrambling observed for Scott erythrocytes was the consequence of decreased lysis of Scott cells versus normal cells initiated by Ca²⁺ plus PIP2 (see Fig 2).

The extensive lysis observed upon addition of Ca²⁺ to PIP2-loaded erythrocytes raises the possibility that any lipid scrambling resulting from PIP2/Ca²⁺ is masked by the sensitivity of the erythrocytes to the presence of this detergent-like lipid. To investigate this possibility in a protein-free membrane, unilamellar lipid vesicles composed of PC, PS, and cholesterol (18:2:5 molar ratio) were prepared with or without 1 mole percent PIP2, and the transbilayer movement of NBD-PC was examined. To monitor membrane integrity of these artificial lipid vesicles, NBD-PC was first incorporated into both sides of the bilayer and fluorescence intensity measured before and after addition of dithionite, which rapidly reduces NBD in the external leaflet to the corresponding nonfluorescent amine derivative. In the presence of or absence of PIP2 with or without Ca²⁺, 47% to 50% of the NBD-PC was resistant to dithionite, indicating that neither Ca²⁺ nor PIP2 nor a combination of the two cause extensive destabilization of the bilayer because dithionite was not able to gain access to NBD-PC residing in the inner leaflet. To measure transbilayer movement, NBD-PC was added only to the external leaflet of vesicles prepared with or without PIP2, and development of resistance to dithionite reduction determined. As shown in Fig 6, NBD-PC fluorescence intensity decreased in the vesicle interior with roughly linear kinetics at a rate of somewhat less than 10%/h and was unaffected by inclusion of Ca²⁺ in the incubation medium, the presence of PIP2 in the vesicles, or a combination of both.

**DISCUSSION**

In these experiments, we show that (1) PIP2-loading of the erythrocyte membrane can result in a Ca²⁺-dependent transmembrane movement of NBD-PC and NBD-PS. These results are comparable with those previously obtained using spin-labeled probes. A lipid scrambling effect of the Ca²⁺-PIP2 complex was always observed in conjunction with increased cell lysis. NBD-lipids in PIP2-loaded erythrocytes that do not undergo hemolysis exhibit little to no rearrangement of these phospholipids through interaction of Ca²⁺ with outer leaflet PIP2. (2) The lytic action of the Ca²⁺-
PIP₂ complex obeys similar kinetics and concentration dependence as those observed for the apparent scrambling of membrane phospholipid. Furthermore, hemolysis does not occur in PIP₂-loaded cells in the presence of EGTA. (4) Ca²⁺-induced hemolysis of PIP₂-loaded erythrocytes was observed in the absence of NBD lipids, indicating that the lytic property of PIP₂ was unrelated to the fluorescent probe used to monitor phospholipid movement. Therefore, we conclude that the apparent transmembrane movement of phospholipids that arise through interaction of Ca²⁺ with PIP₂-loaded cells is related to induced lytic breakdown of the membrane, rather than a process of accelerated transmembrane lipid movement specifically mediated by a Ca²⁺-PIP₂ complex, as was recently proposed. Under conditions of cell lysis, such apparently accelerated movement of membrane phospholipids could arise through lipid rearrangement during membrane rupture, the exposure of inner leaflet PS in membranes that do not reseal, or lipid movement catalyzed by elevated intracellular Ca²⁺ because of increased permeability of the PIP₂-loaded membrane to this ion. In this context it is of note that PIP₂-loaded Scott cells are relatively resistant to this action of externally-added Ca²⁺. This suggests that lipid scrambling observed under these conditions reflects interaction of Ca²⁺ that leaks across the PIP₂-membrane with intracellular components that normally serve to mediate transmembrane lipid rearrangement and not a direct scrambling of lipid mediated through interaction of external Ca²⁺ with external PIP₂.

Porcine erythrocytes, like Scott erythrocytes, are defective in their response to Ca²⁺ influx, neither shedding microvesicles nor redistributing their membrane lipids. However, unlike Scott syndrome porcine platelets are indistinguishable from normal human platelets in their ability to scramble lipids upon activation (E.M.B., unpublished observation, September 1994). Such tight association between microvesicle formation and loss of lipid asymmetry led to the proposal that membrane fusion events occurring as part of the shedding process are responsible for transient disturbances of the bilayer structure, allowing lipids to equilibrate over both membrane leaflets. There is now evidence that microvesicle formation might be the result rather than the cause of lipid scrambling, possibly facilitated by unequal rates of inward and outward movement of lipids.

The absence of Ca²⁺-induced loss of lipid asymmetry in Scott and porcine erythrocytes might result from active transport of scrambled aminophospholipids from the outer to the inner membrane leaflet. However, ATP-dependent aminophospholipid translocase activity in Scott and porcine erythrocytes was not different from that observed in normal human erythrocytes, and was also normally inhibited by intracellular Ca²⁺. A second mechanism, mediation by Ca²⁺ in association with PIP₂ of scrambling of phospholipids, was also tested using several approaches: measuring PIP₂ content, loading cells with PIP₂, and using model vesicles containing PIP₂. We found the level of PIP₂ in Scott and porcine erythrocytes was the same or higher than the level found in normal human erythrocytes. In addition, the metabolic-resistant pool was also normal or elevated in these cells. These results imply that the simple combination of PIP₂ and Ca²⁺ in erythrocytes does not constitute the scrambling, a conclusion consistent with the fact that the PIP₂ pool in platelets is 5 to 10 times smaller than in erythrocytes, even though Ca²⁺-induced lipid scrambling in platelets is much faster. A similar conclusion can be drawn from artificial vesicles prepared with PIP₂. At a level of 1%, roughly that found in the erythrocytes, and higher than that present after elevation of cytoplasmic Ca²⁺ (Table 1), Ca²⁺ is incapable of accelerating transbilayer lipid movement.

In conclusion, the present data do not lend support to the view that the mechanism of Ca²⁺-induced redistribution of lipids in the membrane of erythrocytes is exclusively mediated by the action of Ca²⁺ ions on the (metabolic resistant) pool of PIP₂. Although a role for PIP₂ in accelerating transbilayer lipid movement is not ruled out by these experiments, there is no strong experimental reason to postulate such a role. The absence of Ca²⁺-induced phospholipid scrambling despite normal cellular PIP₂ levels in Scott and porcine erythrocytes suggest the involvement of other membrane components as essential prerequisite to catalyze this process.

ACKNOWLEDGMENT
We thank patient MS for donating blood.

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The complex of phosphatidylinositol 4,5-bisphosphate and calcium ions is not responsible for Ca(2+)-induced loss of phospholipid asymmetry in the human erythrocyte: a study in Scott syndrome, a disorder of calcium-induced phospholipid scrambling

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