Effects of Phosphatidylinositol Diphosphate on Phospholipid Asymmetry in the Human Erythrocyte Membrane†

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ABSTRACT: While phospholipid asymmetry has been well characterized in red blood cells (RBCs), controversy exists as to what role PIP_2 plays in cation-induced phosphatidylserine (PS) exposure. We report that PIP_2 can redistribute intracellular cations and thereby lead to a loss of phospholipid asymmetry. Flow cytometry was employed to monitor intracellular cation levels by using the fluorophore Fluo-3 and exposure of PS on the outer surface of the RBC bilayer by using fluorescently labeled annexin V. The addition of PIP_2 to RBCs led to a concentration-dependent increase in cytosolic cations and PS exposure. If RBCs were preincubated with 25 μM neomycin sulfate, an inhibitor of phosphoinositide metabolism, PIP_2-induced PS exposure decreased dramatically. If the RBC buffer system contained 2.5 mM EGTA, PS exposure also decreased significantly, suggesting a competition between intracellular Fluo-3 and extracellular EGTA. Together, these data indicate that (1) PS exposure was found in RBCs that exhibited an increased cytosolic cation concentration available for the fluorophore, Fluo-3, (2) both the level of intracellular cations and the movement of PS from the inner to the outer monolayer were affected by the level of PIP_2 in the bilayer, (3) the cleavage of PIP_2 by a phosphoinositide-specific phospholipase lead to the redistribution of intracellular cations and to an increase in the amount of PS exposed on the outer leaflet of the bilayer, and (4) a transient channel could be formed during the interaction of PIP_2 with the RBC membrane which would then allow the transbilayer movement of phospholipids and cations.

Phospholipids are asymmetrically distributed within the bilayer of a variety of cell membranes (*1*). Within the red blood cell, the choline-containing phospholipids, phosphatidylcholine and sphingomyelin, are mainly located in the outer monolayer, whereas the aminophospholipids, phosphatidylethanolamine (PE)[1] and phosphatidylserine (PS), are mainly (PE) or exclusively (PS) found in the inner monolayer of the membrane bilayer (*2*). Although the asymmetric distribution of phospholipids appears to be well conserved throughout the life of the cell, the organization of phospholipids in the membrane is not static and, at any point in time, reflects the equilibrium level of phospholipid molecular species moving across the bilayer in both directions. Lipid−lipid, lipid−protein, and protein−protein interactions may all have roles in the stabilization of membrane phospholipid organization.

Loss of red cell phospholipid asymmetry becomes apparent when both of the following two events occur: (1) the aminophospholipid translocase or flipase, which transports PS and PE from the outer to the inner leaflet of the red cell membrane, is impaired (*3*, *4*); and (2) the membrane phospholipid organization is scrambled by an additional mechanism (i.e., increased movement of PS to the outer leaflet of the red blood cell). Conditions that lead to scrambling of the phospholipid bilayer include treatment of the red cells with calcium and ionophore (*5*) and bilayer fusion processes, including vesiculation (*6*).

When the level of intracellular red cell calcium is increased, there is a rapid loss of membrane phospholipid asymmetry (*5*, *7*). The involvement of proteins such as the flipase (*8*), the scramblase (*9*), and, perhaps, a phosphorylated protein (*10*), along with the combined effect of calcium and PIP_2, have all been implicated in this process (*11*). However, the involvement of PIP_2 in the calcium-induced scrambling of red cell phospholipid membranes is controversial (*12*−*14*).

The purpose of this paper was to investigate whether the redistribution of intracellular cations and the movement of PS from the inner to the outer monolayer of the red cell membrane could be connected to the amount of PIP_2 in the bilayer. The findings reported in this paper suggest that a mechanism for PIP_2-induced loss of phospholipid asymmetry in the red cell membrane could, indeed, exist.

EXPERIMENTAL PROCEDURES

Materials. Fluorescein isothiocyanate (FITC)-labeled annexin V (10 μg/mL) was purchased from R&D Systems (Minneapolis, MN). PIP_2 (phosphatidylinositol 4,5-diphos-

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[1] Abbreviations: PIP_2, phosphatidylinositol diphosphate; RBC, red blood cell; PS, phosphatidylserine; Streptavidin-PE, Streptavidin-R-phycoerythrin conjugate; ATP, adenosine triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PE, phosphatidylethanolamine; FITC, fluorescein isothiocyanate; Fluo-3 AM, Fluo-3 acetoxymethyl ester; POPC, phosphatidylcholine (1-palmitoyl-2-oleoyl); TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; DMSO, dimethyl sulfoxide; FACScan, fluorescence-activated cell sorter; BSA, bovine serum albumin; KOH, potassium hydroxide; SEM, standard error of the mean.

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phate sodium salt) was obtained from Sigma (St. Louis, MO). Fluo-3 acetoxymethyl ester (Fluo-3 AM) (cell permeant), Biotin Labeling Kit, and Streptavidin-R-phycoerythrin (Streptavidin-PE) conjugate (1 mg/mL) were from Molecular Probes (Eugene, OR). Phosphatidylcholine (1-palmitoyl-2-oleoyl) (POPC) was from Avanti Polar Lipids (Alabaster, AL). All other reagents were obtained from Sigma.

Preparation of Erythrocytes. Human erythrocyte suspensions were prepared from fresh human venous blood collected in EDTA, after informed consent was obtained from laboratory volunteers. Erythrocytes were separated from plasma by low-speed centrifugation (1200*g*, 5 min), washed 3 times either with isotonic saline (0.9% NaCl) or with buffer C (10 mM Hepes, 80 mM KCl, 70 mM NaCl, pH 7.4), and then diluted to the appropriate hematocrit with the respective solutions.

ATP Depletion of the Red Blood Cells. Erythrocytes, at a final hematocrit of 16%, were incubated in the ATP depletion buffer (10 mM Tris-HCl, 144 mM NaCl, 10 mM inosine, 6 mM iodoacetamide, pH 7.4) for 90 min at 37 °C. After 90 min, the cells were washed 3 times in buffer A (80 mM KCl, 70 mM NaCl, 0.15 mM $MgCl_2$, 10 mM Hepes, 0.1 mM EGTA, pH 7.4), and then 1 time in buffer B (80 mM KCl, 70 mM NaCl, 0.15 mM $MgCl_2$, 10 mM Hepes, 0.1 mM EGTA, 10 mM inosine, 5 mM pyruvate, pH 7.4). This treatment decreased intracellular red blood cell ATP levels to less than 5% of the control red cell ATP values. Control red blood cells were also prepared along with the ATP-depleted cells by incubation in buffer containing 10 mM Tris-HCl, 144 mM NaCl, pH 7.4.

ATP Measurements. Red blood cell ATP levels were assayed after precipitation of 20 μL of a washed 10% red cell suspension in 200 μL of extraction buffer (10% TCA/100 mM Tris−acetate, 2 mM EDTA, pH 7.8, 3/2, v/v). The mixture was vortexed vigorously and then incubated on ice for 20 min, with occasional vortexing. After 20 min, the mixture was spun in a microfuge (13000*g*, 2 min). Next, 20 μL of the resultant supernatant was aliquoted into 980 μL of cold 100 mM Tris−acetate, 2 mM EDTA, pH 7.8. The sample was either analyzed immediately or stored at −80 °C for up to 3 months prior to measurement. No differences were found as the result of storage.

ATP levels were assayed using an ATP bioluminescent assay kit (Sigma #FLAA; Sigma, St. Louis, MO). The actual assay procedures were performed as outlined within the kit instructions. Chemilumunescence measurements were performed using the ATP measurement channel of a Lumi-Dual Aggregometer (Chrono-Log Corp., Havertown, PA). Final assay values were calculated in nanomoles of ATP per gram of hemoglobin. Hemoglobin values were determined in each sample after conversion to cyanomethemoglobin using Drabkin's reagent and spectrophotometric determination at 540 nm (*15*).

N-Ethylmaleimide (*NEM*) *and Neomycin Sulfate Treatment of Red Blood Cells*. Control (with ATP) and ATP-depleted red blood cells were preincubated in buffer C containing 10 mM NEM for 30 min at 37 °C, washed twice in buffer C, and then PIP_2-loaded in the presence or absence of calcium. Similarly, control and ATP-depleted red blood cells were preincubated in buffer C containing 25 μM neomycin sulfate for 10 min at 37 °C, washed twice in buffer C, and then PIP_2-loaded in the presence or absence of calcium.

Preparation of Fluo-3-Loaded Red Blood Cells. Control (with ATP) and ATP-depleted red cells, at a final concentration of 0.1%, were incubated in buffer B, containing a final concentration of 1 μM Fluo-3 AM and 0.1% dimethyl sulfoxide (DMSO), for 2 h at 37 °C. Subsequently, the tubes were spun in a Sorvall RT6000B centrifuge (1200*g*, 5 min). Next, the cells were washed twice in buffer C, and then resuspended in buffer C to a final red cell concentration of approximately 0.4%.

Flow Cytometry Measurements. Samples were analyzed on a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using Cell Quest software (Becton Dickinson). Ten thousand events per sample were acquired to ensure the adequate measurement of mean fluorescence levels. The light scatter and fluorescence channels were set at a logarithmic gain. The forward angle light scatter setting was E-1 with a threshold of 36. The red cell population was defined by size in forward and side scatter plots. Only events that correlated with "intact red blood cells" were analyzed for fluorescence intensity. Since the experimental conditions slightly changed the forward scatter characteristics of the population in the flow cytometer, the gated regions were adjusted accordingly. Mean fluorescence intensities were expressed in the linear mode, and positive fluorescence was defined by comparison with appropriate control samples. All experiments were conducted at room temperature (22−25 °C).

Determination of the Presence of Free Cytosolic Cations by Fluo-3 Measurements. Fluo-3-loaded control and ATP-depleted red blood cells were incubated at room temperature for 30 min in the presence or absence of calcium and/or PIP_2. The final percentage of red cells and the final concentration of EGTA in each incubation tube were 0.1% and 0.1 mM, respectively (total volume in each incubation tube, 2 mL). After 30 min, an aliquot from each incubation tube was transferred to a microfuge tube and spun in a microfuge (13000*g*, 20 s), the supernatant was removed, and the resultant red cell pellet was resuspended in buffer C to a final red cell concentration of approximately 0.04%.

Buffer-incubated red cells were used to define the background fluorescence for each assay (marker M1, Figure 1). No shift in fluorescence was found in these cells under any of the experimental conditions. The population of red cells loaded with Fluo-3 AM showed an increase in fluorescence (marker M2, Figure 1), as compared to cells that did not contain Fluo-3. Calcium binding to Fluo-3, as induced by incubation of red cells with the calcium ionophore A23187 in a calcium-containing buffer, led to an expected significant increase in fluorescence (marker M3, Figure 1). The relative number of cells that was found in the region marked by M3 was defined as the "% of Fluo-3^+ RBCs".

Determination of PS Exposure by FITC−Annexin V Measurements. Buffer-incubated control and ATP-depleted red blood cells were incubated at room temperature for 30 min in the presence or absence of calcium and/or PIP_2. The final percentage of red cells and the final concentration of EGTA in each incubation tube were 0.2% and 0.1 mM, respectively (total volume in each incubation tube, 2 mL). After 30 min, an aliquot from each incubation tube was transferred to a microfuge tube and spun in a microfuge (13000*g*, 20 s), the supernatant was removed, and the resultant red cell pellet was resuspended in buffer C to a

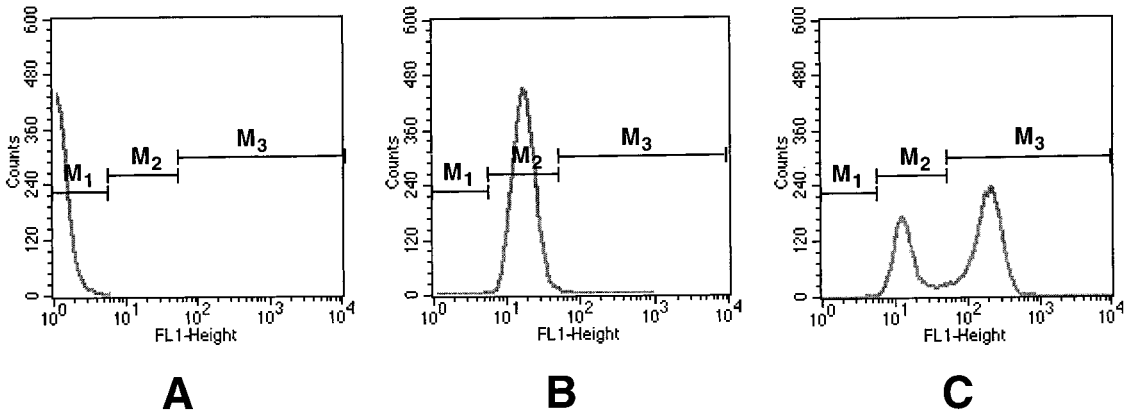

FIGURE 1: Fluo-3 measurements. (A, Left panel) Buffer-incubated red blood cells were used to define the background fluorescence for each assay, and were identified by the marker M1. (B, Middle panel) Fluo-3-loaded red cells were indicated by marker M2. (C, Right panel) The population of red cells containing Fluo-3 which was bound to cations such as calcium was indicated by marker M3, and was subsequently referred to as the "Fluo-3^+ RBCs (%)".

final red cell concentration of approximately 0.2%. PS exposure was then measured as described previously (*5*).

In a separate series of experiments, after the 30 min incubation of cells in the presence or absence of calcium and/or PIP_2, control and ATP-depleted red blood cells were washed 3 times in buffer C containing 3% BSA, then washed twice in buffer C, and finally resuspended in buffer C to a final red cell concentration of approximately 0.04%. Non-BSA treated (buffer C-washed) red cells were also prepared.

A negative control (i.e., 0 μM PIP_2 and 0 μM $CaCl_2$) was used to define background fluorescence for each assay series, and, as a negative background for each experimental condition, red cells were used which were not labeled with FITC−annexin V. The population of red cells labeled with FITC−annexin V, above background, was defined as the "% of annexin V labeled RBCs".

The treatment of red blood cells at a 30% hematocrit with 10 mM NEM (37 °C, 30 min) followed by incubation (37 °C, 1 h) with 1 mM calcium and 4 μM calcium ionophore A23187 generated a cell population in which 75−95% of the cells were labeled with FITC−annexin V (*5*). These "positive" red cell preparations were used to evaluate the activity of each stock solution of FITC−annexin V, and the performance of the FACScan instrument.

Purification and Biotin Labeling of Annexin V. Recombinant annexin V was purified from an *Escherichia coli* expression system by phospholipid affinity chromatography (Ando et al., 1989). Biotinylated annexin V was then prepared by using the procedures as outlined within the kit instructions of a Biotin Labeling Kit (Molecular Probes).

Double Label Experiments. Fluo-3-loaded control and ATP depleted red blood cells were incubated at room temperature for 30 min in the absence of calcium and in the presence of 0 or 10 μM PIP_2. The final percentage of red cells and the final concentration of EGTA in each incubation tube were 0.2% and 0.1 mM, respectively (total volume in each incubation tube, 2 mL). After 30 min, an aliquot from each incubation tube was transferred to a microfuge tube and spun in a microfuge (13000*g*, 20 s), the supernatant was removed, and then the resultant red cell pellet was resuspended in buffer C to a final red cell concentration of approximately 0.2%. Next, an aliquot of the 0.2% red cell suspension from each experimental condition was incubated for 30 min at room temperature in buffer C which contained 1.2 mM $CaCl_2$ and 64 ng/mL biotinylated annexin V (2 μL of a 1:50 dilution of the stock biotinylated annexin V conjugate in 10 mM Hepes, 100 mM NaCl, 5 μg/mL ovalbumin, pH 7.0) (total volume in each incubation tube, 0.5 mL). After 30 min, each incubation tube was spun in a microfuge (13000*g*, 20 s), the supernatant was removed, and then the cells were resuspended in an equivalent volume of buffer C (0.5 mL) which contained 1.2 mM $CaCl_2$ and 4 μg/mL Streptavidin-PE (2 μL of a 1 mg/mL solution). The tubes were then incubated for 30 min at room temperature. After 10 min, each incubation tube was spun in a microfuge (13000*g*, 20 s), the supernatant was removed, and then the cells were resuspended in an equivalent volume of buffer C (0.5 mL) which contained 1.2 mM $CaCl_2$. Fluo-3 and phycoerythrin fluorescence were then measured simultaneously in the FACScan flow cytometer.

At each experimental condition, the appropriate red cell suspension was incubated under the following four separate conditions: with biotinylated annexin V and with Streptavidin-PE, with only biotinylated annexin V, with only Streptavidin-PE, and without any biotinylated annexin V or Streptavidin-PE.

Electronic compensation of the instrument was set to exclude any overlap of the two emission spectra. Specifically, compensation settings were adjusted using the following controls: (1) Fluo-3-loaded red cells, which had been preincubated with 13 μM PIP_2 in the presence of 0 μM free calcium, and then further incubated in the absence of both biotinylated annexin V and Streptavidin-PE; (2) buffer-incubated red cells, which had been preincubated with 13 μM PIP_2 in the presence of 0 μM free calcium, and then further incubated in the presence of both biotinylated annexin V and Streptavidin-PE; and (3) buffer-incubated red cells, which had been preincubated with 0 μM PIP_2 in the presence of 0 μM free calcium, and then further incubated in the absence of both biotinylated annexin V and Streptavidin-PE (i.e., unstained red cells).

Fluo-3-loaded red cells, which had been preincubated with 0 μM PIP_2 in the presence of 0 μM free calcium, and then further incubated in the absence of both biotinylated annexin V and Streptavidin-PE, were used to define the vertical setting which intersects the FL-1 (or *x* axis) and is the gate between the population of red cells loaded with Fluo-3 and those red cells which contained Fluo-3 bound to cations (i.e.,

Fluo-3^+ RBCs). Next, buffer-incubated red cells, which had been preincubated with 0 μM PIP_2 in the presence of 0 μM free calcium, and then further incubated in the absence of both biotinylated annexin V and Streptavidin-PE, were used to define the horizontal setting which intersects the FL-2 (or *y* axis) and is the gate between the population of red cells labeled with Streptavidin-PE and those red cells which are not.

Potassium Hydroxide (KOH) Treatment of the Fluo-3 AM Probe. The Fluo-3 AM ester (50 μg/vial) was reconstituted with a volume (44 μL per each 50 μg vial) of dimethyl sulfoxide (DMSO) (final Fluo-3 AM concentration, 1 mM). Next, an equal volume of methanol (MeOH) (44 μL per each 50 μg vial) and then 25 μL of a 2 N solution of KOH were added to this solution (final KOH-treated Fluo-3 concentration, 392 μM). The vial was then covered with foil and incubated at room temperature for 1 h. This treatment generated the form of the Fluo-3 probe which was present after intracellular red cell esterase digestion.

Fluorometric Measurements of the Fluo-3 Probe. An aliquot of the Fluo-3 probe was added into an EGTA-containing buffer system (buffer C) in a magnetically stirred cuvette, and base line fluorescence was recorded in a fluorometer. The final concentrations of EGTA and of Fluo-3 were 0.1 mM and 0.392 μM, respectively. The final pH of each assay solution was approximately 7. Next, a predetermined amount of a stock solution of calcium chloride or zinc acetate was injected into the cuvette, and then the fluorescence was recorded for a total of 5 min. All experiments were conducted at room temperature (22−25 °C). Excitation was at 488 nm, and fluorescence emission was measured at 525 nm (Perkin-Elmer LS-5B Luminescence Spectrometer, Palo Alto, CA).

Calculation of Final and Free Calcium Concentrations. The free calcium concentrations in our EGTA-containing buffer systems were calculated according to Schoenmakers et al. (*16*). The computer-based program renders calcium concentrations that are correct from the 10 nM to millimolar range. In those conditions in which we omitted the addition of calcium to the 0.1 mM EGTA buffer system, the concentration of free calcium was lower than 10 nM, which for all practical purposes will be defined as "0 μM free calcium" throughout this paper.

Phospholipid Measurements. The phospholipid concentrations of the stock PIP_2 solutions were determined by phosphorus analysis (*17*).

Microscopy Studies. Red blood cells were fixed in 1% glutaraldehyde in buffer C, and then examined by Nomarsky light microscopy. Images were collected using a video camera connected to a Macintosh computer (Apple, Cupertino, CA).

Surface Balance Measurements. Surface film pressure was measured using a platinum Wilhelmy dipping plate (wetted length, 40 mm; Krüss, Charlotte, NC) within a Beckman LM500 microbalance (Beckman, Palo Alto, CA) which was interfaced to a Macintosh computer (Apple, Cupertino, CA). A 50 mL Teflon trough (surface area, 65.5 cm^2) was placed in a maximally humidified temperature-controlled chamber at 25 °C. The surface of the subphase buffer (100 mM Tris-HCl, pH 7.4) was cleaned by aspiration, and then a monolayer film of POPC, from a dichloromethane solution, was spread at the air−water interphase, and surface pressure was recorded. After an appropriate equilibrium period at the desired initial surface pressure (i.e., less than 20 mN/m), a solution of PIP_2, from a dichloromethane solution, was carefully added dropwise to the lipid interphase, and then surface pressure was recorded. Next, after a second equilibrium period, a solution of BSA (4%, final concentration) was injected into the subphase and under the monolayer, and then surface pressure was recorded.

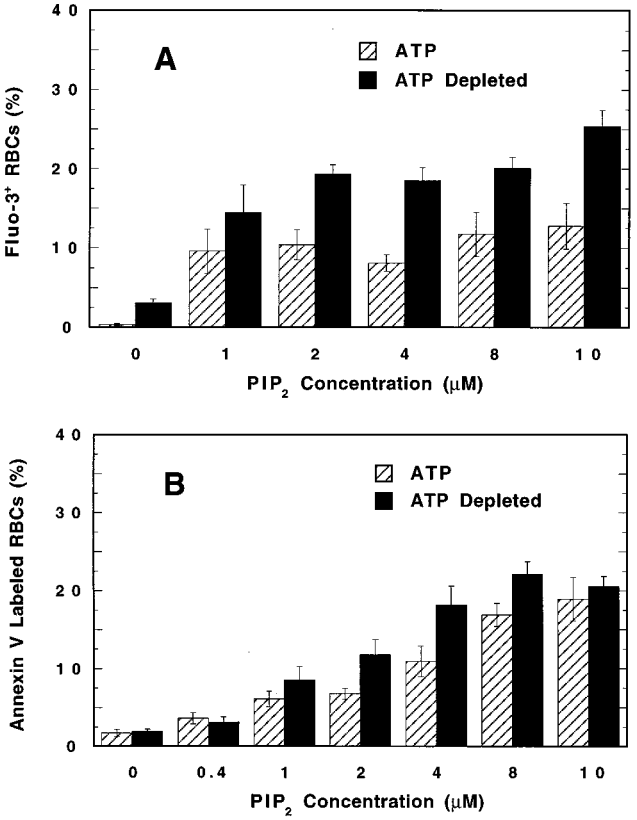

FIGURE 2: Effect of PIP_2 on cation-induced fluorescence and PS exposure. Control and ATP-depleted red blood cells were incubated in the presence of 0 μM free calcium, and the final PIP_2 concentration of each incubation mixture was varied from 0 to 10 μM. (A, Top) 0 μM free calcium, Fluo-3^+ RBCs (%). (B, Bottom) 0 μM free calcium, annexin V labeled RBCs (%). The Fluo-3 values at each experimental condition are the mean ± the SEM of four experiments and represent the "Fluo-3^+ RBCs (%)", as measured in the flow cytometer. The FITC−annexin V values at each experimental condition are the mean ± the SEM of four experiments and represent the "annexin V labeled RBCs (%)", as measured in the flow cytometer.

RESULTS

Effect of PIP_2 on Cation-Induced Fluorescence and PS Exposure. Fluo-3-loaded control and ATP-depleted RBCs were incubated in an EGTA-containing buffer system at an extracellular free calcium concentration of 0 μM, as the final PIP_2 concentration in each incubation mixture was varied from 0 to 10 μM. Figure 2A shows that when the free calcium concentration was kept constant at 0 μM, the percent of Fluo-3^+ RBCs increased as the final PIP_2 concentration of each incubation mixture was increased up to 1 μM. The population of RBCs which showed cation-induced fluorescence did not increase as PIP_2 concentrations were further increased to 10 μM. ATP depletion, prior to incubation with PIP_2, significantly expanded the percent of RBCs with increased intracellular cations.

Similarly, control and ATP-depleted RBCs were incubated in an EGTA-containing buffer system at an extracellular free calcium concentration of 0 μM, as the final PIP_2 concentrations within each incubation mixture were varied from 0 to 10 μM. Next, the fraction of RBCs that exposed PS on the outer surface of the RBC was determined by flow cytometry using FITC−annexin V. Figure 2B shows that when the free calcium concentration was kept constant at 0 μM, the percent of annexin V-labeled RBCs increased as the final PIP_2 concentration in each incubation mixture was varied from 0 to 10 μM. ATP depletion, prior to incubation with PIP_2, seemed to increase the fraction of RBCs that exposed PS.

Figure 2 shows that cytosolic cations available for interaction with the Fluo-3 probe increased if RBCs were incubated with PIP_2, and that these cations levels increased even further if the RBCs were depleted of ATP. In addition, the percent of RBCs that exposed PS increased if RBCs were incubated with PIP_2. Taken together, these data suggested that a subpopulation of RBCs existed which had an increased level of cytosolic cations available for interaction with Fluo-3 and an increased amount of PS exposed on the outer surface of the red cell membrane.

Effect of PIP_2 Incorporation into Red Cell Membranes and Lipid Monolayers. The Fluo-3 and FITC−annexin V data suggest that PIP_2 can interact with the red cell membrane and set off an event, or series of events, that will lead to a redistribution of cytosolic cations and to the movement of PS from the inner to outer monolayer of the red cell membrane. To monitor the incorporation of PIP_2 within the red cell membrane, we analyzed the morphology of RBCs which were incubated under several specific conditions. At an extracellular free calcium concentration of 0 μM and a final PIP_2 concentration of 10 μM, morphological studies indicated that most of the RBCs exhibited echinocytic shapes (data not shown). This finding substantiated previously reported findings (*12*), and indicated that PIP_2 molecules had, indeed, been incorporated into the red cell bilayer.

To further illustrate the incorporation of PIP_2 from an aqueous medium into a lipid bilayer, we performed surface balance studies to measure whether PIP_2 could be incorporated into a lipid monolayer. As indicated by the significant increase in surface pressure shown in Figure 3A at indicator b, PIP_2 was rapidly incorporated into a POPC monolayer. Furthermore, the addition of PIP_2 into the subphase and under the monolayer also produced an increase in surface pressure, whereas POPC did not. This indicated that PIP_2, in contrast to other long-chain phospholipids such as PC, was able to transfer through the water phase. It also indicated that PIP_2 could be removed from the monolayer by the addition of an appropriate lipid binding system. To test whether PIP_2 could be extracted from a lipid monolayer, defatted albumin, a well-known lipid binding protein, was added to the subphase of this same monolayer system. As indicated by the significant decrease in surface pressure shown in Figure 3A at indicator c, PIP_2 could be removed from a lipid monolayer. Moreover, at a surface pressure of 30 mN/m, a non-PIP_2-containing POPC monolayer was not affected by the addition of BSA to the subphase (data not shown). The rapid extraction of PIP_2 from a monolayer system suggested that it might also be possible to extract PIP_2 from the red cell membrane. Therefore, in the next series of experiments,

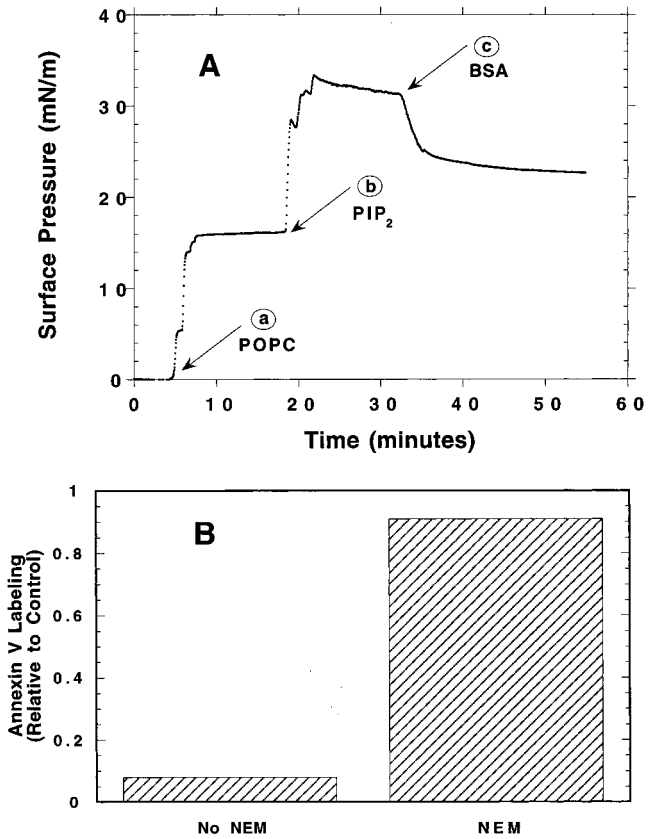

FIGURE 3: (A) Effect of PIP_2 incorporation into lipid monolayers. Surface activity measurements. The surface of the subphase buffer (100 mM Tris-HCl, pH 7.4) was cleaned by aspiration, and then a monolayer film of POPC was spread at the air−water interphase, and surface pressure was recorded (a). After an appropriate equilibrium period, a solution of PIP_2 was carefully added dropwise to the lipid interphase, and then surface pressure was recorded (b). Next, after a second equilibrium period, a solution of BSA (4%, final concentration) was injected into the subphase, and then surface pressure was recorded (c). (B) Effect of BSA on PIP_2-induced PS exposure. Control and ATP-depleted red blood cells, which were or were not pretreated with 10 mM *N*-ethylmalemide at 37 °C for 30 min, were incubated in the presence of 0 μM free calcium and 10 μM PIP_2. The FITC−annexin V values are the mean of two experiments and represent the amount of annexin V labeling relative to the respective control (non-BSA-washed) samples, as measured in the flow cytometer.

PIP_2-loaded control and ATP-depleted RBCs were washed 3 times with 3% BSA, fixed, and then prepared for examination using light microscopy. The results showed that most of the RBCs in these preparations now exhibited a normal, biconcave shape and few echinocytes were seen (data not shown).

To test whether the removal of PIP_2 from these RBCs could alter PS exposure, RBCs were incubated with 10 μM PIP_2 at an extracellular free calcium concentration of 0 μM, and then PS exposure was measured after the RBCs were washed in the presence or absence of 3% BSA. Figure 3B shows that control (non-NEM-treated) RBCs which were washed with BSA had significantly less PS exposed than control RBCs which had not been washed with BSA; the percent of annexin V-labeled RBCs decreased by approximately 90%. Together, these data indicated that PS exposure could be altered or decreased upon treatment of RBCs with BSA and, therefore, upon the removal of PIP_2 from the red cell membrane.

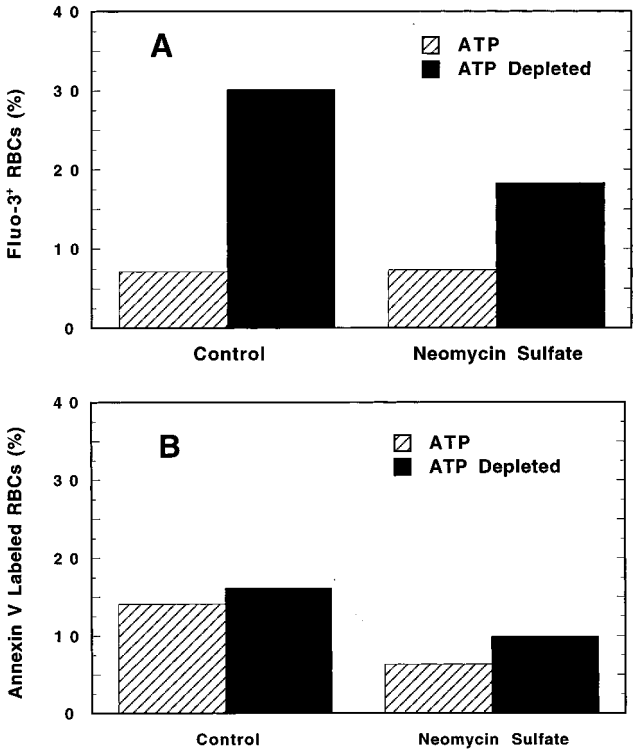

FIGURE 4: Effect of neomycin sulfate on PIP_2-induced cation-induced fluorescence and PS exposure. Control and ATP-depleted red blood cells, which were or were not pretreated with 25 μM neomycin sulfate at 37 °C for 10 min, were incubated in the presence of 0 μM free calcium and 10 μM PIP_2. (A, Top) Fluo-3 data. (B, Bottom) FITC−annexin V data. The Fluo-3 values at each experimental condition are the mean of two experiments and represent the "Fluo-3^+ RBCs (%)", as measured in the flow cytometer. The FITC−annexin V values are the mean of two experiments and represent the "annexin V labeled RBCs (%)", as measured in the flow cytometer.

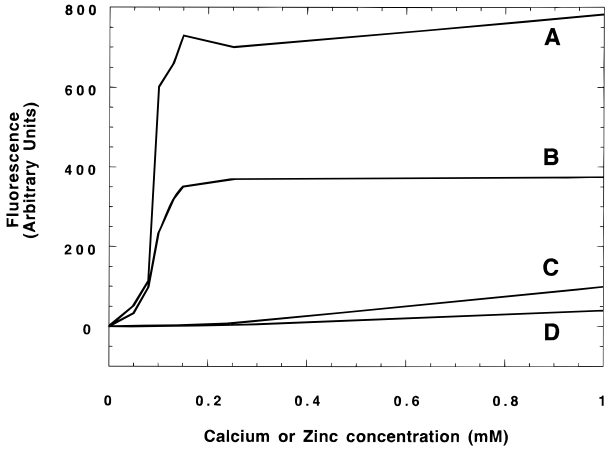

FIGURE 5: Fluorescent measurements of the interaction of Fluo-3 with various divalent cations in the presence of an ion chelator. KOH-treated Fluo-3 AM (which renders Fluo-3) was incubated in an EGTA-containing buffer system as the final calcium, zinc, or magnesium concentration of each incubation mixture was varied from 0 to 1 mM. Within each experimental series, the final concentration of EGTA in the buffer system was also varied. Fluorescent measurements were performed in a Perkin-Elmer LS-5B Luminescence Spectrometer. (A) 0.1 mM EGTA/calcium; (B) 0.1 mM EGTA/zinc; (C) 2.5 mM EGTA/calcium; (D) 2.5 mM EGTA/zinc and 0.1 or 2.5 mM EGTA/magnesium.

The decrease in annexin V labeled RBCs after BSA treatment implicated the involvement of the magnesium, ATP-dependent, aminophospholipid translocase or flipase which transports PS from the outer to the inner RBC monolayer. An active flipase could correct the loss of phospholipid asymmetry by translocating PS back to the inner monolayer after the removal of PIP_2 from the red cell membrane. Since the flipase can be inhibited by the sulfhydryl reagent *N*-ethylmaleimide (NEM), RBCs were pretreated with NEM prior to the incubation with PIP_2, and then tested in the FITC−annexin V assay. Figure 3B shows that, in contrast to the non-NEM-treated cells, BSA washing of the RBCs which had been pretreated with NEM did not decrease the level of annexin V labeled RBCs. Therefore, if RBCs were pretreated with NEM, the flipase was no longer able to correct any PIP_2-induced alterations in phospholipid asymmetry.

Effect of Neomycin Sulfate on PIP_2-Induced Cation-Induced Fluorescence and PS Exposure. Figure 2 shows that the interaction of PIP_2 with the RBC bilayer can scramble membrane phospholipids through a mechanism that involves an increase in cytosolic cations under conditions in which the RBCs were incubated in an extracellular buffer system which contained EGTA and a free calcium concentration of 0 μM. Therefore, to explain the cation-induced fluorescence results, we decided to further investigate mechanisms which might involve the redistribution of intracellular RBC divalent cations. Since the activation of phospholipase C or D and the subsequent cleavage of PIP_2 are crucial steps in the inositide signaling pathways in other cell types, we investigated the effects of neomycin sulfate, an inhibitor of phosphoinositide metabolism, on PIP_2-induced PS exposure. Figure 4A shows that if ATP-depleted RBCs were preincubated with 25 μM neomycin sulfate, the cation-induced fluorescence caused by PIP_2 decreased significantly. Figure 4B shows that the percent of annexin V labeled RBCs also decreased significantly when red cells were preincubated with 25 μM neomycin sulfate. This decrease in PS exposure occurred in both control and ATP-depleted RBCs. Together, these data suggest that the cleavage of PIP_2 by a phosphoinositide-specific phospholipase C or D is an important step in the series of events that lead to the exposure of PS on the outer surface of the red cell membrane.

Fluorescent Measurements of the Interaction of Fluo-3 with Various Divalent Cations in the Presence of an Ion Chelator. Since the intracellular RBC free calcium concentration is normally low (i.e., less than 1 μM) and most of the intracellular calcium is tightly bound, we needed to substantiate whether the increase in Fluo-3 fluorescence and subsequent increase in PS exposure could be attributed to a redistribution of calcium. Furthermore, the RBC does contain a substantial amount of zinc (*18*) and magnesium, both of which could bind to Fluo-3 and, thus, increase its fluorescence. To underscore this point, KOH-treated Fluo-3 AM (which renders the RBC post-esterase form of Fluo-3) was incubated in an EGTA-containing buffer system as the final calcium, zinc, or magnesium concentration of each incubation mixture was varied from 0 to 1.0 mM (Figure 5). Within each experimental series, the final concentration of EGTA in the buffer system was also varied; EGTA concentrations of 0.1 or 2.5 mM were used. Figure 5 shows that when the buffer system contained 0.1 mM EGTA, fluorescence values increased significantly at concentrations approximating 0.1 mM calcium or zinc (Figure 5A,B).

Under these conditions, both cations became available to bind Fluo-3, and the Fluo-3 fluorescence reached a plateau around a final calcium or zinc concentration of 0.2 mM. As was reported before (*19*), calcium gave higher fluorescence values than did zinc. When the magnesium concentration was varied in this same 0.1 mM EGTA-containing buffer system, the fluorescence values did not increase and remained low over the entire range of magnesium concentrations tested (i.e., from 0 to 1 mM) (Figure 5D). At 2.5 mM EGTA, neither calcium, zinc, nor magnesium was available for Fluo-3 binding, and, consequently, the fluoresence values were low (Figure 5). Taken together, these data indicate that the increase in the Fluo-3 signal from PIP_2-loaded RBCs cannot necessarily be attributed to a redistribution of only red cell calcium. Therefore, the redistribution of intracellular divalent cations other than calcium, in particular zinc, could also be responsible for the increase in cation-induced fluorescence in PIP_2-loaded RBCs.

Effect of Free Calcium on A23187-Induced Cation-Induced Fluorescence and PS Exposure. Since the calcium ionophore A23187 is effective in scrambling red cell phospholipid organization by increasing intracellular calcium, experiments were performed to see whether any apparent similarities existed between the effects of A23187 and those of PIP_2 on red cell membranes (compare Figure 6A,B to Figure 2A,B). In contrast to PIP_2, A23187 was not able to cause an increase in cation-induced fluorescence or PS exposure in control or ATP-depleted RBCs which were incubated in a buffer system containing an extracellular free calcium concentration of 0 μM. However, if the extracellular free calcium levels were raised to 25 μM, the A23187-treated control and ATP-depleted RBCs showed a dramatic increase in the percent of Fluo-3^+ RBCs and a significant increase in the percent of annexin V labeled cells. Next, if the extracellular free calcium levels were further increased to 900 μM, the percent of annexin V labeled cells increased dramatically while high levels of cation-induced fluorescence were maintained. If RBCs were pretreated with NEM and then incubated in the presence of 900 μM extracellular free calcium, the percent of Fluo-3^+ RBCs remained elevated, and the number of FITC−annexin V labeled cells increased significantly. Interestingly, if the extracellular EGTA-containing buffer system contained 1 mM zinc or magnesium instead of 1 mM calcium (i.e., 900 μM free calcium), the level of RBC annexin V labeling was virtually absent (i.e., less than 1%). This significant decrease in PS exposure, relative to the amount found when calcium was used (Figure 6B), was observed whether control or ATP-depleted RBCs were pretreated with NEM or not (data not shown). However, under the same conditions, the percent of Fluo-3^+ RBCs remained elevated, and reflected the differences in K_D values and in the fluorescence values of the complexes for each respective divalent cation and Fluo-3 (*19*). For example, if the A23187-treated control and ATP-depleted RBCs were incubated in a EGTA/buffer system which contained 1 mM zinc or 1 mM magnesium, the levels of cation-induced fluorescence were approximately 2 (zinc) or 0.5 (magnesium) times the values obtained had 1 mM calcium been used instead. Together these data show that the increase in PS exposure in A23187-treated RBCs was initiated by an increase in cytosolic calcium ions, not by an increase in intracellular zinc or magnesium ions. Moreover, these data suggest that calcium plays a central role in PIP_2-induced PS exposure.

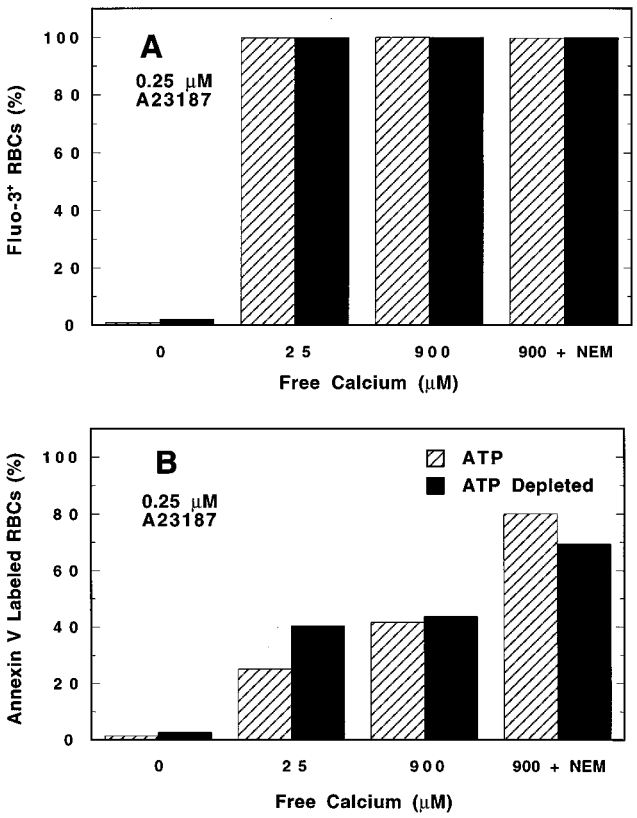

FIGURE 6: Effect of free calcium on A23187-induced cation-induced fluorescence and PS exposure. Control and ATP-depleted red blood cells were incubated in the presence of 0.25 μM A23187, and the free calcium concentration of each incubation mixture was varied from 0 to 900 μM. (A, Top) Fluo-3 data. (B, Bottom). FITC−annexin V data. The Fluo-3 values at each experimental condition are the mean of two experiments and represent the "Fluo-3^+ RBCs (%)", as measured in the flow cytometer. The FITC−annexin V values are the mean of two experiments and represent the "annexin V labeled RBCs (%)", as measured in the flow cytometer.

Comparisons of Cation-Induced Fluorescence and PS Exposure within the Same Red Cell Population. The data shown in Figures 2, 4, and 6 suggested that a subpopulation of RBCs existed which contained an increased cytosolic cation concentration available for interaction with Fluo-3 and which also exposed PS. Since the spectral overlap of Fluo-3 and FITC fluorescence did not allow simultaneous measurement of these probes, we decided to test this hypothesis by using two-color flow cytometric analyses. Fluo-3-loaded control and ATP-depleted red blood cells were incubated in an EGTA-containing buffer system at an extracellular free calcium concentration of 0 μM and at final PIP_2 concentrations of 0 or 10 μM. Next, cells were incubated with biotinylated annexin V and Streptavidin-PE, and then the Fluo-3 and phycoerythrin fluorescence values within each condition were measured simultaneously. The addition of PIP_2 led to an increase in Fluo-3^+ and in phycoerythrin−annexin V labeled RBCs (Figure 7). ATP depletion, prior to PIP_2 loading, significantly expanded the percent of Fluo-3^+ RBCs. NEM pretreatment of control and ATP-depleted RBCs, prior to PIP_2 loading, also significantly increased both the percent of Fluo-3^+ RBCs and the percent of PS-exposed RBCs. No significant fluorescence was observed in cells which were incubated in the absence of PIP_2 (data not

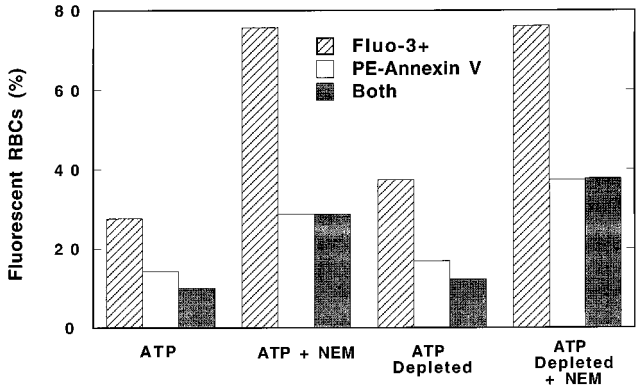

FIGURE 7: Comparisons of cation-induced fluorescence and PS exposure within the same red cell population. Fluo-3-loaded control and ATP-depleted red blood cells, which were or were not preincubated with NEM, were incubated in an EGTA-containing buffer system at an extracellular free calcium concentration of 0 μM and at a final PIP_2 concentration of 10 μM. Next, cells were incubated with biotinylated annexin V and Streptavidin-PE, and then the Fluo-3 and phycoerythrin fluorescence values within each condition were measured simultaneously. The Fluo-3 values are the mean of two experiments and represent the "Fluo-3^+ RBCs (%)", as measured in the flow cytometer. The phycoerythrin-labeled annexin V values are the mean of two experiments and represent the "annexin V labeled RBCs (%)", as measured in the flow cytometer.

shown). Only a portion of the Fluo-3^+ cells were found to expose PS by labeling with annexin V; however, virtually all of the cells that were labeled with annexin V were also positive for Fluo-3. Therefore, these data indicate that PS exposure was found in cells that also exhibited an increased cytosolic cation concentration available for Fluo-3.

Effect of an Ion Chelator on Cation-Induced Fluorescence and PS Exposure. To test whether an ion chelator in the extracellular medium could also compete with intracellular Fluo-3 for RBC cations, Fluo-3-loaded control and ATP-depleted red blood cells were incubated in a buffer system which contained higher levels of EGTA (i.e., 2.5 instead of 0.1 mM). Figure 7 shows the results when control or ATP-depleted RBCs, which were or were not pretreated with NEM, were incubated in a buffer system containing 0.1 mM EGTA, 0 μM free calcium, and 10 μM PIP_2. ATP depletion of the RBCs, prior to PIP_2 loading, increased the level of cation-induced fluorescence. NEM pretreatment of control and ATP-depleted RBCs, prior to PIP_2 loading, increased the levels both of cation-induced fluorescence and of PS exposure. If, however, the extracellular buffer system contained 2.5 mM EGTA, there was a significant decrease in the percent of PS-exposed RBCs. The percent of annexin V labeled control and ATP-depleted RBCs decreased to less than 2 and 5%, respectively. Furthermore, if the RBCs were pretreated with NEM, then the percent of annexin V labeled control and ATP-depleted RBCs decreased to less than 7 and 12%, respectively. Apparently, the extracellular EGTA and intracellular Fluo-3 probe were competing with each other for any free cations which became available as a consequence of PIP_2-loading in the absence of extracellular free calcium. These data suggest that a transient channel or pore could be formed during the interaction of PIP_2 with the red cell membrane, which would then allow the transbilayer movement of phospholipids and cations.

DISCUSSION

Previously, Sulpice et al. (*12*) used PIP_2-loaded red cells and inside-out vesicles to show that PIP_2 was required in red cell calcium-induced phospholipid scrambling. Although external calcium had no effect on phospholipid redistribution in normal red blood cells, calcium could trigger phospholipid redistribution either from the internal or from the external side of the membrane, provided that enough PIP_2 was present on that side. Their results also showed that spermine, a positively charged polyamine, inhibited calcium-induced phosphatidylcholine scrambling in large unilamellar vesicles which contained PIP_2, as well as in PIP_2-loaded red blood cells (*13*). Bevers et al. (*14*) reported that PIP_2-loaded red cells produced a calcium-induced increase in prothrombinase activity and an increase in the apparent transbilayer movement of NBD-phospholipids. However, they do not believe that a calcium−PIP_2 complex is responsible for the loss of red cell phospholipid asymmetry. This is because elevation of intracellular red cell calcium levels was unable to induce phospholipid scrambling when red blood cells from a Scott syndrome patient, which contained a normal content of PIP_2, were tested. Furthermore, PIP_2 loading of Scott red blood cells also did not result in calcium-induced scrambling.

We hypothesized that in the absence of extracellular calcium, PIP_2 could interact with the red cell membrane and cause a redistribution of intracellular red cell cations. This could be due to an imbalance in the normal equilibrium of phosphoinositides in the red cell membrane as the result of increased PIP_2 levels. Indeed, the Fluo-3 and FITC−annexin V data reported in this paper showed that PIP_2 induced a significant increase in cation-dependent fluorescence within the RBC and an increase in PS-mediated annexin V binding to the outer monolayer of the red cell membrane. Since an apparent increase in cytosolic cations occurred even under conditions in which extracellular calcium was virtually absent, PIP_2 was apparently able to mobilize and redistribute cations from sites within the red cell. Taken together, the results indicate that both the level of intracellular cations and the movement of PS from the inner to the outer monolayer may be connected to the amount of PIP_2 in the bilayer, and, thus, suggest a mechanism for cation-induced loss of phospholipid asymmetry. In addition, the double-label experiments show that a subpopulation of red cells exists which exhibit both an increase in cytosolic cations available for interaction with the Fluo-3 probe and an increased amount of PS exposed on the outer surface of the red cell membrane. Virtually all RBCs that exposed PS also exhibited a cytosolic increase in cations (i.e., Fluo-3^+/PS^+ RBCs). However, a population of RBCs also existed which exhibited an increase in cation-induced fluorescence but did not expose PS (i.e., Fluo-3^+/PS^- RBCs). Several options can be considered to explain these findings. First, the levels of cation-induced fluorescence, as determined by the fluorophore Fluo-3, are not quantitative measurements. Perhaps, a threshold level of intracellular cations is actually required to initiate phospholipid membrane scrambling. Second, the data presented in this paper show that both zinc and calcium will effectively form a fluorescent complex with Fluo-3. The red cell contains 5.6 μmol of calcium/L of RBCs. Of this calcium, 4 μmol is not extractable with EDTA in the presence of ionophore, and can be defined as tightly bound calcium.

In contrast to other cell types, the red cell does not seem to have a calcium pool from which calcium can become readily available. The red cell also contains 129 μmol of zinc/L of RBCs, of which 122 μmol is tightly bound (*18*). While A23187 will induce loss of phospholipid asymmetry in the presence of calcium, this was not the case if zinc or magnesium was present within the extracellular buffer system. Perhaps, the cation-induced fluorescence of the Fl^+/PS^- cells was caused primarily by the zinc/Fluo-3 complex, while in the case of Fl^+/PS^+ RBCs the increase in fluorescence was caused by calcium. Therefore, it is tempting to assume that an intracellular redistribution of calcium is central to our observation of PIP_2-induced phospholipid scrambling. Last, a redistribution or imbalance in calcium may be merely one step in a series of events which, in turn, lead to the loss of phospholipid asymmetry. At this time, it is also unclear whether the increase in PS exposure is due to a direct interaction between intracellular red cell cations and a scramblase (*9*), or the result of events that lead to phosphorylation of membrane proteins which, in turn, affected red cell phospholipid asymmetry. For example, cells which do not expose PS may have an increased level of intracellular calcium but may not have completed any additionally needed cellular events (i.e., phosphorylation and/or dephosphorylation of cytosolic or membrane proteins). However, a direct connection between phosphoinositide metabolism, cation levels, and scrambling is indicated by the fact that an inhibitor of phosphoinositide metabolism, neomycin sulfate, counteracted the effects observed. Although this finding tentatively points to a role of the phospholipase C in phosphoinositide pathways in the exposure of PS on the red cell surface, it is important to note that neomycin sulfate is an inhibitor not only of phospholipase C but also of phospholipase D. Since the red cell does contain calcium-induced phospholipase D activity, we must also take the possible involvement of this enzyme into consideration (*20*).

At physiological pH and in the absence of divalent cations, PIP_2 is negatively charged (*21*). Recent evidence has shown that the addition of calcium to phosphatidylcholine/PIP_2 artificial membranes increases the lateral diffusion of PIP_2 (*22*). Sulpice et al. (*13*) reported that calcium triggered a partial phospholipid redistribution in phosphatidylcholine large unilamellar vesicles which were prepared with 5% PIP_2. Although Bevers et al. (*14*) did not detect the same response in their PIP_2-containing lipid vesicles, the final amount of PIP_2 within their vesicles was probably less than 1%, which could account for this discrepancy.

Our data suggest that the presence of PIP_2 in the red cell membrane allows the formation of a pore through which cations can move from the cytosol to the extracellular medium. It is possible that such a pore would also allow the movement of phospholipids across the bilayer. Such a possible mechanism is depicted in Figure 8. We hypothesize that negatively charged PIP_2 molecules might form a pore within the red cell membrane, which could act as a lipid scrambling entity by allowing the rapid movement of phospholipids across the bilayer and by forming a pore for cations. It could well be that positively charged cations, such as calcium, would play a role in the formation of such a hypothetical pore. Furthermore, the transbilayer redistribution of phospholipids would be rapid at these sites, and the kinetics of this movement could approach those of the lateral movements of phospholipids in the plane of the bilayer. As long as the aminophospholipid translocase or flipase and the ATP-dependent calcium pump are active, PS will be moved inward and calcium will be pumped outward. Thus, red cell PS asymmetry and low cytosolic calcium will be restored. The importance and, perhaps, uniqueness of this hypothesis is that phospholipids and calcium could be transported both in and out of the red cell through the lipid phase. This hypothesis could also be used to explain the previous findings of Sulpice et al. (*13*).

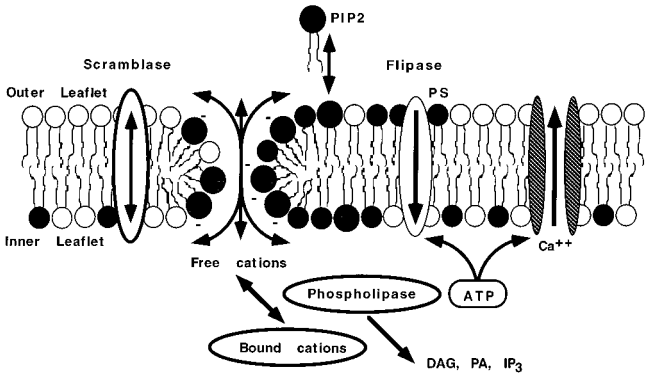

FIGURE 8: Membrane model of the proposed effects of PIP_2 on the human red blood cell bilayer. The interaction of PIP_2 with the red cell membrane could (1) form a pore within the membrane which could act as a lipid scrambling entity by allowing the rapid movement of phospholipids across the bilayer of the red blood cell membrane and by forming a pore for cations and/or (2) activate membrane-bound phospholipase C or D molecules which, in turn, could hydrolyze PIP_2 to diacylglycerol (DAG), phosphatidic acid (PA), and inositol 1,4,5-triphosphate (IP_3). DAG and IP_3 have been implicated as regulators of protein kinase C and intracellular calcium release, respectively, in other cell types (*23*).

Our data also support a second hypothesis in which the interaction of PIP_2 with the red cell membrane could activate phospholipases which would result in the formation of products such as diacylglycerol (DAG), phosphatidic acid (PA), and inositol 1,4,5-triphosphate (IP_3). DAG and IP_3 have been implicated as regulators of protein kinase C and intracellular calcium release, and are important mediators in signal transduction in other cell types (*23*). Such mechanisms are less clearly defined in the red cell. The exposure of PS on the outer surface of the red blood cell may parallel the processes of programmed cell death or apoptosis which are found in other cell types. Apoptosis, defined by characteristic morphologic alterations and DNA fragmentation, can be accompanied by exposure of PS on the outer cell surface, and has also been shown to be calcium-dependent (*24*). Furthermore, since the hydrolysis of PIP_2 by phospholipase C can activate important intracellular signaling mechanisms characteristic of apoptosis, phosphatidylinositol metabolism has also been implicated in this process (*25*). Therefore, our findings regarding the loss of phospholipid asymmetry in red cells may be applicable, in a general mechanistic sense, to the series of cellular events taking place in apoptosis in other cell types.

In summary, our data indicate that PIP_2 can affect distribution of cations in the red cell and induce a loss of phospholipid asymmetry, and if the aminophospholipid translocase is inactive, RBCs will continue to expose PS. This loss of phospholipid asymmetry in the RBC membrane

could lead to abnormal membrane function and subsequent recognition and removal of the red cells from the peripheral circulation (*26*). Loss of phospholipid asymmetry is observed in the early stages of programmed cell death or apoptosis (*27*). Although strictly speaking the red cell does not undergo apoptosis, it does have a limited lifespan and is efficiently removed from the circulation. Since aged RBCs show an increased PS exposure, recognition of PS might play a role in this process (*26*). Therefore, we suggest that phosphoinositide metabolism and calcium levels could be some of the underlying reasons for the removal of all cells that expose PS.

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