Calcium Induces Phospholipid Redistribution and Microvesicle Release in Human Erythrocyte Membranes by Independent Pathways†

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ABSTRACT: The increase in intracellular Ca^{2+} concentration in erythrocytes and platelets results in simultaneous phospholipid scrambling and microvesicle shedding. Microvesicle formation involves membrane fusion events which were proposed either to be tightly linked to phospholipid transversal redistribution or to occur by a separate mechanism. We report here that in erythrocytes incubated in high K^+ medium, or in resealed ghosts, phospholipid scrambling can be fully induced by intracellular Ca^{2+} without microvesicle formation. Furthermore, in ghosts resealed in the presence of spermine, intracellular Ca^{2+}, at low concentration, was able to induce microvesicles, whereas scrambling was drastically inhibited. Surprisingly, in spermine-containing ghosts prepared from erythrocytes of a patient with a bleeding disorder, due to a lack of Ca^{2+}-induced phospholipid scrambling and vesicle shedding (characterized as a Scott syndrome), Ca^{2+} also promoted microvesicle release. Data show that phospholipid scrambling and microvesicle production, although closely regulated, proceed by independent pathways.

Microvesicle release from plasma membrane plays an important role in the blood cell homeostasis (*1*, *2*) and involves fusion events (*3*, *4*). Microvesicle release is generally accompanied by the loss of transversal phospholipid asymmetry leading to externalization of phosphatidylserine (PS).[1] Both processes have been extensively studied in erythrocytes and occur in a number of circumstances, including platelet activation and apoptosis. PS exposure may serve as a signal for blood cell elimination by reticuloendothelial system (*5*−*7*) or, in activated platelets, plays an important role in blood clotting by creating a procoagulant surface (*7*). In erythrocytes, microvesicle release and phospholipid scrambling occur during cell aging (*8*) and in disease states (*9*) or in in vitro conditions, such as prolonged ATP depletion (*10*), spectrin oxidation (*11*), intercalation of amphipaths such as dimyristoyl-phosphatidylcholine into the outer leaflet of the membrane bilayer (*12*), or increase in intracellular Ca^{2+} induced by an ionophore (*13*).

The mechanisms at the origin of phospholipid redistribution and microvesicle shedding and their inter-relationships are still a subject of debate. Several mechanisms are proposed to account for phospholipid scrambling: direct interactions of Ca^{2+} with anionic phospholipids (*14*, *15*) and particularly with phosphatidylinositol 4, 5-bisphosphate (PIP_2) (*16*, *17*), protein phosphorylation (*18*), and/or involvement of specific protein(s) referred to as "scramblase". A protein fraction, partially purified from erythrocyte or platelet membranes and reconstituted in liposomes, induces a limited phospholipid redistribution in the presence of Ca^{2+} (*19*, *20*). A similar protein fraction has been also found in erythrocytes from a patient with Scott syndrome, an inherited bleeding disorder characterized by a coagulation defect and a lack of Ca^{2+}-induced phospholipid scrambling. Surprisingly, this protein fraction possesses similar properties (Ca^{2+} and pH sensitivity) to induce phospholipid scrambling, as that prepared from normal erythrocytes (*21*). It was thus deduced that presumably other factors could inhibit Ca^{2+}-induced phospholipid scrambling in patient cells. As hypothesized in our previous study, these factors could be polycations (spermine) or spermine-like proteins (*17*). In a recent study, a 37 kDa protein, able to induce phospholipid scrambling, was cloned and a recombinant protein was expressed (*22*). Scrambling activity in cells could depend not only on the amount of the active protein present in the membranes, but

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[1] Abbreviations: PS, phosphatidylserine; PIP_2, phosphatidylinositol 4, 5-bisphosphate; BSA, bovine serum albumin; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; MLVs, multilamellar vesicles; ESR, electron spin resonance; FITC, fluorescein isothiocyanate; DMSO, dimethylsulfoxide; RPE, R phycoerythrin.

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also on a regulatory cofactor enhancing phospholipid scrambling (*22*). A possible candidate is PIP_2, as we have previously proposed (*16*, *17*).

Membrane phospholipid asymmetry in microvesicles was reported either to be lost or to be maintained, depending on the experimental conditions. Using a prothrombinase assay after treatment of erythrocytes or platelets with Ca^{2+} and ionophore, PS was found to be externally exposed in vesicles as well as in remnant cells (*23*). However, in other studies using a phospholipase A_2 to assess phospholipid asymmetry, no change was found in vesicles shed from erythrocytes (*24*).

Zwaal et al. (*25*) have proposed that Ca^{2+}-induced scrambling of membrane phospholipids either resulted from, or triggered, the formation of inverted micelles at sites where membrane fusion allowed the release of the vesicles. These mechanisms were deduced from an observation made in the platelets and erythrocytes from a patient with Scott syndrome, which do not exhibit vesicle shedding and full phospholipid scrambling when activated by Ca^{2+} (*26*, *27*). However, in platelets, phospholipid randomization induced by Ca^{2+} could occur in the absence of vesicle release and only this latter process required protease activation and fusion processes (*28*−*31*). In erythrocytes, inhibition of Ca^{2+}-induced K^+ efflux reduced the formation of microvesicles (*13*), without affecting the phospholipid scrambling (*32*, *33*), indicating that scrambling was not dependent on microvesicle release. The relationship between phospholipid scrambling and vesicle formation (or cell fragmentation) was also questioned in apoptotic cells (*34*).

In the present study, we show that scrambling and microvesicle formation are independent events in erythrocyte ghosts, prepared either from a normal individual or from a patient with a Scott-like syndrome, recently characterized in a French family (*35*). The features of this disease are essentially the same as those of the previously reported Scott syndrome (*26*, *27*): lack of Ca^{2+}-induced phospholipid scrambling and microvesicle formation in platelets, lymphocytes, and erythrocytes. In normal ghosts, phospholipid scrambling was maximal with only minimal microvesicle release. Conversely, when the ghosts contained spermine, microvesicles were shed in the virtual absence of phospholipid redistribution. Unlike the microvesicles released from intact erythrocytes after Ca^{2+}/ionophore treatment, those produced by Ca^{2+} in spermine-containing ghosts did not exhibit external PS exposure. In addition, microvesicle shedding could be also induced without scrambling, in ghosts prepared from patient erythrocytes and containing spermine.

MATERIALS AND METHODS

Materials. Bovine serum albumin (fatty acid free) (BSA) (A 7511), spermine (S 4264), and calcium ionophore A23187 (C 7522) were obtained from Sigma (France), annexin V-FITC from Boehringer Mannheim (France), and R phycoerythrin (RPE)-conjugated antihuman glycophorin A monoclonal antibody and RPE-conjugated irrelevant antibody from Dako (Denmark). Spin-labeled phospholipids with a short (C5) βchain bearing a nitroxide probe, phosphatidylcholine (PC*), sphingomyelin (SM*), phosphatidylethanolamine (PE*), and (PS*), were synthesized as previously described (*36*).

Erythrocytes and Resealed Ghosts. Blood was withdrawn from healthy volunteers, heparinized, and centrifuged (1300*g*, 10 min, 4 °C). The packed erythrocytes were washed three times and resuspended in Na medium or K medium (see below). To prepare the sealed ghosts, the cells were washed in potassium phosphate (KP) medium [137 mM KCl, 2.7 mM NaCl, 10 mM K_2HPO_4, 8.5 mM KH_2PO_4 (pH 7.4)] and lysed, at 1:20 volume ratio of cells to buffer, by incubation at 4 °C in KP medium diluted to 1:5 and supplemented with 1 mM $MgCl_2$ and 0.1 mM EGTA (*14*, *17*). These conditions allow ghosts to retain the native asymmetric transbilayer distribution of phospholipids (*14*, *37*). When required, spermine was added to the lysis buffer and the pH was readjusted to 7.4. After 60 min, isotonicity was restored by adding an appropriate volume of 5-fold concentrated KP medium, and ghosts were resealed by warming to 37 °C for 60 min. Resealed ghosts were isolated by centrifugation (1300*g*, 15 min, 4 °C) and washed twice in Na medium without spermine (see below). The integrity of the sealed membranes (ghosts, remnants, or microvesicles) was checked in independent experiments, by measuring the release of hemoglobin during a 90 min incubation at 37 °C of control or spermine-loaded ghosts, in the presence of ionophore and either 1 mM $CaCl_2$ or 100 μM EGTA (see below). Hemoglobin leakage, assessed by measuring the absorbance in the supernatants ($\lambda = 540$ nm), was less than 2% of the initial ghost hemoglobin content, regardless of the presence of spermine or of $CaCl_2$ into the ghosts.

Blood samples from a 71-year-old patient suffering from an inherited bleeding disorder were obtained after informed consent. This disorder has been shown to be similar to the Scott syndrome (*35*). Cells and ghosts were prepared as described above.

Transbilayer Redistribution of Spin-Labeled PC or PS* and Microvesicle Shedding in Erythrocytes and Resealed Ghosts.* Erythrocytes or resealed ghosts were resuspended at a 30% hematocrit (corresponding to a membrane phospholipid concentration of 1.2 mM), either in Na medium [145 mM NaCl, 10 mM Hepes (pH 7.4)] or in K medium [90 mM KCl, 55 mM NaCl, 10 mM Hepes (pH 7.4)]. Suspensions were mixed at 20 °C with ionophore A23187 (8 μM final concentration), dissolved in dimethyl sulfoxide (DMSO), and with spin-labeled phospholipids, either PC* or PS* (0.8% of total phospholipid), solubilized in the incubation medium. Measurement of PC* redistribution was started after 1 min of incubation at room temperature. Measurement of PS* redistribution was started after internalization of the probe in the inner leaflet of the intact erythrocytes, by a preincubation at 37 °C for 60 min. The suspensions were transferred to 37 °C (time 0 of the kinetics) and incubated in the presence of $CaCl_2$ or EGTA. As cells or ghosts can shed microvesicles, inward PC* or outward PS* translocation rates were determined from the amount of probes remaining in the remnant cells, after removal of the vesicles and of BSA-extractable PC* or PS* by centrifugation. At given time intervals, aliquots (70 μL) were withdrawn and mixed at 4 °C, with 50 μL of incubation medium with or without 4% BSA. After 1 min, 700 μL of cold medium was added and the suspension was centrifuged. The conditions of centrifugation (30 s, 8000*g*) were strictly reproduced in all of the experiments and led to the same criteria of separation (size, density) of microvesicles from remnants as those described by Allan et al. (*9*, *13*). When observed under a light microscope, normal or pathologic ghosts exhibited the same

spiculated shape, due to ATP dilution during ghost preparation. Their morphology was not modified either after incubation with ionophore and 20 μM Ca^{2+} or after spermine incorporation. Microvesicles were observed in spermine-loaded ghosts only after Ca^{2+} treatment. The size of the microvesicles was about 500 nm, consistent with previous observations (*13*). Cross contamination between remnant and microvesicle populations was estimated to be less than 5%. The supernatants, containing the microvesicles, were removed and extracted with acidic $CHCl_3/CH_3OH$ for determination of the total phospholipid content by phosphorus assay (*38*). The cell pellets were lysed in 30 μL of water, and the amount of associated PC* or PS* was assayed, after reoxidation by ferricyanide, by electron spin resonance (ESR) spectroscopy using a Bruker ER 200D spectrometer (*36*). The intensity of the central peak of the signal measured in the BSA-treated pellets compared to that of the corresponding untreated pellets gave the percentage of spin label in the inner monolayer. The signal intensity measured at each time in the BSA-untreated pellets, compared to that at time 0, allowed the estimation of the membrane loss corresponding to the release of microvesicles. The values obtained by this latter method were the same as those measured by phospholipid assay in the supernatants. The similarity between the values obtained by both methods also indicates that the spin-labeled PC was not selectively localized in the vesiculated membranes, but homogeneously distributed between microvesicle and remnant membranes.

Flow Cytometry. The formation of microvesicles induced by Ca^{2+} ionophore treatment in ghosts was initially identified by light scatter, and further distinguished from electronic noise and debris by the RPE-conjugated anti-glycophorin A antibody (nonspecific binding was tested with RPE-conjugated irrelevant antibody). PS exposure was detected by the specific binding of FITC-conjugated annexin V (annexin V-FITC).

Erythrocyte ghosts (control or loaded with 2 mM spermine) were prepared as described above. Erythrocytes or ghosts were resuspended in Na medium and incubated at 37 °C with 8 μM A23187. The reaction was started by the addition of either 1 mM $CaCl_2$ or 100 μM EGTA and incubation of the samples at 37 °C for 60 min. The reaction was stopped by transferring 20 μL aliquots in a medium containing 2% BSA, to extract Ca^{2+} ionophore. After 30 min at room temperature, 10 μL aliquots (5×10^6 erythrocytes or resealed ghosts) were incubated for 10 min in the dark with saturating concentrations of annexin V-FITC (4 μg/mL) and RPE-conjugated anti-glycophorin A antibody, in the presence of 2 mM Ca^{2+}. Immediately after incubation, samples were diluted by adding 450 μL of Na medium containing 2 mM Ca^{2+}. Samples were analyzed using a Becton Dickinson FACSort flow cytometer (Becton Dickinson, France). In each sample run, at least 10 000 events were acquired.

RESULTS

Ca^{2+} Can Induce Phospholipid Scrambling independently of Microvesicle Shedding. Erythrocytes, suspended in Na or K medium and loaded with the PC* probe, were incubated in the presence of A23187 and either 1 mM EGTA or 1 mM $CaCl_2$. The extent of PC redistributed into the internal membrane leaflet was measured in BSA-extracted pellets. After 90 min without $CaCl_2$, 10−15% of the probe was internalized, in agreement with the known asymmetric distribution of endogenous PC. In the presence of $CaCl_2$, PC* was fully randomized (scrambling) as reflected by the plateau value around 50% reached by internal PC* (data not shown). Figure 1 shows that the extent of Ca^{2+}-induced redistribution (calculated as the difference between the values with and without $CaCl_2$) was not affected by the cationic composition of the medium (37% ± 6% and 40% ± 2% in Na and K medium, respectively). However, microvesicle release, expressed as the percentage of cell phospholipids recovered in the supernatants, was significantly reduced in K medium when compared to Na medium (10% ± 2% and 35% ± 3%, respectively, $p < 0.01$). In ghosts resealed on their cytosol diluted to 1/20, ionophore treatment in Na medium containing only 20 μM $CaCl_2$ induced PC* scrambling (30% ± 4%), but a low percentage of released microvesicles (less than 10%).

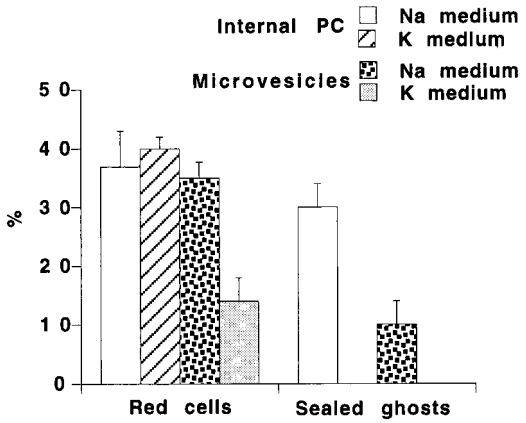

FIGURE 1: Ca^{2+}-induced redistribution of PC* and microvesicle production can occur independently. Erythrocytes suspended in Na medium or K medium were loaded with the PC* probe and incubated with A23187 and either 1 mM EGTA or 1 mM $CaCl_2$. After 90 min of incubation, cell suspensions were treated with BSA, diluted with ice-cold Na medium or K medium, and centrifuged to collect the microvesicles. Internal PC* was estimated by measuring the ESR signal in the pellets. The percentage of the probe redistributed by Ca^{2+} was calculated from the difference between the percentage of PC* present in the inner leaflet with and without Ca^{2+}. The amount of microvesicles, recovered from the supernatant and estimated by phospholipid assay, is expressed as a percentage of the total phospholipids in the initial cell suspension. Ghosts were prepared by hypotonic lysis, sealed, and resuspended in Na medium. They were loaded with the PC* probe, and incubated with A23187 and either 100 μM EGTA or 20 μM $CaCl_2$. The percentage of the probe redistributed by Ca^{2+} was obtained as described for the erythrocytes. Data shown are means ±SE of 3 experiments.

Spermine Inhibits Ca^{2+}-Induced Phospholipid Scrambling but Promotes Microvesicle Shedding in Resealed Ghosts. Ghosts were resealed either without or with 2 mM spermine and incubated with A23187 and 500 μM $CaCl_2$. Figure 2 shows that inclusion of spermine induced a 2-fold increase in the release of microvesicles. In parallel, spermine reduced the Ca^{2+}-induced PC* redistribution measured in the remnant ghosts, as previously reported (*14*, *17*). The same experiments were performed at different concentrations of $CaCl_2$ and of spermine (Figure 3). At 20 μM $CaCl_2$, spermine effects on PC* scrambling and microvesicle release were inversely correlated as a function of the concentration of spermine (Figure 3A). This negative correlation was less

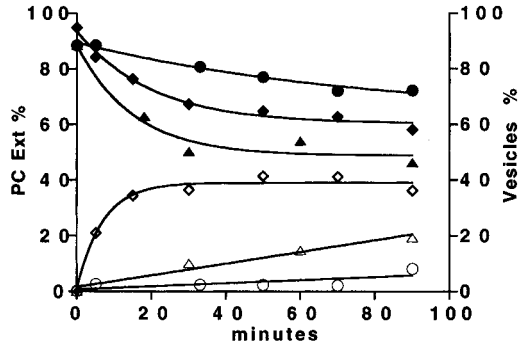

FIGURE 2: Spermine inhibits Ca^{2+}-induced inward redistribution of PC* but favors microvesicle release in sealed ghosts. Ghosts were prepared by hypotonic lysis in diluted KP medium without (circles, triangles) or with 2 mM spermine (diamonds), sealed, and resuspended in Na medium. They were loaded with the PC* probe and incubated with A23187 and either 100 μM EGTA (circles) or 500 μM $CaCl_2$ (triangles, diamonds). Internal PC* was estimated by measuring the ESR signal in the BSA-extracted pellets. The percentage of the probe in the outer leaflet (closed symbols) was calculated from the difference to 100%. Microvesicle release (open symbols) is expressed as a percentage of the total phospholipids in the initial cell suspension. Data shown are from 1 experiment representative of 3 experiments.

apparent at 500 μM (Figure 3B). At 1 mM $CaCl_2$, the inhibitory effect of spermine on PC* redistribution was suppressed, although its stimulating effect on the formation of microvesicles was maintained (Figure 3C).

Figure 4 shows the redistribution of endogenous PS on the outer membrane leaflet of ghosts, assessed from the binding of annexin V-FITC. Flow cytometry allows one to quantify simultaneously, from the size parameter, microvesicles and remnant ghosts, respectively, in lower and upper quadrants and, from the fluorescence intensity, the percentage of particles with or without externally exposed PS, in right and left quadrants, respectively. In control ghosts (Figure 4, A and B), 100 μM $CaCl_2$ induced PS redistribution without significant vesicle formation, whereas in ghosts resealed in the presence of spermine, PS exposure was inhibited in remnants, but the formation of microvesicles was drastically enhanced (Figure 4, C and D). Microvesicles and remnant ghosts or cells constituted clearly two different populations as shown from the distribution of the size parameter FSC-H (ordinate) (Figure 4, A−F). Surprisingly, the vesicles produced by ghosts containing spermine, in contrast to those obtained from ionophore-treated erythrocytes (Figure 4, E and F), were not fluorescent indicating that scrambling could be drastically inhibited even in microvesicles. However, the sensitivity of the cytometer is dependent on the number of photons emitted by each particle, and consequently could be dependent on the particle size. To test this possibility, multilamellar vesicles (MLVs) in a size range (0.1−1 μm) compatible with that of the microvesicles were prepared to contain various amounts of PS. At the annexin V-FITC concentration used, binding became detectable from a threshold of 6% PS in the phospholipid mixture used to form the MLVs (results not shown). In erythrocyte membranes, PS amounts to 16% of total phospholipids or 32% of the phospholipids in the inner leaflet, where it is confined. This method allows the detection of 6% of externally exposed PS in the microvesicle membrane, that is, 20% of the total PS pool. Conversely, the absence of a fluorescent signal indicates that PS externalization is

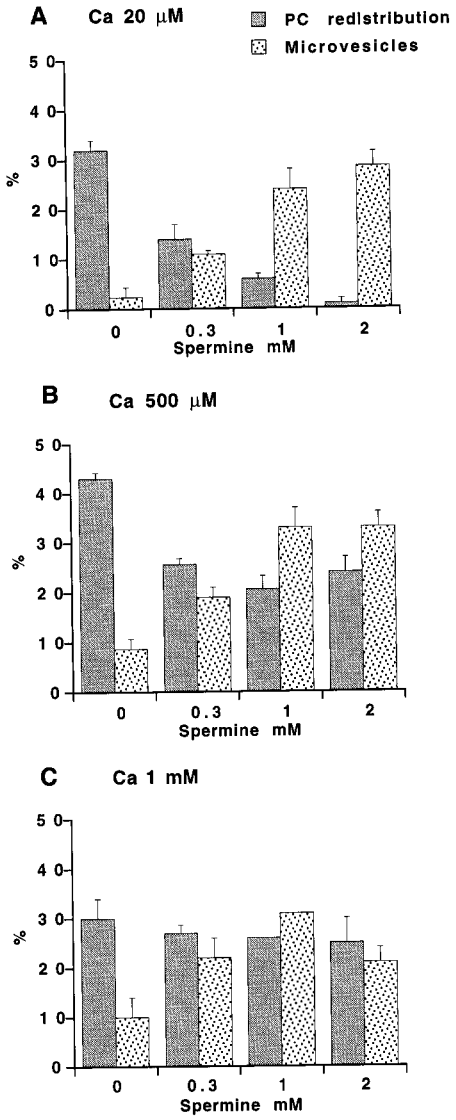

FIGURE 3: Spermine concentration dependence of the inhibition of inward redistribution of PC* and of stimulation of microvesicle release in sealed ghosts at various Ca^{2+} concentrations. Ghosts were prepared by hypotonic lysis with various concentrations of spermine, sealed, and resuspended in Na medium. They were loaded with the PC* probe and incubated for 90 min with A23187 and various concentrations of $CaCl_2$. Aliquots were treated with BSA, diluted with cold Na medium, and centrifuged to collect the microvesicles. Internal PC* was estimated by measuring the ESR signal in the pellets. Ca^{2+}-induced PC* redistribution was calculated as described in the legend of Figure 1. Microvesicle release is expressed as a percentage of the total phospholipids in the initial cell suspension. Values shown are means ±SE of 3−5 experiments or means of 2 experiments.

less than 20% of the total pool. Although ghost lysis was less than 2%, even when they were resealed on spermine (see Materials and Methods), the possibility was tested that a small leakage of spermine would have led to block binding of annexin V to exposed PS on the microvesicles and remnant ghosts. Intact red cells were incubated with A23187 and 1 mM $CaCl_2$ for 90 min, conditions which promote microvesicle formation with externally exposed PS (see Figure 4, E and F). Spermine (0.5−2 mM) was then added to samples of the suspension, which were further incubated for 30 min at 20 °C, before the addition of annexin V-FITC and flow cytometry analysis. The percentage of fluorescent microparticles and the maximal fluorescence intensity were

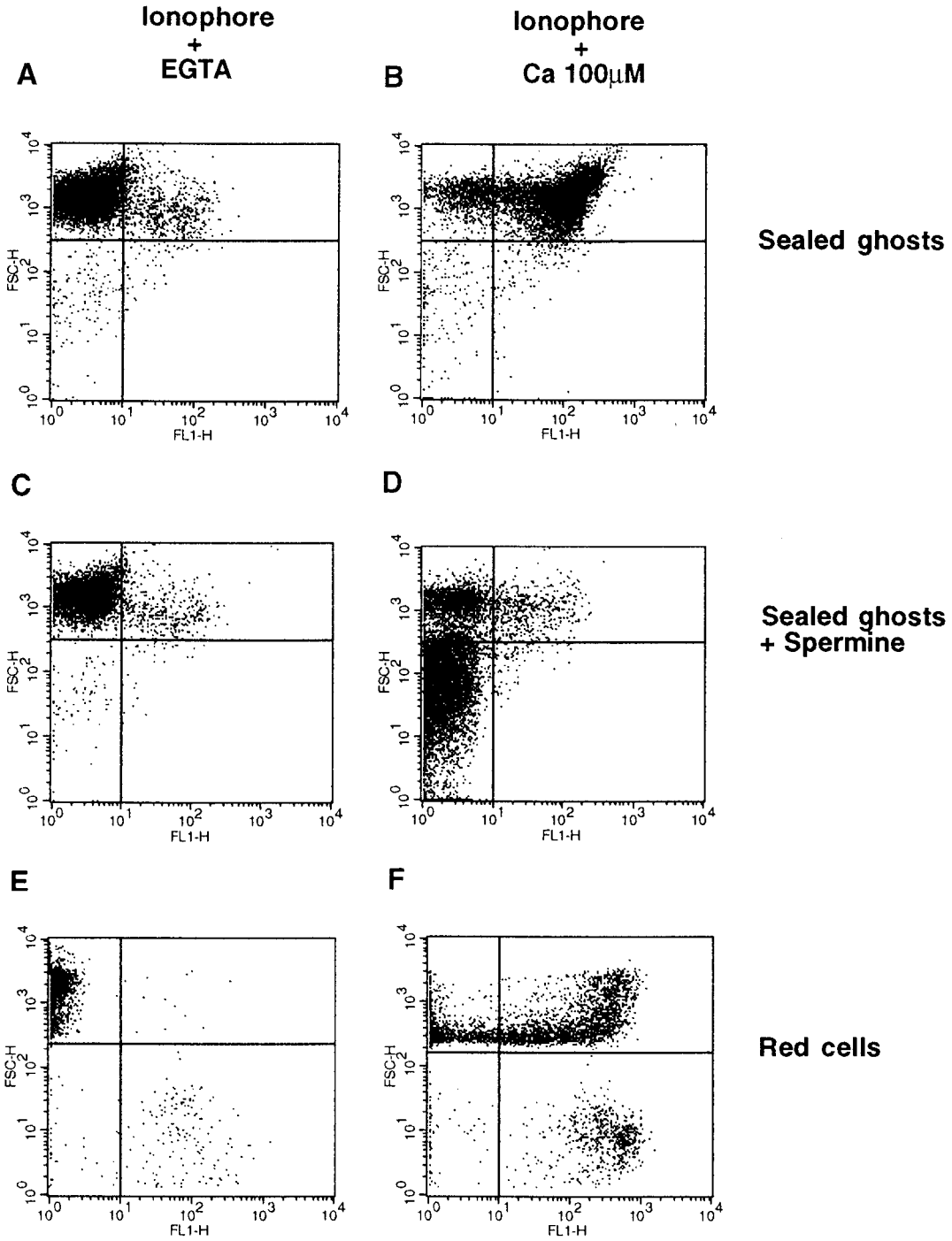

FIGURE 4: Ca^{2+}-induced redistribution of endogenous PS in microvesicles and in remnant ghosts measured by annexin V-FITC binding and flow cytometry analysis. Dot plot representations showing fluorescence (FL1-H) versus forward light scatter (FSC-H) in arbitrary units. Ghosts were prepared by hypotonic lysis in diluted KP medium without (A, B) or with (C, D) 2 mM spermine, sealed, and resuspended in Na medium. They were incubated for 90 min with A23187 and either 100 μM EGTA (A, C) or 100 μM $CaCl_2$ (B, D). Erythrocytes, washed in Na medium, were incubated for 90 min with A23187 and either 100 μM EGTA (E) or 500 μM $CaCl_2$ (F). After extraction of the ionophore with BSA, annexin V-FITC and RPE-conjugated anti-glycophorin A antibody were added to an aliquot of ghosts in the presence of 2 mM $CaCl_2$ and the suspension (6×10^8 cells/mL) was analyzed by FACS. The same treatment was used for erythrocytes (E and F) but without ionophore extraction. Data shown are from 1 experiment representative of 5−10 experiments.

not affected by the presence of spermine in the incubation medium, showing that spermine did not prevent annexin V binding.

The effects of different concentrations of $CaCl_2$ on phospholipid redistribution and microvesicle release in control and in spermine-containing ghosts were determined by different methods. In Figure 5, A and B, PC redistribution was measured using a spin-labeled phospholipid analogue and production of microvesicles by phospholipid assay. In Figure 5, C and D, flow cytometry analysis was used to estimate endogenous PS redistribution and microvesicle release. ESR studies allow one to measure the mean value of the extent of the spin-labeled PC redistributed in the inner leaflet of the remnant cells. FACS analysis of annexin V-FITC labeled cells provides the percentage of cells which have externalized endogenous PS. Although the parameters

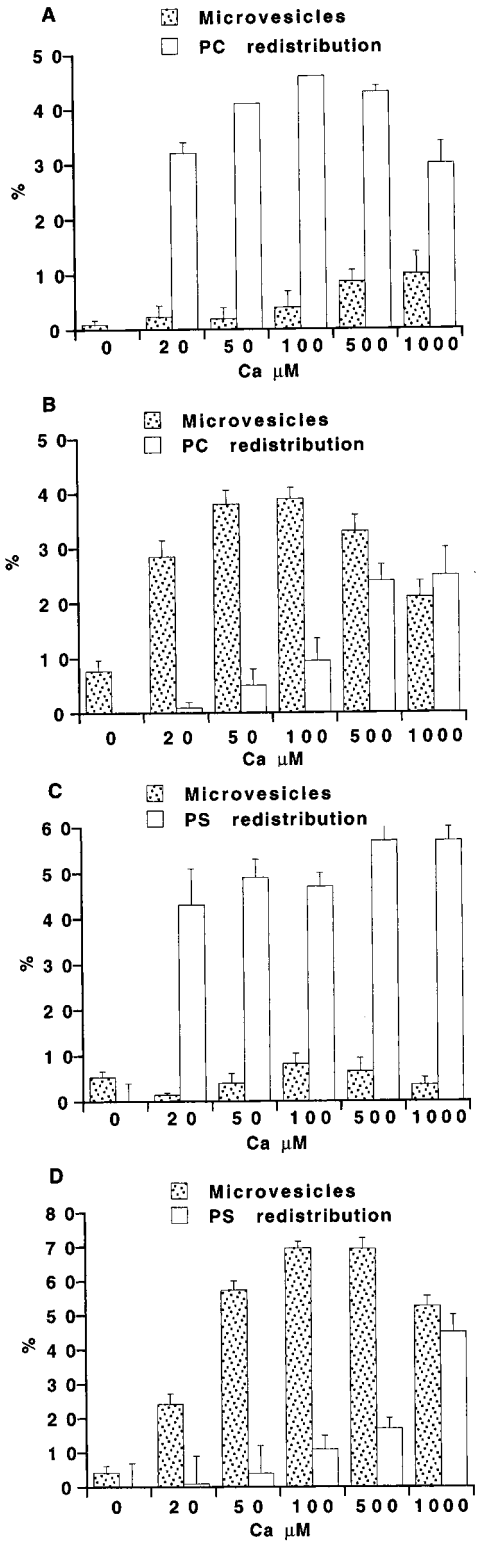

FIGURE 5: Ca^{2+} concentration dependence of phospholipid redistribution and microvesicle production in spermine-containing ghosts. Comparison of the results obtained by two methods. Ghosts were prepared by hypotonic lysis in diluted KP medium without (A, C) or with (B, D) 2 mM spermine, sealed, and resuspended in Na medium. They were incubated for 90 min with A23187 and various concentrations of $CaCl_2$. In A and B, PC* redistribution was measured by ESR in remnant ghosts, and microvesicle release by phospholipid assay. In C and D, annexin V-FITC was added to an aliquot of ghosts and the suspension (6×10^8 cells/mL) was analyzed by FACS as described in Materials and Methods. Data shown are means ±SE of 5−10 values obtained in independent experiments.

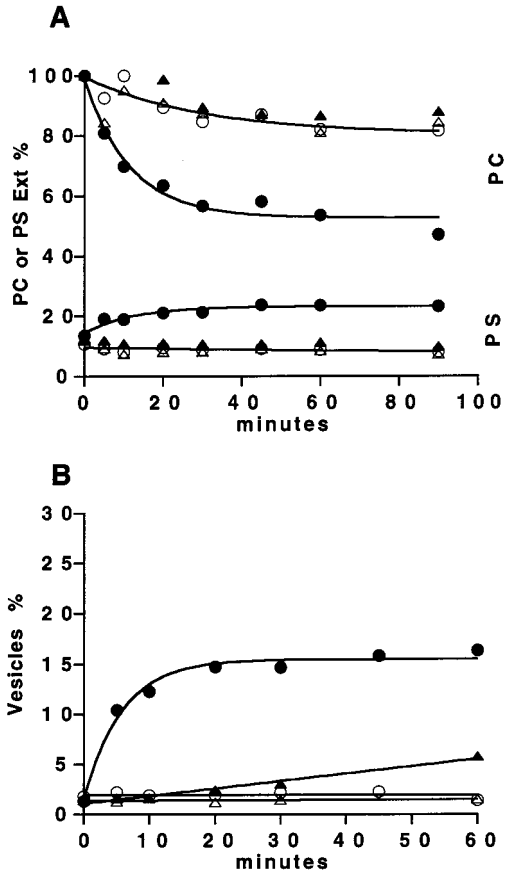

FIGURE 6: Ca^{2+}-induced inward redistribution of PC* and outward redistribution of PS* (A) and microvesicle release (B) in control and patient erythrocytes. Erythrocytes from a control subject (circles) or from a patient with a Scott-like syndrome (triangles) were suspended in Na medium and loaded for 1 min with the PC* probe or for 60 min with the PS* probe. After addition of A23187 and either 100 μM EGTA (open symbols) or 1 mM $CaCl_2$ (closed symbols), suspensions were transferred to 37 °C (time 0). At different time intervals, aliquots were extracted with BSA and centrifuged. The percentage of the probe in the outer leaflet was determined by measuring the ESR signal in BSA supernatants. Microvesicle release is expressed as a percent of the total phospholipids in the initial cell suspension.

measured by the two methods were not the same, they conveyed convergent information. Similarly, phospholipid assays in the supernatant of centrifuged suspensions permit the quantification of the amount of microvesicles regardless of the number shed per ghost, whereas the number of microvesicles, expressed as the percentage of total particles and deduced from gated forward light scatter, will depend on the number of particles shed per ghost. However, the data obtained by the two methods, although quantitatively different, led to the same conclusions. In control ghosts (Figure 5, A and C), $CaCl_2$ at concentrations up to 1 mM was unable to induce massive vesicle shedding, whereas PC and PS redistribution was maximal at 20 μM $CaCl_2$. By contrast, in ghosts containing 2 mM spermine (Figure 5, B and D), PC* and PS* redistribution was drastically inhibited at low Ca^{2+} concentration, but microvesicles were released at a $CaCl_2$ concentration as low as 20 μM.

Spermine Promotes Ca^{2+}-Induced Microvesicle Shedding in Ghosts Prepared from the Erythrocytes of a Patient with a Scott-Like Syndrome. In contrast to normal erythrocytes, there was almost no Ca^{2+}-induced PC and PS redistribution

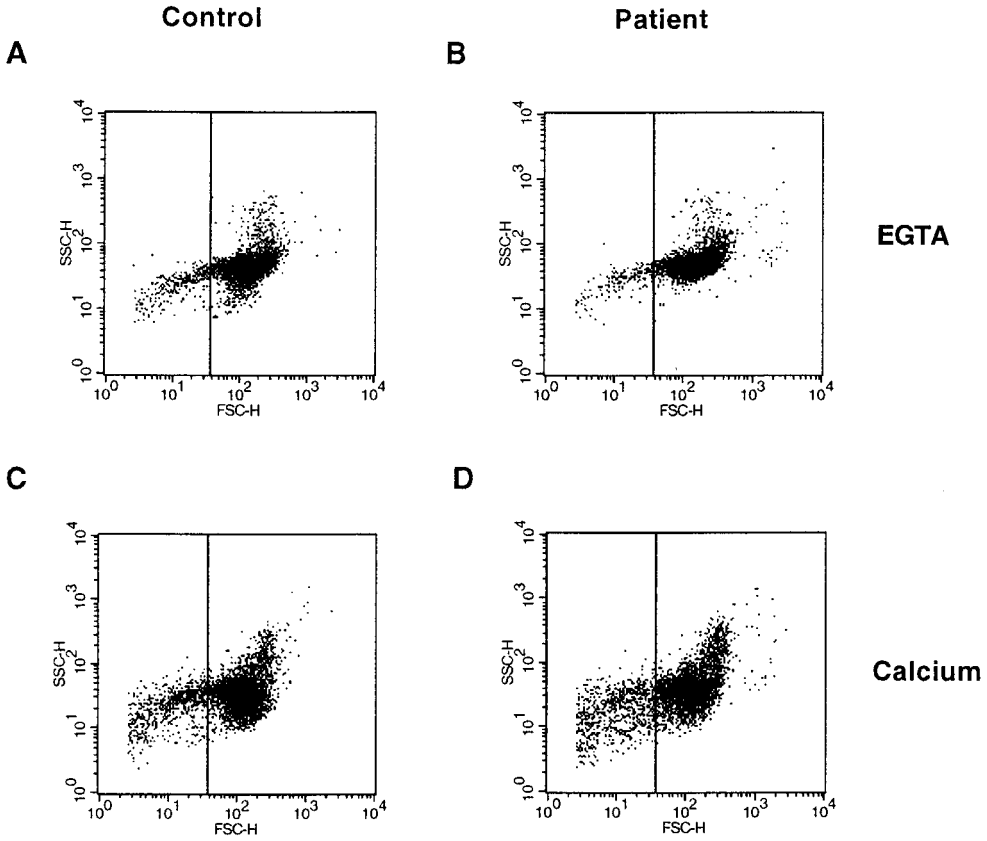

FIGURE 7: Effect of spermine on Ca^{2+}-induced microvesicle shedding in control and patient ghosts. Dot plot representations showing right-angle light scatter (SSC-H) versus forward light scatter (FSC-H) (size parameter), in arbitrary units. Ghosts were prepared from erythrocytes of a normal subject or of a patient with a Scott-like syndrome, sealed in the presence of 2 mM spermine, suspended in Na medium, and incubated for 60 min with A23187 and either 100 μM EGTA (A, B) or 1 mM $CaCl_2$ (C, D). These patterns correspond to a single analysis representative of results obtained in duplicate in the same experiment.

in the erythrocytes from a patient with a Scott-like syndrome, as measured with the spin-labeled probes (Figure 6A) and by the binding of ^{125}I-annexin V, which was 100-fold lower than in normal erythrocytes (D. Geldwerth, personnal communication). Likewise, microvesicle production, measured by phospholipid assay, which amounted to 15% of the membrane phospholipids in control erythrocytes, was drastically reduced in patient cells (Figure 6B).

As in control ghosts without spermine (Figure 4), Ca^{2+}-induced formation of microvesicles, assayed by flow cytometry, was very low in the patient ghosts (2.5%, data not shown). Figure 7 shows dot plots of forward-angle versus right-angle light scatter obtained from ghosts prepared either from control or patient erythrocytes and resealed in the presence of spermine. They both ranged in the same forward-angle scatter gate (Figure 7, A and B). Ca^{2+} treatment resulted in a significant microparticle formation both in control and in patient ghosts, amounting to about 20% of the total particles (remnant ghosts + microparticles) (Figure 7, C and D). Therefore, the defect in Ca^{2+}-induced microvesicle release in the patient ghosts is not related to the lack of phospholipid scrambling, since it can be overcome by the presence of spermine.

DISCUSSION

Our results show that Ca^{2+}-induced phospholipid transmembrane scrambling and microvesicle shedding are independent processes.

In agreement with previous data obtained in platelets (*28*−*31*), we have found that in erythrocytes or ghosts, under particular conditions, Ca^{2+} could induce phospholipid scrambling without, or with only minimal, release of microvesicles. In one of these situations (erythrocytes incubated in high K^+ medium), the rate of lipid diffusion was even higher than that usually observed when microvesicles were produced (in high Na^+ medium; results not shown). Conversely, under conditions of drastic inhibition of phospholipid scrambling (ghosts resealed on spermine), microvesicles were shed to a significant extent after Ca^{2+} addition. Interestingly, whereas the dose-dependent inhibition of phospholipid scrambling by spermine was highly dependent on the Ca^{2+} concentration, the dose-dependent stimulation of microvesicle production was much less affected by the Ca^{2+} concentration (see Figures 3 and 5). Inhibition of scrambling by spermine was detected not only in the remnant cells, but also in the microvesicles produced from ghosts containing spermine, as shown by the lack of the binding of annexin V-FITC, which reflects the lack of PS exposure on the outer membrane leaflet (Figure 4D). The absence of PS detection in the microvesicles was not due to their small size, since annexin V-FITC binding was observed with the microvesicles produced after Ca^{2+}/ionophore treatment of erythrocytes (Figure 4E) or platelets, or with MLVs of the same size range as that of the microvesicles (results not shown). A possible leakage of spermine from the ghosts leading to block annexin V binding can be excluded since (1) ghost membrane

integrity was preserved during the incubation, as demonstrated by the absence of hemolysis, and (2) spermine added in ionophore-treated red cell suspensions did not prevent annexin V binding either to remnant cells or to microvesicles. Altogether these observations strongly argue against a coupling between scrambling and microvesicle formation. Further evidence was provided in the case of the patient ghosts, which constitutively lack both Ca^{2+}-induced phospholipid scrambling (*26*) and microvesicle release (our data), but became capable of shedding microvesicles when resealed in the presence of spermine.

The mechanisms at the origin of the effect of spermine remain hypothetical. Although the concentration of spermine (0.3−2 mM) used in these experiments is nonphysiologic—in red cells the spermine concentration is about 20 μM (*14*)—this polyamine is a valuable tool to investigate the role of polyphosphoinositides in various processes. Spermine is known to interact with negatively charged proteins and phospholipids, particularly with polyphosphoinositides (*39*−*42*). Spermine enhances the Ca^{2+} sensitivity and the rate of Ca^{2+}-induced fusion of PS/PE large unilamellar vesicles, specifically when they contain PIP_2 (*39*, *42*). Thus, although spermine has been described to stabilize the membrane and to protect ghosts from shear stress-induced membrane fragmentation (*43*), it could increase the Ca^{2+}-induced microvesiculisation by favoring the fusion step required in this process. The formation of microvesicles could depend on a yet unknown, cytosolic or loosely membrane-bound factor, whose concentration is reduced during ghost preparation. This factor could be replaced and/or recruited by spermine. In ghosts, which exhibit numerous spicules, spermine and/or spermine-like factor could facilitate Ca^{2+}-induced fusion of apposing membrane segments, by interacting with PIP_2, especially in the spicule areas. A similar role of PIP_2 in protein recruitment has been recently reported in budding and shedding of vesicles from the trans-Golgi network (*44*, *45*).

In conclusion, our data do not support the proposal that microvesicle formation is the result or the cause of a redistribution of phospholipids. They argue in favor of independent mechanisms governing both Ca^{2+}-induced phospholipid scrambling and microvesicle formation. Furthermore, microvesicle formation, and thus membrane fusion, without phospholipid redistribution in ghosts containing spermine, is consistent with the observation that the influenza virus fused with liposomes or erythrocytes without phospholipid redistribution (*46*, *47*). In any case, the underlying mechanism could involve the transient formation of a stalk, according to a previously described model of fusion (*48*, *49*). Hemifusion of the apposing (internal) leaflets would occur at first, prior to fusion. No contact would be formed between inner and outer leaflets, avoiding bilayer scrambling.

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