Transverse Redistribution of Phospholipids during Human Platelet Activation: Evidence for a Vectorial Outflux Specific to Aminophospholipids

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ABSTRACT: The redistribution kinetics of phospholipids during human platelet activation by calcium ionophore were investigated to determine the specificity of the observed phospholipid outflux [Bassé et al. (1993) *Biochemistry 32*, 2337]. (1) Two double-labeling experiments were performed with a combination of equal amounts of spin- and fluorescently-labeled phosphatidylserine and phosphatidylcholine. During A23187-induced activation, 50% of the internal phosphatidylserine analogs were rapidly ($t_{1/2}$ < 1 min) reexposed on the platelet surface while no reciprocal influx of the external phosphatidylcholine analogs was observed. (2) Treatment with chlorpromazine allowed the internalization of 20% of external spin-labeled sphingomyelin or spin-labeled phosphatidylcholine, without either inducing platelet activation or interfering with aminophospholipid translocase activity or with A23187-induced activation (dense granule secretion and vesicle shedding). During A23187-induced activation, none of the previously internalized choline head phospholipids were exposed externally, while spin-labeled phosphatidylserine outward movements were similar irrespective of whether platelets were pretreated or not pretreated with chlorpromazine. Our results demonstrated that during strong platelet activation (1) the PL excess in the internal leaflet, due to the probe addition, is not responsible for their outflux; (2) the rapid aminophospholipid outflux is definitely a vectorial outflux not counterbalanced by a rapid reciprocal influx of choline head phospholipids (i.e., not scrambling); and (3) the vectorial outflux is specific for aminophospholipids since previously internalized sphingomyelin and phosphatidylcholine did not move outward. This suggests that the specific vectorial outflux of aminophospholipids could be catalyzed by a "reverse aminophospholipid translocase" activity.

The asymmetric distribution of phospholipids (PLs)[1] in membranes has already been fully described (Op den Kamp, 1979). In platelet plasma membrane, choline head PLs [phosphatidylcholine (PC) and sphingomyelin (SM)] are preferentially located on the outer leaflet, while aminophospholipids (aminoPLs) [phosphatidylserine (PS) and phosphatidylethanolamine (PE)] are concentrated on the inner leaflet (Schick et al., 1976; Chap et al., 1977b). As in red blood cells (Seigneuret & Devaux, 1984; Zachowski et al., 1986), the asymmetric distribution in the platelet plasma membrane is maintained by continuous pumping of aminoPLs from the outer to the inner leaflet by ATP-dependent aminoPL translocase (Suné et al., 1987). During platelet activation, transbilayer movements of aminoPLs were observed to result in a PS- and PE-rich surface that favors the formation of coagulation factor complex (prothrombinasic converting activity); this is of considerable importance in the hemostatic process (Bevers et al., 1982, 1983; Zwaal et al., 1989, 1992). The amount of aminoPL redistributed during the activation process was about 50% of PS and PE; this redistribution occurring in less than 2 min (Bassé et al., 1993). According to our previous work (Bassé et al., 1993), this phenomenon is unlikely to consist of PL scrambling through the paltelet plasma membrane as no rapid PC or SM influx was detected during platelet activation. However, this point was contested in the literature. Some investigators proposed that aminoPLs outflux is accompanied by choline head phospholipid influx resulting in total scrambling of the platelet plasma membrane (Smeets et al., 1994). Furthermore, this short-lasting scrambling was described as a mechanism mediated by vesicle shedding during strong activation (Comfurius et al., 1990; Schroit & Zwaal, 1991). Recently, it was proposed that this scrambling could be mediated by a plasma membrane protein (scramblase) in addition to the vesicle shedding process (Zwaal et al., 1993; Galli & Bevers, 1994).

The aims of this study were (1) to investigate whether aminophospholipids redistribution induced by Ca^{2+}-ionophore activation is due to a generalized scrambling phenomenon (i.e., not mixing of all PLs species resulting in a symmetric distribution of PLs on both sides of the plasma membrane) or due to a short-lasting vectorial outflux of PS and PE accompanied by a long-lasting influx of PC and (2) to investigate whether this short-lasting vectorial outflux is specific to aminoPLs. Our results indicate that the exposure of aminoPLs on the platelet plasma membrane during calcium ionophore-induced activation is not a scrambling

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[1] Abbreviations: (0,2)PS, 1-palmitoyl-2-(4-doxylpentanoyl)glycerophosphoserine; (0,2)PC, 1-palmitoyl-2-(4-doxylpentanoyl)glycerophosphocholine; (0,2)SM, *N*-(4-doxylpentanoyl)-*trans*-sphingenyl-1-phosphocholine; (0,2) refers to the general nomenclature for spin-labeled chains, with 0 and 2 being, respectively, the number of methylene groups after and before the labeled position on the acyl chain; C6-NBD, *N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]aminohexanoic acid; NBD-PC, 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine; NBD-PS, 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphoserine; PL, phospholipid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; CPZ, chlorpromazine; (^{3}H)5-HT, (^{3}H)5-hydroxytryptamine.

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phenomenon but a short-lasting vectorial outflux specific to aminoPLs.

MATERIALS AND METHODS

Materials. Calcium ionophore A23187 and chlorpromazine were obtained from Sigma Chemical Co. (St. Louis, MO); (^{3}H)5-hydroxytryptamine [(^{3}H)5-HT] (8.8 Ci/mmol) was from Amersham (France); and *N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]aminohexanoic acid [C6-NBD], 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine [NBD-PC], and 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphoserine [NBD-PS] were from Avanti Polar-Lipids (USA). All other reagents were of the highest grade available commercially.

Platelets. Platelets were prepared at room temperature from 50 mL of fresh human blood (Centre Regional de Transfusion Sanguine, Montpellier, France) anticoagulated with 0.15 vol of ACD (85 mM trisodium citrate, 111 mM dextrose, 71 mM citric acid) using the erythrocyte cushion procedure as previously described (Valone et al., 1982). Platelet-rich plasma containing autologous erythrocytes (hematocrit 5%) was collected by centrifugation at 1200*g* for 4 min and then mixed with 0.5 vol of buffer A (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.1 mM $MgCl_2$, 0.55 mM dextrose, 5 mM Hepes, pH 6.3). If necessary, at this stage the suspension was incubated with 0.1 μCi/mL (^{3}H)5-HT for 30 min at 37 °C. After the cells were washed twice in buffer A (15 min at 1200*g*), contaminating erythrocytes were removed by centrifugation at 300*g* for 5 min. Platelets were then centrifuged at 1200*g* for 15 min and resuspended in buffer B (136 mM NaCl, 1 mM $CaCl_2$, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM dextrose, 10 mM Hepes, pH 7.4) leading to a final pH value at 7.2. Final counts after staining with Plaxan reagent (Sobioda SA, France) were 2.2×10^9 platelets/mL. Platelets were incubated for 30 min at 37 °C (pH 7) before the addition of 1 mM Ca^{2+} (final concentration) and testing. Platelets were activated at 37 °C by adding calcium ionophore A23187 (1 μM final concentration) in the presence of 1 mM external Ca^{2+} (pH 6.9). Platelet secretion was quantified according to Suné and Bienvenüe (1988), using (^{3}H)5-HT as a marker for dense granules. Radioactivity was measured in the supernatant of the platelet suspension (11000*g* for 3 min) and expressed as a percentage of total radioactivity in the suspension. Platelet vesiculation was monitored by quantifying the vesicles recovered in the supernatant of the centrifuged (11000*g* for 3 min) platelet suspension. Lipids were extracted (Folch et al., 1957), quantified (Barlett & Korn, 1959), and then expressed as a percentage of total PLs. Platelet lysis, as evaluated by lactate dehydrogenase activity (Sigma kit no. 500, St Louis, MO) in the supernatant of activated platelets, did not exceed 6%.

NBD-PLs Labeling and Fluorescence Measurement. NBD-labeled analogs of phosphatidylcholine (NBD-PC) and phosphatidylserine (NBD-PS) added (1−2% of endogenous PLs) to the platelet suspension from a concentrated solution in buffer B were incorporated into the outer plasma membrane leaflet in less than 2 min (data not shown). At different times, 70-μL aliquots of platelet suspension were mixed with 630 μL of a fatty-acid-free solution of bovine serum albumin in buffer A (or in water) when extractions were performed on resting (or activated) platelet suspensions (albumin, final concentration 1.8% w/v). The mixture was incubated at 25 °C for 3 min, allowing the albumin to trap NBD-PL probes remaining on the outer leaflet of the plasma membrane (conditions for albumin-mediated extraction were determined from kinetics at 25 °C where the amounts of extracted NBD-PL reached a plateau after 3-min incubation). After centrifugation for 5 min at 11000*g*, 600 μL of the supernatant [containing albumin-extracted NBD-PL and aqueous-soluble NBD-labeled short-chain acid (C6-NBD) from hydrolyzed NBD-PL] was stored at −20 °C. Simultaneously, samples used to determine the amount of C6-NBD in the platelet suspension were prepared by centrifuging 100-μL aliquots of platelet suspension for 5 min at 140000*g* on an air-driven ultracentrifuge (Beckman Airfuge). A total of 70 μL of the supernatant containing C6-NBD was mixed with 630 μL of a fatty-acid-free solution of bovine serum albumin in buffer A (albumin, final concentration 1.8% w/v) and then stored at −20 °C. An Aminco Bowman luminescence spectrometer was used to measure the fluorescence (excitation 470 nm; emission 534 nm) of 500-μL aliquots mixed with 1.5 mL of a Triton X100 solution (Triton X100, final concentration 0.65% w/v). The amount of C6-NBD was calculated from the supernatant of the 120000*g* centrifuged platelet suspension since C6-NBD is entirely aqueous soluble and not distributed in the membrane bilayers (data not shown). The amount of albumin-extractable NBD-PL was calculated by subtracting the C6-NBD fluorescence from the 11000*g* supernatant fluorescence (NBD-PL + C6-NBD fluorescence) of the albumin-extracted platelet suspension. The NBD-PL on the outer leaflet of the plasma membrane (NBD-PL extractable) was then expressed as a percentage of total NBD-PL remaining in the platelet suspension at the same time. The amount of hydrolyzed NBD-PL (C6-NBD) in the platelet suspension was expressed as a percentage of total NBD-PL added at time zero.

Spin-Labeling and ESR Experiments. Spin-labeled analogs of phosphatidylserine {1-palmitoyl-2-(4-doxylpentanoyl)-glycerophosphoserine [(0,2)P]}, phosphatidylcholine {1-palmitoyl-2-(4-doxylpentanoyl)glycerophosphocholine [(0,2)-PC]}, and sphingomyelin {*N*-(4-doxylpentanoyl)-*trans*-sphingenyl-1-phosphocholine [(0,2)SM]} were synthesized as described previously (Davoust et al., 1983). The redistribution of the paramagnetic probes previously incorporated in the outer leaflet of the plasma membrane was quantified as follows (Morrot et al., 1989; Bassé et al., 1992). Spin-labeled analogs were added (1−2% of endogenous PL) to the platelet suspension at time zero from a concentrated solution in buffer B. Spin-labeled PLs [(0,2)PLs] were incorporated into the outer plasma membrane leaflet in less than 1 min (Seigneuret & Devaux, 1984). At different times, 70-μL aliquots of platelet suspension were mixed with 25 μL of a fatty-acid-free solution of bovine serum albumin in buffer A (albumin, final concentration 0.8% w/v). The mixture was incubated on ice for 1 min to allow the albumin to trap probes remaining on the outer leaflet of the plasma membrane. After centrifugation for 2 min at 11000*g*, 50 μL of the supernatant (containing albumin-extracted spin labels and aqueous-soluble spin-labeled short-chain acid from the hydrolyzed probes) was added to 5 μL of 100 mM potassium ferricyanide to reoxidize any reduced spin labels. The signal of the reoxidized solution was measured on a Bruker ECS 106 ESR spectrometer. The ESR spectra were

then analyzed to determine the respective amounts of (0,2)-PLs and hydrolyzed probe. The amount of hydrolyzed probe was expressed as a percentage of total (0,2)PLs added at time zero, while the amount of (0,2)PLs present in the outer leaflet of the plasma membrane was expressed as a percentage of the nonhydrolyzed (0,2)PLs remaining in the platelet suspension [calculated by subtracting the amount of hydrolyzed probe (measured for each spectrum) from the total (0,2)PLs added at time zero].

Chlorpromazine Treatment. The chlorpromazine (CPZ) concentration used to treat the platelet suspension was defined according to the three following criteria: (1) it did not induce more than 15% release of dense granules, (2) it induced scrambling sufficient to internalize a significant amount of externally located (0,2)SM or (0,2)PC, and (3) it did not interfere with aminoPL translocase. A suitable CPZ concentration (150 μM) was selected from a concentration series ranging from 0 to 500 μM in steps of 50 μM. To prevent local concentration, the CPZ was added to the platelet suspension from a weakly concentrated solution in buffer B (600 μM). The extent of platelet lysis following incubation with CPZ (<5%) was determined from cytoplasmic lactate dehydrogenase activity in the supernatant of the platelet suspension.

RESULTS

Redistribution Kinetics of NBD- and Spin-Labeled Phospholipids during Platelet Activation. Is Renewed Exposure of PS Analogs a Scrambling Phenomenon? To support previous results indicating that the renewed exposure of aminoPLs is not a scrambling phenomenon in platelets, we performed double kinetic experiments using fluorescent (NBD) and spin-labeled [(0,2)] analogs: NBD-PC and (0,2)-PS (Figure 1A) or (0,2)PC and NBD-PS (Figure 1B). Double labeling enabled us (1) to monitor the redistribution kinetics of aminoPLs and choline head PLs at the same time in the same platelet suspension and (2) to suppress the initial imbalance in the plasma membrane due to one or another probe. PC analogs are incorporated entirely in the outer leaflet of the platelet plasma membrane within 2 min and remain so for at least 1.5 h without internalization (NBD-PC) (data not shown) or redistributed slowly ($t_{1/2}$ > 20 min) to the inner leaflet [(0,2)PC] (Bassé et al., 1992). PS analogs are incorporated in the outer leaflet of the plasma membrane within 2 min (data not shown), and their subsequent internalization to the inner leaflet is both rapid ($t_{1/2}$ < 5 min) and ATP-dependent (Seigneuret & Devaux, 1984; Zachowski et al., 1986; Suné et al., 1987; Connor et al., 1992). Before activation, more than 95% of PC analogs were located on the outer leaflet of the plasma membrane, and the PS analogs redistribution was equilibrated with 100% [(0,2)PS] and 90% (NBD-PS) on the inner leaflet. Less than 2 min after activation by A23187 in the presence of 1 mM calcium, 50% of the PS analogs were reexposed on the outer leaflet of the plasma membrane and 10% of the PC analogs were internalized (Figure 1A,B). This new distribution of PS and PC analogs remained stable for at least 40 min except for (0,2)-PC, which redistributed to the inner leaflet more rapidly than in unactivated platelets to level off as previously described (Bassé et al., 1993) at 60% on the outer leaflet 1 h after activation. As platelet activation by A23187 caused the concentration of intracellular calcium to increase, we also monitored hydrolysis of the probes (data not shown).

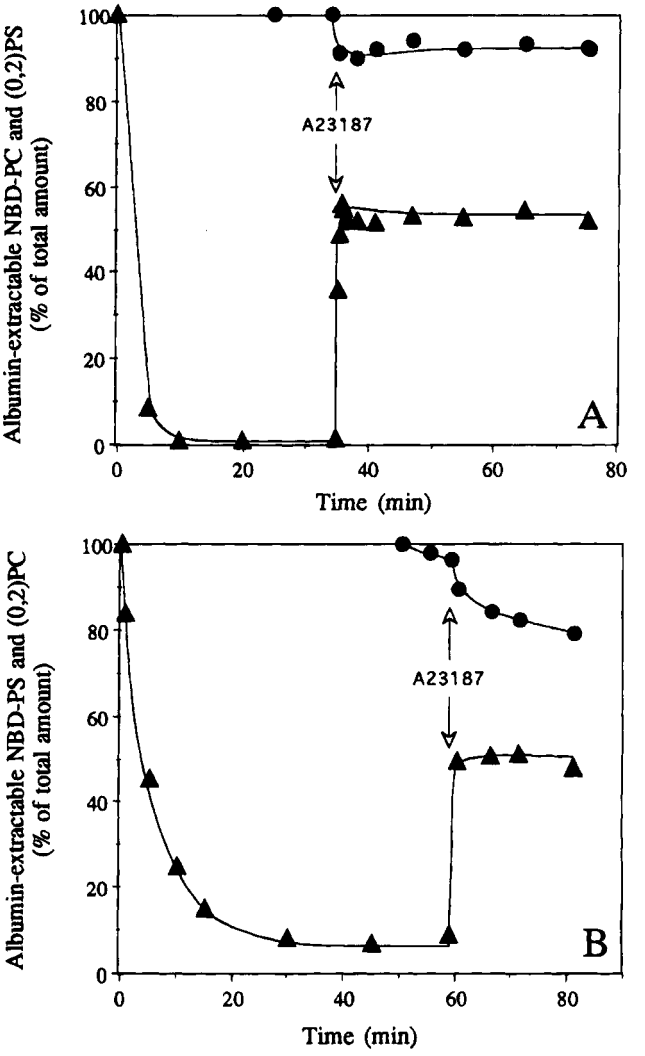

FIGURE 1: Double-labeling experiments: Redistribution kinetics of NBD- and spin-labeled analogs during platelet activation by A23187 at 37 °C. (0,2)PS (A) or NBD-PS (B) were added to platelet suspension (1−2% of endogenous phospholipids) and allowed to internalize at 37 °C for 35 and 60 min, respectively. An equal amount of NBD-PC (A) or (0,2)PC (B) was added to the same platelet suspension 10 min before activation allowing total incorporation of the probe into the platelet plasma membrane. Activation was then performed by the addition of A23187 (final concentration 1 μM) in the presence of 1 mM Ca^{2+} (arrows). Amounts of PS (▲) and PC (●) analogs recovered in the supernatant of the albumin-extracted platelet suspensions were quantified from fluorescence or ESR signals and expressed as a percentage of total NBD- or spin-labeled phospholipids remaining in the platelet suspension at the same time (for calculations, see Materials and Methods). The data shown were obtained in a single experiment. Two other experiments gave similar curves.

Redistribution of PS analogs to the plasma membrane inner leaflet of resting platelets was accompanied by hydrolysis: 10% of (0,2)PS by 35-min and 30% of NBD-PS by 60 min. NBD-PC and (0,2)PC were not affected by hydrolysis in the resting platelets. Following activation, the rate of hydrolysis of (0,2)PS increased and then leveled off after 40 min at 30% of the (0,2)PS added initially while NBD-PS hydrolysis remained at 30% of total probe added at time zero. NBD-PC and (0,2)PC hydrolysis increased smoothly following activation: 10% of NBD-PC by 40 min and 10% of (0,2)PC by 20 min respectively.

(^{3}H)5-HT Release and Vesicle Shedding during A23187 Activation of Chlorpromazine-Treated Platelets. As a control

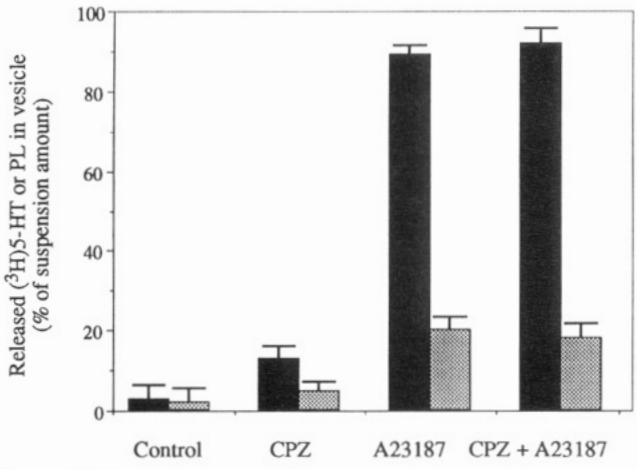

FIGURE 2: Vesiculation and (^{3}H)5-HT release after treatment with chlorpromazine and A23187 activation. Phospholipids in vesicles (gray) and released (^{3}H)5-HT (black) were measured in the supernatant of platelet suspension after various treatments. Untreated platelets (control), platelets incubated at 37 °C with 150 μM chlorpromazine for 20 min (CPZ), platelets 10 min after activation by 1 μM A23187 in the presence of 1 mM Ca^{2+} at 37 °C (A23187), and platelets incubated for 10 min with chlorpromazine at 37 °C and then activated for 10 min by 1 μM A23187 in the presence of 1 mM Ca^{2+} at 37 °C (CPZ + A23187). The data shown were obtained in a single experiment. Two other experiments gave similar curves.

of platelet activation, dense granule release and vesicle shedding were measured after platelets were treated by chlorpromazine (CPZ) and activated by A23187 in the presence of 1 mM calcium. Treatment with CPZ induced very weak (^{3}H)5-HT release (<15%) and vesicle shedding (<5%) and did not interfere with A23187-induced activation (Figure 2). In fact, 90% of the (^{3}H)5-HT previously incorporated into dense granules and 20% of total PLs in suspension were recovered in the supernatant of the platelet suspension following the release of dense granules and the shedding of vesicles induced by A23187 activation of CPZ-treated or untreated platelets.

Redistribution Kinetics of Previously Internalized (0,2)-PS, (0,2)SM, and (0,2)PC during A23187 Activation of Chlorpromazine-Treated Platelets. Is Renewed Exposure of (0,2)PS due to Specific Outflux? The redistribution kinetics of (0,2)PS in resting platelets indicated that (0,2)PS was entirely and rapidly ($t_{1/2}$ < 5 min) redistributed on the inner leaflet of the plasma membrane (Figure 3A). The asymmetrical distribution of (0,2)PS in the inner leaflet was not modified by CPZ treatment; this indicates that 150 μM CPZ did not interfere with aminoPL translocase and hence allowed (0,2)PS (externally located due to CPZ-induced scrambling) to be pumped back from the outer to the inner leaflet in less than 5 min (Rosso et al., 1988). Moreover, the redistribution kinetics of (0,2)PS following A23187-induced activation of CPZ-treated platelets were not modified in comparison with the situation following activation of untreated platelets [in both cases, 50% of initially internal (0,2)PS were reexposed within 2 min]. This new distribution remained stable for at least 20 min.

The redistribution kinetics of (0,2)SM in resting platelets not treated with CPZ indicated that (0,2)SM remained entirely located on the outer leaflet of the plasma membrane (Figure 3B). When resting platelets were treated with 150 μM CPZ, 20−25% of initially external (0,2)SM redistributed rapidly (10 min) to the internal leaflet of the plasma membrane. Following platelet activation by calcium ionophore A23187, 7−10% of externally located (0,2)SM were rapidly internalized, regardless of whether platelets were previously treated or not with CPZ (Figure 3B). This new distribution remained stable for at least 20 min.

The internalization of (0,2)PC in resting platelets not treated with CPZ was a slow mechanism ($t_{1/2}$ > 20 min) leveling off at 70−80% of probes on the outer leaflet after 1−1.5 h redistribution (Figure 3C) (Bassé et al., 1992). When resting platelets were treated wit 150 μM CPZ, 20−25% of initially external (0,2)PC redistributed to the inner leaflet of the platelet plasma membrane in 10 min (Figure 3D). Following A23187-induced activation, (0,2)PC redistribution kinetics accelerated slightly (Figure 3C,D), regardless of whether platelets were treated or not by CPZ, and leveled off at 60% of (0,2)PC on the outer leaflet 40 min after activation if CPZ-treated or 1 h after activation if not treated (Bassé et al., 1993). It should be noted that the rate of (0,2)-PC internalization after A23187-induced activation is slow ($t_{1/2} \approx$ 5−10 min) (Figure 3C,D) in comparison with the rate of (0,2)PS externalization ($t_{1/2}$ < 1 min) (Figure 3A).

Dashed curves (Figure 3B−D) were given for comparison and show the amounts of (0,2)PC and (0,2)SM that would be extractable after A23187 addition if these PLs should move in the same way as (0,2)PE. These data were drawn from Bassé et al. (1993), showing that about 50% of internally located (0,2)PE moved rapidly ($t_{1/2}$ < 1 min) to the external leaflet during A23187-induced activation, whatever the amount of previously internalized (0,2)PE. After this fast outflux, a slow influx occurred with the same rate for all phospholipid analogs studied.

Spin-labeled hydrolysis (not shown) in CPZ-treated and untreated platelets did not exceed 3% for (0,2)SM even after A23187 activation. (0,2)PC hydrolysis was lower than 5% in resting platelets and increased (10% in 20 min) when platelets were activated. (0,2)PS hydrolysis leveled off at 10−15% after complete internalization of the probe and leveled off at 25−30% 20 min after A23187 activation.

In conclusion, in resting platelets not treated with CPZ, (0,2)PS was entirely and rapidly internalized ($t_{1/2}$ < 5 min), (0,2)SM remained entirely on the outer leaflet, and (0,2)PC redistributed slowly ($t_{1/2}$ > 20 min) to the inner leaflet to level off at 70−80% on the outer leaflet 60−90 min after its addition to the platelet suspension. Following CPZ treatment, (0,2)PS remained internally located while 20−25% of spin-labeled choline head phospholipids were rapidly internalized. Finally, in less than 2 min after A23187-induced activation, 50% of (0,2)PS were very rapidly reexposed ($t_{1/2}$ < 1 min) on the outer leaflet. By contrast, only 7−10% of (0,2)SM were rapidly ($t_{1/2} \approx$ 1−2 min) internalized while (0,2)PC internalization was slightly accelerated ($t_{1/2} \approx$ 5−10 min) to level off at 60% on the outer leaflet 40−60 min after A23187 activation.

DISCUSSION

Blood platelets are implicated in the coagulation process, hemostasis, and thrombogenic diseases. The activation process has therefore been the subject of many investigations. Of the different agents used to induce platelet activation *in vitro* (ADP, collagen, thrombin, collagen plus thrombin, calcium ionophore), some (collagen plus thrombin, calcium ionophore) induce exposure of aminoPLs, PS, and PE on

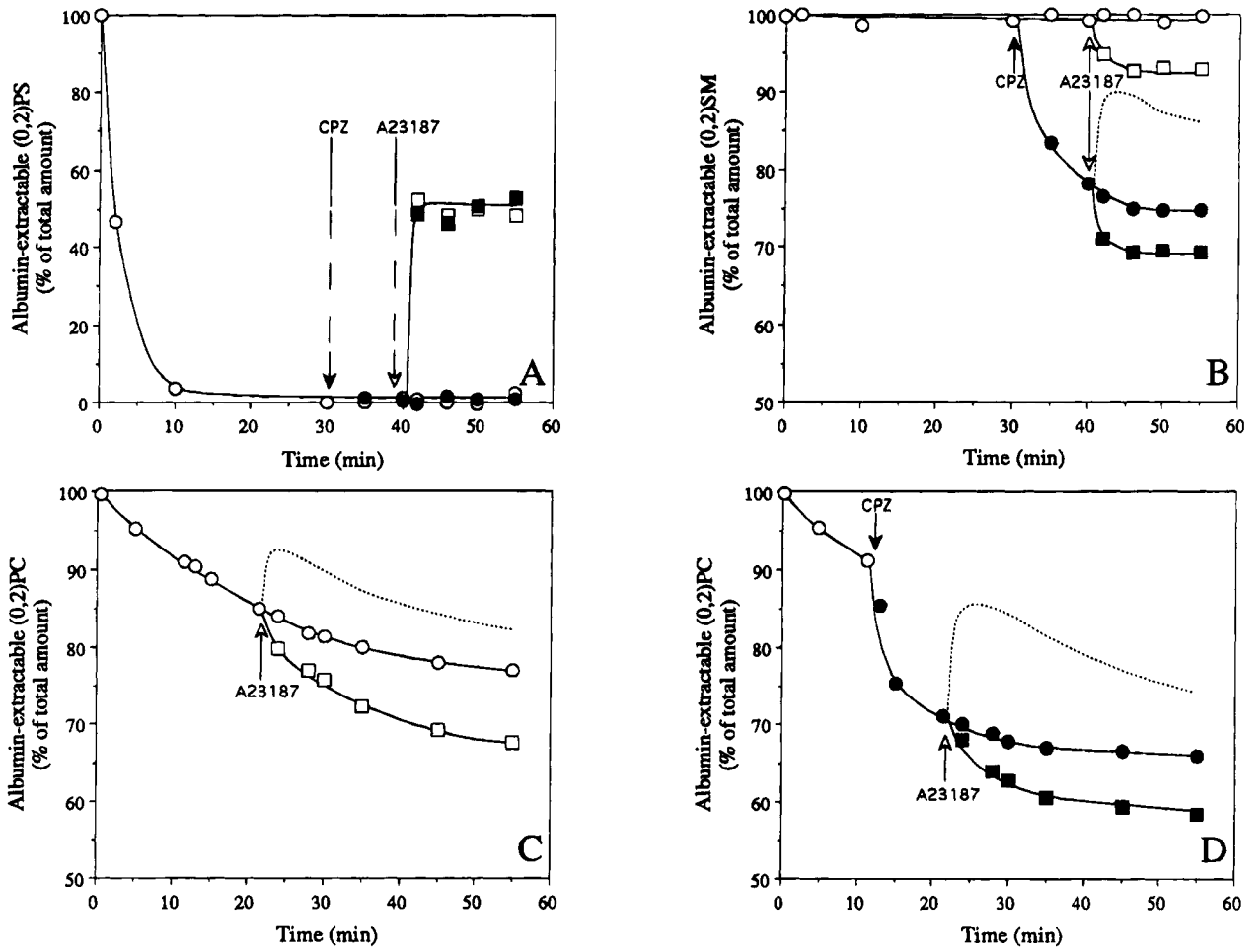

FIGURE 3: Redistribution kinetics of (0,2)PS, (0,2)SM and (0,2)PC after treatment with chlorpromazine and A23187-activation. (0,2)PS (A), (0,2)SM (B), or (0,2)PC (C and D) were added (1−2% of endogenous phospholipids) to the platelet suspension and allowed to distribute at 37 °C for 30, 30, and 10 min, respectively. Platelets were then incubated (●, ■) or not (○, □) with 150 μM chlorpromazine for 10 min at 37 °C and then activated (□, ■) by 1 μM A23187 in the presence of 1 mM Ca^{2+} (arrows). Spin-labeled phospholipid amounts recovered in the supernatant of the albumin-extracted platelet suspension were quantified from ESR signals and expressed as a percentage of total spin-labeled phospholipids remaining in the platelet suspension at the same time (for calculations, see Materials and Methods). Broken lines are simulations on the basis of previously observed redistribution kinetics of spin-labeled phosphatidylethanolamine where 50% of the non-albumin-extractable probes became albumin-extractable within less than 2 min following A23187-induced activation (see Discussion). Note that scales are 0−100% in (A) and 50−100% in (B−D). The data shown were obtained in a single experiment. Two other experiments gave similar curves. A fourth experiment where activation was performed at pH 7.4 (buffer B with 50 mM Hepes) gave the same results.

the outer leaflet of the plasma membrane (Bevers et al., 1983; Bassé et al., 1993). Initially, the aminoPLs are maintained in the inner leaflet of the plasma membrane by ATP-dependent aminoPL translocase, which may be inhibited by an increase in concentration of intracellular calcium (Zachowski et al., 1986). Activated platelets with a PS- and PE-rich surface then allow a coagulation factor complex (prothrombinasic converting activity) to form at their surface: this triggers the coagulation cascade (Bevers et al., 1982, 1983). Although the exposure of aminoPLs has been correlated in time with the appearance of prothrombinasic activity (Bevers et al., 1983), vesicle shedding (Comfurius et al., 1990), and submembrane cytoskeleton proteolysis (Fox et al., 1983, 1985; Comfurius et al., 1985), the mechanism responsible for this has not yet been elucidated. Some investigators have suggested that membrane scrambling occurs during vesicle shedding. AminoPLs outside exposure would then be the cumulative effect of membrane scrambling, submembrane cytoskeleton proteolysis (rupture of aminoPL−cytoskeleton interactions), and inhibition of aminoPL translocase (Comfurius et al., 1990; Schroit & Zwaal, 1991). More recently, the existence of a membrane protein responsible for phospholipid scrambling (scramblase) was proposed by the same group (Zwaal et al., 1993; Galli & Bevers, 1994; Smeets et al., 1994). Conversely, we showed (Bassé et al., 1993) that (1) outflux of aminoPL is maintained even in the absence of submembrane cytoskeleton proteoysis and vesiculation; (2) no influx of choline head PLs could be observed; and (3) the initial asymmetric labeling of the plasma membrane with choline head PLs is preserved in activated platelets and in vesicles shed during activation. These conclusions are at variance with the hypothesis that aminoPL exposure is a scrambling phenomenon (mediated by a scramblase or by vesicle shedding), resulting in the equal distribution of all PLs on both sides of the platelet plasma membrane after activation.

To demonstrate that aminoPL exposure was not the consequence of excess aminoPL on the plasma membrane's inner leaflet, resulting from the addition of (0,2)PS, we performed two double-labeling experiments in platelet plasma membrane.

When added to platelet suspension, more than 95% of the PS analogs (spin- or fluorescently-labeled PS) were redistributed to the inner leaflet of the plasma membrane through

an ATP-dependent mechanism. To counterbalance this excess of PL in the inner leaflet, an equal amount of PC analogs (fluorescently- or spin-labeled PC) was added to the suspension. In the absence of activation, more than 95% of the PC analogs remained externally located for at least 10 min. The platelets were then activated by the addition of calcium ionophore A23187, and the redistribution kinetics of the two probes were monitored simultaneously in the same experiment (Figure 1). As there was no difference between the exposure of PS analogs in these two experiments and that already observed (Bassé et al., 1993), we conclude that the initial imbalance due to added PS analogs had no effect on their outflux. In fact, 50% of internally located PS analogs were reexposed less than 2 min after activation. The influx of externally located PC analogs was neither as rapid nor as extensive as the PS analogs outflux, contrary to the requirements of a scrambling mechanism. Only very slight scrambling (<10% of total NBD-PC) was observed for NBD-PC while (0,2)PC influx kinetics were slightly accelerated. Therefore, the redistribution of phospholipids following A23187 addition involved two different mechanisms: one responsible for the rapid and massive exposure of PS analogs on the outer leaflet during the activation process (less than 2 min) and another responsible for the slower long-lasting redistribution of (0,2)PC following activation. These results provide firm evidence against the scrambling theory proposed to explain the rapid and extensive outflux of aminoPLs. NBD-PC cannot redistribute passively between the two leaflets of the platelet (Figure 1A) and erythrocyte (Colleau et al., 1991) plasma membranes. In this case, very short-lasting scrambling can be observed, involving less than 10% of NBD-PC and perhaps being mediated by vesicle shedding. Moreover, spin-labeled hydrolysis occurred during internalization of the two PS analogs in resting platelets while no hydrolysis of PC analogs occurred as long as they remained externally located (i.e., before activation). The increase in (0,2)PS hydrolysis during activation indicated that (0,2)PS which remained on the inner leaflet was rapidly hydrolyzed, presumably by phospholipase A2, which is activated by increased intracellular calcium concentration during activation (McKean et al., 1981; Van Kuijk et al., 1987). By contrast, the hydrolysis of PC analogs is in accordance with the moderate internalization of these probes.

It should be emphasized that the results of these two experiments were essentially the same irrespective of the label (fluorescent or spin) attached to the phospholipid backbone. This demonstrates that the PL movements were mainly dependent on the head group structure and that the difference in the kinetics and amplitude of the transmembrane movement of NBD- and spin-labeled lipids (Colleau et al., 1991; Connor et al., 1992) were not observed in our case. This allows us to generalize our results to endogenous PC and PS molecules in platelet plasma membrane.

We then investigated whether the observed short-lasting vectorial outflux was specific to aminoPLs. This required a non-aminoPL probe to be incorporated into the inner leaflet of the plasma membrane. As the (0,2)SM and (0,2)PC remained mainly in the outer leaflet of nonactivated platelets, we used CPZ (Ferrell et al., 1988; Rosso et al., 1988) to scramble a significant amount of these probes (20−30%) without inducing platelet activation.

CPZ treatment did not induce platelet activation (<15% dense granule release and <5% PLs in vesicles) nor modify platelet responses when platelets were activated by A23187 (<90% dense granule release and ≈20% PLs in vesicles) (Figure 2). The redistribution kinetics of (0,2)PS (Figure 3A) were similar in both CPZ-treated and untreated platelets indicating that, at the concentration used, CPZ had no effect on the short-lasting vectorial outflux of aminoPL (Rosso et al., 1988). Following A23187-induced activation, we did not observe any outflux of (0,2)SM or (0,2)PC as would be expected (dashed curves in Figure 3B−D) if the short-lasting outflux related to 50% of internally located probes (Bassé et al., 1993) was not specific for aminoPLs. In fact, we did not observe any fast outflux ($t_{1/2}$ < 1 min) but a slight and slower influx of (0,2)SM (7−10% after 10 min) and an accelerated redistribution of (0,2)PC ($t_{1/2} \approx 5-10$ min). This was observed both in CPZ-treated and in CPZ-untreated platelets. As previously proposed for NBD-PC in Figure 1, the slight influx of (0,2)SM following activation could be mediated by very limited PL scrambling, possibly during vesicle shedding. Since no vectorial outflux was observed for (0,2)SM or (0,2)PC during A23187-induced platelet activation, we therefore concluded that the vectorial outflux was specific to (0,2)PS. In fact, spin-labeled PE has been shown to be exposed at the same rate and to the same extent as (0,2)PS during platelet activation (Bassé et al., 1993). Finally, as (0,2)PLs are known to be good reporters of endogenous PL movements and distributions (Seigneuret & Devaux, 1984; Tilley et al., 1986), we conclude that during strong activation with calcium ionophore, endogenous aminoPLs are exposed on the platelet surface by means of a short-lasting and specific vectorial outflux. According to Siegel et al. (1994), the fusion between two membranes proceeds through a very small (some nanometers) and very short-lived (about 5 ms) intermediate called stalk (Yeagle, 1994). The time that the PL arrangement allows for the mixing of the two leaflets must be even shorter than the life span of the stalk. Therefore, despite the numerous fusion events that occur (degranulation, vesicle shedding), it is not too surprising to observe only very weak lipid scrambling during platelet activation.

To prevent a misunderstanding between the results we obtained in this work and those reported by Smeets et al. (1994), the following points should be clarified. After platelet activation by thrombin-thapsigargin (Smeets et al., 1994) or calcium-ionophore A23187 (this work and Bassé et al., 1993), a slow PC influx occurred ($t_{1/2} \approx 10$ min in our hands and 2−3 min according to Smeets et al.). In addition, we observed the same $t_{1/2}$ value when (0,2)PC was added either 10 min after (Bassé et al., 1992) or 20 min before (Figure 3C) A23187 treatment. An even smaller (this work) or slower (Smeets et al., 1994) influx of SM was observed. Taken together, the small differences observed between Smeets and ourselves could result from the various chemical structures of the spin- and NBD-labeled probes (PC and SM analogs) or the various back-exchange procedures (temperature, incubation time).

The main discrepancy concerns only PS redistribution during platelet activation. When platelets are activated by A23187, (0,2)PS moves rapidly ($t_{1/2}$ < 1 min) from the inner to the outer leaflet of the plasma membrane (this work) in agreement with the increase in prothrombinase activity (Comfurius et al., 1990), which itself is related to the amount of endogenous PS on the outer leaflet of the plasma membrane (Bevers et al., 1983; Comfurius et al., 1985;

Connor et al., 1989). On the other hand, when platelets are activated by thrombin−thapsigargin (Smeets et al., 1993) or thrombin−collagen (Comfurius et al., 1990), the rate of endogenous and NBD-labeled PS outfluxes is slower and seems to be of the same order of magnitude as for PC influx. Finally, the SM influx rate during activation by thrombin−thapsigargin is consistently slower than the PC and PS rates, and as noticed by Smeets et al. (1994), the resulting imbalance in PL distribution at the beginning of the activation could be in favor of filopodia formation and vesicle shedding. The fact that vesicle shedding is more pronounced during A23187 activation than during thrombin−collagen activation (Sims et al., 1989; Zwaal et al., 1992) is consistent with a larger imbalance in PL distribution, resulting from a faster aminophospholipid outflux than that observed during activation induced by thrombin−thapsigargin or thrombin−collagen.

To summarize, the redistribution of fluorescently-labeled PS and PC during thrombin−thapsigargin-induced activation are of the same order of magnitude while the slower SM redistribution induces a PL imbalance in favor of moderate vesicle shedding (Smeets et al., 1994). During A23187-induced activation, the PS outflux is accelerated while PC and SM influxes remain similar to those in thrombin−thapsigargin-induced activation. The more pronounced imbalance resulting from this more rapid aminophospholipid outflux would induce more marked vesicle shedding.

According to our results, the PL transverse movement during A23187-induced activation can be summarized by an outflux of 50% of internally located PS and PE in less than 2 min and during the same time a little scrambling inducing internalization of 5% of PC and SM. Taking into account the relative amount of each phospholipid species (Chap et al., 1977a; Perret et al., 1979) and the phospholipid contribution due to plasma membrane-granule fusion, the PL distribution (outside/inside) expressed as a percentage of total PL in plasma membrane after the first minute of activation is thus: SM (21.8/3), PC (14.8/22.2) PS (5.7/4.8), and PE (16.6/11.1), giving an overall PL balance of 59/41. Due to this net outflux, the external leaflet contains an excess of PL (possibly modulated by redistribution of cholesterol, inositol phospholipids, lyso derivatives and diacylglycerol), which can be adapted only by some very short curvature radius area, as observed for example in the filopodia (Bassé et al., 1994). When the filopodia are stabilized by inhibition of calpain, maintaining a strong interaction between the plasma membrane and the cytoskeleton, the platelet shape is strongly spiculated. By contrast when filamin, talin, and heavy chain myosin are proteolyzed, up to 20% of the cell PL appear in small vesicles, and the remnant platelets adopt a rounded shape (Bassé et al., 1994). After 1 min, the imbalance slowly disappears as the membrane is much less stable in activated platelets (Bassé et al., 1992).

To date, no mechanism has been described to explain the specific transbilayer movement of aminoPLs from the inner to the outer leaflet of the platelet plasma membrane, although a number of hypothesis have been proposed. In red blood cells, following the influx of calcium, the asymmetric distribution of aminoPLs is also lost. However, this occurs at a much slower rate (>20 min) than in platelets (<2 min) (Comfurius et al., 1990; Williamson et al., 1992). Various mechanisms such as plasma membrane scrambling during the shedding of vesicles (Comfurius et al., 1990) and calcium- and phosphatidylinositol 4,5-bisphosphate-dependent PL scrambling (Sulpice et al., 1994) have been proposed to account for aminoPL exposure in red blood cells. Recent studies have demonstrated that the anion exchanger of the erythrocyte membrane (band 3) could be a flip-floppase for anionic amphiphiles (Ortwein et al., 1994; Vondenhof et al., 1994). Also, rapid PL flip−flop has been described in a model membrane containing pore-forming peptides (melittin or synthetic peptides inducing bilayer perturbation) (Fattal et al., 1994). However, all these mechanisms lead to a nonvectorial and nonhead group structure-dependent flip-flop of PLs, in other words, to scrambling. On the other hand, we have demonstrated (Gaffet et al., 1995) that neither actin or tubulin polymerization nor submembrane skeleton proteolysis influence the aminoPLs outflux during platelet activation. We infer from these results that calcium-induced catalytic activity (perhaps enzyme mediated) in the platelet plasma membrane is likely to be responsible for the fast vectorial outflux of aminoPLs.

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