Translocation of Spin-Labeled Phospholipids through Plasma Membrane during Thrombin- and Ionophore A23187-Induced Platelet Activation†

François Bassé,*,‡ Patrick Gaffet,‡ Francine Rendu,§ and Alain Bienvenüe‡

URA 530 CNRS, CP 107, Université Montpellier II, F-34095 Montpellier Cedex 05, France, and INSERM CJF 9101, Faculté de Pharmacie, 4, avenue de l'Observatoire, F-75270, Paris Cedex 06, France

Received October 19, 1992; Revised Manuscript Received December 14, 1992

ABSTRACT: After incorporation of spin-labeled phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine analogues in the outer leaflet of the plasma membrane in resting platelets, more than 90% amino-head analogues accumulated within 30 min in the inner leaflet by aminophospholipid translocase activity, while choline analogues mostly remained on the outer leaflet. Platelets were then activated by thrombin or Ca^{2+} ionophore A23187. No outward movement of internally located spin-labeled aminophospholipids was observed during thrombin-induced activation, whereas the influx of externally located probes increased slightly. During A23187-mediated activation, similar slightly increased influx was observed, while 40–50% of the initially internally located aminophospholipids could then be extracted from the outer leaflet. This sudden exposure on the outer face was dependent on an increase in intracellular Ca^{2+} and achieved in less than 2 min at 37 °C. Inhibition of translocase activity by *N*-ethylmaleimide did not induce any aminophospholipid outflux. When probes were incorporated on the outer face of the plasma membrane in resting platelets, they were still fully accessible from the extracellular medium after A23187-induced activation. Moreover, they were distributed between the vesicles and remnant platelets in proportion to the external membrane phospholipidic content in each structure. This suggested that no scrambling of plasma membrane leaflets occurred during the vesicle blebbing. Moreover, the spin-labeled aminophospholipids exposure rate and amplitude were unchanged when vesicle formation was inhibited by the calpain inhibitor calpeptin. These results indicate that loss of asymmetry thus inducing generation of a catalytic surface is not the consequence of vesicle formation. Conversely, we propose that vesicle shedding is an effect of PL transverse redistribution and calpain-mediated proteolysis during activation.

One of the most important steps in platelet activation is the generation of a catalytic membrane surface on which some coagulation factors interact with each other (Davie et al., 1991). The presence of aminophospholipids (aminoPLs),[1] and more specifically of phosphatidylserine (PS), on the outer leaflet of the plasma membrane allows the conversion of factor X to factor Xa and the association of the latter with factor Va (Bevers et al., 1983). This association forms the prothrombinase complex, which catalyzes prothrombin hydrolysis into thrombin. This leads to fibrin formation and generation of an insoluble fibrin clot that strengthens the platelet plug. Generation of a procoagulant membrane surface is concomitant with secretion of intracellular granules content and shedding of vesicles. These latters are formed by plasma membrane budding and blebbing (Fox et al., 1991).

Since aminoPLs are mostly absent from the outer leaflet of the plasma membrane in resting platlets (Bevers et al., 1983; Suné & Bienvenüe, 1988), the plasma membrane has to undergo large modifications during activation in order to externally expose aminoPLs. In unstimulated platelets, aminoPLs are continuously pumped back from the outer to the inner leaflet of the plasma membrane by membrane aminoPL translocase, thus maintaining phospholipidic transverse asymmetry (Suné & Bienvenüe, 1988). In a previous report (Bassé et al., 1992), we showed that translocase activity was completely inhibited in platelets activated by A23187, but not when activation was induced by thrombin, in agreement with similar investigations using fluorescent PL analogues (Tilly et al., 1990). Translocase was also shown to be inhibited when platelets were activated by thrombin + collagen (Comfurius et al., 1990). This inhibition is required to stop continuous aminoPLs displacement from the outer to the inner leaflet of the plasma membrane due to translocase activity, which would thwart the formation of a catalytic membrane surface. On the other hand, net displacement of aminoPLs occurs during activation, leading to the exposure of aminoPLs on the outer face. The presence of PS on the outer leaflet of the plasma membrane in activated platelets has been investigated using phospholipase attack (Bevers et al., 1983), PS-dependent binding of a placental anticoagulant protein (Thiagarajan & Tait, 1991), measurement of the prothrombinase activity (Comfurius et al., 1990), and direct evaluation of the internalization of paramagnetic analogues of PL (Bassé et al., 1992). Loss of PL transverse asymmetry, and thus formation of catalytic sites on the platelet surface during activation, was reported to be due to the formation of transient nonbilayer lipid phases at the point where vesicles bud from the plasma membrane (Sims et al., 1989; Comfurius et al., 1990). However, net displacement of PLs through the plasma membrane during platelet activation is poorly documented, mainly because of the lack of direct measurements of PL movements.

† This work was supported by the Centre National de la Recherche Scientifique (URA 530) and by grant from the University of Montpellier II. F.B. is a recepient of a grant from the Direction des Recherches Etudes et Techniques (FR 89/247).

* Author to whom correspondence should be addressed.

‡ URA 530 CNRS.

§ INSERM CJF 9101.

[1] Abbreviations: PL, phospholipid; (0,2)PS, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine; (0,2)PE, 1-palmitoyl-2-(4-doxylpentanoyl)-phosphatidylethanolamine; (0,2)PC, 1-palmitoyl-2-(4-doxylpentanoyl)-phosphatidylcholine; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; (^{3}H)5-HT, (^{3}H)5-hydroxytryptamine; NEM, *N*-ethylmaleimide.

0006-2960/93/0432-2337$04.00/0

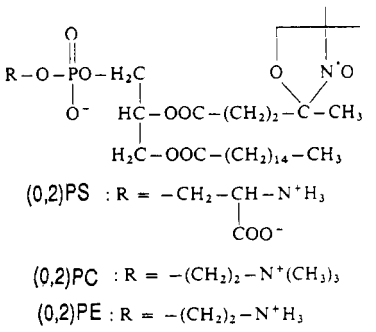
The aim of this study was to investigate PL movements during platelet activation using paramagnetic analogues of endogenous PLs. Moreover, we assessed the cause and effect relationship between PS external exposure and vesicle formation. The present results show that generation of the catalytic surface is due to displacement of 40–50% of aminoPLs initially situated on the cytoplasmic leaflet of the plasma membrane and probably of the granule membrane. This work provides strong evidence that this sudden outflux of PL is the cause rather than the consequence of vesicle shedding.

MATERIALS AND METHODS

Materials. Calcium ionophore A23187 and thrombin were obtained from Sigma Chemical Co. (St. Louis, MO). Calpeptin was from Novabiochem (Switzerland). All other reagents were of the highest grade commercially available.

Isolation of Platelets. Platelets were prepared at room temperature from 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 previously described erythrocyte cushion procedure (Bassé et al., 1992). Platelets were finally resuspended in a Hepes buffer (136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM dextrose, 10 mM Hepes, pH 7.4) at 2×10^9 platelets/mL. Platelets were incubated for 30 min at 37 °C before any experimentation. When necessary, the membrane permeant calpain inhibitor calpeptin (Tsujinaka et al., 1988; Fox et al., 1990) was added at the beginning of this incubation. Unless otherwise specified, all experiments were performed at 37 °C.

Spin-Labeling and ESR Experiments. The following spin-labeled phospholipids, synthesized as previously described (Seigneuret & Devaux, 1984), were used:



Incorporation and evaluation of externally situated paramagnetic probes were performed as previously described (Bassé et al., 1992; Morot et al., 1989), with minor modifications. Briefly, spin-labels (1–2% of endogenous PLs) were added to the platelet suspension at zero time from a concentrated solution in Hepes buffer. Analogues incorporated into the outer membrane leaflet in less than 1 min (Seigneuret & Devaux, 1984). At specific times, 70-μL aliquots of the platelet suspension were drawn and mixed with 25 μL of fatty acid free bovine serum albumin solution in Hepes buffer (BSA, final amount 0.8% w/v). The mixture was incubated on ice for 30 s and then centrifuged 2 min at 11000*g*. This procedure was sufficient to extract all the external labeled phospholipids, regardless of probe polar head, as shown in control experiments. Each sample was completed with 10% vol of 100 mM potassium ferricyanide in order to reoxidize any reduced spin-label. The fraction of spin-labels extractable from the outer leaflet of the platelet plasma membrane was evaluated by ESR measurements performed on a Bruker ECS 106 spectrometer, by evaluating the intensity of the central line of the ESR spectra. Long-lasting incubation of platelets with spin-labels led to the appearance of three narrow peaks in the spectra, corresponding to the hydrolysis of ester bound at position 2 of the spin-labeled phospholipids. This hydrolysis was mainly inhibited in erythrocytes by prior incubation with diisopropyl fluorophosphate, without any effect on aminophospholipid translocase activity (Morot et al., 1989). In platelets, this product strongly perturbs translocase activity and the shape of these cells, without any obvious effect on probe hydrolysis (unpublished observations). Thus, determinations of the central line magnitudes were automatically corrected by evaluation of the hydrolyzed component, taking the ratio between the low-field and central line amplitudes of the spectra into account. Incubations at 37 °C for 2 h led to hydrolysis of 10–15% of the initially incorporated spin-labeled phospholipids. Modifications of transverse distribution during activation were reported to the initial distribution of intact analogues before activation. The data presented are from a single experiment representative of at least three separate experiments.

Activation Procedures. Extraction of phospholipids for ESR measurements was possible only if the entire external side of the platelet plasma membrane remained accessible to bovine serum albumin. This required conditions where no (micro)-aggregation would occur during the activation procedure. This was controlled in each experiment by light microscopy. Briefly, thrombin (5 units/mL) activation was performed without stirring in the presence of 5 mM EGTA; for A23187 activation (final concentration 1 μM), the Hepes buffer was supplemented with 1 mM $CaCl_2$ or 2 mM EGTA. When the experiments were performed at 20 °C or 10 °C, the platelet suspension was slowly brought to the desired temperature before the addition of A23187.

Platelet secretion was measured according to Suné and Bienvenüe (1988), using (^{3}H)5-hydroxytryptamine [(^{3}H)5-HT, specific activity 1.8 MBq/mmol] (Amersham) as a dense granule marker. Platelet lysis, evaluated by measuring lactate dehydrogenase activity in the supernatant using the Sigma No. 500 kit (St. Louis, MO) (Cabaud & Wroblewski, 1958; Suné & Bienvenüe, 1988), never exceeded 2% in any of the performed experiments.

Vesicle Isolation and Quantification. Vesicle formation was stopped by adding 5 mM EGTA to the platelet suspension. Vesicles were recovered in the supernatant of the centrifugated (11000*g* for 2 min) platelet suspension (Comfurius et al., 1990). Lipids were extracted according to Folch et al. (1957). PL amounts were evaluated according to Rouser et al. (1970).

Spin-Label Incorporation. When spin-labels are added to heterogeneous suspension composed of remnant platelets and vesicles, the amount of probe incorporated in each structure was proportional to the external surface of each structure. Moreover, postactivation localization of previously incorporated spin-labels, i.e., their distribution between vesicles and remnant platelets, can be compared to the external surface of each structure. We therefore used plasma membrane/whole cell PL content ratios of 60% and 80% in resting and activated platelets, respectively, as previously described (Perret et al., 1979; Bevers et al., 1983). Then, the mentioned PL contents in the vesicles were compared to the plasma membrane PL content of the whole cell. BSA extraction of external located spin-labels on isolated vesicles was performed in the same way as described for platelets, excepted that BSA supernatants were isolated by high-speed centrifugation (160000*g* for 5 min) using an Airfuge (Beckman) ultracentrifuge. This

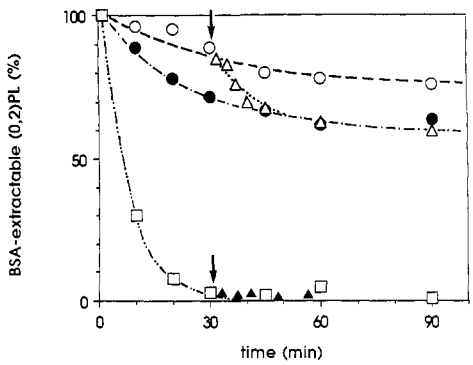


FIGURE 1: Modification in BSA-extractable (0,2)PC and (0,2)PS during thrombin-induced activation at 37 °C. (0,2)PC (○, ●) and (0,2)PS (□) were added at zero time to resting (○, □) or thrombin-activated platelets (●); 30 min after the incorporation of spin-labeled PLs, thrombin (5 units/mL) was added (arrow) to the suspension of resting platelets in the presence of 5 mM EGTA. BSA-extractable (0,2)PS (▲) and (0,2)PC (△) were then monitored.

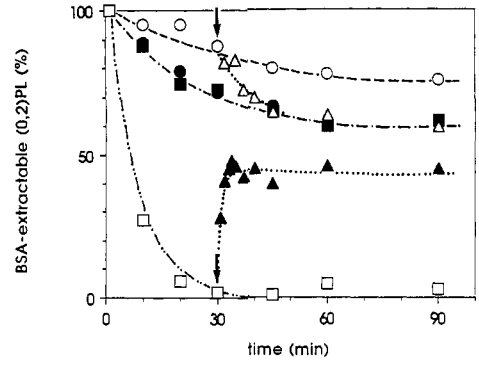


FIGURE 2: Modification in BSA-extractable (0,2)PC and (0,2)PS during ionophore A23187-induced activation at 37 °C. (0,2)PC (○, ●) and (0,2)PS (□, ■) were added at zero time to resting (○, □) or A23187-activated platelets (●, ■); 30 min after the incorporation of spin-labeled PLs, resting platelets were activated by the addition (arrow) of A23187 (1 μM) in the presence of 1 mM Ca^{2+}. BSA-extractable (0,2)PS (▲) and (0,2)PC (△) were then monitored.

procedure led to correct assignment of probe location, since previous experiments ruled out the existence of an increase in basal flip-flop rates of phospholipids in the membrane of shed vesicles (Bassé et al., 1992).

Analysis of Proteins. Platelet proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions (Wiedmer et al., 1990), using a 7.2% homogeneous gel (LKB 2050, Pharmacia). Proteins were stained with Coomassie blue.

RESULTS

Movement of Spin-Labeled Phospholipids during Thrombin-Mediated Platelet Activation. When resting platelets were incubated at 37 °C in the presence of spin-labeled PLs, more than 95% of the initially incorporated (0,2)PS was displaced from the external to the internal leaflet after 30 min (Figure 1). This was in agreement with the reported high rate of transverse reorientation of PS in the platelet plasma membrane (Suné & Bienvenüe, 1988; Bassé et al., 1992). Concurrently, 85–90% of the initially incorporated (0,2)PC was extractable by BSA (Figure 1). At this time (i.e., after 30 min) the platelets were activated by adding thrombin (5 units/mL) to the cell suspension in the presence of 5 mM EGTA. In these conditions, the release of (^{3}H)5-HT reached 75–85% of the total content. No (0,2)PS could be extracted after thrombin-induced activation, indicating that no outward displacement of this aminoPL occurred. Similar activation was performed in the presence of 0.8% BSA in order to trap any transient exposure of the probe. It should be noted that BSA extraction was faster than translocase-mediated relocation of external PS (15 s were sufficient to extract the whole external probe by BSA, whereas the half-time of PS transverse reorientation was about 4 min). Even though very little time (as low as 15 s) elapsed after the addition of thrombin, no spin-labeled PS could be detected in the cell supernatant containing BSA. In contrast, thrombin activation modified the transverse mobility and equilibrium distribution of (0,2)PC (Figure 1). Immediately after the addition of thrombin, (0,2)PC molecules internalized faster, leading to identical distribution whether the platelets were activated before or during probe reorientation.

Movement of Spin-Labeled Phospholipids during Ionophore A23187-Mediated Platelet Activation. The same procedure as described above was performed, so as to incorporate (0,2)PC and (0,2)PS on the outer and the inner leaflet, respectively. A23187-mediated activation (1 μM final concentration) was performed in the presence of 1 mM Ca^{2+}. This procedure led to massive release (80–85% of total (^{3}H)5-HT), vesicle formation (20–25% of the whole cell phospholipids recovered in the vesicles), and calpain-mediated proteolysis of high molecular weight proteins, as previously described (Comfurius et al., 1985). The translocation kinetic of (0,2)-PC (Figure 2) during A23187-induced activation was comparable to that observed during thrombin-mediated activation (Figure 1), i.e., slightly increased transverse mobility and a higher proportion of internally situated probes at equilibrium than in resting platelets. By contrast, A23187-induced platelet activation provoked a very rapid increase in the proportion of extractable (0,2)PS, since 40–45% of the initially incorporated probes was extractable within 1 min after A23187 addition (Figure 2). The presence of labeled PS on the BSA-accessible face (i.e., external leaflet) was stable for up to 1 h. Moreover, the rate and the amplitude of (0,2)PS outward displacement were unaffected if the platelets were activated by A23187 when the time between probe incorporation and activation was longer (up to 2 h). When ionophore A23187 was added to resting platelets in the presence of 2 mM EGTA, no (0,2)-PS exposure was observed, whereas transverse reorientation of (0,2)PC was slightly increased, similar to the result when A23187 was added in the presence of external Ca^{2+}. An external Ca^{2+} concentration of higher than 0.2 mM was required to induce an observable outward (0.2)PS displacement, as shown in preliminary experiments (not presented).

Movement of (0,2)PE Induced by A23187-Mediated Platelet Activation during Internalization of the Probe. The (0,2)PE transverse reorientation through the plasma membrane is slower than that of (0,2)PS in resting platelets (Suné & Bienvenüe, 1988), as for several different membranes (Zachowski & Devaux, 1990). After incorporation of labeled PE, the equilibrium state was reached within 45 min, with 95% of probes being located on the inner leaflet (Figure 3D). This slow reorientation rate allowed activation of cells at different steps during transverse distribution of the probe. Figure 3 presents the movement of (0,2)PE when A23187 (1 μM final concentration, in the presence of 1 mM Ca^{2+}) was added to activate platelets at 10, 15, 20, or 51 min after the initial incorporation of (0,2)PE. Regardless of the time of A23187 addition, activation induced sharp increases (<2 min) in extractable (0,2)PE. The amount of extractable (0,2)PE depended on the time at which activation was performed, in relation to the transverse distribution of this probe at a given activation time. Quantitatively, extracted (0,2)PE was equal to the sum of externally located probes and half of the internally

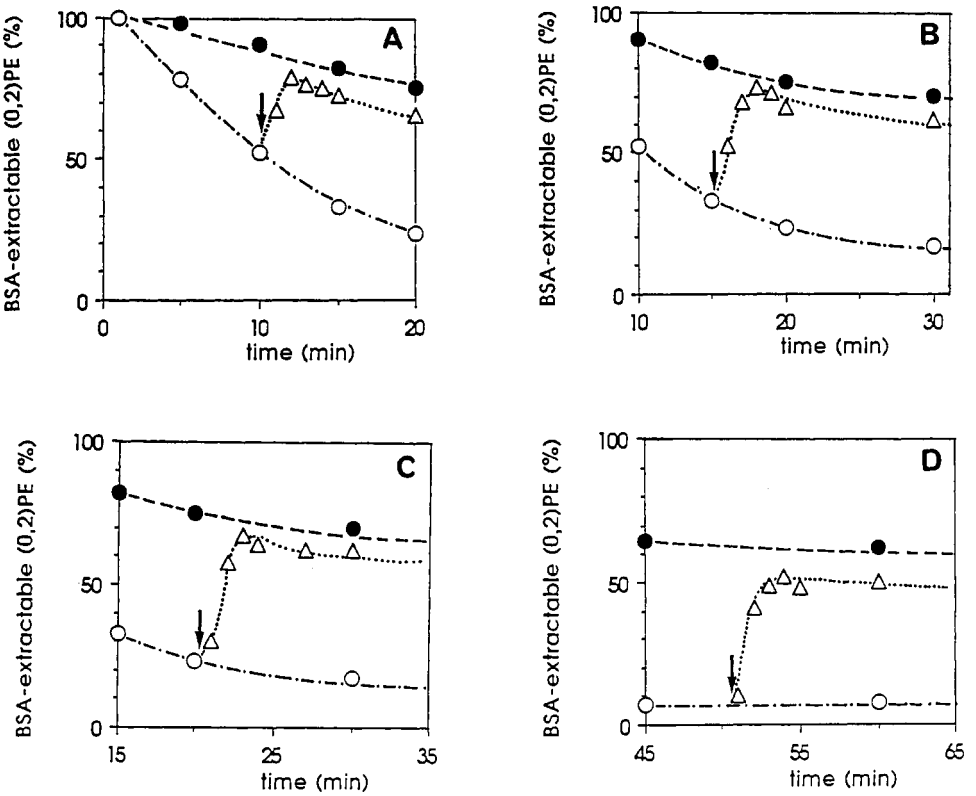


FIGURE 3: Modification in BSA-extractable (0,2)PE during ionophore A23187-induced activation at 37 °C. (0,2)PE was added at zero time to resting (○) or A23187-activated platelets (●). Activation of resting platelets was performed (A) 10 min, (B) 15 min, (C) 20 min, or (D) 51 min after the incorporation of (0,2)PE by adding A23187 (1 μM) (arrows) to the suspension in the presence of 1 mM Ca^{2+}. BSA-extractable (0,2)PE (△) was then monitored.

located probes at the time of activation. When activation was induced after a long period of time, enough to let (0,2)PE reach the equilibrium state (i.e., after 51 min, Figure 3D), outward movement of (0,2)PE was similar to that of (0,2)PS in terms of rate and amplitude (Figure 2). Once (0,2)PE was on the outer leaflet, transverse distribution of this spin-label slowly reached equilibrium state, resulting in about 50% extractable probe. Note that the rates of slow relaxation following activation were identical to those measured in preactivated platelets (Figure 3).

Transbilayer Movement of Spin-Labeled Phospholipids during Vesicle Formation. Spin-labeled PLs can be used as markers for the two plasma membrane leaflets in resting platelets. Two different experiments were performed to investigate the scrambling phenomenon putatively occurring during vesicle formation. In the first one, spin-labeled PC was added to a platelet suspension which had been activated with 1 μM A23187 in the presence of 1 mM Ca^{2+}, i.e., to a mixture of remnant platelets and vesicles. The proportions of labeled PLs extractable from isolated vesicles were correlated with the vesicle PL content (see Methods), indicating that the spin-labels incorporated into both vesicles and remnant platelet membranes without selectivity (Table I, line A). In the second experiment, (0,2)PC or (0,2)PS were added to resting platelets 30 min before A23187-induced platelet activation. This time was sufficient to allow (0,2)PS to reach the internal leaflet, as shown in control experiments, whereas 90% of (0,2)PC remained on the outer leaflet. The amount of (0,2)PC extractable from isolated vesicles was proportional to the PL content in these structures, although the platelets had been activated after probe incorporation (Table I, line B). When (0,2)PS had been previously incorporated into platelets, 40–45% of the probes was extractable from the whole suspension after the A23187-mediated activation (Figure 2). The amount of extractable (0,2)PS from vesicles and remnant platelets was directly proportional to the PL content of these structures (Table I, line C).

Table I: Comparison between the Phospholipid Content and the BSA–Extractable Amount of (0,2)PLs in Shedded Vesicles

experimental conditions[a]	PL in vesicles (% of whole cell)	(0,2)PLs extractable from vesicles (% of whole suspension)
A	24	23
B	23	19
C	25	24

[a] Conditions: (A) Platelets were activated by A23187 (1 μM), and (0,2)PC was added 5 min later. Vesicles were isolated by centrifugation and further analyzed for their PL content and BSA-extractable spin-labels. The amount of BSA-extractable spin-labels was compared to that of spin-labels extracted from the whole suspension, i.e., remnant cells and vesicles. (B) (0,2)PC was added to resting platelets. Platelets were activated by 1 μM A23187 30 min later. After 5 min, vesicles were isolated and analyzed as described above. (C) Same as (B), but with (0,2)PS instead of (0,2)PC. The amount of BSA-extractable (0,2)PS was compared with that of (0,2)PS extracted from the suspension after the activation.

Influence of N-Ethylmaleimide Treatment. NEM inhibits translocase activity in erythrocytes (Zachowski et al., 1986; Daleke & Huestis, 1989; Morot et al., 1989). This property was used to evaluate the consequence of translocase inhibition on the aminoPL exposure in resting platelets. The inhibitory effect of NEM on translocase activity in resting platelets was investigated by measuring the (0,2)PS translocation rate through the plasma membrane. NEM was added to the cell suspension 10 min before the addition of spin-labels. The initial rate was determined graphically. Translocase activity was almost completely inhibited with 2 mM NEM (Figure 4 insert). NEM (2 mM) not only inhibited translocase activity but also inhibited A23187- (1 μM) induced granule secretion

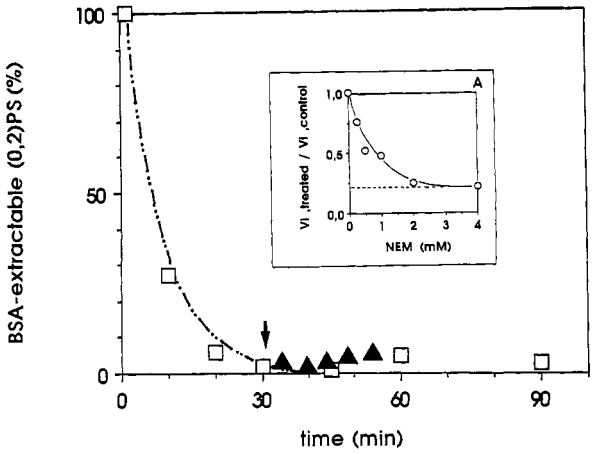


FIGURE 4: Effect of NEM addition on BSA-extractable (0,2)PS. (0,2)PS was added to resting platelets at zero time and the BSA-extractable proportion of these probes was monitored (□); 30 min after the incorporation of spin-labeled PS, 2 mM NEM was added (arrow) to the platelet suspension, and BSA-extractable (0,2)PS was monitored (▲). Insert: platelets were incubated for 5 min at 37 °C in the presence of various NEM concentrations. Then, (0,2)PS was added to these suspensions. The initial rates of decrease in BSA-extractable spin-labels (V_i) were plotted versus the NEM concentration. The dashed line corresponds to the initial rate in platelets activated by 1 μM A23187 in the presence of 1 mM Ca^{2+}.

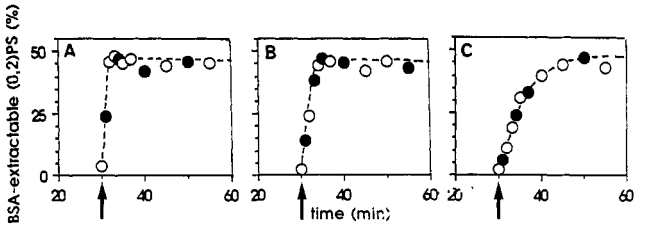


FIGURE 5: Effect of temperature and calpain inhibition on BSA-extractable (0,2)PS during A23187-induced activation. After the platelets had been preincubated (●) or not (○) with 50 μg/mL calpeptin for 30 min at 37 °C, (0,2)PS was added to the cell suspension. After another 30-min incubation at 37 °C, the platelet suspension was slowly brought to 20 °C (B) or 10 °C (C) or left at 37 °C (A). A23187 (1 μM) was then added to the suspension in the presence of 1 mM Ca^{2+} (arrows), and the BSA-extractable (0,2)PS was monitored (●, ○).

by 50%. On the other hand, the addition of NEM to a platelet suspension previously incubated with (0,2)PS for 30 min did not modify (for the first 15 min) the amount of extractable spin-labeled PS previously located in the inner leaflet of the plasma membrane (Figure 4). Longer incubations led to higher proportions of extractable (0,2)PS but were accompanied by secretion of dense granules, suggesting further perturbation of the cell by NEM treatment.

Influence of Calpain Inhibition on (0,2)PS Movement and Vesicle Shedding during A23187 Activation. As shown above, the outward movement of aminoPLs was very rapid (1 min) when platelet activation was performed at 37 °C (Figures 2 and 3). In order to slow down this outflux, so as to be able to measure slight modifications of this outward movement, platelets having previously internalized (0,2)PS were activated at lower temperatures. Platelets were incubated for 30 min at 37 °C in the presence of (0,2)PS before activation induced by 1 μM A23187 in the presence of 1 mM Ca^{2+}. Regardless of the temperature at which it was performed (37 °C, 20 °C, or 10 °C), activation induced similar secretion (75–80% of the initially taken up (^{3}H)5-HT was released), vesicle formation (20–25% of the whole cell PLs were found in the vesicles) and cytoskeleton proteolysis (not shown). The rate of appearance in extractable (0,2)PS decreased with the temperature (Figure 5), but the equilibrium level (40–45% of initially incorporated paramagnetic probes) did not change. In the same conditions, degranulation, vesiculation, and proteolysis were slowed down at 20 °C or 10 °C, occurring with similar kinetics to the exposure of (0,2)PS on the outer leaflet (not shown).

Similar experiments were carried out on platelets pretreated with 50 μg/mL calpeptin. Calpeptin-mediated proteolysis of filamin, talin, and myosin heavy chain was inhibited by this product, as shown by the intact protein pattern after A23187-induced activation (Figure 6A). In agreement with previous reports (Fox et al., 1990, 1991), inhibition of calpain activity strongly reduced vesicle formation during activation. Whatever the temperature, 6–8% of whole cell PLs was found in the supernatant of platelets activated after calpeptin preincubation, instead of 20–25% with nontreated cells (Figure 6B). However, calpain inhibition did not influence the rate and final amplitude of (0,2)PS displacement from the inner to the outer leaflet after the A23187-induced activation (Figure 5).

DISCUSSION

In the present study, we used paramagnetic PL analogues as markers for the reorganization of internal and external faces of the plasma membrane during platelet activation. Rapid translocation of aminoPL analogues from the outer to the inner leaflet occurred within resting platelets ($t_{1/2}$ about 4 min and 10 min, for (0,2)PS and (0,2)PE respectively; Figure 1–3) in agreement with previous reports (Suné & Bienvenüe, 1988). At equilibrium, aminoPL analogues were mainly located in the inner face (>95% and about 90% for (0,2)PS and (0,2)PE, respectively), whereas limited reorientation of choline head-group PL analogue (0,2)PC left these probes almost exclusively in the outer leaflet (Figures 1–3). In conditions where platelets were activated 30 min after the addition of paramagnetic analogues, the spin-labeled aminoPLs and choline-head PLs were suitable markers for the endogenous PLs initially located in the inner and outer monolayers, respectively. Two different activation agonists were used, namely thrombin or Ca^{2+} ionophore A23187, differing in their ability to induce the generation of a catalytic surface as determined by prothrombinase activity measurements (Bevers et al., 1983).

Both agonist-mediated platelet activations (thrombin or Ca^{2+} ionophore A23187) induced a slight increase in the rate of transmembrane reorientation of external (0,2)PC, resulting in transmembrane distribution of this probe identical to that measured in platelets activated before spin-label incorporation (Figures 1 and 2). As previously mentioned (Bassé et al., 1992), the slight increase in the movement of externally located probes could be the result of some changes in membrane composition and/or structure related to activation. By contrast, the outward movements of internally situated spin-labeled PLs (PS and PE) differed depending on the agonist used, in agreement with the different abilities of the two agonists to induce a procoagulant platelet surface (Bevers et al., 1983). Thrombin-mediated activation of platelets did not lead to any external exposition of (0,2)PS (Figure 1), not even transiently, in line with the lack in prothrombinase activity on the thrombin-activated platelet surface (Bevers et al., 1983). By contrast, when platelets were activated by A23187 after the incorporation of (0,2)PLs, both labeled PS and PE suddenly appeared on the outer face (Figures 2 and 3), consistent with the establishment of a catalytic surface (Comfurius et al., 1990). The whole displacement phenomenon was achieved

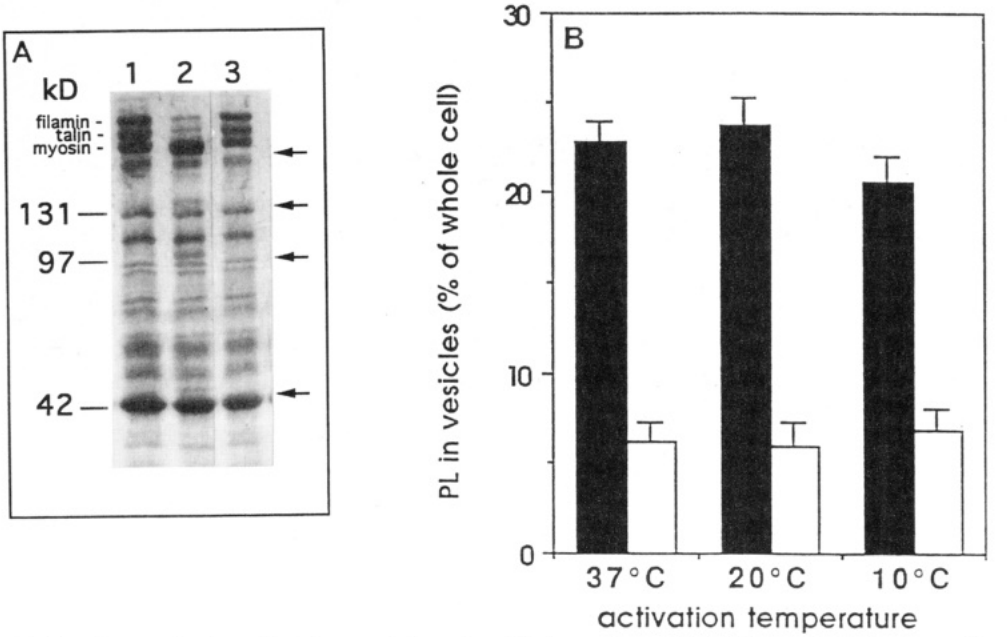


FIGURE 6: Effect of calpeptin on calpain-mediated proteolysis and vesicle formation. (A) SDS–PAGE of platelets activated at 37 °C by 1 μM A23187 in the presence of 1 mM Ca^{2+}. Lane 1: resting platelets. Lane 2: activated platelets. Lane 3: platelets activated after a preincubation with 50 μg/mL calpeptin. Arrows indicate the greatest modifications in the protein pattern after activation. Similar protein patterns were observed when platelets were activated at 20 °C or 10 °C. (B) Effect of the calpain inhibition on vesicle formation. After 30 min of incubation in the presence or absence of calpeptin (50 μg/mL) at 37 °C, platelets were slowly brought to the desired temperature and further activated by adding 1 μM A23187 in the presence of 1 mM Ca^{2+}. PL content in vesicles was compared to the whole cell PL content. Columns: dark, without calpeptin pretreatment; white, after a pretreatment with 50 μg/mL calpeptin. Data represent mean ± SEM of triplicate measurements.

within 1 min, with identical proportions of initially incorporated (0,2)PS or (0,2)PE (40–45%) being extracted after activation.

As mentioned above, spin-labeled PLs were previously noted to be good reporters for endogenous PLs. Indeed, similar general conclusions were drawn concerning PL transverse mobility or distribution in erythrocytes or platelets when paramagnetic (Suné & Bienvenüe, 1988; Morot et al., 1989; Devaux, 1990; Bassé et al., 1992), fluorescent (Tilly et al., 1990), radiolabeled (Daleke & Huestis, 1989), or endogenous PLs (Roelofsen et al., 1987) were used. Hence, one can assume that outward and inward movements of spin-labeled PLs during platelet activation described in this work correctly reflected the movement of endogenous PLs. Nevertheless, small quantitative differences between paramagnetic and endogenous PLs in their mobilities during activation cannot be completely ruled out because of the shorter β-chain in paramagnetic PLs.

The present experiments provide new information on the mechanism by which aminoPLs appear on the extracellular face of the plasma membrane. First, we directly observed aminoPLs outward movement dependence on an increase in intracellular Ca^{2+} concentration, according to a previous report showing that external exposition of PS after activation is dependent on an increase in intracellular Ca^{2+} (Verhallen et al., 1987). As is the case for vesiculation (Wiedmer et al., 1990), calpain-mediated proteolysis (Verhallen et al., 1987), and inhibition of translocase activity (Comfurius et al., 1990; Tilly et al., 1990; Bassé et al., 1992), high intracellular Ca^{2+} concentrations were required to induce the outflux of spin-labeled aminoPLs. Second, the sudden outflux was not fully specific, since PS and PE analogues were exposed at a similar rate and amplitude (Figures 2 and 3). Third, inhibition of translocase activity was not the only prerequisite for transmembrane displacement of aminoPLs. Indeed, although exposition of aminoPLs was concomitant with inhibition of translocase activity (Tilly et al., 1990; Bassé et al., 1992), the addition of NEM to the platelet suspension, at a concentration that inhibited translocase activity (Figure 4, insert), failed to induce any movement of internally located probes to the outer leaflet (Figure 4). Accordingly, translocase inhibition (by addition of 1 μM A23187 in the presence of 1 mM Ca^{2+}) failed to induce sudden large modifications of transmembrane asymmetry in red blood cells (Schewe et al., 1992). We also confirmed that the secretion events were not involved in the loss of asymmetry, as demonstrated by the absence of any transient appearance of (0,2)PS on the outer leaflet during thrombin-mediated activation. Tilly et al. (1990) proposed that fusion-induced scrambling of membrane PLs occurring during secretion could be counterbalanced by an increase in aminoPL translocase activity. If this was the case, this scrambling process would not concern a major number of endogenous PLs, since we did not observe any appreciable amounts of spin-labeled PS on the outer leaflet, even transiently.

This brings up the question of the origin of endogenous aminoPLs appearing on the outer face during activation. Could they originate either from the inner leaflet of the plasma membrane or from the granule merging with the plasma membrane during secretion? The merging of plasma and granule membranes does not induce any exposition of PS alone, as shown by the absence of any catalytic properties of the cell surface after secretion (Comfurius et al., 1985; Sims et al., 1989; Tilly et al., 1990). This suggests that endogenous PS in the granule membrane is mainly located on the cytoplasmic leaflet, as observed in other intracellular membranes (Zachowski et al., 1989; Zachowski & Morot Gaudry-Talarmain, 1990). On the other hand, granule and plasma membrane fusion is one of the earliest steps in activation (Karniguian et al., 1990; Bachelot et al., 1992), contrary to generation of the catalytic surface and vesicle shedding. Indeed, it was possible to induce secretion without modifying transverse asymmetry (Figure 1) and inducing a procoagulant surface (Bevers et al.,

1983). In addition, membrane granule associated protein GMP 140 (Berman et al., 1986) is found in vesicles shedded from platelets activated by C5b-9 (Sims et al., 1988; Wiedmer et al., 1990). This indicates that granule and plasma membranes are undistinguishable after secretion, and subsequently both submitted to the same loss of asymmetry, and that aminoPLs involved in catalysis of the coagulation cascade could also originate from the granule membranes. Consequently, spin-labeled PLs appearing on the outer leaflet during A23187-induced activation could partly originate from the granule membranes, provided that the short-chain paramagnetic PLs were distributed between the internal membranes because of their high hydrosolubility. However, such a distribution of labeled PLs in the internal membranes has never been demonstrated. Nevertheless, it cannot be excluded that some probes reached internal membranes other than the plasma and granule membranes. Other nongranule internal membranes represent 20% of the total cell PL content (Peret et al., 1979). Thus, the amount of probes involved in the transmembrane rearrangement might be reduced by 20%. Consequently, if the distribution of labeled PLs within the internal membranes occurred, the proportion of aminoPLs located on the cytoplasmic leaflet of the plasma and granule membrane in resting platelets being displaced to the external face of the plasma membrane can be assessed at 50% (instead of 40–45%).

The most commonly reported mechanism for the loss of membrane asymmetry is PL scrambling at the fusion locus between the apposed plasma membrane leaflets during the vesicle blebbing (Sims et al., 1989; Comfurius et al., 1990; Bevers et al., 1992). As pointed out above, the net outflux of aminoPLs during the activation was not accompanied by a reciprocal influx of PLs. Accordingly, the amount of exposed (0,2)PE was roughly equal to the outside located probe augmented by half of the inside located probe before activation, regardless of the time between probe incorporation and activation (Figure 3). This suggests that no net inward displacement of externally situated (0,2)PE counterbalanced the outward movement and that no mixing between external and internal leaflets occurred during the transverse redistribution of PLs. To further investigate the relationship between vesicle formation and aminoPL outflux during A23187-mediated activation, we compared the proportion of externally located probes and the PL content of the formed vesicles (Table I). In fact, if phospholipid scrambling occurs at the site of vesicle shedding, one would expect to observe a strongly modified phospholipid transverse repartition into vesicles, since these structures are really close to the shedding site. When spin-labels were located in the external monolayer of the platelet plasma membrane before activation, as was the case for (0,2)PC, they conserved their external location after activation into isolated vesicles and remnant cells, thus being unaffected by vesicle formation. Similarly, transverse distribution of (0,2)PS was the same in the vesicles and remnant platelets. These results suggest that vesicle formation did not itself greatly perturb the transverse PL distribution. This was confirmed by the aminoPL exposition rate during A23187-induced activation of platelets previously incubated with calpeptin. Calpeptin inhibited calpain-mediated degradation of high molecular weight cytoskeletal proteins (Figure 6A) and strongly reduced vesicle formation (Figure 6B), whereas it did not affect either the rate or amplitude of (0,2)PS outflux during the A23187-induced activation (Figure 5). This is in agreement with the fact that the rise in prothrombinase activity was not affected by calpain inhibition (Comfurius et al., 1990). Hence, aminoPL exposition on the outer face was not due to PL scrambling during vesicle formation. The relationship between calpain-mediated proteolysis and vesicle formation is still controversial. Some discrepancies between different reports could be due to the use of different calpain inhibitors or to the different technical procedures used to monitor vesicle formation: prothrombinase activity in the platelet supernatant (Comfurius et al., 1990), flow cytometry after binding of a GP Ib specific antibody (Wiedmer et al., 1990), and densitometry of ^{3}H-labeled glycoproteins on platelet supernatant fluorogram (Fox et al., 1991). In the present study, vesicle formation was quantitatively evaluated by measuring the PL content in the supernatant of activated platelets. Results obtained with this experimental procedure (i) provide evidence that vesicle shedding strongly depended on calpain activity, in agreement with Fox et al. (1990, 1991) and (ii) ruled out the earlier proposal that the loss of PL asymmetry could be the result of membrane scrambling during vesicle formation (Sims et al., 1989; Comfurius et al., 1990; Bevers et al., 1992).

Vesicles are clearly formed by shedding of the plasma membrane, probably after the secretion events, since the PL and protein contents of vesicles are quite similar to those of the plasma membrane of platelets after secretion (Sandberg et al., 1982; Sims et al., 1988). Several authors have shown that changes in the plasma membrane bilayer balance can influence platelet shape (Ferrell et al., 1988; Suné & Bienvenüe, 1988), as is the case for erythrocytes (Seigneuret & Devaux, 1984; Daleke & Huestis, 1989), in agreement with the bilayer couple model proposed by Sheetz and Singer (1974). Incorporation of amphiphile molecules in the outer leaflet was shown to induce vesiculation of the platelet plasma membrane (Kobayashi et al., 1984; Bevers et al., 1987). Outward movement of aminoPLs during activation, not counterbalanced by reverse PL movement, resulted in excess PLs in the outer leaflet. Consequently, expansion of this leaflet may be related to the formation of very small bending radius shapes like filopodias (Ferrel et al., 1988; Suné & Bienvenüe, 1988), supported by actin filaments in platelets (White, 1991). On the other hand, some submembrane proteins like actin-binding protein and talin have a very important role in maintaining platelet shape by capping actin filaments and anchoring membrane glycoproteins (Wilkinson, 1991). Their proteolysis by calpain during activation thus modifies interactions between the cytoskeleton and the plasma membrane (Davies et al., 1976; Truglia & Stracher, 1981; Fox, 1985; Beckerle et al., 1989), leading to easier vesiculation of this membrane. Indeed, the inhibition of calpain strongly decreased the amount of vesiculation. In summary, membrane budding could be caused by the net PL outflux while vesicles should be shed provided the interactions between the membrane and the cytoskeleton are modified. In agreement with this proposal, it was reported that erythrocytes bud and shed vesicles when the membrane PL transverse distribution is perturbed, e.g., by aging, certain disease states, ATP depletion, or A23187 treatment (Beaudoin & Grondin, 1991).

The molecular mechanism of aminoPL outflux is still unclear. It appears to occur independently of calpain-mediated proteolysis (see above) and without ATP consumption, since ATP depletion in platelets does not modify the rise in prothrombinase activity induced by A23187 (Comfurius et al., 1990). The present study suggests that the mechanism involved is not related to a scrambling phenomenon during vesicle formation. On the contrary, it seems likely that vesiculation is caused by budding of the plasma membrane due to the large excess of PLs on its outer monolayer and to

calpain-mediated proteolysis of submembranous cytoskeletal proteins.

ACKNOWLEDGMENT

We are grateful to the personnel of the Centre Regional de la Transfusion Sanguine in Montpellier for their kind assistance and to J. Sainte-Marie for her critical review of the manuscript.

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