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PHOSPHOLIPID TRANSVERSE MOBILITY MODIFICATIONS IN PLASMA MEMBRANES OF ACTIVATED PLATELETS: AN ESR STUDY

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SUMMARY: Spin labeled phospholipid analogs were used to directly study changes in aminophospholipid translocase activity in activated platelets. In thrombin-activated platelets, the translocase activity was slightly stimulated, whereas no vesicle formation or proteolysis of cytoskeletal protein occurred. Ca^{2+} ionophore A23187-mediated activation produced vesiculation and proteolysis. Additionally, the translocase activity was completely inhibited, probably due to a sharp rise the intracellular Ca^{2+} concentration, as shown when platelets were activated in the presence of various A23187 and Ca^{2+} concentrations and by the recovery of the translocase activity when Ca^{2+} was complexed with EGTA. No translocase activity was found in vesicles. Whereas vesiculation and translocase inhibition can occur independently of proteolysis, this later accentuated the shedding phenomenon.

The procoagulant property of the plasma membrane of activated platelets was demonstrated to be related to the presence of aminophospholipids (aminoPLs), and more potentially of phosphatidylserine (PS), on the outer leaflet [1,2]. In resting platelets [3], as is the case in many eukaryotic cells [4], phosphatidylserine is exclusively located on the inner face of the plasma membrane. The presence of PS on the external membrane leaflet of activated platelets, studied by different methods [2,5,6], could be due to blockade of the aminoPLs translocase, which actively catalyses the outward-inward movement of PS and phosphatidylethanolamine (PE) [4]. In order to directly investigate modifications in translocase activity in activated platelets, we measured PL movements using paramagnetic probes in electron spin resonance (ESR) experiments. The relationships between translocase inhibition and the platelet activation process, vesicle shedding and proteolysis were investigated using two activation inducers (calcium-ionophore A23187 and thrombin) that produce or not a procoagulant surface on platelets [1].

MATERIALS AND METHODS

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

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Biological: Platelets were isolated from fresh human blood anticoagulated with 0.15 vol of ACD (85 mM trisodium citrate, 111 mM dextrose, 71 mM citric acid), using the erythrocyte cushion procedure described by Valone et al.[7]. Briefly, centrifugation steps were performed in 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, and 0.15 vol of ACD at pH 6.4) in the presence of autologous erythrocytes (hematocrit 5%). After washing the cells twice in buffer A, contaminating erythrocytes were removed by centrifugation at 200 x g for 10 min. Platelets were centrifuged at 1000 x g for 15 min and resuspended in buffer B (136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM dextrose, 10 mM Hepes, pH 7.4) at a concentration of 2 x 10^9 platelets/ml. Platelets were allowed to settle for 30 min at 37°C before experimentation. When necessary, the membrane permeant calpain inhibitor calpeptin [8] was added at the beginning of this incubation.

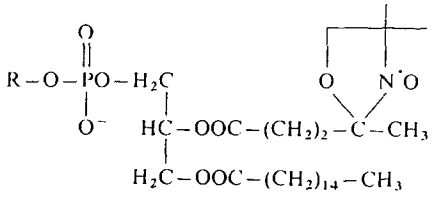
Activation procedures: Extraction of phospholipids for ESR measurements was only possible when the whole external side of the platelet plasma membrane remained accessible to bovine serum albumin (see later). Thrombin activation was thus performed in such a way that no visible aggregation occurred, in the presence of 5 U/ml thrombin and 5 mM EGTA without stirring; for A23187 activation (1 µM final concentration), buffer B was completed with 1 mM $CaCl_2$. When adding EGTA, the free Ca^{2+} concentration was calculated by taking the previously published association constants into account [9,10].

Secretion was measured according to Suné, using (^{3}H)5-HT (5-hydroxytryptamine) (s.a. 1.8 MBq/mmol, Amersham) as a marker for dense granules [3]. Platelet lysis, evaluated by measuring the lactate deshydrogenase activity in supernatant (Sigma kit No 500, St. Louis, USA) [11], never exceeded 2% in any of the performed experiments.

Isolation of vesicles: Vesicles were recovered in the supernatant of centrifuged (11000 x g for 2 min) platelet suspensions [5]. Lipids were extracted according to Folch's method [12]. Phospholipids were measured by spectrophotometry, according to Rouser et al. [13], and compared to the whole cell phospholipid content.

Analysis of cytoskeletal proteins: Platelet proteins were solubilized and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described [14], using a 7.2% homogeneous gel (LKB 2050, Pharmacia, Sweden) stained with Coomassie blue. For analysis of the vesicles protein content, supernatants of activated platelet suspensions were centrifuged at 160000 x g for 5 min. The pellets were solubilized and the proteins separated in the same way as above.

ESR experiments: The following spin-labeled PLs, synthesized as previously described [15], were used:



(0,2)PS R = $-CH_2-CH(COO^-)-N^+H_3$

(0,2)PC R = $-(CH_2)_2-N^+(CH_3)_3$

(0,2)PE R = $-(CH_2)_2-N^+H_3$

Incorporation and evaluation of externally situated paramagnetic probes were performed as previously described [16]. Briefly, 10 min after activation, spin-labels (2% of endogenous PLs) were added to the platelet suspension from a concentrated solution in buffer B. After different incubation times at 37°C, spin-labels remaining on the outer leaflet were extracted by bovine serum albumin at 4°C, reoxidized with ferrycianide and evaluated by ESR. Determination of central line magnitudes were automatically corrected by evaluation of the hydrolysed component [3,16], taking the ratio between the low-field and the 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. When ESR experiments were performed on vesicles, the spin-label concentration was 2-4 µM, and the suspensions were centrifuged at 160000 x g for 5 min at 4°C after BSA-mediated extraction of externally situated spin-labels. Data in figures represent results of at least 4 experiments. The percentage of translocase activity inhibition was calculated considering the initial kinetic rates, Vo and Vi, in resting and completely activated platelets, respectively, and Vm, the initial rate of the kinetic considered,

$$\% \text{ inhibition} = 100 \times (Vo - Vm) / (Vo - Vi).$$

RESULTS AND DISCUSSION

Both activation inducers, thrombin and ionophore A23187, produced similar secretion (70-80% of the dense granule content), but the results were very different for inhibition of translocase, vesicle formation and breakdown of cytoskeletal proteins.

In thrombin-activated platelets, measurements of the transverse mobility of (0,2)PS (Fig. 1 a) and (0,2)PE (not shown) revealed a slightly increased translocase activity compared to resting platelets. This phenomenon could be observed 5 min after thrombin addition. Moreover, the passive movement of (0,2)PC, not related to the translocase activity [17], was increased (Fig.1 b) suggesting that the dynamic membrane properties were modified. This might also have been responsible for the increase in translocase activity (see above), since it was clearly shown that some membrane modifications, such as cholesterol depletion [17] or incorporation of benzyl alcohol [16], increase the translocase activity. Thrombin-induced activation did not lead to vesicle formation, as measured by the absence of PL in the cell supernatant, nor to protein breakdown (Fig. 1 c), in agreement with previous studies [18].

In ionophore A23187-activated platelets (1 μM A23187 and 1 mM Ca^{2+}), translocase activity was strongly inhibited (Fig. 2 a). No differences in the rate of reorientation and the transverse repartition at equilibrium state of the three spin-labeled PLs were detected. Proteolysis (Fig. 2 b) and vesiculation occurred, leading to a substential fragmentation of the membrane system, since 22-25% of the initial cell PLs were found in the vesicles. Protein patterns of vesicles were similar to those of remnant cells, whether or not proteolysis had been inhibited by prior incubation of platelets with calpeptin (Fig. 2 b).

Hence, ESR experiments with spin-labeled PLs allowed direct measurement of the translocase activity after platelet activation and the results were in agreement with those of previous studies using fluorescent probes [19]. Moreover, they confirmed that the exposition of a catalytic surface for the prothrombinase complex on the platelet plasma membrane was in line with the inhibition of the translocase activity maintaining the aminoPLs on the outer layer of activated platelet membrane.

Platelets were activated with different ionophore concentrations in varying external Ca^{2+} conditions (Fig. 3), to determine whether the inhibition of the translocase activity was related to an influx of external Ca^{2+}, as is the case for vesicle formation and proteolysis [14]. In the presence of 1 mM Ca^{2+}.

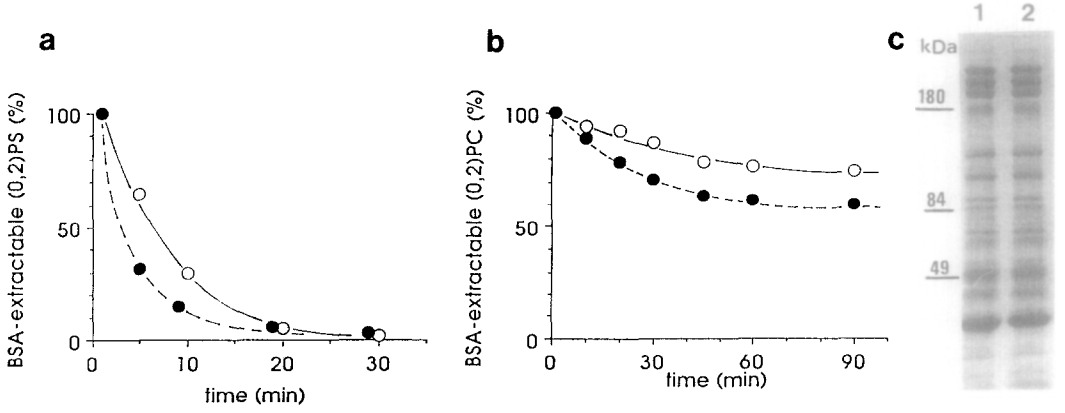


Fig. 1. Transverse reorientation of (0,2)PS (a) and (0,2)PC (b) in thrombin-activated (●) or resting platelets (○). Note the different time-scales. (c) SDS-PAGE of resting (1) and thrombin-activated platelets (2).

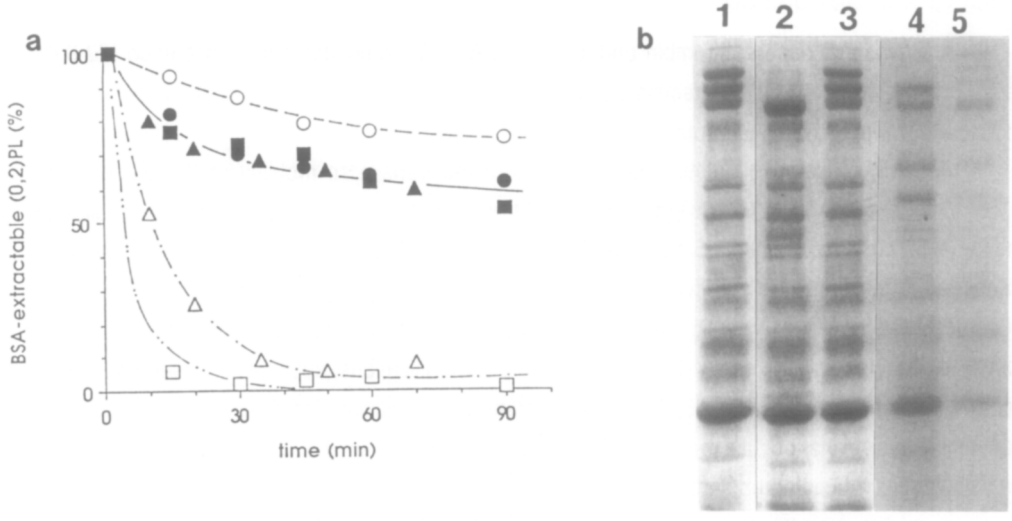
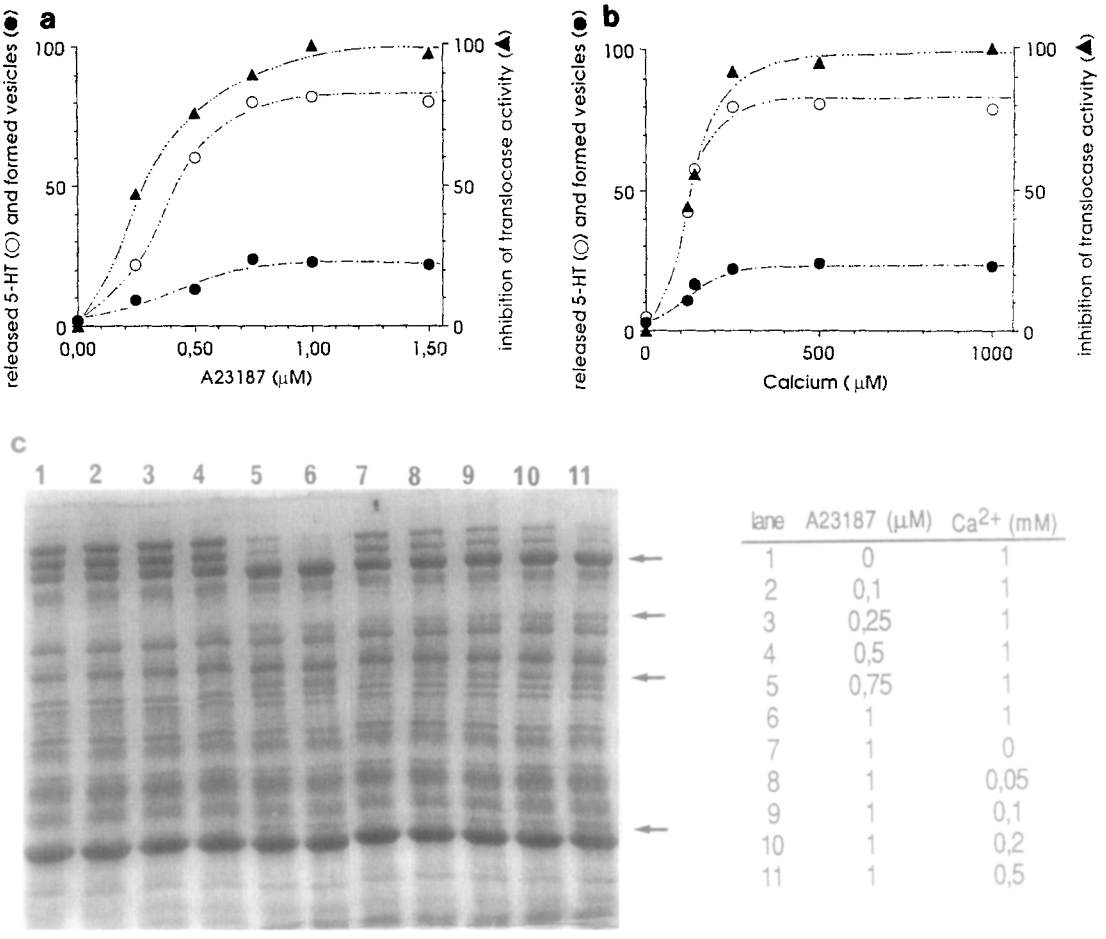


Fig. 2. (a) Transverse reorientation of (0,2)PS (squares), (0,2)PE (triangles) and (0,2)PC (circles) in A23187-activated (full symbols) or resting platelets (empty symbols). (b) SDS-PAGE of resting (1), A23187-activated platelets (2), A23187-activated platelets after preincubation in the presence of 100 µg/ml calpeptin (3), and of vesicles induced by A23187-activation of platelets which had been (4) or not (5) preincubated with 100 µg/ml calpeptin.

degranulation, vesicle formation, inhibition of translocase (Fig. 3 a) and proteolysis (Fig. 3 c) were maximal with 0.75 µM A23187. However, no proteolysis was detected in the presence of 0.5 µM A23187, although vesiculation and (^{3}H)5-HT release occurred and translocase activity was mostly inhibited. In the presence of 1 µM A23187, free Ca^{2+} concentrations above 100 µM induced vesicle shedding and inhibition of translocase activity. This activity was completely inhibited with 250 µM Ca^{2+}, with shedding of vesicles being maximal (Fig. 3 b), in agreement with Wiedmer et al. [14]. Regardless of the A23187 and Ca^{2+} concentrations, the protein patterns in the vesicles, when present, were identical to those in remnant cell (not shown).

The concentration of external Ca^{2+} (250 µM) required to induce full activation was quite high relative to the intracellular free Ca^{2+} concentrations in resting platelets (100 nM) and in physiologically activated platelets (0,5 µM) [1]. It is possible that the influx of external Ca^{2+} during A23187-activation led to invasion of the whole cell by free Ca^{2+}, whereas physiological activation only induced local Ca^{2+} increases. Moreover, A23187 concentrations higher than 0.5 µM were necessary to activate platelets in the presence of 1 mM Ca^{2+} (Fig. 3 b). The high number of ionophore molecules per cell even in the presence of low A23187 concentrations (< 0.5 µM) suggested that the final intracellular Ca^{2+} concentration was higher than 250µM. The velocity of Ca^{2+} invasion could thus be an important parameter for the platelet response.

The dependence on Ca^{2+} was also shown by the restoration of translocase activity inhibited after A23187-induced activation by adding excess external EGTA (2 mM) (Fig. 4 a). Nevertheless, the location of (0,2)PS in the inner layer was only partially recovered after a few minutes, since 25% of the labeled PS remained extractable by BSA. According to Comfurius et al. [5], inhibition of calpain and complexation of Ca^{2+} were both required to inhibit the prothrombinase activity of A23187-activated platelets. We therefore investigated the effect of calpeptin, a calpain inhibitor [8], on the EGTA-mediated restoration of translocase activity. The addition of 2 mM EGTA 2 min after A23187 to platelets preincubated in the presence of 100



lane	A23187 (μM)	Ca^{2+} (mM)
1	0	1
2	0,1	1
3	0,25	1
4	0,5	1
5	0,75	1
6	1	1
7	1	0
8	1	0,05
9	1	0,1
10	1	0,2
11	1	0,5

Fig. 3. (a) Effect of A23187 concentration on (^{3}H)5-HT release (○), vesicle formation (●), and inhibition of translocase activity (▲) in the presence of 1 mM Ca^{2+} (see Methods). (b)same experiment as a), but in the presence of 1 μM A23187 and various amounts of free Ca^{2+}. (c) SDS-PAGE of platelets activated under the conditions described in the figure.

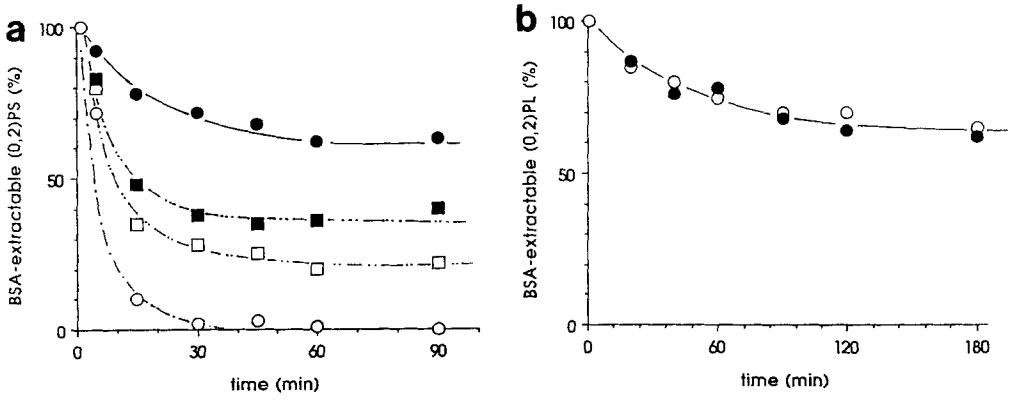


Fig.4. (a) Effect of EGTA on the transverse reorientation of (0,2)PS in A23187-activated platelets: resting platelets (○), A23187-activated platelets (●), activated platelets in the presence of 2 mM EGTA after (■) or not (□) preincubation in the presence of 100 μg/ml calpeptin. (b) transverse reorientation of (0,2)PS (○) and (0,2)PC (●) in isolated vesicles.

μg/ml calpeptin restored the translocase activity, but to a lesser extent than when added to untreated platelets. Our results agree with those of Wiedmer et al. , who also failed to inhibit expression of prothrombinase activity and vesicle formation by inhibiting calpain action [14], but contrast with those of Comfurius et al. [5], suggesting that the relationship between prothrombinase activity and external localization of PS is questionable in some cases.

In vesicles, the kinetics of relocation of (0,2)PS or (0,2)PC were similar, and led to a symmetric distribution of these PLs in the vesicle membrane (Fig. 4). The same results were obtained when 2 mM EGTA was added to the vesicle suspension in order to complex free Ca^{2+}. This complexing of Ca^{2+} is thus not sufficient to restore their translocase activity. Since the major platelet proteins are present in vesicles [14,20], it seems likely that the vesicles also contain the translocase protein. Since the vesicles were shown to be devoid of ATP (unpublished results), the ATP-dependent translocase could not function. When spin-labels were added to A23187-activated platelets, the probes were incorporated into vesicles and cell membranes in proportion to their PL content. In the presence of EGTA, probes incorporated in remnant cells (75-80%) were quickly displaced from the outer leaflet, while those incorporated in the vesicles (20-25%) remained on the external side of the membrane. This would explain the apparent EGTA-induced incomplete reversion shown in Fig. 4 a.

Although the proteolysis of some cytoskeletal proteins is clearly an important mechanism of platelet activation, its role remains controversial [1,14,20]. Our experiments showed that: i) protein patterns were identical in vesicles and remnant cells (Fig. 2 b); ii) the inhibition of calpain by calpeptin (Fig. 3 c) led to reduced vesicle formation (4-6% of whole cell PLs versus 20-25% in the absence of calpeptin) during A23187-activation; iii) under some experimental conditions (0.5 μM A23187 and 1 mM Ca^{2+}), platelets were activated (including vesicle formation) without any proteolysis (Fig. 3 a and c). Moreover, the extent of translocase inhibition was independent of previous calpain inhibition (not shown). The translocase activity was restored by complexing free Ca^{2+} but to a lesser extent when the platelets had been preincubated in the presence of calpeptin (Fig. 4 a). Hence, vesicle formation and inhibition of translocase appeared concomitantly, in terms of the activation conditions, and quite independently of proteolysis. However, when proteolysis occurred, the vesicle shedding was increased. Vesicles appeared to be unable to actively translocate aminoPLs. Physiologically, these structures could be involved in scattering catalytic sites for the coagulation cascade around vascular injuries.

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