Reconstitution of Phospholipid Scramblase Activity from Human Blood Platelets†

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Received March 20, 1996; Revised Manuscript Received April 25, 1996⊗

ABSTRACT: Cellular activation, accompanied by elevation of cytoplasmic Ca^{2+} levels, can induce a progressive loss of plasma membrane phospholipid asymmetry, resulting from increased transbilayer movement (flip-flop) of phospholipids. While this process has been demonstrated in a variety of different cells, it is most active in blood platelets. In order to test whether this lipid scrambling process is mediated by a membrane protein, platelet membranes were solubilized in cholate and fractionated by anion exchange chromatography, and fractions were reconstituted into phospholipid vesicles by detergent dialysis in the presence of small amounts of fluorescent (NBD) phospholipids. Using dithionite reduction to monitor the transbilayer location of NBD phospholipids, it was shown that addition of Ca^{2+} and ionomycin to vesicles reconstituted with a particular fraction results in transbilayer movement of the fluorescent phospholipid analogs from the vesicle's inner to outer leaflet. Lipid vesicles reconstituted in the absence of membrane protein, or reconstituted with another platelet membrane protein fraction, were devoid of this activity. Heating the active fraction or incubating it with pronase or the SH reagent pyridyldithioethylamine markedly diminished the ability of the vesicles to translocate fluorescent phospholipid analogs across the bilayer in response to Ca^{2+} and ionophore. These results argue that a membrane protein (or proteins) from blood platelets is required to catalyze Ca^{2+}-induced transbilayer movement of phospholipids, suggesting its (or their) involvement in the loss of lipid asymmetry that can occur during cellular activation.

Transbilayer phospholipid asymmetry is a well-known characteristic of mammalian plasma membranes: the outer leaflet of the lipid bilayer is rich in the cholinephospholipids, sphingomyelin and phosphatidylcholine (PC),[1] whereas the aminophospholipids, phosphatidylethanolamine and phosphatidylserine (PS), preferentially reside in the inner leaflet [Bretscher, 1972; reviewed in Schroit and Zwaal (1991), Zachowski (1993), and Williamson and Schlegel (1994)]. The existence of an ATP-dependent aminophospholipid translocase, which concentrates aminophospholipids in the inner membrane leaflet (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Connor & Schroit, 1988), suggests that the orientation of these lipids is a critical aspect of cell function. Whereas phospholipid asymmetry is generally maintained during the life span of a cell, cellular activation can induce rapid transbilayer movement of the lipids (flip-flop) and randomization of the phospholipid distribution, as was first demonstrated for blood platelets (Bevers *et al*., 1983). This lipid scrambling process results in surface exposure of PS which promotes blood coagulation (Zwaal *et al*., 1978; Rosing *et al*., 1985), as well as cell−cell recognition and phagocytosis (Schroit *et al*., 1985; Schlegel & Williamson, 1987; Fadok *et al*., 1992; Verhoven *et al*., 1995). Since rapid loss of phospholipid asymmetry is not induced by simple inhibition of aminophospholipid translocase, it has been suggested (Zwaal *et al*., 1993) that a separate activity (phospholipid scramblase) mediates rapid flip-flop of all the major phospholipid classes in response to elevation of intracellular Ca^{2+} levels (Williamson *et al*., 1992; Smeets *et al*., 1994). We describe here the reconstitution of a membrane protein fraction from human platelets with properties of this phospholipid scramblase activity.

MATERIALS AND METHODS

Platelet Membrane Proteins. Human platelets, isolated as described previously (Bevers *et al*., 1983), were suspended in TEMS buffer (50 mM Tris, 0.2 mM EGTA, 1 mM Mg^{2+}, 120 mM NaCl, pH 7.5) at a concentration of 5×10^8/mL. PMSF and EGTA were added to this suspension to a final concentration of 0.5 mM. Platelets were subjected to three cycles of freezing and thawing. Membrane material from 10^9 platelets was collected by centrifugation (10 min, 10 000*g*). Pellets were solubilized in TEMS containing 5% cholate (w/v) and 0.5% Triton X-100 (w/v). The detergent insoluble membrane skeleton (mainly composed of myosin, MW 220 kDa, and actin, MW 43 kDa) was removed by centrifugation. Solubilized material was applied to a QAE ion exchange column of 20 mL (Dowex AG 1-X8, Bio-Rad), equilibrated with TEMS containing 5% cholate (TEMS-cholate). After elution of nonbound protein with TEMS-cholate, a linear NaCl gradient was applied, from 0 to 3 M

† Supported by Grant AI 26641 (P.W. and R.A.S.) from the United States Public Health Service.

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PC, phosphatidylcholine; PIP-2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PDA, pyridyldithioethylamine; PMSF, phenylmethylsulfonyl fluoride.

S0006-2960(96)00685-X CCC: $12.00

NaCl in TEMS-cholate, total volume 100 mL. Optical density at 280 nm was recorded.

Reconstitution Procedure. A lipid stock solution in chloroform/methanol (1/1; v/v) containing 9 mM PC (Sigma, from egg yolk), 1 mM PS (Sigma, from bovine brain), 2.5 mM cholesterol, and 20 μM NBD-PS (or NBD-PC, both obtained from Avanti Polar Lipids, Alabaster, AL) was prepared [NBD-PS: 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-oleoyl-*sn*-glycero-3-phosphoserine]. 100 μL of this solution was dried by flushing with nitrogen. 1 mL of a QAE-column sample was added to the lipid film. After solubilization, the mixtures were allowed to equilibrate at 37 °C for 60 min. The samples were dialyzed overnight in the dark against 25 mL of TEMS at room temperature, followed by two times 3 h against 1 L of TEMS. Finally, to all samples was added 1 g of washed Bio-Beads SM2 (obtained from Bio-Rad and prepared by washing with methanol followed by extensive washing with distilled water) to remove traces of cholate and Triton X-100. The tubes were rotated overnight in the dark. Resulting vesicles were examined by cryoelectron microscopy as described (Frederik *et al*., 1991).

Measurement of Scramblase Activity. The amount of NBD lipid residing in or appearing at the outer surface of reconstituted vesicles was assessed using the membrane impermeable reducing agent sodium dithionite ($Na_2S_2O_4$) which converts the NBD group to the nonfluorescent 7-amino derivative (McIntyre & Sleight, 1991). To 2 mL of TEMS in a fluorimeter cuvette (37 °C) was added 50 μL of the vesicle suspension, giving a final lipid concentration of approximately 25 μM. Ionomycin was added to a final concentration of 0.5 μM, followed 30 s later by 7.5 mM dithionite. After 60 s to allow reduction of the NBD groups present in the outer leaflet, recording of the residual fluorescent signal was begun. After 1 min, either calcium (1 mM final concentation) or EGTA (0.2 mM) was added. The traces shown were obtained by subtracting the values for fluorescence intensity (sampled at 2 s intervals) in the absence of Ca^{2+} (I_t^{EGTA}) from the corresponding values obtained after the addition of Ca^{2+} to a final concentration of 1 mM (I_t^{Ca}) and then reducing the original initial fluorescence (I_0) by the resulting difference (in formula: $I_t = I_0 - \{I_t^{Ca} - I_t^{EGTA}\}$). The resulting curves represent the Ca^{2+}-inducible change in the rate of NBD reduction, interpreted as outward movement of NBD-PS or NBD-PC.

Electrophoresis. SDS−PAGE was carried out according to the procedure of Laemmli (1970), using 1.5 mm slab gels. Briefly, total membrane material was solubilized in sample buffer [100 mM Tris, 5 mM EDTA, 2% SDS (w/v), 5 mM dithiothreitol, 10% glycerol (w/v), pH 8]. Samples from the QAE-column were extensively dialyzed against distilled water to remove cholate and treated with Bio-Beads SM2 to remove last traces of detergent including Triton X-100. After dialysis, the proteins were concentrated by precipitation with 4% (w/v) trichloroacetic acid, followed by solubilization in and dialysis against TEMS. To 100 μL of these samples was added 25 μL of five-times concentrated sample buffer prior to electrophoresis.

RESULTS AND DISCUSSION

Human platelet membranes were solubilized in sodium cholate and fractionated by anion exchange chromatography

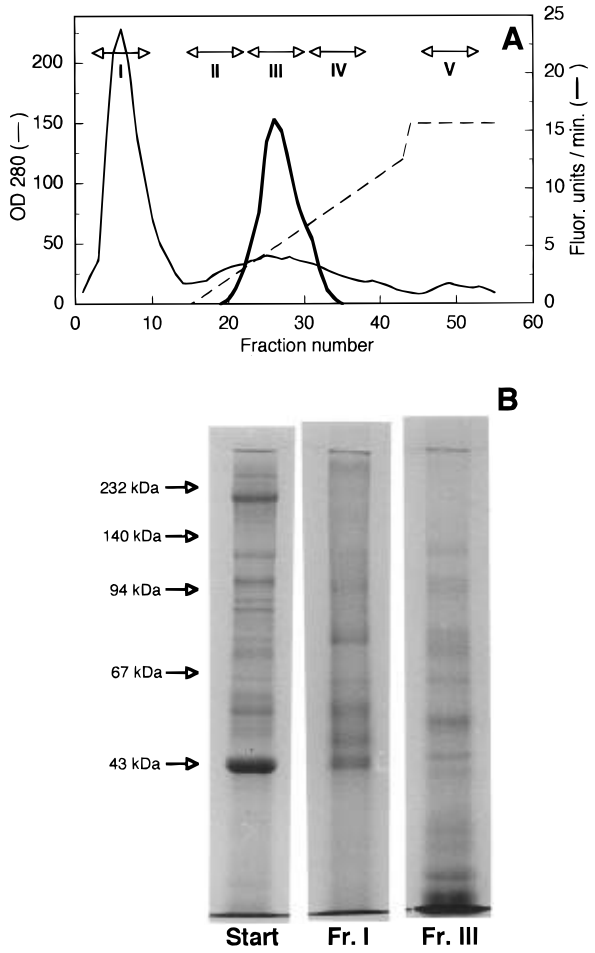

FIGURE 1: Fractionation of platelet plasma membrane proteins. Panel A, elution with NaCl gradient of bound proteins from a QAE column (salt gradient is shown as the dashed line). Protein content was measured by the optical density at 280 nm; scramblase activity is measured as initial change in fluorescence intensity as described in Materials and Methods. Pooled fractions are indicated by roman numerals. Panel B, gel electrophoretic pattern of total membrane proteins before solubilization (start), protein not bound to the QAE-column (Fr. I) and the proteins in the active fraction (Fr. III).

(Figure 1A). SDS−PAGE of the different column fractions revealed that most of the platelet membrane proteins with a molecular weight above 43 kDa are present in fraction I, whereas the active (see below) fraction III is clearly enriched in proteins between 20 and 50 kDa (Figure 1B). Pooled fractions were reconstituted into defined lipid vesicles, containing 2 mol % of the fluorescent lipid analog NBD-PS, followed by extensive dialysis and treatment with Bio-Beads to remove detergent. In all cases, this protocol resulted in formation of unilamellar vesicles with a diameter of approximately 20−40 nm (Figure 2).

The presence of Ca^{2+}-inducible scramblase activity in the reconstituted vesicles was tested by measuring the movement of NBD-PS from the inner leaflet to the vesicle surface induced by the addition of Ca^{2+} and the Ca^{2+} ionophore ionomycin. The method used was based on the principle (McIntyre & Sleight, 1991) that the fluorescent NBD moiety in phospholipids in the external leaflet can be reduced to the nonfluorescent 7-amino derivative by reaction with dithionite, a membrane impermeant anion. After reconstitution, about 30% of the added NBD-PS was in the internal leaflet of impermeable vesicles, as judged by its resistance to externally added dithionite. In vesicles without protein,

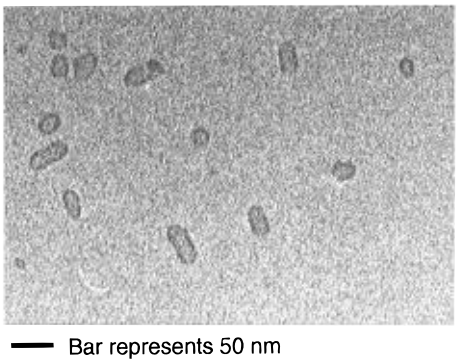

FIGURE 2: Reconstituted vesicles visualized by cryoelectron microscopy.

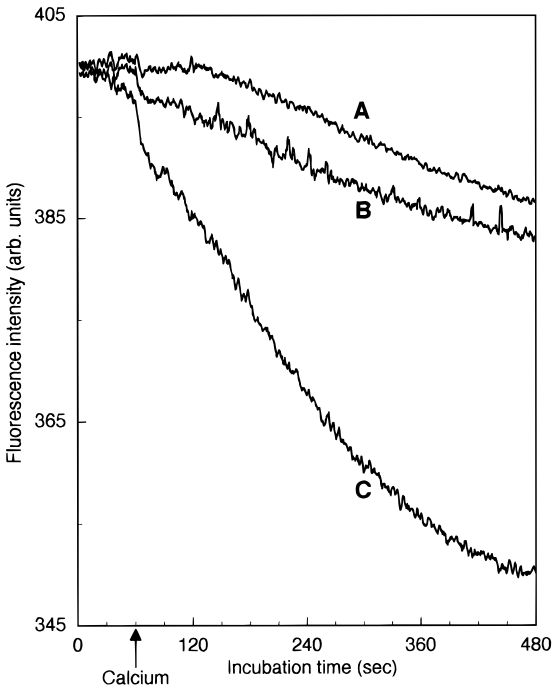

FIGURE 3: Ca^{2+}-dependent reduction in fluorescent signal of reconstituted vesicles. Trace A, no protein reconstituted. Trace B, non-bound protein from the QAE column (fraction I) reconstituted. Trace C, top fraction of the activity profile (fraction III) reconstituted. For details see Materials and Methods.

this resistant probe is slowly reduced in the continued presence of dithionite (Figure 3), presumably reflecting a combination of slow probe migration to the vesicle surface and slow penetration of the dithionite to the vesicle interior. As also shown in Figure 3, in vesicles reconstituted with one of the fractions from the QAE column (fraction III), addition of Ca^{2+} in the presence of ionomycin induces an instantaneous elevation in the rate of reduction of the fluorescent signal, suggesting that the rate of NBD-PS movement to the surface is elevated. This increase in the rate of probe reduction was insensitive to the concentration of external dithionite (data not shown), indicating that it does not reflect more rapid penetration of the dithionite into the vesicle interior. Identical treatment of the vesicles with ionophore in medium lacking Ca^{2+} does not induce an increase in the rate of NBD-PS reduction. Also, treatment with Ca^{2+} and ionomycin of vesicles reconstituted with protein fractions other than fraction III does not appreciably induce this activity (Figure 3).

It has been recently proposed that a complex between Ca^{2+} and the metabolic-resistant pool of phosphatidylinositol 4,5-bisphosphate (PIP-2) mediates Ca^{2+}-induced lipid scrambling in erythrocyte membranes, without involvement of a membrane protein (Sulpice *et al.*, 1994). However, addition of 1 mol % of PIP-2 to the lipid mixture prior to reconstitution with the various protein fractions had no effect on the observed activity, suggesting that this lipid was neither the source of the observed activity, nor a required cofactor. This agrees with findings on intact cells which also exclude a major role for PIP-2 in the process of lipid scrambling (Bevers *et al.*, 1995). To test whether the active principle in fraction III is a protein, the mixture was subjected to heat treatment or incubation with pronase prior to the reconstitution procedure. In both cases, the capacity to induce rapid surface exposure of NBD-PS following addition of ionomycin was markedly reduced (Figure 4). In addition, treatment of fraction III with the SH-reactive compound PDA resulted in marked inhibition of the scrambling activity (Figure 4), which is consistent with observations on platelets where treatment with PDA also resulted in a substantial but not complete loss of scramblase activity (Williamson *et al.*, 1995). The present observations strongly suggest that the activity in fraction III is a protein. Moreover, when the protein-to-lipid ratio during reconstitution was changed, a corresponding change in the scrambling activity was observed (data not shown). The observation that surface exposure of NBD-PS in vesicles containing fraction III does not occur to any appreciable extent in the presence of Ca^{2+} without ionomycin suggests that this protein is largely reconstituted in the same orientation as in platelet membranes.

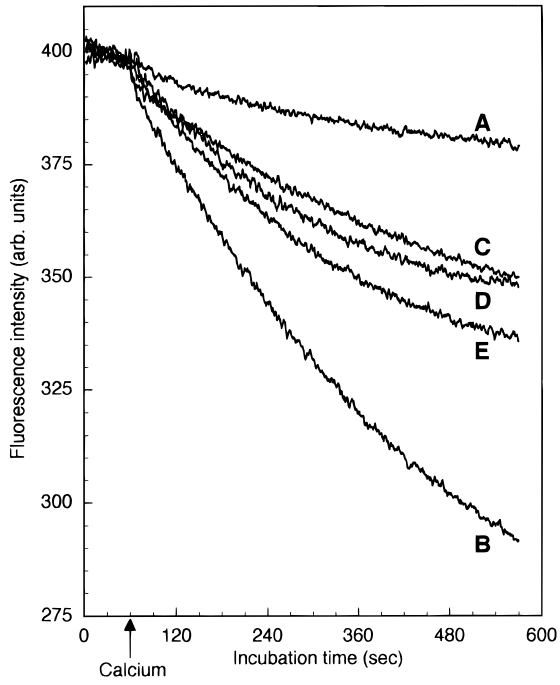

FIGURE 4: Effects of heat, proteolysis, or blocking of SH-groups on scramblase activity. Prior to reconstitution, samples of the most active fraction from a QAE column procedure (fraction III) were treated in different ways. Trace A, no protein reconstituted. Trace B, fraction III incubated for 60 min at 37 °C. Trace C, fraction III incubated for 60 min with 5 mM PDA at 37 °C. Trace D, fraction III incubated for 60 min at 100 °C. Trace E, fraction III incubated for 60 min at 37 °C with 0.1 mg of pronase/mL. The samples were reconstituted immediately after treatment and scramblase activity was measured as described in Materials and Methods.

In both erythrocytes (Williamson *et al*., 1992) and platelets (Smeets *et al*., 1994; Williamson *et al*., 1995) the Ca^{2+}-induced scrambling activity, unlike aminophospholipid translocase, is ATP-independent and moves zwitterionic lipids at the same rate as PS. To test whether the activity in fraction III shares this property, the reconstitutions were carried out with lipid mixtures containing NBD-PC instead of NBD-PS. Addition of ionomycin in the presence of Ca^{2+} resulted in externalization of this lipid as well, at rates similar to those seen with NBD-PS, indicating that the lipid-translocating activity in the reconstituted vesicles, like the *in situ* activity, does not discriminate between these lipid classes.

The decrease in fluorescence signal in about 8 min, which results from addition of Ca^{2+}/ionophore to vesicles reconstituted with fraction III, varies between 10% and 25% of the starting signal in different experiments (cf. Figures 3 and 4). This observation implies that the level of activity is lower than that found in the original platelet membrane, where Ca^{2+}-dependent lipid movement has been shown to result in an almost complete scrambling of lipids within 2 min, as evidenced by the ability of the activated platelets to promote prothrombinase activity (exposure of endogenous PS) or externalize previously internalized NBD-PS (Comfurius *et al*., 1990; Williamson *et al*., 1995). Moreover, storage of the fractions prior to reconstitution, even in the presence of protease inhibitors and thiols, leads to gradual loss of activity (after reconstitution) over a period of 48 h, suggesting that a substantial amount of the activity may have been lost during isolation. However, reconstitution of the active protein fraction in lipid vesicles results in partial protection against inactivation.

Attempts to further purify the protein have thus far resulted in inactive preparations. Moreover, the electrophoresis patterns of fractions II and IV (not shown) have a heterogeneous character and exhibit considerable overlap with the protein pattern of fraction III (Figure 1B), thus precluding assignment of scramblase to a specific protein band. Also, it cannot be excluded that active scramblase is composed of different protein subunits that may appear in separate fractions upon further purification. However, the methods described form the basis for further isolation and molecular characterization of the protein(s) responsible for scramblase activity. An improved understanding of this activity will be essential to elucidating the mechanisms and consequences of the activation of lipid scrambling that occurs in activated blood platelets (Bevers *et al*., 1983), Ca^{2+}-loaded normal erythrocytes (Williamson *et al*., 1992), diabetic erythrocytes (Wilson *et al*., 1993), apoptotic lymphocytes (Fadok *et al*., 1992; Verhoven *et al*., 1995), and tumorigenic cells (Connor *et al*., 1989). It will also be of interest to learn whether the scramblase is involved in the genetic defect in lipid scrambling in blood platelets underlying Scott syndrome, a bleeding disorder (Kojima *et al*., 1994; Toti *et al*., 1996).

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BI9606859