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Biochimica et Biophysica Acta 1195 (1994) 281–286

BBA Biochimica et Biophysica Acta

Calcium-induced transbilayer scrambling of fluorescent phospholipid analogs in platelets and erythrocytes

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Received 26 May 1994

Abstract

The non-random distribution of phospholipids in the plasma membrane of human platelets and erythrocytes is at least partially maintained by the ATP-dependent aminophospholipid translocase, but can be disturbed by a calcium-induced scrambling of lipids. Using fluorescent NBD-phospholipid analogs, we demonstrate that in both cells the aminophospholipid translocase has a slightly higher preference for the naturally occurring L-isomer of the polar headgroup of phosphatidylserine as compared to the D-isomer. Calcium-induced outward movement of internalized phosphatidylserine probe, however, is not affected by the stereochemical configuration of the serine headgroup and is virtually identical to both the inward and outward movement of the phosphatidylcholine probe. The data also indicate that both in platelets and red blood cells the calcium-induced transbilayer movement is bidirectional and involves all major phospholipid classes, with reorientation rates of sphingomyelin being appreciably lower than that of the other phospholipid classes. While our results largely support earlier observations on red cells, they clearly differ from a recent study on platelets which suggested that calcium-induced scrambling is restricted to aminophospholipids and would not involve cholinephospholipids. The present results indicate that the same mechanism is responsible for calcium-induced lipid scrambling in red blood cells and platelets.

Keywords: Flip-flop; Phospholipid; NBD-phospholipid; Aminophospholipid translocase; Phosphatidylserine; Membrane asymmetry

1. Introduction

A characteristic feature of blood cell membranes is that the various phospholipid classes are distributed in a non-random fashion over both halves of the bilayer. In quiescent cells, the choline containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM), preferentially reside in the outer leaflet, whereas the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are predominantly present in the inner leaflet [1,2]. Maintenance of membrane phospholipid asymmetry is controlled by an aminophospholipid translocase, a putative integral membrane protein which selectively transports PE and PS from the outer to the inner leaflet of the plasma membrane at the expense of ATP [3–10]. In addition, interaction of aminophospholipids, in particular PS, with the membrane skeleton may contribute to the maintenance of phospholipid asymmetry [11–16], but the importance of this contribution has been disputed [17–19].

Although the non-random distribution of lipids is maintained throughout the life span of the cell, certain conditions may give rise to an appreciable loss of lipid asymmetry in red blood cells and platelets [20–25]. The mechanisms which govern this scrambling process are poorly understood. An increase of the cytoplasmic calcium concentration seems to be an essential requirement. The tight coupling between loss of lipid asymmetry and shedding of microvesicles from the plasma membrane has led to the suggestion that membrane fusion processes during microvesicle formation are responsible for lipid scrambling [22,23]. This hypothesis has been challenged, however, by several investigators who proposed that lipid scrambling may be the cause rather than the effect of microvesicle formation [25–27]. While it has been suggested that Ca-induced rearrangement of the lipids is mediated by a membrane protein [27,28], it has recently been proposed that

Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PLS, phosphatidyl-L-serine; PDS, phosphatidyl-D-serine; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl.

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SSDI 0005-2736(94)00205-3

this process depends on the interaction of calcium with phosphatidylinositol 4,5-bisphosphate, and does not require the involvement of a specific membrane protein [29].

Lipid scrambling serves an important physiological function: in platelets this process leads to exposure of anionic phosphatidylserine molecules that provide binding sites for the assembly of enzyme complexes essential for the blood clotting process [2,30]. In addition, surface exposure of phosphatidylserine also forms a signal for rapid removal of cells by macrophages [31,32].

Transbilayer movement of phospholipids in cells can be studied by means of exogenously added lipid probes which are readily incorporated in the plasma membrane. The use of spin-labeled and fluorescent phospholipid analogs in human erythrocytes has revealed that Ca-induced transbilayer movement permits diffusion of all major phospholipid classes and does not distinguish between the two types of probes [24]. In contrast, studies with human platelets employing spin-labeled probes have suggested that ionophore-activation results in a sudden transmembrane outflux of aminophospholipids which is not counterbalanced by a reciprocal influx of cholinephospholipids [25]. While this may suggest that the mechanism of transbilayer reorientation in platelets is different from that in red cells, this is difficult to reconcile with observations on Scott syndrome, a bleeding disorder where Ca-induced scrambling of the lipids is equally deficient in both platelets and erythrocytes [33]. The purpose of the present study was to asses, under comparable conditions, the in- and outward transbilayer movement of different fluorescent phospholipid analogs in platelets and erythrocytes following Ca-influx. With respect to the transbilayer movement of PS, we also investigated the influence of the stereochemical configuration of the serine headgroup.

2. Materials and methods

2.1. Chemicals

Calcium ionophore A23187, ionomycin, fatty acid-free human- and bovine serum albumin, D-serine and thapsigargin were from Sigma (St. Louis, MO). The thrombin-specific chromogenic substrate S2238 was from AB Kabi Diagnostica (Stockholm, Sweden).

2.2. Proteins

Bovine prothrombin was purified as described by Owen et al. (1974). Bovine factor Xa was a kind gift of Dr. Rosing (Department of Biochemistry, University of Limburg) and was prepared according to Fujikawa et al. [34]. Bovine factor Va was obtained from Dr. Wagenvoort (Department of Biochemistry, University of Limburg) and was purified according to Lindhout et al. [35].

2.3. Lipids

1-Oleoyl-2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)caproyl-*sn*-glycero-3-phosphocholine (18:1/C6-NBD-PC), 1-palmitoyl-2-(C6-NBD)-*sn*-glycero-3-phosphocholine (16:0/C6-NBD-PC), and the corresponding phosphatidyl-L-serine analogs (18:1/C6-NBD-PLS and 16:0/C6-NBD-PLS) were obtained from Avanti Polar (Alabaster, AL). The two phosphatidylserine analogs containing the D-isomer of serine (18:1/C6-NBD-PDS and 16:0/C6-NBD-PDS) were synthesized by base exchange of the corresponding NBD-PC probes catalyzed by phospholipase D from *Streptomyces* species [36] in the presence of octyl glucoside as described before [37]. NBD-sphingomyelin (NBD-SM) was obtained from Molecular Probes (Eugene, OR).

2.4. Isolation of cells

Human blood from healthy volunteers was freshly drawn on acid-citrate-dextrose (ACD). Washed platelets were obtained by differential centrifugation, as described before [20]. Platelets were resuspended in Hepes buffer composed of 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose, adjusted to pH 7.4 with NaOH, and used within 4 h at a concentration of $2 \cdot 10^8$/ml. Washed erythrocytes were obtained by differential centrifugation, resuspended in a Tris buffer composed of 50 mM Tris and 120 mM NaCl, adjusted to pH 7.5 with HCl, and used within 24 h at a concentration of $2 \cdot 10^8$/ml. Shortly before activation of the cells, $CaCl_2$ was added to a final concentration of 3 mM in the case of platelets or 1 mM in the case of erythrocytes. Platelet activation by thapsigargin plus thrombin was performed as described before [39]. All experimental procedures with platelets were performed in Hepes buffer, and those with erythrocytes in Tris buffer.

2.5. NBD-phospholipid studies

Transbilayer movement of NBD-labeled phospholipids was measured using the back-exchange procedure described by Connor and Schroit [7]. Briefly, NBD-labeled phospholipids were added to a final concentration of 1 μM from 1000-fold concentrated stock solutions in dimethyl sulfoxide or ethanol to cell suspensions of $2 \cdot 10^8$/ml, corresponding to approx. 1.25 mol% of the endogenous platelet- and red cell phospholipids. To determine the amount of total fluorescence at a given time point after addition of the NBD-phospholipid, 100-μl aliquots of cell suspension were mixed with 1 ml buffer (pH 7.5). To remove NBD-phospholipid from the outer cell membrane monolayer, 1 ml of the same buffer supplemented with 1% (w/v) bovine serum albumin was used. After centrifugation for 3 min at $12000 \times g$, pelleted cells were solubilized in 1 ml of 1% (w/v) Triton X-100. NBD fluorescence was measured (λ_{ex} 472 nm, λ_{em} 534 nm) on a

Shimadzu RF-5001PC spectrofluorometer (Shimadzu Europe, Duisburg, Germany). NBD-signal remaining in the cellular fraction after extraction with bovine serum albumin was considered to be present at the cytoplasmic leaflet of the cell membrane and is referred to as percentage of non-exchangeable NBD-phospholipid after comparison with the total fluorescence at the same time point. In experiments with activated cells, inward movement (flip) of lipid probes was measured by adding the probes just before the activation procedure. To determine outward movement (flop), quiescent cells were preloaded with NBD-lipid for time periods indicated, and subsequently washed with 1% BSA to remove residual probe from the external leaflet after which the activation procedure was started.

For unknown reasons, the fluorescence intensity of the NBD-lipid probes incorporated in platelets decreased in time. A 2-h incubation resulted in approx. 70% loss of fluorescence intensity, independent of the type of lipid probe used. This loss could be reduced to approx. 5% in 2 h, by pretreating the platelets with 200 μM phenylmethylsulfonyl fluoride during the first washing procedure. This treatment did not affect the functionality of the platelets as verified by their response to thrombin. Loss of fluorescence intensity was not observed with erythrocytes.

3. Results

3.1. Inward transport of NBD-phospholipids in non-acivated cells

Inward transport of NBD-PS is dependent on the activity of the aminophospholipid translocase, a putative integral membrane protein, demonstrated in a variety of cells, particularly in red blood cells and platelets. In order to investigate whether the stereochemical configuration of the serine moiety of phosphatidylserine affects the transport kinetics of this protein, we have compared the inward movement of NBD-labeled phosphatidyl-L-serine (NBD-PLS) and its D-stereoisomer (NBD-PDS) in platelets and erythrocytes. Fig. 1 shows the percentage non-exchangeable NBD-lipid as a function of time in quiescent human platelets. A small but consistent difference in the extent and rate of transport was observed between 18:1/C6-NBD-PLS and 18:1/C6-NBD-PDS. The equilibrium distribution of the 18:1/C6-NBD-PLS was constantly found to be higher (85%) than that observed for the PDS-analog (75%). Halftimes of translocation were reproducibly found to be slightly faster for NBD-PLS (approx. 3 min) than for NBD-PDS (approx. 4 min), although this difference did not reach significance. We have previously shown that in comparison to the 18:1 derivative, the rate of inward transport of 16:0/C6-NBD-PS is lower both in platelets and in erythrocytes [9]. When the 18:1 analogs of both stereoisomers were replaced by their respective 16:0

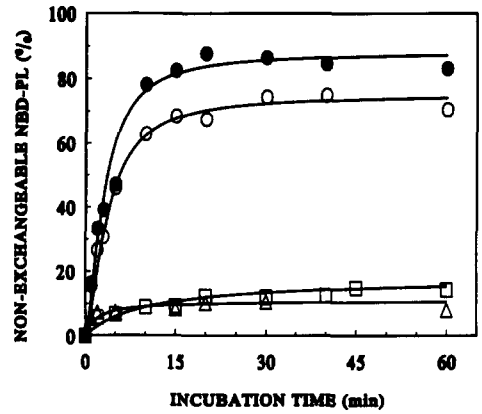

Fig. 1. Inward translocation of 18:1/C6-NBD-phospholipids in quiescent human blood platelets. 18:1/C6-NBD-phospholipids were added to platelet suspensions ($2 \cdot 10^8$/ml) to a final concentration of 1 μM. At different time points, percentage of non-exchangeable NBD-phospholipid (NBD-PL) was determined as described in Section 2. Symbols: ●, NBD-PLS; ○, NBD-PDS; □, NBD-PC; △, NBD-SM. Data shown are from a single experiment representative of five independent experiments so performed.

analogs, a similar difference in transport between the L- and D-isomer was found (data not shown). As expected, minor amounts of NBD-labeled choline-phospholipids were found to migrate to the inner monolayer: 15% of the NBD-PC and 10% of the NBD-SM were found to be non-exchangeable after 60 min incubation (Fig. 1).

The inward movement of the same NBD-phospholipids in erythrocytes is shown in Fig. 2. 18:1/C6-NBD-PLS was translocated with a halftime of 14 min, which is in good agreement with the literature [7,9,38]. However, the rate of inward transport of the 18:1/C6 NBD-PDS was found to be considerably lower than that observed for the L-isomer: the time required for half maximal translocation was estimated to be 36 min. Also, a consistent difference in the equilibrium distribution was observed: 83–85% of the NBD-PLS was present in the inner leaflet after overnight

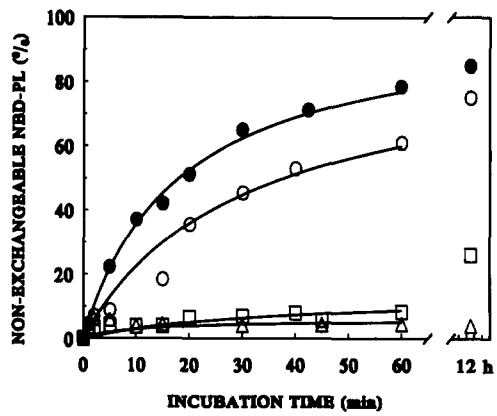

Fig. 2. Inward translocation of 18:1/C6-NBD-phospholipids in washed human erythrocytes. Experimental procedures were the same as for Fig. 1. Symbols: ●, NBD-PLS; ○, NBD-PDS; □, NBD-PC; △, NBD-SM. Data shown are from a single experiment representative of four independent experiments so performed.

incubation, versus 74–79% of the NBD-PDS. A very similar difference between PLS and PDS inward transport in erythrocytes was found when the palmitoyl derivatives of the NBD-analogs were compared (data not shown). In addition, Fig. 2 shows the passive diffusion of NBD-PC and NBD-SM over the bilayer. At equilibrium (after overnight incubation), approx. 25% of the NBD-PC and 4% of the NBD-SM were non-exchangeable, closely reflecting the distribution of the endogenous PC and SM.

3.2. Bidirectional movement of NBD-phospholipids upon cell activation

Platelet activation has been shown to result in surface exposure of PS, the extent of which depends on the platelet activation procedure [20,30]. Although platelet activation by thrombin causes minor exposure of PS, a pretreatment of the platelets with the Ca-ATPase inhibitor thapsigargin strongly amplifies the platelet procoagulant response, indicating substantial loss of PS asymmetry [39]. In contrast to platelet activation by thrombin alone, this condition was also found to inhibit aminophospholipid translocase activity in platelets. To monitor outward movement during activation, platelets were first loaded with 18:1/C6 NBD-PS for 40 min resulting in 75–85% of the probe being internalized (cf. Fig. 1). Residual NBD-PS present in the outer leaflet was removed by BSA extraction in order to start platelet activation with 100% of the NBD-PS inside. When these platelets were activated by sequential addition of thapsigargin and thrombin, approx. 40% of both stereoisomers of NBD-PS became externalized with a half-time of about 2 min (Fig. 3). In unstimulated platelets or platelets treated with either thrombin or thapsigargin, the

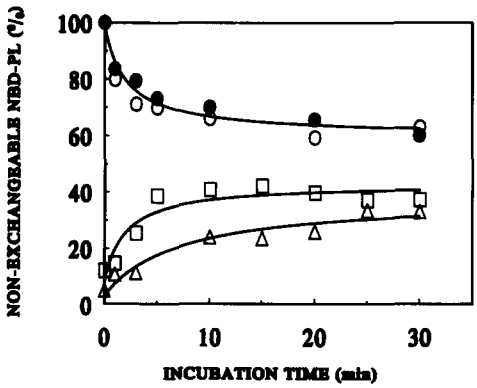

Fig. 3. Activation-induced scrambling of 18:1/C6-NBD-phospholipids in human platelets. For measuring outward movement, platelets were loaded with NBD-PLS or NBD-PDS for 40 min and residual probe was removed from the outer leaflet by BSA extraction prior to platelet activation as described in Section 2. For measuring inward movement, NBD-PC or NBD-SM were added directly before starting platelet activation. Platelet activation was started by adding 100 nM thapsigargin followed by 4 nM thrombin in Hepes buffer containing 3 mM Ca^{2+}. Symbols: ●, NBD-PLS; ○, NBD-PDS; □, NBD-PC; △, NBD-SM. Data presented are from two separate experiments, representative of four experiments thus performed.

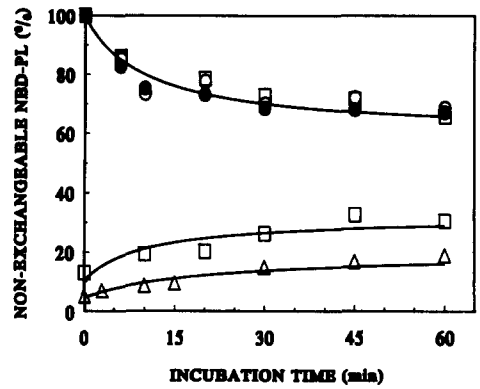

Fig. 4. Activation-induced scrambling of 18:1/C6-NBD-phospholipids in human erythrocytes. For measuring outward movement, red cells were loaded overnight with NBD-PLS, NBD-PDS or NBD-PC and residual probe was removed from the outer leaflet by BSA extraction prior to addition of 3 μM Ca-ionophore in the presence of 1 mM $CaCl_2$ as described in Section 2. For measuring inward movement, NBD-PC or NBD-SM were added directly before addition of ionophore and calcium. Symbols: ●, NBD-PLS; ○, NBD-PDS; □, NBD-PC; △, NBD-SM. Please note that in the case of NBD-PC, both inward- (lower curve) and outward movement (upper curve) are depicted. Data presented are from two separate experiments, representative of three experiments thus performed.

spontaneous outward movement of the probe was less than 5% in 30 min, presumably because the aminophospholipid translocase is not inhibited under these conditions [39]. No appreciable difference in rate or extent of exposure was found between the two stereoisomeric forms of NBD-PS during platelet activation.

Since the passive diffusion rates of NBD-PC and NBD-SM are very low (cf. Fig. 1), prolonged incubation times with concomitant loss of platelet function would be required to accumulate sufficient amounts of the probes at the inner leaflet for studying outward movement during cell activation. Therefore, the inward movement of NBD-PC and NBD-SM was investigated during thrombin activation of thapsigargin-treated platelets. Whereas in quiescent platelets less than 10% of the 18:1/C6-NBD-PC and 5% of the NBD-SM migrated to the inner leaflet, the activation procedure led to a rapid inward movement of approx. 40% of the NBD-PC and about 30% of the NBD-SM. Also, the rate of inward migration of the two probes was clearly different with a halftime of 2–3 min for NBD-PC and about 7 min for NBD-SM.

Activation of platelets with the Ca-ionophore ionomycin (or A23187) also resulted in rapid exposure of previously internalized 18:1/C6-NBD-PS to approx. 40%, with no appreciable difference between the two stereoisomers (data not shown). Likewise, increased inward movement of NBD-PC and NBD-SM was observed under these conditions, with the same difference in halftimes as observed for the activation procedure with thrombin plus thapsigargin. Identical results were obtained when the 18:1/C6 NBD-lipids were substituted by the corresponding 16:0/C6-NBD-lipid analogs, indicating that scram-

bling of these probes during cell activation was not dependent on the acyl chain.

Treatment of erythrocytes with ionophore in the presence of extracellular calcium is also known to cause loss of lipid asymmetry [21,23,24], although this process occurs less rapidly and to a lesser extent as compared to platelets. As depicted in Fig. 4, a gradual exposure of previously internalized 18:1/C6-NBD-analogs of PLS, PDS and PC was observed after addition of calcium ionophore to red cells, without appreciable difference in rate or extent of outward movement. Approx. 33% of the internalized NBD-lipids became exposed at the outer surface in 60 min. Furthermore, 16:0/C6-NBD lipid analogs exhibited the same behavior as the corresponding 18:1/C6-NBD lipids (data not shown). When erythrocytes were treated with Ca-ionophore in the presence of NBD-SM or NBD-PC, both probes moved to the inner leaflet but at different rates (Fig. 4). Whereas inward movement of NBD-PC reaches approx. 30% after 45 min, inward movement of NBD-SM barely reaches 20% after 60 min. It is also obvious from Fig. 4 that rate and extent of inward movement of NBD-PC is similar to rate and extent of outward movement of both NBD-PS and NBD-PC. Although aminophospholipid translocase activity is inhibited by increased levels of intracellular calcium [40], it is not clear whether this inhibition is instantaneous. Therefore, the inward movement of NBD-PS upon activation with ionophore was not considered in these studies.

4. Discussion

The first evidence for the existence of a protein-mediated inward transport of aminophospholipids was found in erythrocytes by Seigneuret and Devaux [3]. Meanwhile, aminophospholipid translocase activity has been demonstrated in a variety of cells [41] and many of its properties have been elucidated, although the identity of the protein is still a matter of debate. Important evidence for the protein nature of the transporter came from a study by Martin and Pagano [6] showing the inability to transport the unnatural D-isomers of the glycerol backbone of PS and PE. The present paper demonstrates that the stereospecificity of the serine headgroup of PS is less critical, although the extent, and in the case of red cells also the rate, of NBD-PDS inward transport was consistently found to be lower than that observed for the L-isomer. These results suggest that there is a slight preference of the aminophospholipid translocase for the naturally occurring form of PS, although it cannot be excluded that minor structural differences in headgroup conformations exist between the two diastereomers of PS [42] that contribute to the observed difference in inward translocation. It was recently suggested that both PS-diastereomers translocate equivalently across human erythrocyte membranes [43]. However, these authors assessed lipid translocation by monitoring erythrocyte morphology changes which may not be sensitive enough to discern minor differences.

Calcium-loading of platelets and erythrocytes results in loss of lipid asymmetry as was shown previously [20,21,24,44]. Contrary to the aminophospholipid translocase activity, the calcium induced transbilayer movement was not selective for the stereochemical configuration of the polar headgroup of PS. The present findings also demonstrate that during activation of platelets or ionophore-treatment of red cells, scrambling of lipids as visualized by fluorescent lipid probes, is not restricted to PS but involves the other phospholipid classes as well. The data are consistent with previous findings which showed an increased bidirectional movement of trimethylammoniumdiphenylhexatriene (TMA-DPH) in the membrane of activated platelets [45], and confirm earlier studies in platelets where phospholipases were used to establish changes in lipid asymmetry following activation [20]. While the latter studies suggested that all the phospholipids, including SM, tended to adopt a more random distribution over both membrane leaflets, it should be emphasized that the approach with phospholipases is not suited to monitor actual rates of transbilayer movement of the lipids.

This aselective character of the scrambling process has been previously observed in erythrocytes, irrespective of whether spin-labeled lipid analogs or fluorescently labeled lipids were used to monitor this process [24]. The present data obtained with NBD-lipids show that under virtually identical conditions both erythrocytes and platelets scramble their membrane lipids with similar specificity, suggesting that the same mechanism is operative in both cells. In agreement with the findings of Williamson et al. [24] on erythrocytes, we observed that both in platelets and red cells the inward rate of transbilayer movement of SM is appreciably lower than the outward rate of PS, the latter being approximately equal to the flip-flop rate of PC. It is tempting to speculate that at least during the initial stage of cell activation, the lower flip rate of SM may produce a mass imbalance in favour of the outer leaflet which leads to the eversion of plasma membrane that could facilitate the formation of microvesicles shed from the surface.

The present results obtained with human platelets clearly differ from those of Bassé et al. [25] who reported that Ca-ionophore-induced platelet activation led 40–50% of the internalized spin-labeled PS-probe to become externalized, which was not counterbalanced by a roughly equal inward migration of the PC-probe. From the present data, it can be concluded that the behavior of NBD-PS in platelets closely resembles that of the spin-labeled PS, but there appears to be a clear discrepancy regarding the inward movement of the PC probes. This could be due either to different properties of the lipid probes or to differences in experimental conditions. Differences in translocation between fluorescent- and spin-labeled probes have been observed for PE. Whereas spin-labeled PE is rapidly transported by aminophospholipid translocase in

human erythrocytes, NBD-PE is not [38]. However, this analog was not employed in this study even though it has been observed in ionophore treated red cells that transbilayer scrambling of NBD-PE is not appreciably different from that of spin-labeled PE [24]. Therefore, it seems unlikely that the different observations of Bassé et al. [25] are due to a markedly different behaviour of spin-labeled lipids compared to fluorescent lipid analogs. It should be mentioned that the present study employs physiological platelet concentrations of $2 \cdot 10^8$/ml, whereas Bassé et al. [25] used a 10-times higher cell concentration which in our hands gives rise to a considerable and rapid decrease in pH (due to excessive lactate production) when buffered with 10 mM Hepes. Also, activation of $2 \cdot 10^9$ platelets/ml with 1 μM ionophore may not be that efficient as activation of platelets at $2 \cdot 10^8$/ml, particularly if the platelet suspension at the higher concentration would be more acidic. Whether or not this would influence transbilayer scrambling of lipid analogs remains uncertain.

Provided that NBD-lipids are indeed reliable reporters of the Ca-induced scrambling of endogenous phospholipids, the present observations are fully consistent with the observations on a patient with a moderately severe bleeding disorder (Scott syndrome), where scrambling of the lipids and thus exposure of procoagulant PS is equally deficient in the platelets and the red blood cells [33]. Although the actual mechanism of the scrambling process remains open, it seems very unlikely that both platelets and erythrocytes would be affected if the mechanisms were different.

Acknowledgements

We thank Dr. Patrick Williamson for helpful discussions and critical reading of the manuscript.

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