Comparison between Ca^{2+} -induced scrambling of various fluorescently labelled lipid analogues in red blood cells

David W. C. DEKKERS, Paul COMFURIUS, Edouard M. BEVERS¹ and Robert F. A. ZWAAL

Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands

Treatment of red blood cells with calcium and ionomycin causes activation of the lipid scramblase, a putative membrane protein catalysing flip-flop of (phospho)lipids. Various fluorescent 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] caproyl (C_6 -NBD) analogues were tested for transbilayer movement across the plasma membrane of red blood cells. Among these phospholipid analogues were phosphatidylgalactose, phosphatidylmaltose and phosphatidylmaltotriose, which were obtained from C_6 -NBD-phosphatidylcholine by phospholipase D-catalysed transphosphatidylolation. The inward movement after the

onset of scrambling was monitored by extraction of the non-internalized probe with BSA. We demonstrate that both the amino group and the size of the headgroup determine the kinetics of lipid scrambling, and that lipids with a ceramide backbone migrate much more slowly than glycerophospholipids with the same headgroup.

Key words: phospholipase D, erythrocyte, lipid asymmetry, lipid transport, scramblase.

INTRODUCTION

The phospholipids of the membrane of human red blood cells (RBCs) are asymmetrically distributed over both leaflets. The choline-phospholipids phosphatidylcholine (PC) and sphingomyelin are mainly present in the outer leaflet, whereas the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) are located predominantly on the inside (reviewed in [1]). Maintenance of this transversal lipid distribution is important for the function of the plasma membrane as a selective barrier between the intra- and extra-cellular environments. However, collapse of lipid asymmetry can occur to serve a function in physiological processes, such as recognition by phagocytes and blood coagulation. This transverse reorganization of lipids was first observed in blood platelets [2,3] and later in other cell types [4–9]. An increase in intracellular calcium is a requisite for the onset of the scrambling process and concomitant inhibition of the aminophospholipid translocase activity [10–12]. Using phospholipases, Bevers et al. [3] showed that the major phospholipid classes become randomized when platelets are activated with calcium/ionophore. In subsequent studies, we and others showed that 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] caproyl (C_6 -NBD)-labelled lipid analogues can be used as reporter molecules for measuring transbilayer movement [13–18]. A possible explanation for the non-selectivity of the scrambling process is a model in which a transmembrane proteinaceous pore is formed, facilitating migration of the polar headgroup of the lipids across the hydrophobic core of the bilayer while keeping the acyl chain moieties in the core of the membrane [19]. One way to evaluate this hypothesis is to look for differences in the rate of scrambling as a function of the size of the headgroup. For this purpose, we have synthesized a number of NBD-labelled lipid analogues varying in the size of their polar

headgroups, and subsequently measured their kinetics of transbilayer movement in Ca^{2+} -loaded RBCs.

MATERIALS AND METHODS

Materials

C_6 -NBD-labelled PC, PS and PE, and C_6 -NBD-*sn*-sphingomyelin (Spm) were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). C_6 -NBD-galactosylceramide (GalCer) and C_6 -NBD-glucosylceramide were gifts from D. Hoekstra (University of Groningen, Groningen, The Netherlands). BSA, β -methyl-cyclodextrin and phospholipase D (*Streptomyces chromofuscus*; EC 3.1.4.4) were purchased from Sigma (St Louis, MO, U.S.A.). All other reagents were of the highest grade available commercially.

Synthesis of C_6 -NBD-labelled phospholipids

Synthesis of C_6 -NBD-labelled phosphatidyl-*N*-methyl-ethanolamine (MePE), phosphatidyl-*N,N*-dimethyl-ethanolamine (DiMePE), phosphatidylgalactose (PGal), phosphatidylmaltose (PMal) and phosphatidylmaltotriose (PTriose) was carried out using phospholipase D-catalysed base exchange as described by Comfurius and Zwaal [20]. Briefly, 1 mg of C_6 -NBD-PC was dissolved in 2 ml of diethyl ether (solution A). A saturated solution of reagent X (containing a primary OH group, see Figure 1) in a sodium acetate/ CaCl_2 buffer (each 100 mM, final concentration), adjusted to pH 5.6 with acetic acid, was used as coupling reagent (solution B). Solutions A and B were mixed together and, while stirring, phospholipase D was added (7.5 i.u.). The mixture was incubated for 45 min at 30 °C with vigorous stirring in a tightly closed glass tube. The products were

Abbreviations used: C_6 -NBD, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] caproyl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MePE, phosphatidyl-*N*-methyl-ethanolamine; DiMePE, phosphatidyl-*N,N*-dimethyl-ethanolamine; PS, phosphatidylserine; PGal, phosphatidylgalactose; PMal, phosphatidylmaltose; PTriose, phosphatidylmaltotriose; Spm, sphingomyelin; GalCer, galactosylceramide; RBC, red blood cell.

¹ To whom correspondence should be addressed (e-mail em.bevers@bioch.unimaas.nl).

Name Structure X

PC $-\text{CH}_2-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$

PE $-\text{CH}_2-\text{CH}_2-\text{NH}_3^+$

MePE $-\text{CH}_2-\text{CH}_2-\text{NH}_2^+(\text{CH}_3)$

DiMePE $-\text{CH}_2-\text{CH}_2-\text{NH}^+(\text{CH}_3)_2$

PS $-\text{CH}_2-\text{CH}(\text{C}(=\text{O})\text{O}^-)-\text{NH}_3^+$

PGal

PMal

Structure of C₆-NBD-Sphingomyelin molecule

Structure of C₆-NBD-Ceramide molecule

Name Structure X

GalCer

GluCer

Figure 1 Structure of C₆-NBD-labelled phosphoglyceride molecules

GluCer, glucosylceramide.

extracted, separated by TLC and quantified by determination of phosphorus as described by Verkleij et al. [21].

Isolation of RBCs

Blood was obtained from healthy volunteers by venipuncture, using acid citrate dextrose (0.18 M glucose/0.08 M trisodium citrate/0.052 M citric acid) as an anticoagulant in a final ratio of 1:5 (v/v). RBCs were collected by centrifugation and washed with Hepes buffer (containing 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 10 mM Hepes, 0.2 mM EGTA and 5 mM glucose, adjusted to pH 7.5). Cells were finally resuspended in the same buffer at a concentration of 5×10^8 RBCs/ml and used within 1 day.

Inward movement of C₆-NBD-labelled lipid analogues

After addition of C₆-NBD-labelled probe at a final concentration of 2 μM, RBCs were incubated at 37 °C for 2 min and the scrambling process was started by adding Ca²⁺ (1 mM, final concentration) and ionomycin (2.5 μM, final concentration). At different time intervals, 200 μl aliquots were removed and diluted in 850 μl of ice-cold Hepes buffer (to stop lipid scrambling) with or without 2% (w/v) BSA. After 20 min of extraction with BSA at 4 °C, cells were sedimented at 12000 g for 30 s and the pellets were solubilized in 2 ml of 1% (w/v) Triton X-100. The amount of internalized probe was determined by comparing the fluorescence intensity associated with the cells before and after extraction of the probe from the outer monolayer with BSA. The fluore-

scence intensities were measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm. Integrity of the lipid probes was verified with TLC. No degradation of the probes occurred during the course of the experiments.

Outward movement of C₆-NBD-labelled lipid analogues

RBCs were incubated overnight at 20 °C in the presence of 0.5–5.0 μ M C₆-NBD-labelled probe. When the outer leaflet was depleted of NBD-labelled probe by BSA, the amount of probe remaining on the inside was approx. 1% of the endogenous lipids. Only C₆-NBD-labelled PC, PE, PS, MePE and DiMePE could be used for this approach, because insufficient quantities of the other probes reached the inner leaflet to allow accurate measurement of outward movement (see the Discussion). After 2 min at 37 °C, cells were activated with 1 mM CaCl₂ and 2.5 μ M ionomycin and the outward movement was measured using the BSA back-exchange protocol as described for inward movement. No degradation of the probes occurred during the course of the experiments.

Cholesterol depletion of RBCs by β -methyl-cyclodextrin

RBCs (5×10^8 /ml) were incubated with 3.3 mM β -methyl-cyclodextrin at 37 °C for 90 min [22]. Cells were washed three times in Hepes buffer and resuspended at 5×10^8 RBCs/ml. Samples were taken for cholesterol and phosphate determination. Cholesterol content was determined enzymically by the cholesterol oxidase assay, using a commercial kit (Boehringer Mannheim, Mannheim, Germany). Phospholipid concentration was determined by the method of Fiske and Subbarow as described in [23].

RESULTS

Synthesis of C₆-NBD-labelled lipid analogues

TLC of the different C₆-NBD-lipid analogues is shown in Figure 2. For coupling carbohydrates, the yield of synthesis decreases with increasing size of the substituting molecule. e.g. PGal >

Figure 2 TLC of the purified C₆-NBD-labelled lipid probes

To discriminate between C₆-NBD-labelled phosphatidic acid (PA), PC, PGal, PMal and MePE, the products were first purified by TLC on a silica gel 60 plate using chloroform/methanol/acetic acid/water (45:20:12:1, by vol.) as a solvent, whereas for PTriose the same solvent was used, but in the respective proportions 45:20:18:1 (by vol.). The dark areas in the spots are due to self-quenching of the NBD group.

Figure 3 Inward movement of fluorescent glycerophospholipid analogues in calcium/ionophore-treated human RBCs

C₆-NBD-labelled PC (■), PE (◆), PS (▲), PGal (□), PMal (△) and PTriose (●) were used. Data shown are from a representative experiment out of four so performed; i.e. with RBCs taken from one donor on one day, repeated three times with cells from other donors.

PMal > PTriose, accompanied by increased phosphatidic acid formation. The yields of synthesis for PGal, PMal, PTriose and MePE were 75, 29, 16 and 90%, respectively. Although maltose and maltotriose have two and three primary OH groups, respectively, available for coupling in the transphosphatidylation reaction, we suspect that due to steric hindrance only one of the primary OH groups is the target for coupling. TLC analysis showed two major spots in the final reaction mixture, identified as the phosphatidyl-carbohydrate and phosphatidic acid. Which of the primary OH groups of maltose and maltotriose was used for coupling was not determined further, since this will not influence the size of the polar head group.

Inward movement of C₆-NBD-labelled lipid analogues

Although the glycerophospholipids (PGal, PMal and PTriose) are rather soluble in water, they were rapidly and virtually completely incorporated into the RBC membrane. Once incorporated, these probes are completely extractable by BSA, indicating their presence in the outer leaflet of the plasma membrane (results not shown). Figure 3 shows the inward movement of various NBD-labelled phospholipid analogues upon Ca²⁺-induced scrambling. The rate and extent of movement are dependent on the size of the headgroup. The extent of inward movement of glycerophospholipids in calcium/ionophore-treated RBCs, in descending order, was as follows: C₆-NBD-PE > C₆-NBD-PC > C₆-NBD-PS > C₆-NBD-PGal > C₆-NBD-PMal > C₆-NBD-PTriose. Although no quantitative data are available with respect to the size of the polar headgroup of the different lipids used here, it may be assumed, based on the chemical structures, that the observed differences in rate of scrambling result from differences in size of the polar headgroup. The lipid with the largest headgroup, PTriose, hardly reached the inner leaflet, whereas the lipid with the smallest headgroup, PE, migrated rapidly over the membrane. Whether or not differences in scrambling rate are also influenced

Figure 4 Comparison of inward movement of fluorescent sphingosine-backbone lipid analogues with glycerophospholipid analogues in calcium/ionophore-treated human RBCs

C_6 -NBD-labelled PC (■), PGal (□), GalCer (○) and Spm (×) were used. Data shown are from one representative experiment out of three so performed; i.e. with RBCs taken from one donor on one day, and repeated twice with cells from other donors.

by dehydration energy of water molecules surrounding the polar headgroup cannot be deduced from the present experiments. In the absence of calcium the inward movement of C_6 -NBD-labelled lipid probes, with the exception of the aminophospholipids, is negligible within the time course of the experiments. C_6 -NBD-labelled phosphatidic acid could not be used in this study since it becomes metabolized to diacylglycerol [24,25]. It should be mentioned that although the rates and extents of Ca^{2+} -induced

lipid scrambling showed some variation between different donors, this did not affect the relative differences between the various NBD-labelled lipid probes.

Although C_6 -NBD-Spm has the same headgroup as C_6 -NBD-PC, it hardly undergoes scrambling when the cells are challenged with calcium (Figure 4). In addition, the inward movement of GalCer was found to be slower than that of PGal, also suggesting that lipids with a ceramide backbone were scrambled much more slowly than glycerophospholipids with the same polar headgroup. Binding of Spm to cholesterol may hamper participation in lipid scrambling. To investigate this possibility, RBCs were pre-incubated with β -methyl-cyclodextrin, which results in a depletion of approx. 30% of the total cholesterol content without haemolysis. However, Ca^{2+} -induced lipid scrambling of Spm and PC in these cholesterol-depleted RBCs was not appreciably different from that in control cells.

Outward movement of C_6 -NBD-labelled lipid analogues

In Figure 5, the kinetics of Ca^{2+} -induced inward and outward movement of C_6 -NBD-PS, C_6 -NBD-PC and C_6 -NBD-PE were compared. While inward and outward migration of C_6 -NBD-PC were virtually identical, it appears that the initial inward rate of movement of C_6 -NBD-PS was significantly slower than its outward movement. The reverse picture is observed for C_6 -NBD-PE, where inward movement considerably exceeds the outward movement. These differences are not due to different amounts of probe present in the inner or outer leaflet at the start of the experiments. Since PC varies from PE only by its 3-fold methylated amino group, the effect of progressive methylation on the scrambling behaviour of C_6 -NBD-PE was determined. Figure 6 shows that mono-methylation of the amino group of PE changes the scrambling behaviour of this lipid probe to become indistinguishable from that of C_6 -NBD-PC, with respect to both inward and outward movement. The same result was obtained with DiMePE. In an effort to measure the outward movement of the glycerophospholipid analogues, we used two different protocols aimed at incorporating sufficient fluorescent label on the inside of RBC membranes to allow measurement of outward movements. Unfortunately, neither protocol was useful in providing

Figure 5 Comparison of inward (▲, ■, ◆) and outward (△, □, ◇) movement of C_6 -NBD-labelled lipids

C_6 -NBD-PS (▲, △), C_6 -NBD-PC (■, □) and C_6 -NBD-PE (◆, ◇) were used. Left-hand panel, PS ($n = 3$); central panel, PC ($n = 3$); right-hand panel, PE ($n = 3$). Results are shown as means $\pm$ S.D. from three experiments, performed with three different donors.

Figure 6 Effect of methylation of PE on inward (■,◆,▲) and outward (□,◇,△) movement

C₆-NBD-PC (■,□), C₆-NBD-PE (◆,◇) and C₆-NBD-MePE (▲,△) were used (all $n = 3$). For reasons of clarity, the inward and outward movements of DiMePE are not shown, because they were indistinguishable from those with PC and MePE. Results are shown as the means $\pm$ S.D. from three experiments, performed with three different donors.

information on the outward movement of these fluorescent lipid probes (see the Discussion).

DISCUSSION

The collapse of lipid asymmetry has been ascribed to the activation of a calcium-dependent lipid scramblase [26]. It is increasingly appreciated that the scramblase is a protein. Reconstitution of protein fractions from both platelet and erythrocyte membranes showed Ca²⁺-induced lipid scrambling in artificial membranes [26,27]. Due to the non-specificity of the scramblase with respect to the polar headgroup of the lipids, a model has been postulated in which a pore is formed by Ca²⁺-induced assembly of membrane protein subunits, facilitating bidirectional movement of all lipids [19]. In such a model, scrambling can be envisaged as movement of the lipid polar headgroup through a central aqueous channel, while the acyl chain moieties diffuse along hydrophobic interfaces between the protein subunits.

The relation between the size of the polar headgroup and the rate of inward movement observed in the present study is not inconsistent with the pore-forming model described above. Migration of the polar headgroup through an aqueous pore raises the question of whether the permeability for small molecules such as choline or monosaccharides will also be increased in RBCs under scrambling conditions. In the case of a non-aqueous pore, the observed order in rates of scrambling may reflect differences in dehydration energy for the various probes used in this study. In addition to size restriction, migration through a pore may also be dependent on the charge of the headgroup moiety. In the present study, only neutral lipid probes and probes with anionic polar headgroups have been studied. Bevers et al. [28] have shown that the positively charged amphipath trimethylammonium diphenyl hexatriene is able to cross the plasma membrane of platelets only under scrambling

conditions. These observations suggested that the charge of the lipid headgroup is of little importance for Ca²⁺-induced lipid scrambling.

Differences in the rate of scrambling are not related exclusively to the size of the lipid polar headgroup. Spm, having the same choline headgroup as PC, hardly participates in the scrambling process, at least in RBCs. Similarly, the rate of scrambling of GalCer was appreciably less than that of PGal, despite its somewhat smaller headgroup (galactose instead of phosphogalactose). These findings may indicate diminished recognition by the scramblase of lipids with a ceramide backbone. Alternatively, a decreased rate of scrambling of the ceramide-derived lipids could reflect their preferential localization in rafts [29]. Associations in rafts with other membrane lipid components such as cholesterol could restrict these lipids' access to scrambling sites. This, however, is not supported by the present findings showing that a 30% decrease in the membrane cholesterol content does not increase Ca²⁺-induced transbilayer movement of Spm, although it cannot be excluded that the extent of cholesterol depletion was insufficient to make ceramide-derived lipids available for scrambling.

In contrast with RBCs, a significant rate of inward movement of C₆-NBD-Spm was found in platelets activated with calcium/ionophore [13]. Moreover, the kinetics of the outward movement of both C₆-NBD-Spm and C₆-NBD-GalCer in scrambling platelets were found to be indistinguishable from those of C₆-NBD-PC [14,30]. A possible explanation could be that the platelet plasma membrane contains less Spm and cholesterol and/or is less rich in raft domains. Alternatively, one may speculate that RBCs and platelets possess different scramblases, although this seems rather unlikely considering that in Scott syndrome the Ca²⁺-induced scrambling of lipids is impaired in both cell types [31].

For C₆-NBD-PC the rates of outward and inward movement are virtually the same (Figure 5, central panel), whereas this does not hold for the aminophospholipids: inward scrambling of C₆-NBD-PE is much faster and occurs to a greater extent than its outward movement (Figure 5, right-hand panel). The opposite was found for C₆-NBD-PS, where the initial rate of outward movement exceeds that of inward movement, although the difference in extent between inward and outward movement was much less pronounced (Figure 5, left-hand panel). This suggests that the presence of an amino group (and possibly also a carboxy group) has a significant effect on the scrambling behaviour of the phospholipid. This is confirmed by the observation that monomethylation of the free amine of PE makes the kinetics of scrambling of this probe indistinguishable from those of PC (Figure 6). It should also be emphasized that the C₆-NBD-PE probe may not be a very reliable reporter molecule. In contrast with C₁₂-NBD-PE or C₄-spin-labelled PE, anomalous behaviour of C₆-NBD-PE was observed in studies on the aminophospholipid translocase activity [16,18].

In an attempt to study the effect of the presence of an amino group in more detail, we also synthesized C₆-NBD-phosphatidylgalactosamine by phospholipase D-catalysed transphosphatidylation. However, approx. 15–20% of the probe was bound irreversibly to the membrane, which hampered interpretation of the results (results not shown). Similar irreversible binding was found for C₆-NBD-phosphatidylglucose and C₆-NBD-glucosylceramide, making these probes unsuitable for these studies (results not shown).

Although the presence of phospholipase D in RBCs is well recognized [32], the influence of phospholipase D in our experimental set-up seems to be negligible, since no detectable NBD-labelled phosphatidic acid formation occurred during the

time course of the experiments. Additionally, Selle et al. [32] concluded that in RBCs the calcium-dependent control of phospholipase D prevents a significant breakdown of PC at low physiological calcium concentrations.

It is well known that, during the scrambling process, microvesiculation occurs [6]. However, the amount of membrane material shed in the form of microvesicles was estimated previously to amount to less than 10% of the total RBC membrane [6]. Moreover, measurement of NBD fluorescence in the supernatant of the cells in the absence of BSA revealed that, for each lipid probe, less than 5–10% resides in the microvesicles, produced during the course of the experiment. This suggests that no selective incorporation of the NBD-labelled lipid analogues in microvesicles occurs, and that microvesicle formation does not influence the relative differences between scrambling kinetics of the probes.

Since the spontaneous movement of the glycopospholipid probes was too low to accumulate sufficient fluorescence signal in the inner leaflet to allow reliable measurement of their outward movement, two different protocols were examined. In one protocol, we used resealed ghosts in which probe was trapped in the inner leaflet. In the other protocol, probes were allowed to reach the inner leaflet under scrambling conditions, followed by removal of calcium to stop the scrambling process. However, both approaches were not useful due to strongly enhanced transbilayer movements of all the probes. Apparently, the membranes of resealed ghosts or pre-scrambled RBCs have different characteristics, which are no longer comparable with the membrane of the unperturbed RBC.

The group of Hamon et al. [33] showed that ABC1, an ATP-binding-cassette transporter, is involved in Ca^{2+} -induced scrambling of PS and not of PC. Because the process of lipid scrambling in RBCs is not coupled directly to ATP hydrolysis, whereas transport by ABC1 is ATP-dependent, it is conceivable that these proteins have different functions. Bassé et al. [34] have suggested that the scramblase is specific for aminophospholipids. They showed that in calcium/ionophore-activated platelets PS is redistributed rapidly within 1 min, whereas movement of PC hardly occurs. Furthermore, Bettache and co-workers [35] showed that Ca^{2+} -induced scrambling of PS is defective in platelets from a patient with Scott syndrome, whereas the minor redistribution of PC in the patients' cells was found to be similar to that of control cells. However, these observations are not in concordance with those of Smeets et al. [13] and Williamson et al. [14], who showed that PC scrambling in platelets is indistinguishable from PS scrambling, and that scrambling of both lipids is impaired in Scott cells [31]. The present findings with RBCs do not seem to be in line with the apprehension that lipid scrambling is independent of the headgroup moiety, as was previously assumed. It should be emphasized, however, that this notion was based primarily on experiments with platelets, where the high rates of lipid scrambling may have obscured differences in the kinetics of scrambling of the various lipid probes.

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