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Out-to-in translocation of butanetriol-containing phospholipid analogs in human erythrocyte membrane

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Abstract

Fluorescent butanetriol-containing phospholipid analogs were synthesized by replacing the glycerol moiety in 1-hexadecanoyl-2-[6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoyl]-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoserine and 1-hexadecanoyl-2-[12-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyl]-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoserine by the 1,3,4-butanetriol residue, and their out-to-in translocation in the human erythrocyte membrane studied by 'back exchanging' the outer surface-incorporated phospholipids using bovine serum albumin. The results of these studies indicate that the replacement of the glycerol moiety by the 1,3,4-butanetriol residue in aminophospholipids does not effect their out-to-in translocation in the human erythrocyte membrane. Furthermore, since earlier study by Arora and Gupta (Biochim. Biophys. Acta 1324 (1997) 47–60) has shown that the conformation of the 1,3,4-butanetriol phospholipids possess the backbone conformation similar to that of glycerophospholipids, it is suggested that besides the normal phospholipid polar head-group, a normal phospholipid interface conformation may also be required for the aminophospholipid–translocase interactions.

Keywords: Aminophospholipid translocase; Phospholipid conformation; Butanetriol analog; Erythrocyte membrane

1. Introduction

Transbilayer movement of membrane phospholipids is a fundamental process of membrane biogenesis and cell growth [1,2]. It is now well established that these movements, at least in the plasma membrane, are mediated by the membrane-bound proteins [2–4]. While some of these proteins translocate

Abbreviations: TLC, thin-layer chromatography; FAB, fast atom bombardment; EDTA, *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; s, singlet; d, doublet; b, broad; m, multiplet; BSA, bovine serum albumin; C_6-NBD-PC, -PE, -PS, 1-hexadecanoyl-2-[6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoyl]-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoserine; C_{12}-NBD-PC, -PE, -PS, 1-hexadecanoyl-2-[12-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyl]-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoserine; C_6-NBD-bPC, -bPE, -bPS, 1-hexadecanoyloxy-3-[6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyloxy]-(3*R*)-but-4-yl-phosphocholine, -phosphoethanolamine, -phosphoserine; C_{12}-NBD-bPC, -bPE, -bPS, 1-hexadecanoyloxy-3-[12-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyloxy]-(3*R*)-but-4-yl-phosphocholine, -phosphoethanolamine, -phosphoserine; PBS, phosphate-buffered saline (10 mM phosphate, 140 mM NaCl, pH 7.4); HS buffer, HEPES-saline buffer (20 mM HEPES, 137 mM NaCl, 3 mM KCl, 10 mM glucose, pH 7.4)

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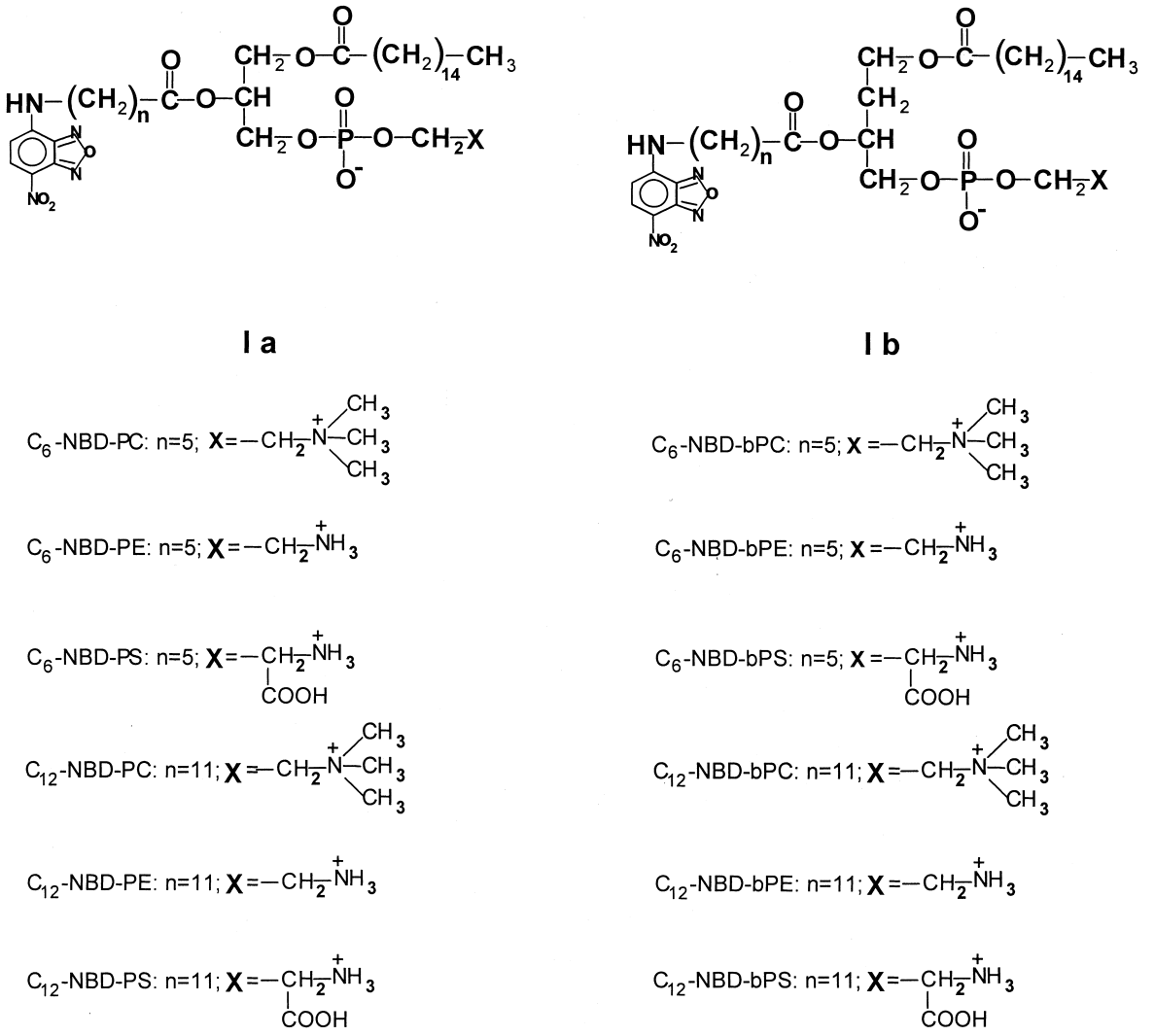

Fig. 1. Molecular structures of NBD-labeled PC, PE and PS analogs wherein the glycerol moiety has been replaced by 1,3,4-butanetriol residue.

only a specific class of phospholipids [5–7], non-specific translocating proteins have also been reported [5,8].

Out-to-in translocation of aminophospholipids in human erythrocytes is mediated by an ATPase-like protein [9–11]. Several studies have shown that this protein translocates PS at a faster rate than PE, but it does not translocate PC under identical conditions [2,3]. Furthermore, it has been reported that this aminophospholipid translocase recognizes not only the amino group on the lipid polar moiety, but also the glycerol and the ester bonds [12]. To further investigate the role of glycerol backbone in aminophospholipids–translocase interactions, we replaced the glycerol residue by the 1,3,4-butanetriol moiety in diacylglycerophospholipids and then studied the out-to-in translocation of modified phospholipids (Fig. 1) in intact erythrocytes. The results of these studies clearly demonstrate that insertion of one methylene residue between the carbons that carry the two acyl chains in aminophospholipids (PE and PS) does not significantly effect their out-to-in movements in the erythrocyte membrane.

2. Materials and methods

2.1. Materials

All the reagents and solvents used in the study were of the highest purity available. L-Serine, ethanolamine, calcium chloride, HEPES, sodium acetate, *n*-octyl-β-D-glycopyranoside, Triton X-100, Tris-HCl, BSA and EDTA were purchased from Sigma. CF-11 was bought from Whatman, Germany and silica gel (60–120 mesh) was from Sisco, Bombay.

2.2. General methods

Purity of various reaction intermediates was checked by TLC on silica gel G-60 plates. Homogeneity of phospholipids was established by both TLC and HPLC. TLC plates were developed in chloroform/methanol/water (65:25:4, v/v/v) mixture and the phospholipid spots visualized by staining the plate with iodine vapor followed by Molybdenum blue spray [13]. Two-dimensional TLC was performed on precoated Merck aluminium-backed silica gel G-60 plates. The plates were developed in chloroform/methanol/ammonia (65:25:4, v/v/v) in one dimension followed by chloroform/methanol/acetone/acetic acid/water (40:8:16:9:4, v/v/v/v) in a direction at right angles to the first dimension. The spots were visualized as described above. HPLC was performed using Waters HPLC pump (Model 510) along with Waters R401 differential refractometer or Waters fluorescence detector (Model 420-AC) interfaced with Waters System Interface Module. Chromatograms were analyzed by using baseline 810 software. Analytical HPLC was performed on an Altex Ultrasphere C18 reverse-phase column (4.6×250 mm, particle size 5 μm) using methanol/chloroform/water (87:13:6, v/v/v) containing 176 mM choline chloride, at a flow rate of 1.0 ml/min, as the elution system, whereas preparative HPLC was carried out on an LKB TSK-ODS-120T semiprep column (7.8×300 mm, particle size 10 μm) using methanol/chloroform/water (90:10:4, v/v/v) at a flow rate of 2 ml/min as the eluent.

Phospholipids were purified by preparative TLC, Sephadex LH-20 column (2.5×100 cm) chromatography, and preparative HPLC. The Sephadex LH-20 column was eluted with chloroform/methanol (1:1, v/v) mixture at a flow rate of about 60 ml/h. All the phospholipids thus-purified exhibited single spot on TLC and single peak on analytical HPLC, and were characterized by NMR and mass spectrometry analyses. ^{1}H-NMR spectra were recorded on a Bruker ACP 300 FT-NMR spectrometer, and the chemical shifts have been expressed in parts per million down-field from tetramethylsilane. Positive ion FAB mass spectrometry was performed on a Jeol JMS-SX 102 FAB mass spectrometer equipped with JMA-DA 6000 data system.

2.3. Phospholipids

1,3-Dihexadecanoyloxy-*rac*-but-4-yl-[2-(trimethylammonium)ethyl] phosphate was prepared as described earlier [14]. It was treated with *Naja naja* snake venom (Sigma) and the resulting 1-hexadecanoyloxy-3-hydroxy-(3*R*)-but-4-yl-[2-(trimethylammonium)ethyl] phosphate was isolated in pure form by following the published procedure [15]. Reaction of this lysolipid with 6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoic acid and 12-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminododecanoic acid in the presence of 1,1′-carbonyldiimidazole [16] afforded C_6-NBD-bPC (Fig. 1) and C_{12}-NBD-bPC (Fig. 1) respectively. Similarly, C_6-NBD-PC and C_{12}-NBD-PC were prepared using 1-hexadecanoyl-*sn*-glycero-3-phosphocholine as the starting material, which in turn was prepared as described earlier [17]. C_6-NBD-PC: FAB mass 772 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.49 (d, $J=9$ Hz, 1H), 6.23 (d, $J=9$ Hz, 1H), 5.28–5.21 (m, 1H), 4.37–4.29 (b, 2H), 4.17–4.08 (m, 2H), 4.03–3.97 (m, 2H), 3.85–3.75 (b, 2H), 3.30 (s, 9H). C_{12}-NBD-PC: FAB mass 856 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OH$) δ 8.50 (d, $J=9$ Hz, 1H), 6.19 (d, $J=9$ Hz, 1H), 5.27–5.20 (m, 1H), 4.30–4.20 (b, 2H), 4.18–4.10 (m, 2H), 4.02–3.97 (m, 2H), 3.63–3.56 (b, 2H), 3.22 (s, 9H). C_6-NBD-bPC: FAB mass 786 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.44 (d, $J=9$ Hz, 1H), 6.14 (d, $J=9$ Hz, 1H), 5.08–5.01 (m, 1H), 4.27–4.21 (b, 2H), 4.14–4.04 (b, 2H), 4.02–3.99 (m, 2H), 3.58–3.49 (b, 2H), 3.14 (s, 9H), 2.03–1.82 (m, 2H). C_{12}-NBD-bPC: FAB mass 870 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.43 (d, $J=9$ Hz, 1H), 6.14 (d, $J=9$ Hz, 1H), 5.06–5.00 (m, 1H), 4.29–4.21 (b, 2H), 4.16–4.05 (b, 2H), 4.03–4.01 (m,

2H), 3.56–3.46 (b, 2H), 3.14 (s, 9H), 2.04–1.82 (m, 2H).

C_6-NBD-PS, C_6-NBD-bPS, C_6-NBD-PE, C_6-NBD-bPE, C_{12}-NBD-PS, C_{12}-NBD-bPS C_{12}-NBD-PE and C_{12}-NBD-bPE (Fig. 1) were prepared by transesterification of their corresponding NBD-PC and NBD-bPC analogs using published procedure [18]. C_6-NBD-PS: FAB mass 774 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.47 (d, $J=8.7$ Hz, 1H), 6.16 (d, $J=8.7$ Hz, 1H), 5.38–5.26 (m, 1H), 4.36–4.24 (b, 2H), 4.20–4.13 (b, 2H), 4.04–3.95 (m, 2H), 3.65–3.58 (b, 1H). C_6-NBD-bPS: FAB mass 788 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.44 (d, $J=9$ Hz, 1H), 6.11 (d, $J=9$ Hz, 1H), 5.32–5.24 (m, 1H), 4.37–4.23 (b, 2H), 4.20–4.12 (b, 2H), 4.03–3.97 (m, 2H), 3.65–3.59 (b, 1H), 2.33–2.10 (m, 2H). C_{12}-NBD-PS: FAB mass 858 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.48 (d, $J=8.7$ Hz, 1H), 6.21 (d, $J=8.7$ Hz, 1H), 5.22–5.16 (m, 1H), 4.34–4.26 (b, 2H), 4.20–4.08 (b, 2H), 4.07–4.01 (m, 2H), 3.62–3.53 (b, 1H). C_{12}-NBD-bPS: FAB mass 872 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.37 (d, $J=9$ Hz, 1H), 6.09 (d, $J=9$ Hz, 1H), 5.07–5.01 (m, 1H), 4.29–4.21 (b, 2H), 4.14–4.04 (b, 2H), 4.03–3.97 (m, 2H), 3.59–3.46 (b, 1H), 1.98–1.82 (m, 2H). C_6-NBD-PE: FAB mass 730 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.43 (d, $J=8.7$ Hz, 1H), 6.13 (d, $J=8.7$ Hz, 1H), 5.22–5.15 (m, 1H), 4.35–4.24 (b, 2H), 4.22–4.10 (b, 2H), 4.05–3.98 (m, 2H), 3.61–3.47 (b, 2H). C_6-NBD-bPE: FAB mass 744 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.46 (d, $J=9$ Hz, 1H), 6.16 (d, $J=9$ Hz, 1H), 5.29–5.21 (m, 1H), 4.38–4.26 (b, 2H), 4.24–4.13 (b, 2H), 4.06–3.99 (m, 2H), 3.64–3.51 (b, 2H), 2.31–2.09 (m, 2H). C_{12}-NBD-PE: FAB mass 814 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.44 (d, $J=8.7$ Hz, 1H), 6.12 (d, $J=8.7$ Hz, 1H), 5.16–5.07 (m, 1H), 4.38–4.25 (b, 2H), 4.20–4.11 (b, 2H), 4.10–4.03 (m, 2H), 3.67–3.49 (b, 2H). C_{12}-NBD-bPE: FAB mass 828 (MH^+); ^{1}H-NMR ($CDCl_3/CD_3OD$) δ 8.45 (d, $J=9$ Hz, 1H), 6.13 (d, $J=9$ Hz, 1H), 5.13–5.06 (m, 1H), 4.36–4.24 (b, 2H), 4.19–4.09 (b, 2H), 4.06–3.99 (m, 2H), 3.67–3.49 (b, 2H), 2.02–1.81 (m, 2H).

2.4. Erythrocytes

Human erythrocytes from freshly drawn healthy human donor blood in acid citrate-dextrose were washed three times with PBS. The buffy coat was removed and the erythrocytes were resuspended in PBS. The suspension was passed through a CF-11 column to remove any remnants of leucocytes. In all the experiments, the fresh human erythrocytes from single donor were used.

2.5. ATP depletion of erythrocytes

Erythrocytes were starved of energy by incubating them in a buffer composed of 50 mM Tris-HCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 90 mM NaCl, 0.2 mg/ml streptomycin and 200 IU/ml penicillin (pH 7.4) at 37°C [11] under nitrogen atmosphere. Aliquots were taken at the required intervals. ATP levels in fresh and starved erythrocytes were determined using the hexokinase/glucose-6-phosphate dehydrogenase assay of Lamprecht and Trautschold [29].

2.6. Translocation of lipids from outer-to-inner leaflet

Erythrocytes (2×10^8 cells) were suspended in 1 ml of ice cold HS buffer. NBD-labeled lipid analog in ethanol (1 μg/10 μl) was then added to the erythrocyte suspension (2×10^8 cells/ml) in HS buffer, mixed rapidly and incubated at 0°C for 20 min. After incubation, the erythrocytes were washed two times with ice cold HS buffer, and then resuspended in 1 ml of the same buffer. It was incubated at 37°C and aliquots (100 μl) were drawn at the required intervals. To each aliquot was then added 1 ml of HS buffer containing 1% BSA. The mixture was incubated at 0°C for 10 min to remove the lipid located in the outer surface of the cells (back exchange). Cells incubated with the C_{12} analogs were processed similarly except that for back exchange cells were incubated with 10% BSA for 20 min at 0°C. The cells were pelleted and washed 6 or 7 times with 1 ml each time of HS buffer to remove traces of BSA. The cell pellet was solubilized in 500 μl of 1% Triton X-100, and the fluorescence intensity was measured. The fraction of lipid translocated from outer-to-inner leaflet was determined by comparing the amounts of lipid remaining in the cells before and after back exchange to BSA. Steady state fluorescence of NBD labeled lipids was measured on a Perkin Elmer LS 5000 spectrofluorometer at an emission wavelength of 521 nm (excitation, 470 nm) at room temperature using 10 nm slit widths.

3. Results

Out-to-in movements of phospholipids was monitored by incorporating fluorescent phospholipids in the human erythrocytes, and then back-exchanging the thus-incorporated lipids to BSA at different time intervals [19]. The fluorescent phospholipids were synthesized by acylating the corresponding lysolipids with NBD-containing C_6 or C_{12} fatty acids, as reported earlier [16,18]. All the phospholipids (Fig. 1) were purified to homogeneity and characterized by NMR and mass spectrometry, as described in Section 2.

Fig. 2 shows that both C_6-NBD-PC and C_{12}-NBD-PC failed to translocate from the outer-to-inner leaflet of erythrocyte membrane bilayer, which is quite in agreement with the earlier reports [19,20]. Like PC, the corresponding butanetriol analogs, viz. C_6-NBD-bPC and C_{12}-NBD-bPC, also failed to translocate across the membrane bilayer. Consistent with the earlier observations [19,21,22], C_6-NBD-PE very poorly migrated to the inner monolayer, but its corresponding C_{12} derivative translocated at least up to 75% to the inner leaflet of the erythrocyte membrane bilayer (Fig. 3). Almost identical results were observed by employing the corresponding butanetriol analogs in place of C_6-NBD-PE and C_{12}-NBD-PE (Fig. 3). Similarly, no significant differences were ob-

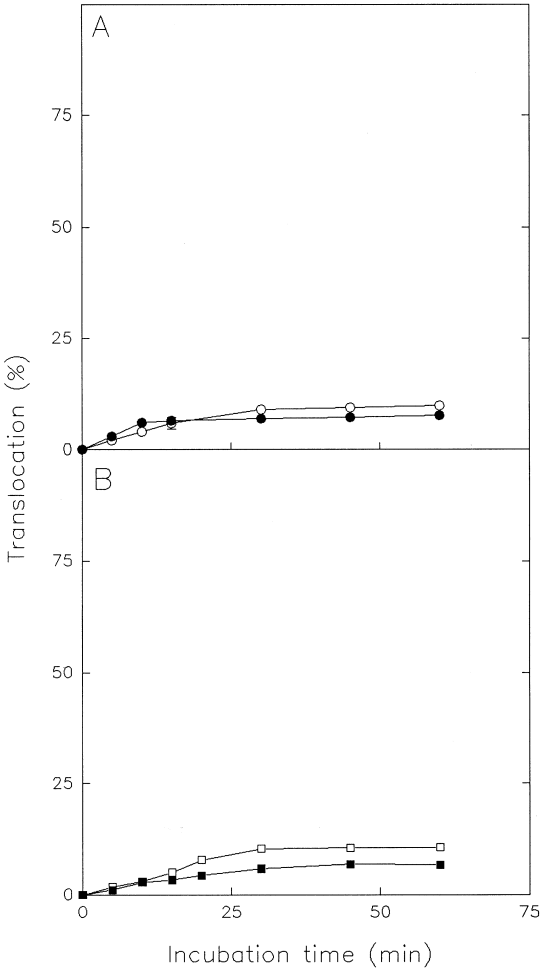

Fig. 2. Translocation of PC analogs from outer to the inner membrane leaflet of human erythrocytes at 37°C. (A) Solid circles, C_6-NBD-PC; open circles, C_6-NBD-bPC. (B) Solid squares, C_{12}-NBD-PC; open squares, C_{12}-NBD-bPC. Values shown are the mean of 3–5 observations ± S.D.

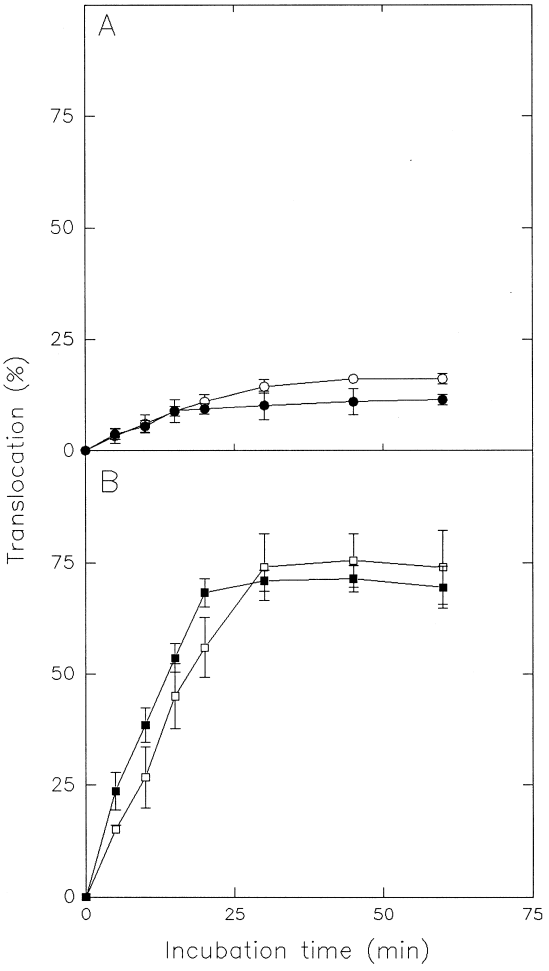

Fig. 3. Translocation of PE analogs from outer to the inner membrane leaflet of human erythrocytes at 37°C. (A) Solid circles, C_6-NBD-PE; open circles, C_6-NBD-bPE. (B) Solid squares, C_{12}-NBD-PE; open squares, C_{12}-NBD-bPE. Values shown are the mean of 5–7 observations ± S.D.

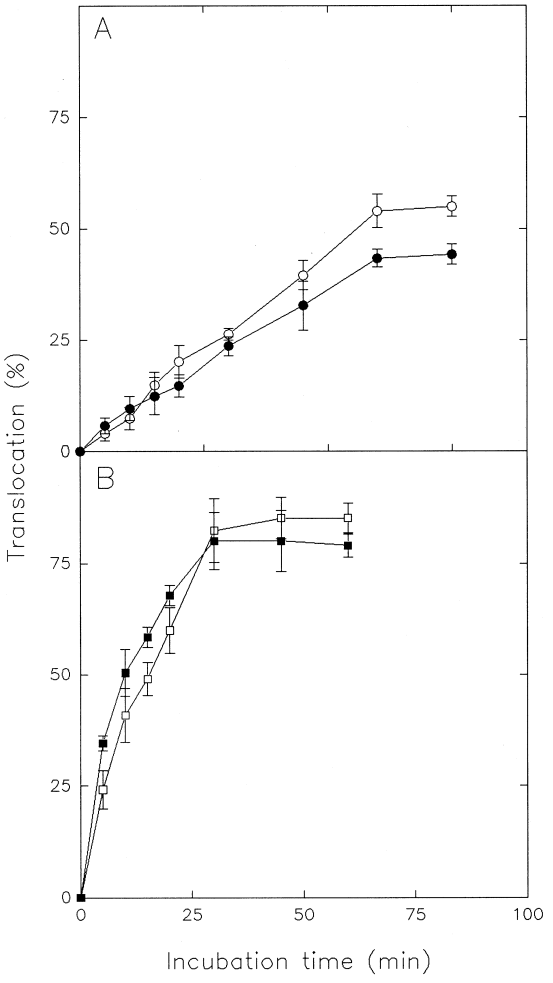

Fig. 4. Translocation of PS analogs from outer to the inner membrane leaflet of human erythrocytes at 37°C. (A) Solid circles, C_6-NBD-PS; open circles, C_6-NBD-bPS. (B) Solid squares, C_{12}-NBD-PS; open squares, C_{12}-NBD-bPS. Values shown are the mean of 5–7 observations ± S.D.

served between the PS (C_6-NBD-PS and C_{12}-NBD-PS) and the corresponding butanetriol analogs (C_6-NBD-bPS and C_{12}-NBD-bPS) in the extent of their out-to-in movements (Fig. 4).

To examine whether the out-to-in translocation of butanetriol-containing phospholipids, like the glycerophospholipids [9–11], is influenced by the cellular ATP levels, we depleted the human erythrocytes of ATP and then studied the out-to-in movement of C_{12}-NBD-PS and C_{12}-NBD-bPS. Cellular ATP was depleted by incubating the erythrocytes under starving conditions, as described earlier [11]. The ATP levels dropped to about 25% in 6 h and to less than 10% in 18 h. Fig. 5 shows that the extent of out-to-in translocation of both C_{12}-NBD-PS and C_{12}-NBD-bPS decreased with a decrease in the cellular ATP content, and was almost abolished by starving the cells of energy for 18 h.

4. Discussion

Present study demonstrates that replacement of the glycerol backbone by the 1,3,4-butanetriol residue in PC, PE and PS does not effect their out-to-in movements in the erythrocyte membrane. This is in contrast to the earlier study which concluded that besides the polar head-group, glycerol residue and the ester groups are also required for translocation of PE and PS to the inner monolayer [12]. Since this conclusion was based on the very poor translocation of the ceramide-containing PS derivatives in the erythrocyte membrane, we suggest that this behavior may well be attributed to a tighter association of these derivatives with the translocase because of the H-bonding character of the ceramide residue [23].

It would seem that the formation of a productive complex between the translocase and aminophospholipids besides requiring the polar head-group also requires the interface conformation typical to the glycerophospholipids [24,25]. This is based on our earlier study which showed that the conformational preference around the butanetriol C2–C3 bond in butanetriol-containing phospholipids is almost identical to that observed with the glycerol C1–C2 bond in phosphatidylcholines [14]. Also it is consistent with the previous study of Martin and Pagano [26] wherein they showed that the D-isomers of NBD-PE and NBD-PS are not transported by the aminophospholipid translocase. Furthermore, it may find support from the work of Morrot et al. [12], who in spite of the substitution of the glycerol moiety in PS by the ceramide residue, could still observe detectable translocation of the PS analogs, as contrast to sphingomyelins, in the erythrocyte membrane. Furthermore, we suggest that the C1 ester moiety may not play a crucial role in the glycerophospholipid–translocase interactions, since the preferred conformation of the butanetriol-containing phospholipids is such wherein the additional methylene, rather than the C1 methylene, serves as the proximal beginning of the primary acyl chain [14], which should result in

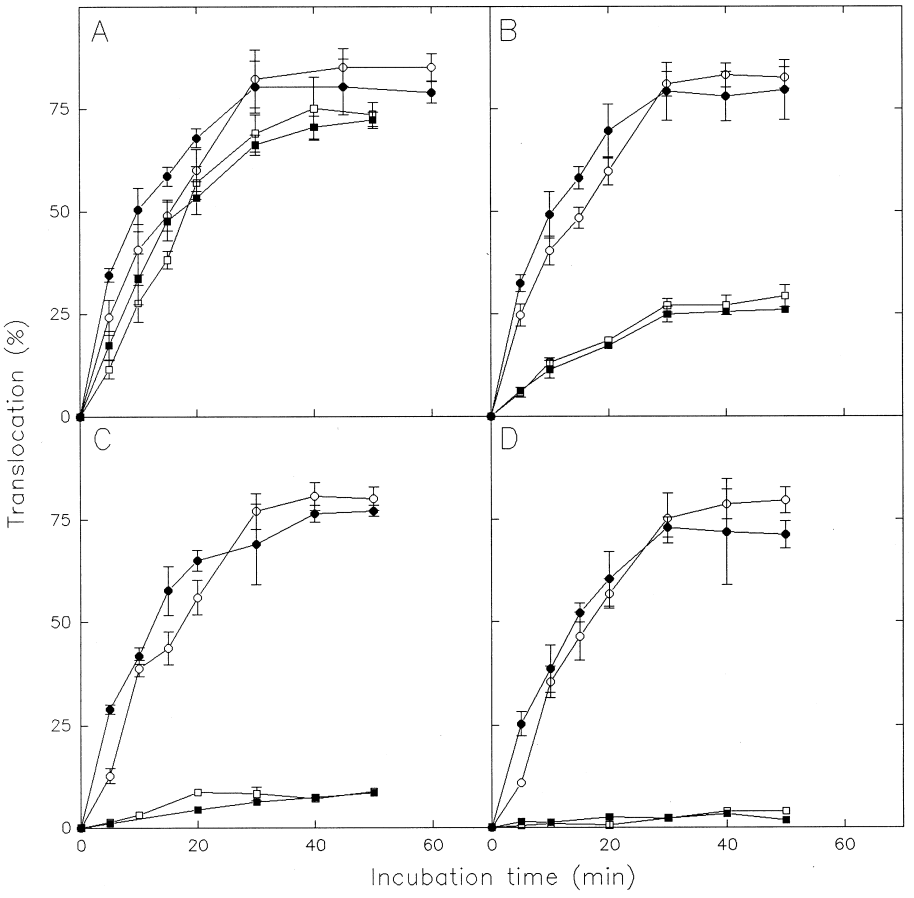

Fig. 5. Effect of ATP depletion on translocation of C_{12}-NBD-bPS from outer to the inner membrane leaflet of human erythrocytes at 37°C. Fresh erythrocytes were divided into two parts. One part was incubated, for varying time intervals, in a buffer composed of 50 mM Tris-HCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 90 mM NaCl, 0.2 mg/ml streptomycin and 200 IU/ml penicillin (pH 7.4) at 37°C under nitrogen and the other part was incubated under the same conditions in HS buffer. The cells were washed and then suspended in HS buffer (without glucose) and out-to-in translocation of C_{12}-NBD-PS and C_{12}-NBD-bPS was measured. Solid circles, out-to-in translocation of C_{12}-NBD-PS in erythrocytes incubated under normal conditions; solid squares, out-to-in translocation of C_{12}-NBD-PS in erythrocytes incubated under starving conditions; open circles, out-to-in translocation of C_{12}-NBD-bPS in erythrocytes incubated under normal conditions; open squares, out-to-in translocation of C_{12}-NBD-bPS in erythrocytes incubated under starving conditions. (A) 0 h incubation; (B) 6 h incubation; (C) 12 h incubation; (D) 18 h incubation. Values shown are the mean of 3–5 observations ± S.D.

displacement of the C1 ester by one additional methylene in the phospholipid structure [24,25].

It is interesting to observe that the out-to-in translocation of the aminophospholipids that carry C_6 acyl chain at the C2 position of the glycerol moiety in glycerophospholipids or at the C3 position of the butanetriol residue in butanetriol-containing phospholipids was significantly smaller than that of the corresponding C_{12} fatty acyl chain-containing phospholipids, which is quite in agreement with the earlier findings [19,21]. This result may suitably be explained in the light of the earlier observations of Chattopadhyay and London [27]. These authors have shown that the NBD group attached to a flexible acyl chain in phospholipids tend to loop back to the surface due to its polar nature. This 'looping back' may cause greater distortion of the phospholipid conformation in case of C_6-NBD-PE or C_6-NBD-PS due to very short acyl chain length, as compared to C_{12}-NBD-PE or C_{12}-NBD-PS, which in turn could lead to poor interactions between the aminophospholipid and translocase.

Earlier studies have shown that the erythrocyte membrane aminophospholipid translocase specifi-

cally recognizes the phospholipid polar head group [12]. This behavior of the protein is quite similar to that of the other phospholipid-specific transfer proteins [28]. In case of PC-specific exchange protein, it has been suggested that this protein transfers only those PC derivatives which have normal polar head-group configuration [28]. It is therefore likely that the aminophospholipid translocase, besides recognizing the phospholipid backbone conformation, also recognizes the specific polar head-group configuration. This is well supported by our earlier findings [14] that butanetriol-containing phospholipids besides possessing the backbone conformation similar to that of glycerophospholipids also possess similar polar head-group configuration.

In conclusion, this study demonstrates that replacement of the glycerol residue in PE and PS by the 1,3,4-butanetriol moiety does not effect the ATP-dependent out-to-in translocation of these phospholipids in the human erythrocyte membrane. Furthermore, it suggests that this translocation, besides requiring the specific phospholipid polar head-group, may also require specific phospholipid backbone conformation.

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