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Phosphatidylserine headgroup diastereomers translocate equivalently across human erythrocyte membranes

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Abstract

The natural chiral phospholipid substrates for the plasma membrane aminophospholipid translocator are L-α-phosphatidyl-L-serine and L-α-phosphatidylethanolamine. The glyceric D-stereoisomers of these lipids, D-α-phosphatidyl-L-serine and D-α-phosphatidylethanolamine, are not translocated (Martin, O.C. and Pagano, R.E. (1987) J. Biol. Chem. 262, 5890–5898). We have synthesized a diastereomer of phosphatidylserine, L-α-phosphatidyl-D-serine, to study the effects of headgroup stereochemistry on translocation. The diastereomer was synthesized as the dilauroyl (12:0) species, and the translocation was monitored by human erythrocyte morphology changes at 25°C and 37°C. Unlike other phosphatidylserine stereoisomers, L-α-phosphatidyl-D-serine is translocated to the same degree as the natural L,L-isomer. Incorporation of apparently equal amounts of the L,D- and L,L-diastereomers does produce minor quantitative differences in the cell morphological response, possibly as a result of differences in lipid packing of the two isomers.

Key words: Phosphatidylserine stereochemistry; Aminophospholipid translocator; Erythrocyte morphology

1. Introduction

When mammalian erythrocytes are treated with exogenous amphipaths, echinocytic or stomatocytic shape changes result as the amphipaths incorporate into the outer or inner monolayer of the plasma membrane [1]. The choline phospholipids phosphatidylcholine (PC) and sphingomyelin (SM) remain primarily in the outer monolayer, while amino phospholipids such as phosphatidylethanolamine (PE) and the anionic lipid phosphatidylserine (PS) tend to be sequestered in the inner leaflet [2,3].

The process by which the aminophospholipids 'flip' from the outer to the inner monolayer and are sequestered there is due at least in part to the activity of a presumably integral membrane protein, the aminophospholipid translocator [4]. This translocator, or 'flippase', has not yet been identified with certainty, but parameters of its activity have been determined indirectly. For example, the translocator is inhibited by vanadate, low temperatures, and sulfhydryl-active reagents [3–6], and shows higher affinity for PS than PE [3].

Additionally, the translocator is stereospecific with respect to the α carbon of the glycerol backbone of lipids: PS and PE having the unnatural D-glycerol configuration are not translocated from the outer monolayer in fibroblasts [7]. In this study, we further examine the stereochemical requirements of the red blood cell aminophospholipid translocator. We find that the stereochemistry of the PS headgroup does not affect the activity of the translocator. The diastereomers L,L-DLPS and L,D-DLPS are recognized and translocated equally. However, incorporation of equal amounts of the two diastereomers does produce subtle quantitative differences in the cell morphological response, possibly as a result of minor differences in lipid packing of the two isomers.

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Abbreviations: DLPS, dilauroylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; CMC, critical micelle concentration; DPH, diphenylhexatriene; MI, morphological index.

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2. Materials and methods

2.1. Chemicals

Unless otherwise specified, all biochemicals were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. DLPS synthesis

1,2-dilauroyl-*sn*-glycero-3-phospho-L-serine (L,L-DLPS) was synthesized according to the method of Comfurius and Zwaal [8]. 1,2-dilauroyl-*sn*-glycero-3-phospho-D-serine (L,D-DLPS) was synthesized by the method of Hermetter et al. [9], with the following additions or modifications: (i) The sodium salt of phosphatidic acid was used directly without conversion to the pyridinium salt. (ii) The coupling solvent, pyridine, was purified by refluxing over potassium hydroxide for 90 min, followed by fractional distillation. Purified pyridine was stored under nitrogen and over calcium hydride. Purification of the pyridine was found to be critical to the success of the coupling reaction. (iii) Final purification of the L,D-DLPS was achieved by column chromatography on silica gel as described in Woolley and Eibl [10]. In later experiments, chromatography on cation-exchange resin (CM-52 cellulose, Whatman, Clifton, NJ) [8] proved to be a preferable method for separating and purifying DLPS synthesized with these modifications. DLPS synthesis via this route proceeds without racemization [9,11].

L,L-DLPS and L,D-DLPS, synthesized and purified as described above, were dried in vacuo and prepared as 3.25 mM stock solutions in chloroform. Stock solutions were sealed and stored at −20°C. DLPS concentrations were determined by phosphate assay [12].

2.3. Cells

Red blood cells were obtained from healthy adult volunteers by venipuncture and collected into citrate. The erythrocytes were isolated and washed free of other cells and serum components by repeated centrifugations (5000 × *g*, 5 min) in 150 mM NaCl. Isolated erythrocytes were resuspended in phosphate-buffered saline (138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 mM glucose (pH 7.4)), stored at 5°C, and were used no more than 12 h after isolation. Before any experiment, cells were rewarmed to room temperature or to 37°C for at least 30 min.

2.4. Vesicle preparation

Small unilamellar vesicles of either L,L-DLPS or L,D-DLPS were prepared by first evaporating stock solutions under a stream of argon and then placing the dried lipids under vacuum for at least 2 h. Phosphate buffer (140 mM NaCl, 10 mM NaH_2PO_4, 5 mM glucose (pH 7.4) [NaCl/P_i/glucose]) was then added to yield final concentrations of 250 μM. The suspensions were vortexed for one minute, then sonicated under argon for 45 min in a bath sonicator.

2.5. Cell-vesicle incubations

Packed red blood cells were suspended to hematocrit 50 in 250 μM L,L-DLPS or L,D-DLPS (final vesicle lipid concentration in the suspension 125 μM). Incubations were carried out at room temperature or 37°C for 1–2 h, as specified in figure legends. At the times indicated in figure legends, 1–2 μl aliquots were removed and fixed in 100 μl glutaraldehyde (1% in 150 mM NaCl). Morphologies of the erythrocytes were assessed by light microscopy of fixed samples. Echinocytes were given scores of + 1 to + 5, reflecting the degree of crenation; discocytes were scored 0; and stomatoctyes were assigned scores of −1 to −4, depending on the degree of invagination [3,13]. The average score of a field of 100 cells is defined as the morphological index (MI). Numerous fields were scored until the standard deviation in the MI was reduced to ±0.2 or less.

2.6. Vanadate inhibition of aminophospholipid translocation

Packed erythrocytes (200 μl) were treated with 800 μl sodium orthovanadate solution (125 μM Na_3VO_4 in NaCl/P_i/glucose). The suspension was agitated at room temperature for 20 min, then cells were pelleted by centrifugation (6500 × *g*, 5 min). The supernatant was removed, and cells were resuspended to hematocrit 50 in 90 μM L,L-DLPS or L,D-DLPS (final PS concentration in suspension 45 μM) and sodium vanadate (100 μM). (Vanadate was added first, as a concentrate.) Samples were incubated at room temperature for 90 min. At time intervals indicated in figure legends, 1–2 μl aliquots were removed and glutaraldehyde fixed. Fixed samples were observed by light microscopy and morphological indices were calculated.

2.7. Extraction of outer monolayer DLPS

DOPC/DPPG vesicles (97:3; 20 mM total lipid) were prepared as described [14], using a probe sonicator (Heat Systems, Inc., Farmingdale, NY, Model XL2010) rather than a bath sonicator. After 20 min sonication, vesicles were centrifuged (30000 × *g*, 10 min) to remove probe titanium particles. Control cells (discs) and stomatocytes formed by incubation with L,L-DLPS or L,D-DLPS for one hour were treated with

DOPC vesicles as described [14] for 60 min. Samples were fixed with glutaraldehyde and observed by light microscopy; MI's were calculated.

2.8. Determination of PS critical micelle concentration

The critical micelle concentration (CMC) of DLPS was calculated according to Marsh and King [15]. Disregarding possible effects of headgroup stereochemistry, the CMC of DLPS at room temperature, pH 7, and in 150 mM NaCl in water was calculated to be 0.56 μM.

The CMC's of L,L-DLPS and L,D-DLPS were measured using diphenylhexatriene (DPH) fluorescence as described by Chattopadhyay and London [16].

3. Results

3.1. Morphological responses of red blood cells treated with DLPS diastereomers at 25°C

The shape changes of red blood cells incubated with 250 μm L,L-DLPS (the natural DLPS diastereomer) have been described previously [3]; the morphological timecourse (Fig. 1) is characterized by an initial echinocytosis followed by stomatocytosis. Erythrocytes incubated with 250 μM L,D-DLPS showed a similar response, with the difference that the extents of both echinocytosis and stomatocytosis were less (Fig. 1). The differential stomatocytosis induced by the two diastereomers was stable on incubation for several hours.

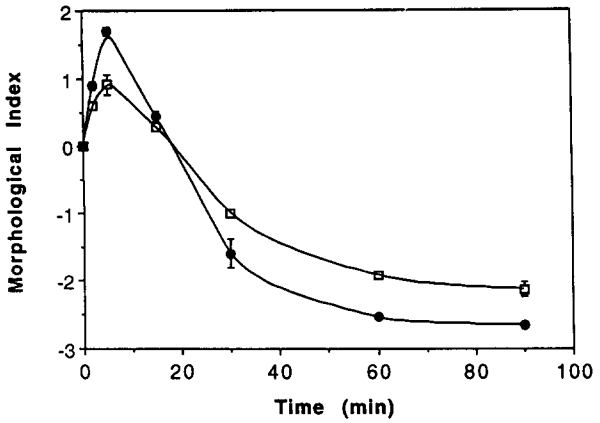

Fig. 1. Red blood cell shape change upon incubation with L,L- or L,D-DLPS vesicles at 25°C. Packed cells were treated with an equal volume of 250 μM L,L-DLPS (●) or 250 μM L,D-DLPS (□). At the times indicated, aliquots were removed and fixed and MI's were calculated, based on the scoring protocol described in Materials and methods. Data points without apparent error bars reflect counting errors less than the size of the data symbols. Figures are representative results from three or four repeated experiments.

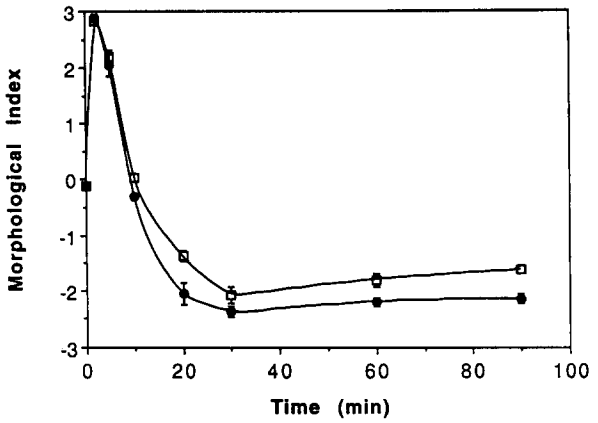

Fig. 2. Red blood cell shape change upon incubation with L,L-(●) or L,D-DLPS (□) vesicles at 37°C. Other conditions were as in Fig. 1. At the times indicated, aliquots were removed and fixed and MI's were calculated.

3.2. Determination of the CMC's of DLPS diastereomers

Differences in cell morphology responses to the DLPS diastereomers might reflect differential lipid incorporation, perhaps arising from differing aqueous phase solubilities [17]. Such different solubilities would be evident as differences in the CMC's of the lipids. The CMC's of the two diastereomers were determined by fluorescence assay of DPH, the CMC value being given by the lipid concentration at which DPH is solubilized and thus fluoresces [16]. No difference was found in the CMC values of L,L-DLPS and L,D-DLPS (data not shown); at pH 7, 150 mM NaCl, and room temperature, the CMC was found to be 0.6 μM in each case. This result corresponded closely to the calculated value for these conditions (0.56 μM).

3.3. Morphological responses of red blood cells treated with DLPS diastereomers at 37°C

The morphological changes of red blood cells incubated with 250 μM L,L-DLPS and L,D-DLPS were examined at the higher temperature of 37°C. As at 25°C, both diastereomers produced initial echinocytosis, followed by reversion to discocytes and then stomatocytes (Fig. 2). However, at 37°C, the initial difference in echinocytosis seen at lower temperatures was not evident. The L,D-diastereomer eventually induced less extensive stomatocytosis than the natural isomer, as was found at 25°C. The magnitude of the difference in the final stomatocytosis was consistently smaller at 37°C than at 25°C.

3.4. Treatment of L,L- and L,D-DLPS-induced stomatocytes with DOPC vesicles

Differences in cell morphological responses to PS diastereomers might reflect less complete inner mono-

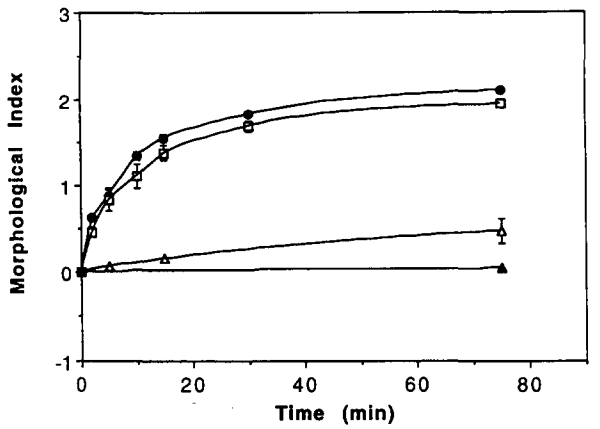

Fig. 3. Shape change of vanadate-inhibited red cells upon incubation with L,L- or L,D-DLPS vesicles at 25°C. Cells were treated with 100 μM sodium vanadate, followed by exposure to 90 μM L,L-DLPS (●), 90 μM L,D-DLPS (□) or 0 μM DLPS (△). At the times indicated, aliquots were removed and fixed and MI's were calculated. Control cells (▲) without vanadate or lipid treatment did not change shape.

layer sequestration of L,D-DLPS. Incubation of amphipath-loaded erythrocytes with an excess of vesicles composed of DOPC, a non-transferable lipid, extracts any transferable amphipath residing in the membrane outer monolayer ('back-extraction') [14]. This treatment was used to investigate the differing steady state stomatocytosis in cells treated with the DLPS diastereomers. Both L,L- and L,D-DLPS-generated stomatocytes back-extracted with DOPC remained stomatocytic with only a slight increase in stomatocytosis due to extraction of cholesterol [13] (data not shown). This failure to revert to discocytes indicated that the PS isomers were equally accessible to the exofacial phase.

3.5. Morphological responses of vanadate-inhibited red blood cells treated with DLPS diastereomers

The differing degree of echinocytosis in response to L,L-DLPS and L,D-DLPS at 25°C was investigated by inhibiting the aminophospholipid translocator, thus focusing on the echinocytic stage. Incubation of vanadate-inhibited erythrocytes with L,L-DLPS (90 μM) produced echinocytosis which reached a steady state after 45 min (Fig. 3). Vanadate-inhibited cells treated with L,D-DLPS crenated more slowly and to a lesser extent, but the difference was minor (Fig. 3). At 37°C, cells treated with the L,D-isomer crenated slightly more slowly, but reached the same steady state morphology (data not shown). Incubation of cells with vanadate alone produced a slight echinocytosis, presumably due to general ATPase inhibition.

4. Discussion

Phosphatidylserine is unique among the abundant phospholipids in that it has two chiral carbon centers. All natural phospholipids contain the stereocenter conventionally designated L at the middle (α) carbon of the glycerol backbone. Naturally occurring PS contains the additional chiral center in the L-serine headgroup. The sequestration of native PS in the inner monolayer of cell plasma membranes is achieved at least in part by the activity of an aminophospholipid translocating enzyme [3,4,7,18,19]. This 'flippase' also transports PE from the outer to inner membrane leaflet, but more slowly and less completely than PS, resulting in less pronounced asymmetry in PE's steady state distribution. This difference in transporter activity could be due to a stereochemical affinity for the diastereomeric structure of the PS headgroup.

This possibility was examined by comparing translocator activity for the natural lipid L,L-DLPS and its headgroup diastereomer L,D-DLPS. Since aminophospholipid translocator activity is not influenced by lipid acyl chain composition, both isomers were synthesized as dilauroyl (12:0) species; short acyl chain lipids transfer from source liposomes into cells at convenient rates. The incorporation and eventual disposition of such lipids in red cell plasma membranes are monitored readily through morphology change progressions [14].

When erythrocytes were treated with equal concentrations of the diastereomeric PS's, both induced transient echinocytosis followed by reversion to stomatocytic forms. These shape changes reflect incorporation of the foreign lipid into the cell outer monolayer, followed by translocation to the inner monolayer [14]. At 37°C (Fig. 2), both lipids were translocated at the same initial rate, as evinced by the slope of the echinocyte-to-discocyte transitions. Thus, the aminophospholipid translocator activity is not sensitive to specific stereochemistry in the PS headgroup. This is unexpected, since the demonstrated requirement for L-stereochemistry at the glycerol chiral center (demonstrated in fibroblasts [7]) is cited as evidence that the translocator, in general, is an enzyme [20]. The present results indicate that the recognition site for lipid headgroups is permissive in a major aspect of their structure.

The subtle morphological differences in cell response to L,L- and L,D-DLPS may reveal stereochemical specificity in other membrane properties. In general, the unnatural isomer, L,D-DLPS, induces less extensive shape alterations than the naturally-occuring lipid. At 25°C, this distinction is evident in the degree of transient spiculation, the rate of reversion, and the final extent of stomatocytosis at steady state (Fig. 1). At 37°C, initial spiculation is sufficiently rapid that this

difference is not evident (Fig. 2), but at steady state the translocated L,D-DLPS induces slightly less extensive stomatocytosis at the higher temperature as well. At both temperatures, the morphology difference at steady state persists for many hours.

Similarly, erythrocytes pretreated with vanadate respond slightly differently to incorporation of the two lipid isomers. Vanadate inhibits the aminophospholipid translocator, leaving subsequently incorporated PS trapped in the membrane outer monolayer, and cells remain echinocytic (Fig. 3). Echinocyte formation is slightly slower in vanadate-treated cells for L,D-DLPS than for L,L-DLPS, and the ultimate steady-state morphology is slightly different (Fig. 3). Both differences are more apparent at 25°C than at 37°C. Thus, the differential effects of the diastereomers are evident when the lipids are confined to the membrane outer monolayer, where direct interaction with cytoskeletal components is not possible.

A possible source of these disparities is a systematic difference in the quantity of incorporated lipid. If less L,D-DLPS were introduced, both extremes of shape response would be expected to be less extensive, and the translocation rate might be reduced. However, there is no obvious basis for differential lipid transfer. The lipid concentrations in donor vesicles were essentially identical and the water solubilities (critical micelle concentrations) of the two isomers were indistinguishable. Unless the cell membrane possesses a previously undisclosed mechanism that discriminates in favor of the L,L-diastereomer, interbilayer lipid transfer should proceed identically for the two lipids.

A second condition that would produce differing steady-state stomatocytosis for the two isomers is differential sequestration; i.e., the unnatural isomer might not be transported as completely as the natural lipid, or might be less well anchored at cytofacial binding sites and therefore more susceptible to transbilayer scrambling. In either situation, outer monolayer L,D-DLPS should be accessible to extraction by exogenous vesicles. Such extraction would be evident as a cell morphology change: L,D-DLPS treated cells should become more stomatocytic if a static population of outer monolayer lipid is accessible to extraction. If, as is likely, such a population is in slow equilibrium with inner monolayer DLPS [21], extraction of outer leaflet lipid would eventually induce reversion to discocytes. This was not found; stomatocytic L,L- or L,D-DLPS-treated cells remain stomatocytic when exposed to DOPC vesicles.

These observations render it unlikely that the two DLPS diastereomers are incorporated, translocated, or sequestered differently. The observed morphology differences may instead reflect differential interactions with membrane components. ^{2}H- and ^{31}P-NMR studies have revealed small differences in the head group conformations of L,L- and L,D-DMPS and DOPS [22]. Such structural differences might affect packing interactions and alter the effective area a lipid occupies in the monolayer.

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