Biochimica et Biophysica Acta, 1066 (1991) 37–42

ADONIS 000527369100254D

BBAMEM 75272

Transbilayer movement of phosphatidylserine in erythrocytes. Inhibitors of aminophospholipid transport block the association of photolabeled lipid to its transporter

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(Received 17 January 1991)

Key words: Phosphatidylserine; Lipid asymmetry; Red blood cell

The ability to cross-link [^{125}I]iodo-azido-phosphatidylserine (^{125}I-N_3-PS) to the putative 32-kDa aminophospholipid transporter of human red blood cells (RBC) has been examined by SDS-PAGE. In the absence of transport inhibitors, ^{125}I-N_3-PS preferentially labeled the 32-kDa polypeptide, whereas treatment of the cells with pyridyldithioethylamine (PDA), a potent inhibitor of the aminophospholipid translocase, abrogated the association of the probe to this protein. ATP-depletion, low temperature, and diamide or 5,5'-dithiobis(2-nitrobenzoic acid), inhibitors that oxidize an endofacial sulfhydryl distinct from the PDA-sensitive site (Connor, J. and Schroit, A.J. (1990) Biochemistry 29, 37–43), also blocked association of the PS analogue to the protein. Once ^{125}I-N_3-PS became associated with the transporter, however, only PDA was able to partially displace it. These data suggest that sulfhydryl reactive reagents inhibit PS transport by blocking the association of PS with its transporter, a process that is also ATP- and temperature-dependent.

Introduction

The asymmetric distribution of PS in RBC [1,2] is maintained by an ATP- and temperature-dependent, cysteine-containing, aminophospholipid-specific translocase [3,4]. Recent studies have suggested that this protein, which is responsible for the intrabilayer movement of PS, is a 32-kDa integral membrane polypeptide [5–7] associated with the Rh blood group system [8]. This was concluded from experiments showing that monoclonal Rh antibodies immunoprecipitate 32-kDa polypeptides specifically labeled with a transportable photoactivatable PS analogue, ^{125}I-N_3-PS [5], and an inhibitor of PS transport, ^{125}I-labeled PDA [6].

Although the translocase is responsible for the specific movement of PS from the outer to the inner membrane leaflet, lipid transport is dependent upon at least two distinct sulfhydryl sites, one that resides in the bilayer membrane and a second complementary site that resides at the cells' endofacial surface [9]. Both sulfhydryls must be reduced for transport to proceed [9].

Since PDA appears to bind directly to the hydrophobic transmembrane portion of the transporter, it is likely that PDA-mediated inhibition of PS transport involves direct modification of the 32-kDa polypeptide. On the other hand, it is unclear how oxidation of the endofacial sulfhydryl site(s) inhibits the generation of PS asymmetry.

To determine how oxidation of these critical sulfhydryls inhibits PS transport, we investigated the effects of ATP-depletion and sulfhydryl reagents (that selectively modify the hydrophobic or endofacial sites), on the ability of ^{125}I-N_3-PS to associate with the 32-kDa polypeptide. Depletion of ATP or oxidation of either sulfhydryl site inhibited the association of ^{125}I-N_3-PS with the 32-kDa polypeptide.

Abbreviations: ^{125}I-PDA, *N*-[3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionyl]pyridyldithioethylamine; BSA, bovine serum albumin; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellmans' reagent); DTT, dithiothreitol; ^{125}I-N_3-PS, -PC, 1-oleoyl-2-[[[3-(3-[^{125}I]iodo-4-azidophenyl)propionyl]amino]caproyl]phosphatidyl-serine, -choline; PBS, phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 10 mM Na/K phosphates, 10 mM glucose, pH 7.4); PDA, pyridyldithioethylamine; PS, phosphatidylserine; RBC, human red blood cells.

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Material and Methods

Materials and routine procedures

Carrier-free ^{125}I-N_3-PC (spec. act. approx. 2000 Ci/mmol) was synthesized as previously described [5]. ^{125}I-N_3-PS was prepared by phospholipase D-catalyzed base exchange [10] from the PC analogue. Diamide, DTNB and NaF were from Sigma Chemical Co. (St. Louis, MO), and ^{125}I-labeled Bolton-Hunter reagent (spec. act. approx. 2000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). PDA and ^{125}I-PDA were synthesized as previously described [6]. Human RBC were obtained from healthy volunteers by venipuncture into heparinized saline and washed with PBS. All experiments were carried out under red safelights.

Translocation of ^{125}I-N_3-lipid in RBC

RBC (2×10^8 cells/ml) were incubated in the dark with ^{125}I-N_3-PS at 37°C. At the indicated time points aliquots were removed, and the fraction of lipid transported from the outer leaflet to the inner leaflet was determined by assessing the residual radiation (lipid transported to the inner leaflet) of RBC back-exchanged with 5% BSA in PBS for 15 min on ice. BSA was removed by centrifugation and the fraction of photolabeled lipid remaining in the cells was determined by comparing the radiation to RBC not back-exchanged. The effects of PDA, sodium azide/deoxyglucose, NaF, DTNB, and diamide on the transport of ^{125}I-N_3-PS was determined using the conditions described in Table I. After washing, the cells were incubated with ^{125}I-N_3-PS or ^{125}I-N_3-PC (approx. 0.1 μCi in EtOH; final EtOH concentration $< 0.5\%$) for 45 min at 37°C in the absence or presence of DTT (5 mM). The ability of the cells to transport the photolabeled lipids was then determined.

Photolysis of RBC

RBC were rapidly mixed with ^{125}I-N_3-PS or ^{125}I-N_3-PC (approx. 1 μCi) in EtOH (final EtOH concentration $< 0.5\%$) in the dark. The cells were then incubated at 37°C for 20 min, washed twice with ice-cold PBS, transferred to 1.0 cm quartz cuvettes, and irradiated with constant mixing for 3 min 12 cm from an Osram HBO 100W/2 super-pressure mercury lamp. The cells were then washed with PBS. To identify the proteins labeled with the ^{125}I-N_3-lipids, ghosts were prepared by hypotonic lysis (5 mM phosphate buffer, pH 8.0), solubilized with SDS, and subjected to SDS-PAGE using an 11% separating and 6% stacking gel with the discontinuous buffer system of Laemmli [11]. The gels were stained with Commassie R-250, dried, and autoradiographed with Kodak XAR-5 X-ray film. To quantify the fraction of ^{125}I-N_3-lipid bound to individual polypeptides, films were analyzed by densitometry or cut into 0.25 cm strips and directly assessed for radiation by scintillation counting.

Effect of transport inhibitors on ^{125}I-N_3-PS binding to the translocase

RBC (2×10^8 cells/ml) were incubated with PDA, diamide, DTNB, NaF, or azide/deoxyglucose, under the conditions described in Table I. The cells were then incubated with the ^{125}I-N_3-lipids at 37°C for 20 min, photolyzed, and prepared for electrophoresis as described above. Alternatively, RBC (2×10^8 cells/ml) were incubated in the dark with ^{125}I-N_3-PS for 20 min at 37°C, treated with the inhibitors, and then photolyzed. In some experiments, RBC were labeled with ^{125}I-PDA as described previously [6].

Effect of temperature on the binding of ^{125}I-N_3-PS to the transporter

RBC (2×10^8 cells/ml) were incubated with ^{125}I-N_3-PS for 30 min on ice. The cells were then washed, and aliquots were incubated for an additional 20 min at on ice or at 37°C. The association of ^{125}I-N_3-PS with the 32-kDa polypeptide was determined by autoradiography after photolysis and SDS-PAGE.

Results

Transbilayer movement of ^{125}I-N_3-PS in RBC

Similar to the transport of PS [4] and its spin-labeled [12] or fluorescent analogues [13], the movement of ^{125}I-N_3-PS across the bilayer membrane of RBC was rapid (Fig. 1), ATP-dependent, and inhibited by a variety of sulfhydryl-reactive reagents (Table I). Table I shows that inhibition of ^{125}I-N_3-PS transport by the specific sulfhydryl oxidants, PDA, DTNB and diamide, was reversible after reduction with DTT.

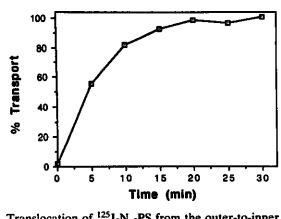

Fig. 1. Translocation of ^{125}I-N_3-PS from the outer-to-inner leaflet of RBC. RBC were incubated with ^{125}I-N_3-PS at 37°C. The fraction of lipid transported to the inner leaflet was determined at various intervals by back-exchange to BSA as described in Materials and Methods. Representative experiment of three.

TABLE I

Effect of inhibitors on the translocation of ^{125}I-N$_3$-PS

RBC ($2 \cdot 10^8$/ml) were incubated with the indicated inhibitors under the following conditions: PDA, 2 mM at 4°C for 30 min; DTNB, 5 mM at 37°C for 3 h (without glucose); diamide, 5 mM at 20°C for 30 min. Cells were also treated with sodium azide (10 mM) and deoxyglucose (50 mM) at 37°C for 3 h or with sodium fluoride (10 mM) at 37°C for 30 min, which reduced ATP by > 95% of control values (determined by luceferase/luciferin assay). After washing, the cells were incubated with ^{125}I-N$_3$-lipid for 45 min at 37°C and assessed for translocation as described in Materials and Methods. Representative of three experiments. Deviation between experiments was < 5%.

Inhibitor	% Translocation		
	^{125}I-N$_3$-PS		^{125}I-N$_3$-PC
	− DTT	+ DTT	
Control	84	86	19
PDA	14	90	18
Azide/deoxyglucose	10	11	14
DTNB	12	84	17
Diamide	11	88	17
NaF	10	–	16

Effect of transport inhibitors on the association of ^{125}I-N$_3$-PS with the 32-kDa polypeptide

There are several mechanisms by which inhibitors of aminophospholipid transport could block the transbilayer movement of PS. They might, for example, operate by inhibiting the association of the lipid substrate to the transporter, and/or by inhibiting its movement across the bilayer membrane. To examine the possible mechanism of PDA-mediated inhibition of PS transport, we determined whether PDA directly affected association of the translocating substrate, (^{125}I-N$_3$-PS), to the 32-kDa polypeptide. Fig. 2 shows that both ^{125}I-PDA and ^{125}I-N$_3$-PS preferentially labeled the 32-kDa polypeptide, while as previously seen, ^{125}I-N$_3$-PC labeled most RBC proteins randomly. Pretreatment of RBC with PDA blocked both the binding of ^{125}I-PDA and the association of ^{125}I-N$_3$-PS to the 32-kDa polypeptide, suggesting that both probes bind the same protein. Interestingly, the distribution of ^{125}I-N$_3$-PS in PDA-treated cells appeared to be very similar to the distribution of ^{125}I-N$_3$-PC in control cells (Figs. 2 and 5). As expected, PDA did not affect the distribution of ^{125}I-N$_3$-PC.

Similar to the effect of PDA, oxidation of endofacial sulfhydryls with DTNB or diamide also inhibited cross-linking of ^{125}I-N$_3$-PS to the 32-kDa polypeptide

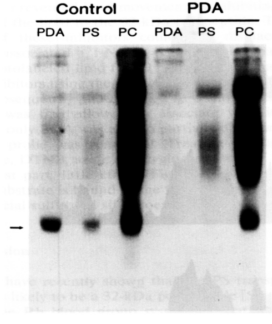

Fig. 2. Labeling of RBC with ^{125}I-N$_3$-lipid and ^{125}I-PDA. Control RBC or cells pretreated with PDA (2mM for 30 min at 4°C) were labeled with ^{125}I-PDA, ^{125}I-N$_3$-PS, or ^{125}I-N$_3$-PC as described. The cells were then photolyzed, subjected to SDS-PAGE, and autoradiographed. Free ^{125}I-N$_3$-lipid at the gel front (lipid not cross-linked to protein) is not shown. Arrow is the position of the 31 kDa molecular weight marker.

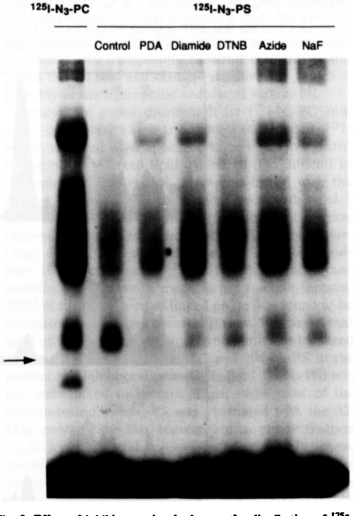

Fig. 3. Effect of inhibitor preincubation on the distribution of ^{125}I-N$_3$-PS crosslinked to RBC. RBC were pretreated with the indicated inhibitors as described in Table I. The cells were then incubated with ^{125}I-N$_3$-PS for 20 min at 37°C, photolyzed, subjected to SDS-PAGE, and autoradiographed.

Fig. 4. Effect of temperature on ^{125}I-N_3-PS labeling of the 32-kDa polypeptide. RBC were incubated with ^{125}I-N_3-PS for 30 min on ice and then kept on ice or transferred to 37°C for an additional 20 min. After photolysis the cells were subjected to SDS-PAGE, and the distribution of the lipid analog was determined by autoradiography.

(Fig. 3), a finding consistent with its inability to be transported (Table I). In addition, treatment of RBC with these reagents also resulted in an apparent random distribution of ^{125}I-N_3-PS throughout the membrane, a pattern similar to that obtained with ^{125}I-N_3-PC. Surprisingly, identical results were obtained in cells depleted of ATP with either sodium azide/deoxyglucose or fluoride (Fig. 3) and cells incubated with the lipid analog at 0°C (Fig. 4). This suggests that ATP and elevated temperature as well as reduced sulfhydryls, are critical for the association of PS to its transporter.

To quantify the amount of ^{125}I-N_3-lipid cross-linked to RBC membrane proteins, electrophoretically separated polypeptides were directly analyzed for bound lipids by scintillation counting (Fig. 5). The autoradiographs show basic differences between the labeling patterns of ^{125}I-N_3-PC and ^{125}I-N_3-PS. While the PC analogue appeared to uniformly label a wide range of membrane proteins, the PS analogue, under normal conditions, preferentially labeled the 32-kDa PS transporter. Interestingly, patterns similar to the apparent random distribution of ^{125}I-N_3-PC emerged when ^{125}I-N_3-PS transport was inhibited by PDA, DTNB, diamide or azide/deoxyglucose (see Table I), suggesting that these inhibitors block substrate recognition by the

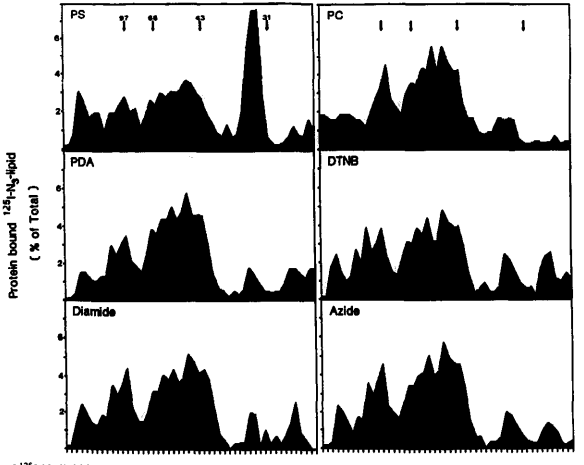

Fig. 5. Analysis of ^{125}I-N_3-lipid binding to RBC proteins in inhibitor-treated cells. SDS-PAGE gels prepared as described in Fig. 3 were cut into 0.25 cm strips and binding was quantified by scintillation counting. To normalize the results among the groups, the data were plotted as the fraction of total protein-bound radiation. The data have has not been corrected for background radiation. Arrows mark the positions of the indicated molecular weight standards.

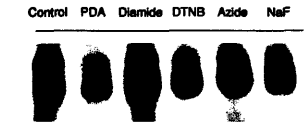

Fig. 6. Effect of inhibitors on the distribution of ^{125}I-N_3-PS preincubated with RBC. RBC were incubated in the dark with ^{125}I-N_3-PS for 20 min at 37°C. The cells were then incubated with the indicated inhibitors under the conditions described in Table I and photolyzed, solubilized, subjected to SDS page, and autoradiographed. The percent of ^{125}I-N_3-PS bound was determined by scintillation counting of the 32-kDa region. The autoradiograph shows the 32-kDa region only.

transporter. That the inhibitors blocked the association of ^{125}I-N_3-PS to the 32-kDa site was verified by determining the fraction of protein-associated lipid bound to the transporter. In control cells, approx. 30% of the protein-associated ^{125}I-N_3-PS localized in the 32 kDa region, whereas less than 5% of the protein-associated PS analogue bound to the same region in inhibitor-treated cells.

Disassociation of ^{125}I-N_3-PS from the 32-kDa polypeptide by PDA

Pretreatment of RBC with various transport inhibitors prevented lipid movement by inhibiting association of the lipid to the 32-kDa polypeptide. To determine if these inhibitors could also displace transporter-associated ^{125}I-N_3-PS, RBC were incubated with the photolabeled lipid for 20 min at 37°C, treated with the inhibitors using the conditions described in Table I, and subsequently photolyzed. Fig. 6 shows that if ^{125}I-N_3-PS was first allowed to associate with its transporter, only PDA was able to partially displace it (51% of the probe was removed). Treating the cells with diamide, DTNB, azide/deoxyglucose, or NaF, had, for the most part, little effect (Fig. 6). This suggests that once substrate is bound to the transporter, oxidation of endofacial sulfhydryl sites does not result in its dissociation.

Discussion

We have recently shown that the PS transporter in RBC is likely to be a 32-kDa polypeptide [5] associated with the Rh blood group system [8] that contains at least one PDA-sensitive cysteine residue critical to its activity [6]. In addition, a second independent site must also be reduced for transport to proceed [9]. This DTNB and diamide-sensitive site is on the endofacial surface and is distinct from the hydrophobic PDA-sensitive site. Although this endofacial sulfhydryl has not been identified, inhibition of transport is associated with cross-linking of cytoskeletal proteins without directly affecting the 32-kDa PDA-sensitive transporter [9]. To determine if inhibition of PS transport by sulfhydryl reagents that oxidize either site is similar, the association of photolabeled probes with the 32-kDa transporter in inhibitor-treated RBC was assessed.

Inhibition of transport by oxidation of either sulfhydryl, as well as by ATP-depletion and low temperature, correlated with a dramatic decrease in the fraction of probe cross-linked to the 32-kDa polypeptide. This suggests that the mechanism of transport inhibition, irrespective of the reagent used to achieve it, is accomplished by blocking the association of the lipid substrate to its transporter. It is possible that the inhibition induced by the sulfhydryl reagents is due to a conformational change in the transporter that might prevent recognition of the lipid, but this explanation cannot account for the results obtained by ATP depletion and low temperature. However, ATP may be required for recognition of the substrate, and low temperature may simply restrict lateral diffusion of the probes, resulting in random distribution. Interestingly, only oxidation of the hydrophobic 32-kDa sulfhydryl displaced probe associated with the polypeptide. This may indicate that the PDA-sensitive site is in close proximity to the transporters' substrate recognition site, unlike the DTNB- and diamide-sensitive site, which is probably at the hydrophilic endofacial surface [9].

Analysis of probe distribution for ^{125}I-N_3-PC and, under conditions where transport was inhibited, ^{125}I-N_3-PS, revealed what appeared to be a random distribution of cross-linked lipid. Although it is difficult to determine which proteins were cross-linked with the probes, it appears that the majority was with band 3 and the 70–40 kDa region with only a small fraction (approx. 5%) associated with the 32-kDa polypeptide. Once conditions were conducive for transport, however, the distribution of ^{125}I-N_3-PS among membrane proteins shifted, and a much larger fraction (approx. 30%) of the protein-cross-linked probe was found to be associated with the transporter. It should be noted that most of the photolabeled lipid, whether it was localized in the outer leaflet (^{125}I-N_3-PC and ^{125}I-N_3-PS in the presence of inhibitors) or inner leaflet (^{125}I-N_3-PS) was not cross-linked to protein. Thus, while most of the protein-bound ^{125}I-N_3-PS was associated with the 32-kDa polypeptide this represented a minor fraction (< 3%) of the total cell-associated probe. It seems, therefore, that photolysis captures a window of the transport process by cross-linking a fraction of the photolabeled analogue to the transport protein. Probe not cross-linked by photolysis is both transported and released, where it is free to diffuse in the cells' inner leaflet. Except for the 32-kDa labeling, this results in probe distributions remarkably similar to that of probe

resident in the cell's outer leaflet (^{125}I-N_3-PC: Fig. 5).

These data support previous observations [5,7] that the PS-specific phospholipid transporter in RBC is a 32-kDa integral membrane polypeptide. This is particularly evident from the finding that PDA completely inhibited the association of ^{125}I-N_3-PS with this polypeptide. Surprisingly, other inhibitors of lipid transport that operate via a mechanism independent of the 32-kDa polypeptide [9] also prevented the association of ^{125}I-N_3-PS with the protein. These findings imply close association of the 32-kDa transporter with a complementary endofacial component, possibly an ATPase [14,15] or other ATP-utilizing enzyme, suggesting that the maintenance of aminophospholipid asymmetry is dependent upon a complex of functionally dependent proteins.

Acknowledgments

We thank Karen Gillum for technical assistance and Peter Coderre for helpful discussions. This work was supported by National Institutes of Health grant DK 41714.

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