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Transfer of pyrene-labelled diacyl-, alkylacyl-, and alkenylacyl-glycerophospholipids from vesicles to human blood platelets

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The present study was aimed at investigating the spontaneous transfer of fluorescently labelled serine- and choline-glycerophospholipids from unilamellar vesicles to resting human blood platelets. The most effectively transferred phospholipids were pyrene-phosphatidylserine (PS) and the ether analogues of choline-glycerophospholipids, e.g., pyrene-alkylacyl- and pyrene-1'-alkenylacyl-glycerophosphocholines (plasmalogens). Transfer of pyrene-diacyl-glycerophosphocholine and pyrene-phosphatidic acid was almost not detectable under the same experimental conditions. The fast intermembrane PS-transfer could be explained by the very high degree of adsorption of PS donor vesicles to the platelet plasma membrane. The short halftime of transfer rate (12–14 min) and the high incorporation (1.08–2.16% of total platelet glycerophosphocholines) observed for ether choline-phospholipids in contrast to pyrene-PS (20 min, 0.8% of total platelet PS), could be interpreted in terms of their bulk membrane properties.

Introduction

Phospholipids (PLs) in the blood stream are subject to dynamic exchange either between (i) lipoproteins, (ii) lipoproteins and various cells, and between (iii) different cell membranes. The most abundant PLs of mammalian lipoprotein surfaces and plasma cell membranes are diacyl-glycerophospholipids. Transfer of this PL subclass is facilitated by specific lipid transfer proteins [1]. Previous studies have shown that alkylacyl- and alkenylacyl-glycerophospholipids which are present in lipoproteins and, at higher concentrations in cellular membranes [2] are also highly transferable between artificial membranes [3] and biological membranes. The latter observations were made with red blood cells [4] and cultured human skin fibroblasts [5,6] as biological acceptor membrane systems.

Choline and ethanolamine glycerophospholipids are the predominant lipid components of human blood platelets comprising 40 mol% (choline glycerophospholipid) and 23–28 mol% (ethanolamine glycerophospholipid) of total platelet membrane PLs. The choline lipid fraction consists of approx. 80% diacyl- and 20% alk(en)ylacyl-glycerophosphocholine (GPC), whereas the ethanolamine lipid fraction contains approx. 65% ether-lipids [7–10]. In platelet membranes, ether-lipids have been found to incorporate high concentrations of $\omega - 3$ fatty acid (eicosapentaenoic acid and docosahexaenoic acid) after dietary supplementation [11,12]. This indicates that alkenylacyl/alkylacyl-GPLs might play a subtle role in the ability of platelets to aggregate and release of eicosanoids. Since platelet membrane PLs are predominantly involved in various membrane-mediated processes of hemostasis and thrombosis [13], this study was carried out with a 2-fold purpose in mind. First, we wanted to assess if the transfer of ether-PLs from PL vesicles is facilitated when compared to its diacyl-counterparts. Secondly, we addressed the question as to how and to what extent PL transfer from vesicles to platelets depends on the headgroup structure.

For this purpose we synthesized fluorogenic diacyl-, alkenylacyl-and alkylacyl-GPC as well as diacyl-glycerophosphate and diacyl-glycerophosphoserine. Pyrene-labelled lipids can be considered as versatile probes to monitor membrane lipid transport [14]. Thus, the re-

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spective model compounds contained the pyrenedecanoyl residue instead of the natural acyl chain at the *sn*-2 position of the glycerol backbone. 1-Palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphoserine (pyrene-PS) was prepared by phospholipase D-catalyzed transphosphatidylation of 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine (pyrene-PC) [15] in the presence of L-serine and separated from unreacted pyrene-PC and the by-product 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphate (pyrene-PA) by TLC (Silica gel H, Merck) using $CHCl_3$/CH_3OH/25% NH_4OH (20:80:5, v/v/v) as a mobile phase. R_f values were: pyrene-PS, 0.7; pyrene-PC, 0.24; pyrene-PA, 0.21. Zones containing pyrene-PS and pyrene-PA were scraped off the plates and extracted with $CHCl_3$/CH_3OH (1:4, v/v). Pyrene-PC was prepared by treatment of dipalmitoyl-PC with phospholipase A_2 [16] and subsequent acylation of the resulting lyso-compound with pyrenedecanoic acid [5]. 1-*O*-(1'-Alkenyl)-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine (pyrene-alkenylacyl-GPC, pyrene-choline plasmalogen) was prepared from 1-*O*-(1'-alkenyl)-*sn*-glycero-3-phosphocholine (alkenyl-GPC, choline lysoplasmalogen) by acylation with pyrenedecanoic acid [5,17,18]. After catalytic hydrogenation of 1-*O*-(1'-alkenyl)-*sn*-glycero-3-phosphocholine prepared from beef heart PLs [21], 1-*O*-alkyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine (pyrene-alkylacyl-GPC) was prepared as described for pyrene-choline plasmalogen [5,18].

Donor vesicles were prepared from fluorogenic PLs by the ethanol injection method [19]. The vesicle suspension showed high pyrene excimer fluorescence at 480 nm and low pyrene monomer fluorescence at 380 and 400 nm. The fluorescence of pyrene-labelled lipids is concentration-dependent showing blue and green emission maxima at low and high label concentrations, respectively [14,15]. Simple binding and/or adsorption of labelled PL vesicles to cell membranes would leave the fluorescence signal at 380 nm and 480 nm unchanged. Transfer of pyrene-labelled PLs from vesicles into platelet membranes was determined from the continuous increase in pyrene monomer fluorescence intensity at 380 nm due to dilution of labelled lipid by unlabelled lipids present in the platelet plasma membrane. Isolation of platelets was performed by gel filtration of platelet-rich plasma on Sepharose 2B column [20].

After incubation of intact resting gel-filtered platelets (GFPs) with pyrene-PL vesicles at 25°C (up to 2 h) GFPs were separated from unbound vesicles by centrifugation. The amount of platelet-associated pyrene-lipid was determined from the fluorescence intensity (excitation and emission wavelengths were 342 and 380 nm, respectively) obtained after extraction of platelet membrane lipids with $CHCl_3$/CH_3OH (2:1, v/v) [21] and subsequent separation of lipids by thin-layer chromatography [18]. Up to 95% of the labelled PL-species associated with platelets was present in the corresponding unlabelled lipid fraction (Table I). This indicates that degradation of pyrene-lipids was insignificantly low within the time of incubation.

TABLE I

Identification of pyrene-labelled PLs associated with GFPs (isolated from four different platelet donors) after incubation with PL vesicles

The difference to 100% was found in: (a) neutral lipids; (b) neutral lipids, PC, and PS; (c) PA; (d) neutral lipids, traces in phosphatidylethanolamine. GFPs and unilameller vesicles composed of the respective pyrene-labelled PLs were incubated for 60 min. After sonication of GFPs and extraction of platelet lipids (including pyrene-labelled lipids associated with GFPs) with $CHCl_3$/CH_3OH (2:1, v/v) [21], PLs were separated and identified by two-dimensional TLC ($CHCl_3$/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O, 55:20:10:10:5, by vol.) on Silica 60 H plates (Merck) and identified using total beef heart PL as a standard.

Pyrene-labelled PLs in donor vesicles	% recovery from lipid extract
Pyrene-PC	92±4% PC (a)
Pyrene-PA	96±2% PA (b)
Pyrene-PS	87±3% PS (c)
Pyrene-alkenylacyl-GPC	96±3% PC (d)
Pyrene-alkylacyl-GPC	95±2% PC (d)

By comparing the rates of transfer of labelled PLs from vesicles to resting GFPs it could be shown that pyrene-PS, pyrene-alkylacyl- and pyrene-alkenylacyl-GPC were rapidly incorporated with halftimes of transfer rates of 20 min, 14 min and 12 min, respectively (Fig. 1). Transfer of pyrene-diacyl-PC was almost not detectable under the same experimental conditions (Fig. 1). As pyrene-PA is also poorly transferred from vesicles to resting platelets (similar as observed for diacyl-GPC), it must be concluded that a net negative charge which is present on the PL headgroup of PS and PA is not the determining factor for the transfer of PLs into platelets. By comparing transfer rates of readily transferred PLs (PS and alk(en)ylacyl-GPCs) to platelets, ether-PLs with a zwitterionic polar headgroup are transferred slightly faster than PS.

To further investigate whether the transfer of labelled PLs might depend on the different extent of adsorption of donor vesicles to platelets, GFPs were incubated (5–30 min) with pyrene-labelled PL vesicles at 25°C. After separation of platelets from unbound donor vesicles by centrifugation and subsequent extraction of lipids with chloroform/methanol (2:1, v/v) [21] pyrene fluorescence intensities were measured. Pyrene-PS showed the highest extent of adsorption (up to 70% of total fluorescent lipids) when compared to pyrene-PA (25%), pyrene-diacyl-GPC (22%), pyrene-alkylacyl-GPC (17%) or pyrene-alkenylacyl-GPC (15%) (Fig. 2).

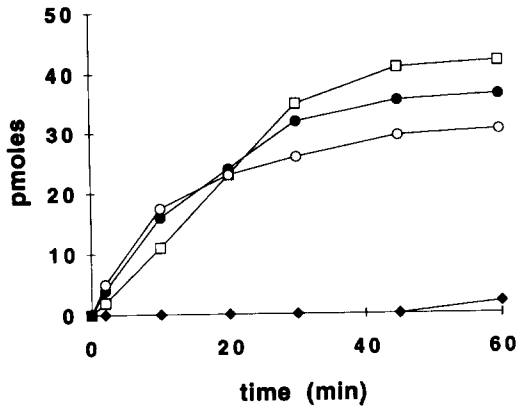

Fig. 1. Transfer of pyrene-labelled PLs from vesicles to GFPs ($1 \cdot 10^8$/3 ml Tyrode's solution [20]) at 25°C. □, Pyrene-PS; ●, pyrene-alkylacyl-GPC; ○, pyrene-alkenylacyl-GPC; ♦, pyrene-diacyl-GPC. Pyrene-lipid concentration was 6 nmol/3 ml in the assay system. PL transfer was determined from the increase of fluorescence intensity at 380 nm using a Shimadzu RF-540 spectrofluorometer interfaced to an IBM computer. Excitation (ex) wavelength was 342 mn. Slit widths were 20 nm (ex) and 5 nm (emission), respectively. Samples were kept under an argon atmosphere during measurement. Concentration of pyrene-labelled PLs was estimated from the fluorescence intensity at 380 nm, using pyrenedecanoic acid as a standard. Results shown here are the means ± S.D. of three experiments, each in duplicate.

In order to determine the effect of PL composition in the donor vesicles on the transfer of individual PLs, vesicles were prepared containing mixtures of pyrene-PS and palmitoyloleoyl phoshatidylcholine (POPC) at molar ratios of 1:2 and 1:5. The absolute amount of pyrene-PS in the sample volume rather than the relative concentration of labelled PS determines the rate and the extent of PS transfer into GFPs (data not shown). This might be taken as evidence that (i) PS may form clusters in PC-containing vesicles at least upon adsorption to cells and that (ii) PS transfer rates depend on the extent of PS adsorbed to platelets. In contrast to pyrene-PS, the rate of pyrene-choline plasmalogen transfer is reduced in the presence of unlabelled POPC (data not shown) reflecting dilution of pyrene-plasmalogen within the outer leaflet of donor vesicles which in turn leads to a change of bulk membrane properties.

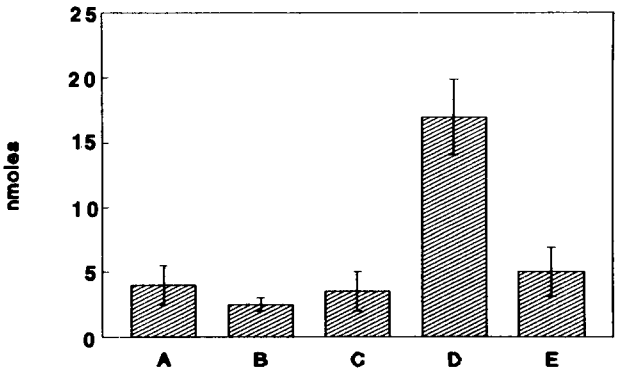

Fig. 2. Adsorption of pyrene-PL vesicles to platelets. GFP suspensions ($2 \cdot 10^8$ GFPs/3 ml) were incubated for 10 min (25°C) with vesicles containing 20 nmol pyrene-diacyl-GPC (A), pyrene-alkenylacyl-GPC (B), pyrene-alkylacyl-GPC (C), pyrene-PS (D), or pyrene-PA (E). Shown are the amounts of bound lipid as determined from the monomer fluorescence intensities (380 nm) of pyrene-PLs extracted from GFPs with $CHCl_3/CH_3OH$ (2:1, v/v) [21]. Values are given as means ± S.D. of three experiments, each in duplicate.

According to Fig. 1, approx. 20 to 40 pmol of fluorescent lipids per 35.6 ± 0.99 nmol total platelet PL per $1 \cdot 10^8$ platelets [7,8,22] can be incorporated as determined from the continuous time-dependent increase in pyrene-monomer fluorescence intensity. As platelet PS accounts for approx. 10 mol% of total platelet membrane PLs [8,22] pyrene-PS amounts up to 0.8% of total platelet PS. As ether-PLs account for up to 20% of total platelet PC [8,10] the amounts of pyrene-ether GPCs incorporated into the platelet membrane (under the same experimental conditions as described for pyrene-PS) were 1.08–2.16% of total platelet alkylacyl-plus alkenylacyl-GPC content.

Summarizing the results of the present study we could show that PS is easily adsorbed to and incorporated into platelet plasma membranes. Ether choline-glycerophospholipids (pyrene-alkenylacyl-and alkylacyl-GPCs) are also effectively taken up from vesicles into platelets, but adsorption was by far lower when compared with PS, but similar when compared with diacyl-GPC or PA. From our data it may be deduced that ether-GPCs may represent a subclass of easily exchangeable membrane lipids. In addition to ether lipids prepared from natural sources containing predominantly 16-carbon hydrophobic side chains [23] we also used defined molecular ether-PL species containing only 1-*O*-hexadec(en)yl moieties which were purified by HPLC [24]. Almost the same halftime of transfer rates were found for both substrates indicating that we measured effects that were specific for ether lipids.

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References

1 Zilversmit, D.B. (1983) Methods Enzymol. 98, 565–573.
2 Das, A.K., Holmes, R.D., Wilson, G.N. and Hajra, A.K. (1992) Lipids 27, 401–405.
3 Szolderits, G., Daum, G., Paltauf, F. and Hermetter, A. (1991) Biochim. Biophys. Acta 1063, 197–202.
4 Prenner, E., Paltauf, F. and Hermetter, A. (1990) Proc. SPIE 1204, 605–610.
5 Loidl, J., Schwabe, G., Paschke, E., Paltauf, F. and Hermetter, A. (1990) Biochim. Biophys. Acta 1049, 75–84.
6 Van der Veer, E., Van der Weide, D., Heijmans, H.S.A. and Hoekstra, D. (1993) Biochim. Biophys. Acta 1146, 294–300.
7 Holub, B. (1984) Can. J. Biochem. 62, 342–351.

8 Adams, G.A. (1985) in The Platelets: Physiology and Pharmacology (Longenecker, G.L., ed.), pp. 1–14, Academic Press, London.
9 Natarajan, V., Zuzarte-Augustin, M., Schmid, H.H.O. and Graff, G. (1982) Thromb. Res. 30, 119–125.
10 Mueller, H.W., Purdon, A.D., Smith, J.B. and Wykle, R.L. (1983) Lipids 18, 814–819.
11 Holub, B.J., Celi, B. and Skeaff, C.M. (1988) Thromb. Res. 50, 135–143.
12 Aukema, H.M. and Holub, B.J. (1989) J. Lipid Res. 30, 59–64.
13 Zwaal, R.F.A., Comfurius, P. and Bevers, E. (1992) Biochim. Biophys. Acta 1180, 1–8.
14 Pownall, H.J. and Smith, L.C. (1989) Chem. Phys. Lipids 50, 191–211.
15 Somerharju, P.J., Virtanen, J.A., Eklund, K.K., Vainio, P. and Kinnunen, P.K.J. (1985) Biochemistry 24, 2773–2781.
16 Mason, J.T., Broccoli, A.V. and Huang, C.H. (1981) Anal. Biochem. 113, 96–101.
17 Hermetter, A. and Paltauf, F. (1982) Chem. Phys. Lipids 30, 47–53.
18 Paltauf, F. and Hermetter, A. (1991) Methods Enzymol. 197, 134–149.
19 Kremer, J.M.H., Van der Esker, M.W.J., Pathmamanoharan, C. and Wiersema, P.H. (1977) Biochemistry 16, 3932–3935.
20 Malle, E., Sattler, W., Prenner, E., Leis, H.J., Hermetter, A., Gries, A. and Kostner, G.M. (1991) Atherosclerosis 88, 193–201.
21 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–511.
22 Kawasaki, T., Kambayashi, J., Mori, T. and Kosaki, G. (1984) Thromb. Res. 36, 335–344.
23 Schmid, H.H.O. and Takahashi, T. (1968) Biochim. Biophys. Acta 164, 141–147.
24 Loidl, S. (1991) Thesis, Technische Universität Graz, Graz.