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Topological distribution of aminophospholipid fatty acids in trout intestinal brush-border membrane

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The transbilayer distribution of aminophospholipids in trout intestinal brush-border membrane has been investigated using trinitrobenzene sulfonic acid (TNBS). In the middle intestine, phosphatidylethanolamine (PE) is symmetrically distributed between the two leaflets while 68% of the phosphatidylserine (PS) are located in the inner membrane leaflet. In the posterior intestine, 64% of the PE and 69% of the PS are located in the inner membrane leaflet. When asymmetrically distributed, the inner species of PE and PS have a higher content of 22:6($n-3$) than the outer ones. This asymmetric distribution of docosahexaenoic acid in trout intestinal brush-border membrane might be related to the rod-like shape of the microvillus membrane and to its metabolism to hydroxylated derivatives.

Introduction

In most biological membranes of eukaryotic cells, phospholipids are the major class of lipids. These molecules play an important role in membrane properties, such as fluidity [1], permeability [2] and enzyme activities [3].

The intestinal brush-border membrane is a highly specialized plasma membrane responsible for the digestive and absorption functions of the enterocyte. It is easy to purify [4] and the closed vesicles retain the original orientation of the membrane [6]. In view of the important functions of this membrane requiring specific phospholipids, the intramembrane distribution of the different phospholipids would imply a role in the control of the vectorial processes occurring through this membrane.

In a previous paper [4] we reported some differences in the fatty acid composition and the fluidity of trout brush-border membranes isolated from the middle or posterior intestine. Trout phospholipids are very rich in 22:6($n-3$) [7], which has been recently demonstrated to play an important fluidizing role in the membranes [8]. These observations prompted us to investigate the intramembrane distribution of aminophospholipids, in relation to their fatty acid composition, in trout intestinal brush-border membrane. The probe we used was the trinitrobenzene sulfonate (TNBS) a well-known non-permeant reagent for amino groups [9,10].

Materials and Methods

Fish and membrane preparation

Rainbow trout (250–350 g) were fed, for 3 months, a linolenic acid-rich diet prepared as pre-

Abbreviations: TNBS, trinitrobenzenesulfonic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine.

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viously described [4]. Trout used for the kinetic studies of TNBS labelling were fed a commercial pelleted diet (Universal-Kraftfutterwerk, Kehl, F.R.G.). Intestinal brush-border membranes were prepared from the middle and the posterior part of the intestine according to an original procedure, yielding pure and closed membranes with low amounts of contaminating organelles and therefore suitable for biochemical studies [4,5]. For the experiments, membranes of similar purity were selected according to the alkaline phosphatase enrichment (13–18-fold).

Trinitrophenylation of the aminophospholipids

The labelling of aminophospholipids by TNBS was carried out as described by Rothmann and Kennedy [11]. Brush-border membranes were suspended in 170 mM $NaHCO_3$ (pH 8.00). 15 mM TNBS in 5% $NaHCO_3$ (pH 9.00) was added to the membrane suspension, to give a final concentration of 1 mM. 400 μg protein were sufficient for each incubation time. The reaction was performed at 0°C and stopped by addition of 10 μl ethanolamine before lipid extraction [12].

Phospholipid analysis

The separation of trinitrophenylated aminophospholipids from the non-reactive ones has been performed by two-dimensional TLC on pre-coated silica gel plates (Merck No. 5721). Methyl acetate/1-propanol/chloroform/methanol/0.25% KCl (25 : 25 : 28 : 10 : 7, by vol.) was used for the first dimension, chloroform/methanol/acetic acid/water (90 : 40 : 12 : 2, by vol.) was used for the second one. This original procedure allowed accurate separation of all membrane phospholipids.

After detection with primulin under ultraviolet light [13], the reacted and the non-reacted aminophospholipids were eluted from the silica gel with chloroform/methanol/water (5 : 5 : 1, by vol.). An alkaline hydrolysis at room temperature before methylation [14] was necessary to discriminate phospholipid fatty acids from co-migrating glycolipid ones. Fatty acid methyl esters were analysed as previously described [4]. Data are expressed as percent molar distribution. The amount of each phospholipid was estimated using heptadecanoate as an internal standard, their average molecular weight being computed from their fatty acid composition.

Enzymatic radioiodination

Lactoperoxidase catalyzes the radioiodination of the phospholipids localized at the cell surface [15] as well as in the outer leaflet of synthetic membranes [16]. This was used to ascertain that, in our experimental conditions, TNBS reacts with external aminophospholipids exclusively. The plasma membrane preparations were labelled as previously described [17], except that neither sodium thiosulfate nor hydrophobic gel filtration were used since free iodine species did not comigrate with the labelled phospholipids in the two-dimensional TLC system used. Omission of lactoperoxidase reduced almost entirely the phospholipid radioiodination. Subsequent to radioiodinated phospholipid chromatography, thin-layer plates were exposed to X-ray films (Agfa-Gevaert, AFW Curix RP2) in X-ray film cassettes (Philips-Massiot). At the appropriate time films were developed (Agfa-Gevaert, G1500 and fixed (Agfa-Gevaert, G350). The silica gel containing aminophospholipids was scraped and counted in a gamma counter (ICN Tracerlab GS 500).

Results

When brush-border membranes from trout fed a commercial diet were incubated with 1 mM

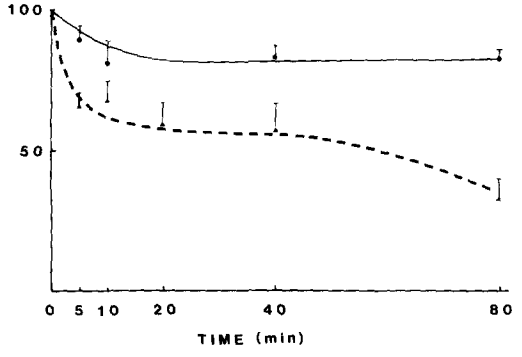

Fig. 1. Time course for the trinitrophenylation of aminophospholipids of intestinal brush-border membrane from rainbow trout. The extent of the reaction is expressed as aminophospholipids (mol%) remaining underivatized. Incubation at 0°C with 1 mM TNBS. Values are mean ± S.E. of three determinations. ●, PS; ▲, PE.

TABLE I

DISTRIBUTION OF AMINOPHOSPHOLIPIDS (mol%) BETWEEN THE TWO LEAFLETS OF INTESTINAL BRUSH-BORDER MEMBRANE

Results are means ± S.E. for three preparations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for differences between the two leaflets (Student's t-test).

	Middle intestine		Posterior intestine	
	internal leaflet	external leaflet	internal leaflet	external leaflet
PE	53.96 ± 6.86	46.04 ± 6.86	63.76 ± 3.01 **	36.24 ± 3.01
PS	68.06 ± 7.73 *	31.94 ± 7.73	68.80 ± 1.30 ***	31.20 ± 1.30

TNBS, the conversion of ethanolamine and serine phospholipids into their trinitrophenylated derivatives reached a plateau between 30 and 50 min (Fig. 1). After a 40 min treatment, about half of the PE (46%) from middle intestine membranes was derivatized, while only one-third of the PS (32%) reacted with TNBS. However, in membranes from the posterior intestine, about one-third of both PE (36%) and PS (32%) was derivatized (Table I). When 40 min TNBS treatment was performed after the lactoperoxidase catalyzed radioiodination, 70% of the total spotted radioactivity was detected on the PE and PS derivative spots while underivatized PE and PS were not radioactive; thus, in our experimental conditions, external aminophospholipids were trinitrophenylated. When the TNBS treatment was performed in the presence of Triton X-100 (0.05%), almost the total membrane PE and PS (about 87%) was derivatized.

In the brush-border membrane of the middle intestine (Table II), only PS presented fatty acid composition differences between both leaflets, the internal one presenting higher percentages of 18 : 0 and 22 : 6($n-3$) and lower amounts of 16 : 0 and 18 : 1($n-9$) than the external one.

In the brush-border inner membrane leaflet of the posterior intestine (Table III), both PE and PS

TABLE II

AMINOPHOSPHOLIPIDS FATTY ACID COMPOSITION (mol%) OF THE INTERNAL AND EXTERNAL LEAFLETS FROM MIDDLE INTESTINE BRUSH-BORDER MEMBRANE.

Values are means ± S.E. of three preparations. * $P < 0.05$, ** $P < 0.01$ for differences between both leaflets (Student's t-test). Fatty acids contributing less than 1% were omitted. Σ, sum of all the fatty acids of the indicated species.

Fatty acid	PE		PS	
	internal leaflet	external leaflet	internal leaflet	external leaflet
16 : 0	20.02 ± 1.03	19.02 ± 3.09	16.27 ± 2.42 **	29.50 ± 0.82
18 : 0	15.58 ± 0.48	13.96 ± 0.54	29.89 ± 2.06 **	15.24 ± 1.23
Σ(sat)	40.94 ± 1.30	37.78 ± 3.77	50.46 ± 1.70	52.08 ± 1.25
16 : 1	5.56 ± 0.54	4.71 ± 0.29	4.36 ± 1.10	12.17 ± 3.11
18 : 1	16.70 ± 0.84	16.73 ± 1.02	9.20 ± 2.74 *	21.47 ± 0.93
Σ($n-9$)	24.06 ± 0.67	23.34 ± 0.98	15.76 ± 2.21 **	36.34 ± 1.81
18 : 2	4.35 ± 0.67	6.44 ± 1.26	1.15 ± 0.20	3.23 ± 0.85
Σ($n-6$)	7.02 ± 0.38	9.77 ± 1.06	4.28 ± 0.35	4.18 ± 0.99
18 : 3	2.92 ± 0.28	3.79 ± 0.58	–	2.02 ± 0.19
20 : 5	2.40 ± 0.30	2.80 ± 0.60	–	–
22 : 6	19.96 ± 1.36	19.94 ± 3.04	25.37 ± 2.72 **	4.57 ± 0.76
Σ($n-3$)	25.27 ± 1.65	19.13 ± 4.19	29.53 ± 3.17 **	7.42 ± 0.59
Unsat/sat	1.45 ± 0.08	1.71 ± 0.28	0.99 ± 0.07	0.93 ± 0.05
$n-3/n-6$	4.00 ± 0.16	3.01 ± 0.38	6.95 ± 0.71 **	1.98 ± 0.43

TABLE III

AMINOPHOSPHOLIPIDS FATTY ACID COMPOSITION (mol%) OF THE INTERNAL AND EXTERNAL LEAFLETS FROM POSTERIOR INTESTINE BRUSH-BORDER MEMBRANE

Values are means ± S.E. on three preparations. * $P < 0.05$, ** $P < 0.01$ for differences between both leaflets (Student's t-test). Σ, sum of all the fatty acids of the indicated species.

Fatty acid	PE		PS	
	internal leaflet	external leaflet	internal leaflet	external leaflet
16:0	20.40 ± 0.80	23.65 ± 2.19	20.16 ± 4.13	31.57 ± 0.59
18:0	15.93 ± 0.44	14.00 ± 0.97	24.49 ± 4.68	13.52 ± 0.88
Σ(sat)	41.20 ± 1.01	45.89 ± 2.83	49.90 ± 0.63	51.75 ± 3.88
16:1	5.32 ± 0.70	7.45 ± 1.05	5.30 ± 0.43 *	14.18 ± 1.99
18:1	15.78 ± 0.16	16.91 ± 0.56	12.44 ± 3.38	21.41 ± 1.39
Σ($n-9$)	23.21 ± 1.77	29.55 ± 2.48	19.92 ± 2.93	38.13 ± 2.65
18:2	2.58 ± 0.21	5.22 ± 2.32	1.94 ± 0.55	3.22 ± 0.43
Σ($n-6$)	4.93 ± 0.34	7.34 ± 2.10	4.74 ± 0.51	4.29 ± 1.23
18:3	1.86 ± 0.43	2.26 ± 0.22	0.98 ± 0.38	1.75 ± 0.19
20:5	2.90 ± 0.40	1.60 ± 0.40	–	–
22:6	21.84 ± 1.88 *	10.72 ± 2.88	19.72 ± 3.45 **	2.91 ± 0.84
Σ($n-3$)	30.36 ± 2.48	17.24 ± 4.21	25.46 ± 2.81 **	5.81 ± 1.48
Unsat/sat	1.44 ± 0.06	1.20 ± 0.14	1.01 ± 0.03	0.96 ± 0.15
$n-3/n-6$	6.27 ± 0.86	2.83 ± 0.98	5.55 ± 0.93 *	1.39 ± 0.05

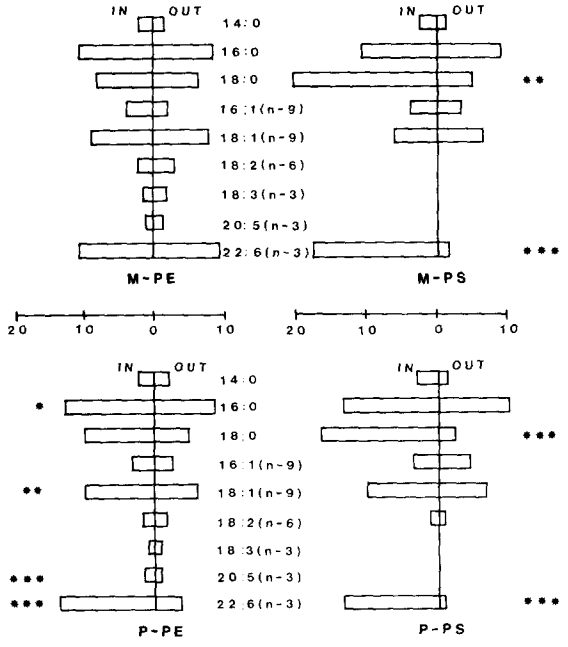

Fig. 2. Fatty acid distribution (mol%) between inner and outer leaflets of trout intestinal brush-border membrane. Fatty acids contributing less than 2% in the whole membrane phospholipid are omitted. M-PE, PE of brush-border membrane from the middle intestine. M-PS, PS of brush-border membrane from the middle intestine, P-PE, PE of brush-border membrane from the posterior membrane. P-PS, PS of brush-border membrane from the posterior intestine. IN, inner leaflet; OUT, outer leaflet. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for differences between both leaflets (Student's t-test).

were enriched in 22:6($n-3$); furthermore, PS presented a low percentage of 16:1($n-9$).

These differences in the fatty acid distribution for PE and PS between both membrane leaflets are emphasized when considering the contribution of each leaflet to the fatty acid composition of the whole membrane (Fig. 2). The most important contribution to 16:0, 18:1($n-9$), 20:5($n-3$) and 22:6($n-3$) PE content in the posterior intestine originates from the internal leaflet, while in the middle intestine both leaflets have a similar contribution.

Discussion

Since radioiodination results indicate that TNBS reacts with all the external aminophospholipid molecules and does not permeate, as has already been shown for chick brain microsomes [17], this reagent appears to be suitable for use in the study of the topological distribution of PE and

PS in trout brush-border membranes. The partial labelling of aminophospholipids obtained with Triton X-100 treatment might reflect the presence of aminophospholipids which are tightly bound to integral proteins, and hence are not accessible to the reagent, as has already been reported [18,19]. However, our results clearly indicate that the aminophospholipids are asymmetrically distributed in trout brush-border membrane.

Brush-border membranes of middle and posterior intestine of trout have already shown differences in their biophysical properties as well as in their fatty acid composition [4], their enzyme activities [5] and their absorption properties [20]. The present results also indicate some differences in the topological distribution of the aminophospholipids in the brush-border membranes from the middle and posterior parts of the intestine. PE, which represents 19.3 ± 0.8 and $20.2 \pm 0.8\%$ of the brush-border membrane phospholipids from the middle and posterior intestine, respectively, is symmetrically distributed in the middle intestine while in the posterior intestine most of it is located in the inner membrane leaflet. A similar asymmetric topological distribution of PE has been described in membranes of erythrocytes [21], human platelets [22], microsomes of chick brain [17] and rat liver [23], gastric mucosa [24] and rabbit enterocytes [25]. PS, which represents 16.3 ± 1.5 and $18.4 \pm 2.7\%$ of the middle and posterior intestine phospholipids, respectively, is for more than 30% an external phospholipid in both intestinal parts. In contrast, other biological membranes display a more internal location (80–100%) [17,19,21,26]. The diet probably influences PS distribution since only 18% (Fig. 1) were present in the external membrane leaflet when trout are fed a commercial diet (kinetic study of TNBS labelling).

As far as the fatty acid composition of PE and PS is concerned, striking differences appear with regard to their topological location. Aminophospholipids, which form about 40% of the trout brush-border membrane phospholipids contain more than 70% of the total $22:6(n-3)$ (unpublished data) which is mainly located in the internal membrane leaflet. Moreover, our findings demonstrate a differential distribution of PE and PS $22:6(n-3)$ content between middle and posterior intestine membrane inner leaflets: it was relatively high for PE in the posterior intestine membrane preparation, whereas for PS it was high in both membrane preparations. A higher fatty acid unsaturation of the internal leaflet of plasma membranes has also been reported in murine fibroblasts [19,27] and synaptosomes [26]. Although the functional significance of this asymmetry is still unknown, one can hypothesize that the preferential location in the inner leaflet of molecular species of PE and PS rich in polyunsaturated fatty acids is related to the rod-like shape of the microvillus membrane, as has been suggested for the spicule formation of erythocytes [9]. Furthermore, as for arachidonic acid in plasma membrane of human platelet [22], the preferential location of $22:6(n-3)$ at the cytoplasmic side of the brush-border membrane is likely to be of great physiological importance. Recently, 14-lipoxygenase activities have been described in the cytosolic compartment of trout tissues [28]; thus, one might expect that its preferential substrate, docosahexaenoic acid, should be mainly located in the inner leaflet of the membrane. The particular location of this fatty acid, described here for the first time, might enable a more efficient coupling between its release by phospholipase A_2 action and its further transformation to hydroxylated derivatives.

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