Lipid Asymmetry in Rabbit Small Intestinal Brush Border Membrane As Probed by an Intrinsic Phospholipid Exchange Protein

G. Lipka, J. A. F. Op den Kamp,[‡] and H. Hauser*

Laboratorium für Biochemie, ETH Zürich, ETH-Zentrum, CH-8092 Zürich, Switzerland, and Department of Lipid Biochemistry, State University of Utrecht, NL-3584 CH Utrecht, The Netherlands

Received June 27, 1991; Revised Manuscript Received September 19, 1991

ABSTRACT: All classes of phospholipids present in brush border membrane are exchanged in a 1:1 ratio for egg phosphatidylcholine when brush border membrane vesicles from rabbit small intestine are incubated with small unilamellar vesicles of egg phosphatidylcholine. The exchange reaction exhibits biphasic kinetics similar to those of the hydrolysis of brush border membrane phospholipids by phospholipase A_2 and sphingomyelinase C. In both reactions there is an initial fast phase followed by a markedly slower one. The phospholipid exchange appears to be catalyzed by intrinsic brush border membrane protein(s), while the digestion by phospholipases is mediated by externally added enzymes. From a comparison of the kinetics of phospholipid exchange and phospholipid hydrolysis, the following conclusions can be drawn: Both sets of experiments indicate the presence of two phospholipid pools differing in the rate of phospholipid exchange and hydrolysis. Except for sphingomyelin, the size of the two phospholipid pools derived from phospholipid exchange is in good agreement with that derived from phospholipid hydrolysis. This is the main finding of this work, and on the basis of this result the two lipid pools are tentatively assigned to phospholipid molecules located on the outer and inner layer of the brush border membrane. The slow rate of phospholipid exchange reflects the rate of transverse or flip-flop movement of phospholipids. The half-time of this motion is ~8 h for isoelectric (neutral) phospholipids such as phosphatidylethanolamine and ~80 h for negatively charged phosphatidylserine and phosphatidylinositol. Isoelectric phospholipids (phosphatidylcholine, phosphatidylethanolamine) are preferentially located on the inner (cytoplasmic) side (to about 70%) while the negatively charged phospholipids are more evenly distributed: 55–60% are located on the inner side.

We reported that the absorption of cholesterol and phosphatidylcholine by small intestinal brush border membrane vesicles from either micelles or small unilamellar phospholipid vesicles is protein mediated. If micelles are used as donor particles, there is net transfer of cholesterol and phosphatidylcholine from donor to acceptor membrane (Thurnhofer & Hauser, 1990a,b; Thurnhofer et al., 1991). If, however, small unilamellar phospholipid vesicles are used as the donor containing radiolabeled cholesterol or phosphatidylcholine, these lipids are evenly distributed at equilibrium between the lipid pools of the donor and acceptor vesicles (Thurnhofer & Hauser, 1990a,b). Furthermore, we proved that phosphatidylcholine transferred in this way to brush border membrane is indeed incorporated in the lipid bilayer of this membrane (Mütsch et al., 1986). That phosphatidylcholine is exchanged between small unilamellar egg phosphatidylcholine vesicles and brush border membrane can be shown by labeling brush border membrane with radioactive dipalmitoylphosphatidylcholine and measuring the rate of the back-reaction (Mütsch et al., 1986). In the context of phosphatidylcholine exchange between brush border membrane vesicles and small unilamellar egg phosphatidylcholine vesicles, the question arises whether there is a strict 1:1 exchange of egg phosphatidylcholine for intrinsic phosphatidylcholine of brush border membrane or whether other lipids of brush border membrane can participate in this exchange interaction. The first part of the work described here is primarily concerned with this question. It is shown that not only all phospholipid classes of brush border membrane are exchanged but also cholesterol and glycolipids. The exchange of brush border membrane phospholipids is biphasic, i.e., part of the phospholipid is exchanged at significantly faster rates than the rest of the phospholipids. We tentatively assign the two pools of lipids detected by lipid exchange to lipid molecules located on the outer and inner half of the brush border membrane. The second part of the paper describes experiments designed to check the assignment. For this purpose brush border membrane vesicles are digested with different phospholipases. This approach has been widely used in the past to determine the asymmetric distribution of phospholipids in plasma membranes [for reviews, see Op den Kamp (1979), Etemadi (1980), and Zachowsky and Devaux (1990)]. The main finding of this work is that, except for sphingomyelin, the asymmetric distribution of brush border membrane phospholipids derived from phospholipid exchange is in good agreement with the results obtained by digesting brush border membrane vesicles with phospholipases.

Materials and Methods

Bee venom phospholipase A_2 was obtained from Sigma (St. Louis, MO) and used without further purification. Sphingomyelinase C from *Staphylococcus aureus* was purified according to Zwaal et al. (1975) and stored at a concentration of 11 IU/10 μL. Trypsin from bovine pancreas was purchased from Boehringer (Mannheim, FRG), egg phosphatidylcholine from Lipid Products (Surrey, U.K.), spin-labeled stearic acid with the 4,4-dimethyl-3-oxazolidinyloxy group (doxyl group) attached to C-atom 5 from Molecular Probes (Junction City, OR), 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-phosphatidylcholine from Avanti Polar Lipids (Pelham, AL), deoxycholic acid and D-mannitol from Merck (Darmstadt, FRG), Triton X-100 from Sigma (St. Louis, MO), and EDTA from Siegfried AG (Zofingen, Switzerland). Orcinol monohydrate (purum) and

* To whom correspondence should be addressed.
[‡] Department of Lipid Biochemistry, State University of Utrecht.

0006-2960/91/0430-11828$02.50/0

organic solvents of grade puriss. were from Fluka (Buchs, Switzerland).

The sodium salt of deoxycholic acid was made by neutralizing the acid with NaOH. Deoxycholate was purified by preparative TLC[1] (silica gel plates 60 F-254 from Merck, Darmstadt, FRG; thickness of silica gel: 2 mm) using ethyl acetate/CH_3OH/CH_3COOH 70:20:10 (v/v/v) as the solvent. All lipids used in this study were pure by TLC standard.

Brush Border Membrane Vesicles. Brush border membrane vesicles were prepared from rabbit small intestine according to Hauser et al. (1980) and characterized as described in this reference. The resulting membrane vesicles were suspended in buffer (10 mM Hepes adjusted to pH 7.3 with Tris, both from Sigma, St. Louis, MO, containing 0.3 M D-mannitol and 1 mM EDTA). The amount of total lipid of brush border membrane was determined gravimetrically after extraction of the lipid according to Bligh and Dyer (1959). Protein concentrations were determined with bicinchoninic acid from Pierce using bovine serum albumin as a standard (Smith et al., 1985).

Incubation of Brush Border Membrane Vesicles with Small Unilamellar Vesicles of Egg Phosphatidylcholine. Small unilamellar vesicles of egg phosphatidylcholine were produced by microtip sonication as described before (Thurnhofer & Hauser, 1990a). Brush border membrane vesicles at 6–10 mg of total lipid/mL were incubated with egg phosphatidylcholine vesicles at 4–7 mg/mL at room temperature. At timed intervals, aliquots of 0.2 mL of the incubation mixture were centrifuged at 100000*g* for 10 min in order to quantitatively separate brush border membrane from small unilamellar egg phosphatidylcholine vesicles. Lipids of both pellet and supernatant were extracted using the method of Bligh and Dyer (1959).

Treatment of Brush Border Membrane Vesicles by Phospholipases. Brush border membrane vesicles (8 mg of protein/mL; total volume 0.2 mL) suspended in buffer containing 5 mM $CaCl_2$ were incubated with bee venom phospholipase A_2 (1.3 IU/mL) at 30 °C. Under the same conditions except that the buffer contained 5 mM $MgCl_2$ instead of $CaCl_2$, brush border membrane vesicles were digested with sphingomyelinase C from *S. aureus* (1.6 IU/mL). In either case the digestion was stopped after timed intervals by adding 10 mM ice-cold EDTA. Brush border membrane vesicles were pelleted and extracted for total lipids as described above. In control experiments, brush border membrane vesicles were digested with phospholipase A_2 and sphingomyelinase C as described above except that the brush border membrane vesicles were treated with 0.2% deoxycholate at 0 °C prior to the activation of the lipases by raising the temperature to 30 °C.

TLC Analysis of Lipid Extracts. Lipid extracts were prepared according to Bligh and Dyer (1959). Extracted lipids were dried under a gentle stream of N_2 and the lipid residue was dried in vacuo to constant weight. The dry lipid was dissolved in $CHCl_3$/CH_3OH (2:1 v/v), and an aliquot of the solution was applied to a 20 × 20 cm silica gel 60 F-254 plate from Merck (Darmstadt, FRG). Lipids were usually separated by one-dimensional TLC. Two-dimensional TLC was used in the experiments with phospholipase A_2 in order to separate phosphatidylserine from lysophosphatidylethanolamine. Lipids (~1 mg) were applied as bands of ~1-cm width for one-dimensional TLC and as spots for two-dimensional TLC. The standard solvent for one-dimensional TLC was $CHCl_3$/CH_3OH/H_2O/CH_3COOH 65:50:4:1 (v/v/v/v). The separation of phospholipids by two-dimensional TLC was carried out as described by Bowyer and King (1977). $CHCl_3$/CH_3OH/CH_3COOH/H_2O 55:35:3:2 (v/v/v/v) was used in the first dimension and $CHCl_3$/CH_3–CO–CH_3/CH_3OH/CH_3COOH/H_2O 45:16:15:11:6 (v/v/v/v) was used in the second one. Lipids were stained with iodine vapor, and, after fading of the iodine staining, silica gel containing lipids was scraped off the plate and the phospholipids were digested with perchloric acid (70%) and analyzed for phosphate (Chen et al., 1956). Glycolipids were identified by staining with orcinol spray (200 mg of orcinol dissolved in 100 mL of 75% H_2SO_4; Skipski & Barclay, 1969).

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out using the Mini-Protean II dual slab cell from Bio-Rad (Glattbrugg, Switzerland). Gels containing 8% polyacrylamide were cast as described in the Bio-Rad instruction manual. Gels were run at 150 V using an electrode buffer with the composition given in the manual. Bio-Rad low molecular weight markers were used as standard. Proteins were visualized by staining with 0.1% solutions of Coomassie Brillant blue R 250 in an aqueous solution containing 40% CH_3OH and 10% acetic acid (Serva Feinbiochemica, Heidelberg, FRG).

ESR Spin-Labeling. Brush border membrane vesicles were labeled with 5-doxylstearate or spin-labeled phosphatidylcholine as follows: a solution of the spin label in $CHCl_3$ was taken to dryness in a round-bottom glass flask using a rotary evaporator, and the resulting lipid film was dried under high vacuum for 1 h. About 1 mL of brush border membrane vesicles (23–30 mg of protein/mL; 13–16 mg of total lipid/mL) suspended in buffer was added to the dry lipid film, and the uptake of spin label by brush border membrane was monitored by ESR; 98% of the spin-labeled fatty acid and 93% of the spin-labeled phosphatidylcholine were incorporated in the lipid bilayer of brush border membrane after incubating for about 2 h. The amount of spin label dried down was such that the final mole ratio of spin label to brush border membrane lipid was 1:130. The brush border membrane suspension (50 μL) was filled into a glass capillary (internal diameter 1 mm), and ESR spectra were recorded at room temperature at 9.2 GHz using a Varian X-band spectrometer (model E-104A) fitted with a variable temperature control.

Results

Characterization of Brush Border Membrane. Brush border membrane vesicles were routinely prepared from frozen rabbit small intestine according to Hauser et al. (1980). The major phospholipids were phosphatidylethanolamine (35 wt %) and phosphatidylcholine (33%), amounting to about two-thirds of the total phospholipid, with sphingomyelin (11%), phosphatidylserine (6%), and phosphatidylinositol (13%) making up the remaining one-third. The cholesterol content of our preparation was 0.047 mg of cholesterol/mg of protein, and the lipid/protein weight ratio was 0.52. These values are in good agreement with the analytical data of brush border membrane published previously (Hauser et al., 1980). Regarding the stability of brush border membrane, it is important to determine the content of lysophosphatidylethanolamine and lysophosphatidylcholine. Preparations used in this study had lysophospholipid contents of less than 3% each, usually 2%; if the lysophospholipid contents exceeded 3%, preparations were discarded.

Lipid Exchange between Brush Border Membrane Vesicles and Small Unilamellar Egg Phosphatidylcholine Vesicles.

[1] Abbreviations: TLC, thin-layer chromatography; EDTA, disodium salt of ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

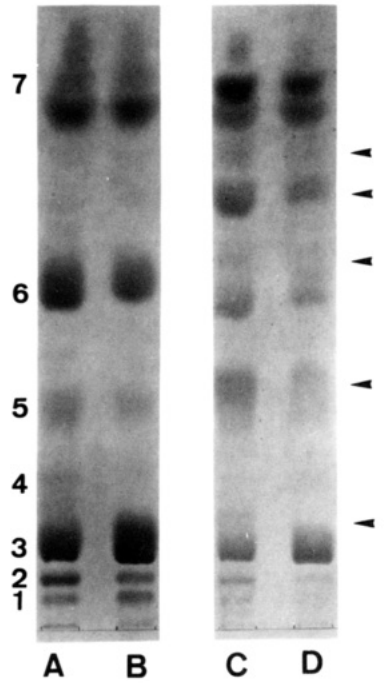

FIGURE 1: TLC chromatograms of phospholipid extracts. Lipid extracts were made from brush border membrane vesicles and small unilamellar egg phosphatidylcholine vesicles before and after incubation of the two vesicle populations as described under Materials and Methods. An aliquot of the lipid extract corresponding to ~1 mg of total lipid was applied to the TLC plate (20 × 20 cm; silica gel 60 F-254 from Merck, Darmstadt, FRG) as a band of ~1-cm width using a Hamilton syringe. $CHCl_3/CH_3OH/H_2O/CH_3COOH$ 65:50:4:1 (v/v/v/v) was used as the solvent. Lipids were made visible by exposing the TLC plate to iodine vapor. (Lanes A and C) Total lipids extracted from brush border membrane vesicles prior to incubation. (Lanes B and D) Total lipids extracted from small unilamellar vesicles (5.6 mg/mL) that had been incubated with brush border membrane vesicles (8.3 mg of total lipid/mL) at room temperature for 18 h. Lanes A and B were stained with iodine, lanes C and D with orcinol. The latter reagent detected glycolipids as blue-violet bands (marked by arrows). Bands: lysophosphatidylcholine (1), sphingomyelin (2), phosphatidylcholine (3), phosphatidylserine and lysophosphatidylethanolamine (4), phosphatidylinositol (5), phosphatidylethanolamine (6), and cholesterol (7).

The first series of experiments reported here is based on a previous observation: if brush border membrane vesicles are incubated with small unilamellar vesicles made of egg phosphatidylcholine, egg phosphatidylcholine is incorporated into the lipid bilayer of the brush border membrane (Mütsch et al., 1986; Thurnhofer & Hauser, 1990b). It is a second-order reaction, and the mechanism is protein-mediated, collision-induced lipid exchange between small unilamellar vesicles of egg phosphatidylcholine and brush border membrane vesicles (Mütsch et al., 1986; Thurnhofer & Hauser, 1990b). It is still unknown whether egg phosphatidylcholine is strictly exchanged for brush border membrane phosphatidylcholine or whether the exchange reaction involves other membrane phospholipids as well. Results summarized in Figure 1 shed light on this question. Brush border membrane vesicles were incubated with small unilamellar egg phosphatidylcholine vesicles at room temperature for 18 h. After incubation, lipids were extracted from brush border membrane as well as from egg phosphatidylcholine vesicles as described under Materials and Methods and separated by TLC (Figure 1). Lanes A and C are one-dimensional chromatograms of the lipid extracts from untreated brush border membrane, i.e., from brush border membrane prior to incubation with egg phosphatidylcholine vesicles; lanes B and D (Figure 1) show chromatograms of lipids extracted from egg phosphatidylcholine vesicles that were incubated with brush border membrane at room temperature for 18 h. Lipids were stained with iodine (lanes A and B) and orcinol (lanes C and D). With the latter reagent, glycolipids appear as blue-violet (see arrows in Figure 1C and D) and phospholipids as brown bands. Unfortunately, the relative intensities of the lipid bands are not reproduced correctly in this photograph. The TLC pattern of the lipid extract from egg phosphatidylcholine vesicles after incubation with brush border membrane resembles qualitatively that of the lipid extract of brush border membrane. It is clear from Figure 1 that the lipid exchange reaction is by no means confined to brush border membrane phosphatidylcholine. On the contrary, practically all phospholipid classes contained in brush border membrane were incorporated in egg phosphatidylcholine vesicles in the course of lipid exchange. Furthermore, in addition to phospholipids, cholesterol and also glycolipids were transferred from brush border membrane to egg phosphatidylcholine vesicles as a result of lipid exchange (Figure 1). The lipid extract of egg phosphatidylcholine vesicles prior to incubation with brush border membrane yielded a single band of phosphatidylcholine (data not shown). The lipid extract of brush border membrane after incubation with phosphatidylcholine vesicles for 24 h was quite different from that shown in Figure 1A. The dominant band was that of phosphatidylcholine, amounting to 80% of the total phospholipid (data not shown). In this experiment, brush border membrane vesicles (6.4 mg of total lipid/mL) were incubated with egg phosphatidylcholine vesicles (6.4 mg/mL) at room temperature for 24 h; after this period of time, 1.9 mg of egg phosphatidylcholine was transferred from egg phosphatidylcholine vesicles to brush border membrane. From the analytical composition of the incubation medium, it is calculated that the distribution of phosphatidylcholine is at equilibrium, i.e., phosphatidylcholine is evenly distributed between the lipid pools of brush border membrane and egg phosphatidylcholine vesicles. At equilibrium 38% of the total egg phosphatidylcholine present in the incubation medium should be incorporated in the lipid pool of brush border membrane, and this value was indeed verified by experiment. If we assume that phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, and phosphatidylserine are exchanged 1:1 for phosphatidylcholine, it is calculated that at equilibrium the total amount of all four phospholipids transferred to egg phosphatidylcholine vesicles is 0.9 mg/mL. This number is borne out by experiment and has to be compared with 1.9 mg/mL of egg phosphatidylcholine that are transferred in exchange of phospholipids and possibly other lipids. The TLC analysis in Figure 1 reveals that cholesterol is also transferred from brush border membrane to egg phosphatidylcholine vesicles. Assuming that cholesterol is exchanged for phosphatidylcholine on a 1:1 basis and that this exchange reaches equilibrium after 18 h, about 0.75 mg of egg phosphatidylcholine would be transferred in exchange for cholesterol from egg phosphatidylcholine vesicles to brush border membrane. This assumption is supported by experiment: after incubation of brush border membrane with egg phosphatidylcholine vesicles for 10 h, 57% of the cholesterol of brush border membrane was present in egg phosphatidylcholine vesicles. This is close to the equilibrium value of 63%. The total amount of phosphatidylcholine transferred from egg phosphatidylcholine small unilamellar vesicles to brush border membrane amounts then to ~1.6 mg/mL. The difference between 1.9 and 1.6 mg/mL is probably accounted for by exchange of some glycolipids. The TLC chromatogram in Figure 1 provides evidence that glycolipids (marked by arrows) present in the outer half of

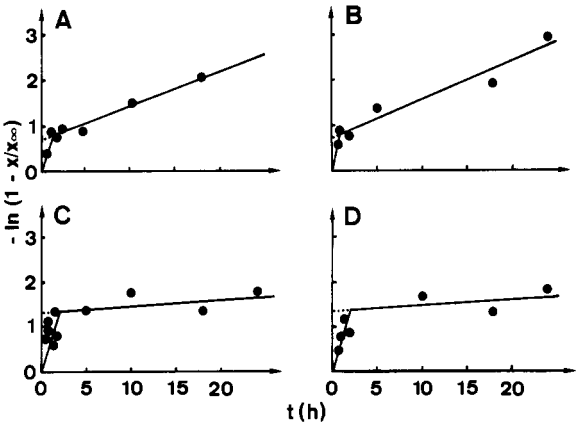

FIGURE 2: Kinetics of phospholipid exchange between brush border membrane vesicles and small unilamellar egg phosphatidylcholine vesicles. Brush border membrane vesicles (donor) at 6–10 mg of total lipid/mL were incubated with small unilamellar vesicles (acceptor) at 4–7 mg of lipid/mL at room temperature. The weight ratio of total lipid of acceptor to donor was kept constant at 0.7. The time course of phospholipid transfer was linearized according to eq 1. (A) Sphingomyelin, (B) phosphatidylethanolamine, (C) phosphatidylserine, and (D) phosphatidylinositol. The solid lines were fitted to the experimental points by linear regression analysis. Each point in the graph represents the average of two or three measurements.

the bilayer of brush border membrane are indeed exchanged for egg phosphatidylcholine. In another experiment brush border membrane vesicles (8.3 mg of total lipid/mL) were incubated with egg phosphatidylcholine vesicles (5.6 mg/mL) at room temperature for 18 h. The lipids of both brush border membrane and egg phosphatidylcholine vesicles were extracted and analyzed by TLC. It was found that 46% of the phosphatidylethanolamine, 48% of sphingomyelin, 40% of phosphatidylinositol, and about 30% of phosphatidylserine of brush border membrane were transferred to egg phosphatidylcholine vesicles. It is calculated that, under the experimental conditions used, 44% of each phospholipid of brush border membrane would be transferred to egg phosphatidylcholine vesicles at equilibrium. The comparison of theory and experiment indicates that isoelectric but not acidic phospholipids are equilibrated between the lipid pools of the brush border membrane and egg phosphatidylcholine vesicles after 18 h.

The time course of the phospholipid exchange between brush border membrane and small unilamellar egg phosphatidylcholine vesicles is shown in Figure 2. The kinetics of lipid exchange were treated according to (MacLean & Phillips, 1984; Mütsch et al., 1986)

$$-\ln (1 - x/x_\infty) = k_1[(a + b)/a]t \qquad (1)$$

where x and x_∞ represent the fractional transfer of phospholipid from brush border membrane as the donor to small unilamellar vesicles as the acceptor at time t and at equilibrium, respectively, a and b are the lipid concentrations of acceptor and donor particles, respectively, and k_1 is the pseudo-first-order rate constant. The exchange of phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, and phosphatidylserine for phosphatidylcholine (Figure 2A–D) was plotted according to eq 1. The exchange of all four phospholipids is biphasic: there is an initial fast exchange, and after about 90 min there is a break in the curve due to a significant drop in the rate of phospholipid exchange (Figure 2). From the slopes of the solid lines, pseudo-first-order rate constants were derived (Table I). For all phospholipids, similar k_1 values were obtained for the initial fast exchange corresponding to half-times of about 2 h. This is consistent with the rate constant measured for egg phosphatidylcholine transfer from small unilamellar egg phosphatidylcholine vesicles to brush border membrane. The pseudo-first-order rate constants k_1 for the slow exchange were significantly smaller. The k_1 values for the slow exchange of the two isoelectric phospholipids, phosphatidylethanolamine and sphingomyelin, were smaller by a factor of about 5 compared to the initial fast exchange; those for the negatively charged phospholipids, phosphatidylserine and phosphatidylinositol, dropped even by a factor of 40–50 (Table I). Extrapolation of the straight lines representing the slow phase of exchange to time zero yields values $-\ln(1 - x/x_\infty)_{t\to 0}$ as the intercept on the y-axis. From these extrapolated values, the fraction of readily exchangeable phospholipid can be calculated. Values thus obtained for the fraction of readily and less readily available phospholipid pools are summarized in Table II.

Table I: Pseudo-First-Order Rate Constants k_1 and Half-Times $t_{1/2}$ for Phospholipid Exchange

	fast exchange[a]		slow exchange	
phospholipid	k_1 (h^{-1})	$t_{1/2}$ (h)	k_1 (h^{-1})	$t_{1/2}$ (h)
sphingomyelin	0.36 ± 0.07	1.9 ± 0.4	0.073 ± 0.005	9 ± 0.6
phosphatidylethanolamine	0.39 ± 0.05	1.8 ± 0.2	0.085 ± 0.006	8 ● 0.6
phosphatidylserine	0.39 ± 0.08	1.8 ± 0.4	0.009 ± 0.006	75 ± 50
phosphatidylinositol	0.39 ± 0.03	1.8 ± 0.2	0.008 ± 0.006	85 ± 60

[a] The exchange of phospholipids between brush border membrane vesicles as the donor and small unilamellar egg phosphatidylcholine vesicles as the acceptor is biphasic, i.e., an initial fast phase of exchange is followed by a slow phase of lipid exchange. The data are derived from Figure 2.

Table II: Asymmetric Distribution of Phospholipids in the Brush Border Membrane[a]

	phospholipid exchange		digestion with lipases	
phospholipid	outer layer (%)	inner layer (%)	outer layer (%)	inner layer (%)
sphingomyelin	31 ± 2	69 ± 2	63 ± 3	37 ± 3
phosphatidylcholine	n.d.	n.d.	32 ± 2	68 ± 2
phosphatidylethanolamine	28 ± 2	72 ± 2	34 ± 3	66 ± 3
phosphatidylserine	42 ± 3	58 ± 3	44 ± 9	56 ± 9
phosphatidylinositol	40 ± 4	60 ± 4	40 ± 10	60 ± 10

[a] Values are presented as the mean ± standard deviation.

Digestion of Brush Border Membrane Vesicles with Phospholipases. The following experiments were carried out in order to shed light on the nature of the two phospholipid pools revealed by the phospholipid exchange interaction described above (Figure 2). Brush border membrane vesicles were digested with either phospholipase A_2 or sphingomyelinase C. The kinetics of phospholipid hydrolysis by these enzymes are depicted in Figure 3. Semilogarithmic plots of unhydrolyzed phospholipids (%) as a function of time reveal the biphasic character of the hydrolysis of brush border membrane phospholipids. The data for the hydrolysis of phosphatidylethanolamine and phosphatidylcholine were very similar to those reported by Barsukov et al. (1986) and were therefore not included in Figure 3. The initial fast rate of phospholipid hydrolysis appears to be identical with the rate of hydrolysis observed after treating brush border membrane with deoxycholate (open circles and dashed lines in Figure 3). Under these conditions, 90% or more of the phospholipids were hydrolyzed in a pseudo-first-order reaction within the first 10 min. The hydrolysis of phosphatidylserine is slightly slower with 80% of the phospholipid being hydrolyzed after 10 min. Pseudo-first-order rate constants for the initial fast phase of hydrolysis were derived from the slopes of the dashed lines in

Table III: Pseudo-First-Order Rate Constants k_1 and Half-Times $t_{1/2}$ of Phospholipid Hydrolysis by Lipases

phospholipid	phospholipase	fast reaction		slow reaction	
		k_1 (h^{-1})	$t_{1/2}$ (h)	k_1 (h^{-1})	$t_{1/2}$ (h)
sphingomyelin	sphingomyelinase C	14.4	0.048	0.62 ± 0.06	1.1 ± 0.1
phosphatidylcholine	phospholipase A_2	10.1	0.069	1.04	0.67
phosphatidylethanolamine	phospholipase A_2	12.2	0.057	1.15	0.60
phosphatidylserine	phospholipase A_2	9.6	0.072	0.71	0.98
phosphatidylinositol	phospholipase A_2	13.2	0.053	0.57 ± 0.04	1.20 ± 0.09

Table IV: Effect of Intrinsic and Extrinsic Lipases on the Composition of Isoelectric Phospholipids of Brush Border Membrane Vesicles

	phospholipid composition (%) of brush border membrane vesicles[a]									
	buffer		buffer + 5 mM Ca^{2+}		buffer + 5 mM Ca^{2+} + phospholipase A_2			buffer + 5 mM Mg^{2+} + sphingomyelinase C		
time (min)	PC	PE	PC	PE	PC	PE	SM	PC	PE	SM
0	31.5	38.9	32.8	38.9	32.8	34.6	11	32.8	34.6	11.0
10			32.6		21.7	19.4	11.7	32.6	33.7	3.7
20	32.0	38.1	33.0	32.9	15.0	15.5		33.0	25.3	3.0
30							11.7			
40			31.8		12.4	12.9		31.8	21.5	2.6
45	31.5	39.5		31.3						

[a] Brush border membrane vesicles (8 mg of protein/mL) were dispersed in buffer with and without Ca^{2+} and incubated at 30 °C. The experiment was repeated after adding either phospholipase A_2 (1.3 IU/mL) or sphingomyelinase C (1.6 IU/mL).

Figure 3. Similar rate constants k_1 are obtained for the initial fast hydrolysis ranging between 9.6 and 14.4 h^{-1} corresponding to half-times of 2.9–4.3 min (Table III). Within the first 10 min a break in the rate of phospholipid hydrolysis occurs (Figure 3). Pseudo-first-order rate constants k_1 for the second slow phase of hydrolysis were derived from a linear regression analysis of the data points obtained after 10 min and are included in Table III. These k_1 values are more than one order of magnitude smaller than those for the initial fast hydrolysis. Extrapolation of the straight line representing the slow phase of hydrolysis to time zero (see dotted lines in Figure 3) yields the size of the phospholipid pool that is less readily accessible to lipases (Table II). The results for phosphatidylethanolamine and phosphatidylcholine are included in this table although the original graphs are not presented for reasons mentioned above. Table II shows that the isoelectric glycerophospholipids, phosphatidylethanolamine and phosphatidylcholine, are asymmetrically distributed between readily accessible and less accessible lipid pools: 30–35% of these phospholipids are present in the readily accessible pool (pool I), giving a lipid weight ratio pool I/pool II of ~0.5. This result agrees within experimental error with published data (Barsukov et al., 1986). The distribution of sphingomyelin between the two pools differs from that of the glycerophospholipids: the main portion (~65%) is present in the readily accessible lipid pool. The two negatively charged phospholipids, phosphatidylserine and phosphatidylinositol, exhibit a more even distribution, with about 40% in pool I and a lipid wt ratio pool I/pool II of 0.67.

The digestion of brush border membrane vesicles with phospholipase A_2 and sphingomyelinase C was carried out at 30 °C in the presence of 5 mM Ca^{2+} and 5 mM Mg^{2+}, respectively. Millimolar concentrations of Ca^{2+} or Mg^{2+}, particularly at elevated temperatures, might activate intrinsic lipases, and the hydrolysis of phospholipids might affect the membrane stability. To shed light on this question, the composition of phosphatidylcholine and phosphatidylethanolamine of brush border membrane vesicles dispersed in buffer containing 5 mM Ca^{2+} at 30 °C was monitored as a function of time. Phosphatidylcholine was not hydrolyzed at 30 °C in the presence or absence of Ca^{2+} (Table IV). In contrast, phosphatidylethanolamine was hydrolyzed by ~20% in 45 min in the presence of Ca^{2+}. Table IV also contains information pertinent to the question of lipase specificity. Phospholipase A_2 hydrolyzed all phospholipids, but not sphingomyelin, while sphingomyelinase C attacked both sphingomyelin and phosphatidylethanolamine but not phosphatidylcholine. Phosphatidylethanolamine was hydrolyzed by sphingomyelinase C by about 40% in 40 min.

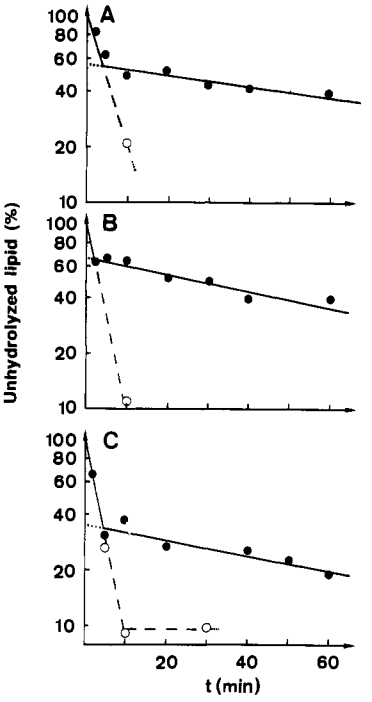

FIGURE 3: Hydrolysis of brush border membrane phospholipids by extrinsic lipases. Brush border membrane vesicles (8 mg of protein/mL) in buffer were incubated with either phospholipase A_2 (1.3 IU/mL) or sphingomyelinase C (1.6 IU/mL) at 30 °C. Lipids were extracted and analyzed by TLC as described under Materials and Methods. Unhydrolyzed phospholipid (%) remaining in the brush border membrane is plotted semilogarithmically as a function of time (min) (full circles). Each point represents the average of three experiments. The time course of the digestion of phosphatidylserine and phosphatidylinositol by phospholipase A_2 is shown in panels A and B, respectively, and that of sphingomyelin by sphingomyelinase C in panel C. In control experiments, brush border membrane vesicles (8 mg of protein/mL) in buffer containing 0.2% deoxycholate were digested with the same quantities of enzyme as described above. The time course of phospholipid digestion in the presence of deoxycholate is given by dotted lines (open circles).

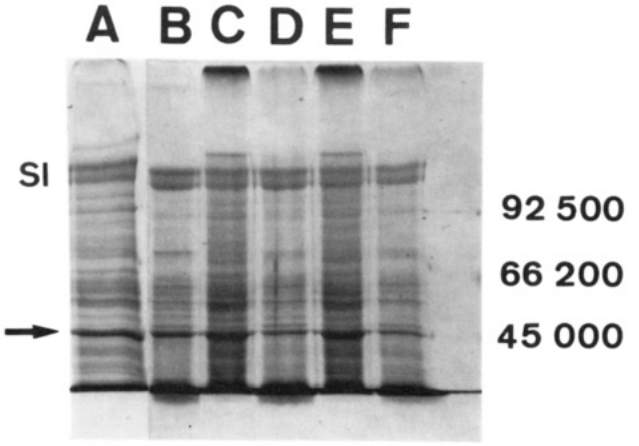

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns. Electrophoresis experiments on 8% polyacrylamide gels were performed as described under Materials and Methods. Protein bands were stained with Coomassie blue. (Lane A) Brush border membrane vesicles (8 mg of protein/mL) dispersed in buffer containing 5 mM $MgCl_2$ were incubated at 30 °C for 15 min. (Lane B) Brush border membrane vesicles treated as described for lane 1 were cooled to room temperature and digested with trypsin (0.15 mg/mL) for 2 h. (Lane C) Brush border membrane vesicles (8 mg of protein/mL) in buffer containing 5 mM $MgCl_2$ were incubated with sphingomyelinase C (1.6 IU/mL) at 30 °C for 15 min. The hydrolysis was stopped by adding ice-cold EDTA (25 mM). (Lane D) Brush border vesicles were treated as described for lane C and then digested with trypsin (0.15 mg/mL). (Lanes E and F) Brush border membrane vesicles were treated as in lanes C and D, respectively, except that the buffer contained 0.4% Triton X-100 in order to disrupt brush border membranes. The arrow marks the actin band. SI is sucrase–isomaltase.

From an inspection of Table II, it is clear that the values for the sizes of the two lipid pools derived from phospholipid exchange agree well with those derived from phospholipid digestion. The exception is sphingomyelin. From the lipid exchange experiment, sphingomyelin appears to be distributed similarly to the isoelectric glycerophospholipids, with about 30% of the phospholipid being present in the readily accessible pool. The result obtained with sphingomyelinase C is at variance with this result, indicating that about 65% of the sphingomyelin is present in the readily accessible lipid pool. In an attempt to explain this discrepancy, the following experiment was carried out. The membrane integrity of brush border vesicles was monitored with trypsin before and after digestion of the brush border membrane vesicles with sphingomyelinase C. The cytoskeletal protein actin located on the internal side of brush border membrane was used as a marker protein. It was shown before that trypsin neither crosses intact brush border membrane (Klip et al., 1979a) nor brush border membrane treated with phospholipase A_2 (Barsukov et al., 1986). Figure 4 shows sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of brush border membrane before and after various treatments. Lanes A–F were scanned with a densitometer, and intensities were normalized with respect to sucrase-isomaltase (SI, see Figure 4, lane A). Comparison of the normalized intensities of actin in lanes A and B confirm that trypsin does not digest actin when the enzyme is added to intact brush border membrane vesicles dispersed in buffer containing 5 mM $MgCl_2$ at 30 °C. Further, digestion of brush border membrane with sphingomyelinase C has practically no effect on actin. Activation of endogenous proteinases by the sphingomyelinase C treatment which in turn might digest actin can be ruled out. If, however, brush border membrane vesicles were treated with sphingomyelinase C followed by digestion with trypsin, the polyacrylamide gel pattern in lane D was obtained. The normalized intensity of the actin band is reduced by a factor of 2, indicating that the sphingomyelinase C treatment affected the membrane integrity and as a result the cytoskeletal protein actin became accessible to trypsin. The effect of trypsin on actin in this case (lane D) is similar to that observed when brush border membrane vesicles were disrupted with Triton X-100 prior to trypsin digestion (lane F).

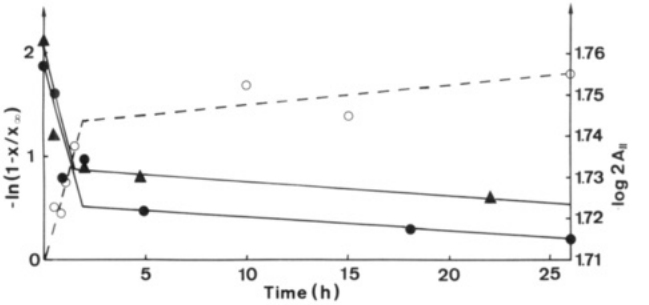

FIGURE 5: Time course of the hyperfine splitting constants $2A_{\parallel}$. Brush border membranes were spin labeled with 5-doxylstearic acid and 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-phosphatidylcholine as described under Materials and Methods. Spin-labeled brush border membrane vesicles were incubated with small unilamellar egg phosphatidylcholine vesicles at room temperature, and after timed intervals brush border membrane vesicles were separated from small unilamellar phospholipid vesicles by centrifugation at 100000*g* for 10 min. The brush border membrane pellet was resuspended in the same volume of buffer, and ESR spectra were recorded. For comparison, the time course of phosphatidylinositol transfer from brush border membrane to egg phosphatidylcholine vesicles shown in Figure 2D is included in this figure (see dashed line). (●) Log $2A_{\parallel}$(G) of 5-doxyl stearic acid; (▲) log $2A_{\parallel}$ of spin-labeled phosphatidylcholine.

ESR Spin-Labeling. Brush border membrane vesicles incubated with egg phosphatidylcholine vesicles take up egg phosphatidylcholine. It was shown that under the experimental conditions used the egg phosphatidylcholine uptake is substantial, amounting to 80% of the total phospholipid content. Evidence was presented previously that phosphatidylcholine taken up by brush border membrane is indeed incorporated in the lipid bilayer of brush border membrane (Mütsch et al., 1986). The incorporation of large quantities of egg phosphatidylcholine is expected to affect the membrane fluidity, and this expectation is borne out by ESR spin-labeling experiments. Brush border membrane vesicles were spin-labeled with 5-doxylstearic acid or spin-labeled phosphatidylcholine as described under Materials and Methods. The values for the hyperfine splitting constants measured at room temperature for 5-doxylstearic acid and spin-labeled phosphatidylcholine incorporated in brush border membrane were $2A_{\parallel}$ = 57.5 G and $2A_{\parallel}$ = 58 G, respectively, in good agreement with published values (Hauser et al., 1982). Spin-labeled brush border membrane vesicles were then incubated with small unilamellar egg phosphatidylcholine vesicles under conditions as described above. After timed intervals, brush border membrane vesicles were separated from small unilamellar vesicles by centrifugation at 100000*g* for 10 min. The pellet of brush border membrane vesicles was resuspended in the same volume of buffer, and ESR spectra were recorded. ESR spectra were also recorded of small unilamellar vesicles contained in the supernatant. As expected from experiments reported here (cf. Figure 2) and also previously (Mütsch et al., 1986), both spin labels distributed between the two lipid pools. Figure 5 shows the time course of the hyperfine splitting constant $2A_{\parallel}$ of both spin labels present in brush border membrane in a semilogarithmic plot. Both labels gave similar biphasic relationships which should be compared to the biphasic curves obtained for phospholipid transfer from brush border membrane to egg phosphatidylcholine vesicles (cf. Figure 2). For comparison, the time course of phosphatidylinositol transfer (Figure 2D)

is included in Figure 5 (dashed line). In all biphasic curves shown in Figure 5, the break point between the two phases is between 1.5 and 2 h. From such a comparison, it is clear that the spin labels present in brush border membrane sense the change in fluidity produced by the incorporation of egg phosphatidylcholine. After incubation of brush border membrane with egg phosphatidylcholine vesicles for 24 h, the hyperfine splitting constants decreased markedly to $2A_{\parallel}$ = 51.9 G for 5-doxylstearic acid and $2A_{\parallel}$ = 53 G for the spin-labeled phosphatidylcholine. These values are similar to those obtained when the spin labels are present in pure egg phosphatidylcholine bilayers. The ESR spectra of the supernatant containing the small unilamellar phospholipid vesicles were also recorded as a function of time. The hyperfine splittings $2A_{\parallel}$ decreased slightly with time, but the values were similar to those measured for pure egg phosphatidylcholine bilayers (data not shown).

Discussion

The brush border membrane of the small intestine is a highly specialized part of the plasma membrane engaged in digestive and absorptive functions. Our preparation of brush border membrane vesicles has been shown to be relatively homogeneous with respect to size (Perevucnik et al., 1985) and to be oriented right side out to at least 90% (Klip et al., 1979b). The rather uniform orientation of brush border membrane vesicles is essential for obtaining physiologically relevant results. A prerequisite for obtaining meaningful results in phospholipid exchange reactions as reported here is that other mechanisms of phospholipid transfer do not interfere with the actual measurement. This requirement is by and large fulfilled: (i) It was shown that the absorption of small unilamellar egg phosphatidylcholine vesicles by brush border membrane is negligible (Mütsch et al., 1986). (ii) There is some fusion between brush border membrane and egg phosphatidylcholine vesicles, but it is confined to a level that does not interfere with the interpretation of the phospholipid exchange. (iii) Shedding of phospholipid vesicles from brush border membrane can be ruled out. If brush border membrane vesicles are stored at room temperature for 24 h, i.e., if they are incubated in the absence of small unilamellar egg phosphatidylcholine vesicles, then fatty acids, lysophosphatidylcholine, lysophosphatidylethanolamine, and some glycolipids, but no cholesterol and diacylphospholipids are released into the supernatant (data not shown).

The two series of independent experiments indicate that there are two pools of phospholipids in brush border membrane. These two pools differ in their rate of phospholipid exchange and in their accessibility to phospholipases. The main result of this work is that the size of the two pools derived from phospholipid exchange agrees well with that determined by phospholipase treatment (see Table II). For sphingomyelin the two methods give conflicting results. In this case we cannot differentiate between two possibilities: (i) The exchange of the externally located sphingomyelin is hampered, and for unknown reasons only part of the external sphingomyelin is involved in the fast phospholipid exchange. (ii) Sphingomyelinase C treatment renders the brush border membrane partially permeable to the lipase, giving rise to artificially high values for sphingomyelin. We favor the second explanation in the light of the control experiments shown in Figure 4. Sphingomyelinase cleaves sphingomyelin to phosphocholine and ceramide which remains associated with the bilayer and which may have a destabilizing effect on the membrane (Verkleij et al., 1973). The problems and possible pitfalls involved in the determination of phospholipid asymmetry of biological membranes using phospholipases and phospholipid exchange proteins are well known and have been reviewed in detail [cf. Bergelson and Barsukov (1977), Op den Kamp (1979), Etemadi (1980), and Krebs (1982)]. Ignoring the sphingomyelin result for the time being, we tentatively assign the readily accessible lipid pool to lipid molecules located on the outer layer of brush border membrane and the less readily available pool to lipid molecules located on the cytoplasmic side. This assignment appears to be justified considering the good agreement of the pool sizes derived from the two independent methods (Table II). Furthermore, such an assignment is based on the assumption that brush border vesicles do not entrap smaller vesicles and small membrane fragments. That this is indeed the case has been shown by electron microscopy before (Kessler et al., 1978).

The rate constants for the fast exchange of brush border membrane phospholipids are similar (Table I) and, within experimental error, consistent with the rate constant of phosphatidylcholine transfer from small unilamellar egg phosphatidylcholine vesicles to brush border membrane. This result supports the notion of a 1:1 phospholipid exchange between brush border membrane and small unilamellar egg phosphatidylcholine vesicles. The reduction in the rate of phospholipid exchange observed after about 2 h (Figure 2) is not due to inactivation of the exchange protein(s). For instance, the exchange of cholesterol between small unilamellar egg phosphatidylcholine vesicles and brush border membrane is a fast monophasic process over 10–15 h until equilibrium is reached (unpublished observation). The slow rate of phospholipid exchange probably reflects the rate of replenishing of external phospholipid molecules removed from brush border membrane by exchange. Replenishing of external phospholipid is achieved by the transverse or flip-flop movement of phospholipids, and the rate constants for slow exchange are therefore characteristic of this process. For the isoelectric phospholipids, the half-time of the flip-flop motion is ~8 h, and for the negatively charged phospholipids the half-time values are increased by a factor of about 10 (Table I). Our value for the flip-flop motion of isoelectric phospholipids is in good agreement with that reported for phosphatidylcholine in erythrocyte membranes [for a review, see Zachowsky and Devaux (1990)].

The rate constants for the initial fast rate of phospholipid hydrolysis in the presence of phospholipase A_2 are of the same order but not identical to those reported previously [Table III and Barsukov et al. (1986)]. The present rate constants are smaller by a factor of ~2, and this decrease in the rate of phospholipid hydrolysis may reflect the greater stability of brush border membrane vesicles used here. The rate constants (half-times) for the slow process of phospholipid hydrolysis are also similar to those reported before (Barsukov et al., 1986). These half-times are significantly shorter, at least one order of magnitude compared to the half-times for the slow phospholipid exchange as evident from a comparison of Table I and Table III. Furthermore, similar values are obtained for isoelectric and negatively charged phospholipids (Table III). The relatively short half-times observed under these conditions are probably artificial. The flip-flop motion seems to be facilitated in the presence of phospholipase A_2, and this is so in spite of the fact that even extensive phospholipid hydrolysis with phospholipase A_2 apparently does not affect the integrity of brush border membrane (Barsukov et al., 1986). Phospholipid hydrolysis by phospholipase A_2 generates lysophospholipids which could induce higher flip-flop rates. Phospholipases are known to partially penetrate the lipid bi-

layer, and this in turn might create local packing defects facilitating phospholipid flip-flop. In this context, it is interesting to note that the flip-flop rate of phosphatidylcholine depends on the type of phospholipase used (Venien & Grimellec, 1988).

The experiments with deoxycholate (Figure 3) lend support to the assignment of the two lipid pools. Addition of deoxycholate to brush border membrane vesicles at concentrations that are insufficient to solubilize brush border membrane vesicles but sufficient to render the membrane permeable to proteins (Klip et al., 1979b) makes all phospholipids accessible to phospholipases in a single fast reaction step (cf. dashed lines in Figure 3). Sphingomyelin is, however, not totally hydrolyzed: the remaining 10% that are unaffected may represent phospholipid intimately associated with integral membrane protein, e.g., phospholipid present in the boundary layer of these proteins, and as a result these phospholipids may not be susceptible to hydrolysis.

The phospholipid distribution in brush border membrane derived from the two methods employed here may be summarized as follows: the neutral (isoelectric) phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and possibly also sphingomyelin are preferentially located on the cytoplasmic side, i.e., only about 30% of each phospholipid is readily exchangeable, whereas the negatively charged phospholipids are nearly equally distributed between the two halves of the membrane. A similar asymmetric distribution of phosphatidylethanolamine and phosphatidylcholine was reported for rabbit kidney brush border membrane (Venien & Grimellec, 1988). However, the negatively charged phospholipids exhibit a much more asymmetric distribution in this membrane with 82–87% being located on the cytoplasmic side. It should be mentioned that the total lipid composition of rabbit kidney brush border membrane is different from that of intestinal brush border membrane.

The phospholipid exchange between brush border membrane and small unilamellar egg phosphatidylcholine vesicles merits discussion. It was shown that this reaction is abolished by treating brush border membrane vesicles with proteinases, and on the basis of this finding it was concluded that the phospholipid exchange is protein mediated (Thurnhofer & Hauser, 1990b). The work presented here shows that if one molecule of phosphatidylcholine is transferred from small unilamellar egg phosphatidylcholine vesicles to brush border membrane, phospholipids and also cholesterol and glycolipids located in the outer layer of brush border membrane are transferred back in exchange to egg phosphatidylcholine vesicles. This very likely occurs as a 1:1 exchange. The protein responsible for this exchange reaction was shown to recognize and bind cholesterol and phosphatidylcholine (Thurnhofer & Hauser, 1990a,b). The exchange experiments described here suggest that we are dealing with (a) nonspecific lipid transfer protein(s). The phospholipid exchange can lead, at least under certain conditions, to a massive accumulation of phosphatidylcholine in brush border membrane and in turn to a depletion of intrinsic brush border membrane lipids. The accumulation of extrinsic phosphatidylcholine in brush border membrane is such that the membrane fluidity and related properties change significantly. The depletion of intrinsic phospholipids and the concomitant replacement by extrinsic phospholipids are hardly physiological processes. It has been suggested that unilamellar phospholipid vesicles are present in the upper small intestine and lipid absorption takes place from these vesicles as donor particles. If correct, phospholipid vesicles cannot make up a significant proportion of donor particles, i.e., the major donor particles must be mixed bile salt micelles. We could show that contrary to phospholipid vesicles there is a net transfer of cholesterol and other lipids from mixed micelles to brush border membrane (Thurnhofer & Hauser, 1990a,b). In the latter case lipids such as dietary lipids move into one direction from the donor to the acceptor, and only such a process is physiologically meaningful.

Therefore, the accumulation of extrinsic lipids in brush border membrane as reported here cannot represent a physiological process: it leads to drastic changes in membrane properties and may eventually result in the loss of membrane functions. In order to prevent the accumulation of dietary lipids in brush border membrane during lipid absorption, dietary lipids must be transported from the outer to the inner layer of the bilayer and finally to the cytosol of the enterocyte. These processes would have to be fast enough in order to avoid the possibly disastrous effect of lipid accumulation. It is therefore quite likely that both steps, the lipid flip-flop as well as the release of the lipid into the cytosol, are protein mediated, at least for some of the dietary lipids. Experiments to shed light on this question are currently in progress.

Registry No. Phospholipase A_2, 9001-84-7; sphingomyelinase C, 9031-54-3.

References

Barsukov, L. I., Bergelson, L. D., Spiess, M., Hauser, H., & Semenza, G. (1986) *Biochim. Biophys. Acta 862*, 87–99.

Bergelson, L. D., & Barsukov, L. I. (1977) *Science 197*, 224–230.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Bloj, B., & Zilversmith, D. B. (1976) *Biochemistry 15*, 1277–1283.

Bowyer, D. E., & King, J. P. (1977) *J. Chromatogr. 143*, 473–490.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Etemadi, A.-H. (1980) *Biochim. Biophys. Acta 604*, 423–475.

Hauser, H., Howell, K., Dawson, R. M. C., & Bowyer, D. E. (1980) *Biochim. Biophys. Acta 602*, 567–577.

Hauser, H., Gains, N., Semenza, G., & Spiess, M. (1982) *Biochemistry 21*, 5621–5628.

Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M., & Semenza, G. (1978) *Biochim. Biophys. Acta 506*, 136–154.

Klip, A., Grinstein, S., & Semenza, G. (1979a) *J. Membr. Biol. 51*, 47–73.

Klip, A., Grinstein, S., & Semenza, G. (1979b) *FEBS Lett. 99*, 91–96.

Krebs, J. J. R. (1982) *J. Bioenerg. Biomembr. 14*, 141–157.

McLean, L. R., & Phillips, M. C. (1984) *Biochemistry 23*, 4624–4630.

Mütsch, B., Gains, N., & Hauser, H. (1986) *Biochemistry 25*, 2134–2140.

Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48*, 47–71.

Perevucnik, G., Schurtenberger, P., Lasic, D. D., & Hauser, H. (1985) *Biochim. Biophys. Acta 821*, 169–173.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provezano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.

Skipski, V. P., & Barclay, M. (1969) *Methods Enzymol. 14*, 530–598.

Thurnhofer, H., & Hauser, H. (1990a) *Biochemistry 29*, 2142–2148.

Thurnhofer, H., & Hauser, H. (1990b) *Biochim. Biophys. Acta 1024*, 249–262.
Thurnhofer, H., Schnabel, J., Betz, M., Lipka, G., Pidgeon, C., & Hauser, H. (1991) *Biochim. Biophys. Acta 1064*, 275–286.
Venien, C., & Le Grimellec, C. (1988) *Biochim. Biophys. Acta 942*, 159–168.
Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, A., & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 323*, 178–193.
Zachowsky, A., & Devaux, P. F. (1990) *Experientia 46*, 644–656.
Zwaal, R. F. A., Roelofsen, B., Comfurius, P., & van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406*, 83–96.

Picosecond Rotation of Small Polar Fluorophores in the Cytosol of Sea Urchin Eggs†

N. Periasamy, M. Armijo, and A. S. Verkman*
Departments of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, California 94143-0532

Received June 12, 1991; Revised Manuscript Received September 19, 1991

ABSTRACT: A new fluorescence method to measure viscosity in cell cytosol [Fushimi, K., & Verkman, A. S. (1991) *J. Cell Biol. 112*, 719–725] has been applied to determine fluid-phase viscosity in sea urchin eggs. Freshly harvested eggs from *Lytechinus pictus* were loaded with the dyes 2,7-bis(2-carboxyethyl)-5-(and-6-)carboxyfluorescein (BCECF), 6-carboxyfluorescein (6CF), fluorescein, or calcein. Fluorescence lifetimes and anisotropy decay were measured in single eggs by multiharmonic, frequency-domain microfluorometry using a 1–2-μm focused laser spot and 25× air objective. In calibration solutions consisting of glycerol in pH 8 buffered sea water, probe lifetime was single exponential and probe rotation was isotropic with a single correlation time which increased linearly with viscosity in the range 1–3.6 cP. In eggs at 22 °C, there were single lifetimes (in nanoseconds) of 3.6 (BCECF), 3.4 (6CF), 3.2 (fluorescein), and 3.3 (calcein). Probe rotation in eggs had two components, a fast component (in picoseconds, mean ± SE, 10–18 eggs) of 568 ± 39 (BCECF), 311 ± 21 (6CF), 313 ± 15 (fluorescein), and 516 ± 44 (calcein) and a slow component of 10–40 ns. The fractional amplitude of the fast component, corresponding to unbound dye, was 0.72–0.81. Apparent viscosities of fluid-phase cytoplasm (centipoises) given by the four different probes were in good agreement: 2.3 ± 0.2 (BCECF), 2.1 ± 0.1 (6CF), 2.5 ± 0.1 (fluorescein), and 2.3 ± 0.2 (calcein). The viscosity in cytosol of sea urchin eggs (2.1–2.5 cP) is thus relatively low, yet significantly greater than that of water (1 cP) or cytosol in cultured fibroblasts (1.2–1.4 cP). Taken together with previous results, the increased viscosity in egg cytosol may be a consequence of the high concentration of small organic osmolytes required to maintain cell volume.

Cell cytoplasm is a complex compartment consisting of water, dissolved solutes, and organized macromolecular structures and organelles (Fulton, 1982; Stossel, 1982; Porter, 1984; Gershon et al., 1985; Luby-Phelps et al., 1986). The rotational and translational dynamics of solute molecules depend upon the viscous properties of the cellular environment as well as upon solute geometry and specific interactions with intracellular components. A particularly important quantity is the microviscosity of "fluid-phase" cytoplasm, defined as the viscosity detected by a very small probe molecule which does not interact with intracellular macromolecular and lipid structures. Fluid-phase cytoplasmic viscosity is an important determinant for the kinetics of diffusion-limited transport and enzymatic processes and for the energetics of cell motility (Taylor & Fechheimer, 1982; Clegg, 1984b; Cameron et al., 1988; Luby-Phelps et al., 1988).

A number of approaches have been applied to estimate fluid-phase viscosity in cell cytoplasm. Although there is evidence that cytoplasm is a viscous gellike compartment [for reviews, see Keith (1973) and Porter (1984)], more recent studies of the motions of small molecules suggest that cytoplasm has many properties of simple aqueous solutions. Studies of probe translational motion in cell cytoplasm by fluorescence recovery after photobleaching and electron spin resonance methods gave viscosities as low as 2–6 cP when probe size was extrapolated to zero (Luby-Phelps et al., 1986; Salmon et al., 1984; Wojcieszyn et al., 1981); measurements of probe translational diffusion would overestimate fluid-phase viscosity because of probe binding to and collisions with intracellular structures and the existence of intracellular barriers such as cytoskeleton and organelles (Keith et al., 1977b). Probe rotation would be influenced to a lesser extent by physical barriers; however, probe binding could strongly hinder rotational mobility. Electron spin resonance studies of the rotation of small spin-labeled probes gave viscosities of 2 to >50 cP (Keith et al., 1977a; Lepock et al., 1983; Mastro & Keith, 1984). Because fluid-phase viscosity would be overestimated by each of the measurements above, it was predicted that the actual fluid-phase viscosity of cytosol, at least in some cells, is <2 cP.

We introduced an approach recently to estimate fluid-phase viscosity in cell cytosol on the basis of the picosecond rotational

† This work was supported by Grants DK39354, DK17054, HL42368, and DK35124 from the National Institutes of Health and a grant from the National Cystic Fibrosis Foundation. Dr. Verkman is an Established Investigator of the American Heart Association.
* Address correspondence to this author.

0006-2960/91/0430-11836$02.50/0