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The uptake of phosphatidylcholine by small intestinal brush border membrane is protein-mediated

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Brush border membrane vesicles prepared from rabbit small intestine are essentially free of basolateral membranes and nuclear, mitochondrial, microsomal and cytosolic contaminants. The resulting brush border membrane is unstable due to intrinsic lipases and proteinases. The PC transfer between small unilamellar lipid vesicles or mixed lipid micelles as the donor and the brush border membrane vesicles as the acceptor is protein-mediated. After proteolytic treatment of brush border membrane with papain or proteinase K the PC transfer activity is lost and the kinetics of PC uptake are similar to those measured with erythrocytes under comparable conditions. Evidence is presented to show that the PC transfer activity resides in the apical membrane of the enterocyte and not in the basolateral part of the plasma membrane. Furthermore, the activity is localized on the external surface of the brush border membrane exposed to the aqueous medium with its active centre probably not in direct contact with the lipid bilayer of the membrane. Proteins released from brush border membrane by proteolytic treatment catalyze PC exchange between different populations of small unilamellar vesicles. Furthermore, these protein(s) bind(s) PC forming a PC-protein complex.

Introduction

Previously we reported the exchange of PC from small unilamellar vesicles as the donor to intestinal brush border membrane vesicles as the acceptor [1]. Spin-labeled and radioactive PC were used and the transfer of these labeled molecules was shown to be an exchange reaction leading to an even distribution of PC between the donor and acceptor pool of lipids at equilibrium with half times of the order of hours. Evidence was presented to show that the labeled PC originally present in small unilamellar vesicles is transferred to brush border membrane and indeed incorporated in the lipid bilayer of this membrane [1,2]. The PC exchange is a second order reaction and its mechanism was shown to be collision-induced [1].

Very little is known about how dietary lipids interact with the apical or brush border membrane of enterocytes, how they are incorporated into the external layer of this membrane, how they diffuse across the relatively tightly packed brush border membrane and by which mechanism they are released into the cytosol of the enterocyte. The biogenesis of membrane lipids is also far from being solved: it is still unknown how membrane lipids are delivered from their site of synthesis to their site of final destination and how the usual asymmetric distribution of membrane lipids is generated and maintained during extensive intracellular membrane traffic. Evidence has accumulated that shows that membrane lipids similar to membrane proteins are transported between various intracellular membranes and the plasma membrane. However, little is known about the mechanism by which lipid sorting is accomplished, i.e., the mechanism by which lipids reach their site of destination (for a review, see Ref. 3).

Here we report that the uptake of PC by small intestinal brush border membrane from either mixed micelles or small unilamellar vesicles is protein-mediated. The aim of the present study is to characterize in detail the uptake of PC by brush border membrane

Abbreviations: DEAE, diethylaminoethyl; DPPC, 1,2-dipalmitoyl-sn-phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NADH, reduced form of nicotinamide adenine dinucleotide; PC, phosphatidylcholine; PI, phosphatidylinositol; TLC, thin-layer chromatography; TLCK, *N*α-tosyl-L-lysine chloromethylketone hydrochloride; Tris, tris(hydroxymethyl)aminoethane.

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vesicles with respect to the kinetics, the activation energy and the mechanism.

Materials and Methods

Materials

Egg PC, egg lyso PC, egg phosphatidic acid, ox brain phosphatidylserine and wheat germ phosphatidylinositol were purchased from Lipid Products (Surrey, U.K.), 1,2-dipalmitoyl-*sn*-phosphatidyl[*N-methyl*-^{14}C]-choline (^{14}C-DPPC, specific activity 58 mCi/mmol) and 1,2-dipalmitoyl-*sn*-phosphatidyl[*N-methyl*-^{3}H]choline (^{3}H-DPPC, specific activity 76 Ci/mmol) from Amersham (Amersham, U.K.), *sn*-phosphatidyl-3-[*myo-inositol*-2-^{3}H(n)] (^{3}H-PI, specific activity 5.0 Ci/mmol) from Du Pont de Nemours International (Regensdorf, Switzerland), papain (EC 3.4.22.2) from papaya latex as a suspension of crystals in 50 mM sodium acetate pH 4.5 either from Sigma (St. Louis, MO) or Boehringer (Mannheim, F.R.G.), 2,3-dihydroxy-1,4-dithiolbutane, soybean trypsin inhibitor type I-S and bovine lung aprotinin from Sigma, yeast hexokinase and lyophilized proteinase K from Tritirachium album from Boehringer, TLCK, phenylmethylsulfonyl fluoride, *N*-ethylmaleimide and 5-dimethylamino-1-naphthalenesulfonyl chloride from Fluka (Buchs, Switzerland), 3-isothiocyano-1,5-naphthalene disulfonic acid (disodium salt), methyl isocyanate and 2,4-dinitrofluorobenzene from Pierce (Oud-Beijerland, The Netherlands), iodoacetic acid from Merck (Darmstadt, F.R.G.), iodoacetamide and sodium dodecyl sulfate from BDH (Poole, U.K.), pepstatin and chymostatin from Protein Research Foundation (Japan) and bacitracin from Dr. Grossmann AG Pharmaca (Allschwil-Basel, Switzerland). All other chemicals used were of analytical grade. The water for preparing aqueous solutions and lipid dispersions was double-distilled in a quartz apparatus.

1,2-Dipalmitoyl-*sn*-phosphatidylethanolamine and 1,2-dihexadecyl-*sn*-phosphatidylethanolamine were synthesized by Mr. R. Berchtold (Biochemisches Labor, Bern). 1-Palmitoyl-2-[9,10-^{3}H]palmitoyl-*sn*-phosphatidylcholine (^{3}H-DPPC, specific activity 85 Ci/mol) was prepared as described in Ref. 1. 1,2-Dipalmitoyl-*sn*-phosphatidyl[*N-methyl*-^{3}H(U)]choline was synthesized from 1,2-dipalmitoyl-*sn*-phosphatidylethanolamine (10 mg) which was dissolved at 50 °C in 1 ml $CHCl_3$/CH_3OH (1 : 1, by vol.) containing 50 mg $NaHCO_3$. To this solution a 40-fold excess of ^{3}H-CH_3I (specific activity 101 Ci/mol) in 1.5 ml toluene was added and the mixture was stirred at room temperature for about 100 h. After completion of the reaction 0.4 ml H_2O and 0.4 ml $CHCl_3$ were added and the organic phase was separated from the aqueous phase using a separation funnel. The organic solvent was removed on the rotary evaporator and the radioactive phosphatidylcholine (specific activity 96 Ci/mol) was further purified by preparative TLC. 1,2-Dihexadecyl-*sn*-phosphatidyl[*N-methyl*-^{3}H(U)]choline was synthesized as described above except that 1,2-dihexadecyl-*sn*-phosphatidylethanolamine was used as the starting material. Spin-labeled 1-palmitoyl-2-(5-doxylpalmitoyl)-*sn*-phosphatidylcholine (5-doxyl-PC) and 1-palmitoyl-2-(8-doxylpalmitoyl)-*sn*-phosphatidylcholine (8-doxyl-PC) were synthesized as described before [4]. All lipids used in this study were pure by TLC standard [5].

Methods

Preparation of brush border membrane vesicles

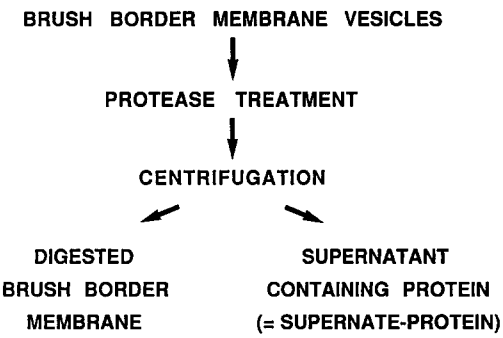
Brush border membrane vesicles were prepared by the Mg-EGTA procedure [5] from rabbit small intestines that were stored at −80 °C prior to preparation. The resulting brush border membrane vesicles were suspended in 10 mM Hepes-Tris buffer (pH 7.3), 0.3 M D-mannitol, 5 mM EDTA, 0.02% NaN_3 (buffer A) and characterized as described previously [5]. They were fairly homogeneous with respect to size with an average diameter of about 150 nm [6]. The method of preparation used here was shown to yield brush border membrane vesicles free of nuclear and mitochondrial membranes as well as cytosolic contaminants [7,8]. The presence of basolateral membranes was assessed by determining Na^+/K^+-ATPase activity. Contamination of brush border membrane with microsomal membranes was determined by monitoring KCN-insensitive NADH oxidoreductase activity [9]. The cytosol of brush border membrane was prepared as described before [5]. It is identical to the supernatant obtained after the first high-speed centrifugation of brush border membrane vesicles at $48\,000 \times g$ for 15 min. The cytosol which may contain degradation products released from brush border membrane during preparation was tested for phospholipid exchange activity.

Preparation of basolateral membranes from rabbit small intestine

Basolateral membranes were prepared and purified according to Ref. 10. Crude basolateral membranes were suspended in 10 vol% Percoll (from Pharmacia) and purified by density gradient centrifugation at $48\,000 \times g$ for 60 min at 4 °C. The density gradient formed spontaneously upon centrifugation of the membrane suspension in 10 vol% Percoll. After centrifugation the density gradient was fractionated and analyzed for protein content and Na^+/K^+-ATPase activity [10].

Preparation of erythrocytes and erythrocyte ghosts

Erythrocytes and erythrocyte ghosts were prepared essentially as described before [11,12]. Erythrocytes of fresh human blood (from the Swiss Red Cross Blood Transfusion Service, Zürich) were pelleted by centrif-



Scheme I.

ugation at 350 × *g* for 10 min and resuspended in 5 mM sodium phosphate buffer (pH 7.0) containing 0.154 M NaCl at 4° C. The resulting erythrocytes were washed by repeating the above procedure two to three times. For the production of ghosts, human erythrocytes were hemolyzed by diluting their suspension with 20 volumes of 5 mM sodium phosphate buffer at 0° C. After 10 min the ghosts were pelleted by centrifugation at 12000 × *g* for 5 min and the pellet was washed once with the same buffer.

Proteolytic treatment of brush border membrane vesicles

Papain digestion. The digestion of brush border membrane vesicles with either papain or proteinase K was carried out as shown in Scheme I. For the papain digestion a published procedure [13] was used as modified below. Papain was activated by diluting 0.2 ml of the papain suspension (25 mg/ml) with 60 μl of 0.5 M potassium phosphate buffer (pH 6.8) containing 50 mM 2,3-dihydroxy-1,4-dithiolbutane and 10 mM EDTA. The resulting papain solution was saturated with N_2 and incubated at room temperature for 30 min. A suspension of brush border membrane vesicles in buffer A (3 ml, 25 mg protein/ml) were mixed with 0.4 ml of the 0.5 M potassium phosphate buffer (pH 6.8) described above and 0.21 ml of the activated papain solution. The resulting brush border membrane suspension (21 mg membrane protein/ml) containing papain at 1.1 mg/ml (28 U/ml or 1.3 U/mg brush border membrane protein) and 6.2 mM 2,3-dihydroxy-1,4-dithiolbutane was thoroughly flushed with N_2 and incubated either at room temperature or 37° C. The reaction was stopped by adding 0.19 ml of a 0.1 M solution of TLCK in H_2O (final concentration about 5 mM) and cooling the reaction mixture to approx. 4° C. The papain-treated brush border membrane vesicles were separated from the supernatant by centrifugation at 120000 × *g* for 30 min at 4° C. The supernatant was decanted from the brush border membrane pellet; proteins present in the supernatant will be referred to as supernate-protein. The brush border membrane pellet was washed once more with buffer A by resuspending the pellet in about 25 ml buffer A and repeating the centrifugation.

Digestion with proteinase K. To brush border membrane vesicles dispersed in buffer A proteinase K was added (18 mg membrane protein/ml and 2.7 mg proteinase K/ml, 55 U/ml, in a total volume of 1.1 ml) and the mixture was incubated at room temperature for 2 h. The reaction was stopped by adding 40 ml of buffer A cooled to 4° C. The digested brush border membrane vesicles were separated from supernate-protein and washed as described under papain digestion.

Storage of brush border membrane vesicles

Brush border membrane vesicles were prepared from rabbit small intestine and suspended in buffer A as described above. Excess brush border membranes which were not used immediately after preparation were frozen and stored at −35° C. Upon thawing and incubating brush border membrane suspension at temperatures ≥ 4° C membrane proteins were liberated even in the absence of extrinsic proteinases. At the end of the incubation time 20 volumes of 0.01 M sodium phosphate buffer (pH 7.3) containing 0.14 M NaCl, 2.5 mM EDTA and 0.02% NaN_3 (buffer B) were added, and the proteins released were separated from brush border membrane vesicles by centrifugation at 120000 × *g* for 30 min. The proteins present in the supernatant will also be referred to as supernate-protein. Hexokinase activity was determined in the presence of increasing amounts of brush border membrane vesicles according to Ref. 14.

Preparation of small unilamellar vesicles and micelles

Small unilamellar vesicles of egg PC were produced by sonicating 2.5 ml of the phospholipid dispersion in buffer A with a microtip sonicator (Branson B-30) for 90 min under standard conditions as specified in Ref. 15. These vesicles were used as acceptor vesicles in experiments measuring PC exchange between two different populations of small unilamellar vesicles. Small unilamellar vesicles used as donors consisted of either pure egg PC or egg PC/egg phosphatidic acid (85 : 15, w/w) containing a trace amount of radiolabeled PC. They were made by sonicating 0.65 ml phospholipid dispersion in buffer A in a bath sonicator (Laboratory Supplies, Hicksville, NY) for 2.5 h at room temperature under Ar. Micellar dispersions consisting of egg lyso PC/egg PC (3 : 2, w/w) in buffer A containing a trace amount of radiolabeled PC were prepared as described before [16].

PC uptake by brush border membrane vesicles

Brush border membrane vesicles were incubated with donor particles which were either small unilamellar phospholipid vesicles or mixed phospholipid micelles containing radiolabeled or spin-labeled PC. Unless stated otherwise the incubation was carried out at room temperature. After timed intervals 0.1 ml of the incuba-

tion medium were diluted with 0.05 ml buffer A and donor particles were separated from brush border membranes by centrifugation at $80000 \times g$ for 10 min in a Beckman airfuge. The donor particles remaining in the supernatant were analyzed for radioactivity using a Beckman LS 7500 liquid scintillation counter. Spin-labeled lipid dispersions were made as described before [1]. The concentration of spin label in the donor particle was chosen such that its ESR spectrum was a simple spin-exchange line. Spin label transferred to brush border membrane gave rise to an anisotropic ESR spectrum superimposed on the spin-exchange line. The spectral intensity was used to quantitate the lipid transfer from donor to acceptor (see below). The ESR method of monitoring lipid transfer from donor to acceptor membrane has the advantage that donor and acceptor particles need not be separated prior to recording the ESR spectra. Control experiments were carried out in which the brush border membranes were spun down after incubation with donor particles at $80000 \times g$ for 10 min. In this way brush border membranes were separated quantitatively from the donor particles. After resuspending the brush border membrane pellet in buffer the ESR spectrum was recorded and signal intensities were measured by double integration. No difference in results was observed regardless whether or not donor and acceptor particles were separated before recording the ESR spectra.

Determination of PC exchange activity in the supernatant (supernate-protein)

The papain-treatment of brush border membrane or storage of brush border membrane at 4°C described caused the release of membrane proteins into the supernatant which exhibit PC exchange activity between two different populations of small unilamellar phospholipid vesicles. The donor vesicles consisted of egg PC/egg phosphatidic acid (85 : 15, w/w) and trace amounts of ^{3}H-DPPC whereas the acceptor vesicles were made of pure egg PC. Both donor and acceptor vesicles were dispersed in buffer A and sonicated as described above. The two populations of small unilamellar vesicles were mixed so that the final total phospholipid concentration of donor vesicles was 0.1 mg/ml and that of acceptor vesicles 1 mg/ml. The mixture (0.12 ml) was incubated at room temperature in the absence and presence of supernate-protein, i.e., protein liberated from brush border membrane. After timed intervals 0.1 ml of the incubation mixture were applied to a DEAE-Sepharose CL-6B column (column size 2×0.6 cm, from Pharmacia). The negatively charged donor vesicles were retained while the isoelectric acceptor vesicles were eluted from the column. The quantity of radiolabeled PC transferred from donor to acceptor vesicles was determined by counting 0.5 ml aliquots of the eluate in a Beckman LS 7500 liquid scintillation counter. It should be noted that some vesicle fusion will not interfere with this assay. Fusion between one donor and one acceptor vesicle will lead to a reduction in the surface charge density of the donor vesicles by a factor of about 2 while fusion between the same kind of vesicles has no effect on lipid composition. Control experiments showed that vesicles containing half of the egg phosphatidic acid of our original donor vesicles are still retained on the DEAE-Sepharose CL-6B column and hence do not contribute to the radioactivity determined in the acceptor vesicles.

Analytical procedures

Lipid phosphorus was determined according to Chen et al. [17], the protein content was determined according to Lowry et al. [18] or alternatively with bicinchoninic acid from Pierce [19], with bovine serum albumin as the standard. The lipid content of brush border membrane vesicles was determined gravimetrically after extraction into hexane-2-propanol (3 : 2, by vol., Ref. 20) and evaporation of the organic solvent. Lipid extraction with $CHCl_3/CH_3OH$ (2 : 1, by vol.) and one-dimensional TLC analysis of lipids was carried out as detailed in Ref. 5. The solvents used for TLC analysis were: $CHCl_3/CH_3OH/H_2O$/acetic acid (65 : 50 : 4 : 1 and 55 : 35 : 3 : 2, by vol.). Cholesterol was determined with Cholesterol Merckotest from Merck.

ESR measurements

ESR spectra were recorded at 9.2 GHz with a Varian X-band spectrometer (Model E-104A) fitted with a variable temperature control. The temperature inside the 1 mm diameter sample capillary was measured to ± 0.5 C° by using a thermocouple. ESR signal intensities were determined by double integration of the ESR spectrum using a Bruker BNC-12 computer interfaced with a Varian X-band spectrometer.

Results

Characterization of brush border membrane preparation

The method of preparation used here [5] yields brush border membrane vesicles essentially free of basolateral membranes and of microsomes. Basolateral membranes were prepared and purified by density gradient centrifugation on a Percoll gradient according to Scalera et al. [10]. The activity of Na^+/K^+-ATPase, which was used as a marker enzyme for the basolateral plasma membrane, was measured in all fractions eluted from the Percoll density gradient. The fractions that exhibited Na^+/K^+-ATPase activity had no PC exchange activity.

Possible contamination of our brush border membrane with microsomes was checked by using KCN-insensitive NADH oxidoreductase as marker enzyme [9]. Since no oxidoreductase activity was detected in our

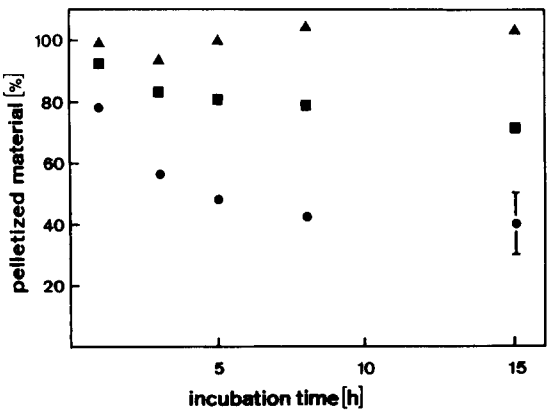


Fig. 1. Stability of rabbit small intestinal brush border membrane at 37 °C. Brush border membrane vesicles were prepared as described in Materials and Methods. The vesicles were suspended in Hepes-Tris buffer pH 7.3 (20 mg protein/ml) and incubated at 37 °C. After timed intervals 0.9-ml aliquots of the brush border membrane suspension were pipetted into 5 ml Hepes-Tris buffer (pH 7.3). The brush border membrane vesicles were spun down at 32000 × *g* for 30 min and the pellet was resuspended in the same buffer. The resulting dispersion was analyzed for protein (■) according to Lowry et al. [18] and for lipids. For lipid analysis the membrane suspension was extracted with $CHCl_3/CH_3OH$ (2:1, by vol.) and the total phospholipid content (●) and cholesterol (▲) were determined in the lipid extract (see Materials and Methods).

brush border membrane preparation, we can conclude that this preparation is essentially free of microsomes.

The cytosol of brush border membrane containing possibly degradation products was analyzed for phospholipid exchange activity. It exhibited phosphatidylinositol and PC exchange activity between two populations of small unilamellar vesicles. It catalyzed the exchange of ^{3}H-PI and ^{3}H-DPPC between small unilamellar egg PC/egg phosphatidic acid vesicles (85 : 15, w/w, 0.1 mg total lipid/ml) as the donor and small unilamellar egg PC vesicles (1 mg/ml) as the acceptor. These results are in agreement with a previous report [21].

Stability of brush border membrane preparation

It was reported before [2] that small intestinal brush border membrane vesicles prepared as described in Materials and Methods are not stable at room temperature and even less so at elevated temperatures. Evidence for the instability of our brush border membrane preparation is presented in Fig. 1. Upon incubation at 37 °C increasing amounts of both phospholipid and protein were degraded and released into the supernatant. The material liberated from brush border membrane remained in the supernatant when brush border membrane vesicles were centrifuged to a pellet at 32000 × *g* for 30 min. After incubation at 37 °C for 15 h about 60% of membrane phospholipid were degraded and 30% of membrane protein were released into the supernatant (Fig. 1). In contrast, no cholesterol was found in the supernatant indicating that cholesterol was not degraded and remained associated with brush border membrane. Inspection of Table I (top) shows that freshly prepared brush border membrane vesicles were susceptible to phospholipid hydrolysis and degradation even at low temperatures. Extraction of brush border membrane lipids after incubation at 37 °C for 17 h and TLC analysis of the lipid extract revealed that amino phospholipids are primarily affected. Half of phosphatidylethanolamine and practically all of phosphatidylserine were degraded (bottom panel). The presence of EDTA markedly slowed down the degradation of phospholipids indicating that the degradation is due to lipases.

The following experiments suggest that the liberation of membrane proteins is not due to extrinsic proteinases

TABLE I

Stability of brush border membrane

Brush border membrane vesicles were incubated at different temperatures for 17 h and the total phospholipid content was determined (top panel). The phospholipid composition of brush border membrane vesicles (BBMVs) was determined before and after incubation at 37 °C for 15 h (bottom panel).

Freshly prepared brush border membrane vesicles were dispersed in buffer A (35 mg protein/ml) and incubated at various temperatures for 17 h. After incubation 9 volumes of buffer A were added and brush border membranes were spun down by centrifugation at 40000 × *g* for 30 min. The pellet was resuspended in buffer, lipids were extracted as described in Ref. 5 and the phospholipid content of the lipid extract was determined by phosphate analysis [17]. In a control experiment phospholipids were extracted from freshly prepared brush border membranes. The phospholipid content determined in this lipid extract was taken as 100%.

Incubation temperature (°C)	Total phospholipid content (%)
4	90
22	84
37	50
37 in the presence of 10 mM EDTA	87

Also included in the table is the phospholipid composition of brush border membrane vesicles. Brush border membrane vesicles suspended in buffer A (25 mg protein/ml) were stored at −35 °C prior to the experiment. After thawing of the brush border membrane suspension lipids were extracted before and after incubation at 37 °C for 15 h. The phospholipid analysis was carried out as described in Ref. 5.

Phospholipid	Phospholipid composition (%)	
	BBMVs before incubation (control)	BBMVs after incubation at 37 °C for 15 h
PC + phosphatidylinositol	38	63
Phosphatidylethanolamine	35	22
Sphingomyelin	10	12.5
Lyso PC	3	2.5
Phosphatidylserine + lyso-phosphatidylethanolamine	11	2

present in our membrane preparation as contaminants. The activity of hexokinase, which is particularly susceptible to proteolytic attack [22], was monitored in the presence of increasing concentrations of brush border membrane. The hexokinase activity remained unaffected when the enzyme was incubated with excess brush border membrane up to a protein weight ratio of brush border membrane protein/hexokinase = 800 at 20°C for 2 h. In one preparation of brush border membrane soybean trypsin inhibitor (0.15 mg/ml) was added to all media right from the beginning of the preparation. No difference between the resulting brush border membrane vesicles and a standard brush border membrane preparation was observed regarding the liberation of membrane protein.

In an attempt to inhibit the intrinsic brush border membrane proteinases, a suspension of brush border membrane in buffer A (4.2 mg protein/ml) was incubated at 0°C with an inhibitor cocktail containing aprotinin (1.9 μg/ml), pepstatin (8.7 μg/ml), chymostatin (8.7 μg/ml), phenylmethylsulfonyl fluoride (44 μg/ml), EDTA (22 mM) and bacitracin (87 μg/ml). After incubation for 45 min the brush border membrane was pelleted by centrifugation at 120000 × *g* for 30 min and protein was determined in the supernatant. The supernatant contained about 10% membrane proteins and this value agreed within the experimental error with the control which was treated in the same way except that the above mixture of compounds was replaced by an equal volume of buffer A. Resuspending the brush border membrane pellet in buffer with and without inhibitor cocktail and repeating the incubation as described above produced a similar result. Another 15% of membrane protein were released into the supernatant regardless whether the proteinase inhibitors were present or not.

Proteolytic treatment of brush border membrane vesicles

Proteolytic treatment of brush border membrane with papain or proteinase K liberated a significant proportion of the total membrane protein. The exact amount depended on experimental conditions, such as for instance, temperature, specific activity of protein, incubation time. For instance, papain treatment at room temperature for 45 min liberated about 40% of the membrane protein, while the same treatment of brush border membrane vesicles in the absence of papain led to a 15% loss of membrane protein (cf. Fig. 1). Proteinase K treatment at room temperature for 45 min released about 50% of the membrane protein.

PC uptake by brush border membrane

Fig. 2 shows the kinetics of PC transfer from mixed egg lyso PC/egg PC micelles (3:2, w/w) as the donor to brush border membrane vesicles as acceptor. The time course of the decrease in ^{3}H-DPPC present in the donor micelles was linearized (see inset of Fig. 2) and pseudo-first-order rate constants were derived using established procedures [1,23–25]. The ^{3}H-DPPC exchange between small unilamellar vesicles as the donor and brush border membrane vesicles as the acceptor was analyzed in the same way. The pseudo-first-order rate constants for ^{3}H-DPPC transfer (exchange) derived from this analysis are summarized in Table II.

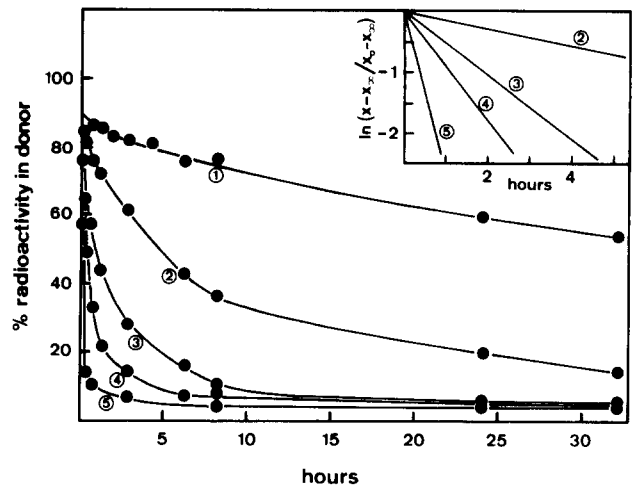


Fig. 2. Time course of PC transfer from phospholipid mixed micelles (lyso egg PC/egg PC (3:2, w/w)) as donor to brush border membrane vesicles as acceptor. The radioactivity expressed as % remaining in the micelles is plotted as a function of time. The phospholipid mixed micelles at 0.15 mg total lipid/ml labeled with a trace amount of ^{3}H-DPPC were incubated with brush border membrane vesicles of different concentrations at room temperature: 0.19 (1), 1.9 (2), 3.8 (3), 7.6 (4), and 15.1 (5) mg brush border membrane lipid/ml. After timed intervals brush border membrane vesicles were separated from the donor micelles by centrifugation at 80000 × *g* for 15 min. Phospholipid micelles remained in the supernatant and the radioactivity associated with them was determined by scintillation counting.

The pseudo-first-order rate constants obtained for the PC transfer from mixed micelles to brush border membrane were similar to those measured for PC exchange between small unilamellar vesicles and brush border membrane. The k_1 value for the exchange of radiolabeled ^{3}H-DPPC agrees well with the k_1 value measured for the exchange of spin-labeled PC (Table II). The k_1 values for PC transfer (exchange) increased linearly with acceptor concentration indicating that this reaction is of second order. This is true for ^{3}H-DPPC transfer from phospholipid micelles to brush border membrane (Fig. 3) as well as for PC exchange between unilamellar lipid vesicles and brush border membrane (Table II). As discussed in detail previously [1] the kinetic data are consistent with a mechanism involving collision-induced transfer (exchange) of PC. Despite the same reaction order there were significant differences in the mechanism depending on the nature of the donor particle. There was net transfer of PC from mixed micelles as the donor: practically all PC was incorpo-

rated in the lipid bilayer of brush border membrane at equilibrium. This was contrasted by PC exchange between small unilamellar vesicles as the donor and brush border membrane as the acceptor. At equilibrium PC was evenly distributed between the lipid pools * of the donor vesicles and that of brush border membrane. The expected equilibrium values calculated on the basis of the lipid concentration of donor and acceptor agreed well with the experimentally determined values (data not shown, see also Ref. 1).

Evidence for lipid exchange between small unilamellar vesicles and the lipid bilayer of brush border membrane was obtained as follows. Brush border membrane vesicles were labeled with ^{3}H-DPPC by incubation with sonicated egg PC vesicles containing ^{3}H-DPPC. The resulting brush border membrane vesicles containing ^{3}H-DPPC were separated from sonicated egg PC vesicles by centrifugation at $40000 \times g$ for 30 min and incubated with egg PC vesicles containing a trace amount of ^{14}C-DPPC under standard conditions (see Materials and Methods). The double-labeling allowed us to measure k_1 values simultaneously for the forward and backward reaction of PC exchange. The k_1 value for the forward reaction determined at an acceptor/donor lipid weight ratio of 1 was 0.031 h^{-1} (Table II) and under comparable conditions the k_1 value for ^{3}H-DPPC transfer from brush border membrane vesicles to sonicated egg PC vesicles was 0.033 h^{-1} ($t_{1/2} = 21$ h).

After proteolytic treatment by either papain or proteinase K, the brush border membrane lost its ability to incorporate PC (Table II). After treatment of brush border vesicles with papain, k_1 values were measured that were some 30 to 50 times smaller than the values measured in original brush border membrane vesicles. The half-times $t_{1/2}$ measured after papain treatment were on the order of 100 hours and similar to the half-times characteristic of ^{3}H-DPPC exchange between small unilamellar egg PC vesicles as donor and erythrocytes and erythrocyte ghosts as the acceptor membrane (Table II). The rate constants k_1 are similar to the values measured for the exchange of 1-palmitoyl-2-oleoyl-PC between two populations of small unilamellar vesicles [25]. Brush border membrane vesicles treated with proteinase K gave the lowest k_1 values that were measured. Apparently, proteinase K treatment practically abolished protein-mediated PC uptake in brush border membrane (Table II). However, there was a remarkable difference between papain and proteinase K treatment. While no recovery of PC exchange activity was observed when supernate-protein was added to papain treated brush border membrane, proteinase K treated brush border membrane at least partially regained its capacity of taking up PC in the presence of supernate-protein (Table II).

Brush border membranes were pretreated with enzyme inhibitors (1 mM) for 20 min at 4°C and washed twice to remove excess inhibitor. Brush border membrane vesicles pretreated with *N*-ethylmaleimide, $CuSO_4$, 5-dimethylamino-1-naphthalenesulfonyl chloride or 3-isothiocyano-1,5-naphthalene disulfonic acid (disodium salt) caused a 2-fold decrease in the PC exchange between brush border membrane vesicles and small unilamellar egg PC vesicles. Other inhibitors such as methyl isocyanate, 2,4-dinitrofluorobenzene, 2,3-dihydroxy-1,4-dithiolbutane, TLCK, iodoacetic acid and iodoacetamide had no effect on the PC exchange compared to the control.

The temperature dependence of the pseudo-first order rate constants of ^{3}H-DPPC exchange between small unilamellar egg PC vesicles and brush border membrane vesicles was measured and the data plotted as an Arrhenius plot. A linear relationship between 0 and 35°C was thus obtained (data not shown) from which the activation energy ΔE for PC exchange was derived as $\Delta E = 57 \pm 2$ kJ/mol (13.6 ± 0.5 kcal/mol).

The PC transfer to brush border membrane vesicles from both mixed phospholipid micelles and small unilamellar egg PC vesicles obeyed Michaelis-Menten kinetics. In either case, if the initial velocity v of PC transfer was plotted as a function of increasing concentrations of donor lipid, v approached a limiting value which is the maximum velocity v_{max} (data not shown). This behaviour is characteristic of saturation kinetics. The data can be plotted in form of the Lineweaver-Burk or the Eadie-Hofstee equation which are both linear transforms of the Michaelis-Menten equation [23]. From the linear relationships thus obtained (data not shown) values for K_M (Michaelis constant) and v_{max} were derived. Both linear transforms gave similar results: for PC transfer from mixed micelles to brush border membrane $K_M = 0.90$ mM and $v_{max} = 0.9$ nmol/mg protein per min = 15 pmol/mg protein per s, for PC exchange between small unilamellar vesicles and brush border membrane the values were $K_M = 1.5$ mM and $v_{max} = 1.3$ nmol/mg protein per min = 22 pmol/mg protein per s.

Supernate-protein

The proteins liberated from brush border membrane by either incubation or papain treatment revealed PC exchange activity between two different populations of small unilamellar vesicles. The time course of ^{3}H-DPPC exchange between sonicated egg PC/egg phosphatidic (85 : 15, w/w) as the donor and sonicated egg PC vesicles as the acceptor in the presence and absence of supernate-protein is depicted in Fig. 4. The time curves were linearized as described in Fig. 2 and pseudo-first order rate constants were derived from the resulting linear

* The lipid pool of brush border membrane vesicles was shown to amount to (0.3 × protein concentration in mg/ml) [1].

TABLE II

Pseudo-first-order rate constants k_1 for the transfer (exchange) of ^{3}H-DPPC between mixed micelles or small unilamellar vesicles (SUVs) as the donor and brush border membrane vesicles (BBMVs) as the acceptor

Donor	Acceptor	Lipid weight ratio acceptor/donor	k_1 (h^{-1}) [a]	$t_{1/2}$ (h) [a]	Experimental [b] conditions
SUVs of egg PC	BBMVs	1	0.031	22	
		10	0.28	2.5	
		9	0.24	2.9	
		14	0.43	1.6	
Egg lyso PC/ egg PC mixed micelles	BBMVs	1.3	0.05	14	
		3.9	0.13	5.3	
		13	0.49	1.4	
		26	0.84	0.82	
		52	1.1	0.63	
		103	2.5	0.27	
SUVs of egg PC	BBMVs papain digested	9.1	0.0067 ±0.0007	100 ±10	BBMVs were treated with papain and then washed twice with buffer A prior to uptake measurement of ^{3}H-DPPC
SUVs of egg PC	BBMVs papain digested	9.1	0.0063	110	BBMVs were papain-treated as discussed above. To the mixture of treated BBMVs and egg PC SUVs supernate-protein (1.8 mg/ml) was added
SUVs of egg PC	BBMVs after incubation at room temperature and washing	14	0.023	30	BBMVs were incubated at room temperature for 2 h and washed twice
SUVs of egg PC	BBMVs treated with proteinase K	14	0.00061	1100	BBMVs were treated with proteinase K and washed twice with buffer A prior to ^{3}H-DPPC uptake
SUVs of egg PC	erythrocytes [c]	13	0.0060 ±0.0009	120 ±15	
SUVs of egg PC	erythrocytes [c]	13	0.0060 ±0.0009	120 ±15	in the presence of supernate-protein at 0.67 mg/ml
SUVs of egg PC	erythrocytes [c]	13	0.0048	140	uptake measured at 29°C
SUVs of egg PC	erythrocyte ghosts	13	0.0083	83	
	erythrocyte ghosts	13	0.0073	95	in the presence of supernate-protein at 0.67 mg/ml
SUVS of egg PC	BBMVs treated with proteinase K	14	0.016	43	to the incubation mixture supernate-protein was added at 2.0 mg/ml
SUVs of egg PC	BBMVs treated with proteinase K	14	0.096	7.2	to the incubation mixture supernate-protein was added at 11.8 mg/ml
SUVs of egg PC	BBMVs	20	0.55	1.3	5-doxyl-PC was exchanged between donor and acceptor
	BBMVs	20	0.58	1.2	
SUVs of egg PC	BBMVs	10	0.23	3.0	the donor vesicles were labeled with 1,2-dihexadecyl-*sn*-phosphatidyl[*N-methyl*-^{3}H]choline

[a] Pseudo-first-order rate constants k_1 were derived from linearized plots as shown in the inset of Fig. 2. The straight lines represent least-squares fit to the experimental data (for the equations used and details see Ref. 1). The error in the k_1 determination was usually 5 to 10% unless stated otherwise. Half-times $t_{1/2}$ were calculated from k_1: $t_{1/2} = \ln 2/k_1$.

[b] Unless stated otherwise all experiments were carried out at room temperature (22 ± 1°C) and the phospholipid transferred (exchanged) between donor and acceptor particles was ^{3}H-DPPC.

[c] Erythrocyte lipid was calculated according to Ref. 26, i.e., 1% hematocrit is equivalent to 60 μM total lipid.

TABLE III

Pseudo-first order rate constants k_1 for the exchange of ^{3}H-DPPC between two different populations of small unilamellar vesicles (SUVs)

Donor	Acceptor	Lipid weight ratio acceptor/donor	k_1 (h^{-1})	$t_{1/2}$ (h)	Experimental conditions
SUVs of egg PC/ egg phosphatidic acid (85 : 15, w/w)	SUVs of egg PC	10	0.0063 ±0.0015	110 ±25	without supernate-protein
		10	0.037	19	with supernate-protein, 0.016 mg/ml
		10	0.26	2.7	with supernate-protein, 0.071 mg/ml
		10	1.11	0.62	with supernate-protein, 0.28 mg/ml
Ox brain phosphatidylserine dispersion (unsonicated)	SUVs of egg PC	10	0.0072	95	without supernate-protein
		10	0.057	12	with supernate-protein, 0.25 mg/ml
		10	0.27	2.5	with supernate-protein, 0.87 mg/ml

relationships. The k_1 values were independent of acceptor/donor lipid weight ratio indicating that the PC exchange is a true first-order reaction. The k_1 values in the absence of supernate-protein were about 0.006 h^{-1} ($t_{1/2}$ on the order of 100 h) similar to values published for 1-palmitoyl-2-oleoyl-PC exchange between two populations of small unilamellar vesicles (Ref. 25, Table III). The presence of supernate-protein mediated PC exchange between the two populations of small unilamellar vesicles. The k_1 values increased approximately linearly with protein concentration (Table III). When unsonicated dispersions of negatively charged vesicles of ox brain phosphatidylserine were used as donor particles the k_1 values were significantly reduced compared to negatively charged small unilamellar vesicles (Table III). The supernate-protein failed to catalyze ^{3}H-DPPC exchange between the following systems: (a) small unilamellar donor vesicles (egg PC/egg phosphatidic acid = 85 : 15, w/w) and large multilamellar egg PC vesicles as acceptor (data not shown), (b) small unilamellar egg PC vesicles and erythrocytes or erythrocyte ghosts (Table II). Apparently, erythrocytes and erythrocyte ghosts behaved in these exchange experiments like brush border membrane vesicles after proteolytic treatment.

The following series of experiments provided evidence that the PC exchange activity measured in the

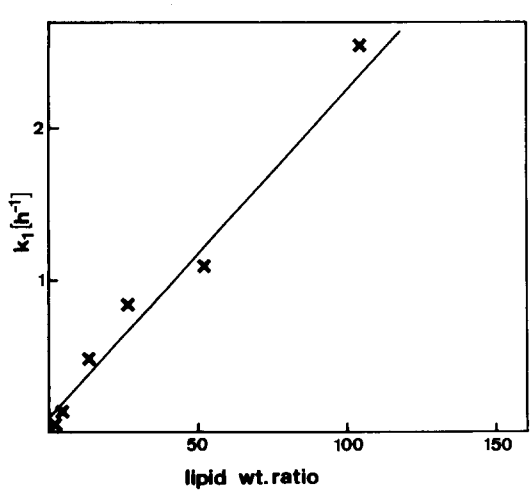


Fig. 3. Pseudo-first-order rate constants k_1 (h^{-1}) as a function of the weight ratio of brush border membrane lipid to phospholipid present in mixed micelles. The k_1 values were derived from the data presented in Fig. 2. k_1 values were determined for the following wt. ratios of brush border membrane lipid to micellar lipid: 1.3; 13; 26; 52; 103. The solid line represents the least-squares fit to the experimental data ($r^2 = 0.98$).

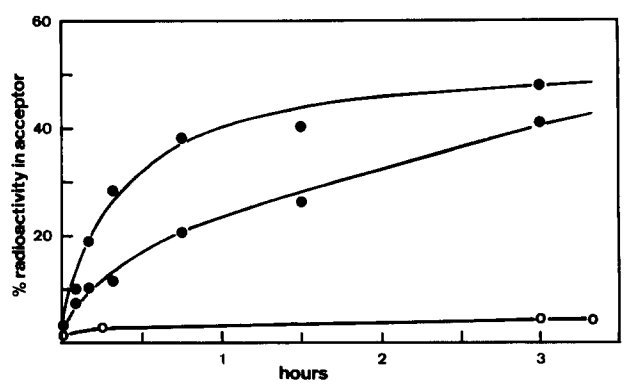


Fig. 4. ^{3}H-DPPC exchange between two populations of small unilamellar vesicles. Donor and acceptor vesicles both dispersed in buffer A were produced by sonication as described under Materials and Methods. Donor vesicles (egg PC/egg phosphatidic acid (85 : 15, w/w)) containing ^{3}H-DPPC and acceptor vesicles (egg PC) were mixed so that the final concentrations were 0.1 mg lipid/ml and and 1 mg lipid/ml, respectively (acceptor/donor wt. ratio = 10). The mixture was incubated at room temperature in the absence (○) or presence (●) of supernate-protein (0.14 mg protein/ml (middle), 0.28 mg/ml (top)). After timed intervals donor and acceptor vesicles were separated by DEAE-Sepharose CL-6B chromatography and the radioactivity transferred from donor to acceptor was determined in a Beckman LS 7500 liquid scintillation counter.

supernatant of brush border membrane either after storage or after digestion with papain is due to protein(s):

(I) The activity was non-dializable and was sedimented by centrifugation at 120000 × *g* for 15 h. (II) The activity was destroyed after briefly boiling. Plotting the activity in the supernatant vs. temperature gave a sigmoidal heat denaturation curve (data not presented) which showed that the PC exchange activity is reduced to 50% at 58 ± 1° C. (III) The activity was precipitated with $(NH_4)_2SO_4$ (2.7 M, 50% saturation). Upon exhaustive dialysis to remove $(NH_4)_2SO_4$, approx. 95% of the precipitate was redissolved, however, the activity regained was 45 ± 10%. (IV) No PC exchange activity was observed in buffer containing urea at concentrations ≥ 5 M. Removal of urea by exhaustive dialysis restored the activity to 88 ± 8%. (V) Exposure to 0.1% (3.5 mM) sodium dodecyl sulfate for 5 min at room temperature and subsequent removal of the detergent by exhaustive dialysis led to the complete loss of activity indicating that sodium dodecyl sulfate denatured the protein irreversibly. (VI) After proteinase K treatment of the supernatant the activity was reduced to about 20%. (VII) The PC exchange activity present in the supernatant exhibited a well defined pH-maximum at pH = 7.1 (data not shown).

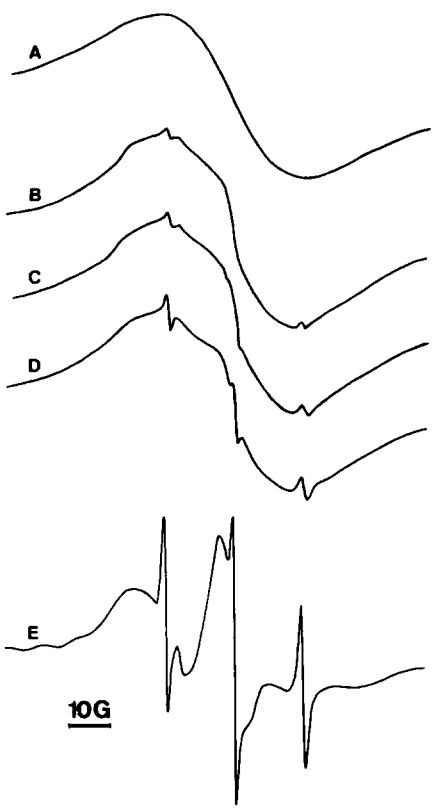


Fig. 5. ESR spectra of 8-doxyl-PC at 20° C. 50 µl of a $1.3 \cdot 10^{-4}$ M solution (= 6.5 nmol) of 8-doxyl-PC in $CHCl_3/CH_3OH$ (2:1, by vol.) were taken to dryness in the ESR glass capillary tube and the resulting phospholipid film was dried in vacuo. The ESR spectrum of the dry 8-doxyl-PC film is shown in (A). Afterwards the film was incubated with 80 µl of a solution of supernate-protein in buffer B (10 mg/ml) at 20° C and ESR spectra of the resulting dispersion were recorded after 15 min (B), 40 min (C) and 21 h (D). After recording spectrum D the dispersion was centrifuged at 500 × *g* for 5 min to pellet the multilamellar 8-doxyl-PC particles. The supernatant containing supernate-protein gave spectrum E.

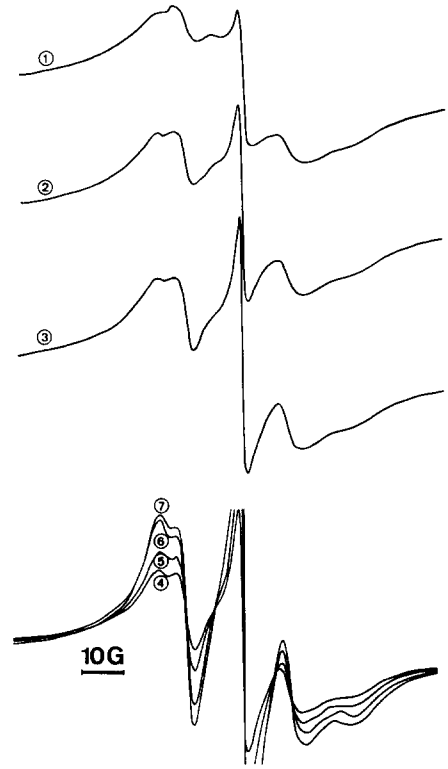


Fig. 6. ESR spectra of 8-doxyl-PC incorporated into egg PC bilayers. A film of 8-doxyl-PC on the glass wall of the ESR capillary tube was produced as described in the legend to Fig. 5. The dry 8-doxyl-PC film was incubated with 50 µl of a sonicated egg PC dispersion in buffer B (0.11 mg egg PC/ml) in the presence of supernate-protein (3.2 mg protein/ml). ESR spectra 1–7 of the egg PC dispersion were recorded at room temperature after incubation with supernate-protein for 4 min (1), 15 min (2), 20 min (3), 40 min (4), 70 min (5), 120 min (6) and 200 min (7).

The protein(s) liberated from brush border membrane by incubation or by papain treatment can be shown to bind PC. This is demonstrated in Fig. 5. Bilayers of 8-doxyl-PC were deposited on the glass wall of the ESR capillary tube and the ESR spectrum was recorded. A typical Heissenberg spin exchange spectrum (top spectrum, Fig. 5A) was obtained. A similar spectrum to that in Fig. 5A was recorded if buffer B was added to the dry film and the phospholipid was dispersed by incubation at 20° C for about 15 min. If, however, the dry 8-doxyl-PC film was incubated with buffer B containing supernate-protein, spectra B–D were obtained (Fig. 5). With increasing incubation time some fine structure appeared superimposed on the spin exchange spectrum. Multilamellar 8-doxyl-PC vesicles that formed upon adding the aqueous medium were readily pelleted by centrifugation at 500 × *g* for 10 min. The ESR spectrum obtained with the supernatant shown

in Fig. 5E is characteristic of rapid but anisotropic motion. The sharp three-line spectrum superimposed on the anisotropic spectrum is due to some free spin label. The maximum hyperfine splitting derived from this spectrum is $A_{\parallel} = 26.4$ G. This value and the line shape of the ESR spectrum indicate that the spin group is strongly immobilized. Upon addition of 25 mM sodium ascorbate to this sample the isotropic component of the spectrum disappeared immediately while the intensity of the anisotropic component remained unaffected or decreased only slightly within 30 min (data not shown).

The spectral series shown in Fig. 6 was obtained when supernate-protein together with a sonicated egg PC dispersion containing small unilamellar egg PC vesicles were added to the dry film of 8-doxyl-PC. The to spectrum (Fig. 6) is a supposition of two ESR spectra, (1) a spin exchange spectrum due to the 8-doxyl-PC present as a film deposited on the glass wall of the ESR capillary tube or as multilamellar vesicles and (2) an anisotropic spectrum characteristic of 8-doxyl-PC incorporated in egg PC bilayers. With increasing incubation time the spectral intensity of the anisotropic component grew at the expense of the spin exchange spectrum (Fig. 6, top to bottom). The maximum hyperfine splitting in spectra 6 and 7 was $A_{\parallel} = 23.2$ G which is typical for liquid crystalline egg PC bilayers.

Discussion

Characterization and stability of brush border membrane vesicles from rabbit small intestine

The method of preparation used here to prepare brush border membrane was shown previously to yield membrane vesicles free of nuclear, mitochondrial and cytosolic contaminants [7,8]. By using proper marker enzymes, we were able to show that our brush border membrane preparation is also free of basolateral membranes and microsomes. The cytosolic fraction of enterocytes as prepared here exhibit PC and PI exchange activity between two different populations of small unilamellar vesicles in agreement with a report in the literature [21]. However, from the experiments described here it cannot be decided whether this activity is genuine, i.e., whether it really is of cytosolic origin or whether it is due to proteins originally present in the brush border and released from the membrane by proteolysis during preparation.

An obvious disadvantage of our brush border membrane preparation is its instability. As reported before [5,27–29] the apical membrane of the enterocyte is rich in phospholipases and proteases [29]. Both phospholipase A_2 and B are present and the former has been reported to have the highest specific activity in intestinal brush border membrane [27]. Above room temperatures massive hydrolysis and degradation of phospholipids was observed (Fig. 1, Table I) whereby the amino phospholipids, phosphatidylserine and phosphatidylethanolamine were primarily affected (Table I). Phospholipases require Ca^{2+} for their activation and although we strictly avoided the addition of Ca^{2+}, the quantity of Ca^{2+} required for activation seems to be present in brush border membrane, presumably as membrane-bound Ca^{2+}. The phospholipase activity was markedly reduced in the presence of excess EDTA (Table I). By adding EDTA to the buffer and working at low temperatures ($\leq 4°C$) it was possible to control the phospholipase activity or keep it at least at a reasonably low level (Table I).

It is clear from the data presented that controlling the proteolytic activity of brush border membrane is difficult. Extrinsic proteinases can probably be ruled out as the source of this activity. The experiments with hexokinase and trypsin inhibitor are pertinent to this question. Both experiments suggest that the cleavage of membrane protein and the subsequent release of proteins and peptides into the supernatant are not due to extrinsic proteinases adhering to brush border membrane, e.g., proteinases of pancreatic origin. The proteolytic activity observed can therefore be attributed mainly to intrinsic, membrane-bound proteinases. Our experiments indicate that the proteolytic activity is hardly affected in the presence of standard proteinase inhibitors or mixtures of these inhibitors. Adding a mixture of approved proteinase inhibitors to brush border membrane cooled to 0°C slowed down proteolysis but did not abolish it. The mechanism by which the activity of the intrinsic proteinases is controlled and such enzymes are switched on and off in the native brush border membrane is still unknown. It seems that in our membrane preparation the control of these intrinsic proteinases and also of lipases and other enzymes is impaired. As a result membrane phospholipids and proteins are hydrolyzed leading to the self destruction of the membrane structure. This process is dramatic at elevated temperatures, e.g., 37°C. Previously we showed [29] that solubilization of brush border membrane in detergents such as bile salts, Triton X-100, lyso PC induces non-specific proteolysis. Some membrane proteins were more susceptible to hydrolysis than others. Under lytic conditions all membrane proteins including those on the cytoplasmic side appeared to be hydrolyzed. Actin located on the cytoplasmic side of brush border membrane was used as an internal marker protein, and it was shown that proteolysis of actin was only induced at detergent concentrations sufficient to cause disruption of the brush border membrane [29]. Based on data reported here and this previous report [29] it seems probable that the intrinsic proteinases are located on the exoplasmic side of the brush border membrane and oriented towards the lumen of the small intestine.

PC uptake by brush border membrane vesicles

Inspection of Table II reveals that the PC uptake by brush border membrane is enhanced compared to erythrocytes and erythrocyte ghosts. Lipid analysis of the lipid extract of brush border membrane at the end of the PC exchange experiment clearly shows that PC was incorporated as such and was not hydrolyzed prior to incorporation into the brush border membrane. This is also consistent with the finding that the ether compound 1,2-dihexadecyl-*sn*-PC exhibited uptake behaviour similar to that of ester compounds (Table II).

The PC exchange (transfer) between small unilamellar vesicles or mixed lipid micelles as the donor and brush border membrane vesicles as the acceptor is protein-mediated. This conclusion is primarily based on the observation that the enhanced exchange (transfer) activity is lost after proteolytic treatment of brush border membrane with extrinsic proteinases like papain or proteinase K. After proteolytic treatment with papain brush border membrane behaves like erythrocytes regarding the PC uptake. In both membrane systems similar rate constants (half times) were observed (Table II). Papain treatment reduced (increased) the rate constants (half-times) by 30 to 50 times. Our results of the PC uptake by erythrocytes are in good agreement with published data [26]. Proteinase K-treated brush border membrane has practically lost its ability to incorporate PC (Table II). It should be noted that the k_1 value for ^{3}H-DPPC uptake by proteinase K-treated brush border membrane is about 25-times smaller than the k_1 value measured at 37 °C for the exchange of 1-palmitoyl-2-oleoyl-PC between two populations of small unilamellar vesicles [25]. As pointed out by McLean and Phillips [25], it reflects the PC exchange via diffusion of PC monomers through the aqueous phase. A remarkable difference was observed between proteinase K-treated and papain-treated brush border membrane vesicles. PC-exchange was partially restored if supernate-protein was added to proteinase K-treated brush border membrane vesicles. In contrast, no exchange activity was measured when supernate-protein was added to papain-treated brush border membrane vesicles. The difference could be rationalized by postulating that the more extensive proteolysis by proteinase K makes the membrane surface of the digested brush border membrane accessible to the supernate-protein. The effect of the extrinsic proteinases papain and proteinase K to brush border membrane is in principle the same as that of the intrinsic proteinases of brush border membrane. The difference observed between extrinsic and intrinsic proteinases is of a quantitative nature. The effect of incubation of brush border membrane vesicles at 4 °C under conditions where the intrinsic proteinases were shown to be active, is that the PC exchange is slowed down markedly (Table II).

The notion that PC exchange between lipid particles and brush border membrane is protein-mediated is supported by the observations that the PC transfer is saturable; and that the PC exchange is significantly reduced in the presence of enzyme inhibitors such as *N*-ethylmaleimide, $CuSO_4$, 5-dimethylamino-1-naphthalenesulfonyl chloride or 3-isothiocyano-1,5-naphthalenedisulfonic acid (disodium salt).

Unambiguous evidence is presented showing that the PC exchange activity resides in the apical membrane of the enterocyte and not in the basolateral membrane. The possibility that the PC exchange activity is due to membranous contaminants, e.g., contamination with endoplasmic reticulum or alternatively, that it is due to residual cytosol can be safely ruled out as mentioned before.

The exchange (transfer) activity is probably associated with an intrinsic membrane protein of the brush border membrane located on the exoplasmic side of the brush border membrane. This is indicated by the proteolytic treatment with extrinsic proteinases like papain. Proteinases of the size of trypsin or greater were shown to have access to proteins that are located exclusively on the external surface of brush border membrane vesicles. Proteins located on the internal surface became only accessible to proteolysis after detergent solubilization of the membrane [29]. There is ample evidence that the PC exchange between lipid particles and brush border membrane is affected by the action of intrinsic proteinases. It was shown previously [29] that the intrinsic proteinases can attack internal proteins, i.e., proteins located on the inner surface and oriented towards the vesicle cavity, only after disruption of the integral membrane structure by detergent solubilization [29]. The temperature dependence of the PC exchange is pertinent to the question of the location and orientation of the PC exchange protein in brush border membrane. The Arrhenius plot for the PC exchange yields a straight line over the temperature range 0–35 °C. This range covers the gel-to-liquid crystal phase transition which has been shown to be between 10 and 30 °C for this kind of brush border membrane [2]. The linear Arrhenius plot suggests that the active centre of the PC exchange protein is located on the external side of the lipid bilayer of brush border membrane. Integral proteins with an active centre embedded in the lipid bilayer are expected to sense the lipid bilayer fluidity and changes in this fluidity as they occur at the lipid phase transition. As a result integral membrane proteins of this type exhibit a kink in the Arrhenius plot that coincides with the phase transition temperature [2,30,31]. The straight-line relationship of the Arrhenius plot suggests therefore that the active centre of the PC exchange protein is located outside the lipid bilayer. Based on this evidence together with the results of extrinsic and intrinsic proteinases we propose the PC exchange protein to be a peripheral protein located on the external surface of the

brush border membrane, i.e., the surface exposed to the lumen of the intestine. We place it tentatively outside the lipid bilayer, at least its active centre. This does not rule out the possibility that the protein is anchored to the membrane by a domain spanning the lipid bilayer. The proteolytic experiments are consistent with such a mode of anchoring: the PC exchange protein would then be an integral membrane protein with its active centre being exposed on the external membrane surface. Presently no details are known concerning the actual size of the protein and related to it the distance between the active centre and the membrane surface.

The treatment of brush border membrane with papain liberates protein(s) which are released into the supernatant. These proteins exhibit PC exchange activity between two populations of small unilamellar vesicles. That this kind of activity is indeed due to a protein is unambiguously documented by the denaturation induced by boiling or in the presence of ≥ 5 M urea or 3.5 mM concentration of sodium dodecyl sulfate, by proteolysis with proteinase K, the precipitation with 2.7 M $(NH_4)_2SO_4$ and by the sharp maximum of the PC exchange activity at pH 7.1. The protein(s) liberated from the brush border membrane resembled soluble lipid exchange proteins in several respects. As evident from Table III, the supernate-protein mediated ^{3}H-DPPC exchange between two different kinds of small unilamellar phospholipid vesicles regardless whether donor or acceptor vesicles were neutral or had a negative surface charge (surface potential). However, the protein failed to catalyze ^{3}H-DPPC exchange between small unilamellar vesicles as donor and large multilamellar egg PC dispersions, erythrocytes and erythrocyte ghosts as the acceptor particles. Erythrocytes as well as erythrocyte ghosts behaved in this test like papain-treated brush border membranes. More importantly, the protein(s) released into the supernatant by proteolysis bind(s) PC as evident from Figs. 5 and 6. The maximum hyperfine splitting derived from the ESR spectrum $A_{\parallel} = 26.4$ G indicates that the PC spin label is strongly immobilized in the presence of protein. Furthermore, the spin label probably sits in a hydrophilic pocket of the protein and is therefore not readily accessible to the water-soluble reducing agent sodium ascorbate. The protein(s) present in the supernatant also catalyze(s) the incorporation of PC spin label into egg PC bilayers (Fig. 6). We note that the supernate-proteins contain no phospholipase activity; hence, the possibility that the PC exchange activity is associated with a phospholipase activity can be ruled out.

Our experiments do not allow us to comment on the specificity of the PC exchange (transfer) protein. This question is a fundamental one and must be tackled before one can speculate on the function of the protein. Presently work is in progress which is addressed to the specificity of this protein, particularly to the question whether it specifically recognizes choline phospholipids or whether it is less specific and recognizes several lipid classes.

Acknowledgements

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