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Effect of Micellar Lipids on Rabbit Intestinal Brush-Border Membrane Phospholipid Bilayer Integrity Studied by ^{31}P NMR

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Summary. The effect of biliary salts and fatty acids on the bilayer structure of rabbit intestinal brush-border membranes was studied using the nonperturbing probe ^{31}P NMR. The broad, asymmetric lineshape of the ^{31}P NMR spectrum of isolated brush-border vesicles demonstrates that their component phospholipids are organized in extended bilayers. These membranes are not significantly perturbed by incubation with physiological concentrations of biliary salts (3, 9, 18 mM), demonstrating that the vesicles are highly stable, corresponding to their biological function. However, the emergence of a narrow peak superimposed on the broad lineshape indicates that a small proportion of the membrane phospholipids has reached isotropic motion, which may correspond to external or internal micellar structures. Incubation with mixed micelles of fatty acids and taurochlorate show that long-chain fatty acids enhance the membrane-perturbing effect of taurocholate while short-chain, water-soluble fatty acids do not, suggesting a difference in the absorption mechanisms.

Key Words intestine · brush border · phospholipid · biliary salt · fatty acid · ^{31}P NMR

Introduction

It is well known that bile salts play a key role in such biological functions of the gut as absorption and digestion. Conjugated bile acids are secreted into the small intestine and are absorbed by active transport mechanisms from the ileum [3, 10], and jejunum [25]. Their physiological activities come from their ability to form micelles. Within the intestinal lumen, bile acids interact with lipases and assist the lipolysis and absorption of fats by the formation of mixed micelles [14]. They can therefore solubilize all kinds of molecules insoluble in water. Fatty acids are thus absorbed by the epithelium of the small intestine, but the mechanism of their absorption by the intestinal mucosa will vary according to the chain length [32].

Biliary salts are powerful detergents frequently used in the solubilization, as in the reconstitution, of biological membranes [13]. It has been proved that such salts can produce dramatic membrane damage, suggesting that physical modifications can occur in the membrane structure during transport and absorption. Hemolysis of intact erythrocytes was obtained with only 1 mM bile salt concentrations [6, 22]. However, biliary salts naturally occur in fairly high amounts—up to 20 mM—in the intestinal lumen and in the gallbladder.

Until now, the various studies conducted on intestinal brush-border membranes have provided very little information on the physical modifications which may occur on absorption or transport of micellar compounds. Thus, the effect of biliary salt concentrations, which regulate the rate of uptake fatty acids by intestinal epithelium, on the stability and integrity of these membranes has not yet been investigated.

In order to detect possible perturbations of membrane structure in contact with micellar solutions, we have used the noninvasive ^{31}P nuclear magnetic resonance (NMR) spectroscopy [26]. This method provides considerable information on the structural arrangement of phospholipids in membranes [8, 17]. Furthermore, intestinal brush-border membranes, which possess a very high plasma membrane surface compared with other cell membranes can be extracted and purified with high yields so that reasonable signals can be obtained in a short period of time.

In this study, we observed the perturbation of the structure and integrity of isolated intestinal brush-border membranes after their incubation with micelles of biliary salts and fatty acids of various chain lengths. We found that brush-border membranes exhibited high stability in contact with biliary salts compared with other membranes [31]. The bilayer structure is not strongly disturbed even

when taurocholate concentrations reach 20 mM. However, it is shown that the slight membrane perturbation caused by mixed micellar solutions of biliary salts and fatty acids is dependent on the fatty acid chain length. These results are discussed in relation to absorption and transport mechanisms. It has been proved that for procaryotic cells, the uptake of exogenous deuterated fatty acids into *Escherichia coli* cytoplasmic membranes [9, 18] produces a marked increase in fluidity, but until now, few examples of NMR studies on the membrane transport of exogenous molecules have been reported.

Materials and Methods

Rabbit Brush-Border Membrane Preparation

The homogenization and centrifugation were all carried out at 4°C in a cold room.

Rabbit jejunum and duodenum (50 g) were first removed immediately after the death of the animals, freed from most attached mesenteric and adipose tissue, washed with ice-cold saline buffer, everted and frozen (−30°C). Brush-border membrane were then prepared principally according to references [12] and [19]. Briefly, the intestine sample was cut and thawed slowly in 200 ml of buffer A containing 5 mM EGTA [ethyleneglycol-bis-(β-aminoethylether), N,N′ tetraacetic acid], 20 mM Tris HCl and 300 mM mannitol at pH 7.3; then mixed in a Waring Blendor at 40% of the low speed for 2 min. The preparation was filtered through a Buchner funnel to remove muscle and mucus. The homogenate was diluted six times with cold distilled water and submitted to the high speed in the Waring blendor for 2 min. 10 mM solid $MgCl_2$ was then added to precipitate the membranes and the preparation was kept under a low-speed stirring device for 20 min. The preparation was centrifuged (3000 × *g*, 15 min) and the pellet removed. The supernatant was then centrifuged (27,000 × *g*, 30 min) and the pellet containing the brush-border membranes was suspended in 50 ml of buffer B (10 mM Tris HCl, 50 mM mannitol, 5 mM EGTA, pH 7.3) and homogenized in a Potter Elvehjem. It was recentrifuged (38,000 × *g*, 15 min), and the final pellet was suspended in the same buffer B and collected immediately for NMR and biochemical studies.

Preparation of Micellar Solutions

Micelles of Biliary Salts

Na-taurocholate or Na-cholate (Merck) at 30, 90 and 180 mM concentrations were dissolved in buffer B at pH 7.3 after a thorough mixing in a magnetic stirring device (1 hr at 37°C).

Micelles of Biliary Salts and Fatty Acids

Caproic acid (C6 : 0), lauric acid (C12 : 0) or oleic acid (C18 : 1) (Merck) at concentrations of 90 mM were mixed in Na-taurocholate 90 mM in buffer B at pH 7.3.

Incubation

Micelles were incubated with the brush-border vesicles just before the NMR studies were conducted. One part of a micellar solution was added to nine parts of brush-border vesicle preparation. The total volume in an NMR tube was 2 ml containing 50 mg of proteins.

Lipid Analysis

Total lipids were extracted from the brush-border vesicle incubations with 20 volumes of chloroform/methanol 1 : 1 vol/vol after mixing in an ultraturrax mixer for one min. The mixtures were kept at room temperature for 1 hr with some stirring. The solvents were removed, then 20 volumes of chloroform/methanol 2 : 1 vol/vol were added and the extraction was repeated. After filtration of the proteins, the solvents were pooled, then evaporated to dryness. Total lipid extracts were dissolved in chloroform/methanol 1 : 1 vol/vol and aliquots were taken for phosphorus analysis and for chromatography on silica gel thin-layer plates. TLC plates 250 μm, 10 × 20 cm (Merck) were activated at 120°C one hour before use. Plates were run in different solvent mixtures. Chloroform/methanol/water 65 : 35 : 6 vol/vol/vol was routinely used for the separation of phospholipids. Spots were visualized with iodine vapor and compared to standard phospholipids. The areas corresponding to different phospholipids were scraped off and eluted from silica gel according to reference [27], with some modifications. A first extraction in chloroform/methanol/water 45 : 45 : 10 vol/vol/vol was repeated twice; then, a second extraction in chloroform/methanol/water 20 : 70 : 10 vol/vol/vol, was also repeated twice. The efficiency of extraction was greater than 95%.

Biochemical Assays

Inorganic phosphorus was determined colorimetrically from a modification of the technique of Rouser et al. [30] with a standard solution of potassium dihydrogeno-phosphate (Merck). Proteins were assayed with the Folin phenol reagent according to Lowry et al. [23], using bovine serum albumin as standard.

^{31}P NMR Analysis

Proton noise decoupling ^{31}P NMR spectra were obtained on a BRUKER WM 250 at 101.24 MHz. A volume of 2 ml containing the sample preparation was transferred in a 10-mm NMR tube and the sample was kept at 37°C for 15 min. The spectra were accumulated from up to 6000 transients using a 90° pulse (20 μsec), or 25 kHz sweep width, and a 0.5-sec interpulse delay using gated proton noise decoupling (5 W input power during the 0.0819 sec acquisition time). Exponential multiplication was used to smooth the spectra, with the resultant line broadening listed in the figures. For determination of isotropic spectra amount as a percentage of total bilayer, the area of each peak was cut out and weighed. It was previously established that the weight per unit area of chart paper was constant. In addition, the surfaces under each spectral component were calculated and the results were similar to those obtained by weighing.

Results

^{31}P NMR Spectra of Phospholipids in Brush-Border Membranes and Micelles

Figure 1 shows the results of ^{31}P NMR measurements of phospholipids under two conditions. An asymmetrical lineshape with a low field shoulder characteristic of phospholipids in a bilayer form can be seen in the control experiment for brush-border membranes at 37°C (Fig. 1*A*). Two narrow peaks representing less than 5% of the total spectrum are superimposed on the bilayer signal. The spectrum obtained with phosphatidylcholine (1 mM) incorporated in a micellar solution of taurocholate (9 mM) can be seen in Fig. 1*B*. Fast tumbling of micelles causes effective motional averaging resulting in an isotropic ^{31}P NMR signal corresponding to one of the two narrow peaks observed in Fig. 1*A*. The other peak, at high frequency may correspond to phosphorylated molecules entrapped in the vesicles.

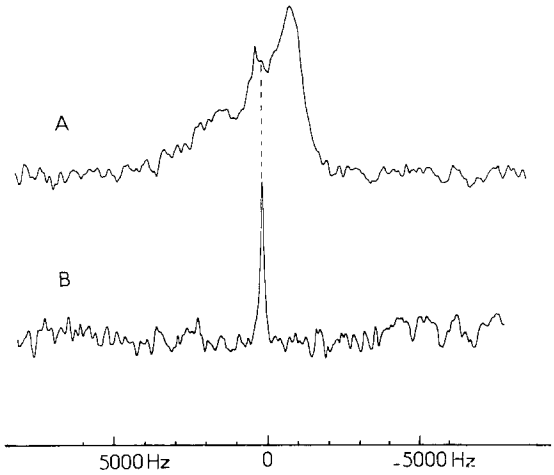

Fig. 1. 101.24 MHz ^{31}P NMR spectra of: (*A*) Intestinal brush-border membranes in buffer B (Tris HCl, mannitol, EGTA, pH 7.3) at 37°C. (*B*) Mixed micelles of egg yolk phosphatidylcholine (1 mM) and taurocholate (9 mM) in the same buffer at 37°C

Effect of Biliary Salts

Figure 2 shows the ^{31}P NMR spectra obtained after incubation of brush-border membranes with increasing amounts of micelles of taurocholate (3, 9 and 18 mM). The last two concentrations were chosen because they correspond to *in vivo* biliary salt concentrations either in the intestine (9 mM) or in pure bile (18 mM). Increasing concentrations of taurocholate result in the emergence of an anisotropic signal characteristic of the motional averaging of phospholipids. The phospholipids can only come from the brush border, and therefore part of these phospholipids are in the form of mixed micelles or in small vesicles in which tumbling and lateral diffusion in the curved bilayers cause isotropic molecular motion of the molecules. Measurement of the effective chemical shift anisotropy $\Delta\sigma$ in the bilayer signal of the mixtures shows that no significant variation of this parameter was detected with increasing concentration of taurocholate.

The quantitative interpretation of the spectra given in Fig. 2 demonstrates that only about 5% of the bilayer structure is lost for 3 mM taurocholate, about 10% for 9 mM and about 18% for 18 mM. This demonstrates that the bilayer configuration is preserved up to high molar concentrations of taurocholate (18 mM taurocholate corresponds to a taurocholate/phospholipid ratio of 2:1). Similar, but slightly lower, results were obtained when the taurocholate was replaced by cholate (*data not shown*).

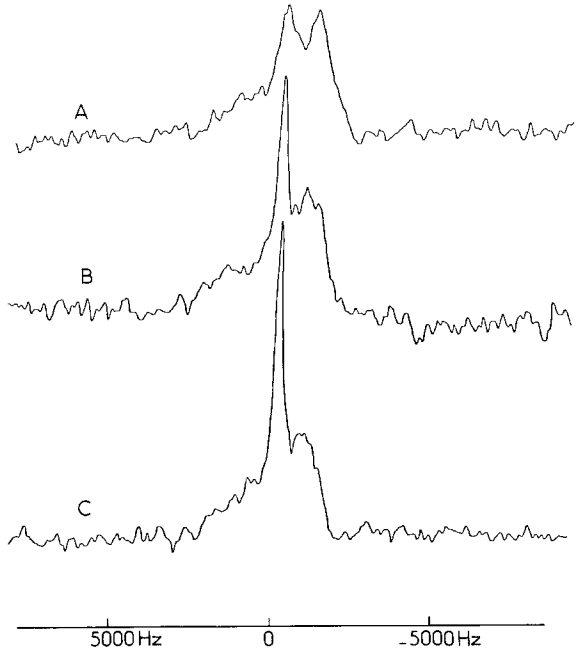

Fig. 2. 101.24 MHz ^{31}P NMR spectra of brush-border membranes incubated with micelles of taurocholate in buffer B at 37°C. Taurocholate concentrations: (*A*) 3 mM; (*B*) 9 mM; (*C*) 18 mM

In order to further investigate the origin of the isotropic peak superimposed on the bilayer signal, samples were centrifuged at 35,000 × *g* for 30 min after incubation with taurocholate. After resuspending the pellets, ^{31}P NMR spectra were once again recorded, both of the suspended pellet and of the supernatant. The pellet showed a bilayer-type

Table. Brush-border vesicles' phospholipid distribution[a]

	Brush-border vesicles				
	Control experiments without micelles	Micelles with taurocholate (9 mM)	Micelles with taurocholate 9 mM and fatty acids		
			C6:0	C12:0	C18:1
Phosphatidylethanolamine (+ phosphatidylserine[b])	30.6	33.9	34.8	34	35
Phosphatidylcholine (+ phosphatidylisositol[b])	47	49.5	50	49.8	53.5
Sphingomyelin	14.4	10.5	8.4	9	9.6
Monoacylphosphatidylcholine	2.9	2.5	3.4	4	4.4
Other phospholipids[c]	5.1	3.1	3.6	3.1	3.9

[a] The phospholipid composition determined by inorganic phosphorus analysis following resolution of the phospholipid classes by TLC and elution of the fraction as described in Materials and Methods. Values are expressed as a percentage of total phospholipidic phosphorus. Results are the mean of two experiments.
[b] Not separated on the TLC plate from PE or PC.
[c] Mainly phosphatidic acid and monoacylphospholipids other than monoacylphosphatidylcholine.

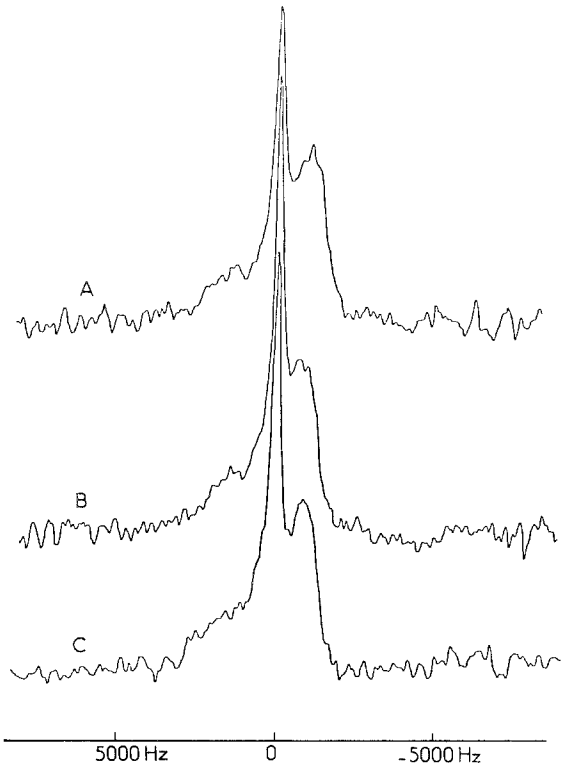

Fig. 3. 101.24 MHz ^{31}P NMR spectra of brush-border membranes incubated with both taurocholate (9 mM) and fatty acids (9 mM) in buffer B at 37°C. (*A*) caproate; (*B*) laurate; (*C*) oleate

spectrum with an isotropic signal of less than 5%, while the supernatant produced only isotropic signals. This indicates that most of the lipids responsible for the isotropic signal are not associated with the larger structures.

Effect of Mixed Micelles

Since biliary salts are essential *in vivo* to the absorption of fats by the formation of mixed micelles, we thought it of interest to examine the membrane-perturbing properties of fatty acids dispersed in taurocholate (9 mM). Three fatty acids of different chain lengths are chosen: caproate, laurate and oleate. The concentrations used (9 mM) correspond to *in vivo* intestinal fatty acid concentrations. Figure 3 shows the ^{31}P NMR spectra of the brush-border membrane after incubation with mixed micelles of taurocholate and fatty acids. In each assay, there is an increase in the isotropic component of the ^{31}P NMR spectrum. However, when caproate is added (Fig. 3*A*), a slight increase in the isotropic peak is observed compared with the spectrum of the control experiment in which the membrane was incubated with taurocholate (9 mM) alone.

The percentage of phospholipids in an isotropic state reached 18% with laurate (Fig. 3*B*) and oleate (Fig. 3*C*). This means that these two fatty acids strongly amplified (up to 9%) the membrane perturbation caused by taurocholate.

As has been shown in Fig. 1*B*, mixed micelles of taurocholate and phospholipids give rise to isotropic motion. However, when these mixed micelles are incubated with the brush-border membranes, the spectrum obtained was identical to Fig. 2*B*.

In order to eliminate possible chemical degradation of membranes during their incubation with mixed micelles, the phospholipid composition of brush-border membranes was controlled at the end of each experiment (Table). The phospholipid

distribution does not change significantly: the main diacyl-phospholipids are phosphatidylcholine (~50%) and phosphatidylethanolamine (~33%). The monoacylphosphatidylcholine remains a minor phospholipid (less than 5%).

Discussion

According to our knowledge of fat absorption by intestinal epithelium, the micellar solutions of intestinal contents from the duodenum and jejunum are composed of biliary salts, short-, medium- or long-chain fatty acids, monoglycerides, diacyl and monoacylphosphoglycerides, cholesterol, etc. . . [24, 28]. Brush-border membranes from rabbit duodenum and jejunum cells (the upper part of the small intestine), absorb dietary short-, and medium- and long-chain fatty acids from micellar solutions [2, 5, 32]. However, biliary salts are further extensively absorbed in the distal part of the intestine (ileum) after disruption of the micellar solution [20, 33]. In our preparation, brush-border membranes were isolated from duodenum and jejunum so that lipid absorption was preferentially an absorption of fatty acids rather than biliary salts.

Our experiments were conducted with 2 ml of membrane suspension, which corresponds to 12.5 mg of phospholipids and 50 mg of membrane proteins. The total vesicle volume, 0.4 ml, was estimated from the volume of the membrane pellet after centrifugation at 43,000 × *g* for 30 min and from the ratio of Na^+_{in}/Na^+_{out} determined by ^{23}Na NMR of these membranes [1]. Assuming a mean vesicle diameter of 1,500 Å [19], several square meters of brush-border membrane are incubated with 10 mg (9 mM) of taurocholate. These conditions are of the same order as in an *in vivo* situation.

Using ^{31}P NMR spectroscopy, we have recently proved [34] the integrity of the phospholipid bilayer of rabbit brush-border vesicles when they are obtained from a precipitation step using $MgCl_2$ and EGTA. These membranes gave rise to a large asymmetrical signal typical of phospholipids organized in bilayers (Fig. 1*A*). In this study, we demonstrate that the structure and integrity of the phospholipid bilayer is retained when the membranes are incubated with biliary salts, fatty acids or phospholipids. No change in the phosphorus chemical shift anisotropy is detected, which confirms that most of the vesicles are not broken during incubation. The large asymmetrical ^{31}P signal represents at least 82 to 95% of the total spectrum whatever the micellar compounds in the incubation medium.

It is known that a high detergent concentration, greater than physiological concentrations, solubilizes a brush-border membrane preparation [13, 21]. In our experiments, even the highest concentration of taurocholate (18 mM), which corresponds to a molar ratio taurocholate/membrane phospholipids of 2 : 1 is not high enough to disorganize the brush-border structure. This result contrasts with experiments conducted with egg phosphatidylcholine or dipalmitoyl phosphatidylcholine vesicles [4, 35] where a molar ratio detergents/phospholipids of 1 : 1 leads to the formation of mixed micelle systems. Furthermore, it has been demonstrated that at the concentrations used in these experiments, bile salts produce extended hemolysis of erythrocytes [31]. The high stability of brush-border membranes in contact with natural detergents could be related to the high proportion of cholesterol (the molar ratio cholesterol/phospholipids is about 1 : 1) which is known to oppose the effect of detergents on membrane structure [16, 35].

The emergence of the isotropic signal when taurocholate micelles are added to brush-border membranes is probably due to diacylglycerophospholipids and sphingomyelin which have been lost from the bilayer to form small vesicles or mixed micelles with detergents. This is demonstrated by the presence of isotropic phosphoreted components in the supernatant after centrifugation of brush-border membranes incubated with taurocholate. However, the fact that part of the ^{31}P isotropic signal originates from inverted micelles located inside the brush-border membranes cannot be excluded. It has been demonstrated that in model membrane systems bile salt taurocholate can act as a micellar ionophore for Pr^{3+} by a transbilayer movement of inverted micelles {Pr(bile salt)$_4$} [15, 16]. Furthermore, the formation of inverted micelles or hexagonal phases is related to the nature of the phospholipids. Unsaturated phosphatidylethanolamine [7] or glycolipids [29] facilitate the emergence of such structures in model membranes. Curiously, brush-border membranes are particularly rich in these two lipids [12]. It is known that glycolipids are situated on the outside of the lipid bilayer and easily form micelles; these polar lipids enhance contact with exogenous micellar compounds. Physical perturbation of natural membranes by detergents, as well as by phospholipases or proteases [11] can lead to a modification in the activity of lipid-dependent proteins associated with these membranes. If we consider the lipid profile of these membranes, the very low proportion of lyso-phospholipids indicates that brush-border membrane phospholipase A_2 and sphingomyelinase stay inactive; the monoglycerophospholipids or phosphorylcholine amounts cannot, therefore, explain the ^{31}P isotropic signal.

The experiments (Fig. 3) with fatty acids 9 mM

and taurocholate 9 mM, correspond to the current concentration of these acids in micelles of intestinal contents *in vivo* [24]. The brush-border membranes from rabbit duodenum and jejunum cells absorb dietary short-, medium- and long-chain fatty acids from micellar solutions. However, it is accepted that short-chain fatty acids may enter without the help of such micelles [32]. Moreover, in the enterocytes, short-chain fatty acids remain free, while medium- and long-chain fatty acids are re-esterified to give triglycerides. We have demonstrated that caproate does not modify the ^{31}P NMR spectrum of the control experiment (taurocholate alone). In contrast, laurate and oleate soluble micelles increased the proportion of the isotropic component to a value obtained with taurocholate 18 mM alone. No detectable modification of membrane structure was observed with caproate, which confirms the difference in the absorption mechanism of short-chain, water-soluble fatty acids. One explanation of the strong effect of laurate and oleate could be that fatty acids enhance the detergent effect of taurocholate on brush-border membranes. Alternatively, the increase in the isotropic peak could be due to inverted micelles formed during the absorption of these insoluble fatty acids. It is not possible, at the moment, to say which of these two hypotheses is the more probable.

Until now, little has been known about the structure and physical state of intestinal brush-border membranes. In the light of our results, we conclude that the bilayer structure of intestinal brush-border membranes is highly stable as would be expected from their biological function. However, long-chain fatty acids in mixed micelles can partially destabilize these membranes thus perhaps facilitating their transport across the membrane. This demonstrates that ^{31}P NMR is a powerful, noninvasive method for observing physical perturbations which can occur in membranes.

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