Biochimica et Biophysica Acta 939 (1988) 145–150
Elsevier

BBA 73915

Membrane fluidity and lipid composition of rat small intestinal brush-border membranes during postnatal maturation

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(Received 7 October 1987)

Key words: Brush-border membrane; Membrane fluidity; Fluorescence polarization; (Rat intestine)

Fluidity and lipid composition of rat small intestinal brush-border membranes (BBM) were studied during maturation in five age groups: newborns, sucklings (1–3 weeks), weaned (4–6 weeks), juveniles (8–10 weeks), and adults (12 weeks). Brush-border membrane fluidity was measured by steady-state fluorescence polarization. Fluorescent probes used were: 1,6-diphenyl-1,3,5-hexatriene, 1-(4-(trimethylammonium)phenyl)-6-phenyl-1,3,5-hexatriene, and a set of *n*-(9-anthroyloxy) fatty acids. Fluorescence anisotropy measured with all fluorophores was increased in adult versus newborn rats ($P < 0.004$). The weight ratio of saturated to *cis*-unsaturated fatty acids increased from birth to the suckling age ($P < 0.0004$). The cholesterol to phospholipid molar ratio increased from birth to the weaned age ($P < 0.0001$). Cholesterol to protein ratio and phospholipid to protein ratio decreased after the weaned age ($P < 0.004$). The results not only describe maturational changes of brush-border membranes but also give a better understanding of the correlations between biophysical and biochemical data in biological membranes.

Introduction

Biophysical studies on small intestinal brush-border membranes (BBM) during postnatal maturation have been a topic of increasing interest in recent years. Pang et al. [1] examined rabbit BBM by electron spin resonance and found a more ordered environment in adults versus newborns. Neu et al. [2], Schwarz et al. [3,4], and Brasitus et al. [5] studied rat and rabbit BBM from the suckling [2–4] and postweaned [5] age onward; they found an increase in 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence anisotropy (i.e. decrease in membrane fluidity) [2–5], an increasing cholesterol to phospholipid molar ratio [4,5], a decreasing saturated to unsaturated fatty acid weight ratio [4], and a decreasing double-bond index [5] during postnatal BBM maturation.

Various biochemical findings in BBM correlate with different aspects of membrane fluidity [6,7]. Therefore, we measured fluorescence anisotropy in rat BBM with several different fluorescent probes monitoring different characteristics of membrane fluidity: DPH is mainly located in the hydrophobic membrane interior, reflecting the static parameter of membrane fluidity [8]. The cationic analogue 1-(4-(trimethylammonium)phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) is pre-

Abbreviations: 16-AP, 16-(9-anthroyloxy)palmitic acid; 6-, 7-, 9-, and 12-AS, 6-, 7-, 9-, and 12-(9-anthroyloxy)stearic acid; BBM, (small intestinal) brush-border membranes; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-(trimethylammonium)phenyl)-6-phenyl-1,3,5-hexatriene.

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dominantly located in the more outer membrane regions [9,10]. A set of long chain fatty acids with the fluorophore 9-anthroyloxy attached to various positions of the fatty acid chain monitors membrane fluidity at graded depths within the bilayer, to a large extent reflecting the dynamic parameter of membrane fluidity [11–13].

In parallel to these biophysical studies, we examined the same BBM preparations for cholesterol, phospholipid, and protein content and determined the fatty acid distribution patterns, thus being able to correlate biochemical data to different biophysical phenomena.

Materials and Methods

Preparation of small intestinal rat BBM

BBM were prepared from Sprague-Dawley rats (WIGA-Charles River Laboratories, Sulzfeld, F.R.G.) right after birth and in weekly intervals up to the age of 12 weeks. A modification of the Ca^{2+} precipitation method was used [14]. Intestinal scrapings of rats over three weeks of age, and short pieces of intestine of rats under three weeks of age were homogenized in 500 mM mannitol and 10 mM Hepes buffer (pH 7.5) (Sigma Chemical Co., St. Louis, MO). After filtration through a fine nylon mesh (40 μm mesh size, Nybolt DIN 130-40, Swiss Silk Bolting Ltd., Zurich), $CaCl_2$ was added to give a final concentration of 10 mM at 4°C. After a 15 min centrifugation at $2500 \times g$ the pellet was discarded. The supernatant was further centrifuged at $30000 \times g$ for 30 min, the pellet was suspended in 100 mM mannitol and 10 mM Hepes buffer (pH 7.5), and recentrifuged at $30000 \times g$. Protein determination was carried out according to Lowry et al. [15]. Yield of BBM preparations was between 1.7 and 2.0% of initial protein content. Enzyme activities were determined according to Dahlquist [16]. Enrichment factors were calculated from enzyme activity per mg protein of final BBM preparations, compared to initial homogenates. Lactase was determined in BBM of newborn and of suckling (1–3 weeks) rats, saccharase was determined in BBM of weaned (4–6 weeks), juvenile (8–10 weeks), and of adult (12 weeks) rats. Enrichment factors of BBM preparations were (means ± S.D.): 16.0 ± 0.14 (newborns), 19.1 ± 2.55 (sucklings), 21.7 ± 2.10 (weaned), 17.9 ± 2.90 (juveniles), and 19.6 ± 2.10 (adults), respectively. BBM preparations were essentially free of DNA, and purity had additionally been checked by electron microscopy. Freshly prepared BBM were used for biophysical studies, they were frozen and stored at −20°C for biochemical studies.

Fluorescence polarization

Fluorescence anisotropy was measured at 27°C as described previously [17]. The fluorescent probes used were DPH (Sigma Chemical Co., St. Louis, MO), TMA-DPH, and five long-chain saturated fatty acids with the fluorophore 9-anthroyloxy attached to various positions of the fatty acid chain: 6-, 7-, 9-, 12-(9-anthroyloxy) stearic acids (6-, 7-, 9-, 12-AS), and 16-(9-anthroyloxy)palmitic acid (16-AP) (Molecular Probes, Inc., Junction City, OR). Membrane suspensions labeled with DPH (0.4 μM) were incubated for 10 min (27°C), those with TMA-DPH (0.1 μM) for 1 min (27°C), and those with anthroyloxy probes (0.4 μM) for 50 min (27°C). Total fluorescence intensity of each respective probe was monitored for each BBM maturation step in order to detect changes in fluorescence lifetime of the respective probes according to Brasitus et al. [5]. In none of the probes such changes were observed. The optical density ($\lambda = 365$ nm) of the labeled BBM suspensions during measurement was 0.161 ± 0.008 absorbance units.

Lipid and fatty acid composition

BBM were homogenized by sonication, the homogenized samples were lyophilized and reconstituted in small volumes of water. Lipids were extracted and washed as described by Folch et al. [18]. The washed lipid extracts were dried under nitrogen, redissolved in small volumes of chloroform/methanol (2:1, by vol.) and stored under pure nitrogen at −20°C.

Cholesterol content was estimated by applying the method of Sperry and Brand [19]. Lipid phosphorus was determined by the method of Fiske and Subbarow [20] as modified by Herschkowitz et al. [21].

Fatty acid methyl esters were prepared from the total lipid extract by mild acid methanolysis using the Instant Methanolic HCl Kit (No. 18053,

Alltech Europe Applied Science Labs, Nazareth/EKE, Belgium). The fatty acid methyl esters were extracted from the reaction mixture into *n*-hexane, dried under nitrogen, redissolved in *n*-hexane, and injected into a chromatograph equipped with a SP 2330 capillary column (0.32 mm i.d., 60 m length) and a flame ionization detector. Operating conditions of the capillary column were in a temperature programme mode (starting temperature 150 °C, increasing to 220 °C at a rate of 4 °C/min). Each fatty acid methyl ester was identified by its retention time using pure fatty acid methyl ester preparations (obtained from Alltech Europe Applied Science Labs) as standards. Eluting fatty

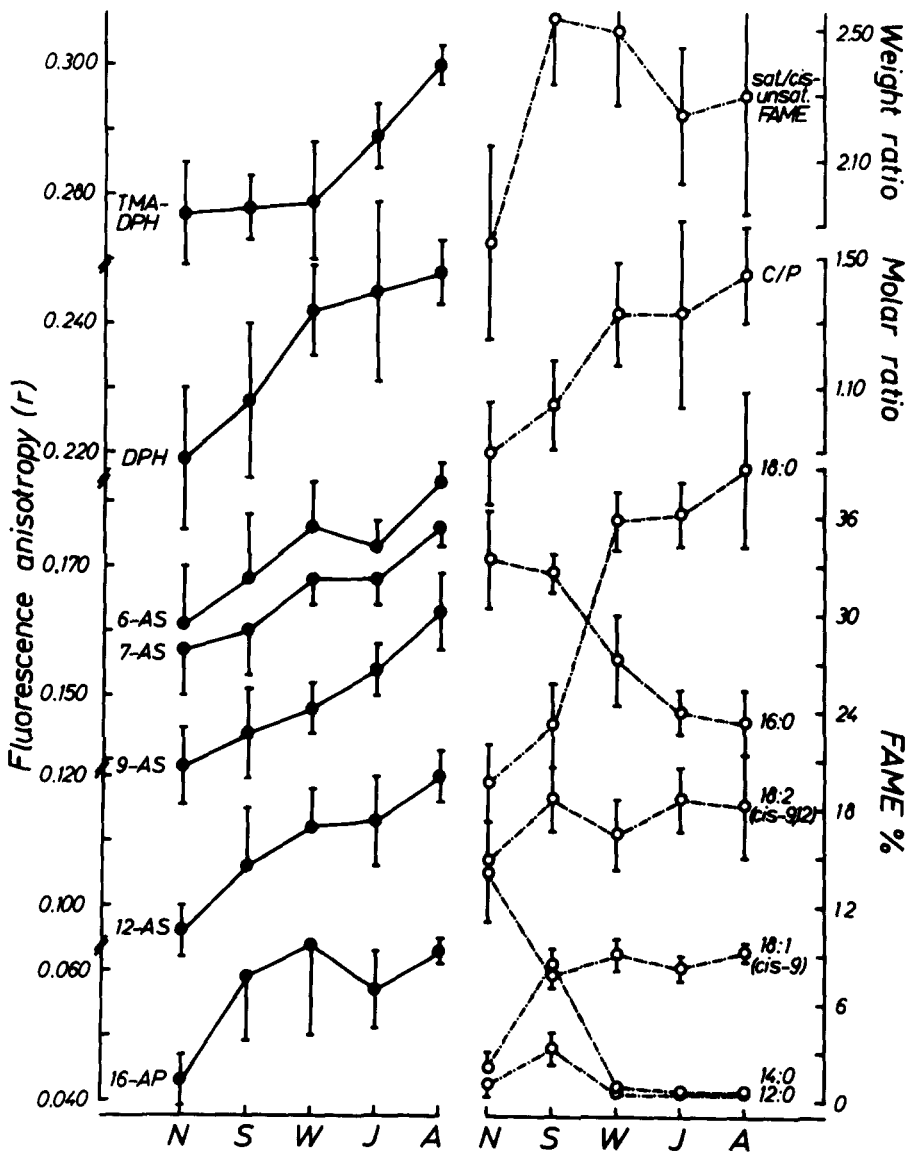

Fig. 1. Fluorescence anisotropy and lipid composition of rat small intestinal BBM during maturation (N ≙ newborns; S ≙ sucklings, 1–3 weeks; W ≙ weaned, 4–6 weeks; J ≙ juveniles, 8–10 weeks; A ≙ adults, 12 weeks). Left side (●———●): fluorescence anisotropy data when measured with different fluorescent probes. Right side (○·–·–·○): biochemical data; in the upper part weight ratio of saturated to *cis*-unsaturated fatty acid methyl esters (satd./*cis*-unsatd. FAME) and cholesterol to phospholipid (C/P) molar ratio, in the lower part weight % of the major fatty acid methyl esters (FAME %).

acid methyl esters were quantified by integrating all peaks from C12:0 to C18:2. Results were expressed as weight % of all fatty acid methyl esters analyzed.

Statistical analysis

Statistical analysis was done by one-way analysis of variance with linear contrasts for comparison of subgroups [22]. Results were expressed as means ± S.D. The respective biophysical and biochemical data were combined to five age groups: newborns ($n = 6$), sucklings (1–3 weeks, $n = 8$), weaned (4–6 weeks, $n = 10$), juveniles (8–10 weeks, $n = 7$), and adults (12 weeks, $n = 4$).

Results

Fluorescence anisotropy of BBM

Fluorescence anisotropy measured with all the fluorophores was increased in BBM of adult versus newborn rats ($P < 0.0002$ for 6-AS, 7-AS, 9-AS, 12-AS, TMA-DPH, and DPH, respectively; $P < 0.004$ for 16-AP, Fig. 1). When measured with the different fluorescent probes, fluorescence anisotropies increased during maturation in different ways (Fig. 1).

From birth to the suckling age, the rise of fluorescence anisotropy measured with all fluorescent probes was significant only for 12-AS and 16-AP ($P < 0.008$). From birth to the weaned age, DPH fluorescence anisotropy showed a steep increase ($P < 0.0003$) and no significant increase thereafter. From birth to the weaned age, TMA-DPH fluorescence anisotropy showed no increase, but a steep increase from the weaned to the adult age ($P < 0.0001$). As discussed below, the differences of the fluorescence anisotropy changes when using different probes can be correlated to specific biochemical changes in BBM during maturation.

Lipid and fatty acid composition of BBM

From birth to the suckling age, the relative contents of *cis*-unsaturated fatty acids C16:1(*cis*-9) (newborns: 2.0 ± 0.7; sucklings: 0.5 ± 0.1; weaned: 0.5 ± 0.1; juveniles: 0.6 ± 0.1; adults: 0.5 ± 0.1 fatty acid methyl ester %) and C18:1(*cis*:9) (Fig. 1) decreased ($P < 0.0001$), the relative content of the fatty acid C18:2(*cis*-9,12) slightly increased in the same time ($P < 0.02$, Fig. 1).

TABLE I

THE CHOLESTEROL TO PROTEIN AND PHOSPHOLIPID TO PROTEIN RATIOS DURING BBM MATURATION

Age groups of BBM (weeks)	Cholesterol to protein (μg/mg)	Phospholipid to protein (μg/mg)
Newborns	117.9 ± 28.3	270.3 ± 44.5
Sucklings (1–3)	133.4 ± 16.9	265.9 ± 36.9
Weaned (4–6)	190.5 ± 34.4 [a]	296.9 ± 38.5 [a]
Juveniles (8–10)	114.3 ± 21.8	182.2 ± 40.5
Adults (12)	147.2 ± 22.1 [a]	210.8 ± 27.8 [a]

[a] $P < 0.004$ for adults versus weaned.

The relative contents of the saturated fatty acids showed a decrease of C16:0 and a simultaneous increase of C18:0 (adults versus newborns: $P < 0.0001$, Fig. 1). C12:0 and C14:0 were increased in sucklings versus newborns ($P < 0.0001$, Fig. 1). The weight ratio of saturated to *cis*-unsaturated fatty acids showed a steep increase from birth to the suckling age ($P < 0.0004$) and no significant change thereafter (Fig. 1). The *trans*-unsaturated fatty acids C16:1(*trans*-9), C18:1(*trans*-9), and C18:2(*trans*-9,12) were present only in trace amounts (less than 0.4% each, respectively). From birth to the weaned age, the cholesterol to phospholipid molar ratio showed a steep increase ($P < 0.0001$) and no significant increase thereafter (Fig. 1). After the weaned age, the cholesterol to protein ratio and the phospholipid to protein ratio decreased (Table I).

Discussion

When examining BBM of rat small intestine, we found an increase of fluorescence anisotropy (i.e. a decrease of membrane fluidity) during maturation from newborns to adults (Fig. 1). This increase has to be explained by corresponding biochemical changes. Comparing the curves of the fluorescence anisotropy changes with the biochemical data we would like to discuss three different explanations in particular.

(I) From birth to the suckling age, the weight ratio of saturated to *cis*-unsaturated fatty acids steeply increased mainly due to a steep decrease of

C18 : 1(*cis*-9). This correlates with an increase of fluorescence anisotropy from birth to the suckling age when measured with 12-AS and 16-AP. Thus, the fluorescence anisotropy in the hydrophobic membrane interior when measured with labels reflecting the dynamic parameter of membrane fluidity seems to be influenced by the increase of the ratio of saturated to *cis*-unsaturated fatty acids or by the decrease of C18 : 1(*cis*-9), 16-AP being the more sensitive probe for this phenomenon (Fig. 1). These results are in accordance with the data of Kitagawa et al. [23] who examined the influence of incubation in *cis*-unsaturated fatty acids on the fluorescence anisotropy of bovine platelets and demonstrated decreasing values of 12-AS fluorescence anisotropy after incubation in C18 : 1(*cis*-9).

The relative content of C16 : 0 decreased during maturation, C18 : 0 simultaneously increased (Fig. 1). This might indicate a fatty acid chain elongation during maturation. According to Kitagawa et al. [23], changes of saturated fatty acid composition do not influence membrane fluidity.

(II) From birth to the weaned age, the cholesterol to phospholipid molar ratio showed a fairly steep increase and no significant increase thereafter. This increase of the cholesterol to phospholipid ratio is strikingly parallel to the increase of fluorescence anisotropy when measured with DPH (Fig. 1). Our data on DPH fluorescence anisotropy are in accordance with the results of Neu et al. [2] and Schwarz et al. [3,4] demonstrating an increase of DPH fluorescence anisotropy in weaned versus suckling rabbit and rat BBM, our data on cholesterol to phospholipid ratio are in accordance with the results of Schwarz et al. [4] demonstrating an increase of cholesterol to phospholipid ratio in weaned versus suckling rabbit BBM. Our data on DPH fluorescence anisotropy and cholesterol to phospholipid ratio after the weaned age are not in accordance with the results of Schwarz et al. [4] and Brasitus et al. [5] who found a significant increase of DPH fluorescence anisotropy and cholesterol to phospholipid ratio also in the postweaned period. Van Blitterswijk et al. [24,25] demonstrated a close relationship between the cholesterol to phospholipid ratio and DPH fluorescence anisotropy in studies on several plasma membranes and liposomes. The striking parallelism between the increase of the cholesterol to phospholipid ratio and the DPH fluorescence anisotropy during maturation of BBM fits in with the data of Van Blitterswijk et al. [24,25], probably indicating a close if not a causal relationship between the cholesterol to phospholipid ratio and DPH fluorescent anisotropy. The changes of TMA-DPH fluorescence anisotropy behaved quite differently: the TMA-DPH values remained constant from birth to the weaned age and showed a steep increase thereafter. Therefore, the increase of the cholesterol to phospholipid ratio during maturation cannot be the explanation for the change of TMA-DPH fluorescence anisotropy.

(III) After the weaned age, the cholesterol to protein ratio and the phospholipid to protein ratio decreased (Table I), probably indicating an increase of the protein content of BBM. Thus, the increase of TMA-DPH fluorescence anisotropy may be mainly due to the increase of the protein content after the weaned age. This assumption is compatible with the fact that proteins are membrane rigidifiers [6]. It would imply that TMA-DPH, rather than DPH, is sensitive for influences of proteins on membrane fluidity.

Summing up, we found that the ratio of saturated to *cis*-unsaturated fatty acids increased during postnatal maturation of BBM from birth to the suckling age, the cholesterol to phospholipid ratio increased from birth to the weaned age, the protein content probably increased from the weaned to the adult age. These biochemical findings correlate with the decrease of BBM fluidity during maturation best documented with the labels 16-AP, DPH, and TMA-DPH.

Acknowledgments

We thank Dr. G. Beron, University of Hamburg, for his helpful discussions concerning the statistics. The excellent technical assistance of B. Gellermann, M. Knorre, and I. Wernicke is gratefully acknowledged. This study was supported by the Deutsche Forschungsgemeinschaft (Ko 756, Ste 305).

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