Biochimica et Biophysica Acta 856 (1986) 610–614
Elsevier

BBA 73044

A comparison of brush-border membranes prepared from rabbit small intestine by procedures involving Ca^{2+} and Mg^{2+} precipitation

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(Received December 17th, 1985)

Key words: Brush-border membrane preparation; Membrane precipitation; Ca^{2+}; Mg^{2+}; (Rabbit intestine)

Brush-border membranes were isolated from rabbit small intestine by procedures involving precipitation of undesired membranes with either 10 mM $MgCl_2$ or 10 mM $CaCl_2$. The membranes were compared on the basis of marker enzyme content and lipid composition. Ca^{2+}-prepared membranes displayed a greater enrichment of alkaline phosphatase and sucrase activity compared to homogenate than did the Mg^{2+}-prepared membranes. The former also displayed an impoverishment of $(Na^+ + K^+)$-ATPase activity, the specific activity of which increased several-fold in Mg^{2+}-prepared membranes. Membranes prepared with Ca^{2+} were characterized by a lower phosphoacylglycerol-protein ratio and a higher phosphatidylethanolamine-phosphatidylcholine ratio. Although lysophosphoacylglycerols accounted for about 6% of the total phospholipids in these membranes compared to 2% in Mg^{2+}-prepared membranes, the free fatty acid content was similar in both types of membranes. It was concluded that Ca^{2+} prepared membranes were less contaminated by basolateral membranes than were Mg^{2+}-prepared membranes and the use of Ca^{2+} did not notably enhance degradation of endogenous lipids by brush-border membrane phospholipase A.

Introduction

Brush-border membranes from small intestine of avian and mammalian species have been conveniently prepared by homogenizing mucosal scrapings and precipitating undesired organelles such as Golgi apparatus membranes, nuclei, microsomes, mitochondria and basolateral membranes with either 10 mM $CaCl_2$ or $MgCl_2$ [1–9].

One previous report indicated that the better method for preparing bursh-border membrane from rabbit small intestine involved precipitation with $MgCl_2$ rather than with $CaCl_2$ [6]. In this particular study, the use of the latter precipitating agent apparently caused an activation of brush-border membrane phospholipase A which degraded endogenous phosphoglycerides into lysophosphoacylglycerols. Accordingly accumulations of lysophosphatidylcholine (lysoPC) and lysophosphatidylethanolamine (lysoPE) accounting for 16 and 26% of the total lipid phosphorus were found to occur [6], but these levels decreased very substantially when $MgCl_2$ was used a precipitant instead of $CaCl_2$.

These elevated values found for lysophosphoacylglycerols are difficult to reconcile with several other pertinent reports [10–14] which deal, however, with rat rather than rabbit. Firstly, although there appears to be some disagreement as to the substrate specificity of the phospholipase A of microvilli membranes, on the basis of what is known it is unlikely that lysophosphoacylglycerols would normally accumulate in large amounts in

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rabbit brush-border membranes unless very marked species differences exist. Indeed, recent evidence has indicated that the substrate specificity of this enzyme is very narrow, its activity for phosphatidylglycerol being several hundred fold greater than for PC [10,11]. Because of this, suggestions have been made that brush-border phospholipase A is specialized in the breakdown of lipids of plant or bacterial origin [10,11]. On the other hand, if older evidence is accepted, the specificity of this degradative enzyme is broader [12–14]; however, up to pH 7.0–7.5 (the pH used for preparing brush-border membranes) there is notably, mainly phospholipase B activity detectable when tested against PC and little or no lysoPC accumulates [12]. Secondly, other recent studies making use of Ca^{2+} as precipitant have indicated a lysoPC content in rat brush-border membranes of no greater than 3.0–8.7% [15,16]. No lysoPE was reported to occur in these membranes, the analyses of which gave total lipid phosphorus recoveries of 96% after separation of the lipids [15]. These various observations might possibly be reconciled on the basis that a phospholipase A of broad substrate specificity is present in brush-border membrane fractions which would not appreciably degrade endogenous lipids even in the presence of Ca^{2+} unless the membranes were subjected to sufficiently adverse conditions such as freezing and thawing, for example.

Since intestinal brush-border membrane vesicles have become a valuable and widely-used tool for the study of various transport systems, it is of some importance to establish, for any given species, which method of membrane preparation yields the purest and most structurally intact fractions. The present study deals with this problem and compares rabbit brush-border membranes prepared with Mg^{2+} [6,8] and Ca^{2+} [2] as precipitants, in terms of marker enzyme composition and detailed lipid composition.

Materials and Methods

Preparation of brush-border membranes. Female, white, New Zealand rabbits weaned at 4–6 weeks were fed standard Purina rabbit chow up to the time of death. The animals were killed by cervical dislocation and the small intestine was removed, everted and thoroughly washed in physiological saline. The epithelial cells were than scraped off and homogenized in 0.05 M mannitol, when indicated, leupeptin (1 μg/ml) aprotinin (0.11 tyrosine inhibitor units (TIU))/ml and phenylmethylsulfonyl fluoride (PMSF) (30 μM) were all added to the homogenate or to any required wash solution to prevent proteolysis [29–32]. Undesired membranes were precipitated with 10 mM $CaCl_2$ and the brush-border membranes were then isolated as described by Selhub and Rosenberg [2]. Alternatively, the brush-border membranes were isolated by similar methods in which treatments with $CaCl_2$ were substituted by those with $MgCl_2$ [8] or $MgCl_2$ + EGTA [6].

Marker enzyme assays. Succinate dehydrogenase as marker of mitochondria was determined by the method of King [17]. Glucose-6-phosphatase, an indicator of endoplasmic reticulum was measured according to Harper [18]. ($Na^+ + K^+$)-ATPase, a marker of basolateral membranes was determined as described by Scharschmidt et al. [19]. Alkaline phosphatase and sucrase, marker enzymes for the brush-border membrane were determined as reported by Forstner et al. [20] and Dahlqvist [21], respectively.

Lipid separations. Lipids were extracted from brush-border membranes by the method of Bligh and Dyer [22]. Neutral lipids were separated on activated silica gel G plates with petroleum ether (b.p. 60–80°C)/ diethyl ether/ formic acid (75 : 25 : 1.5, v/v/v). Components were revealed by spraying with 0.01% Rhodamine 6G and visualized with an ultraviolet lamp. Phosphoacylglycerols were separated by the two-dimensional TLC system of Bowyer and King [23].

Analytical procedures. Lipid phosphorus was determined by the method of Vaskovsky et al. [24]. Glycolipids were estimated by determining total sphingosine content of lipid extracts [25] and subtracting sphingosine due to sphingomyelin. Cholesterol was determined as described by Bowyer and King [23].

Free fatty acids separated from other lipids by TLC, and total fatty acids from lipid extracts were converted to methyl esters by treatment of samples with dry, 2.5% methanolic HCl at 70°C for 1 h under nitrogen. Butylated hydroxytoluene (BHT) 0.01%, was added to the sample to prevent oxida-

tion. The methyl derivatives were analysed with a Varian 6000 gas chromatograph, equipped with a flame ionization detector, employing a 1.83 meter × 2 mm packed glass column (10% SP-2330 on Chromosorb W, AW 100/20 mesh). The injection temperature, the ion temperature, the nitrogen flow rate, the column pressure and temperature program were: 260°C, 300°C, 7.5 ml/min, 10 lb/in^2 and 160°C for 20 min followed by 8 Cdeg/min to 200°C, respectively. Heptadecanoic acid was used as internal standard.

TLC analysis of the neutral lipid fraction revealed cholesterol and free fatty acids as the main components. Only trace amounts of acylglycerols and cholesterol esters were detected. Consequently cholesterol contents were analysed directly on lipid extracts. Free fatty acid determinations gave less variable results if calculated on the basis of the product of molar cholesterol content and the molar cholesterol/free fatty acid ratio. The latter was determined for each sample by analysing the cholesterol and free fatty acids recovered from the same TLC plate.

Results and Discussion

Results summarized in Table I indicate that membranes prepared by $CaCl_2$ precipitation [2] are purer, on the basis of marker enzyme distribution, than those prepared by Mg^{2+} precipitation [8]. Accordingly, compared to homogenate, the enrichments of alkaline phosphatase and sucrase are some 20–30% greater in the brush-border membranes prepared with Ca^{2+} whereas the specific activity of the $(Na^+ + K^+)$-ATPase was increased several-fold in the Mg^{2+}-prepared membranes but decreased in the other membrane fraction. These differences, although statistically significant, could have been due, at least partly, to a specific activation of proteases or a selective inhibition of marker enzyme activity in the presence of one or the other cation. However, it can be noted that total enzyme recoveries are very similar for sucrase, alkaline phosphatase and $(Na^+ + K^+)$-ATPase as are also the total protein recoveries when either precipitation method was used. In fact, the $(Na^+ + K^+)$-ATPase activity is significantly higher in the Mg^{2+}-derived membranes even when recovery of the total activity in the fractions of the Ca^{2+}-treated preparations was essentially complete. Consequently, the lower activity of $(Na^+ + K^+)$-ATPase and the higher activity of sucrase and alkaline phosphatase in the Ca^{2+} prepared membranes must be indicative of a purer fraction. Compared to homogenate there was no significant enrichment of glucose-6-phos-

TABLE I

MARKER ENZYME AND PROTEIN CONTENT OF BRUSH-BORDER MEMBRANES PREPARED BY Mg^{2+} AND BY Ca^{2+} PRECIPITATION

Proteins are expressed as mg recovered in each fraction. Enzyme activities are expressed as nmol/mg per min. Values are averages ± S.E. 6–25 determinations on membranes from 4–12 animals. Values in the first parentheses are average percent total recoveries ± S.E. obtained from analysis of all the fractions which include a Ca^{2+} or Mg^{2+} precipitate, a brush-border membrane fraction (BBM), a post-BBM supernatant and BBM wash. Values in the second parenthesis represent percent of the recovered activity found in each fraction.

	Homogenate	Mg^{2+}-prepared BBM	Ca^{2+}-prepared BBM	
		− Inhibitors	+ Inhibitors [a]	− Inhibitors
Proteins	1085 ± 17	51 ± 3 (93 ± 1)	52 ± 3 (86 ± 3)	47 ± 1 (92 ± 1)
Sucrase	61 ± 5	748 ± 21 (83 ± 3) (69)	936 ± 52 (82 ± 3) (86)	929 ± 12 (86 ± 3) (77) [b]
Alkaline phosphatase	79 ± 2	902 ± 106 (81 ± 2) (65)		1150 ± 98 (86 ± 2) (73) [c]
$(Na^+ + K^+)$-ATPase	48 ± 7	125 ± 5 (94 ± 3) (12)	35 ± 6 (89 ± 3) (3.5)	19 ± 4 (100 ± 2) (2.5) [b]
Succinate dehydrogenase	91 ± 7	6 ± 1		4 ± 1
Glucose-6-phosphatase	81 ± 3	95 ± 10		108 ± 17

[a] Leupeptin (1 μg/ml) apotinin (11 TIU/ml) and PMSF (30 μM) were added throughout the isolation procedure.
[b] Difference between Mg^{2+}- and Ca^{2+}-prepared membranes: $P < 0.01$.
[c] Difference between Mg^{2+}- and Ca^{2+}-prepared membranes: $P < 0.05$.

TABLE II

LIPID COMPOSITION OF BRUSH-BORDER MEMBRANES PREPARED BY Mg^{2+} AND BY Ca^{2+} ADDITION

The values represent averages ± S.E. of 5–11 determinations on extracts from 5–9 rabbits.

	Membrane fraction (μg/mg protein)	
	Mg^{2+}-prepared	Ca^{2+}-prepared
Cholesterol	61 ± 1	60 ± 2
Free fatty acids	32 ± 10	34 ± 6
Lipid phosphorus	227 ± 12	168 ± 5 [a]
Glycolipid sphingosine	138 ± 4	157 ± 1 [a]

[a] Difference between Mg^{2+}-prepared and Ca^{2+}-prepared membranes: $P < 0.005$.

phatase or succinate dehydrogenase activity in either fraction.

Results in Table II indicate differences in the lipid class composition of both membrane fractions. The content of phospholipid per mg protein is significantly higher in membranes prepared by Mg^{2+} precipitation which is again an indication of basolateral membrane contamination in these fractions since the latter usually have a much higher phospholipid : protein ratio [16,26]. Our results confirm those of Yakymyshyn et al. [7] who also found higher phospholipid/protein ratios in Mg^{2+}-prepared brush-border membranes from rabbit. This type of preparation also had a somewhat lower non-sphingomyelin sphingosine content and it is known that basolateral membranes contain a smaller proportion of glycolipids compared to microvilli membranes [26]. Further analysis of these membranes with respect to glycolipid composition would be required. Cholesterol contents per mg protein were similar in both types of membrane fractions whereas the free fatty acid content was more variable and somewhat greater, although not significantly so, in the Ca^{2+}-prepared membranes. These lipids are known to occur in similar amounts in basolateral and microvilli membranes [16,26]. The fact that the free fatty acid content is not greatly increased as indicated presently or even lower in Ca^{2+}-prepared membranes as reported elsewhere [7] can be taken as evidence that very extensive breakdown of endogenous phosphoacylglycerols by phospholipase A does not occur whether Ca^{2+} or Mg^{2+} was used as precipitant.

TABLE III

PHOSPHOLIPID COMPOSITION OF BRUSH-BORDER MEMBRANES PREPARED BY Mg^{2+} OR BY Ca^{2+} ADDITION

	Fraction (μg/mg protein)		
	Mg^{2+}-prepared [a]	Mg^{2+}-EGTA-prepared [b]	Ca^{2+}-prepared [a]
Phosphatidylethanolamine	108 ± 2	127	85 ± 2 [c]
Phosphatidylcholine	59 ± 4	82	37 ± 1 [c]
Sphingomyelin	31 ± 1	26	13 ± 1 [c]
Phosphatidylinositol	14 ± 2	21	10 ± 1
Phosphatidylserine	31 ± 2	30	24 ± 1 [c]
Lysophosphatidylethanolamine	2 ± 1	nil	5 ± 1
Lysophosphatidylcholine	3 ± 1	nil	6 ± 1

[a] Values represent averages ± S.E. of 7–11 determinations obtained from four rabbits.

[b] Values represent the average of two determinations obtained with a single rabbit.

[c] Difference between Mg^{2+}-prepared and Ca^{2+}-prepared membranes: $P < 0.01$.

Results in Table III compare the phospholipid compositions of our usual membrane fractions together with another fraction prepared with Mg^{2+}-EGTA according to Hauser et al. [6]. Quite remarkably, little or no lysophosphoacylglycerol was found in all three preparations. The lysolipid content was highest in the Ca^{2+}-prepared membranes but accounted for no more than approx. 6% of the total phospholipid content. There appears to be a major difference in the phosphoacylglycerol composition of our brush-border membrane preparations compared to that reported by Hauser et al. even when we used their isolation procedure. The phosphatidylethanolamine-phosphatidylcholine ratios that were obtained were consistently and very significantly higher. Isolations making use of $MgCl_2$ [8] and $MgCl_2$ + EGTA [6] decreased the ratio of 1.8 and 1.5, respectively, compared to a ratio of 2.3 when $CaCl_2$ was used. Although there are no reports comparing the phosphoacylglycerol composition of microvilli and basolateral membranes from rabbit intestine, a comparison made with such membranes isolated from mouse intestine indicated a

high ratio of approx. 2.0–3.0 in the brush-border membranes and a low ratio of approx. 0.5–0.9 in the basolateral membranes [26]. On this basis and because in our studies the same rabbits were used for the Mg^{2+}- and Ca^{2+}-prepared membranes, we conclude again that $MgCl_2$-precipitation methods yielded brush-border membranes which were more heavily contaminated with basolateral membranes. However, on the basis of the PE/PC ratio alone it is not possible to ascertain the relative purity of the brush-border membranes from different preparations since this ratio is known to be affected by the age of the animal and the segment of small intestine from which the membranes originate [27].

As a whole, our results indicate that brush-border membranes prepared from procedures employing $CaCl_2$ as precipitant are purer. Although Ca^{2+} could potentially activate phospholipase A and cause extensive degradation of endogenous phosphoacylglycerols it did so only to a very limited extent in our case since free fatty acid levels were not markedly increased by the use of this cation and lysophosphoacylglycerols were increased to only 6% of the total phospholipids as compared to 2% in Mg^{2+}-prepared membranes. It is uncertain as to why high levels of lysophosphoacylglycerols are sometimes obtained in Ca^{2+}-prepared membranes [6]. An analogous situation may exist with *Escherichia coli* phospholipase A which is normally inactive against endogenous phosphoacylglycerols but becomes active when cells are subjected to adverse conditions [28]. Freezing and thawing of intestinal tissues prior to isolation of membranes might possibly be such an adverse condition which we avoided throughout the present studies.

Acknowledgement

This study was supported by the Medical Research Council of Canada.

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