Analytical Biochemistry **289,** 157–161 (2001)
doi:10.1006/abio.2000.4908, available online at http://www.idealibrary.com on IDEAL®

A Novel Method of Preparation of Small Intestinal Brush Border Membrane Vesicles by Polyethylene Glycol Precipitation

R. Prabhu and K. A. Balasubramanian[1]

The Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College and Hospital, Vellore 632 004, India

Received May 9, 2000

A novel method of brush border membrane vesicle (BBMV) preparation from the small intestinal mucosa using polyethylene glycol (PEG) precipitation has been presented. This preparation is compared with calcium-precipitated BBMVs in marker enzyme enrichment, contamination by other subcellular membranes, transport of glucose, and lipid composition. PEG-precipitated BBMVs are comparable with calcium-precipitated membranes in all parameters except lipid composition and thiol content. PEG-precipitated membranes have more phosphatidylcholine and phosphatidylethanolamine and less lysophosphatidylcholine and lysophosphatidylethanolamine as compared to calcium-precipitated membranes. Diacylglycerol and triacylglycerol content are also high in PEG-precipitated membranes. Alteration in lipid composition indicate the possible activation of lipase and phospholipase by calcium during BBMV preparation, which is not seen in PEG precipitation. Thiol content is almost double in PEG-precipitated membranes as compared to calcium-precipitated membranes. These results indicate that PEG can be used for the preparation of BBMVs in native form from the intestine without any alteration in their structural components, and these membranes show comparable transport activity.

Key Words: **brush border membrane; small intestine; polyethylene glycol; lipid composition.**

Small intestinal brush border membranes contain many hydrolytic enzymes and a number of transport systems that are important for the digestion and absorption of nutrients. Transport across the brush border membranes can be studied *in vitro* using isolated brush border membrane vesicles (BBMV).[2] Various techniques have been employed for the isolation of small intestinal BBMV, which include cell disruption followed by density gradient centrifugation (1), immunoabsorbent chromatography (2), and selective precipitation of other membranes using the divalent cation Ca^{2+} (3, 4) or Mg^{2+} (5). The cation precipitation is a widely used method that is also rapid and subsequent treatment of this isolated BBMV with KCNS selectively removes the cytoskeletal material and improves the purification (6). BBMV prepared from rodent intestine by Ca^{2+} and Mg^{2+} precipitation differ in their lipid composition (7), size and shape, intramembrane particle distribution, and protein composition (8). Phospholipid composition as well as static and dynamic components of fluidity were also found to differ (9). Our earlier work has shown that BBMV prepared by Ca^{2+} or Mg^{2+} precipitation from rat intestine differ in their purity and lipid composition, whereas BBMV prepared from monkey intestine using these cations are similar (7).

One of the problems associated with use of cations, especially Ca^{2+} for BBMV preparation is that Ca^{2+} can activate membrane-associated lipase, phospholipase, and possibly protease, which can alter the structural components of the final membrane preparation. Since structural and functional aspects of BBMV are influenced by their lipid and protein composition (10), it is important to isolate the membranes in the purest form with intact structural features. It is known that poly-

[1] To whom correspondence should be addressed. Fax: 91-416-232035. E-mail: wubalu@hotmail.com.

[2] Abbreviations used: BBMV, brush border membrane vesicles; ATP, adenosine triphosphate; PEG, polyethylene glycol; DAG, deacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GSSG, glutathione, oxidized hydrate.

0003-2697/01 $35.00

ethylene glycol can selectively precipitate certain subcellular organelles (11). In the present study we have used PEG for the preparation of BBMVs from the small intestine and compare the structural and functional properties of PEG-precipitated membranes with Ca^{2+}-precipitated membranes.

MATERIALS AND METHODS

Glucose oxidase, *p*-nitrophenyl phosphate, *o*-dianisidine, ouabain, ATP, glucose 6-phosphate, and lipid standards were obtained from Sigma Chemical Co. (USA). C^{14}-labeled glucose was obtained from Bhaba Atomic Research Centre (Bombay, India), Polyethylene glycol 4000 was obtained from Fluka AG (Switzerland). Millipore membranes (pore size 0.45 μm) were obtained from Millipore (India).

Isolation of Brush Border Membrane Vesicles

Wistar rats weighing 200–250 g, fasted overnight, were killed by cervical dislocation. The small intestine was excised, washed with ice-cold saline, and the mucosa scraped with a glass slide. A 2–3% homogenate of the mucosa was prepared in 2 mM Tris–HCl buffer (pH 7.1) containing 50 mM mannitol. This homogenate was divided into two portions and used for BBMV preparation using PEG and calcium.

Calcium Precipitation Method

BBMVs were prepared by Ca^{2+} precipitation as described (3). Briefly to one portion of the homogenate, solid $CaCl_2$ was added to a final concentration of 10 mM, stirred for 1 min and allowed to stand at 4°C for 15 min, centrifuged at 3000*g* for 15 min, and this supernatant respun at 27,000*g* for 30 min. The pellet obtained was washed twice with suspension buffer (10 mM Tris–HCL buffer, pH 7.1, containing 300 mM mannitol) and finally suspended in the same buffer using a syringe fitted with a 26-G needle.

PEG Precipitation Method

Alternatively, BBMVs were also prepared by PEG precipitation. To another portion of homogenate, a solution of 50% PEG 4000 was added to a final concentration of 10% and stirred for 15 min at 4°C. This was centrifuged at 7500*g* for 15 min and the supernatant was respun at 27,000*g* for 40 min. The pellet was washed twice with suspension buffer (10 mM Tris–HCl buffer, pH 7.1, contains 300 mM mannitol) and finally suspended in the same buffer using a syringe fitted with a 26-G needle.

BBMVs were also prepared from overnight fasted rabbit small intestinal mucosa using calcium and PEG precipitation methods. Protein was estimated using BSA as standard (12).

Enzyme Assays

Activity of alkaline phosphatase (13), maltase and sucrase (14) Na^+ K^+ ATPase (15), glucose 6-phosphatase (16), and arylsulfatase (17) were assayed as described. The enzyme activities are expressed as units/mg protein. (Units are expressed as μmol/min/mg protein for alkaline phosphatase and glucose 6-phosphatase, nmol/h/mg protein for maltase, sucrase, Na^+ K^+ ATPase, and arylsulfatase.)

Lipid Analysis

BBMV lipids were extracted by the Bligh and Dyer method (18). The lower organic phase was evaporated to dryness, resuspended in a small volume of chloroform:methanol (2:1) and used for lipid analysis. Neutral lipids were separated on silica gel G plates using the solvent system hexane:diethylether:acetic acid (80:20:1, v/v). Spots corresponding to standard were identified by iodine exposure and eluted. Cholesterol and cholesteryl ester (19), diacylglycerol, and triacylglycerol (20) were quantitated as described. Free fatty acids were methylated and quantitated by gas chromatography after separation on a 5% EGSS-X column. Individual phospholipids were separated on silica gel H plates using the solvent system chloroform:methanol:acetic acid:water (25:14:4:2, v/v) and quantitated by phosphate estimation after acid hydrolysis (21).

Measurement of Glucose Transport

BBMVs prepared by Ca^{2+} and PEG precipitation were tested for their ability to transport glucose. Uptake measurements were carried out by rapid filtration technique at room temperature as described (22). Briefly, 50 μL of BBMVs corresponding to 100 μg protein was incubated with 150 μL of uptake buffer containing 150 mM NaSCN, 50 μM D-glucose, 0.8 μCi D-[^{14}C]glucose, and 10 mM Hepes (pH 7.5) at varying time intervals. At the end of incubation, the mixture was diluted with 2 mL of ice-cold stop buffer (150 mM NaCl, 10 mM Hepes, pH 7.5) and filtered under constant vacuum. The filter was washed three times with 5 mL of stop buffer and transferred to counting vials. The radioactivity retained in the filter was counted using LKB Rack-Beta Scintillation counter.

Statistics

Data are expressed as mean $\pm$ SD from a minimum of three experiments. Statistical analyses were performed with Student's *t* test to compare the changes.

TABLE 1

Comparison of BBMV from Rat Intestine Prepared by Ca^{2+} and PEG Precipitation Method

	Alkaline phosphatase (sp act)	Sucrase (sp act)	Maltase (sp act)	Na^+K^+ ATPase (sp act)	Arylsulfatase (sp act)	Glucose 6-phosphatase (sp act)
Homogenate	0.071 ± 0.001	0.162 ± 0.003	0.375 ± 0.021	130.77 ± 5.07	0.970 ± 0.025	28.57 ± 1.54
$CaCl_2$-precipitated BBMV	1.052 ± 0.035 (28)	1.770 ± 0.056 (31)	4.090 ± 0.491 (32)	11.14 ± 0.240 (0.26)	0.253 ± 0.009 (0.62)	9.330 ± 0.970 (0.8)
Fold enrichment	14.81	10.92	10.90	—	—	—
PEG-precipitated BBMV	0.891 ± 0.023 (37)	1.470 ± 0.023 (40)	3.675 ± 0.619 (38)	10 ± 1.15 (0.28)	0.190 ± 0.004 (0.6)	5.390 ± 0.412 (0.6)
Fold enrichment	12.55	9.07	9.8	—	—	—

Note. The values in parentheses represent the percentage recovery as compared to homogenate. Each value represents mean ± SD of three separate estimations.

RESULTS

Table 1 shows the comparison of marker enzyme enrichment in BBMVs isolated from rat intestine by Ca^{2+} and PEG precipitation. As can be seen, the enrichment of alkaline phosphatase, sucrase, and maltase are more or less similar in both preparations. Contamination by basolateral membranes and other subcellular fractions was checked by Na^+ K^+ ATPase for basolateral membrane, arylsulfatase for lysosomes, and glucose 6-phosphatase for microsomes. There was very little contamination by these fractions in the BBMV preparation and it is similar in Ca^{2+} and PEG precipitation. BBMVs were also prepared from rabbit intestine using these methods and a similar comparison was made. As shown in Table 2, rabbit BBMVs prepared by both these methods were similar as judged by marker enzymes.

Lipid composition was analyzed in BBMVs prepared by these two methods. Among the neutral lipids there was no difference in cholesterol, cholesterol ester, or free fatty acid content between the two methods of preparation, whereas DAG and TAG were higher in PEG-precipitated membranes than in those precipitated by the Ca^{2+} method (Fig. 1A). Among the phospholipids, increased level of PC and PE and decreased level of lysoPC and lysoPE were seen in PEG-precipitated membranes as compared to Ca^{2+}-precipitated membranes. Other phospholipids were more or less similar by both methods of preparation (Fig. 1B).

The functional aspect of these membranes was tested by glucose uptake. As shown in Fig. 2, membranes prepared by both methods showed similar type of glucose uptake and the peak uptake was seen at 20 s. Thiol content of membranes was also measured in both preparations and as can be seen in Table 3, thiol content was high in PEG-precipitated membranes as compared to calcium-precipitated membranes. This was seen in both rat and rabbit BBMVs and it was almost double in rat BBMVs.

DISCUSSION

Although different methods are available for preparation of BBMVs from the intestine, the Ca^{2+} precipitation is a simple and rapid method used by many investigators. One of the problems associated with calcium precipitation is the possible activation of mem-

TABLE 2

Comparison of BBMV from Rabbit Intestine Prepared by Ca^{2+} and PEG Precipitation Method

	Alkaline phosphatase (sp act)	Sucrase (sp act)	Maltase (sp act)	Na^+K^+ ATPase (sp act)	Arylsulftase (sp act)	Glucose 6-phosphatase (sp act)
Homogenate	0.057 ± 0.001	0.048 ± 0.001	0.063 ± 0.003	95.011 ± 1.511	0.621 ± 0.0025	45.102 ± 3.561
$CaCl_2$-precipitated BBMV	0.866 ± 0.010 (48)	1.491 ± 0.080 (90)	2.040 ± 0.030 (93)	2 ± 0.601 (0.05)	0.116 ± 0.009 (0.78)	8.910 ± 0.508 (0.005)
Fold enrichment	15.19	30.19	32.27	—	—	—
PEG-precipitated BBMV	0.773 ± 0.013 (53)	1.620 ± 0.056 (93)	2.256 ± 0.095 (94)	1.191 ± 0.040 (0.05)	0.109 ± 0.012 (0.633)	8.301 ± 0.056 (0.005)
Fold enrichment	13.56	33.6	35	—	—	—

Note. The values in parentheses represent the percentage recovery as compared to homogenate. Each value represents mean ± SD of three separate estimations.

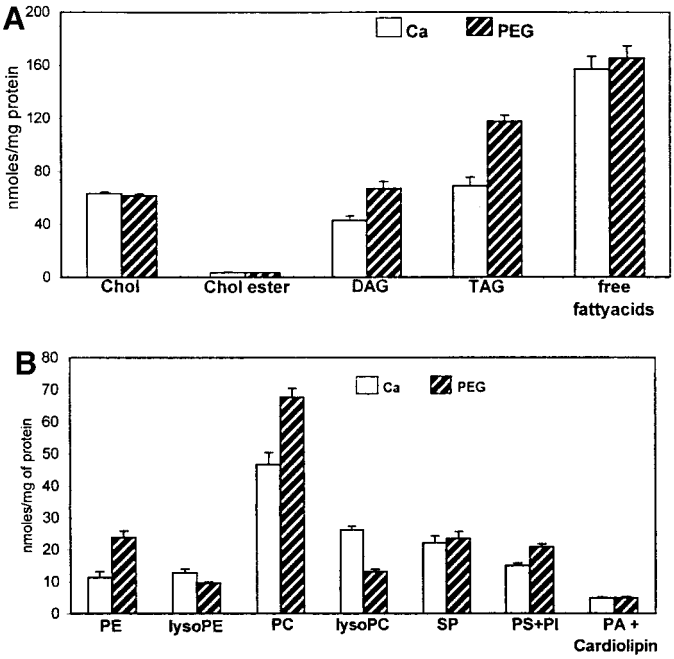

FIG. 1. Lipid composition of BBMV prepared from rat intestine using Ca (□) and PEG (▨) precipitation method. Each value represents mean ± SD of three separate estimates. (A) Neutral lipids (Chol, cholesterol; Chol ester, cholesterol ester; DAG, diacylglycerol; TAG, triacylglycerol). (B) Phospholipids (PE, phosphotidylethanolamine; lysoPE, lysophosphotidylethanolamine; PC, phostidylcholine; lysoPC, lysophosphotidylcholine; SP, sphingomylein; PS + PI, phosphotidylserine + phosphotidylinositol; PA, phosphotidic acid). Each value represents mean ± SD of three separate estimates.

brane-associated lipases, phospholipase, and possibly proteases by divalent metal ions, which is likely to alter the lipid and protein composition and hence structure of the membrane. To overcome this, we have

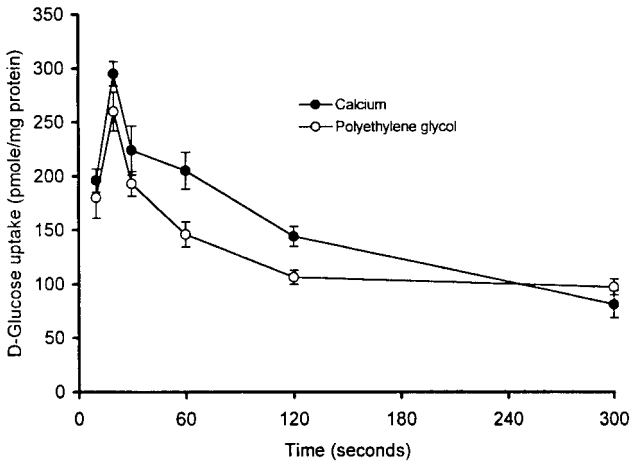

FIG. 2. D-Glucose transport by BBMVs prepared from rat intestine using Ca^{2+} (•) and PEG (○) precipitation method. Each value represents mean ± SD of five separate estimates.

TABLE 3

Total Thiol Content of BBMV from Rat and Rabbit Intestine

	Rat (nmol/mg protein)	Rabbit (nmol/mg protein)
Ca^{2+}-precipitated BBMV	12.59 ± 0.49	21.63 ± 1.62
PEG-precipitated BBMV	23.15 ± 1.42	33.23 ± 0.90

Note. Each value represents mean ± SD of three separate estimations.

used PEG to precipitate out other subcellular organelles and the supernatant can be then used to prepare the BBMVs. PEG has been used earlier to prepare microsomes (11, 23, 24). We have compared the PEG-precipitated membranes with Ca^{2+}-precipitated membranes in marker enzyme enrichment and contamination by other subcellular membranes which showed that PEG-precipitated membranes are comparable to calcium-precipitated membranes. PEG-precipitated membranes also transported [^{14}C]glucose similar to Ca^{2+}-precipitated membranes, suggesting that PEG treatment does not affect the transport properties and is similar to Ca^{2+}-precipitated membranes. Comparison of lipid composition showed some differences between these two preparations. Decreases in DAG, TAG, PC, and PE as well as an increase in lysoPC and lysoPE were seen in Ca^{2+}-precipitated membranes as compared to PEG-precipitated membranes. Other lipids including free fatty acids were similar in both preparations. Alteration in the level of PC, PE, lysoPC, and lysoPE in calcium-precipitated membranes may be due to activation of phospholipases, possibly phospholipase A_2 (25, 26). BBMV is known to contain a phospholipase A_2 but is calcium-independent (27). Intestinal epithelial cells are known to contain Ca^{2+}-activated phospholipase that may degrade membrane phospholipids. Several other reports have shown the presence of lysophospholipids in BBMVs prepared with Ca^{2+} (3, 28).

BBMVs prepared by PEG precipitation also showed increased thiol content as compared to Ca^{2+}-precipitated membranes and is seen both in rat and rabbit BBMVs. Transport of certain compounds across the membrane depends on the presence of reduced thiol groups in the transport proteins. In the present study, although the BBMVs prepared by PEG precipitation had more amount of reduced thiol groups as compared to Ca^{2+}-precipitated membranes, both these membranes showed similar transport function. Two classes of sulphydryl groups have been identified in the intestinal BBMV Na^+ glucose cotransporter. The first class reacts with water-soluble –SH reagents without affecting the cotransporter activity (29). The second class

reacts preferentially with water-insoluble –SH reagents, resulting in noncompetitive inhibition of cotransporter-mediated Na^+ and glucose uptake (30). The similarity in glucose transport between these two preparations even though they differ in total SH group content suggests that essential SH groups are probably still intact in calcium-precipitated membranes. Our earlier work on the modification of rat BBMVs by reaction with GSSG also showed fewer SH groups without alteration in transport properties (31). In conclusion, this study has shown a novel method of BBMV preparation from the intestine using PEG precipitation that avoids degradation of endogenous lipids and phospholipids by intestinal lipase and phospholipase and represents the membrane prepared in natural form.

ACKNOWLEDGMENTS

The Wellcome Trust Research Laboratory is supported by the Wellcome Trust, London.

REFERENCES

1. Eichholz, A., and Crane, R. K. (1965) *J. Cell. Biol.* **26,** 687–690.
2. Carlsen, J., Christiansen, K., and Bro, B. (1982) *Biochim. Biophys. Acta* **689,** 12–20.
3. Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., and Semenza, G. (1978) *Biochim. Biophys. Acta* **506,** 136–154.
4. Schimtz, J., Preisier, H., Maestracci, D., Ghosh, B. K., Cerdar, J. J., and Crane, R. K. (1973) *Biochim. Biophys. Acta* **323,** 98–112.
5. Hauser, H., Howell, K., Dawson, R. M. C., and Bowyer, D. E. (1980) *Biochim. Biophys. Acta* **602,** 567–577.
6. Hopfer, U., Crowe, T. D., and Tandler, B. (1983) *Anal. Biochem.* **131,** 447–452.
7. Ibrahim, S. A., and Balasubramanian, K. A. (1995) *Comp. Biochem. Physiol.* **112**B, 65–69.
8. Bjorkman, D. J., Hagen, C. H., and Trier, J. S. (1986) *Gastroentrology* **91,** 1401–1414.
9. Bjorkman, D. J., and Brigham, E. J. (1990) *Biochem. Biophys. Res. Commun.* **170,** 433–440.
10. Proulx, P. (1991) *Biochim. Biophys. Acta* **1071,** 255–271.
11. Banhegi, G., Lusini, L., Puskas, F., Rossi, R., Fulceri, R., Braun, L., Mile, V., Simplicio, P. D., Mandl, A., and Bendetti, J. (1999) *J. Biol. Chem.* **274,** 12213–12216.
12. Lowry, O. H., Rosebrough, N. J., Farr A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
13. Thambidorai, D., and Bachhawat, B. K. (1977) *J. Neurochem.* **29,** 503–512.
14. Dalhquist, A. (1968) *Anal. Biochem.* **22,** 99–107.
15. Quigley, J. P., and Gotterer, S. G. (1969) *Biochim. Biophys. Acta* **173,** 456–468.
16. Hubscher, G., and West, G. R. (1965) *Nature* **205,** 799.
17. Dodgson, K. S., and Spencer, B. (1953) *Biol. J.* **53,** 444.
18. Bliigh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Phys.* **37,** 911–917.
19. Zaltkis, A., Zak, B., and Boyle, A. J. (1953) *J. Lab. Clin. Med.* **41,** 486–492.
20. Synder, F., and Stephen, N. (1959) *Biochim. Biophys. Acta* **34,** 244–245.
21. Bartlett, G. R. (1959) *J. Biol. Chem.* **234,** 466–468.
22. Tiruppathi, C., Miyamoto, Y., Ganapathi, V., and Leibach, F. H. (1988) *Biochem. Pharmacol.* **37,** 1399–1405.
23. Puskas, F., Braun, L., Marcolongo, P., Csala, M., Fulceri, R., Mandl, A., and Bendetti, J. (1998) *FEBS Lett.* **430,** 293–296.
24. Bangheyi, G., Braun, L., Marcolongo, P., Csala, M., Fulceri, R., Mandl, A., and Bendetti, J. (1996) *Biochem. J.* **315,** 171–176.
25. Gassama-Diagne, A., Fauvel, J., and Chap, H. (1989) *J. Biol. Chem.* **264,** 9470–9475.
26. Gassama-Diagne, A., Rogelle, P., Fauvel, J., Willson, M., Klaebe P., and Chap, H. (1992) *J. Biol. Chem.* **267,** 13418–13424.
27. Pind, S., and Kuksis, A. (1987) *Biochim. Biophys. Acta* **901,** 78–87.
28. Aubry, H., Merrill, A. R., and Proulx, P. (1986) *Biochim. Biophys. Acta* **856,** 610–614.
29. Biber, J., Weber, J., and Semenza, G. (1983) *Biochim. Biophys. Acta* **728,** 429–437.
30. Kilp, A., Grinstein, S., and Semenza, G. (1980) *Biochim. Biophys. Acta* **698,** 100–114.
31. Benard, O., and Balasubramanian, K. A. (1995) *Int. J. Biochem. Cell. Biol.* **27,** 589–595.