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Techniques for isolation of brush-border and basolateral membrane vesicles from dog kidney cortex

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Two methods are reported for renal membrane preparation from the dog kidney cortex. One method is a simultaneous preparation of brush-border (BBMV) and basolateral (BLMV) membranes. Using readily available laboratory equipment, differential centrifugation produced a supernatant which was treated with Mg^{2+}. The Mg^{2+} treatment produced a pellet (crude BLMV) which was added to Percoll and centrifuged to produce purified BLMV. The supernatant after Mg^{2+} treatment eventually yielded pure BBMV after additional Mg^{2+} precipitations. The second method used an acidic medium in conjunction with divalent-cation precipitation to prepare BBMV. Whichever method was used, BBMV and BLMV showed appropriate enzyme and transport activities.

Introduction

Various methods have been used to isolate brush-border and basolateral membrane vesicles (BBMV and BLMV, respectively) from the proximal convoluted tubule cells of dog kidney cortex. Divalent-cation precipitation, using either Mg^{2+} [1,2] or Ca^{2+} [1,3], has been employed to prepare BBMV. Percoll gradients were used to prepare canine BLMV [3,4]. A combination of divalent-cation precipitations and a sucrose gradient was employed by Kinsella and co-workers to prepare BBMV and BLMV from the same homogenate of dog kidney cortex [5]. A simultaneous BLMV and BBMV preparation has also been reported using free-flow electrophoresis [6].

Two methods for preparation of BBMV and BLMV from dog kidney cortex are reported. Sheikh et al. [7] have prepared BBMV and BLMV from rabbit kidney cortex by initially treating the homogenate with Mg^{2+} to separate BBMV from a crude BLMV fraction; purification of BLMV was then attained utilizing a Percoll gradient. In order to adapt this method for the simultaneous preparation of canine BBMV and BLMV, it was necessary to change the Percoll concentration. Compared with previously described canine methods [1–6], our modification has the attributes of being fast, efficient and relatively simple; moreover, it makes use of equipment commonly found in the laboratory (Sorvall RC-5B centrifuge and Sorvall SS-34 rotor).

We have also modified the divalent-cation precipitation method for isolation of BBMV of Blomstedt and Aronson [2] by changing the pH of the medium. This modification has enabled us to eliminate two of the three precipitation steps of the original method and, therefore, to shorten substantially the time interval required for completion of the procedure without sacrificing the purity and quality of the preparation.

Methods

Simultaneous preparation of canine BBMV and BLMV

Both BBMV and BLMV are prepared from dog kidney cortex by a modification of the method of Sheikh et al. [7] which has been developed for the rabbit. The kidneys are perfused and excised. The cortex is removed and homogenized with a Waring Blender for 1 min in the cold room in 250 ml of homogenizing solution composed of 300 mM mannitol/0.1 mM PMSF (phenylmethylsulfonyl fluoride)/1 mM EDTA/18 mM Tris HCl (pH 7.4). Average cortex weight was 33 g (23–45 g range). The homogenate is centrifuged in a Sorvall RC-5B centrifuge and a Sorvall SS-34 rotor at 5000 rpm for 10 min. The supernatant (S_1) is combined

Abbreviations: BBMV, brush-border membrane vesicle; BLMV, basolateral membrane vesicle.

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with 1 percent of its volume of 1 M $MgCl_2$. After 20 min on ice, the $MgCl_2$-treated supernatant (S_1) is centrifuged at 4000 rpm for 10 min. The resulting supernatant (S_2) is used for preparation of BBMV, whereas the corresponding precipitate (P_2) is used for preparation of BLMV.

For preparation of BBMV, supernatant S_2 is centrifuged at 16500 rpm for 20 min. The resulting pellet is resuspended in the homogenizing medium (1/2 of the original volume) and treated with two additional $MgCl_2$ steps according to a method developed by Blomstedt and Aronson [2]. Specifically, sufficient 1 M $MgCl_2$ is added to arrive at a final $MgCl_2$ concentration of 10 mM. After 20 min on ice, the suspension is centrifuged at 6000 rpm for 12 min. The pellet is discarded. The resulting supernatant is centrifuged at 16500 rpm for 20 min. This pellet is resuspended in homogenizing medium and treated with 10 mM $MgCl_2$ as before. After 20 min on ice, the suspension is centrifuged at 7250 rpm for 12 min and the pellet is discarded. The supernatant is centrifuged at 16500 rpm for 20 min. The resulting pellet is the purified BBMV and is resuspended in a small volume of the desired medium.

For preparation of BLMV, the precipitate from the second centrifugation (P_2) is resuspended in homogenizing solution and centrifuged at 20000 rpm for 20 min. This washing step is repeated twice and the resultant pellet is then resuspended in a small volume. The resuspended pellet is mixed with Percoll (7.5 g added to 30.5 g of suspension). This concentration differs from that used for rabbit BLMV [7]. The Percoll suspension is centrifuged in a Sorvall SS-34 rotor at 20000 rpm for 30 min. Two major bands are evident. The upper layer is removed and washed three times by centrifugation in a medium of 300 mM mannitol/1 mM EDTA/2 mM magnesium gluconate/50 mM Hepes (pH 7.5). These centrifugations result in the removal of Percoll, which precipitates as a clear pellet under the membranes. The resulting membrane pellet (the purified BLMV) is resuspended in a small volume of the desired medium.

Preparation of canine BBMV by divalent-cation precipitation at pH 6

Canine BBMV are prepared by a modification of the divalent-cation precipitation method of Blomstedt and Aronson [2]. Cortex from one dog kidney was added to 140 ml of a homogenizing solution composed of 300 mM mannitol/1 mM EDTA/0.1 mM PMSF/50 mM Mes (pH 6) or 50 mM Hepes (pH 7.5). This mixture was homogenized four times for 5 s with a Polytron homogenizer. 35 ml aliquots were combined with 0.35 ml of one of the following: 1 M $MnCl_2$; 1 M $MgCl_2$; 1 M $CaCl_2$; or water. These aliquots were left on ice for 20 min with occasional shaking. The aliquots were then centrifuged in a Sorvall RC-5B centrifuge and a Sorvall SS-34 rotor at 4000 rpm for 10 min. The supernatant was centrifuged at 17000 rpm for 30 min. The resulting pellets were washed twice in pH 7.5 homogenizing medium by centrifugation and resuspension (17000 rpm, 20 min). The pellets (BBMV) were resuspended in a small volume for assay.

Protein determination

In method 1, protein was measured with a commercial Coomassie blue protein assay (Bio-Rad Protein Assay Kit 2, 500-0002), while protein in Method 2 was measured by the Lowry protein assay.

Results

Simultaneous preparation of BBMV and BLMV

Table I shows that the BBMV were enriched 9-fold in a luminal enzyme marker, whereas the preparation was not enriched in enzyme markers for antiluminal

TABLE I

Enzyme enrichments in simultaneously prepared canine BBMV and BLMV

Alkaline phosphatase was measured with a Sigma kit. Na^+/K^+-ATPase was estimated by measuring ouabain-sensitive, K^+-stimulated *p*-nitrophenylphosphatase [8], succinate dehydrogenase was measured according to the method of Pennington [9] and acid phosphatase was measured according to a Sigma kit. Average yield per dog was 15–25 mg BBMV protein and 25–35 mg BLMV protein. Values are means ± S.E. $N = 3$–3.

Organelle	Enzyme marker	Enrichment compared to homogenate	
		BBMV	BLMV
Luminal membrane	alkaline phosphatase	9.0 ± 1.6	0.9 ± 0.4
Basolateral membrane	Na^+/K^+-ATPase	1.8 ± 0.6	20.7 ± 2.6
Mitochondria	succinate dehydrogenase	0.4 ± 0.2	1.1 ± 0.5
Lysosomes	acid phosphatase	2.8 ± 0.2	1.4 ± 0.2

Fig. 1. (A and B) Membrane vesicles were equilibrated in a medium containing 300 mM mannitol/1 mM $MgCl_2$/50 mM Hepes, pH 7.5 (Tris). 10 μl vesicles were added to 50 μl of a medium containing 150 mM NaCl or KCl/1 mM $MgCl_2$/50 mM Hepes, pH 7.5 (Tris) and 50 μM radioactive substrate. Other transport assay details were as described by Hilden and Sacktor [10]. Representative experiments are shown. (C) Membrane vesicles were equilibrated in a medium containing 300 mM mannitol/1 mM EGTA/2 mM magnesium gluconate/50 mM Mes (pH 6). 10 μl BBMV were added to 40 μl of a medium containing 1 mM $^{22}NaCl$/75 mM potassium gluconate or $KHCO_3$/1 mM magnesium gluconate/150 mM mannitol/50 mM Hepes (pH 7.8). At the indicated times, uptake was stopped with 4 ml of a solution containing 75 mM potassium gluconate/150 mM mannitol/1 mM magnesium gluconate/50 mM Hepes (pH 7.8). This mixture was filtered through a 0.65 μm filter and the filter was washed with three 4 ml volumes of the stopping solution. The $^{22}Na^+$ trapped on the filter was counted in a gamma counter. Representative experiments are shown.

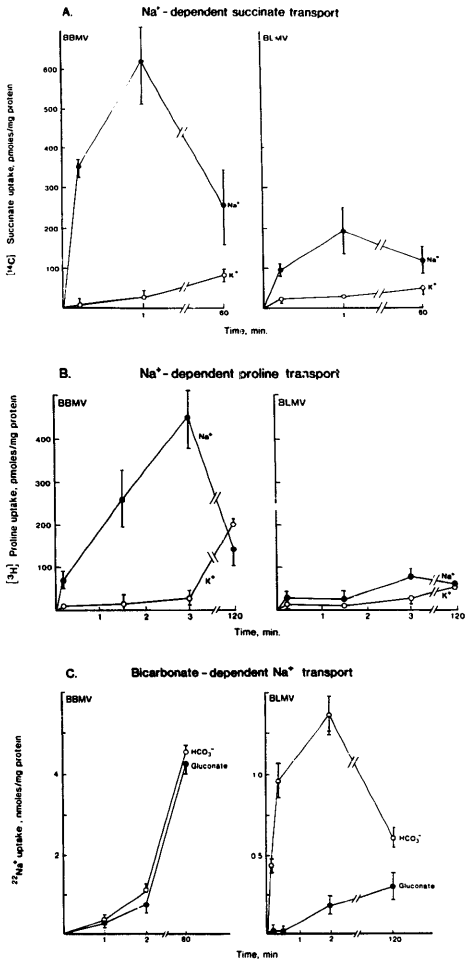
A.
Na+ - dependent succinate transport
BBMV
BLMV
[14C] Succinate uptake, pmoles/mg protein
Na+
K+
Time, min.
B.
Na+ - dependent proline transport
[3H] Proline uptake, pmoles/mg protein
Time, min.
C.
Bicarbonate - dependent Na+ transport
22Na+ uptake, nmoles/mg protein
HCO3-
Gluconate
Time, min

TABLE II

Na^+-dependent transport in simultaneously prepared canine BBMV and BLMV

Values were corrected for uptake in the presence of K^+ as a measure of passive influx. Transport was measured as described by Hilden and Sacktor [10,11] and in Fig. 1. Values are means ± S.E. $N = 3$. Uptake was measured after 10 s transport.

	pmol/mg protein per 10 s		BLMV/BBMV
	BBMV	BLMV	
Succinate	282 ± 96	55 ± 11	0.19
Glutamine	32 ± 9	8 ± 5	0.25
Phosphate	120 ± 32	−5 ± 5	−0.04
Proline	44 ± 9	7 ± 4	0.16

membranes or mitochondria and was slightly enriched in lysosomes. Table I also shows that the BLMV were enriched 21-fold in an antiluminal enzyme marker but not enriched in enzyme markers for luminal membranes, mitochondria or lysosomes. As with other preparation methods, BBMV demonstrated Na^+/H^+ exchange (data not shown) as well as Na^+-dependent transport of succinate, proline, glutamine and phosphate (Figs. 1A and B and Table II). Dog BBMV did not demonstrate Na^+/HCO_3^- cotransport, a transport process recently identified in rabbit renal cortical BLMV, but not BBMV [12,13]. Basolateral membranes from the dog did show this transporter, however (Fig. 1C).

The presence of other Na^+-dependent co-transport systems in dog BLMV is less clear. Succinate uptake was stimulated by Na^+ and showed an overshoot at 1 min as compared with the value at 60 min (Fig. 1A), which was approaching equilibrium. Na^+-dependent proline uptake by BLMV was very small and showed no overshoot (Fig. 1B). The amount of Na^+-dependent uptake of phosphate and glutamine was also very small (Table II).

BBMV preparation by divalent-cation precipitation at pH 6

Significantly, our modification utilizes only one divalent-cation precipitation step as compared with three by the original method. Properties of membrane fractions prepared by different divalent cations at pH 6 or 7.5 are reported in Table III. Values for enrichment in the luminal enzyme marker, γ-glutamyltranspeptidase showed greater purification with divalent-cation precipitation at pH 6 than at 7.5. This was true for Mg^{2+}, Mn^{2+} and Ca^{2+}. Values for enzyme markers of other membrane fractions showed less contamination of preparations made at pH 6 for the antiluminal marker Na^+/K^+-ATPase (Mn^{2+} and Ca^{2+}), the microsomal marker glucose-6-phosphatase and the lysosomal marker (Mg^{2+}, Mn^{2+} and Ca^{2+}). Mitochondrial contamination, as judged by cytochrome-*c* oxidase, appeared to be similar at pH 6 and 7.5. Ouabain-insensitive ATPase (measured at pH 7) was somewhat enriched and may reflect, in part, the activity of another BBMV enzyme marker, alkaline phosphatase. Protein yields with this one-step preparation were significantly greater than in method 1.

Transport systems characteristic of BBMV were also measured. All membranes showed Na^+/H^+ exchange as well as Na^+-dependent glucose uptake (data not shown). Despite higher enzyme enrichment, membranes prepared by Ca^{2+} precipitation showed transport rates which were lower than those of membranes prepared by Mg^{2+} or Mn^{2+} precipitation. This finding is similar to results reported in the rat [25].

Discussion

The assignment of Na^+-dependent uptake of phosphate, proline, succinate or glutamine to BLMV must take into account two considerations: first, transport in membrane vesicles prepared from Percoll gradient frac-

TABLE III

Enzyme enrichments in canine BBMV prepared by divalent-cation precipitation at pH 6 or pH 7.5

Enrichment is calculated by comparison with the activity of the homogenate in pH 7.5 homogenizing medium. Protein yield is calculated on the basis of yield from one dog. Na^+/K^+-ATPase was assayed according to the method of Forbush [23]. Cytochrome-*c* oxidase was measured according to Wharton and Tzagoloff [24]. Values are means ± S.E. $n = 3$–6.

Enzyme marker	Enrichment compared to homogenate							
	pH 6				pH 7.5			
	–	Mg^{2+}	Mn^{2+}	Ca^{2+}	–	Mg^{2+}	Mn^{2+}	Ca^{2+}
γ-Glutamyltranspeptidase	9.2 ± 2.2	11.7 ± 2.6	12.1 ± 2.5	16.5 ± 2.7	4.2 ± 1.4	5.1 ± 1.4	9.4 ± 1.9	8.9 ± 1.2
Na^+/K^+-ATPase	2.8 ± 0.1	2.6 ± 0.4	0.8 ± 0.2	0.7 ± 0.2	1.5 ± 0.0	1.2 ± 0.3	1.6 ± 0.5	1.8 ± 0.3
Glucose-6-phosphatase	3.5 ± 1.1	1.4 ± 0.6	0.6 ± 0.1	0.6 ± 0.1	3.5 ± 1.3	3.3 ± 1.2	2.0 ± 0.6	2.5 ± 0.1
p-Nitrophenyl-*N*-acetyl-β-glucosaminidase	1.2 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	2.0 ± 0.1	2.8 ± 0.2	1.6 ± 0.1	2.1 ± 0.1
Cytochrome *c* oxidase	0.7 ± 0.2	0.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.3	0.4 ± 0.1	0.8 ± 0.2	0.6 ± 0.2
Ouabain-insensitive ATPase	3.7 ± 0.2	4.0 ± 0.6	4.6 ± 0.9	4.6 ± 0.6	1.9 ± 0.4	3.5 ± 0.4	4.6 ± 0.1	4.1 ± 0.2
Yield (mg protein)	147 ± 25	71 ± 10	85 ± 14	79 ± 5	349 ± 34	118 ± 26	196 ± 34	172 ± 12

tions is frequently less than transport in preparations made in the absence of Percoll [11]. For example, we have studied Na^+-dependent glucose transport in rat renal BBMV prepared by Mg^{2+} precipitation of non-BLMV Percoll gradient fractions or by Mg^{2+} precipitation of a cortical homogenate; despite similar enzyme purification, passage through the Percoll gradient reduced the rate of this transport by 20–50% (Hilden, S.A., unpublished data). Second, transport in one membrane fraction may reflect high activity of contaminants rather than an intrinsic transporter. Transport by a BBMV contaminant present in the BLMV preparation will be most easily detected in the most active transport systems (e.g., succinate vs. proline, Figs. 1A and B). However, the method described here may reduce BLMV contamination by BBMV because the crude membranes that are being applied to the Percoll gradient (P_2) have been pre-treated to remove luminal membranes (S_2).

In rabbit BLMV prepared without Percoll, Na^+-dependent succinate uptake was approximately equal to that in BBMV [14]. Na^+-dependent succinate transport has not been previously studied in the dog, but if it is assumed that Na^+-dependent succinate transport is characteristic of dog BLMV, this transport system may be used as a point of reference. BLMV succinate transport at 10 s was 19% of that in BBMV (Table II). Glutamine transport in BLMV was 25% of that in BBMV and may therefore be part of this membrane. Na^+-dependent phosphate transport appears to be absent in BLMV as judged by the absolute magnitude of the Na^+-dependent uptake as well as the comparison with BBMV uptake. Finally, our results do not allow a firm conclusion on whether Na^+-dependent proline uptake is present in dog BLMV.

Our results in dog BLMV can be compared with those of other species as well as other dog preparations. Na^+-dependent succinate (or malate) transport has been reported present in rat and rabbit BLMV, whereas Na^+-dependent proline uptake has been reported absent in rat, dog and rabbit BLMV [14,15]. Na^+-dependent glutamine uptake has been reported in dog and rat BLMV [16,17]. Na^+-dependent and -independent phosphate transport has been found in dog BLMV but rat BLMV phosphate uptake was Na^+-independent [18–21]. Our results in the dog would agree with non-canine experiments suggesting that phosphate transport across the basolateral membrane is not Na^+-dependent [20–22]. It is possible that the divergent results of other investigators reflect substantial contamination of the BLMV preparation with BBMV.

If both transport rates and enzyme purification are taken into consideration, dog BBMV prepared by Mn^{2+} precipitation at pH 6 appear to constitute the best preparation. The main attribute of this modification is that utilization of an acid pH during the divalent-cation precipitation renders one such precipitation step sufficient (as compared with the three steps required in the original method) and, therefore, shortens the preparation time considerably.

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