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ENZYME ACTIVITIES AND SODIUM-DEPENDENT ACTIVE D-GLUCOSE TRANSPORT IN APICAL MEMBRANE VESICLES ISOLATED FROM KIDNEY EPITHELIAL CELL LINE (LLC-PK_1)

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Apical membrane vesicles were isolated from the confluent LLC-PK_1 cells by nitrogen cavitation and Mg/EGTA precipitation methods. The specific activities of marker enzymes for apical membranes were enriched 8- to 18-fold relative to those in the homogenate. D-[^{3}H]Glucose uptake into the vesicles was stimulated in the presence of Na^+ gradient (overshoot phenomenon), and the values of apparent K_m and V_{max} for Na^+-dependent component of D-glucose uptake were 0.3 mM and 5.8 nmol/mg protein per min, respectively.

The use of cell culture techniques permits the study of a relatively homogeneous population of renal epithelial cells under carefully controlled conditions [1]. Confluent LLC-PK_1 pig kidney epithelial cells form domes which are attributed to transepithelial transport of Na^+ and water [2,3]. In addition, the LLC-PK_1 cells express the differentiated characteristic of Na^+-dependent active sugar transport similar to that found in brush-border membranes of proximal tubules [4–6]. Transport studies using intact epithelial cells are complicated by the different transport systems in the apical and basolateral membranes. The present paper describes the preparation of apical membrane vesicles from the confluent LLC-PK_1 cells by the Mg/EGTA precipitation method and their use in investigating Na^+-dependent active D-glucose transport system.

LLC-PK_1 cells [2] at passage 191 obtained from the American Type Culture Collection (ATCC CRL-1392) were grown on 100 mm plastic dishes (Corning) in medium 199 (Flow Laboratories) supplemented with 10% fetal calf serum (Microbiological Associates) without antibiotics, in an atmosphere of 5% CO_2/95% air at 37°C, and were subcultured every 7 days using 0.02% EDTA and 0.05% trypsin. In most experiments, after the inoculation with $1 \cdot 10^6$ cells in 10 ml of culture medium, the cells were fed fresh medium on the 4th day, and were harvested on the 7th day for membrane preparation. The cells reached confluence in the 4–5th day after subculture. In the present study, the cells were used between the 220th and 240th passage.

Apical membrane vesicles were isolated from the LLC-PK_1 cells using some modifications of the preparation methods for brush-border membrane vesicles from rat kidney cortex [7,8]. All subsequent operations were performed at 0–4°C. After removing culture medium, each dish was washed with 10 ml of Dulbecco's phosphate-buffered saline. The cells (40 dishes) were scraped with a

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

rubber policeman into phosphate-buffered saline. The cell suspension was centrifuged at 200 × *g* for 10 min and then suspended in 40 ml of phosphate-buffered saline, followed by centrifugation at 200 × *g* for 10 min. The cells were washed with 40 ml of 300 mM mannitol, 5 mM EGTA and 12 mM Tris-HCl, pH 7.5 and centrifuged again. The packed cell pellet (2 ml) was resuspended in 38 ml of the same buffer, and the cells were gently suspended with five strokes of a loose-fitting Dounce homogenizer. The washed cell suspension was placed in nitrogen cavitation bomb (Parr Instrument Co., Illinois) at 700 lb/inch^2 for 15 min. The homogenate was collected dropwise, diluted 1 : 1 with distilled water, and then added $MgCl_2$ to a final concentration of 7 mM. After 15 min, the sample was centrifuged at 500 × *g* for 12 min (discard pellet 1). The supernate 1 was centrifuged at 48 000 × *g* for 20 min (discard supernate 2). The pellet 2 was resuspended in 40 ml of a buffer containing 150 mM mannitol, 2.5 mM EGTA and 6 mM Tris-HCl, pH 7.5 with a glass/Teflon Potter homogenizer with ten strokes at 1000 rpm, and then added $MgCl_2$ to a final concentration of 7 mM. After 15 min, the sample was centrifuged at 500 × *g* for 12 min (discard pellet 3). The supernate 3 was centrifuged at 48 000 × *g* for 20 min (discard supernate 4). The pellet 4 was resuspended in 20 ml of a buffer containing 100 mM mannitol and 20 mM Hepes-Tris, pH 7.5 (buffer A), and centrifuged at 48 000 × *g* for 20 min (discard supernate 5). The final pellet was resuspended in buffer A by sucking the suspension ten times through a fine needle (0.4 × 19 mm) (approx. 2 mg protein/ml).

Protein was measured by the method of Bradford [9], using the Bio-Rad Protein Assay Kit, with bovine gamma globulin as a standard. Aminopeptidase (EC 3.4.11.2), ($Na^+ + K^+$)-ATPase (EC 3.6.1.3), glucose-6-phosphatase (EC 3.1.3.9) and cytochrome *c* oxidase (EC 1.9.3.1) were assayed by the methods as previously described [10]. Trehalase (EC 3.2.1.28) was measured as described by Dahlqvist [11]. γ-Glutamyltransferase (EC 2.3.2.2) was measured as described by Glossmann and Neville [12]. *N*-Acetyl-β-D-glucosaminidase (EC 3.2.1.30) was measured according to Van Hoof and Hers [13], using a reaction mixture containing 0.15% Triton X-100.

Uptake of D-[1-^{3}H]glucose (Amersham, 5.5 Ci/mmol) by the isolated apical membrane vesicles was measured by a rapid filtration technique. In regular assay the reaction was initiated rapidly by adding 20 μl of buffer A, containing 0.1 mM D-[^{3}H]glucose (50 μCi/ml) plus 200 mM NaSCN or KSCN, to 20 μl of membrane vesicle suspension at 37°C. At the stated time points, the incubation was stopped by diluting a reaction sample with 1 ml of ice-cold stop solution containing 200 mM NaCl and 20 mM Hepes-Tris, pH 7.5. Immediately, the tube contents were poured onto Millipore filters (HAWP, 0.45 μm, 2.5 cm diameter) and washed with 5 ml of ice-cold stop solution. Dried filters were placed in 10 ml of scintillation fluid (toluene/Nissan Nonion/PPO/POPOP) and radioactivity was determined by a liquid scintillation counting. Background was determined by the addition of 20 μl of labeled substrate to 1 ml of ice-cold stop solution containing 20 μl of membrane vesicles. This background value was subtracted from uptake data.

Table I summarizes the recoveries and the specific activities of marker enzymes for apical membranes (aminopeptidase, γ-glutamyltransferase, trehalase), basolateral membranes ($Na^+ + K^+$)-ATPase), endoplasmic reticulum (glucose-6-phosphatase), lysosomes (*N*-acetyl-β-D-glucosaminidase), and mitochondria (cytochrome *c* oxidase) during subcellular fractionation of confluent LLC-PK_1 cells. The final apical membrane fraction contains approx. 8–18% of apical marker enzyme activities in the homogenate. The specific activities of aminopeptidase, trehalase and γ-glutamyltransferase were enriched by a factor of 18.0, 9.2 and 8.2 relative to those in the homogenate, respectively. ($Na^+ + K^+$)-ATPase and *N*-acetyl-β-D-glucosaminidase were also enriched 2.5- and 2.6-fold in the apical membrane fraction, indicating small contamination of the preparation by basolateral membranes and lysosomes. In contrast, the contamination by endoplasmic reticulum and mitochondria were minimal.

The preparation method of brush-border membrane vesicles from mammalian kidney cortex by calcium or magnesium precipitation technique is based on a different interaction of the divalent cation with brush border membranes and other cellular membranes [7,8]. However, the differences

TABLE I

MARKER ENZYME DISTRIBUTIONS DURING PURIFICATION OF APICAL MEMBRANE VESICLES FROM LLC-PK$_1$ CELLS

S.A., specific activity (nmol/min per mg protein) (ΔA_{550}/min per mg protein for cytochrome *c* oxidase). % represents the percentage of the enzyme activity found initially in the homogenate. n.d., not detectable. Each value represents the mean ± S.E. for three or four experiments.

	Amino-peptidase		γ-Glutamyl-transferase		Trehalase	
	S.A.	%	S.A.	%	S.A.	%
Homogenate	22.9 ± 1.1	100	104.2 ± 4.7	100	0.26 ± 0.01	100
Pellet 1	44.3 ± 2.5	63	109.3 ± 10.4	37	0.38 ± 0.06	46
Supernate 2	7.5 ± 0.6	14	10.2 ± 1.3	4	0.45 ± 0.29	24
Pellet 3	70.8 ± 4.3	4	226.5 ± 51.9	2	1.57 ± 1.03	2
Supernate 4&5	36.0 ± 11.6	2	144.9 ± 45.9	2	n.d.	
Apical membrane vesicles	411.4 ± 24.5	18	857.4 ± 53.2	8	2.40 ± 0.35	9

in biochemical and physicochemical properties between the apical and basolateral membranes of LLC-PK cells seem to be less marked than in the proximal tubules [3]. Therefore, in order to get purified apical membranes with high yield, the concentration of $MgCl_2$ was an important factor and was finally fixed at 7 mM.

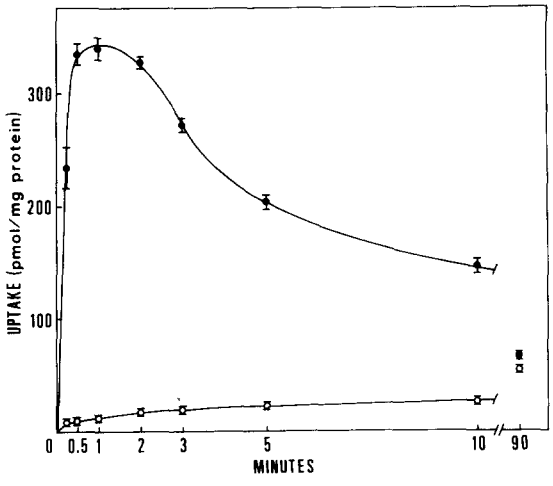

Fig. 1. Time-course of D-glucose uptake by apical membrane vesicles from LLC-PK$_1$ cells. Membrane vesicles were preincubated at 37°C in 100 mM mannitol and 20 mM Hepes-Tris (pH 7.5) for 10 min. The vesicles (20 μl, 23 μg protein) were incubated at 37°C with the substrate mixture (20 μl) containing 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5), 0.1 mM D-[^{3}H]glucose and either 200 mM NaSCN (●) or 200 mM KSCN (○). Final concentration; 0.05 mM D-glucose, 100 mM NaSCN, 100 mM KSCN. Each point represents the mean ± S.E. of three determinations from a typical experiment.

The characteristics of D-glucose transport by apical membrane vesicles were studied in the presence of an initial 100 mM NaSCN gradient or KSCN gradient (extravesicular > intravesicular). As shown in Fig. 1, the simultaneous addition of D-glucose and NaSCN to apical membrane vesicles produced clearly a transient accumulation above the equilibrium level (overshoot phenomenon). The initial rate of D-glucose uptake in the presence of Na^+ gradient was approx. 30-fold that in the presence of K^+ gradient.

Fig. 2 shows the curves for the concentration dependence of D-glucose uptake in the presence of Na^+ or K^+ gradient. The relationship between concentration and rate of uptake was nonlinear in the presence of Na^+ gradient and was linear in the presence of K^+ gradient. In order to obtain the Na^+-dependent component of D-glucose uptake, the uptake observed in the presence of K^+ was subtracted from that found in the presence of Na^+. As shown in Fig. 2 (inset), a Lineweaver-Burk plot of the Na^+-dependent component of D-glucose uptake revealed that the values of apparent K_m and V_{max} were 0.3 mM and 5.8 nmol/mg protein per min, respectively. The apparent K_m value under these conditions was compatible with

$(Na^+ + K^+)$-ATPase		Glucose-6-phosphatase		*N*-Acetyl-β-D-glucosaminidase		Cytochrome *c* oxidase		Protein %
S.A.	%	S.A.	%	S.A.	%	S.A.	%	
28.6±1.7	100	4.4±0.1	100	23.5± 1.4	100	2.9±0.2	100	100
53.9±9.7	71	8.1±1.0	56	39.8± 2.4	52	6.3±0.8	57	32
0.9±0.3	2	2.1±0.4	21	11.3± 0.8	21	0.3±0.0	4	44
56.2±7.6	3	5.6±3.3	2	43.0±11.3	1	8.1±1.3	1	1
n.d.		n.d.		n.d.		n.d.		1
72.0±2.2	3	5.6±0.7	1	60.4±10.4	3	3.8±0.5	1	3

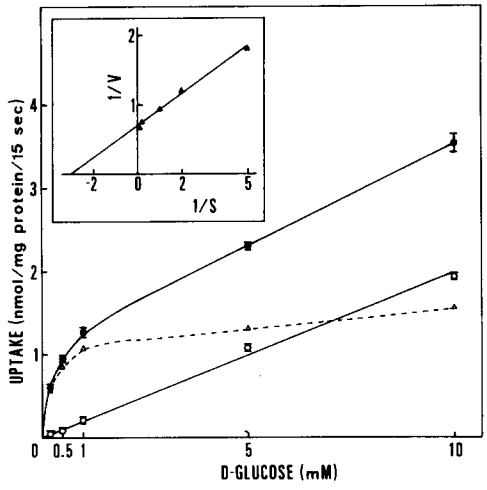

Fig. 2. Relationship between D-glucose concentration and Na^+-dependent and -independent D-glucose uptake by apical membrane vesicles from LLC-PK_1 cells. D-Glucose uptake for 15 s at the concentrations between 0.2 and 10 mM was determined in the presence of 100 mM NaSCN (●) or KSCN (○) as described for Fig. 1. Dashed line (△) represents Na^+-dependent uptake obtained by subtracting uptake in the presence of KSCN (estimated by the regression line) from uptake in the presence of NaSCN at each concentration of D-glucose. Inset shows Lineweaver-Burk plot of Na^+-dependent transport system. Each point represents the mean ± S.E. of three determinations from a typical experiment.

the values for intact LLC-PK_1 cells [4,5].

Recently, Lever [14] and Moran et al. [15] have reported Na^+-dependent hexose transport by apical membrane vesicles from LLC-PK_1 cells. However, there remain some problems to be solved in both papers: (i) lack of transient overshoot phenomenon under initial Na^+ gradient [14], (ii) considerably higher K_m value compared with that for intact LLC-PK_1 cells [14,15], and (iii) using the LLC-PK_1 cells cultured for long-term of 3–4 weeks [15]. Our data have demonstrated that apical membrane vesicles from LLC-PK_1 cells cultured for 1 week retain the characteristic of Na^+-dependent active D-glucose transport with a high-affinity system, observed in intact LLC-PK_1 cells. These discrepancies may be due to the differences of membrane preparation methods and/or cell culture conditions. Biber et al. [8] have reported that membrane vesicles isolated by the Mg/EGTA method seem to possess the better specific transport characteristics than membranes isolated by the calcium precipitation method of Evers et al. [7].

In conclusion, we have developed the preparation of apical membrane vesicles from the confluent LLC-PK_1 cells by the Mg/EGTA precipitation method, retaining the characteristic of Na^+-dependent active D-glucose transport.

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