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H^+-gradient-dependent active transport of tetraethylammonium cation in apical-membrane vesicles isolated from kidney epithelial cell line LLC-PK_1

Ken-ichi INUI, Hideyuki SAITO and Ryohei HORI*
Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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Transport of [^{3}H]tetraethylammonium (NEt_4^+), an organic cation, has been studied by using apical-membrane vesicles isolated from cultured kidney epithelial cell line LLC-PK_1. The uptake of NEt_4^+ by apical-membrane vesicles was osmotically sensitive, time-dependent and saturable. The presence of an H^+ gradient ($[H^+]_i > [H^+]_o$) induced a marked stimulation of NEt_4^+ uptake against its concentration gradient (overshoot phenomenon), and this concentrative uptake was inhibited by $HgCl_2$. These results suggest that apical membranes isolated from the LLC-PK_1 cells retain the transport characteristics of NEt_4^+ similar to those observed in renal brush-border membranes.

The use of cell-culture techniques has permitted the study of a relatively homogeneous population of renal epithelial cells under carefully controlled conditions and has offered advantages for the study of a variety of renal-cell functions, including trans-epithelial transport (Handler *et al.*, 1980). The LLC-PK_1 pig kidney epithelial-cell line (Hull *et al.*, 1976) has been characterized as a model system for the analysis of some epithelial functions in the proximal tubules. The LLC-PK_1 cells form an oriented monolayer with microvilli and tight junctions, exhibit unidirectional transport of salt and water, resulting in dome formation, and contain relatively high activities of proximal-tubule marker enzymes (Mills *et al.*, 1979, 1981; Rabito *et al.*, 1984; Hori *et al.*, 1984). Previous studies have also demonstrated that the LLC-PK_1 cells possess the transport systems for hexose (Rabito & Ausiello, 1980; Mullin *et al.*, 1980; Misfeldt & Sanders, 1981), amino acids (Rabito & Karish, 1982, 1983; Sepúlveda & Pearson, 1982) and phosphate (Rabito, 1983; Biber *et al.*, 1983) similar to those observed in the proximal tubules. Because the analysis of transport mechanisms in intact epithelial cells is complicated by factors such as (i) the simultaneous metabolism of substrates, (ii) the existence of various intracellular compartments, (iii) the different transport systems in the apical and basolateral membranes, and (iv) cell viability, it seemed desirable to examine transport characteristics by using a membrane-vesicle system. In this regard, several laboratories, including our own, have developed the preparations of apical-membrane vesicles from the LLC-PK_1 cells, and demonstrated that the apical membranes retain the characteristics of Na^+-dependent active D-glucose (Lever, 1982; Moran *et al.*, 1982; Inui *et al.*, 1984) and phosphate (Brown *et al.*, 1984) transport observed in renal brush-border membranes (Murer & Kinne, 1980).

However, there is no information about the transportability of organic ions in the LLC-PK_1 cells in relation to the mechanisms of secretion for organic ions in the proximal tubules (Rennick, 1981; Møller & Sheikh, 1982). In the present paper, therefore, we have studied the uptake of NEt_4^+, an organic cation, by apical-membrane vesicles isolated from the LLC-PK_1 cells, and have compared it with the characteristics of NEt_4^+ transport in renal brush-border membranes described previously (Takano *et al.*, 1984). The present results suggest that apical membranes isolated from the LLC-PK_1 cells retain a transport system for NEt_4^+ that has characteristics similar to those of the system observed in renal brush-border membranes. To our knowledge this is the first report of organic-ion transport in LLC-PK_1 cells.

Abbreviation used: NEt_4^+, tetraethylammonium cation.

* To whom correspondence and reprint requests should be sent.

Materials and methods

Materials

[^{3}H]NEt_4^+ bromide (93.0mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Tris, Hepes and Mes were obtained from Nakarai Chemicals (Kyoto, Japan). All other chemicals used for the experiments were of the highest purity available.

Cell culture

LLC-PK_1 cells obtained from the American Type Culture Collection (A.T.C.C. CRL-1392) were grown on 100mm-diameter plastic dishes (Corning) in medium 199 (Flow Laboratories) supplemented with 10% (v/v) fetal-calf serum (Microbiological Associates) without antibiotics, in an atmosphere of CO_2/air (1:19) at 37°C, and were subcultured every 7 days by using 0.02% EDTA and 0.05% trypsin (Inui *et al.*, 1984; Hori *et al.*, 1984). In most experiments, after the inoculation with 1×10^6 cells in 10ml of culture medium, the cells were given fresh medium on day 4 and were harvested on day 7 for preparation of membranes. The cells reached confluence in the days 4–5 after subculture. In the present study, the cells were used between passages 230 and 252.

Isolation of apical-membrane vesicles

Apical-membrane vesicles were isolated as described previously (Inui *et al.*, 1984) from confluent LLC-PK_1 cells by an Mg/EGTA precipitation method. The specific activities of γ-glutamyltransferase and trehalase, the marker enzymes for apical membranes, were enriched 8–9-fold compared with those found in the homogenate. Usually, apical membranes were suspended in a buffer comprising 100mM-mannitol and 20mM-Hepes/Tris, pH7.5. In the H^+-gradient studies for [^{3}H]NEt_4^+ uptake, 20mM-Hepes/Tris was replaced with either 10mM-Mes, pH6.0, or 10mM-Hepes, pH7.5. Protein was measured by the method of Bradford (1976) with the Bio-Rad Protein Assay Kit, and bovine γ-globulin was used as a standard.

Transport studies

The uptake of [^{3}H]NEt_4^+ by the freshly isolated membrane vesicles was measured by a rapid filtration technique (Takano *et al.*, 1984). In the regular assay, the reaction was initiated rapidly by adding 20μl of buffer, containing [^{3}H]NEt_4^+ plus KCl or NaCl, to 20μl of membrane-vesicle suspension (3–6mg of protein/ml) at 25°C. At the stated times, the incubation was stopped by diluting a reaction-mixture sample with 1ml of ice-cold stop solution comprising 200mM-LiCl and 20mM-Hepes/Tris, pH7.5. The tube contents were immediately poured on to Millipore filters (HAWP, 0.45μm-pore-size, 2.5cm diameter) and washed with 5ml of ice-cold stop solution. The radioactivity of dried filters was determined by liquid-scintillation counting. In separate experiments, non-specific adsorption was determined by the addition of labelled substrate mixture to 1ml of ice-cold stop solution containing 20μl of membrane vesicles. This value was subtracted from the uptake data.

Results and discussion

Characteristics of NEt_4^+ transport by apical-membrane vesicles

To ascertain that the uptake of NEt_4^+ by apical membranes represented transport into vesicles rather than membrane binding, the uptake at 60min was measured when the intravesicular space was decreased by increasing the medium osmolarity with mannitol. As shown in Fig. 1, there is a linear relationship between NEt_4^+ accumulation and the reciprocal of the medium

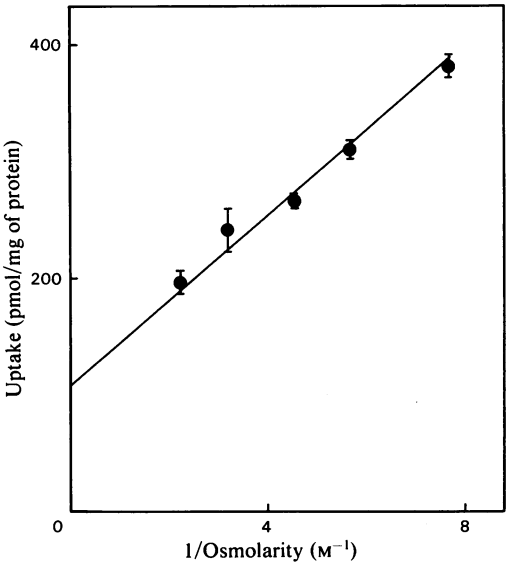

Fig. 1. *Effect of osmolarity on NEt_4^+ uptake by apical-membrane vesicles*

Membrane vesicles, suspended in 100mM-mannitol/20mM-Hepes/Tris(pH7.5)/100mM-KCl, were preincubated at 25°C for 10min. The vesicles (20μl, 57μg of protein) were incubated with the 20μl of substrate mixture [100mM-mannitol/20mM-Hepes/Tris (pH7.5)/100mM-KCl/0.5mM-[^{3}H]NEt_4^+] for 60min. The osmolarity was varied by addition of mannitol and is plotted as its inverse. Each point represents the mean ± S.E.M. for four determinations from a typical experiment.

osmolarity, suggesting that NEt_4^+ entered an interior space. Extrapolation of the line to infinite osmolarity, i.e. to zero intravesicular space, indicated that binding was approx. 30% for NEt_4^+ under the incubation conditions normally used.

Fig. 2 shows the curve for the concentration-dependence of NEt_4^+ uptake by apical-membrane vesicles. The relationship between concentration and rate of uptake was non-linear, providing evidence for saturability. In order to analyse the saturation of NEt_4^+ uptake, the uptake rate was corrected for the non-saturable component. The contribution of the non-saturable uptake could be estimated by employing the straight-line equation generated at higher NEt_4^+ concentrations and subtracting this amount from the total uptake at each concentration. As shown in Fig. 2 (inset), a Lineweaver–Burk plot of NEt_4^+ uptake after correction for the non-saturable component revealed that the values of apparent K_m and $V_{max.}$ were 1.9mM and 1.7nmol/min per mg of protein respectively. The K_m value obtained was comparable with that obtained previously for NEt_4^+ uptake in renal brush-border membraanes (Takano *et al.*, 1984).

Driving force for NEt_4^+ transport

In a previous paper (Takano *et al.*, 1984) we demonstrated that NEt_4^+ transport in renal brush-border membranes is driven by an H^+ gradient via an H^+/NEt_4^+ antiport system. In a similar fashion, the effect of an H^+ gradient on NEt_4^+ uptake by apical-membrane vesicles was studied. As shown in Fig. 3, the presence of an H^+ gradient ($[H^+]_i > [H^+]_o$) induced a marked stimulation of NEt_4^+ uptake against its concentration gradient (overshoot phenomenon). Accumulation of NEt_4^+ in apical-membrane vesicles was maximal at approx. 2min and then decreased. The final level of NEt_4^+ uptake in the presence of the H^+ gradient

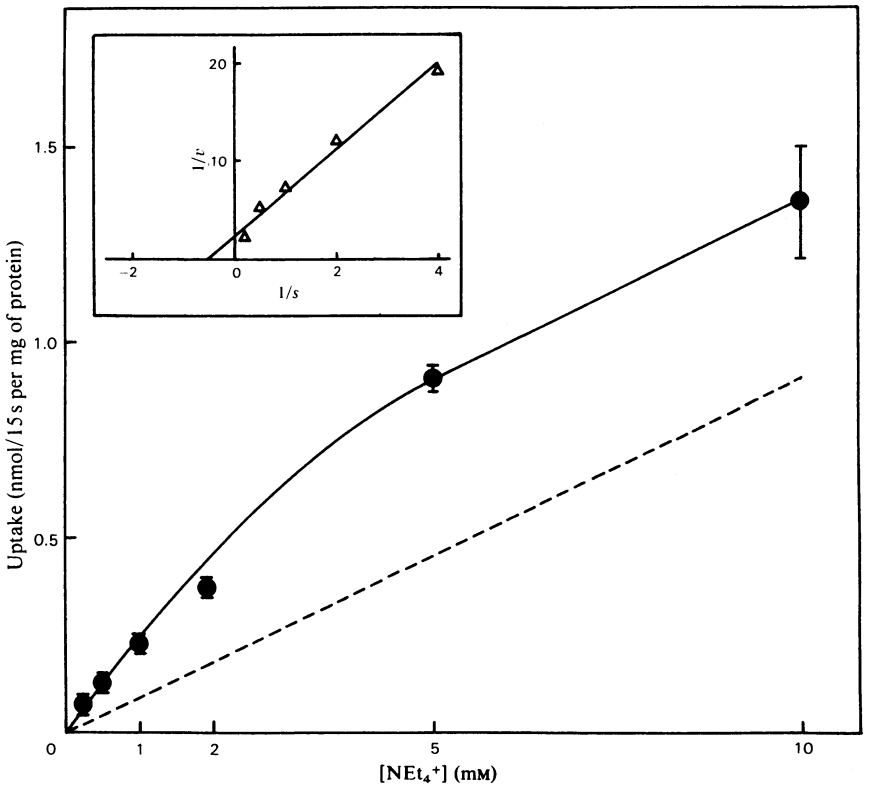

Fig. 2. *Concentration-dependence of NEt_4^+ uptake by apical-membrane vesicles*

The uptake for 15s at concentrations between 0.25 and 10mM was determined. Membrane vesicles (20μl, 68μg of protein), suspended in 100mM-mannitol/20mM-Hepes/Tris, pH 7.5, were incubated at 25°C with 20μl of substrate mixture [100mM-mannitol/20mM Hepes/Tris (pH 7.5)/200mM-KCl/[^{3}H]NEt_4^+]. The broken line represents the non-saturable component and the inset shows the Lineweaver–Burk plot of NEt_4^+ uptake after correction for the non-saturable component as described in the text. Each point represents the mean ± S.E.M. for four determinations from a typical experiment.

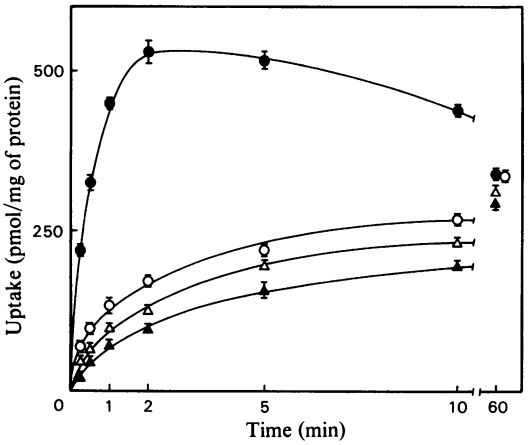

Fig. 3. *Effect of H^+ gradient on NEt_4^+ uptake by apical-membrane vesicles*

Membrane vesicles (20μl, 65μg of protein for pH7.5, 69μg of protein for pH6.0), suspended in 100mM-mannitol/100mM-KCl and either 10mM-Hepes, pH7.5 (○, ▲) or 10mM-Mes, pH6.0 (●, △), were incubated with 80μl of substrate mixture [100mM-mannitol/100mM-KCl/0.125mM-[^{3}H]-NEt_4^+ and either 10mM-Hepes, pH7.5 (○, ●) or 10mM-Mes, pH6.0 (△, ▲)]. Each point represents the mean±S.E.M. for three determinations from a typical experiment.

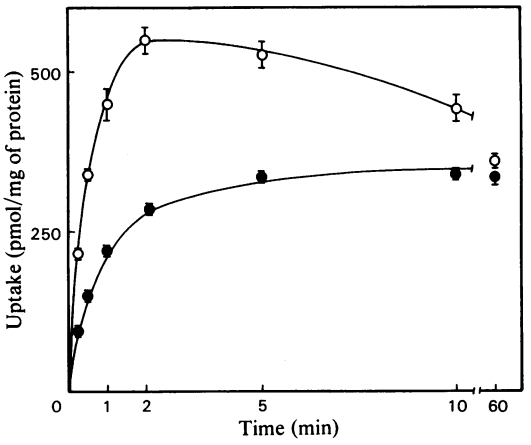

Fig. 4. *Effect of $HgCl_2$ on NEt_4^+ uptake by apical-membrane vesicles*

Membrane vesicles (20μl, 115μg of protein) suspended in 100mM-mannitol/10mM-Mes (pH6.0)/100mM-KCl were incubated with 80μl of substrate mixture [100mM-mannitol/10mM-Hepes (pH7.5)/100mM-KCl/0.125mM-[^{3}H]NEt_4^+ in the presence (●) or absence (○) of 0.125mM-$HgCl_2$]. Each point represents the mean±S.E.M. for three determinations from a typical experiment.

($[H^+]_i > [H^+]_o$) was identical with that attained in the absence of the gradient or in the presence of the reverse gradient ($[H^+]_i < [H^+]_o$).

Furthermore, the effect of $HgCl_2$ on NEt_4^+ uptake by apical-membrane vesicles was examined. As shown in Fig. 4, the concentrative uptake of NEt_4^+ driven by an H^+ gradient was completely inhibited by 0.1mM-$HgCl_2$. In another experiment, $HgCl_2$ also inhibited NEt_4^+ uptake in the absence of an H^+ gradient (results not shown). On the basis of these results, it seemed reasonable to assume that the H^+-gradient-dependent NEt_4^+ transport in apical membranes is a specific carrier-mediated process as well as in renal brush-border membranes.

In conclusion, the present results suggest that the apical-membrane vesicles isolated from the LLC-PK_1 cells retain an H^+/NEt_4^+ antiport system in addition to Na^+-dependent hexose-, phosphate- and amino-acid-transport systems. The transport of NEt_4^+ in apical membranes isolated from LLC-PK_1 cells shows characteristics similar to those of the transport system identified in renal brush-border membranes: (i) the uptake of NEt_4^+ by apical-membrane vesicles is saturable; (ii) it is markedly stimulated by an outwardly directed H^+ gradient; (iii) the concentrative uptake of NEt_4^+ was inhibited by $HgCl_2$. Thus the present findings reinforce the usefulness of the LLC-PK_1 cells as a model epithelium to study organic-ion transport in renal proximal tubules.

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