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Transport of procainamide in a kidney epithelial cell line LLC-PK$_1$

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Transport of procainamide, an anti-arrhythmic drug, was investigated in LLC-PK$_1$ kidney epithelial cell line. The uptake of procainamide by LLC-PK$_1$ monolayers cultured in plastic dishes was temperature-dependent, saturable and inhibited by organic cations such as cimetidine and *N*-acetylprocainamide. An aminocephalosporin antibiotic, cephalexin, also inhibited procainamide uptake, but an organic anion, *p*-aminohippurate, did not. The uptake of procainamide was greater at an alkaline external pH than at an acidic pH. In addition, procainamide uptake increased when intracellular pH was decreased and the uptake decreased when the intracellular pH was increased by ammonium chloride treatment, indicating the involvement of an H^+/procainamide antiport system in apical membrane. The basolateral to apical flux of procainamide across LLC-PK$_1$ monolayers cultured on permeable supports was 2.5-times larger than the apical to basolateral flux, and only the former process was inhibited by other organic cations. These findings suggest that LLC-PK$_1$ cells can transport procainamide by the organic cation transport system and that procainamide is transported unidirectionally from basolateral to apical side across the cell monolayers.

Introduction

Procainamide, an anti-arrhythmic drug, is an organic cation and the kidney plays a major role in its disposition [1,2]. Using rabbit proximal tubules perfused in vitro, McKinney and Speeg [3] reported that procainamide was actively secreted into the tubular lumen by an organic cation transport system. A number of studies using isolated renal brush-border and basolateral membrane vesicles, including those from our laboratory, have provided a great deal of information about the transport mechanisms of organic cations in the kidney. Specific transport of typical organic cations such as tetraethylammonium and N^1-methylnicotinamide has been shown in both basolateral and brush-border membranes [4–7]. In basolateral membrane, organic cations are transported via a carrier-mediated system that is stimulated by the intracellular negative potential [5,8]. In brush-border membrane, the transport of organic cations is driven by an H^+ gradient via an electroneutral H^+/organic cation antiport system [5,9,10]. The transport mechanisms of procainamide in renal plasma membranes should be similar to those of tetraethylammonium [8,11]. However, Sokol and McKinney [8] showed that, in contrast to tetraethylammonium, procainamide did not produce a trans-stimulation of [^{3}H]procainamide transport in rabbit basolateral membrane, and they suggested that there may be a family of related transporters responcible for the initial step in renal tubular secretion of organic cations. Thus, further studies are needed to clarify the transport mechanism of procainamide across renal epithelial cells.

LLC-PK$_1$, a cell line derived from pig kidney [12], possesses a structure and function similar to those of renal proximal tubular cells [13]. We and others have been using this cell line for elucidating transport properties at the cellular level as well as to study the gene expression of some transporters in renal epithelia [14–17]. Recently, LLC-PK$_1$ cells were found to have the ability to transport tetraethylammonium and N^1-methylnicotinamide [18–20]. However, the number of

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studies on organic cation transport using culture cells is still limited, and the involvement of transport mechanisms revealed by membrane vesicle studies (for example, H^+/organic cation antiport in brush-border membrane) has not been established in intact epithelial cells. In addition, in contrast to the membrane vesicle studies, the transport of organic cations which are used for therapeutic purpose has not been tested in LLC-PK_1 cells. In the present study, we studied the transport characteristics of procainamide in LLC-PK_1 cells, either by the cells cultured in plastic dishes or by those cultured on permeable supports.

Materials and Methods

Cell culture

LLC-PK_1 cells obtained from the American Type Culture Collection (ATCC CRL-1392) were cultured in plastic dishes (Corning Glass Works, Corning, NY) in medium 199 (Flow Laboratories, Rockville, MD) supplemented with 10% fetal bovine serum (Microbiological Associates, Bethesda, MD) without antibiotics, in an atmosphere of 5% CO_2/95% air at 37°C, and were subcultured every 4 to 5 days using 0.02% EDTA and 0.05% trypsin [17,21,22]. The cells were used between passages 222 and 245.

Measurement of procainamide uptake by LLC-PK_1 cells

For the uptake experiments, 60-mm dishes were seeded with $4 \cdot 10^5$ cells in 5 ml of complete culture medium (Medium 199 supplemented with 10% fetal bovine serum). The cells were given fresh medium every 2–4 days after inoculation, and were used between the 5th and 7th days (confluence). The uptake of procainamide was measured at 37°C or 4°C (on ice) as previously described [14]. Briefly, the uptake assays were performed in Dulbecco's phosphate-buffered saline (PBS buffer; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). After removal of the culture medium, each dish was washed twice with 5 ml of PBS buffer and allowed to preincubate for 10 min. Then, PBS buffer (2 ml) containing procainamide was added to each dish and the cells were incubated for a specified period. At the end of the incubation period, the medium was immediately aspirated and the dish was rapidly rinsed three times with 5 ml of ice-cold PBS buffer. The cells were scraped with a rubber policeman into 2 ml of ice-cold PBS buffer and homogenized with a Polytron (Kinematica, Kriens-Luzern, Switzerland) at a setting of 7 for 1 min. Procainamide was determined by fluorescence polarization immunoassay.

Measurement of the transepithelial flux of procainamide

The transepithelial procainamide flux was measured in the LLC-PK_1 monolayers cultured in Transwell chambers (Costar, Cambridge, MA). To prepare cell monolayers, cells were seeded at a density of $4 \cdot 10^5$ cells/cm^2 on polycarbonate membrane filters (3 μm pores) in Transwell cell chambers (4.71 cm^2 surface area), and the chambers were placed in six-well, cluster plates. The volume of medium inside and outside the Transwell chambers was 1.5 ml and 2.6 ml, respectively. Fresh medium was replaced every 2–3 days, and the cells were used between the 5th and 7th days.

Cell monolayers were washed with PBS buffer and allowed to preincubate for 10 min at 37°C. Transport measurements were initiated by adding PBS buffer containing procainamide either to the apical or to the basolateral side of the monolayer. After incubation for 15, 30, 45 and 60 min, the medium in the other side was collected (100 μl), and the rate of appearance of procainamide was measured by high-performance liquid chromatography (HPLC) as described below.

Analytical methods

In the measurement of uptake by the cells, procainamide was determined by fluorescence polarization immunoassay using the TDX system (Dainabot, Tokyo, Japan). In the transepithelial transport studies, procainamide was measured by a high-performance liquid chromatograph LC-3A (Shimadzu, Kyoto, Japan) equipped with a fluorescence spectromonitor RF-500 LC (Shimadzu). The conditions used for HPLC were as follows: column, Zorbax-CN 25 cm × 4.6 mm (Shimadzu); mobile phase, 1.3% sodium acetate (pH 5.8)/acetonitrile = 46:54; flow rate, 0.8 ml/min; excitation wavelength, 288 nm; emission wavelength, 356 nm; injection volume, 50 μl; temperature, 40°C. In some experiments, procainamide was measured by the TDX system and by HPLC, and both assay methods gave almost the same results. Protein was determined by the method of Bradford [23], using the Bio-Rad Protein Assay Kit, with bovine γ-globulin as standard.

Materials

Procainamide, *N*-acetylprocainamide, cimetidine and *p*-aminohippurate were purchased from Sigma Chemical (St. Louis, MO). Cephalexin was generously provided by Shionogi (Osaka, Japan). All other chemicals used were of the highest purity available.

Results

The time course of procainamide uptake by LLC-PK_1 cells cultured in plastic dishes was measured at 37°C or 4°C to determine the general transport characteristics. As shown in Fig. 1, the uptake of procainamide at 37°C reached a near-steady state by 10 min. At 4°C, the uptake of procainamide was drastically reduced. Thus, the uptake of procainamide by LLC-PK_1 cells was time- and temperature-dependent.

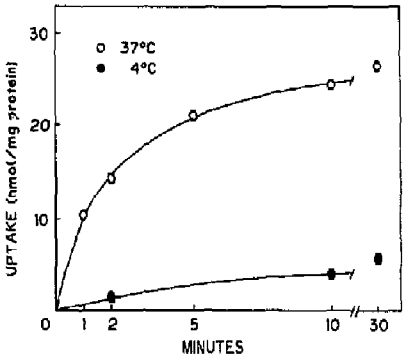

Fig. 1. Time course of procainamide uptake by LLC-PK$_1$ cells. LLC-PK$_1$ cells were incubated in 2 ml of PBS buffer containing 1 mM procainamide for the indicated times at 37°C (○) or at 4°C (●). Each point represents the mean ± S.E. of three determinations.

Fig. 2 shows the time course of procainamide efflux from LLC-PK$_1$ cells preincubated with the drug for 30 min. The efflux of procainamide at 37°C was very rapid and after 10 min, intracellular procainamide was almost undetectable. In contrast, at 4°C, nearly 90% of procainamide remained in the cells even after 30 min incubation.

Fig. 3 shows the concentration dependence of procainamide uptake by LLC-PK$_1$ cells. The relationship between concentration and the rate of uptake approached saturation, but never attained it. An explanation for this phenomenon is that procainamide enters

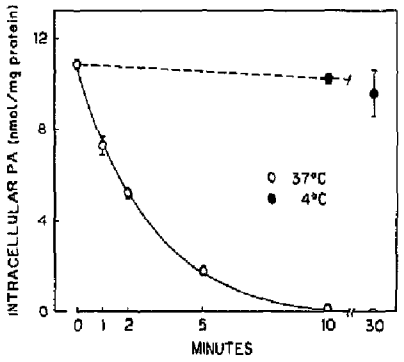

Fig. 2. Efflux of procainamide (PA) from LLC-PK$_1$ cells. LLC-PK$_1$ cells were preincubated at 37°C in 2 ml of PBS buffer containing 0.5 mM procainamide for 30 min. At the end of preincubation, cells were rapidly washed with ice-cold PBS buffer. Then, 5 ml of PBS buffer was added to each dish and the cells were incubated for the indicated times at 37°C (○) or at 4°C (●). At the stated times, procainamide remaining in the cells was determined. Each point represents the mean ± S.E. of three determinations except for two determinations of the uptake at 4°C.

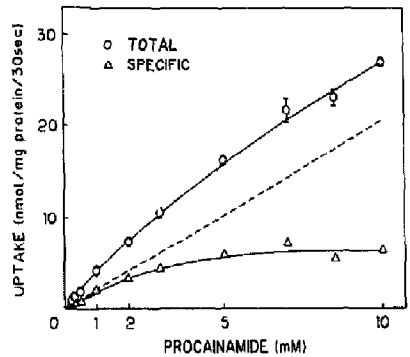

Fig. 3. Concentration dependence of procainamide uptake by LLC-PK$_1$ cells. The uptake for 30 s at concentrations between 0.2 and 10 mM was determined. LLC-PK$_1$ cells were incubated in 2 ml of PBS buffer containing procainamide at 37°C. The osmolarity of the uptake medium was kept constant by adding an adequate concentration of mannitol. Solid lines indicate the total uptake (○) and saturable uptake (△), and dashed line indicates the non-saturable component. Each point represents the mean ± S.E. of three determinations.

the cells by two processes, a saturable and a non-saturable process. Therefore, the initial rate of procainamide uptake can be expressed by the following equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} + k_d[S]$$

where V is the initial uptake rate, [S] is the initial concentration, V_{max} is the maximum uptake rate by a saturable process, K_m is the Michaelis constant, and k_d is the coefficient of simple diffusion (non-saturable process). The contribution of the non-saturable uptake (k_d[S]) was estimated by employing the straight-line equation generated at higher procainamide concentrations, and the saturable uptake was analyzed after subtracting non-saturable uptake from the total uptake at each concentration [4,5,24]. The values of apparent K_m and V_{max} for saturable transport were 3.2 mM and 17.8 nmol/mg of protein per min, respectively.

We examined the effects of other organic ions on the uptake of procainamide by LLC-PK$_1$ cells (Fig. 4). Procainamide uptake was markedly inhibited by organic cations such as cimetidine and *N*-acetylprocainamide (a metabolite of procainamide), although the uptake was not inhibited by *p*-aminohippurate, a typical organic anion.

The effect of medium pH on procainamide uptake was studied (Fig. 5A). Procainamide uptake was greater at pH 8.4 and lower at pH 6.0 compared with the uptake at pH 7.4. We also examined the effect of alterations in intracellular pH produced by ammonium chloride on procainamide uptake. When LLC-PK$_1$ cells are exposed to ammonium chloride (acute treatment),

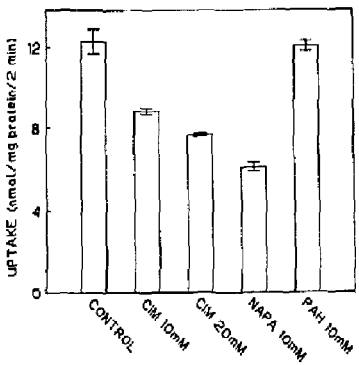

Fig. 4. Effect of various organic ions on procainamide uptake by LLC-PK$_1$ cells. LLC-PK$_1$ cells were incubated in 2 ml of PBS buffer containing 1 mM procainamide for 2 min in the presence or absence of organic ions at the concentrations shown: CIM, cimetidine; NAPA, *N*-acetylprocainamide; and PAH, *p*-aminohippurate. Each value represents the mean ± S.E. of three determinations.

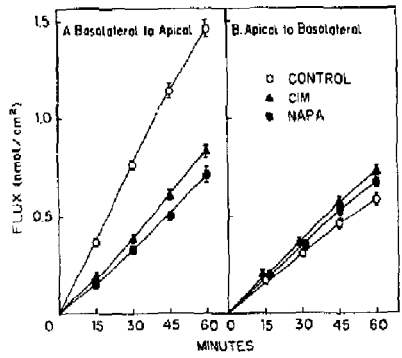

Fig. 6. Transport of procainamide across LLC-PK$_1$ monolayers. (A) Procainamide (20 μM) was added to the basolateral side of cell monolayers in the absence (○) or presence of 10 mM cimetidine (CIM; ▲) or *N*-acetylprocainamide (NAPA; ●), and basolateral to apical fluxes were measured by collecting 100 μl of apical medium at 15, 30, 45, and 60 min. (B) Procainamide (20 μM) was added to the apical side in the absence (○) or presence of 10 mM cimetidine (▲) or *N*-acetylprocainamide (●), and apical to basolateral fluxes were measured by collecting 100 μl of basolateral medium. Each point represents the mean ± S.E. of 4–7 determinations.

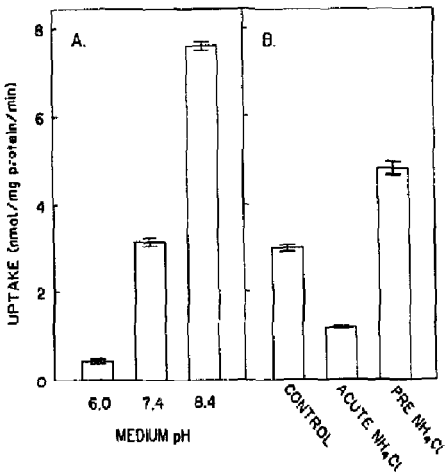

Fig. 5. Effect of medium pH (A) and intracellular pH (B) on procainamide uptake by LLC-PK$_1$ cells. (A) LLC-PK$_1$ cells were incubated in 2 ml of PBS buffer with different pH containing 0.5 mM procainamide for 1 min. The pH of PBS buffer was adjusted to 6.0, 7.4, and 8.4 by adding a solution of hydrochloric acid or sodium hydroxide. Each value represents the mean ± S.E. of five or six determinations. (B) LLC-PK$_1$ cells were preincubated in 5 ml of PBS buffer in the absence (Control, Acute NH_4Cl) or presence (Pre NH_4Cl) of 30 mM NH_4Cl for 20 min. Then, the preincubation medium was aspirated, and the cells were incubated in 2 ml of PBS buffer containing 0.5 mM procainamide for 1 min in the absence (Control, Pre NH_4Cl) or presence (Acute NH_4Cl) of 30 mM NH_4Cl. The osmolarity of the uptake medium was kept constant by adding mannitol. Each value represents the mean ± S.E. of four or five determinations.

intracellular pH rapidly becomes more alkaline. On the contrary, when ammonium chloride is added to the preincubation medium and then is removed (pretreatment), intracellular pH falls [18,25]. As shown in Fig. 5B, procainamide uptake by LLC-PK$_1$ cells was increased by the pretreatment and decreased by the acute treatment of cells with ammonium chloride.

Next, in order to study transepithelial transport of procainamide, we measured the unidirectional flux of procainamide across LLC-PK$_1$ cell monolayers cultured on permeable supports. Fig. 6 shows the basolateral to apical (Fig. 6A) and apical to basolateral (Fig. 6B) flux of procainamide. The basolateral to apical flux of procainamide was about 2.5-times larger than the reverse. Adding cimetidine or *N*-acetylprocainamide to the basolateral side decreased basolateral to apical procainamide flux. On the other hand, apical to basolateral flux was not inhibited by these organic cations.

Fig. 7 shows the cis effect of cephalexin, an aminocephalosporin antibiotic, on the uptake (Fig. 7A) and the unidirectional flux (Fig. 7B) of procainamide. Cephalexin markedly inhibited the uptake of procainamide by LLC-PK$_1$ cells cultured in plastic dishes. On the other hand, the inhibitory effect on the basolateral to apical flux of procainamide was relatively weak.

Discussion

The present results demonstrate that procainamide can be transported by the organic cation transport system in the kidney epithelial cell line LLC-PK$_1$. First,

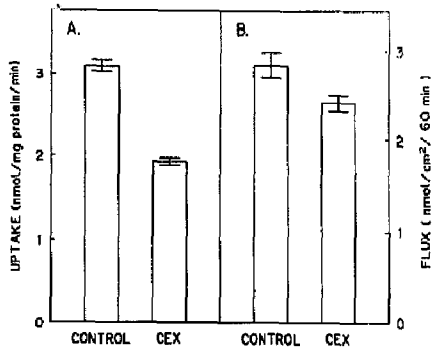

Fig. 7. Effect of cephalexin on uptake (A) and basolateral to apical flux (B) of procainamide in LLC-PK_1 cells. (A) LLC-PK_1 cells grown in plastic dishes were incubated in 2 ml of PBS buffer containing 0.5 mM procainamide for 1 min in the absence or presence of 10 mM cephalexin (CEX). Each value represents the mean ± S.E. of five determinations. (B) Basolateral to apical flux of procainamide (50 μM) across LLC-PK_1 cell monolayers was measured as described in Fig. 6A in the absence or presence of 10 mM cephalexin (CEX, basolateral side). Each value represents the mean ± S.E. of five or six determinations.

the uptake of procainamide by LLC-PK_1 monolayers cultured in plastic dishes was temperature-dependent, saturable and inhibited by organic cations but not by an organic anion. In addition, the uptake was dependent on the medium and intracellular pH. Secondly, basolateral to apical procainamide flux across LLC-PK_1 monolayers cultured on permeable supports was larger than the reverse and was inhibited by organic cations.

In the cellular uptake study, we measured procainamide uptake by LLC-PK_1 monolayers cultured in plastic dishes. The results suggest that the specifically mediated system is involved in procainamide transport in the apical membrane. This finding is consistent with our previous report describing that tetraethylammonium, a typical organic cation, is transported by a carrier-mediated system in apical membrane vesicles isolated from LLC-PK_1 cells [26]. In addition, McKinney et al. [18] reported that tetraethylammonium accumulated inside LLC-PK_1 cells grown on plastic dishes by a saturable, mediated mechanism in the apical cell membrane. Thus, apical membrane of LLC-PK_1 cells should have the organic cation transport system which can transport various substrates such as tetraethylammonium and procainamide, as do the renal brush-border membranes. However, the apparent K_m value of procainamide uptake in the present study was higher than that reported by McKinney and Kunnemann [11] using brush-border membrane vesicles isolated from rabbit kidney ($K_m = 0.54$ mM). The reason for this discrepancy is not clear. It may be due to the difference of experimental systems. For example, McKinney and Kunnemann [11] measured procainamide uptake by brush-border membrane vesicles in the presence of a relatively large pH gradient (1.5 pH unit). Alternatively, the influx of procainamide across the apical membrane should be unphysiological direction and therefore the apparent K_m might become larger in intact cells.

Proton gradient-dependent uphill transport of organic cations (H^+/organic cation antiport) was demonstrated in renal brush-border membrane vesicles from rat, dog and rabbit [5,9,11]. However, the involvement of the H^+/organic cation antiport system has not been well established in intact cells. McKinney et al. [18] showed that tetraethylammonium uptake by LLC-PK_1 cells was influenced by the pH of the incubation medium with greatest uptake occurring at the most alkaline pH. On the contrary, they did not observe the effect of cell pH which was altered by ammonium chloride on tetraethylammonium uptake. Taken together, they discussed that H^+/organic cation antiport should not be the predominant mechanism for tetraethylammonium uptake in apical membrane of LLC-PK_1 cells. In the present study, we also tested the effect of medium pH on procainamide uptake as well as the effect of intracellular pH which was altered by ammonium chloride treatment of cells. In contrast to the results of McKinney et al. [18], procainamide uptake was changed under both experimental conditions, and in a manner which can be explained by H^+/procainamide antiport mechanism. Thus, H^+/organic cation antiport system may be involved in procainamide transport across the apical cell membrane of LLC-PK_1. Recently, Yuan et al. [27] demonstrated the presence of H^+/tetraethylammonium antiport system in the apical membrane of opossum kidney (OK) cells, another cell line derived from the American opossum kidney. Further studies are needed to clarify the role of H^+/organic cation antiport system in the transepithelial transport of procainamide.

Using LLC-PK_1 monolayers cultured on permeable supports, we demonstrated that basolateral to apical procainamide flux was 2.5-times larger than apical to basolateral flux, and only basolateral to apical flux was inhibited by other organic cations. These results indicate that specifically mediated flux of procainamide across LLC-PK_1 monolayers occurs only from the basolateral to apical side, and the apical to basolateral flux represents a non-specific process such as a paracellular transport (leakage through the intercellular lateral space). On the other hand, procainamide uptake by LLC-PK_1 cells cultured in plastic dishes (which should reflect the apical transport) was inhibited by other organic cations as described above. Taken together, these results suggest that, when procainamide was added to the apical side, the drug can enter the cells across the apical membrane but can hardly be transported from intracellular compartment to basolateral

side across the basolateral membrane. We have previously shown that organic cation transport in basolateral membrane, but not in brush-border membrane, was sensitive to the membrane potential [5]. Therefore, the interior negative potential of the cells should restrict the procainamide flux from inside the cells to basolateral side across the basolateral membrane, resulting in the unidirectional transport of procainamide from basolateral to apical side across LLC-PK_1 monolayers. When compared with the renal proximal tubule, this unidirectional transport should correspond to the tubular secretion. Transport of tetraethylammonium and N^1-methylnicotinamide was also shown to be unidirectional in LLC-PK_1 monolayers [19,20].

Cellular uptake and the transepithelial flux of procainamide decreased in the presence of cimetidine or *N*-acetylprocainamide. These results suggest that procainamide can share a common transport system with cimetidine and *N*-acetylprocainamide in LLC-PK_1 cells. Cimetidine, a histamine H_2-receptor antagonist, is transported by organic cation transport systems in rat renal brush-border and basolateral membranes [28]. Several reports have demonstrated that procainamide and cimetidine compete for a common secretory mechanism in proximal tubules perfused in vitro [3], in brush-border membrane vesicles [29], and in isolated perfused rat kidney [30]. *N*-Acetylprocainamide is an active metabolite of procainamide and is mainly excreted unchanged in urine [2,31]. Funck-Brentano et al. [32] demonstrated that the accumulation of *N*-acetylprocainamide during procainamide therapy can alter procainamide elimination in man. They discussed that the pharmacokinetic interaction between *N*-acetylprocainamide and procainamide is due to competition for renal proximal tubule secretion. McKinney and Speeg [3] showed that *N*-acetylprocainamide had an inhibitory effect on procainamide secretion by proximal tubules in vitro. The present study in LLC-PK_1 cells provides direct evidences indicating that the competition for secretion between these organic cations occurs at the cellular level in renal proximal tubules.

We have previously shown that aminocephalosporin antibiotics such as cephalexin and cephradine can be transported by an organic cation transport system in renal brush-border membrane [33]. In brush-border membrane vesicles, aminocephalosporin antibiotics inhibited tetraethylammonium uptake, and the uptake of these drugs were actively driven by an outward H^+ gradient [33]. Thus, aminocephalosporin antibiotics are substrates for H^+/organic cation antiport system in brush-border membrane. On the other hand, cephalexin did not affect tetraethylammonium transport in basolateral membrane [34]. Therefore, it is interesting to compare the effect of cephalexin on the apical uptake and transepithelial flux of procainamide in LLC-PK_1 cells. Cephalexin markedly inhibited the apical uptake of procainamide, but the effect on transepithelial flux was relatively weak. The initial step of the transepithelial flux is the uptake from basolateral side into the cells across basolateral membrane. Therefore, cephalexin may affect procainamide transport in apical membrane in preference to that in basolateral membrane, which is consistent with the finding obtained by membrane vesicle studies [33,34]. Thus, organic cation transport systems in the apical and basolateral membranes of LLC-PK_1 may have similar substrate specificities with those in renal brush-border and basolateral membranes, respectively.

In conclusion, procainamide can be transported in LLC-PK_1 cells by an organic cation transport system. LLC-PK_1 cells should provide a useful model system to study the cellular and transepithelial transport mechanisms of organic cations and drug interaction in renal proximal tubules.

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