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Uptake of L-3,4-dihydroxyphenylalanine and Dopamine Formation in Cultured Renal Epithelial Cells

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ABSTRACT. In the presence of benserazide (50 μM), L-3,4-dihydroxyphenylalanine (L-DOPA) was rapidly accumulated in both LLC-PK_1 and OK cells; equilibrium was attained at 30 min of incubation. For these LLC-PK_1 and OK cells, the analysis revealed a rate constant of inward transport (k_{in} in pmol/mg protein/min) of 3.6 ± 0.4 and 18.1 ± 0.3 and a rate constant of outward transport (k_{out} in pmol/mg protein/min) of 1.0 ± 0.1 and 5.2 ± 0.1, respectively. Nonlinear analysis of the saturation curves for LLC-PK_1 and OK cells revealed a K_m (in μM) of 86 ± 12 and 14 ± 4, respectively. The cellular accumulation of the substrate was temperature-dependent and stereoselective. Aromatic L-amino acid decarboxylase (AAAD) activity was determined in cell homogenates; nonlinear analysis of the saturation curves revealed, for LLC-PK_1 and OK cells, a K_m (in μM) of 1866 ± 107 and 845 ± 153 and a V_{max} (in nmol/mg protein/15 min) of 4.4 ± 0.1 and 0.9 ± 0.1, respectively. In the absence of benserazide, only a limited amount of the L-DOPA taken up was decarboxylated to dopamine in cell monolayers; the K_m value (in μM) for decarboxylation of intracellular L-DOPA in LLC-PK_1 and OK cells was 61 ± 14 and 108 ± 36, respectively. A low amount of newly formed dopamine was found to escape to the apical bathing fluid. This outward transfer of newly formed dopamine was a nonsaturable process up to 300 μM intracellular dopamine. In conclusion, the data presented here show that OK cells are endowed with a more efficient L-DOPA uptake system than LLC-PK_1 cells, but the latter are endowed with a significantly higher AAAD activity than OK cells. In both cell lines, intracellular L-DOPA is rapidly converted to dopamine, some of which diffuses out of the cell. BIOCHEM PHARMACOL **54**;9:1037–1046, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. renal cells; L-DOPA; dopamine; uptake; decarboxylase

In recent years it has been suggested that dopamine of renal origin plays an important role in the regulation of tubular sodium handling, as a result of activation of specific tubular dopamine receptors [1]. The effects of dopamine on tubular sodium absorption are believed to result from inhibition of Na^+-K^+-ATPase and Na^+-H^+ exchanger activities at the level of the proximal nephron [2–4]. The source of dopamine responsible for these natriuretic effects is believed to reside in epithelial cells of the proximal convoluted tubules. These are enriched aromatic L-amino acid decarboxylase (AAAD†) cells which use circulating or filtered L-3,4-dihydroxyphenylalanine (L-DOPA) as a source for dopamine and are believed to be the basic cellular elements of a local nonneuronal dopaminergic system [5, 6]. An interesting characteristic of this system concerns the fact that these epithelial cells, in which the synthesis of dopamine occurs, are also endowed with dopamine receptors, leading to the possibility that dopamine may act as an autocrine/paracrine substance [7].

Early studies have demonstrated that the renal tubular transport of L-DOPA occurs through an energy-dependent and stereoselective carrier-mediated process [8]. More recently, the synthesis of dopamine in tubular epithelial cells has been shown to be closely dependent on extracellular sodium [5], the mechanisms involved in transtubular reabsorption of sodium [9–12], the integrity of the tubular cytoskeleton and the functional integrity of the Na^+-K^+-ATPase [13]. The rise in intracellular levels of cyclic GMP, as induced by the α-human atrial natriuretic peptide, by zaprinast (MB 22,948), a relatively specific guanosine 3′:5′-cyclic monophosphate (cGMP) phosphodiesterase inhibitor, or by 8-bromo cGMP, has also been found to restrict the intracellular availability of L-DOPA and decrease the renal formation of dopamine [14, 15]. However, despite the evidence on an association between the tubular reabsorption of sodium, the levels of cGMP and the uptake of L-DOPA, information is still lacking on the nature and characteristics of the processes involved in the renal formation of dopamine.

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† *Abbreviations:* AAAD, aromatic L-amino acid decarboxylase; cAMP, adenosine 3′:5′-cyclic monophosphate; C_i, intracellular concentration; cGMP, guanosine 3′:5′-cyclic monophosphate; C_o, extracellular concentration; D-DOPA, D-3,4-dihydroxyphenylalanine; k_{in}, rate constant for inward transport; k_{out}, rate constant for outward transport; L-DOPA, L-3,4-dihydroxyphenylalanine.

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Several renal cell lines are often used as physiological model systems of renal proximal tubule function. Both LLC-PK_1 and OK cells express proximal tubule cell-like properties *in vitro* and have been used to study dopamine receptors and the renal actions of the amine. LLC-PK_1 cells have been shown to contain high levels of AAAD and to convert L-DOPA to dopamine in a nonsaturable fashion up to 1 mM L-DOPA [16, 17]. Newly formed dopamine also stimulated adenosine 3′:5′-cyclic monophosphate (cAMP) accumulation in LLC-PK_1, and this autocrine/paracrine effect was attenuated by an equimolar concentration of carbidopa and blocked by the D_1 antagonist Sch 23390 [17]. OK cells convert L-DOPA to dopamine in sufficient amounts to elicit both the maximum dopamine-stimulated cAMP accumulation and inhibition of Na^+-Pi cotransport [18]. Initially, OK cells were demonstrated to express the D_{1A} receptor exclusively, although the expression of both dopamine D_{1A} and D_{1B} receptor subtypes was detected in both the opossum brain and the opossum kidney [19]. More recently, Perrichot et al. [20], while studying the involvement of dopamine receptor subtypes in regulation of renal P_i transport by dopamine, either exogenous or locally synthesised from L-DOPA in OK cells, came to the conclusion that these cells also synthesise dopamine and that the newly formed amine modulates phosphate transport through the activation of both D_1 and D_2 receptors, positively and negatively coupled to adenylate cyclase. It appears, therefore, that both OK and LLC-PK_1 cells may constitute a useful *in vitro* model for the study of renal dopaminergic physiology.

The aim of the present work was to comparatively evaluate the cellular uptake of L-DOPA and its intracellular decarboxylation by AAAD to dopamine in these two renal cell lines. We report here that both LLC-PK_1 and OK cells take up L-DOPA through a saturable, stereoselective and temperature-dependent process when applied from the apical cell border, but OK cells are endowed with a more efficient L-DOPA uptake system than LLC-PK_1 cells. Though LLC-PK_1 cells are endowed with a significantly higher AAAD activity than OK cells, both cell lines rapidly convert intracellular L-DOPA to dopamine, some of which diffuses out of the cell.

MATERIALS AND METHODS

Cell Culture

LLC-PK_1 cells, a porcine-derived proximal renal tubule epithelial cell line, and OK cells, a cell line derived from the kidney of a female American opossum which retains several properties of proximal tubular epithelial cells in culture, were obtained from the American Type Culture Collection and maintained in a humidified atmosphere of 5% CO_2–95% air at 37°C. LLC-PK_1 cells (ATCC CRL 1392; passages 198–206) were grown in Medium 199 (Sigma Chemical Company) supplemented with 100 U/mL penicillin G, 0.25 μg/mL amphotericin B, 100 μg/mL streptomycin (Sigma), 3% foetal bovine serum (Sigma) and 25 mM HEPES (Sigma), whereas OK cells (ATCC CRL 1840; passages 37–43) were grown in minimum essential medium (Sigma) supplemented with 100 U/mL penicillin G, 0.25 μg/mL amphotericin B, 100 μg/mL streptomycin, 10% foetal bovine serum and 25 mM HEPES. The medium was changed every 2 days, and the cells reached confluence after 3–5 days of incubation. For subculturing, the cells were dissociated with 0.05% trypsin-EDTA, split 1:4 and subcultured in Costar flasks with 75- or 162-cm^2 growth areas. For studies in monolayers, the cells were seeded in collagen-treated 24-well plastic culture clusters (diameter 16 mm, Costar) at a density of 40,000 cells per well (2.0×10^4 cells/cm^2). For 24 hr prior to each experiment, the cell medium was free of foetal bovine serum. Experiments were generally performed 2–3 days after cells reached confluence and 6–8 days after the initial seeding, and each cm^2 contained approximately 100 μg of cell protein.

Transport Studies

On the day of the experiment, the growth medium was aspirated and the cells washed with Hanks' medium at 4°C; thereafter, the cell monolayers were preincubated for 15 min in Hanks' medium at 37°C. The Hanks' medium had the following composition (mM): NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris-HCl 0.15 and sodium butyrate 1.0, pH = 7.4. The incubation medium also contained pargyline (100 μM) and tolcapone (1 μM) in order to inhibit the enzymes monoamine oxidase and catechol-O-methyltransferase, respectively; in some experiments, benserazide (50 μM) was also added to the incubation medium in order to inhibit AAAD. During preincubation and incubation, the cells were continuously shaken and maintained at 37°C. Uptake was initiated by the addition of 2 mL Hanks' medium with a given concentration of the substrate under study (L-DOPA, D-3,4-dihydroxyphenylalanine (D-DOPA), benserazide and dopamine); the substrates were applied from the apical cell border. Determination of initial rate of uptake was performed in experiments in which cells were incubated with a nonsaturating concentration of the substrate for 1, 3, 6, 12, 30, 60 and 120 min. Saturation experiments were performed in LLC-PK_1 and OK cells incubated for 6 min with increasing concentrations of the substrates; some experiments were conducted at 4°C. Uptake was terminated by the rapid removal of uptake solution by means of a vacuum pump connected to a Pasteur pipette followed by two rapid washes with cold Hanks' medium and the addition of 250 μL of 0.2 mM perchloric acid; the acidified samples were stored at 4°C before injection into the high pressure liquid chromatograph for the assay of L-DOPA, D-DOPA, benserazide and dopamine. In some experiments, the incubation solution (2 ml) was also collected, acidified with 250 μL of 2 mM perchloric acid and stored at 4°C till the assay of catechol derivatives.

AAAD Preparation and Decarboxylation Studies

Cells were homogenised in 0.5 M phosphate buffer (pH = 7.0) with a Thomas teflon homogeniser and kept continuously on ice. Aliquots of 250 μL of cell homogenate plus 250 μL incubation medium were placed in glass test tubes and preincubated for 15 min. Thereafter, L-DOPA (50 to 10,000 μM) was added to the medium for a further 15 min; the final reaction volume was 1 mL. The composition of the incubation medium was as follows (in mM): NaH_2PO_4 0.35, Na_2HPO_4 0.15, sodium borate 0.11 and pyridoxal phosphate 0.12; tolcapone (1 μM) and pargyline (100 μM) were also added to the medium. The pH of the reaction medium was kept constant at an optimal pH = 7.0 [21]. During incubation, cell homogenates were continuously shaken and gassed (95% O_2 and 5% CO_2) and maintained at 37°C. The reaction was stopped by the addition of 500 μL of 2 M perchloric acid and the preparations kept at 4°C for 60 min. The samples were then centrifuged (200 *g*, 2 min, 4°C), and 500 μL aliquots of the supernatant filtered on Spin-X filter tubes (Costar) were used for the assay of dopamine.

Assay of L-DOPA, D-DOPA, Benserazide and Dopamine

L-DOPA, D-DOPA, benserazide and dopamine were quantified by means of high pressure liquid chromatography with electrochemical detection, as previously reported [22]. The high pressure liquid chromatograph system consisted of a pump (Gilson model 302; Gilson Medical Electronics) connected to a manometric module (Gilson model 802 C) and a stainless steel 5 μm ODS column (Biophase; Bioanalytical Systems) 25 cm in length; samples were injected by means of an automatic sample injector (Gilson model 231) connected to a Gilson dilutor (model 401). The mobile phase was a degassed solution of citric acid (0.1 mM), sodium octylsulphate (0.5 mM), sodium acetate (0.1 M), EDTA (0.17 mM), dibutylamine (1 mM) and methanol (8% v/v), adjusted to pH 3,5 with perchloric acid (2 M) and pumped at a rate of 1.0 mL min^{-1}. The detection was carried out electrochemically with a glassy carbon electrode, an Ag/AgCl reference electrode and an amperometric detector (Gilson model 141); the detector cell was operated at 0.75 V. The current produced was monitored using the Gilson 712 HPLC software. The lower limits for detection of L-DOPA, D-DOPA and dopamine ranged from 350 to 500 fmol.

Cell Water Content

Cell water content was simultaneously measured in parallel experiments using [^{14}C]inulin as extracellular marker and tritiated water as total water marker. Intracellular water obtained by subtracting extracellular water from total water was expressed as microliter of cell water per milligram protein. Subsequently, the cells were solubilised by 0.1% v/v Triton X-100 (dissolved in 5 mM Tris.HCl, pH 7.4) and radioactivity was measured by liquid scintillation counting.

Protein Assay

The protein content of monolayers of LLC-PK_1 and OK cells was determined by the method of Bradford [23], with human serum albumin as a standard.

Cell Viability

Cells were preincubated for 15 min at 37°C and then incubated in the absence or presence of L-DOPA for a further 15 min or 120 min. Subsequently, the cells were incubated at 37°C for 2 min with Trypan blue (0.2% w/v) in phosphate buffer. Incubation was stopped by rinsing the cells twice with Hanks' medium and the cells were examined using a Leica microscope. Under these conditions, more than 95% of the cells excluded the dye.

Data Analysis

The analysis of the time-course of L-DOPA uptake in LLC-PK_1 and OK cells was based on a one-compartment model [24]. The parameters of the equation

$$C_i/C_o = k_{in}/k_{out} \cdot (1 - e^{-k_{out} \cdot t})$$

were fitted to the experimental data by a nonlinear regression analysis using a computer-assisted method [25]. C_i and C_o represent the intracellular and extracellular concentration of the substrate, respectively. k_{in} is the rate constant for inward transport, k_{out} the rate constant for outward transport, and t the incubation time. k_{in} and k_{out} are given in pmol/mg protein/min. A_{max} is defined as the factor of accumulation (C_i/C_o) at equilibrium ($t \rightarrow \infty$). K_m and V_{max} values for the uptake of substrates, as determined in saturation experiments, and decarboxylation of L-DOPA in cell homogenates were calculated from nonlinear regression analysis using the GraphPad Prism statistics software package [25]. The rate constant of outward transfer was determined by the slope of the accumulation of substrates measured by linear regression analysis [26]. Arithmetic means are given with SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student's *t*-test for unpaired comparisons. A *p* value less than 0.05 was assumed to denote a significant difference.

RESULTS

To determine the rate constant of total inward (k_{in}) and outward (k_{out}) transport, LLC-PK_1 and OK cells were incubated in the presence of 0.5 μM L-DOPA for 1, 3, 6, 12, 30, 60 and 120 min (Fig. 1); benserazide (50 μM) was added to the incubation in order to inhibit AAAD. L-DOPA was rapidly accumulated in the cells; equilibrium

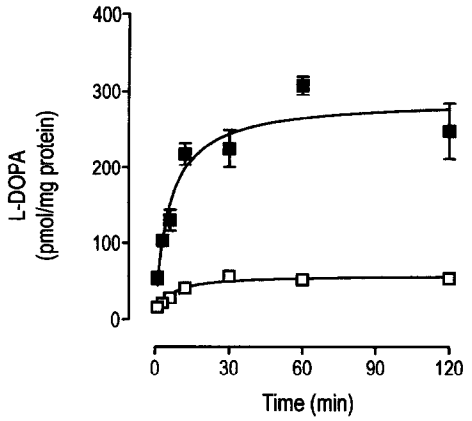

FIG. 1. Time-course of L-DOPA accumulation in LLC-PK$_1$ (open squares) and OK (closed squares) cells. Cells were incubated at 37° with 0.5 μM L-DOPA for 1, 3, 6, 12, 30, 60 and 120 min. Exponential saturation curves were fitted to the experimental data. Symbols represent means of five experiments per group and vertical lines show SEM.

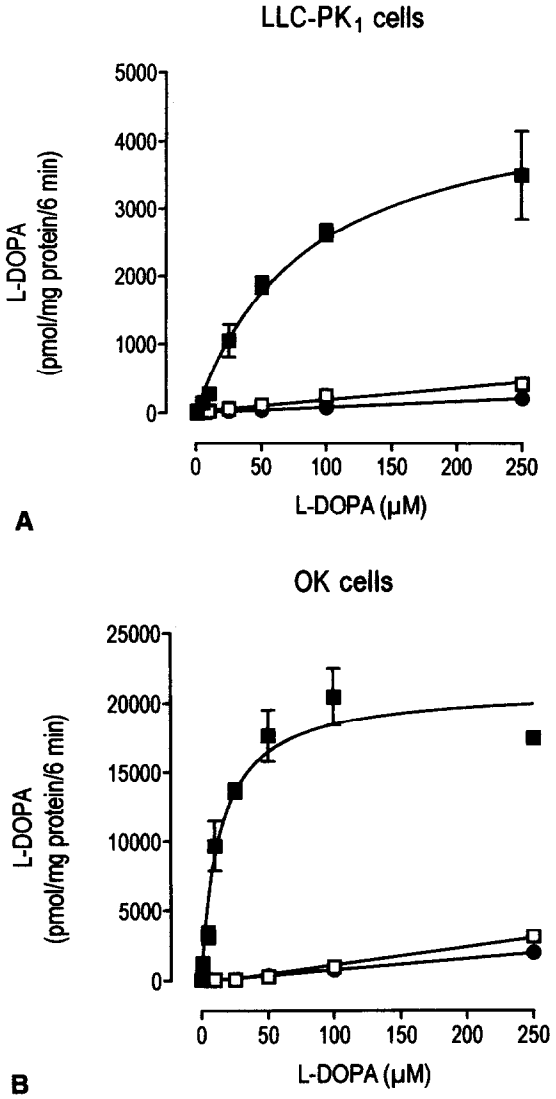

FIG. 2. Accumulation of L-DOPA (squares) and D-DOPA (circles) in (A) LLC-PK$_1$ and (B) OK cells. Cells were incubated for 6 min at 37° (closed symbols) or 4° (open symbols) and increasing concentrations (0.5 to 250 μM) of the substrates were applied from the apical border. Symbols represent means of four to five experiments per group and vertical lines show SEM. Linear coefficient values for LLC-PK1 cells were L-DOPA 4°, $r^2 = 0.967$, N = 32; D-DOPA 37°, $r^2 = 0.998$, N = 20, and for OK cells, L-DOPA 4°, $r^2 = 0.985$, N = 24, D-DOPA 37°, $r^2 = 0.996$, N = 24.

was attained at 30 min of incubation. From the initial rate of uptake, k_{in}, k_{out}, and an equilibrium factor of accumulation (A_{max}) were calculated. The analysis revealed a rate constant of inward transport (k_{in} in pmol/mg protein/min) of 3.6 ± 0.4 and 18.1 ± 0.3, a rate constant of outward transport (k_{out} in pmol/mg protein/min) of 1.0 ± 0.1 and 5.2 ± 0.1 and an equilibrium factor (A_{max}) of 15.9 ± 0.9 and 75.0 ± 5.0 (N = 5) for LLC-PK$_1$ and OK cells, respectively. The intracellular water content of LLC-PK$_1$ and OK cell monolayers was 7.1 ± 0.6 and 6.8 ± 0.7 μL/mg protein (N = 12), respectively. At equilibrium (30 min incubation), the intracellular L-DOPA concentration in LLC-PK$_1$ and OK cells was 7.9 ± 0.4 μM and 37.5 ± 2.5 μM, respectively, at medium concentration of 0.5 μM. This represented a cell concentration of L-DOPA in LLC-PK$_1$ and OK cells that was, respectively, 14.2 ± 1.6 and 66.2 ± 7.3 (N = 8) times higher than the medium concentration. Considering that k_{out} values are directly dependent on the intracellular concentration of the substrate and given that this was substantially greater in OK than in LLC-PK$_1$ cells, the percentage of intracellular L-DOPA leaking out of the cells is approximately the same (13 ± 1% in LLC-PK1 cells and 14 ± 2%in OK cells).

Because in time-course experiments the accumulation for L-DOPA increased linearly with time for several minutes, it was decided in all subsequent experiments to determine the kinetic parameters for the uptake of L-DOPA and other substrates at 6 min incubation time. When L-DOPA was used as the substrate, its accumulation was found to be dependent on the concentration used and to be saturable at 250 μM (Fig. 2). Nonlinear analysis of the saturation curves revealed a K_m of 86 ± 12 μM and a V_{max} of 4873 ± 293 pmol/mg protein/6 min for LLC-PK$_1$ cells and a K_m of 14 ± 4 μM and a V_{max} of 21018 ± 1425 pmol/mg protein/6 min for OK cells. In experiments carried out at 4°, the amount of L-DOPA accumulated in the cells was markedly lower than that observed at 37° and was found to be nonsaturable (Fig. 2). Both LLC-PK$_1$ and OK cells incubated at 37°C with increasing concentrations of D-DOPA instead of L-DOPA were found to accumulate trace amounts of the D-isomer; the cellular accumulation of D-DOPA at the highest concentration used was approximately 5% of the corresponding L-isomer (Fig. 2).

The experiments described above were performed in the presence of benserazide (50 μM) in order to avoid the intracellular decarboxylation of L-DOPA by AAAD. Benserazide is an effective AAAD inhibitor and 50 μM benserazide has been found to completely abolish the decarboxylation of L-DOPA. However, it is not known

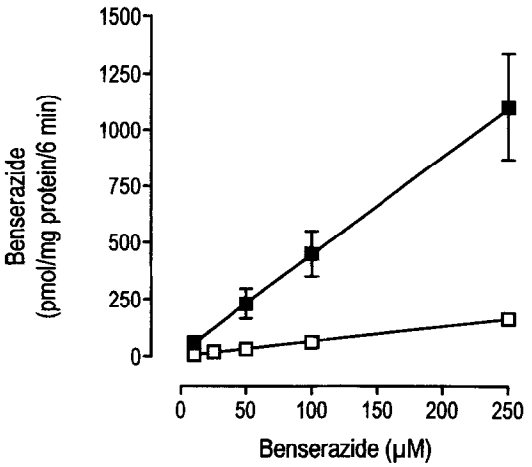

FIG. 3. Accumulation of benserazide in LLC-PK$_1$ (open squares) and OK cells (closed squares). Cells were incubated for 6 min with increasing concentrations (10 to 250 μM) of the substrate. Symbols represent means of four experiments per group and vertical lines show SEM. Linear coefficient values were: LLC-PK$_1$ cells, r^2 = 0.999, N = 20; OK cells, r^2 = 0.999, N = 16.

whether benserazide may also interfere with the cellular uptake of L-DOPA, given the structural similarities between these two compounds. This is, however, almost impossible to test in a cell system endowed with AAAD activity. Therefore, it was decided to study the uptake of benserazide to see whether the accumulation of the AAAD inhibitor in these cells proceeded differently from that of L-DOPA. Incubation of LLC-PK$_1$ and OK cells with increasing concentrations of benserazide also resulted in a nonsaturable accumulation of the compound (Fig. 3); at 250 μM, the amount of benserazide accumulated in the cells was only 5% of the amount of L-DOPA accumulated under similar conditions.

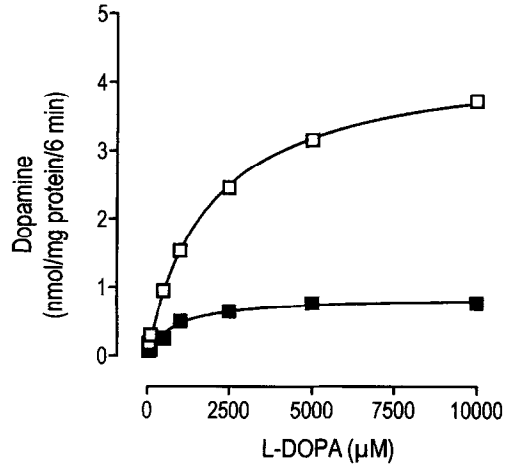

FIG. 4. Saturation curves of aromatic L-amino acid decarboxylase (AAAD) activity in homogenates LLC-PK$_1$ (open squares) and OK (closed squares) cells. AAAD activity is expressed as the rate of formation of dopamine (in nmol/mg protein/15 min) vs. concentration of L-DOPA (μM). Symbols represent means of five experiments per group and vertical lines show SEM.

Incubation of homogenates of LLC-PK$_1$ cells with L-DOPA (50 to 10,000 μM) resulted in a concentration-dependent formation of dopamine (Fig. 4). The decarboxylation process was found to be nonsaturable up to 1 mM L-DOPA and showed a trend for saturation at 2.5 mM L-DOPA. Nonlinear analysis of the saturation curves revealed a K_m (in μM) of 1866 ± 107 and 845 ± 153 and a V_{max} (in nmol/mg protein/15 min) of 4.4 ± 0.1 and 0.9 ± 0.1 for LLC-PK$_1$ and OK cells, respectively.

Because both cell lines were found to be endowed with AAAD activity, it was decided to incubate the cell monolayers with L-DOPA in the absence of benserazide and determine the intracellular concentrations of L-DOPA and newly formed dopamine and the amount of dopamine which had escaped to the incubation medium. Cells were incubated with increasing concentrations of L-DOPA (10 to 500 μM) for 6 min. As shown in Fig. 5, some of the L-DOPA taken up was not decarboxylated to dopamine. This was particularly evident in OK cells, but also occurred

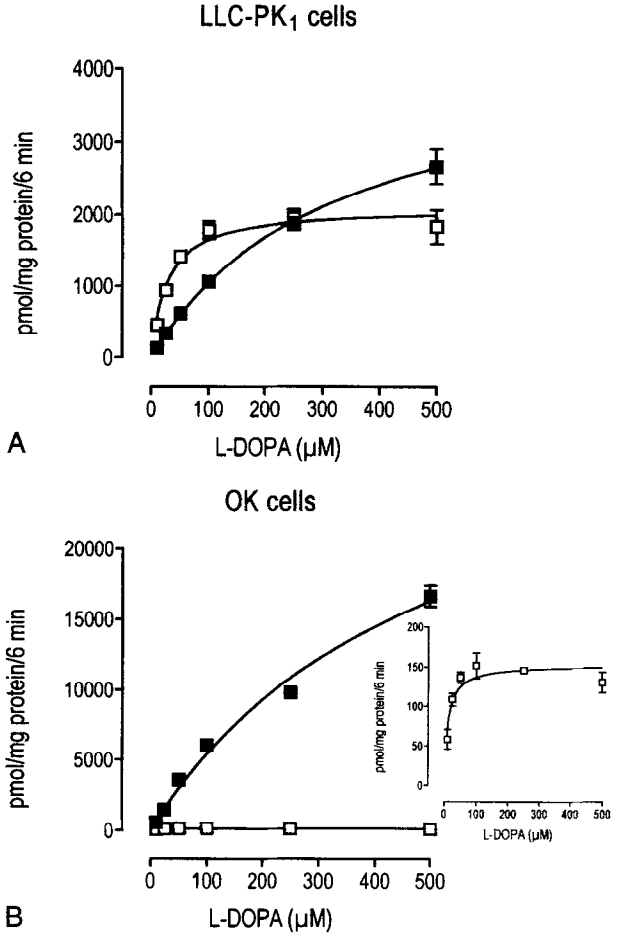

FIG. 5. Intracellular levels of taken up L-DOPA (closed squares) and newly-formed dopamine in (open squares) in (A) LLC-PK$_1$ and (B) OK cells; the inset shows the formation of dopamine in OK cells. Cells were preincubated in the absence of benserazide for 30 min and incubated for 6 min with increasing concentrations of L-DOPA; the substrate was applied from the apical cell border. Symbols represent means of four to five experiments per group and vertical lines show SEM.

TABLE 1. Dopamine/L-DOPA ratios (×100) in LLC-PK$_1$ and OK cells loaded with increasing concentrations of L-DOPA

L-DOPA (μM)	LLC-PK$_1$ cells	OK cells
	dopamine/L-DOPA ratios (×100)	
10	77.6 ± 1.0	8.8 ± 1.5#
25	73.8 ± 0.9	6.9 ± 0.7#
50	69.7 ± 0.8	3.7 ± 0.3*,#
100	62.4 ± 1.7	2.4 ± 0.2*,#
250	51.1 ± 0.6*	1.5 ± 0.1*,#
500	40.5 ± 3.0*	0.8 ± 0.1*,#

Cells were preincubated in the absence of benserazide for 30 min and incubated for 6 min with increasing concentrations of L-DOPA; the substrate was applied from the apical cell border. Results are means ± SEM of four to five experiments per group.

* Significantly different ($P < 0.05$) from values for 10 μM L-DOPA using the Newman–Keuls test.

Significantly different ($P < 0.01$) from corresponding values for LLC-PK1 cells using the Student's *t*-test.

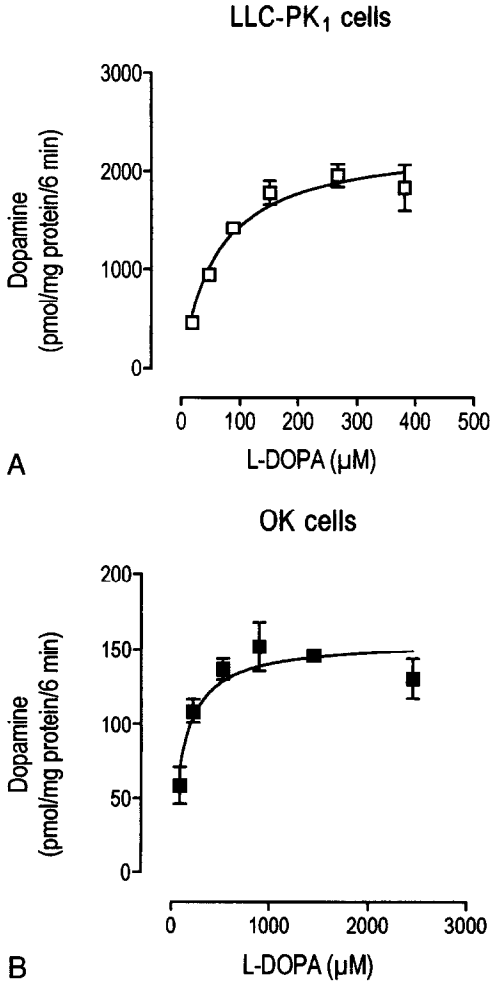

FIG. 6. Decarboxylation of intracellular L-DOPA (L-DOPA accumulated in the cell monolayer plus the total amount of dopamine formed) in (A) LLC-PK$_1$ and (B) OK cells. Cells were preincubated in the absence of benserazide for 30 min and incubated for 6 min with increasing concentrations of L-DOPA; the substrate was applied from the apical cell border. Symbols represent means of four to five experiments per group and vertical lines show SEM.

in LLC-PK$_1$ cells at the highest concentrations of the substrate. This difference as to the ability to decarboxylate intracellular L-DOPA can be better evidenced when dopamine/L-DOPA ratios are analysed (Table 1); in LLC-PK$_1$ cells, these ratios were 50- to 9-fold those in OK cells, indicating that the amount of L-DOPA converted to dopamine in OK cells was substantially lower than in LLC-PK$_1$ cells. This fully agrees with the evidence that AAAD activity, as determined in cell homogenates, was higher in LLC-PK$_1$ cells than that in OK cells. However, despite differences in AAAD activity between these two cell lines, it is interesting to note that in both LLC-PK$_1$ and OK cells the proportion of L-DOPA decarboxylated to dopamine (dopamine/L-DOPA ratios) at the lowest concentration of the substrate (10 μM) was significantly ($P < 0.05$) higher than that observed at 250 and 500 μM L-DOPA; this was particularly evident in OK cells (Table 1) and can be used as an indication of the saturability of the process of decarboxylation of intracellular L-DOPA (see also Fig. 5).

The kinetics of decarboxylation of intracellular L-DOPA was calculated taking into account the amount of L-DOPA accumulated in the cell monolayer plus the total amount of dopamine formed (Fig. 6). The K_m values for decarboxylation of intracellular L-DOPA were 61 ± 14 and 108 ± 36 μM ($P = 0.08$) in LLC-PK$_1$ and OK cells, respectively; these values are significantly lower ($P < 0.05$) than those obtained in experiments in cell homogenates. V_{max} values for the intracellular decarboxylation of L-DOPA in LLC-PK$_1$ cells (2307 ± 164 pmol/mg protein/6 min) were significantly greater ($P < 0.05$) than those in OK cells (156 ± 10 pmol/mg protein/6 min).

A low amount of newly formed dopamine (15 to 26% in LLC-PK1 cells; 21 to 27% in OK cells) was found to escape to the apical bathing fluid. Figure 7 represents the plot between the total dopamine formed (in μmol/L) and the amount of dopamine (in pmol/mg protein/6 min) which had escaped to the incubation medium. As shown in this figure, the outward transfer of newly formed dopamine was a nonsaturable process up to 300 μM intracellular dopamine; the rate constant of outward transfer, as determined by linear regression analysis, was 0.88 ± 0.11 μmol^{-1} ($r^2 = 0.933$; N = 24) for LLC-PK$_1$ cells and 6.80 ± 0.013 μmol^{-1} ($r^2 = 0.999$; N = 24) for OK cells.

We then addressed the question of whether the inward movement of dopamine in LLC-PK$_1$ and OK cells was a saturable or nonsaturable process. The cells were incubated with exogenous dopamine for 6 min; the range of concentrations used (10 to 250 μM) was similar to the intracellular concentrations determined in cells loaded with L-DOPA in the absence of benserazide. The accumulation of exogenous dopamine in both LLC-PK$_1$ and OK cells was found to be nonsaturable (Fig. 8). At 250 μM, the amount of dopamine accumulated in the cells was only 5% of the amount of L-DOPA accumulated under similar conditions; the rate constant of dopamine inward transfer, as determined by linear regression analysis, was 0.57 ± 0.01

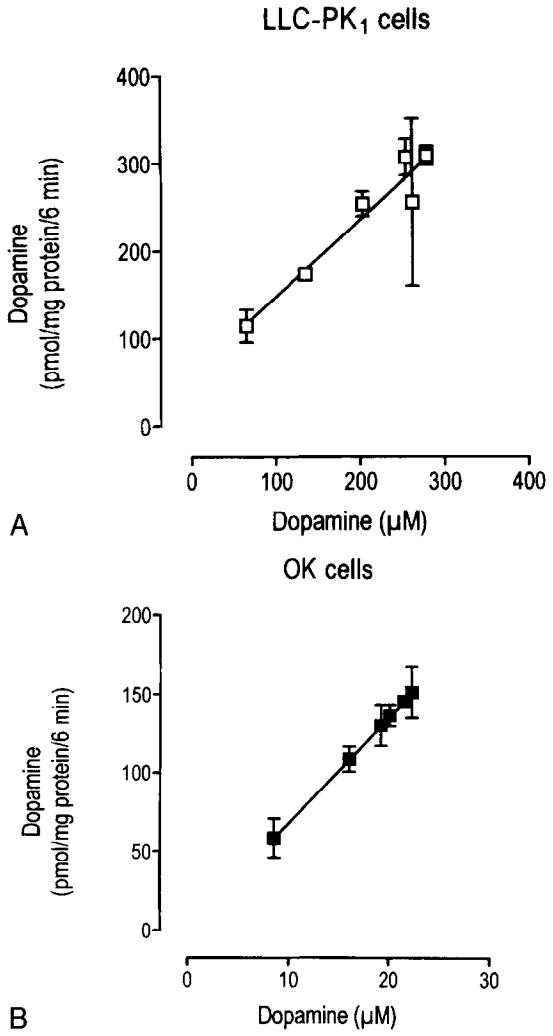

FIG. 7. Plot of total amount of dopamine (in μmol/L) which was formed in (A) LLC-PK$_1$ and (B) OK cells loaded with increasing concentrations of L-DOPA (10 to 500 μM) and the amount of dopamine which escaped to the incubation medium (in pmol/mg protein/6 min). Symbols represent means of five experiments per group and vertical lines show SEM. Linear coefficient values were: LLC-PK$_1$ cells, r^2 = 0.933, N = 30; OK cells, r^2 = 0.999, N = 30.

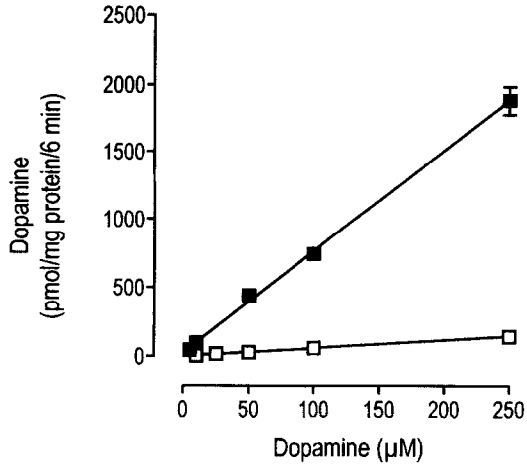

FIG. 8. Accumulation of dopamine in LLC-PK$_1$ (open squares) and OK (closed squares) cells. Cells were incubated for 6 min with increasing concentrations (10 to 250 μM) of the substrate. Symbols represent means of four experiments per group and vertical lines show SEM. Linear coefficient values were: LLC-PK$_1$ cells, r^2 = 0.999, N = 20; OK cells, r^2 = 0.999, N = 20.

μmol^{-1} (r^2 = 0.999; N = 20) for LLC-PK$_1$ cells and 7.41 ± 0.16 μmol^{-1} (r^2 = 0.999; N = 20) for OK cells. This suggests that dopamine enters the cells via a process completely different from that used by L-DOPA, and for each particular cell line, the ease with which dopamine diffuses out of the cell is similar to that with which the amine diffuses into the cell.

DISCUSSION

The data presented here show that LLC-PK$_1$ and OK cells take up L-DOPA efficiently through the apical cell border and several observations demonstrate that this uptake process was a facilitated mechanism. Firstly, steady-state uptake of non-saturating concentrations of L-DOPA showed a curvilinear dependence on incubation time. Secondly, at initial rate of uptake (6-min incubation), the cellular transport of L-DOPA showed a curvilinear dependence on L-DOPA medium concentration, suggesting that the uptake was saturable. Thirdly, the cellular transport of L-DOPA was nearly abolished at 4°, demonstrating that the uptake was energy-dependent. The efficiency of this L-DOPA transport in LLC-PK$_1$ and OK cells can also be evidenced by the ratio of L-DOPA concentration in cellular water to medium concentration. The intracellular L-DOPA concentration at equilibrium was larger than that which could be expected by passive diffusion of L-DOPA. At steady-state of L-DOPA uptake, the mean intracellular concentration of L-DOPA was 14 and 60 times larger in LLC-PK$_1$ and OK cells, respectively, than L-DOPA concentration in the incubation medium. Finally, the finding that D-DOPA transport was nonsaturable indicates that the uptake of L-DOPA is stereoselective, agreeing with previous evidence on the uptake of L-DOPA obtained in isolated rat renal proximal tubules [22, 27].

When nonsaturating concentrations (0.5 μM) of L-DOPA were used in time-course experiments, the result was a rapid accumulation of the substrate until an equilibrium was reached at approximately 30 min of incubation. Under these experimental conditions and considering 1 mg of cell protein, LLC-PK$_1$ and OK cells were found to take up approximately 4 and 18 pmol of L-DOPA per min. Under similar experimental conditions, we were able to demonstrate that Caco-2 cells take up 8 pmol L-DOPA (per mg protein per min) [28]. Other authors [29, 30] have used the same approach to study the uptake of different substrates and found that OK cells have clearance values in the same order of magnitude as those described for L-DOPA. Therefore, it can be suggested that the L-DOPA transporter in LLC-PK$_1$ cells is as efficient in Caco-2 cells, but less efficient than in OK cells; the clearance values are, how-

ever, within the range observed for other substrates. This increased ability of OK cells to take up L-DOPA is confirmed in saturation experiments where it is shown that the affinity of the L-DOPA transporter for the substrate in OK cells is higher than that in LLC-PK1 cells (as evidenced by differences in K_m values). It is possible that this may reflect the different origin of these two renal cell lines.

Another crucial step in the whole process of dopamine formation is the decarboxylation of intracellular L-DOPA. Experiments conducted in cell homogenates showed that LLC-PK_1 cells are endowed with a higher AAAD activity than OK cells, but the efficiency of the decarboxylation process, as indicated by K_m values, was quite similar. When cell monolayers were loaded with L-DOPA in the absence of benserazide, LLC-PK_1 cells were also found to synthesise more dopamine than OK cells. In both cell lines, the process of dopamine formation is rapidly saturated, but a substantial amount of taken up L-DOPA is not converted to dopamine. This is particularly evident in OK cells. The most likely explanation for this finding may have to do with the fact that OK cells are endowed with very low amounts of AAAD activity in comparison with LLC-PK_1 cells. In addition, one should consider that the rate of uptake of L-DOPA in OK cells is substantially greater than that in LLC-PK_1 cells; this may determine that large amounts of L-DOPA would be retained inside the cell without undergoing conversion to dopamine. The opposite would be the case for LLC-PK_1 cells; these cells take up less L-DOPA per unit of time and are endowed with a high AAAD activity. This contrasts with the data obtained while using Caco-2 cells [31]. In this intestinal cell line, the intracellular concentrations of newly formed dopamine are much greater than those of L-DOPA, suggesting that most of the taken up L-DOPA is rapidly converted to dopamine and only residual amounts of L-DOPA escape decarboxylation. Although this apparent discrepant behaviour could be a product of different AAAD activities, the fact is that the kinetic parameters of the enzyme in LLC-PK_1 cells (V_{max} = 4.4 ± 0.1 nmol/mg protein/15 min; K_m of 1866 ± 107 μM) are similar to those observed in Caco-2 cells (V_{max} = 3.93 ± 0.09 nmol/mg protein/15 min; K_m = 780 ± 50 μM). One possible explanation for the different handling of L-DOPA between OK, LLC-PK_1 and Caco-2 cells could be related to the accumulation of L-DOPA in an intracellular compartment devoid of AAAD. AAAD is a cytosolic enzyme and, to our knowledge, there is no information on the presence of such hypothetical intracellular compartments in these cells. These epithelial cells are believed to synthesise dopamine as a result of the availability of L-DOPA in the cytosol. More recently, however, it has been suggested that at least rat renal tubular epithelial cells may be endowed with intracellular compartments where dopamine could be stored [32]; until now, these storage elements have not been identified in LLC-PK_1, OK and Caco-2 cells.

Previous studies have shown that both OK and LLC-PK_1 cells are endowed with the ability to decarboxylate L-DOPA to dopamine and that the newly formed amine was responsible for the activation of specific dopamine receptors as an autocrine/paracrine substance [17, 18]. The present study demonstrates that the efficiency of this decarboxylation process is similar in both cell lines, though more important in LLC-PK_1 cells. In fact, K_m values for AAAD in OK cells were similar to those in LLC-PK_1 cells. The difference in K_m values for decarboxylation of L-DOPA in experiments conducted in cell homogenates and cell monolayers is by a factor of 10. This has been reported in other tissues and is most probably related to the use of different assay mediums [6]; the use of NaH_2PO_4 (0.35 mM), Na_2HPO_4 (0.15 mM) and sodium borate (0.11 mM) instead of Krebs has been demonstrated to decrease the affinity for the substrate but to dramatically increase V_{max} values [6]. It should be stressed, however, that differences in V_{max} values mainly reflect differences in the number of enzyme units in each cell preparation. Obviously, this is an important aspect which one has to have in mind when considering OK and LLC-PK_1 cells as models of the renal dopaminergic system; per mg of protein, the number of AAAD enzyme units in LLC-PK_1 cells is 4.6-fold that in OK cells.

The intracellular fate of newly formed dopamine is another interesting point to discuss. The present study has also evaluated the outward transfer of newly formed dopamine through the apical cell border, and the data obtained clearly show that this transfer is a diffusional process. It is, however, worthwhile to stress the fact that the diffusional inward transfer of exogenous dopamine was found to present similar kinetic characteristics to the diffusional outward transfer of newly formed dopamine. When compared with OK cells, the rate constant of inward transfer in LLC-PK_1 cells (0.57 ± 0.01 vs. 7.41 ± 0.16 μmol^{-1}) is markedly lower; however, a similar difference was also observed for the outward transfer of the amine (OK cells, 6.80 ± 0.01 μmol^{-1}; LLC-PK_1 cells, 0.89 ± 0.12 μmol^{-1}). It is possible that this difference might have to do with the particular characteristics of the apical membrane permeability in these two renal cell lines. However, the finding that dopamine is not actively taken up when applied from the apical cell border is an interesting one and is consistent with the evidence that the renal transport of monoamines is unidirectional from the basolateral to the apical cell border [8]. Although some of the dopamine synthesised in epithelial cells might have access to the renal circulation and escape the kidney through the renal vein [33], most tubular dopamine is believed to leave this cellular compartment through the apical cell border and to be eliminated in the urine. Once in the tubular lumen, dopamine is not thought to diffuse backwards. At least two arguments support this suggestion: firstly, the apical cell border is impermeable to dopamine [8] and, secondly the urinary

excretion of dopamine is independent of the urine flow [34, 35].

In conclusion, the data presented here show that OK cells are endowed with a more efficient L-DOPA uptake system than LLC-PK_1 cells, but the latter are endowed with a significantly higher AAAD activity than OK cells. In both cell lines, intracellular L-DOPA is rapidly converted to dopamine, some of which diffuses out of the cell. Our observations support the use of LLC-PK_1 and OK cells as *in vitro* models for the study of the renal dopaminergic physiology, though this may not reflect what happens in the intact tissue, namely the rat proximal nephron [36].

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