Apical and basolateral uptake and intracellular fate of dopamine precursor L-dopa in LLC-PK₁ cells

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Soares-da-Silva, P., M. P. Serrão, and M. A. Vieira-Coelho. Apical and basolateral uptake and intracellular fate of dopamine precursor L-dopa in LLC-PK₁ cells. Am. J. Physiol. 274 (Renal Physiol. 43): F243–F251, 1998.—The present study was aimed at the uptake of L-3,4-dihydroxyphenylalanine (L-dopa) and its intracellular decarboxylation to dopamine. The accumulation of L-dopa from the apical side in cells cultured in collagen-treated plastic was found to be a saturable process with a Michaelis constant (Kₘ) of 123 ± 17 µM and a maximal velocity (Vₘₐₓ) of 6.0 ± 0.2 nmol·mg protein⁻¹·6 min⁻¹. The uptake of L-dopa applied from either the apical or the basal side was found to be saturable; nonlinear analysis of saturation curves for apical and basal application revealed Kₘ values of 63.8 ± 17.0 and 42.5 ± 9.6 µM and Vₘₐₓ values of 32.0 ± 5.8 and 26.2 ± 3.4 nmol·mg protein⁻¹·6 min⁻¹, respectively. Cell monolayers incubated with L-dopa, applied from either the apical or the basal side, in the absence of benzerazide, led to the accumulation of newly formed dopamine. The intracellular accumulation of newly formed dopamine was a saturable process with apparent Kₘ values of 20.5 ± 8.2 and 247.3 ± 76.8 µM when the substrate was applied from the apical and basal side, respectively. Some of the newly formed dopamine escaped to the extracellular milieu. The basal outward transfer of dopamine was five- to sevenfold of that occurring at the apical side and was uniform over a wide range of concentrations of intracellular dopamine; the apical outward transfer of the amine depended on the intracellular concentration of dopamine and was a nonsaturable process. The apical and basal outward transfers of dopamine were insensitive to cocaine (10 and 30 µM) and GBR-12909 (1 and 3 µM). The accumulation of exogenous dopamine in LLC-PK₁ cells was found to be saturable; nonlinear analysis of the saturation curves revealed for the apical and basal application of dopamine a Kₘ of 17.7 ± 4.3 and 96.0 ± 28.1 µM and a Vₘₐₓ of 2.0 ± 0.1 and 2.2 ± 0.3 nmol·mg protein⁻¹·6 min⁻¹, respectively. However, both cocaine (10, 30, or 100 µM) and GBR-12909 (1 or 3 µM) were found not to affect the uptake of 100 µM dopamine applied from either the apical or the basal cell border. In conclusion, the data presented here show that LLC-PK₁ cells are endowed with considerable aromatic L-amino acid decarboxylase (AADC) activity and transport L-dopa quite efficiently through both the apical and basal cell borders. On the other hand, our observations support the possibility of a basal-to-apical gradient of AADC activity and the possibility that LLC-PK₁ cells might constitute an interesting in vitro model for the study of the renal dopaminergic physiology.

L-3,4-dihydroxyphenylalanine; decarboxylase

ENDOGENOUS DOPAMINE in the kidney has a role in regulating sodium excretion through the activation of specific dopamine receptors located in tubular epithelial cells (1–3, 7, 23). The current view of the renal dopaminergic system is that of a local nonneuronal system constituted by epithelial cells of proximal convoluted renal tubules rich in aromatic L-amino acid decarboxylase (AADC) activity and using circulating or filtered L-3,4-dihydroxyphenylalanine (L-dopa) as a source for dopamine (18, 19, 29). Because the dopamine produced in this area is in close proximity to renal cells that contain receptors for the amine, it has been hypothesized that the amine may act as a paracrine or autocrine substance (25). The relative importance of this system in controlling natriuresis assumes particular relevance in view of the findings that salt-sensitive hypertensive subjects have a defect in renal dopamine production and this may be associated with salt sensitivity of their blood pressure (19). Urinary L-dopa has been found to be greater in salt-sensitive than in salt-resistant hypertensive and normotensive subjects during low- or high-sodium intake. The mean ratio of dopamine to L-dopa has also been found to be subnormal during low- and high-sodium intake, suggesting that salt-sensitive hypertensives might have a reduced capacity to synthesize dopamine (8, 9, 17).

A deficient production of renal dopamine might arise from a reduced delivery of the substrate to the kidney, a reduced uptake of L-dopa into tubular epithelial cells, or a reduced rate of conversion of L-dopa into dopamine. Although the kidney is endowed with one of the highest levels of AADC in the body, the enzyme responsible for the conversion of L-dopa to dopamine, and the plasma levels of L-dopa are in the range of nanomoles per milliliter (12, 37), it is not known which is the rate-limiting step for the synthesis of dopamine in renal tissues. Early studies have demonstrated that the renal tubular transport of L-dopa occurs through an energy-dependent and stereoselective carrier-mediated process (5). More recently, the synthesis of dopamine in tubular epithelial cells has been shown to be closely dependent on extracellular sodium, the mechanisms involved in transtubular reabsorption of sodium (33, 34, 36), and the integrity of the tubular cytoskeleton and the functional integrity of sodium potassium adenosinetriphosphatase (26). The rise of intracellular levels of guanosine 3′,5′-cyclic monophosphate (cGMP), as induced by the α-human atrial natriuretic peptide, by zaprinast (May & Baker 22,948), a relatively specific cGMP phosphodiesterase inhibitor, or by 8-bromocGMP, has also been found to restrict the intracellular availability of L-dopa and decrease the renal formation of dopamine (30, 31). However, despite the evidence of an association among the tubular reabsorption of sodium, the levels of cGMP, and the uptake of L-dopa, information is still lacking on the nature and the characteristics of the rate-limiting step for the renal formation of dopamine. On the other hand, maneuvers that modify the urinary excretion of dopamine have
been demonstrated in parallel with changes with renal AADC activity (14, 22, 27, 32).

The present study was aimed at addressing the question concerning which of the processes, the cellular uptake of L-dopa or its intracellular decarboxylation by AADC, is rate limiting to the formation of dopamine. To obviate technical problems related to the handling of freshly isolated renal tubular epithelial cells, this study was performed in monolayers of LLC-PK1 cells in culture. LLC-PK1 cells express proximal tubule cell-like properties in vitro (16) and have been used for the purpose of studying dopamine receptors and the renal actions of the amine. These cells also have been shown to contain high levels of AADC and to convert L-dopa to dopamine in a nonsaturable fashion for up to 1 mM L-dopa (6, 10, 11). Newly formed dopamine also stimulates adenosine 3',5'-cyclic monophosphate accumulation in LLC-PK1 cells, and this effect was attenuated by an equimolar concentration of carbidopa and blocked by the D2 antagonist Sch-23390 (11). It appears, therefore, that in LLC-PK1 cells, as in epithelial cells of proximal tubules, locally formed dopamine can act as an autocrine/paracrine substance. We report here that LLC-PK1 cells take up L-dopa through a saturable, stereoselective, and temperature-dependent process when it is applied from the apical and basolateral cell border, but the rate-limiting step for the formation of dopamine in these renal epithelial cells depends on the intracellular decarboxylation of L-dopa.

METHODS

Cell culture. LLC-PK1, a porcine-derived proximal renal tubule epithelial cell line that retains several properties of proximal tubular epithelial cells in culture (16), was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in a humidified atmosphere of 5% CO2-95% air at 37°C. LLC-PK1 cells (ATCC CRL 1392; passages 198–206) were grown in medium 199 (Sigma Chemical, St. Louis, MO) supplemented with 100 U/ml penicillin G, 0.25 µg/ml streptomycin (Sigma), 3% fetal bovine serum (Sigma), and 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Sigma). For subculturing, the cells were dissociated with 0.05% trypsin-EDTA, split 1:4, and subcultured in Costar flasks with 75- or 162-cm2 growth areas (Costar, Badhoevedorp, The Netherlands). For uptake studies, the cells were seeded in collagen-treated 24-well plastic culture clusters (16 mm ID, Costar) at a density of 40,000 cells/well (2.0 × 106 cells/cm2) or, depending on the experiment, onto collagen-treated 0.2-µm polycarbonate filters (12 mm ID, Transwell; Costar). The cell medium was changed every 2 days, and the cells reached confluency after 3–5 days of incubation. For 24 h before each experiment, the cell medium was free of fetal bovine serum. Experiments were generally performed 2–3 days after cells reached confluency and 6–8 days after the initial seeding and each square centimeter contained ~130 µg of cell protein. Transport studies. On the day of the experiment, the growth medium was aspirated and the cells were washed with Hanks’ medium at 4°C; thereafter, the cell monolayers were preincubated for 15 min in Hanks’ medium at 37°C. Hanks’ medium had the following composition (in mM): 137 NaCl, 5 KCl, 0.8 MgSO4, 0.33 Na2HPO4, 0.44 KH2PO4, 0.25 CaCl2, 1.0 MgCl2, 0.15 tris(hydroxymethyl)aminomethane hydrochloride, and 1.0 sodium butyrate, pH 7.4. The incubation medium also contained pargyline (100 µM) and tolcapone (1 µM) to inhibit the enzymes monoamine oxidase and catechol O-methyltransferase, respectively; in some experiments, benserazide (50 µM) was also added to the incubation medium to inhibit AADC. During preincubation and incubation, the cells were continuously shaken and maintained at 37°C.

In the first series of experiments, uptake studies were performed in cells cultured in collagen-treated plastic supports, with the substrates being applied from the apical cell border only. Uptake was initiated by the addition of 2 ml Hanks’ medium with a given concentration of the substrate under study. Determination of initial rate of uptake was performed in experiments in which cells were incubated with a nonsaturating concentration of L-dopa during 1, 3, 6, 12, 30, 60, and 120 min. Saturation experiments were performed in LLC-PK1 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 another series of experiments, cells were cultured in polycarbonate supports, with the substrates being applied from the apical or the basal side of the monolayer. The incubation medium used in this series of experiments was similar to that described above; in some experiments, the medium contained benserazide (50 µM) to inhibit AADC. The upper and lower chambers contained 600 µl and 200 µl, respectively. For apical uptake, the uptake solution was added to the upper chamber, whereas for basolateral uptake, the uptake solution was added to the lower chamber. Cells were preincubated for 15 min and thereafter incubated for 6 min in the presence of L-dopa. At the end of incubation, cells were placed on ice, and the medium bathing the apical and basal cell borders was collected, acidified with perchloric acid, and stored at 4°C until assay for L-dopa and dopamine. The cells were washed with ice-cold Hanks’ medium and combined with 0.2 mM perchloric acid (100 and 500 µl in the upper and lower chambers, respectively); the acidified samples were stored at 4°C before injection into the high-pressure liquid chromatograph for the assay of L-dopa and dopamine.

The last series of experiments was also performed in cells cultured in polycarbonate filters and was aimed at testing the effects of cocaine and GBR-12909 on the apical and basolateral outflow of newly formed dopamine and apical and basal uptake of exogenous dopamine. Cells were preincubated for 30 min in the presence of cocaine (10, 30, and 100 µM) or GBR-12909 (1 and 3 µM), and L-dopa (250 µM) or dopamine (100 µM) was then applied from the apical or the basal side of the cell monolayer. At the end of incubation, cells were placed on ice, and the medium bathing the apical and basal cell borders was collected, acidified with perchloric acid, and stored at 4°C until assay for L-dopa and dopamine; cells were handled as mentioned above for determination of intracellular L-dopa and dopamine.

AADC preparation and decarboxylation studies. LLC-PK1 cells were homogenized in 0.5 M phosphate buffer (pH 7.0) with a Thomas Teflon homogenizer and kept continuously on ice. Aliquots of 250 µl cell homogenate plus 250 µl incubation medium were placed in glass test tubes and preincubated for 15 min. Thereafter, L-dopa (50–10,000 µM) was added to the medium for an additional 15 min; the final reaction volume was 1 ml. The composition of the incubation medium was as:

<table>
<thead>
<tr>
<th>Component</th>
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<tbody>
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<tr>
<td>KCl</td>
<td>5 mM</td>
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<tr>
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<tr>
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<tr>
<td>Tris</td>
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<td>pH</td>
<td>7.4</td>
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</table>

The pH was adjusted with NaOH. The incubation medium also contained pargyline (100 µM) and tolcapone (1 µM) to inhibit the enzymes monoamine oxidase and catechol O-methyltransferase, respectively; in some experiments, benserazide (50 µM) was also added to the incubation medium to inhibit AADC.
follows (in mM): 0.35 NaH₂PO₄, 0.15 Na₂HPO₄, 0.11 sodium borate, and 0.12 pyridoxal phosphate; 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 (24). During incubation, cell homogenates were continuously shaken and gassed (95% O₂-5% CO₂) and maintained at 37°C. The reaction was stopped by the addition of 500 µl of 2 M perchloric acid, and the preparations were 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 (35). The high-pressure liquid chromatography system consisted of a pump (model 302; Gilson Medical Electronics, Villiers le Bel, France) connected to a manometric module (Gilson model 802C) and a stainless steel 5-µm ODS column (Biophase; Bioanalytical Systems, West Lafayette, IN) 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 octylsulfate (0.5 mM), sodium acetate (0.1 M), EDTA (0.17 mM), dibutylamine (1 mM), and methanol (8% vol/vol), adjusted to pH 3.5 with perchloric acid (2 M) and pumped at a rate of 1.0 ml/min. 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 analysis revealed a kᵣ of 3.6 ± 0.4 pmol·mg protein⁻¹·min⁻¹, a kₛ of 1.0 ± 0.1 pmol·mg protein⁻¹·min⁻¹, and an Aₘₚ of 15.9 ± 0.9 (n = 8).

In other words, an amount of LLC-PK₁ cells corresponding to 1 mg of protein cleared ~7 µl incubation medium of L-dopa per minute, and, simultaneously, 13% of intracellular L-dopa left the cells. The intracellular water content of cell monolayers was 7.1 ± 0.6 µl/mg protein (n = 12). At equilibrium (30 min incubation), the intracellular L-dopa concentration was 7.9 ± 0.5 µM at medium concentration of 0.5 µM. This represented a cell concentration of L-dopa that was 16 times higher than the medium concentration.

In time-course experiments, the accumulation for L-dopa increased linearly with time for several minutes (Fig. 1). Thus, in all subsequent experiments designed to determine the kinetic parameters for the uptake of

\[ C_i / C_o = k_i / k_o \cdot (1 - e^{-k_o \cdot t}) \]

were fitted to the experimental data by a nonlinear regression analysis, using a computed assisted method (20). Cᵢ and C₀ represent the intracellular and extracellular concentration of the substrate, respectively; kᵢ is the rate constant for inward transport, kₒ is the rate constant for outward transport, and t is the incubation time. Values of kᵢ and kₒ are given in picomoles per milligram protein per minute. Aₘₚ is defined as the factor of accumulation (Cᵢ/C₀) at equilibrium (t → ∞).

Michaelis constant (Kₘ) and maximal velocity (Vₘₚ) 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 (20). The linear rate of accumulation (RA) or outward transfer (RT) was determined by the slope of the accumulation of substrates measured by linear regression analysis (21). Arithmetic means ± SE are given. Statistical analysis was performed by one-way analysis of variance followed by Newman-Keuls test for multiple comparisons. A value of P < 0.05 was assumed to denote a significant difference.

Drugs. Drugs used were L-dopa (Sigma), dopamine hydrochloride (Sigma), GBR-12909 (Research Biochemicals International, Natick, MA), pargyline hydrochloride (Sigma), and tolcapone (kindly donated by the late Prof. Mose Da Prada, Hoffmann La Roche, Basel, Switzerland).

RESULTS

To determine total kᵢ and kₒ, LLC-PK₁ cells were incubated with L-dopa (0.5 µM) for 1, 3, 6, 12, 30, 60, and 120 min in the presence of benserazide (Fig. 1). L-Dopa was rapidly accumulated in LLC-PK₁ cells; equilibrium was attained at 30 min of incubation. From the initial rate of uptake, kᵢ, kₒ, and Aₘₚ were calculated. The analysis revealed a kᵢ of 3.6 ± 0.4 pmol·mg protein⁻¹·min⁻¹, a kₒ of 1.0 ± 0.1 pmol·mg protein⁻¹·min⁻¹, and an Aₘₚ of 15.9 ± 0.9 (n = 8). In other words, an amount of LLC-PK₁ cells corresponding to 1 mg of protein cleared ~7 µl incubation medium of L-dopa per minute, and, simultaneously, 13% of intracellular L-dopa left the cells. The intracellular water content of cell monolayers was 7.1 ± 0.6 µl/mg protein (n = 12). At equilibrium (30 min incubation), the intracellular L-dopa concentration was 7.9 ± 0.5 µM at medium concentration of 0.5 µM. This represented a cell concentration of L-dopa that was 16 times higher than the medium concentration.

In time-course experiments, the accumulation for L-dopa increased linearly with time for several minutes (Fig. 1). Thus, in all subsequent experiments designed to determine the kinetic parameters for the uptake of L-dopa and dopamine.
LLC-PK1 cells incubated for 6 min at 37°C (solid symbols) or 4°C and was found to be nonsaturable (Fig. 2). Cells incubated at 37°C with increasing concentrations of D-dopa at the highest concentration used was 9.6 µM and a \( V_{\text{max}} \) of 6.0 ± 0.2 nmol·mg protein\(^{-1}\)·6 min\(^{-1}\). In experiments carried out at 4°C, the amount of L-dopa accumulated in the cells was markedly lower than that observed at 37°C and was found to be nonsaturable (Fig. 2A). LLC-PK1 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 ~5% of the corresponding L-isomer (Fig. 2A). Results shown in Figs. 1 and 2A were from experiments performed in the presence of benserazide (50 µM), to avoid the intracellular decarboxylation of L-dopa taken up by AADC. Benserazide is an effective AADC inhibitor, and 50 µM benserazide has been found to completely abolish the decarboxylation of L-dopa. However, it is not known whether benserazide may also interfere with the cellular uptake of L-dopa, given the structural similarities between these two compounds. It is, however, almost impossible to test L-dopa uptake in a cell system endowed with AADC activity. Therefore, the apical uptake of benserazide was studied to determine whether the accumulation of the AADC inhibitor in these cells proceeded differently from that of L-dopa. Incubation of LLC-PK1 cells with increasing concentrations of benserazide also resulted in a nonsaturable accumulation of the compound (Fig. 2B); at 500 µM, the amount of benserazide accumulated in the cells was only 5% of the amount of L-dopa accumulated under similar conditions. This suggests that benserazide enters the cells through a process completely different from that used by L-dopa, although it does not prove that benserazide is devoid of effects on the uptake process of L-dopa.

The next series of experiments was performed in cells cultured in polycarbonate filters, and L-dopa was applied from either the apical or the basal border; intracellular L-dopa and L-dopa that escaped to the fluid bathing the basal or the apical cell borders were also measured. The uptake of L-dopa applied from either the apical or basal cell borders was dependent on the concentration and was found to be saturable (Fig. 3); nonlinear analysis of saturation curves for apical and basal application revealed \( K_m \) values of 63.8 ± 17.0 and 42.5 ± 9.6 µM and \( V_{\text{max}} \) values of 32.0 ± 5.8 and 26.2 ± 3.4 nmol·mg protein\(^{-1}\)·6 min\(^{-1}\), respectively.

Incubation of homogenates of LLC-PK1 cells with L-dopa (50–10,000 µM) resulted in a concentration-dependent formation of dopamine (Fig. 4). The decarboxylation process was found to be linear up to 1 mM L-dopa and to become saturated at high concentrations of the substrate (2.5–10 mM L-dopa). Nonlinear analysis of the saturation curves revealed a \( K_m \) of 1,866 ± 107 µM and a \( V_{\text{max}} \) of 4.4 ± 0.1 nmol·mg protein\(^{-1}\)·15 min\(^{-1}\).

Because LLC-PK1 cells were found to be endowed with considerable AADC activity, the cell monolayers were incubated with L-dopa, applied from either the apical or the basal side, in the absence of benserazide, and the intracellular concentrations of newly formed dopamine and the amount of dopamine that had escaped to the fluid bathing the apical and the basal cell...
borders were determined. Cells were incubated with increasing concentrations of L-dopa (5–500 µM) for 6 min. Figure 5 shows the intracellular levels of newly formed dopamine when L-dopa was applied from either the apical or the basal cell border. It is interesting to note that the intracellular accumulation of newly formed dopamine was a rapidly saturable process with an apparent $K_m$ value of $20.5 \pm 8.2 \mu M$ when the substrate was applied from the apical side. By contrast, when the substrate was applied from the basal side, the accumulation of newly formed dopamine in the intracellular compartment was less readily saturable (apparent $K_m$ of $247.3 \pm 76.8 \mu M$). However, intracellular levels of newly formed dopamine attained at 500 µM L-dopa were, under both experimental conditions (apical or basal application of L-dopa), of about the same magnitude. Table 1 shows the percentage of intracellular L-dopa that was decarboxylated to dopamine when the substrate was applied from either the apical or the basal side. As can be observed, the percentage of intracellular L-dopa that undergoes decarboxylation to dopamine decreases progressively with the increase in extracellular L-dopa. Conversely, the decarboxylation of L-dopa applied from the basal side was found to be greater ($P < 0.05$) than that occurring when L-dopa was applied from the apical side (Table 1); this was particularly evident from 5 to 100 µM L-dopa.

Some of the newly formed dopamine was found to escape from the intracellular compartment to the extracellular milieu; this occurred when L-dopa was applied from either the apical or the basal cell side (Fig. 6). The levels of dopamine in the medium bathing the basal cell side were, under both experimental conditions (apical or basal application of L-dopa), considerably greater (5- to 7-fold) than those observed in the medium bathing the apical cell border (Fig. 6). Another interesting difference concerning the basal outward transfer of newly formed dopamine is that apparently it did not depend on the intracellular concentration of the amine (Fig. 6B), whereas the apical outward transfer of dopamine was dependent on the intracellular concentration of dopamine (Fig. 6A). The RT of dopamine, as determined by linear regression analysis, was $0.365 \pm 0.042 \text{ mmol}^{-1} \text{mm}^{-1}$ ($r^2 = 0.950; n = 24$) and $0.425 \pm 0.061 \text{ mmol}^{-1} \text{mm}^{-1}$ ($r^2 = 0.923; n = 24$) for the apical and basal application of L-dopa, respectively.

To define whether the apical and basal outward transfer of newly formed dopamine was mediated through a monoamine transporter, the influence of cocaine (10 and 30 µM) and GBR-12909 (1 and 3 µM) was determined. Cells were loaded with L-dopa (250 µM) applied from the basal side, and the levels of intracellular dopamine and the levels of the amine in the fluid bathing the apical and basal sides were determined. As can be observed in Table 2, both cocaine and GBR-12909 were found not to affect the intracellular levels of newly formed dopamine and the apical or basal outward transfer of the amine.

In the final series of experiments, we addressed the question of the type of inward movement of dopamine in LLC-PK1 cells. Exogenous dopamine (5–250 µM) was applied from the apical or the basal side for 6 min. The accumulation of exogenous dopamine in LLC-PK1 cells was determined by linear regression analysis, was $0.365 \pm 0.042 \text{ mmol}^{-1} \text{mm}^{-1}$ ($r^2 = 0.950; n = 24$) and $0.425 \pm 0.061 \text{ mmol}^{-1} \text{mm}^{-1}$ ($r^2 = 0.923; n = 24$) for the apical and basal application of L-dopa, respectively.

![Fig. 4. Saturation curve of aromatic L-amino acid decarboxylase (AADC) activity in homogenate LLC-PK1 cells. AADC activity is expressed as rate of formation of dopamine (DA; in nmol·mg protein$^{-1}$·15 min$^{-1}$) vs. concentration of L-dopa (µM). Symbols represent means ± SE of 5 experiments/group.](http://ajprenal.physiology.org/Downloaded from http://ajprenal.physiology.org/)

![Fig. 5. Rate of formation of dopamine in LLC-PK1 cells loaded with increasing concentrations of L-dopa. Cells were preincubated in absence of benzerazide for 30 min and incubated for 6 min with increasing concentrations of L-dopa; substrate was applied from either apical (■) or basal (□) cell border. Symbols and vertical lines represent means ± SE, respectively, of 4–5 experiments/group.](http://ajprenal.physiology.org/Downloaded from http://ajprenal.physiology.org/)
cells was found to be saturable (Fig. 7); nonlinear analysis of the saturation curves revealed for the apical and basal application of dopamine a $K_m$ of $17.7 \pm 4.3$ and $96.0 \pm 28.1 \mu M$ and a $V_{max}$ of $2.0 \pm 0.1$ and $2.2 \pm 0.3$ nmol·mg protein$^{-1}$·min$^{-1}$, respectively. However, both cocaine (10, 30, or 100 µM) and GBR-12909 (1 or 3 µM) were found not to affect the uptake of 100 µM dopamine applied from either the apical or the basal cell border (Fig. 8).

**DISCUSSION**

The results presented here show that LLC-PK₁ cells transport L-dopa quite efficiently through both the apical and basolateral cell borders. Intracellular L-dopa levels of newly formed dopamine in intracellular compartment (cell) and in medium bathing apical and basal sides and effect of cocaine and GBR-12909

<table>
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<tr>
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<th>Cell</th>
<th>Apical Fluid</th>
<th>Basal Fluid</th>
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<tr>
<td></td>
<td>Dopamine, nmol/mg protein</td>
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<tr>
<td>Control</td>
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<td>3.5 ± 0.3</td>
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</table>

Results are means ± SE of 4 experiments/group. LLC-PK₁ cells were incubated with 250 µM L-dopa for 6 min, and substrate was applied from basal cell border only. Control values are given in nmol/mg protein and effect of drugs tested is given as percentage of control.
is rapidly converted to dopamine, but the synthesis of the amine in LLC-PK₁ cells becomes a rate-limiting step when high concentrations of L-dopa are used. Intracellular newly formed dopamine leaves the cell through the apical border by a diffusional process, whereas dopamine leaving the cell through the basal cell border appears to be transported readily by a saturable process. Both processes are, however, insensitive to inhibitors of the dopamine transporter, cocaine and GBR-12909. The apical and basal uptake of extracellular dopamine behaves as a saturable process with low affinity for the substrate; this is also insensitive to cocaine and GBR-12909. With consideration of the information provided here on the cell handling of L-dopa and newly formed dopamine and previous evidence on the autorcine/paracrine effects of newly formed dopamine mediated through specific dopamine receptors, it is suggested that these cells constitute a useful in vitro model for the study of the renal dopaminergic physiology.

Several findings demonstrate that the apical uptake of L-dopa into LLC-PK₁ cells was a facilitated mechanism. First, steady-state uptake of nonsaturating concentrations of L-dopa showed a curvilinear dependence on incubation time. Second, at the initial rate of uptake (6 min incubation), the cellular transport of L-dopa showed a curvilinear dependence on L-dopa medium concentration with a Kₘ of 123 µM, suggesting that the uptake was saturable. Third, this accumulation of L-dopa was nearly abolished at 4°C, demonstrating that the uptake was energy dependent. The efficiency of the L-dopa transport in LLC-PK₁ cells also can be evidenced by the ratio of L-dopa concentration in cellular water to medium concentration. It was found that intracellular L-dopa concentration at equilibrium was larger than that which could be expected by passive equilibration of L-dopa. In fact, at steady-state uptake, the mean intracellular concentration of L-dopa was 16 times larger 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. This agrees with previous evidence on the uptake of L-dopa obtained in isolated rat renal proximal tubules. Chan (5) has shown that the uptake of L-dopa in perfused rat renal tubules is energy dependent and stereoselective; similar findings have been also observed in more recent investigations, using suspensions of isolated rat renal tubules loaded with L-dopa (28, 35). Experiments carried out in cells cultured in polycarbonate filters showed that the apical uptake of L-dopa is similar to that observed when the substrate is applied from the basal cell border. It is interesting, however, to observe that the apical uptake of L-dopa in cells cultured in polycarbonate filters differs substantially from that observed in cells cultured in collagen-treated plastic, with the main differences being a lower Kₘ value (32 ± 4 vs. 123 ± 17 µM) and a higher Vₘₐₓ value (32.0 ± 4.4 vs. 5.8 ± 0.2 pmol·mg protein⁻¹·min⁻¹). A possible explanation for this discrepancy may have to do with the different environment of cells cultured in a more physiological system, such as in polycarbonate filters.

The role of the L-dopa transporter in the whole process of dopamine formation is still an open question, i.e., the rate-limiting step in the formation of the amine has not been clearly identified. To defined which of the processes, the uptake process or the decarboxylation process, rate limits the formation of dopamine, LLC-PK₁ cells were incubated in the absence of benserazide, and the intracellular levels of L-dopa and dopamine were determined. As shown in Fig. 5 and Table 1, the process of dopamine formation is rapidly saturable, and a substantial amount of L-dopa taken up is converted to dopamine. This is particularly evident for low concentrations of the substrate (5–50 µM), which most probably are still above the range of physiological levels of L-dopa in renal tissues; plasma levels range between 5 and 10 pmol/ml (37). There are, however, marked differences in this process of intracellular decarboxylation of L-dopa, depending on whether the substrate is applied from the apical or the basal cell border. In fact, saturation of intracellular decarboxylation of L-dopa is readily attained when the substrate is applied from the apical cell border (Kₘ = 20.5 ± 8.2 µM), which contrasts with that observed when the substrate is applied from the basal cell border (Kₘ = 247.3 ± 76.8 µM). This marked difference may suggest the presence at the basolateral cell pole of an intracellular storage compartment devoid of AADC activity, which prevents intracellular L-dopa from being converted to dopamine. The capacity of these hypothetical L-dopa storage compartments appears, however, to be quite limited, since at 500 µM L-dopa, in the extracellular medium, the formation of dopamine attains the same Vₘₐₓ value as when the substrate is applied from the apical cell border. Alternatively, it might suggest a heterogeneous distribution of AADC in the cell, with the predominant pool being concentrated near the apical pole of the cell. This is the most likely explanation and would agree with the evidence obtained in histofluorescence studies showing that L-dopa-induced fluorescence prevails at the apical pole of epithelial cells of rat renal proximal tubules (13).

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 and basal cell borders, and the data obtained clearly show that the former transfer is a diffusional process, whereas the latter may be an easily saturable one. The finding that the outward movement of intracellular dopamine through the apical cell border is nonsaturable within a range of intracellular concentrations of dopamine from 0.3 to 1.4 mM strongly suggests that this is a diffusional process; this can be observed when L-dopa is applied from either the apical or the basal cell border. This contrasts with that observed at the basal cell border; the outward movement of newly formed dopamine was quite stable over a wide range of intracellular concentrations of the amine. This may suggest that the outward transfer of dopamine is a high-affinity, readily saturable process. The experiments carried out in the presence of cocaine and GBR-12909, two potent inhibitors of the dopamine transporter (15), showed that this transfer of dopamine is not sensitive to these inhibi-
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Sides were 0.04-0.06 mmol/l; the corresponding concentrations of dopamine in the apical and basal cell sides, respectively. The highest intracellular concentration of dopamine was 1.36 µg cell protein), these ratios would be 870 and 2,600 for the apical and basal cell sides, respectively. In consideration of the fact that intracellular water ratios. In consideration of the fact that the renal transport of exogenous dopamine applied from the basal cell side was higher than those observed for the apical cell border, considering the outward movement of dopamine. Because the volumes of incubation medium bathing the apical and basal sides differed by a factor of three (200 vs. 600 µl), it could be hypothesized that the higher ability of dopamine to leave the cellular compartment was due to differences in extracellular water-to-intracellular water ratios. In consideration of the fact that intracellular water in cells grown in permeable supports attains a volume of 0.23 µl (7 µmol protein:33 µg cell protein), these ratios would be 870 and 2,600 for the apical and basal cell sides, respectively. The highest intracellular concentration of dopamine was 1.36 ± 0.06 mmol/l; the corresponding concentrations of dopamine in the medium bathing the apical and basal cell sides were 0.04 ± 0.02 and 0.39 ± 0.05 mmol/l, respectively. This clearly suggests that the 870-fold difference between extracellular and intracellular water is enough to allow a gradient to be established.

It is, however, worthwhile to underline the finding that the inward transfer of exogenous dopamine is, in contrast to that found to occur for the outward transfer of the newly formed dopamine, a saturable process when applied from either the apical or the basal cell border. However, the kinetic profile of the uptake process found to occur at the apical cell border presented a higher affinity for the substrate, as evidenced by a fivefold lower K_m value. The affinity of this transporter for exogenous dopamine was, however, lower than that described for the dopamine transporter sensitive to cocaine or GBR-12909. This agrees with the finding that both cocaine (10, 30, and 100 µM) and GBR-12909 (1 and 3 µM) were ineffective in reducing the uptake of dopamine applied either from the apical or the basal cell border. These findings contrast with those obtained in OK cells, renal cells derived from renal tubules of a female American opossum, in which dopamine was demonstrated to be taken up by a saturable transporter when applied from the basal cell border only. The data presented here on the uptake of dopamine by the apical cell border also contrast with the finding that the renal transport of exogenous dopamine is unidirectional from the basolateral to the apical cell border (5). This should be taken into consideration when assuming LLC-PK1 cells as an in vitro model for the study of renal tubular physiology.

In conclusion, the data presented here show that LLC-PK1 cells are endowed with considerable AADC activity and transport L-dopa quite efficiently through both the apical and basal cell borders. Intraacellular L-dopa is rapidly converted to dopamine, and the rate of conversion of L-dopa to dopamine does not appear to rate limit the synthesis of the amine. On the other hand, our observations support the possibility of a basal-to-apical gradient of AADC activity and the possibility that LLC-PK1 cells might constitute an interesting in vitro model for the study of renal dopaminergic physiology.

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