Molecular modulation of inward and outward apical transporters of L-dopa in LLC-PK₁ cells

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Soares-da Silva, P., and M. P. Serrão. Molecular modulation of inward and outward apical transporters of L-dopa in LLC-PK₁ cells. Am J Physiol Renal Physiol 279: F736–F746, 2000.—The present study examined the nature of the apical inward and outward L-3,4-dihydroxyphenylalanine (L-dopa) transporters in LLC-PK₁ cells and whether protein kinases differentially modulate the activities of these transporters. The apical inward transfer of L-dopa was promoted through an energy-dependent and sodium-insensitive transporter (Michaelis constant = 38 μM; maximum velocity = 2608 pmol·mg protein⁻¹·6 min⁻¹). This transporter was insensitive to N′-(methylamino)-isobutyric acid but competitively inhibited by 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BHC; IC₅₀ = 251 μM). Modulators of protein kinase A (cAMP, forskolin, IBMX, and cholera toxin), protein kinase G (cGMP, zaprinast, LY-83583 and sodium nitroprusside), and protein kinase C (phorbol 12,13-dibutirate and chelerythrine) failed to affect the accumulation of L-dopa. The Ca²⁺/calmodulin inhibitors calmidazolium and trifluoperazine inhibited L-dopa uptake (IC₅₀ of 72 and 55 μM, respectively). The inhibitory effect of calmodiazolium on the accumulation of L-dopa was of the noncompetitive type. The organic anion inhibitor DIDS, but not cAMP- or protein kinase C inhibitors, significantly increased L-dopa accumulation, which was mainly due to inhibition of apical outward transfer of L-dopa. It is concluded that LLC-PK₁ cells take up L-dopa over the apical cell border through the L-type amino acid transporter, which appears to be under the control of Ca²⁺/calmodulin-mediated pathways. The apical outward transfer of L-dopa may be promoted through a DIDS-sensitive transport mechanism and appears to be under the tonic control of PTK.

L-3,4-dihydroxyphenylalanine; L-type amino acid transporter; 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid-sensitive transporter; Ca²⁺/calmodulin; protein tyrosine kinase

THE RENAL DOPAMINERGIC SYSTEM is 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 (19, 22, 31). 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 (30). To overcome technical problems related to the handling of freshly isolated renal tubular epithelial cells, LLC-PK₁ cells, which express proximal tubule cell-like properties in vitro (17), have been used to study dopamine receptors and the renal actions of the amine. These cells have been also shown to contain AADC and convert L-dopa to dopamine in a nonsaturable fashion up to 1 mM L-dopa (5, 11, 12). Newly formed dopamine also stimulated cAMP accumulation in LLC-PK₁, and this effect was attenuated by an equimolar concentration of carbidopa and blocked by the D₁-antagonist Sch-23390 (12). 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.

Although the kidney is endowed with one of the highest levels of AADC in the body and plasma levels of L-dopa are in the nanomoles per milliliter range (13, 36), the rate-limiting step for the synthesis of dopamine in renal tissues is still a matter of debate. However, because Michaelis constant (Kₘ) values for L-dopa uptake are 10 times lower than Kₘ values for decarboxylation of L-dopa, it could be possible that L-dopa uptake rather than decarboxylation may limit the rate of formation of dopamine. In a previous report (38), we have concluded that LLC-PK₁ cells take up L-dopa through a saturable, stereoselective, and temperature-dependent process when applied from the apical and basolateral cell border, this being similar to that occurring in rat renal proximal tubules (26, 34). In several epithelia and at the level of brain capillary endothelium, L-dopa and other large neutral amino acids are transported by the L-type amino acid transporter. This is a sodium-independent and 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BHC)-sensitive transporter. Differentiation between A- and L-type transporters is based on sodium dependence and sensitivity to N′-(methylamino)-isobutyric acid (MeAIB) and insensitivity to BHC; the B⁵⁺⁺-type is BHC-sensitive and sodium-dependent transporter (1, 25, 28, 41).

The present study examined the effect of maneuvers that affect cellular sodium and proton gradients and the sensitivity to inhibitors of amino acid transport and inhibitors of organic cation and anion transporters to

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define the nature of the transporters involved in the apical inward and outward transfer of L-dopa. The result of maneuvers that affect molecular mechanisms, namely those concerning protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), Ca²⁺/calmodulin-, and protein tyrosine kinase (PTK)-mediated pathways on L-dopa uptake, was also evaluated. It is reported that LLC-PK₁ cells take up L-dopa over the apical cell border through the L-type amino acid transporter, which appears to be under the control of Ca²⁺/calmodulin-mediated pathways. The apical outward transfer of L-dopa is promoted through a DIDS-sensitive transport mechanism and appears to be under the tonic control of PTK.

METHODS

Cell culture. LLC-PK₁ cells, a porcine-derived proximal renal tubule epithelial cell line that retains several properties of proximal tubular epithelial cells in culture (17), were obtained from the American Type Culture Collection (Rockville, MD). LLC-PK₁ cells (ATCC CRL 1392; passages 198–206) were maintained in a humidified atmosphere of 5% CO₂-95% air at 37°C and grown in Medium 199 (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml treptomycin (Sigma), 3% fetal bovine serum (Sigma), and 25 mM HEPES (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 (internal diameter 16 mm, Costar) at a density of 40,000 cells/well or onto collagen-treated 0.2-μm polycarbonate filter supports (internal diameter 12 mm Transwell, Costar) at a density 13,000 cells per well (2.0 × 10⁴ cells/cm²). The cell medium was changed every 2 days, and the cells reached confluence 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 confluence and 6–8 days after the initial seeding and each squared centimeter contained ~80 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; thereafter, the cell monolayers were preincubated for 30 min in Hanks’ medium at 37°C. The Hanks’ medium had the following composition (mM): 137 NaCl, 5 KCl, 0.8 MgSO₄, 0.33 Na₂HPO₄, 0.44 KH₂PO₄, 0.25 CaCl₂, 1.0 MgCl₂, 0.15 Tris-HCl, and 1.0 sodium butyrate, pH = 7.4. The incubation medium also contained benserazide (50 μM) and tolcapone (1 μM) to inhibit the enzymes AADC and catechol-O-methyltransferase, respectively. Time-course studies were performed in experiments in which cells were incubated with 0.5 μM substrate for 1, 3, 6, and 12 min. Saturation experiments were performed in cells incubated for 6 min with increasing concentrations of L-dopa (10–250 μM). In experiments performed in the presence of different concentrations of sodium, NaCl was replaced by an equimolar concentration of choline chloride. Test substances were applied from the apical side only and were present during the preincubation and incubation periods. During preincubation and incubation, the cells were continuously shaken and maintained at 37°C. Apical uptake was initiated by the addition of 2 ml Hanks’ medium with a given concentration of the substrate. Uptake was terminated by the rapid removal of uptake solution by means of a vacuum pump connected to a Pasteur pipette followed by a rapid wash 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.

Previous studies have shown that some of the L-dopa accumulated in LLC-PK₁ cells can leave the cell through apical outward transporter(s) (38), the inhibition of which leads to an increase in the cellular accumulation of L-dopa (39). Thus in experiments designed to study the effect of drugs that increased the intracellular accumulation of L-dopa, cells were incubated with 25 μM L-dopa applied from the basal cell border and uptake (accumulation in the cell monolayer) and flux (transfer to opposite chamber) were measured over a 6-min period. Test drugs were applied from the apical side only and were present during the preincubation and incubation periods. [³⁵S]sorbitol (0.4 μM) was used to estimate paracellular fluxes and extracellular trapping of L-dopa during L-dopa uptake studies. Paracellular fluxes were estimated dividing concentration of [³⁵S]sorbitol in the apical chamber by the concentration of [³⁵S]sorbitol in the basal chamber. Extracellular trapping was calculated dividing the amount of [³⁵S]sorbitol in the cell monolayer by the amount of [³⁵S]sorbitol in the basal chamber. At the end of incubation, cells were placed on ice and the medium bathing the apical cell border was collected, acidified with perchloric acid, and stored at 4°C till assayed for L-dopa. The cells were washed with ice-cold Hanks’ medium and added 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.

Assay of L-dopa. L-dopa was quantified by means of high-pressure liquid chromatography with electrochemical detection, as previously reported (38). The high-pressure liquid chromatograph system consisted of a pump (Gilson model 302; Gilson Medical Electronics, Villiers le Bel, France) connected to a manometric module (Gilson model 802 C) and a stainless-steel 5-μm ODS column (Biophase; Bioanalytical Systems, West Lafayette, IN) of 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% 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 lower limits for detection of L-dopa ranged from 350 to 500 fmol.

Transport of p-aminophenol. Transport of p-aminophenol (PAH) was initiated by adding Hanks’ medium containing [³H]PAH (3 μM) and [³⁵S]sorbitol (3 μM) to the basal or to the apical side of the monolayers. [³⁵S]sorbitol was used to estimate paracellular fluxes and extracellular trapping of [³H]PAH. For the measurement of transepithelial transport, the medium in the other side was collected after incubation for the specified period of time, and the radioactivity was counted. In time course studies, an aliquot of the medium (100 μl) was collected every 15 min over a period of 60 min, and the aliquot was replaced with an equal volume of Hanks’ medium. The data at 30, 45, and 60 min represent cumulative values. The monolayers were agitated every 5 min during transport measurement. In some experiments, cell mono-
layers were incubated in the presence of unlabeled PAH (1 mM) added from the basal side. At the end of the transport experiment, the medium was immediately aspirated and the filter was washed three times with ice-cold Hanks’ medium. Subsequently, the cells were solubilized by 0.1% vol/vol Triton X-100 (dissolved in 5 mM Tris-HCl, pH 7.4) and radioactivity was measured by liquid scintillation counting.

P-glycoprotein activity. P-glycoprotein activity was measured according to the procedure described by Hollo et al. (15), with minor modifications. In brief, LLC-PK₁ cells cultured in collagen-treated cover slips were incubated in culture medium for 30 min in the absence or the presence of verapamil (25 μM) and genistein (100 μM). Thereafter, the cells were transferred to a Perkin-Elmer cuvette holder (model LS 50) and incubated with Hanks’ medium containing 0.5 μM calcein acetoxymethyl ester (calcein-AM) for a further 5 min at 37°C with continuous stirring. The Hanks’ medium had the following composition (mM): 137 NaCl, 5 KCl, 0.8 MgSO₄, 0.33 Na₂HPO₄, 0.44 KH₂PO₄, 0.25 CaCl₂, 1.0 MgCl₂, 0.15 Tris-HCl, and 1.0 sodium butyrate, pH 7.4. Calcein-AM is highly lipid soluble and rapidly penetrates the plasma membrane and is practically nonfluorescent. By cleavage of the ester bonds, intracellular esterases transform the dye to a hydrophilic and intensely fluorescent free acid form. Fluorescence was measured in a FluoroMax-2 (Jobin Yvon-SPEX, Edison, NJ) spectrofluorometer using excitation and emission wavelengths of 450 and 507 nm, respectively. Time-resolved experiments were started 5 min after the addition of calcein-AM to the medium bathing the cells and lasted for 20 min. In experiments using cells pretreated with verapamil and genistein, the incubation medium also contained verapamil (25 μM) or genistein (100 μM). Calibration of dye concentration was based on the measurements of free calcein fluorescence in the same instrument under identical conditions (in the absence and in the presence of verapamil or genistein). All experiments were repeated five to seven times with different batches of cell monolayers.

Protein assay. The protein content of monolayers of LLC-PK₁ cells was determined by the method of Bradford (2), with human serum albumin as a standard.

Cell viability. Cells cultured in collagen-treated plastic supports were preincubated for 15 min at 37°C and then incubated in the absence or the presence of l-dopa and test compounds for further 6 min. Subsequently the cells were incubated at 37°C for 2 min with trypan blue (0.2% wt/vol) 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, >95% of the cells excluded the dye.

Data analysis. kₘ and maximum velocity (V_max) values for the uptake of l-dopa, as determined in saturation experiments were calculated from nonlinear regression analysis using the GraphPad Prism statistics software package (24). P-glycoprotein activity was determined by the slope of the accumulation of calcein (in pmol/mg protein) measured by linear regression analysis (24). Arithmetic means are given with SE. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference.

Drugs. Actinomycin D, amiloride hydrochloride, BHC, amphotericin B, calmidazolium, chelerythrine chloride, cholora toxin, cycloheximide, N-6,2′-O-dibutyryl cAMP, cGMp, 1,1′-diethyl-2,4′-cyanine (decyanim 24), DIDS, 2,4-dinitrophenol, forskolin, genistein, genistin, IBMX, l-dopa, MeAIB, ouabain, phorbol 12,13-dibutyrate (PDBu), 4α-phorbol 12,13-didecanoate (PDDC), sodium nitroprusside, trifluoperazine dihydrochloride, trypan blue, tyrphostin 1 and tyrphostin 25 were purchased from Sigma, St. Louis, MO. LY-83583 [(E)-phenylamino]-5,8-quinalinedione) and zaprinast were obtained from Research Biochemicals International (Natick, MA) and May & Baker (Dorset, England), respectively. Tolcapone was kindly donated by the late Professor Moise Da Prada (Hoffmann La Roche, Basle, Switzerland). [³⁵S]sorbitol (specific activity 990 GBq/mmol) and [³H]PAH (specific activity 1.8–2.2 GBq/mmol) were purchased from New England Nuclear (Boston, MA).

RESULTS

The accumulation of a nonsaturating concentration (0.5 μM) of l-dopa, in time-course experiments, increased linearly with time for several minutes (Fig. 1A). At 6 min when uptake was linear and considering intracellular water as 7.0 ± 0.7 μl/mg protein (38), the intracellular l-dopa concentration was 4.0 ± 0.4 μM at

![Fig. 1](http://ajprenal.physiology.org/)

**Fig. 1. A:** time course of L-3,4-dihydroxyphenylalanine (l-dopa) in renal tubular epithelial cells (LLC-PK₁). Cells were incubated at 37°C with 0.5 μM of l-dopa and results are levels (in pmol/mg protein) of substrate accumulated. Each point are the mean of 8 experiments/group; vertical lines show SE. **B:** Concentration-dependent accumulation of l-dopa in LLC-PK₁ cells. Cells were incubated for 6 min at 37°C and increasing concentrations (1 to 250 μM) of the substrate were applied from the apical border. Results reflect levels (in pmol·mg protein⁻¹·6 min⁻¹) of accumulated l-dopa. Each point is mean of 4 experiments/groups; vertical lines show SE.
medium concentration of 0.5 μM. This represented a cell concentration of L-dopa that was 8.0 ± 0.8 times higher than the corresponding medium concentration. In a subsequent set of experiments, designed to determine the kinetic parameters of the L-dopa apical transporter, the cells were incubated for 6 min with increasing concentrations (1 to 250 μM) of the substrate (Fig. 1B). Nonlinear analysis of the saturation curve for L-dopa revealed a \( K_m \) value (in μM) of 47 ± 8 and a \( V_{max} \) value (in pmol·mg protein\(^{-1}\)·6 min\(^{-1}\)) of 3,069 ± 224.

To evaluate the metabolic requirements for L-dopa uptake, cells were pretreated with the uncoupling agent 2,4-dinitrophenol or incubated at 4°C. Pretreatment with 2,4-dinitrophenol (1 mM) resulted in a marked reduction in L-dopa (2.5 μM) uptake (Table 1). Similarly, the effect of reducing temperature from 37°C to 4°C during preincubation and incubation was a marked reduction in L-dopa (2.5 μM) accumulation (Table 1).

Reducing extracellular sodium (from 140 mM to 70, 35 and 0 mM) did not affect the accumulation of L-dopa (Fig. 2A). Moreover, in the absence of extracellular sodium (replaced by an equimolar concentration of choline), \( K_m \) and \( V_{max} \) values for L-dopa were similar to those observed in the presence of sodium (Table 2). Maneuvers that affect transepithelial flux of sodium, such as acidification of the extracellular milieu (from pH 7.4 to pH 6.9 or pH 6.4) and the addition of amphoterin B (2.5 μg/ml), amiloride (100 μM), ouabain (500 μM) failed to affect the accumulation of L-dopa (Table 1). MeAIB (1 mM) failed to affect the uptake of L-dopa, whereas BHC produced a concentration-dependent inhibition of L-dopa uptake (IC\(_{50} = 251 ± 26 μM\)) (Fig. 2B). The inhibitory effect of 1 mM BHC on the accumulation of L-dopa was of the competitive type, as evidenced by the increase in \( K_m \) but not \( V_{max} \) values for L-dopa uptake (Table 2). Taken together, these results suggest that the apical inward transfer of L-dopa may be promoted through the BHC-sensitive and sodium-independent L-type amino acid transporter.

Table 1. Effect of manoeuvres that affect energy-dependent process, transepithelial flux of sodium ions and protons and of modulators of PKA, PKG, PKC, and PTK on the uptake of L-dopa (2.5 μM) in LLC-PK\(_1\) cells

<table>
<thead>
<tr>
<th>Energy</th>
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<tbody>
<tr>
<td>37°C</td>
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</tr>
<tr>
<td>4°C</td>
<td>23 ± 2*</td>
</tr>
<tr>
<td>2,4-dinitrophenol</td>
<td>31 ± 4*</td>
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<table>
<thead>
<tr>
<th>pH</th>
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<tbody>
<tr>
<td>pH 7.9</td>
<td>119 ± 4</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>pH 6.9</td>
<td>106 ± 11</td>
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<tr>
<td>pH 6.4</td>
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<table>
<thead>
<tr>
<th>PKA</th>
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<tbody>
<tr>
<td>Control</td>
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</tr>
<tr>
<td>AMP, 0.5 mM</td>
<td>113 ± 6</td>
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<tr>
<td>Forskolin, 50 μM</td>
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</tr>
<tr>
<td>Isobutylmethylxanthine, 0.1 mM</td>
<td>133 ± 6</td>
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<tr>
<td>Cholera toxin, 5 μg/ml</td>
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<table>
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<tr>
<th>PKG</th>
<th>% of Control</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>cGMP, 1 mM</td>
<td>96 ± 9</td>
</tr>
<tr>
<td>Zaprinast, 30 μM</td>
<td>126 ± 21</td>
</tr>
<tr>
<td>LY-83583, 30 μM</td>
<td>135 ± 16</td>
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<tr>
<td>Sodium nitroprusside, 100 μM</td>
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</table>

<table>
<thead>
<tr>
<th>PKC</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>PDBu, 1 μM</td>
<td>116 ± 9</td>
</tr>
<tr>
<td>PDDC, 1 μM</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>Chelerythrine, 10 μM</td>
<td>81 ± 6</td>
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</table>

Values are means ± SE of 4 to 7 determinations/group. Values are percentage of control for accumulation of L-dopa in LLC-PK\(_1\) cells (absolute levels were 143 ± 5 pmol·mg\(^{-1}\)·protein\(^{-1}\)·6 min; \( n = 81 \)). PKA, PKG, PKC: protein kinase A, G, and C, respectively; L-dopa: L-3,4-dihydroxyphenylalanine; PDBu: phorbol 12,13-dibutyrate; PDDC: 4-α-phorbol 12,13 diacetate. Significantly different from corresponding control values (*P < 0.05) using Newman-Keuls test for multiple comparisons.
L-dopa (250 μM) in the growth medium for 24 h affect the L-dopa transporter. Cells were cultured as described in METHODS, and the growth medium was substituted 24 h before experiments by fetal bovine serum (FBS)-free growth medium with added L-dopa (250 μM) or the vehicle. On the day of the experiments, cells were washed with Hanks’ medium and were then preincubated for 30 min followed by incubation with increasing concentrations of L-dopa (10 to 250 μM) for 6 min. Nonlinear analysis of the saturation curve for L-dopa revealed similar \( K_m \) and \( V_{\text{max}} \) values for vehicle- and L-dopa-treated cells (Table 2).

Because neither the affinity nor the number of transport units was affected by prolonged exposure to a saturating concentration of L-dopa, we were then interested to know to which extent inhibition of synthesis of new transporter units would affect the uptake of L-dopa. For this purpose, confluent monolayers were exposed for 3 h to the DNA transcription inhibitor actinomycin D (0.5 μg/ml) or cycloheximide (70 μM) in FBS-free growth medium. Then the cells were allowed to recover for 12 or 24 h in FBS-free growth medium before incubation with L-dopa. As shown in Fig. 3, exposure to both actinomycin D and cycloheximide was found to markedly increase \( P < 0.05 \) the accumulation of L-dopa (2.5 μM) without changes in \( K_m \) values. In contrast, the organic anion PAH was found to produce a slight \( P = 0.09 \) decrease (−20% reduction) in the accumulation of L-dopa (Fig. 4). Though DIDS is a well-known inhibitor of the organic cation and organic anion transporters, respectively. Decynium 24 failed to affect the accumulation of L-dopa, whereas DIDS produced a concentration-dependent increase in the accumulation of a non-saturating concentration (2.5 μM) of L-dopa (Fig. 4). As shown in Table 2, pretreatment of cells with 1 mM DIDS was found to markedly increase \( P < 0.05 \) the accumulation of increasing concentrations of L-dopa without changes in \( K_m \) values. Though more evident, the effects of cycloheximide point in the same direction.

![Fig. 3](http://ajprenal.physiology.org/)

Fig. 3. Effect of pretreatment of LLC-PK1 cells with (A) actinomycin D or (B) cycloheximide on the accumulation of L-dopa (2.5 μM). Cells were exposed for 3 h to actinomycin D (0.5 μg/ml) or cycloheximide (70 μM) in FBS-free growth medium; then, cells were allowed to recover for 12 or 24 h in FBS-free growth medium before incubation with L-dopa. Columns are mean of 8 experiments/group; vertical lines show SE.

![Fig. 4](http://ajprenal.physiology.org/)

Fig. 4. Effect of decynium 24, DIDS, and p-aminobenzvacetic acid (PAH) on accumulation of L-dopa (2.5 μM) in LLC-PK1 cells. Symbols are mean of 4 to 8 experiments/group; vertical lines show SE. *Significantly different from corresponding control values \( (P < 0.05) \).

<table>
<thead>
<tr>
<th>Condition</th>
<th>( K_m ), μM</th>
<th>( V_{\text{max}} ), pmol mg protein (^{-1}) 6 min (^{-1})</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>47 ± 8</td>
<td>3,069 ± 224</td>
</tr>
<tr>
<td>Sodium, 0 mM</td>
<td>38 ± 8</td>
<td>2,608 ± 190</td>
</tr>
<tr>
<td>BHC, 1 mM</td>
<td>130 ± 19*</td>
<td>3,783 ± 244</td>
</tr>
<tr>
<td>DIDS, 1 mM</td>
<td>48 ± 15</td>
<td>4,492 ± 473</td>
</tr>
<tr>
<td>Genistein, 300 μM</td>
<td>28 ± 8</td>
<td>4,376 ± 740</td>
</tr>
<tr>
<td>Calmidazolium, 30 μM</td>
<td>61 ± 21</td>
<td>1,739 ± 215</td>
</tr>
<tr>
<td>L-dopa, 250 μM (24 h)</td>
<td>38 ± 8</td>
<td>3,179 ± 203</td>
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Values are means ± SE of 7 determinations/groups. BHC, 2-amino-1,4-bicyclo-2,2,1-heptane-2-carboxylic acid; \( K_m \), Michaelis constant; \( V_{\text{max}} \), maximum velocity. Significantly different from corresponding control values \((P < 0.05)\) using the Newman-Keuls test for multiple comparisons.
of the organic anion transporter (16), it is unlikely that its effects upon L-dopa uptake might be attributed to the interference with this transporter. First, this cell line has been shown by several groups to have lost the organic anion transporter (16, 23, 27). Second, movement of organic anions is usually performed in the opposite direction (basal-to-apical) to that imposed here for L-dopa. On the other hand, it is difficult to conceive a model in which DIDS promotes the entry of L-dopa into the cell through the apical cell border. By contrast, previous studies have shown that LLC-PK₁ cells are able to extrude L-dopa through apical outward transporter(s) (38), the inhibition of which leads to an increase in the cellular accumulation of L-dopa (39). Thus to clarify these issues it was decided to evaluate whether DIDS decreases the basal-to-apical flux of L-dopa, and second if LLC-PK₁ cells are endowed with the organic anion transporter. Cells cultured in polycarbonate filters were incubated with L-dopa (25 μM) applied from the basal cell border and the basal-to-apical flux measured. As shown in Fig. 5, DIDS (1 mM) markedly reduced the basal-to-apical flux of L-dopa and increased the accumulation of L-dopa in the cell. By contrast, as shown in Fig. 6, the transepithelial transport and the cell accumulation of [³H]PAH was close to that of [¹⁴C]sorbitol, indicating that the apparent transport and accumulation of [³H]PAH represents nonspecific transfer and/or trapping. The basal-to-apical transport and cell accumulation of [³H]PAH and [¹⁴C]sorbitol were not affected by unlabeled PAH. These two sets of experiments indicate that LLC-PK₁ cells were devoid of the organic anion transporter, and the increase in L-dopa accumulation by DIDS depended on its ability to reduce the outward transfer of L-dopa through the apical cell border but was unrelated to its properties as organic anion inhibitor.

Up to this stage, the combined results revealed that in LLC-PK₁ cells the apical inward transfer of L-dopa was promoted through the L-type amino acid transporter and intracellular L-dopa could be extruded by a DIDS-sensitive apical transporter that lacked the properties of an organic anion transporter. The next series of experiments explored the role of intracellular regulatory pathways in the cellular handling of L-dopa applied from the apical cell border. Involvement of a PKA-mediated pathway in the regulation of L-dopa uptake was tested by examining the effect of pretreating LLC-PK₁ cells for 30 min with compounds that are known to increase intracellular cAMP levels. Dibutryl

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Fig. 5. Effect of DIDS (1 mM) on the apical flux and cell accumulation of L-dopa (25 μM) applied from basolateral cell border in LLC-PK₁ cells. Columns are mean of 4 experiments/group; vertical lines show SE. *Significantly different from corresponding control values (P < 0.05).

Fig. 6. Effect of unlabeled PAH (1 mM) on basal-to-apical transports (A) and cell accumulation of 3 μM [³H]PAH and [¹⁴C]sorbitol (B) across LLC-PK₁ monolayers. Each point or column is mean of 4 separate experiments.
cAMP (0.5 mM), forskolin (50 μM), IBMX (0.1 mM), and cholera toxin (5 μg/ml) failed to affect the accumulation of a nonsaturating concentration of L-dopa (Table 1). In another series of experiments, we tested the involvement of a PKG-mediated pathway in the regulation of L-dopa uptake. cGMP (1 mM), zaprinast (30 μM), LY-83583 (30 μM), and sodium nitroprusside (100 μM) failed to affect the accumulation of L-dopa (Table 1). The possible role of PKC in the regulation of L-dopa uptake in LLC-PK1 cells was tested by examining the effect of pretreating cells with either PKC activators or inhibitors. The PKC activator (1 μM), the inactive phorbol ester PDDC (1 μM), and the PKC inhibitor chelerythrine (50 μM) were found to fail to affect the accumulation of L-dopa (Table 1).

In another study, we tested the involvement of PTK in the regulation of L-dopa uptake by LLC-PK1 cells. The PTK inhibitors genistein and tyrphostin 25 were found to increase the accumulation of L-dopa (2.5 μM), whereas their negative controls genistin and tyrphostin 1 were devoid of effect (Fig. 7). At the highest concentrations (300 μM), genistein and tyrphostin 25 significantly (P < 0.05) increased L-dopa accumulation by 112 ± 23% and 37 ± 4%, respectively. As shown in Table 2, pretreatment of cells with 300 μM genistein was found to significantly increase (P < 0.05) the maximal accumulation (V_max) of increasing concentrations of L-dopa without significant changes in K_m values. To test whether the increased accumulation of L-dopa-induced by genistein was due to a reduced outward transfer of intracellular (recently accumulated) L-dopa, cells cultured in polycarbonate filters were incubated with L-dopa (25 μM) applied from basal cell border and the basal-to-apical flux measured. As shown in Fig. 8, genistein (100 μM) markedly reduced the basal-to-apical flux of L-dopa and increased the accumulation of L-dopa in the cell, suggesting that PTK may be tonically active in promoting the phosphorylation of the L-dopa outward transfer. Because P-glycoprotein is one of the transporters involved in the apical outward transfer of L-dopa, it was felt worthwhile to evaluate the effect of genistein upon P-glycoprotein activity in LLC-PK1 cells. P-glycoprotein activity was measured according to the procedure described by Holló et al. (15), using calcine as substrate for P-glycoprotein. Figure 9 shows the accumulation of calcine in LLC-PK1 cells in control conditions and in the presence of verapamil (25 μM) or genistein (100 μM) during a 20-min incubation period. As can be observed in this figure, the P-glycoprotein inhibitor verapamil, but not genistein, markedly (P < 0.05) increased the rate of accumulation of calcine. The lack of effect of genistein on calcine accumulation suggests that the increased accumulation of L-dopa by genistein may be not related to inhibition of P-glycoprotein activity.

In the final series of experiments, the role of Ca^{2+}/calmodulin-mediated pathways in the regulation of L-dopa by LLC-PK1 cells was tested by examining the
The results presented here show that LLC-PK1 cells transport L-dopa quite efficiently through the apical cell border, and several findings demonstrate that this uptake process is a facilitated mechanism. First, steady-state uptake of nonsaturating concentrations of L-dopa showed a linear dependence on incubation time. The efficiency of the L-dopa transport in LLC-PK1 cells can be also evidenced by the ratio of L-dopa concentration in cellular water to medium concentration. The intracellular L-dopa concentration at 6 min, when uptake was linear, was eight times greater than that which could be expected by passive equilibration of L-dopa. Second, at 6 min of incubation the cellular transport of L-dopa showed a curvilinear dependence on substrate medium concentration, suggesting that the uptake was saturable. Thirdly, both low temperature and the uncoupling agent 2,4-dinitrophenol markedly inhibited the uptake of L-dopa. Finally, inhibition of synthesis of new transporter units by the DNA transcription inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide significantly reduced the ability of LLC-PK1 cells to accumulate L-dopa.

The sensitivity of L-dopa uptake to BHC, but not to MeAIB, supports the view that L-dopa inward transfer in LLC-PK1 cells is promoted neither by the A- nor the ASC-type amino acid transporter, but most probably by the L-type amino acid transporter. Other evidence agreeing with this suggestion is that sodium in the incubation appeared to play no role in L-dopa uptake,
with the role of sodium in the formation of renal dopamine. In fact, studies from several groups have shown that the increase in the renal delivery of sodium constitutes the most powerful stimulus for the production of renal dopamine (14, 29, 32). Studies from our group using human and rat kidney slices also showed that L-dopa uptake is a sodium-dependent and ouabain-sensitive process (33, 35), suggesting that the sodium-dependent increase in urinary dopamine depends on enhanced uptake of L-dopa into tubular epithelial cells. More recently, we have shown that OK cells are endowed with sodium-dependent and sodium-independent L-dopa transporters (9, 10), which is currently being investigated in more detail. Altogether, this suggests that LLC-PK1 cells may lack or have lost the sodium-dependent transporter.

The finding that inhibition of the organic cation and the organic anion transporters by decynium 24 and DIDS, respectively, failed to reduce L-dopa accumulation indicates that these transporters are not involved in L-dopa uptake in LLC-PK1 cells. By contrast, DIDS markedly increased L-dopa accumulation; this was a concentration-dependent effect, at the highest concentration (1 mM) resulting in an increase in Vmax values for L-DOPA uptake without changes in Km values (Table 1). Because in these experiments cells were cultured in plastic supports, the most likely explanation is that enhanced accumulation resulted from a reduced apical outward transfer of L-dopa. This is in agreement with findings in experiments carried out in cells cultured in polycarbonate filters, where DIDS produced a marked reduction in basal-to-apical flux of L-dopa and an increase in L-dopa accumulation.

After defining the mechanism of uptake, we then examined the regulation of L-dopa transport in LLC-PK1 cells. We concentrated on intracellular regulatory pathways that have been shown to play an important role in the regulation of uptake of other substrates by epithelial cells (PKA-, PKG-, PKC-, Ca2+/calmodulin- and PTK-mediated pathways). Using specific modulators of these pathways, we found that PKA-, PKG- and PTK-mediated pathways appear to have no role in regulating L-dopa uptake in LLC-PK1 cells. In contrast, antagonists of Ca2+/calmodulin-mediated pathways, such as calmidazolium and trifluoperazine, caused a significant and concentration-dependent reduction in L-dopa uptake. The inhibitory effect of calmidazolium was accompanied by a marked decrease in Vmax values without changes in Km values, which is compatible with a noncompetitive inhibitory profile. This would suggest that calmidazolium might reduce the number of L-type amino acid transporters in the apical membrane. Similar findings were observed in a model of renal epithelial cell model, the HK-2 cells, where calmidazolium and other inhibitors of Ca2+/calmodulin-mediated pathways, such as trifluoperazine and KN-62, were found to markedly reduce riboflavin accumulation (21). In contrast to that observed for inhibitors of Ca2+/calmodulin-mediated pathways, PTK inhibitors genistein and tyrphostin 25 were found to markedly increase L-dopa accumulation in LLC-PK1 cells. This was a concentration-dependent effect, and the inactive analogs genistin and tyrphostin 1 failed to affect the accumulation of L-dopa in LLC-PK1 cells cultured in plastic supports. When tested in cells cultured in polycarbonate filters, the effect of genistein was a marked reduction in basal-to-apical flux of L-dopa, this being accompanied by a large increase in the accumulation of L-dopa. This indicates that PTK-mediated pathways do not interfere with the inward movement of L-dopa but exert a tonic stimulatory effect upon its outward transfer.

The apical outward transfer of L-dopa in LLC-PK1 cells has been suggested to represent a powerful mechanism for the clearance of intracellular L-dopa in steady-state conditions (removing ~13% of intracellular L-dopa at a rate of 1.0 pmol·mg protein−1·min−1; Ref. 38). However, this outward transfer of L-dopa is considerably less dynamic than the inward transfer at initial rate of uptake (3.6 pmol·mg protein−1·min−1; Ref. 38). The data presented here suggest that this outward transfer is a DIDS-sensitive mechanism and is susceptible to modulation by PTK-mediated pathways. Sensitivity of L-dopa outward transfer to DIDS would suggest the involvement of an organic anion transporter; 1) transport from the basal to the apical cell side corresponds to the secretion in renal tubules and 2) DIDS is a well-known inhibitor of the organic anion transporter (16). However, this is quite unlikely to be the case, because this cell line has been shown by several groups to have lost the organic anion transporter (16, 23, 27). As has been previously reported by others (16), the basal-to-apical transport and accumulation of [3H]PAH was not higher than that of [14C]sorbitol, indicating that the apparent transport and accumulation represents nonspecific uptake and/or trapping. In agreement with these results is the finding that PAH failed to increase L-dopa accumulation in LLC-PK1 cells (Fig. 4). It is likely, therefore, that DIDS may have exerted its effects through inhibition of an apical outward transporter rather than the organic anion transporter. This would agree with the results recently reported for the apical secretion of ciprofloxacin by human intestinal Caco-2 epithelia, in which a novel DIDS-sensitive transport mechanism has been suggested to be involved (3). In addition, there is evidence in the literature showing that DIDS and probe-necid block transport of several substrates in LLC-PK1 cells (4, 6, 7, 20, 40). The inhibitory effect of PTK inhibitors genistein and tyrphostin 25 upon the apical secretion of L-dopa may be to some extent connected to this DIDS-sensitive transport mechanism. In fact, genistein has recently been found to effectively inhibit the secretion of anionic substrates of liver canalicular multispecific organic anion transporter in Wistar rats (18) and diminish PAH uptake in S2 segments of rabbit proximal tubules (8). On the other hand, there is evidence suggesting that the apical outward transfer of L-dopa in LLC-PK1 cells may be promoted in part through P-glycoprotein (37, 39). Therefore, an alternative explanation for the inhibitory effect of genistein...
upon the apical secretion of l-dopa could be that resulting from inhibition of P-glycoprotein phosphorylation by PTK. Verapamil, which inhibits P-glycoprotein activity and markedly decreases secretion of l-dopa in LLC-PK1 cells (37, 39), was found to markedly increase calcine accumulation. However, the finding that genistein failed to affect the accumulation of calcine favors the view that modulation of l-dopa outward transfer by PTK may not involve phosphorylation of P-glycoprotein.

In conclusion, it is suggested that LLC-PK1 cells take up l-dopa over the apical cell border through the L-type amino acid transporter, which appears to be under the control of Ca2+/calmodulin-mediated pathways. The apical outward transfer of l-dopa may be promoted through a DIDS-sensitive transport mechanism and appears to be under the tonic control of PTK, the inhibition of which leads to increases in the intracellular accumulation of l-dopa. Taken together the apical membrane in LLC-PK1 cells is endowed with different transporters for the handling of l-dopa at its cytoplasmic and extracellular sides. The fact that all experiments were performed in conditions of AADC inhibition may not reduce the relevance of these findings, because l-dopa uptake process does not rate limit the formation of dopamine; large amounts of taken up l-dopa are not converted to dopamine (38). On the other hand, substance that interfere with the outward transfer of l-dopa may even be beneficial to increase its availability for decarboxylation, leading to enhanced formation of dopamine.

REFERENCES