British Journal of Pharmacology (1996) 118, 741-747

Studies on the pharmacology of the inward transport of L-DOPA in rat renal tubules

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1 The accumulation of L-DOPA in suspensions of renal tubules obtained from male Wistar rats occurred through non-saturable and saturable mechanisms. The kinetics of the saturable component of L-DOPA uptake in renal tubules was as follows: $V_{max} = 46.3 \pm 2.8$ nmol mg^{-1} protein h^{-1} and $K_m = 114.4$ (95% confidence limits; 83.8, 156.2) μM ($n = 5$). The diffusion constant (in nmol^{-1}) of the non-saturable component for the accumulation of L-DOPA was 1.3 (1.1, 1.6; $n = 8$).

2 The effect of 2,4-dinitrophenol was a marked reduction in the tubular uptake of L-DOPA, with an IC_{50} value of 12.1 (4.0, 36.9) μM. Cocaine produced a slight ($P = 0.08$) decrease (22% reduction at 50 μM) in the tubular uptake of L-DOPA. Corticosterone produced a considerable inhibitory effect on the uptake of L-DOPA with an IC_{50} value of 11.0 (3.6, 33.5) μM. The maximal inhibitory effect of probenecid was a 24% decrease in the uptake of L-DOPA; however, the more selective organic anion transport inhibitor 4,4'-diisothiocyantostilbene-2,2'-disulphonic acid (DIDS) was, found not to affect the tubular uptake of L-DOPA. The organic cation transport inhibitor, cyanine 863, was found to produce a marked decrease in the uptake of L-DOPA ($IC_{50} = 2.02$ [1.07, 3.79]). The cyanine derivatives 1,1'-diethyl-2,2'-cyanine (decynium 22) and 1,1'-diethyl-2,4'-cyanine (decynium 24) also potently inhibited L-DOPA uptake with IC_{50} values (in μM) of 0.63 (0.39, 1.01) and 0.10 (0.08, 0.13), respectively, both compounds were found to be more potent than cyanine 863.

3 The inhibitory effect of decynium 24 (0.2 μM) on the tubular uptake of L-DOPA was dependent on the pH of the incubation medium; at pH = 6.5 the accumulation of L-DOPA was reduced up to 37 ± 2% of control values, whereas at pH = 7.4 and pH = 8.2 the accumulation of L-DOPA was reduced by 56 ± 1% and 60 ± 6%, respectively. Cyanine 863 (2 μM), decynium 22 (1 μM) and decynium 24 (0.2 μM) were found to decrease the V_{max} values for the saturable component of L-DOPA uptake without changes in K_m values.

4 It is concluded that the tubular uptake of L-DOPA might be promoted through a mechanism which is dependent on the activity of the organic cation transport system.

Keywords: L-DOPA; renal tubules; uptake; cyanines

Introduction

In the kidney, dopamine plays the role of an autocrine/paracrine hormone regulating sodium and phosphate transport in the proximal nephron (Lee, 1993). The formation of dopamine in renal tissues has been demonstrated to result from the decarboxylation of circulating or filtered L-DOPA in epithelial cells of proximal convoluted tubules (Baines & Chan, 1980; Hagege & Richet, 1985; Goldstein *et al.*, 1989; Zimlichman *et al.*, 1988; Hayashi *et al.*, 1990; Soares-da-Silva & Fernandes, 1990). This type of cell is endowed with a high aromatic L-amino acid decarboxylase (AAAD) activity, but the conversion of circulating or filtered L-DOPA to dopamine in epithelial cells of proximal tubules requires its uptake into this cellular compartment (Soares-da-Silva, 1994). Considering the high activity of AAAD in renal tissues it may be hypothesized that the main factor determining the production in this area concerns the cellular uptake of L-DOPA. However, there is a lack of objective information concerning the mechanism(s) involved and the nature of the process(es) of cell membrane transport of L-DOPA. Early studies on the inward transport of L-DOPA in tubular epithelial cells were conducted in rat microperfused proximal convoluted tubules. Using this preparation, Chan (1976) was able to demonstrate that the cellular uptake of L-DOPA occurred through an energy-dependent and stereo-selective carrier-mediated process, the rate of transport being 2 fmol cm^{-1} s^{-1}. More recently, we have shown that L-DOPA and 3-*O*-methyl-L-DOPA (3-OMDOPA) share the same transporter in order to be taken up into renal tubular cells, with 3-OMDOPA exerting a competitive type of inhibition upon the tubular uptake of L-DOPA (Soares-da-Silva *et al.*, 1994). This effect is similar to that observed at the level of the blood brain barrier (Wade & Katzman, 1975) and may suggest the presence of similar mechanisms for cell inward transfer of L-DOPA in tubular epithelial cells and endothelial cells in brain capillaries.

The aim of the present work was to study the tubular uptake of L-DOPA and to examine the effects of a series of compounds which are known to interfere with the cellular uptake of a wide range of substrates; the compounds tested include monoamine uptake blockers (cocaine and corticosterone), the organic anion inhibitors, probenecid and 4,4'-diisothiocynatostilbene-2,2'-disulphonic acid (DIDS) (Gesek & Friedman, 1995), the organic cation inhibitor, cyanine 863 (1-ethyl-2-[(1,4-dimethyl-2-phenyl-6-pyrimidinylidene)methyl]-quinolinium) (Rennick *et al.*, 1956; Rennick, 1981) and two other cyanine derivatives, 1,1'-diethyl-2,2'-cyanine (decynium 22) and 1,1'-diethyl-2,4'-cyanine (decynium 24), and the uncoupling agent 2,4-dinitrophenol.

Methods

General

Uptake studies of L-DOPA into renal tubules were performed in preparations obtained from male Wistar rats (Biotéro do Instituto Gulbenkian de Ciência, Oerias, Portugal), aged 45-

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60 days and weighing 190–230 g. The rate of tubular uptake of L-DOPA was calculated considering the rate of dopamine formation subtracted from the amount of L-DOPA entering the cells by diffusion.

Preparation of renal tubules

The preparation of renal tubules was based on the techniques previously described and was found to contain predominantly proximal tubules (Soares-da-Silva *et al.*, 1994). In brief, rats were killed by decapitation under the ether anaesthesia and the kidneys removed through a midline abdominal incision, after which they were decapsulated, cut in half and placed in ice-cold Collins solution (containing, mM: KH_2PO_4 15, K_2HPO_4 50, KCl 15, $NaHCO_3$ 15, $MgSO_4$ 60 and glucose 140, pH = 7.4). The outer cortex was cut out with fine scissors and minced with a scalpel into a fine paste. The cortical pase was filtered sequentially through a series of Nybolt nylon sieves, first 180 μm and then 75 μm. Unseparated cortex remained on the upper (180 μm) sieve, while the lower one (75 μm) retained predominantly proximal nephron segments. The sieves were continuously rinsed with cold Collins solution throughout. The retained tubules were then washed off with cold Collins solution and collected into a pellet by centrifugation at 200 *g*, 5 min, 4°C; renal tubules used in incubation experiments were suspended in Hanks' medium. 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. Pargyline (0.1 mM) and tolcapone (1 μM) were also added to the Hanks' medium in order to inhibit the enzymes monoamine oxidase (MAO) and Catechol-*O*-methyltransferase (COMT), respectively (Vieira-Coelho & Soares-da-Silva, 1994).

Uptake studies

The preincubation and incubation of renal tubules (500 μl) were carried out in glass test tubes, continuously shaken, warmed (37°C) and gassed (95% O_2 and 5% CO_2) throughout the experiment. Some experiments were performed to define the kinetic characteristics of the tubular uptake of L-DOPA. The non-saturable component of L-DOPA uptake was determined in experiments conducted in the presence 2,4-dinitrophenol; the saturable component of L-DOPA uptake was derived from the total amount of L-DOPA taken up into the renal tubules (indicated by the amount of dopamine formed), subtracted from the values obtained for the non-saturable component. The renal tubules were preincubated in Hanks medium for 30 min in order to stabilize the preparations and were then incubated for 15 min with increasing concentrations (50 to 2000 μM) of L-DOPA. The incubation was stopped by cooling and 100 μl of 2 M perchloric acid was added to the medium, filtered on Spin-X (Costar) centrifuge filter (0.40 μm) tubes, centrifuged (4 min, 3000 g, 4°C) and the filtered aliquot directly injected into the column of the high performance liquid chromatograph for the determination of dopamine. In experiments conducted in the presence of 2,4-dinitrophenol, an aliquot (300 μl) of the incubation medium containing the renal tubules was immediately transferred to Spin-X (Costar) centrifuge filter (0.40 μm) tubes and centrifuged (4 min, 3000 *g*, 4°C); the renal tubules were washed twice with ice-cold Hanks medium (500 μl). The extraction of L-DOPA from renal tubules was performed by the addition of 500 μl of 0.2 M perchloric acid to the Spin-X centrifuge filter tubes at the end of the second washing period; the centrifuge filter tubes were then centrifuged and 50 μl of the filtered aliquot was injected directly into the column of a high performance liquid chromatograph for the quantification of L-DOPA. The filters were found not to retain L-DOPA or dopamine and the extraction process employed was also found to remove completely both compounds from the renal tubules.

In experiments designed to study the effects of a series of compounds upon the uptake and decarboxylation of L-DOPA, renal tubules were preincubated after 30 min in the presence of the compounds to be tested; after preincubation, renal tubules were incubated for 15 min in Hanks medium with 250 μM L-DOPA, a concentration twice the K_m value as determined in saturation experiments. The effect of compounds under study on the non-saturable component of L-DOPA uptake was determined in parallel experiments conducted in the presence of 2,4-dinitrophenol.

Cell viability

Renal tubules were preincubated for 15 min at 37°C with test drugs (2,4-dinitrophenol, DIDS, cyanine 863, decynium 22, decynium 24, cocaine or corticosterone) and then incubated in the absence or presence of L-DOPA, for further 15 min. Subsequently the tubules were incubated at 37°C for 2 min with trypan blue (0.2% w/v) in phosphate buffer and examined under a Leica microscope. Under these conditions, more than 95% of the tubules excluded the dye.

Decarboxylation of L-DOPA in homogenates of renal tubules

Homogenates of renal tubules were used in experiments designed to evaluate the effects of compounds under study on the decarboxylation of L-DOPA. The tubules were homogenized in the medium used in the decarboxylation reaction with Thomas teflon homogenizers and kept continuously on ice. Aliquots of 250 μl of tubular homogenates plus 250 μl incubation medium were placed in glass test tubes and incubated for 30 min. Thereafter, L-DOPA (0.1 to 20 mM) was added to the medium for 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 and $Na_2B_4O_7$ 0.1; pargyline (100 μM) and tolcapone (1 μM) were also added to the incubation medium in order to inhibit the enzymes MAO and COMT respectively. The incubation medium also contained pyridoxal phosphate (120 μM) as a cofactor for AAAD. The pH of the reaction medium was kept constant at an optimal pH = 7.0 (Shirota & Fujisawa, 1988). During incubation, homogenates of renal tubules were continuously shaken and gassed (95% O_2 and 5% CO_2) and maintained at 37°C; the compounds under study were present during both the preincubation and incubation periods. The reaction was stopped by the addition of 1 ml 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 300 μl aliquots of the supernatant filtered on Millipore microfilters (MF1) were used for the assay of dopamine.

Assay of L-DOPA and dopamine

The assay of L-DOPA and dopamine was performed as previously described (Soares-da-Silva *et al.*, 1994). The high performance liquid chromatograph (h.p.l.c.) 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, U.S.A.) of 25 cm length; samples were injected by means of an automatic sample injector (Gilson model 231) connected to a Gilson diluter (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, a 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 with the Gilson 712 h.p.l.c. software. The lower limits for detection of L-DOPA and dopamine ranged from 350 to 500 fmol.

Protein assay

The protein content of the suspensions of renal tubules and kidney homogenates was determined by the method of Bradford (1976), with human serum albumin used as a standard.

Statistics

V_{max} and K_m values for AAAD activity were calculated from non-linear regression analysis using GraphPad Prism statistics software package (Motulsky *et al.*, 1994). The diffusion constant represents the slope of the accumulation of L-DOPA measured in experiments carried out in the presence of 2,4-dinitrophenol (Neame & Richards, 1972). For the calculation of the IC_{50}s the parameters of the Hill-equation for multisite inhibition were fitted to the experimental data (Segel, 1975). The K_is were calculated from the corresponding IC_{50}s as described by Cheng & Prusoff (1973). Geometric means are given with 95% confidence limits and arithmetic means are given with s.e.mean; n is the number of experiments (=number of animals). Statistical analysis was performed by one-way analysis of variance (ANOVA) using Newman-Keuls multiple comparison test to compare values. A P value of less than 0.05 was considered to denote a significant difference.

Drugs

Drugs used were cocaine hydrochloride (Laboratórios Uquipa, Lisbon, Portugal), corticosterone (Fluka AG, Buchs, SG, Switzerland), cyanine 863 (1-ethyl-2-[(1,4-dimethyl-2-phenyl-6-pyrimidinylidene)methyl]-quinolinium) (Sigma Chemical Company, St. Louis, Mo, U.S.A.), 1.1'-diethyl-2,2'-cyanine (Sigma), 1,1'-diethyl-2,4'-cyanine (Sigma), L-3,4-dihydroxyphenylaline (L-DOPA) (Sigma), 4,4'-diisothiocynatostilbene-2,2'-disulphonic acid (Sigma) 2,4-dinitrophenol (Sigma), dopamine hydrochloride (Sigma), pargyline hydrochloride (Sigma), probenecid (Sigma) and trypan blue (Sigma) Tolcapone was kindly donated by the producer (Hoffmann La Roche, Basle, Switzerland).

Results

Incubation of renal tubules in the presence of increasing concentrations of L-DOPA with added 2,4-dinitophenol (250 μM), results in a linear and concentration-dependent formation of dopamine (Figure 1). When the experiments were carried out in the absence of 2,4-dinitrophenol, the uptake of L-DOPA in renal tubules (indicated by the amount of dopamine formed) was found to be greater than that occurring in the presence of 2,4-dinitrophenol and showed a trend for saturation. The saturable component of L-DOPA uptake (Figure 1) was derived from the total amount of L-DOPA accumulated in renal tubules (indicated by the amount of dopamine formed) from the values obtained in experiments conducted in the presence of 2,4-dinitrophenol. The kinetics of the saturable component of L-DOPA uptake in renal tubules were as follows: $V_{max}=46.3\pm2.8$ nmol mg^{-1} protein h^{-1} ($n=5$) and $K_m=114.4$ (95% confidence limits; 83.8, 156.2) μM ($n=5$). The diffusion constant (in nmol^{-1}) of the non-saturable component for the accumulation of L-DOPA was 1.3 (1.1, 1.6; $n=8$).

The effect of the uncoupling agent, 2,4-dinitrophenol, was a concentration-dependent reduction of the tubular uptake of L-DOPA (basal levels = 4.8 ± 0.4 nmol mg^{-1} protein 15 min^{-1}; $n=6$), with an IC_{50} value of 12.1 (4.0, 36.9) μM; at the highest concentration tested (250 μM), 2,4-dinitrophenol reduced the uptake of L-DOPA by 96% (Figure 2).

The neuronal uptake blocker, cocaine, produced a slight decrease (22% reduction at 50 μM) on the tubular uptake of L-DOPA (basal levels = 12.1 ± 1.5 nmol mg^{-1} protein 15 min^{-1}; $n=6$), but this effect did not attain statistical significance (Figure 3). By contrast, corticosterone, a non neuronal uptake blocker, produced considerable inhibitory effects on the uptake of L-DOPA (basal levels = 4.7 ± 0.4 nmol mg^{-1} protein 15 min^{-1}; $n=6$) with an IC_{50} value of 11.0 (3.6, 33.5) μM; at the highest concentration tested (50 μM), corticosterone reduced the uptake of L-DOPA by 68% (Figure 3).

The organic anion transport inhibitor, probenecid, produced a concentration-dependent decrease of the tubular uptake of L-DOPA (basal levels = 9.3 ± 1.7 nmol mg^{-1} protein 15 min^{-1}; $n=6$); however, at the highest concentration used (50 μM), the effect of probenecid was a 24% decrease in the uptake of L-DOPA (Figure 4). The more selective organic anion transport inhibitor, DIDS was, however, found not to affect the tubular uptake of L-DOPA (Figure 4). On the other hand, the organic cation transport inhibitor, cyanine 863, produced a marked decrease in the uptake of L-DOPA (basal levels = 8.0 ± 0.8 nmol mg^{-1} protein 15 min^{-1}; $n=6$); this was a concentration-dependent effect resulting in 95% reduction of the tubular uptake of L-DOPA at 10 μM cyanine 863 (Figure 4). The cyanine derivatives 1,1'-diethyl-2,2'-cyanine (decynium 22) and 1,1'-diethyl-2,4'-cyanine (decynium 24) also potently inhibited L-DOPA uptake (basal levels = 7.5 ± 1.2

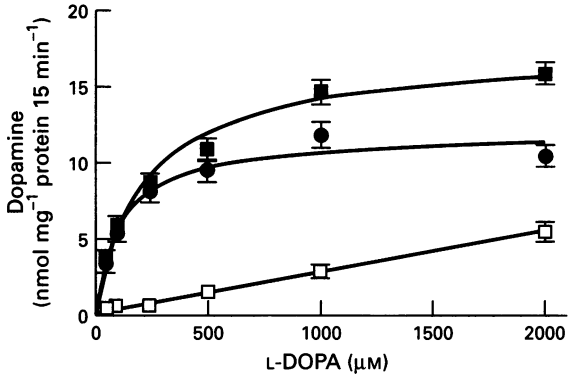

Figure 1 Accumulation of L-DOPA in suspensions of rat isolated renal tubules. The non-saturable component of substrate uptake (□) was obtained in experiments conducted in the presence of 2,4-dinitophenol (250 μM) and was found to be linear with the concentration of the substrate. The saturable component (●) was derived from the total (■) amount of L-DOPA accumulated in renal tubules (indicated by the amount of dopamine formed) minus the values obtained for the non-saturable component. Each point is the mean with s.e.mean of five to eight experiments per group.

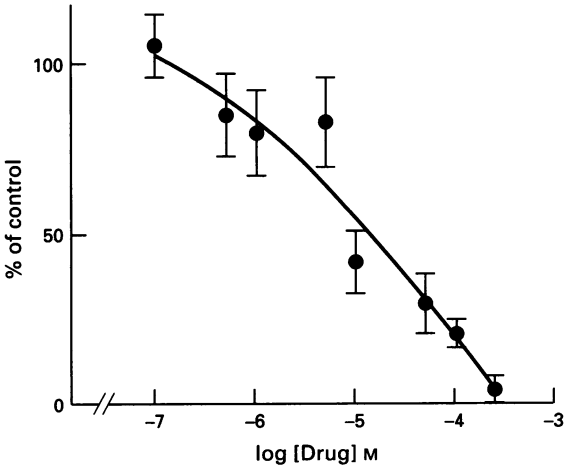

Figure 2 Effect of increasing concentrations of 2,4-dinitrophenol on the saturable uptake of L-DOPA (250 μM) in rat isolated renal tubules. Each point represents the mean with s.e.mean of six experiments per group.

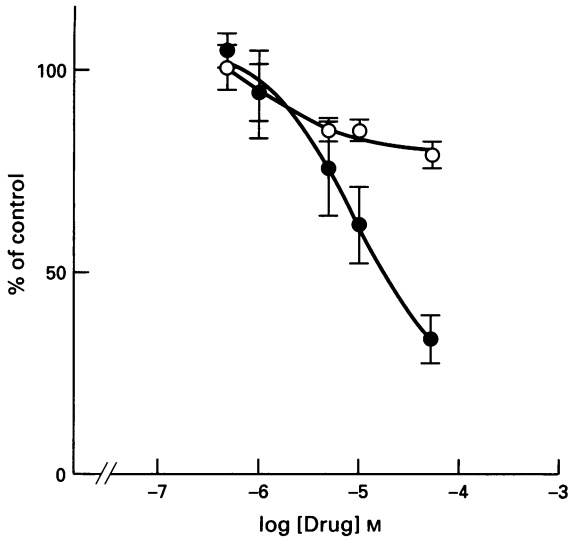

Figure 3 Effect of increasing concentrations of cocaine (○) and corticosterone (●) on the saturable uptake of L-DOPA (250 μM) in rat isolated renal tubules. Each point represents the mean with s.e.mean of six experiments per group.

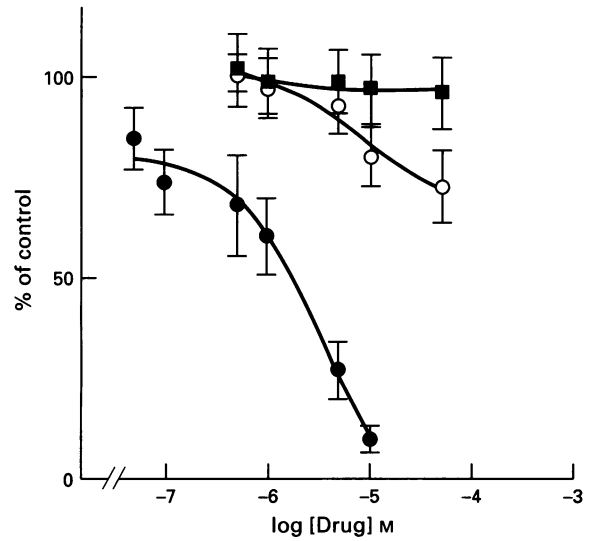

Figure 4 Effect of increasing concentrations of probenicide (○), DIDS (■) and cyanine 863 (●) on the saturable uptake of L-DOPA (250 μM) in rat isolated renal tubules. Each point represents the mean with s.e.mean of six experiments per group.

and 6.5 ± 0.4 nmol mg^{-1} protein 15 min^{-1}, respectively; $n = 5-7$) (Figure 5) with IC_{50} values (in μM) of 0.63 (0.39, 1.01) and 0.10 (0.08, 0.13), respectively; both compounds were found to be more potent than cyanine 863 ($IC_{50} = 2.02$ [1.07, 3.79]).

The inhibitory effect of decynium 24 (0.2 μM) on the tubular uptake of L-DOPA was dependent on the pH of the incubation medium (Figure 6a): at pH = 6.5 the accumulation of L-DOPA was reduced up to $37 \pm 2\%$ of control values (basal levels = 9.3 ± 0.5 nmol mg^{-1} protein 15 min^{-1}; $n = 6$), whereas at pH = 7.4 and pH = 8.2 the accumulation of L-DOPA (basal levels = 10.4 ± 0.8 and 8.5 ± 0.8 nmol mg^{-1} protein 15 min^{-1}, respectively, $n = 6$) was reduced by up to $56 \pm 1\%$ and $60 \pm 6\%$, respectively. Similar effects were obtained with corticosterone (10 μM; Figure 6b); at pH = 6.5 the accumulation of L-DOPA was reduced by up to $32 \pm 2\%$ of control values (basal levels = 9.8 ± 0.7 nmol mg^{-1} protein 15 min^{-1}, $n = 4$), whereas at pH = 7.4 and pH = 8.2 the accumulation of L-DOPA (basal levels = 7.6 ± 0.6 and 7.5 ± 0.4 nmol mg^{-1} protein 15 min^{-1}, respectively, $n = 4$) was reduced by up to $52 \pm 3\%$ and $61 \pm 5\%$, respectively.

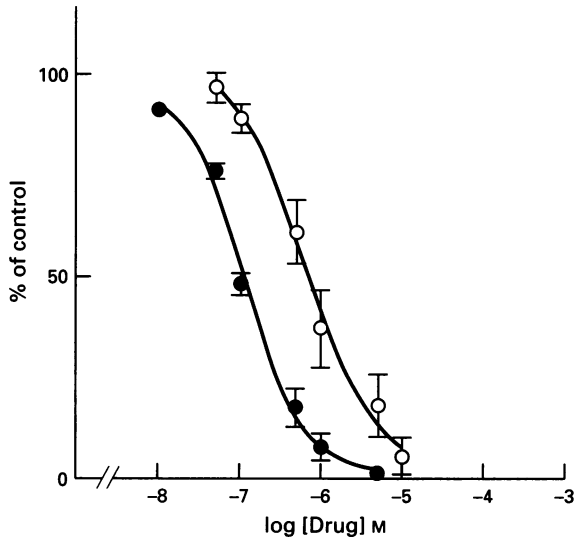

Figure 5 Effect of increasing concentrations of decynium 22 (○) and decynium 24 (●) on the saturable uptake of L-DOPA (250 μM) in rat isolated renal tubules. Each point represents the mean with s.e.mean of five to seven experiments per group.

The effect of cyanine 863 (2 μM), decynium 22 (1 μM), decynium 24 (0.2 μM) and corticosterone (10 μM) on the saturable component of L-DOPA uptake into renal proximal tubules was a decrease in the tubular uptake of L-DOPA. As shown in Table 1, the V_{max} values for the saturable component of L-DOPA uptake in renal tubules incubated in the presence of cyanine 863, decynium 22, decynium 24 and corticosterone were found to be significantly different from those obtained in control conditions. No significant differences were found to occur in K_m values.

Incubation of homogenates of isolated renal tubules, in conditions of COMT and MAO inhibition, with L-DOPA (0.1 to 20 mM) resulted in a concentration-dependent formation of dopamine (Figure 7a); the V_{max} (in nmol mg^{-1} protein h^{-1}; $n = 4$) and K_m (mM) of L-DOPA decarboxylation were 403.6 ± 20.0 and 2.7 (1.5, 3.9; 95% confidence limits). The addition of 2,4-dinitophenol (250 μM), cocaine (50 μM), corticosterone (50 μM), probenecid (50 μM), DIDS (50 μM), cyanine 863 (10 μM), decynium 22 (5 μM) or decynium 24 (5 μM) to the incubation medium was found not to significantly change the decarboxylation of 2 mM L-DOPA (a concentration similar to that of K_m values for AAAD) (Figure 7b).

Discussion

The decision to study the uptake of L-DOPA into renal tubular epithelial cells by measuring the amount of dopamine formed was mainly dependent on the evidence that L-DOPA is easily decarboxylated to dopamine and this reaction is believed to occur exclusively in the intracellular milieu. It is, therefore, expected, that this experimental approach would enable us to exclude the possibility of an accumulation of L-DOPA in non-epithelial cells devoid of AAAD activity or binding of L-DOPA at cellular components. The decarboxylation reaction of L-DOPA in isolated renal tubules shows a trend for saturation at 500 μM L-DOPA and became saturated at 1000 μM of the substrate. In experiments conducted in the presence of 2,4-dinitrophenol, the formation of dopamine showed no trend for saturation, most probably reflecting diffusional uptake of L-DOPA into the cell. Assuming that diffusion of L-DOPA into the tubular cells should be similar in the presence or the absence of 2,4-dinitrophenol, it becomes evident that diffusion accounts for only 8% of the total uptake of L-DOPA at a concentration of the substrate approaching half-saturation concentrations (250 μM). Subtracting the values related to the total entry of L-DOPA with the values obtained in experiments

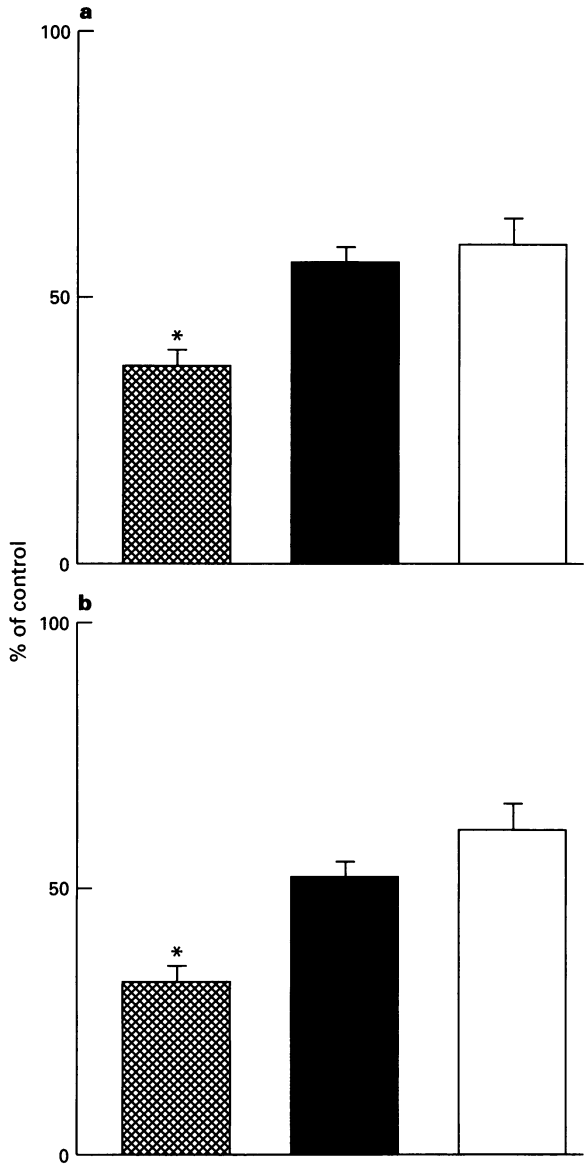

Figure 6 Effect of (a) decynium 24 (0.2 μM) and (b) corticosterone (10 μM) on the saturable uptake of L-DOPA (250 μM) in rat isolated renal tubules at different pH values of incubation medium: pH = 6.5, cross hatched column; pH = 7.4, solid column; pH = 8.2, open column. Each column represents the mean with s.e.mean of four to six experiments per group. Significantly different from values obtained in experiments carried at pH = 7.4 (*$P < 0.02$).

Table 1 V_{max} and K_m values for the saturable component of L-DOPA uptake in rat renal tubules in control conditions and in the presence of cyanine 863 (2 μM), decynium 22 (1 μM), decynium 24 (0.2 μM) and corticosterone (10 μM)

	V_{max} (nmol mg^{-1} protein 15 min^{-1})	K_m (μM)
Control	42.7 ± 2.2	137.7 (105.0, 180.6)
Cyanine 863	22.8 ± 1.7*	105.2 (70.4, 157.5)
Decynium 22	19.7 ± 2.2*	110.8 (82.0, 149.8)
Decynium 24	23.6 ± 3.1*	108.0 (63.7, 183.2)
Corticosterone	20.7 ± 2.3*	106.9 (68.4, 193.5)

Values are arithmetic means ± s.e.mean (V_{max}) or geometric means with 95% confidence limits (K_m) of four experiments per group. *Significantly different from corresponding control values using the Newman-Keuls test.

conducted in the presence of 2,4-dinitrophenol it is possible to obtain the saturable component of L-DOPA uptake with the following kinetic parameters: V_{max} = 46.3 ± 2.8 nmol mg^{-1} protein h^{-1}, k_m = 114.4 μM (83.8, 156.2).

Because L-DOPA is classically considered as an anionic compound and tubular epithelial cells transport easily both organic anions and organic cations it was decided to test the effect of probenecid and DIDS, two organic anion transport inhibitors (Gesek & Friedman, 1995), and the effect of cyanine 863, an organic cation transport inhibitor (Rennick *et al.*, 1956; Rennick, 1981), on the uptake of L-DOPA in isolated renal tubules. In contrast to probenecid, cyanine 893 at 10 μM almost abolished the tubular uptake of L-DOPA; this inhibitory effect of cyanine 863 was found not to occur in experiments conducted in the presence of 2,4-dinitrophenol, suggesting that it selectively interferes with the saturable inward transfer of L-DOPA. Considering that probenecid is not a selective inhibitor of the organic anion transporter (it also produces some inhibition of the organic cation transporter,

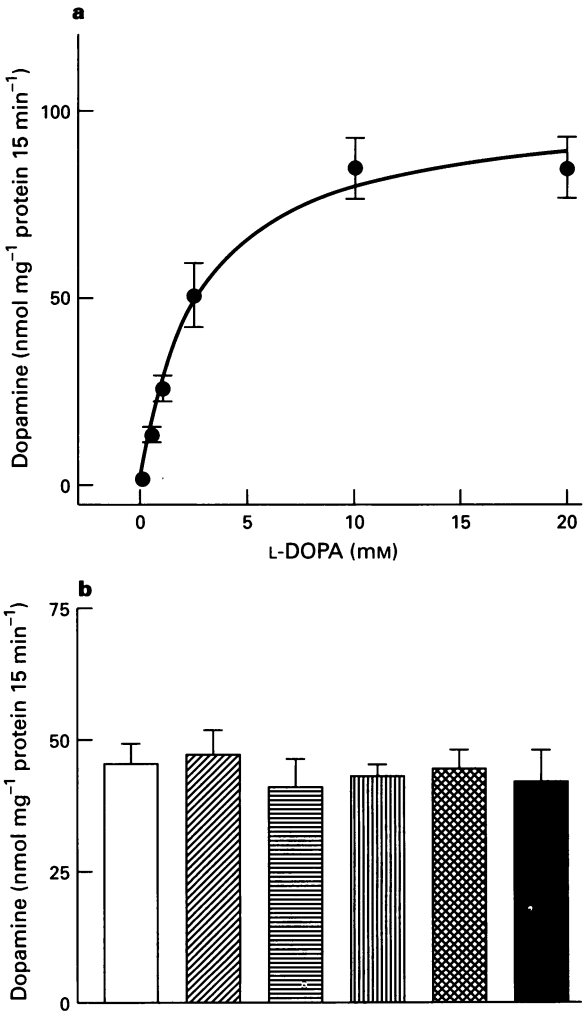

Figure 7 (a) Saturation curve of aromatic L-amino acid decarboxylase (AAAD) activity in homogenates of rat renal cortical tubules. AAAD activity is expressed as the rate of formation of dopamine (in nmol mg^{-1} protein 15 min^{-1}) vs. concentrations of L-DOPA (mM). (b) Effect of 250 μM 2,4-dinitrophenol (hatched column), 50 μM corticosterone (horizontally lined column), 10 μM cyanine 863 (vertically lined column), 5 μM decynium 22 (cross hatched column) and 5 μM decynium 24 (solid column) on the decarboxylation of 2 mM L-DOPA (a concentration similar to the K_m value for AAAD) in homogenate of rat renal cortical tubules; control values are indicated by the open column. Columns represent the mean with s.e.mean of four experiments.

Moller & Sheikh, 1983), these results suggest that in these experimental conditions, L-DOPA is transported through a mechanism which is dependent in the activity of the organic cation transport and the small inhibitory effect of probenecid might be related to the interference upon the organic cation transfer system. In agreement with this view are the results showing that the more selective organic anion transport inhibitor DIDS failed to change the tubular uptake of L-DOPA.

In order to understand fully the pharmacology of this tubular uptake system for L-DOPA two other cyanine derivatives, compounds decynium 24 and decynium 22, were tested. Both compounds were found to be extremely effective in reducing the tubular uptake of L-DOPA, being even more potent than cyanine 863; decynium 22 is twice as potent at cyanine 863 and decynium 24 is by far the most potent compound in inhibiting L-DOPA uptake with an IC_{50} of 0.2 μM. Similarly to cyanine 863, decynium 22 has been recently shown to exert a potent inhibitory effect on the renal transport of the organic cation, tetraethylammonium (Schömig *et al.*, 1993); it is not known whether decynium 24 also inhibits the renal transport of organic cations or not. However, the type of inhibition exerted by cyanine 863, decynium 22 and decynium 24 appears to be of the non-competitive type as indicated by the finding that the reduction in V_{max} values is accompanied by no changes in K_m values. This indicates that L-DOPA and the cyanine derivatives are not competing for the same site of the molecule transporter. This finding together with the result that the inhibitory effect is dependent on the pH of the extracellular medium may constitute important indications of the nature of the mechanisms controlling the entry of L-DOPA in these cells.

The rate of transfer of organic cations has been demonstrated to be dependent on the pH; the higher the pH of the extracellular medium the greater the rate of transfer of cations. The renal secretion of organic cations is secondarily active and is driven by a proton gradient created by the Na^+-H^+ exchanger (Holohan & Ross, 1981). As shown in Figure 6, the inhibitory effect of 0.2 μM decynium 24 was found to be dependent on the pH of the incubation medium, being greater at pH = 6.5 than at pH = 8.2. This finding is in line with that described for the activity of the organic cation transporter, which showed an increased activity at low pH values. Thus, it might be suggested that the inhibitory effect of cyanine derivatives on the uptake of L-DOPA might be related to changes in intracellular pH or the mechanisms responsible for intracellular pH control. In fact this would agree with previous evidence on the role of sodium and the Na^+-H^+ exchanger on the cell inward transport of L-DOPA (Soares-da-Silva & Fernandes, 1992; Soares-da-Silva, 1993; Soares-da-Silva *et al.*, 1993a,b).

Another aspect we think worthwhile discussing concerns the possibility that the inhibitory effect of cyanine 863 on the uptake of L-DOPA is related to the inhibition of the extraneuronal uptake system for catecholamines ($uptake_2$). In fact this compound as well as decynium 22 have been recently demonstrated to inhibit markedly the catecholamine extraneuronal uptake system with an inhibitory potency similar to that shown for corticosterone (Russ *et al.*, 1992). L-DOPA, however, lacks in its molecule the amino group which is believed to be crucial for the binding to the $uptake_2$ transporter. Quite unexpectedly, however, we have found that corticosterone also inhibits the tubular uptake of L-DOPA. Because it is rather unlikely that L-DOPA may serve as substrate for the $uptake_2$ transporter, the only explanation we can put forward for these findings lies in the possibility that corticosterone has some activity at the organic cation transporter or has the ability to alter intracellular pH or the mechanisms responsible for intracellular pH control. In fact, this appears to be the case since the inhibitory effect of corticosterone on L-DOPA uptake was found to be dependent on the pH of the incubation medium, similar to that occurring with decynium 24. This would agree, on the other hand, with the view that the organic cation and the $uptake_2$ transporters share some functional properties, as previously pointed out by Schömig & Schönfeld (1990). As shown for cyanine 863, decynium 22 and decynium 24, corticosterone was also found to produce a non-competitive type of inhibition, as shown by the reduction in V_{max} values with no changes in K_m values.

In conclusion, the results presented here suggest that the tubular uptake of L-DOPA might be promoted through a mechanism that is dependent on the activity of the organic cation transport system.

Supported by grant number PBIC/C/CEN 1139/92 from the Junta Nacional de Investigacão Cietífica e Tecnológica (JNICT). P.C.P.-d.-Ó is the recipient of a fellowship sponsored by the 'American Portuguese Biomedical Research Fund'.

References

BAINES, A.D. & CHAN, W. (1980). Production of urine free dopamine from dopa: a micropuncture study. *Life Sci.*, **26**, 253-259.

BRADFORD, M.M. (1976). A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254.

CHAN, Y.L. (1976). Cellular mechanisms of renal tubular transport of L-DOPA and its derivatives in the rat: microperfusions studies. *J. Pharmacol. Exp. Ther.*, **199**, 17-24.

CHENG, Y.-C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (Ki) and the concentration of the inhibitor which causes 50 per cent inhibition (IC50) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099-3108.

GESEK, F.A. & FRIEDMAN, P.A. (1995). Sodium entry mechanisms in distal convoluted tubules. *Am. J. Physiol.*, **268**, F89-F98.

GOLDSTEIN, D.S., STULL, R., EISENHOFER, G. & GILL, J., Jr. (1989). Urinary excretion of dihydroxyphenylalanine and dopamine during alterations of dietary salt intake in humans *Clin. Sci.*, **76**, 517-522.

HAGEGE, J. & RICHET, G. (1985). Proximal tubule dopamine histofluorescence in renal slices incubated with L-dopa. *Kidney Int.*, **27**, 3-8.

HAYASHI, M., YAMAJI, Y., KITAJIMA, W. & SARUTA, T. (1990). Aromatic L-amino acid decarboxylase activity along the rat nephron. *Am. J. Physiol.*, **258**, F28-F33.

HOLOHAN, P.D. & ROSS, C.R. (1981). Mechanisms of organic cation transport in kidney plasma membrane vesicles: 2. delta pH studies. *J. Pharmacol. Exp. Ther.*, **216**, 294-298.

LEE, M.R. (1993). Dopamine and the kidney: ten years on [editoral]. *Clin. Sci.*, **84**, 357-375.

MOLLER, J.V. & SHEIKH, M.I. (1983). Renal organic anion transport systems: Pharmacological, physiological and biochemical aspects. *Pharmacol. Rev.*, **34**, 315-358.

MOTULSKY, H.J., SPANNARD, P. & NEUBIG, R. (1994). *GraphPad Prism* (version 1.0). San Diego, USA: GraphPad Prism Software Inc.

NEAME, K.D. & RICHARDS, T.G. (1972). *Elementary Kinetics of Membrane Carrier Transport*. Oxford: Blackwell Scientific Publications.

RENNICK, B.R (1981). Renal tubule transport of organic cations. *Am. J. Physiol.*, **240**, F83-F89.

RENNICK, B.R., KANDEL, A. & PETERS, L. (1956). Inhibition of the renal tubular excretion of tetraethylammonium and N'-methylnicotinamide by basic cyanine dyes. *J. Pharmacol. Exp. Ther.*, **118**, 204-219.

RUSS, H., GLIESE, M., SONNA, J. & SCHÖMIG, E. (1992). The extraneuronal transport mechanism for noradrenaline ($uptake_2$) avidly transports 1-methyl-4-phenylpyridinium (MPP^+). *Naunyn-Schmied. Arch. Pharmacol.*, **346**, 158-165.

SCHÖMIG, E., BABIN-EBELL, J. & RUSS, H. (1993). 1,1'-diethyl-2,2'-cyanine (Decyniun 22) potently inhibits the renal transport of organic cations. *Nauyn-Schmied. Arch. Pharmacol.*, **347,** 379–383.

SCHÖMIG, E. & SCHÖNFELD, C.L. (1990). Extraneuronal noradrenaline transport (uptake$_2$) in a human cell line (Caki-1 cells). *Naunyn-Schmied. Arch. Pharmacol.*, **341,** 404–410.

SEGEL, I.H. (1975). *Enzyme Kinetics: Behavior Analysis of Rapid Equilibrium and Steady-State Enzyme Systems.* New York: *John Wiley.*

SHIROTA, K. & FUJISAWA, H. (1988). Purification and characterization of aromatic L-amino acid decarboxylase from rat kidney and monoclonal antibody to the enzyme. *J. Neurochem.*, **51,** 426–434.

SOARES-DA-SILVA, P. (1992). Actin cytoskeleton, tubular sodium and the renal synthesis of dopamine. *Biochem. Pharmacol.*, **44,** 1883–1886.

SOARES-DA-SILVA, P. (1993). Enhanced protein kinase C mediated inhibition of renal dopamine synthesis during high sodium intake. *Biochem. Pharmacol.*, **45,** 1791–1800.

SOARES-DA-SILVA, P. (1994). Source and handling of renal dopamine: Its physiological importance. *New Physiol. Sci.*, **9,** 128–134.

SOARES-DA-SILVA, P. & FERNANDES, M.H. (1990). Regulation of dopamine synthesis in the rat kidney. *J. Autonom. Pharmacol.*, **10,** s25–s30.

SOARES-DA-SILVA, P. & FERNANDES, M.H. (1992). Sodium-dependence and ouabain-sensitivity of the synthesis of dopamine in renal tissues of the rat. *Br. J. Pharmacol.*, **105,** 811–816.

SOARES-DA-SILVA, P., FERNANDES, M.H. & PESTANA, M. (1993a). Studies on the role of sodium on the synthesis of dopamine in the rat kidney. *J. Pharmacol. Exp. Ther.*, **264,** 406–414.

SOARES-DA-SILVA, P., FERNANDES, M.H. & PINTO-DO-O, P.C. (1994). Cell inward transport of L-DOPA and 3-O-methyl-L-DOPA in rat renal tubules. *Br. J. Pharmacol.*, **112,** 611–615.

SOARES-DA-SILVA, P., PESTANA, M. & FERNANDES, M.H. (1993b). Involvement of tubular sodium in the formation of dopamine in the human renal cortex. *J. Am. Soc. Nephrol.*, **3,** 1591–1599.

VIEIRA-COELHO, M.A. & SOARES-DA-SILVA, P. (1994). Sensitivity of liver and kidney catechol-O-methyltransferase to inhibition by tolcapone in developing rats. *Br. J. Pharmacol.*, **112,** 410P.

WADE, L.A. & KATZMAN, R. (1975). 3-O-methylDOPA uptake and inhibition of L-DOPA at the blood brain barrier. *Life Sci.*, **17,** 131–136.

ZIMLICHMAN, R., LEVINSON, P.D., KELLY, G., STULL, R., KEISER, H.R. & GOLDSTEIN, D.S. (1988). Derivation of urinary dopamine from plasma dopa. *Clin. Sci.*, **75,** 515–520.

(Received November 8, 1995
Revised February 7, 1996
Accepted February 9, 1996)