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Evidence for the involvement of P-glycoprotein on the extrusion of taken up L-DOPA in cyclosporine A treated LLC-PK$_1$ cells

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1 The present work has examined the effects of short- (30 min) and long-term (14 h) exposure to cyclosporine A (CsA) on the uptake of L-DOPA, its decarboxylation to dopamine and the cellular extrusion of taken up L-DOPA and of newly-formed amine in monolayers of LLC-PK$_1$ cells.

2 In the presence of benserazide (50 μM), L-DOPA was rapidly accumulated in LLC-PK$_1$ cells (cultured in collagen-treated plastic) attaining equilibrium at 30 min of incubation. Non-linear analysis of the saturation curves revealed a K_m of 113 ± 16 μM and a V_{max} of 5581 ± 297 pmol mg^{-1} protein 6 min^{-1}.

3 In the absence of benserazide, LLC-PK$_1$ cells incubated with increasing concentrations of L-DOPA (10 to 500 μM) for 6 min accumulate newly-formed dopamine by a saturable process with apparent K_m and V_{max} values of 31 ± 6 μM and 1793 ± 91 pmol mg^{-1} protein 6 min^{-1}, respectively. The fractional outflow of newly-formed dopamine was found to be 20%. Up to 200 μM of intracellular newly-formed dopamine, the outward transfer of the amine was found to be a non-saturable process.

4 Short-term exposure to CsA (0.3, 1.0 and 3.0 μg ml^{-1}) was found not to change the intracellular concentrations of newly-formed dopamine, but increased the levels of dopamine in the incubation medium (143% to 224% increase) and the total amount of dopamine formed (31% to 59% increase). Long-term exposure to CsA (0.03 to 3.0 μg ml^{-1}) reduced the total amount of dopamine (15% to 39% reduction) and the intracellular levels of the amine (11% to 56% reduction), without changing dopamine levels in the incubation medium. Both short- and long-term exposure to CsA resulted in a concentration-dependent increase in the fractional outflow of newly-formed dopamine.

5 Short-term exposure to CsA (3.0 μg ml^{-1}) reduced the apical extrusion of intracellular L-DOPA by 15% ($P<0.05$), whereas long-term exposure to CsA reverted this effect and decreased its intracellular availability (15% reduction; $P<0.05$).

6 Detection of P-glycoprotein activity was carried out by measuring verapamil- or UIC2-sensitive rhodamine 123 accumulation. Both UIC2 (3 μg ml^{-1}) and verapamil (25 μM) significantly increased the accumulation of rhodamine 123 in LLC-PK$_1$ cells. A 30 min exposure to CsA was found not to affect the accumulation of rhodamine 123 in the presence of verapamil (25 μM), whereas a 14 h exposure to CsA was found to reduce the accumulation of rhodamine 123.

7 In conclusion, the increase and the reduction in the formation of dopamine after short- and long-term exposure to CsA, respectively, correlate with the effects of the immunosuppressant on the apical cell extrusion of taken up L-DOPA, suggesting the involvement of P-glycoprotein. The effects of CsA on the fractional outflow of newly-formed dopamine appear to be mediated by a different mechanism.

Keywords: Cyclosporine A; L-DOPA; dopamine; LLC-PK$_1$ cells; P-glycoprotein

Introduction

In the rat, chronic administration of cyclosporine A (CsA) is accompanied by antinatriuresis, hypertension and a reduction in daily urinary excretion of dopamine, which appears to result from a reduction in the amount of L-3,4-dihydroxyphenylalanine (L-DOPA) made available to the kidney (Pestana *et al*., 1995). However, when CsA-treated rats are given exogenous L-DOPA an increased accumulation of L-DOPA and newly-formed dopamine is observed. At least two possibilities can be advanced in order to explain this effect: (1) upregulation of the L-DOPA uptake system or (2) decreased cellular extrusion of taken up L-DOPA and newly-formed dopamine. Arguments favouring the hypothesis that CsA treatment upregulates the renal L-DOPA uptake system are the following: (1) V_{max} for L-DOPA uptake in isolated renal tubules is significantly increased without changes in K_m values; (2) no change in L-DOPA diffusion constant values were observed (Pestana *et al*., 1995). The following may favour the hypothesis that CsA treatment decreased the cellular extrusion of L-DOPA and newly-formed dopamine: (1) CsA is a potent inhibitor of P-glycoprotein, an energy-dependent efflux pump (Foxwell *et al*., 1989), (2) L-DOPA in tubular epithelial cells, at physiological pH, behaves as a cationic compound (Pinto-do-O & Soares-da-Silva, 1996) and (3) organic cations and weak organic bases are substrates for P-glycoprotein (Dutt *et al*., 1992).

The present study was aimed to address the question concerning which of the processes — upregulation of the L-DOPA uptake system or decreased cellular extrusion of taken up L-DOPA and of newly-formed dopamine — is in fact responsible for the increased cellular accumulation of L-DOPA and dopamine. In order to obviate technical problems with the accessibility of the substrates (extracellular L-DOPA and intracellular dopamine) to the cellular membrane and the exposure to cyclosporine A, it was decided to perform this study in monolayers of LLC-PK$_1$ cells in culture. LLC-PK$_1$ cells express proximal tubule cell-like properties *in vitro* (Hull *et al*., 1976) and have been used to study dopamine receptors and the renal actions of the amine. These cells have been shown to contain high levels of aromatic L-amino acid decarboxylase (AAAD) and convert L-DOPA to dopamine in a saturable fashion (Dawson & Phillips, 1990; Grenader &

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Healy, 1991; Soares-da-Silva *et al*., 1996). Newly-formed dopamine in LLC-PK$_1$ was demonstrated to stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation, an effect attenuated by an equimolar concentration of carbidopa or blocked by the D_1 antagonist SCH 23390 (Grenader & Healy, 1991); this suggests that locally formed dopamine in LLC-PK$_1$ cells, as in epithelial cells of proximal tubules, can act as an autocrine/paracrine substance. Another reason to use LLC-PK$_1$ cells in the present study has to do with the fact that CsA in these cells is transported in a saturable manner (Saeki *et al*., 1993) and P-glycoprotein is stimulated during chronic exposure to the immunosuppressant, as a mechanism of detoxification (Gardia del Moral *et al*., 1995). We describe here the effects of short- (30 min) and long-term (14 h) exposure to CsA on the uptake of L-DOPA, its decarboxylation to dopamine and the apical cell extrusion of taken up L-DOPA and of newly-formed dopamine in monolayers of LLC-PK$_1$ cells.

Methods

Cell culture

LLC-PK$_1$ cells, a porcine-derived proximal renal tubule epithelial cell line which retains several properties of proximal tubular epithelial cells in culture, were obtained from the American Type Culture Collection (Rockville, MD) and maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. LLC-PK$_1$ cells (ATCC CRL 1392; passages 198–206) were grown in Medium 199 (Sigma Chemical Company, St. Louis, Mo, U.S.A.) supplemented with 100 u ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B, 100 μg ml^{-1} streptomycin (Sigma), 3% foetal bovine serum (Sigma) and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanosulphonic acid (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 cm^2 growth areas (Costar, Badhoevedorp, The Netherlands). For each experiment the cells were seeded in collagen-treated 24 well plastic culture clusters (16 mm internal diameter, Costar) at a density of 40,000 cells per well (2.0×10^4 cells cm^{-2}) or, depending on the experiment, onto collagen-treated 0.2 μm polycarbonate filter supports (12 mm internal diameter Transwell, Costar). 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 foetal bovine serum. Experiments were generally performed 2–3 days after cells reached confluence and 6–8 days after the initial seeding and each cm^2 contained about 100 μg of cell protein. In experiments designed to test the effects of short- and long-term exposure with CsA, the cells were incubated for 30 min and 14 h, respectively, with the immunosuppressant. CsA (0.1 to 3.0 μg ml^{-1}) or the vehicle (olive oil, 0.5% v/v) were added to the cell medium bathing the apical cell border only and the preparations maintained in the incubator for the corresponding period of time. Thereafter, the cells were washed with cold Hank's medium and the process of L-DOPA uptake was initiated, as described below.

Transport studies

On the day of the experiment, the growth medium was aspirated and the cells washed with Hanks' medium at 4°C; thereafter, the cell monolayers were preincubated for 15 min in Hanks' medium at 37°C. The Hanks' medium had the following composition (mM): NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris HCl 0.15 and sodium butyrate 1.0, pH = 7.4. The incubation medium also contained pargyline (100 μM) and tolcapone (1 μM) in order to inhibit the enzymes monoamine oxidase and catechol-*O*-methyltransferase, respectively; in some experiments, benserazide (50 μM) was also added to the incubation medium in order to inhibit AAAD. During preincubation and incubation, the cell monolayers were continuously shaken and maintained at 37°C. Uptake was initiated by the addition of 2 ml Hanks' medium with a given concentration of L-DOPA. Except as specifically indicated the substrate was applied from the apical cell side only; in some experiments the substrate was applied from the basolateral cell border only. Determination of initial rate of uptake was performed in experiments in which cells were incubated with a non-saturating concentration of the substrate for 1, 3, 6, 12, 30, 60 and 120 min. Saturation experiments were performed in LLC-PK$_1$ cells incubated for 6 min with increasing concentrations of L-DOPA. For basolateral uptake, the uptake solution was added to the lower chamber; in these experiments, the uptake solution was added with 0.4 μM [^{3}H]-sorbitol to correct the data for paracellular diffusion. 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 and dopamine. In some experiments, the incubation solution or the medium bathing the apical cell border, when the substrate was applied from the basolateral cell border, was also collected, acidified with 2 mM perchloric acid and stored at 4°C till the assay of catechol derivatives.

Assay of L-DOPA and dopamine

L-DOPA and dopamine were quantified by means of high pressure liquid chromatography with electrochemical detection, as previously described (Soares-da-Silva *et al*., 1994). The high pressure 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) of 25 cm length; samples were injected by means of an automatic sample injector (Gilson model 231) connected to a Gilson dilutor (model 401). The mobile phase was a degassed solution of citric acid (0.1 mM), sodium octylsulphate (0.5 mM), sodium acetate (0.1 M), EDTA (0.17 mM), dibutylamine (1 mM) and methanol (8% v/v), adjusted to pH 3.5 with perchloric acid (2 M) and pumped at a rate of 1.0 ml min^{-1}. The detection was carried out electrochemically with a glassy carbon electrode, an Ag/AgCl reference electrode and an amperometric detector (Gilson model 141); the detector cell was operated at 0.75 V. The current produced was monitored 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.

Assay of P-glycoprotein activity

P-glycoprotein activity was measured according to the procedure described by Fardel *et al*. (1996) and Mechetner & Roninson (1992), with minor modifications. In brief, LLC-PK$_1$ cells cultured in collagen-treated plastic 24 wells were incubated for 30 min in the presence of UIC2 (Immunotech, Marseille, France; 3 μg ml^{-1}) or verapamil (25 μM) and then

incubated with 100 μM rhodamine 123 for a further 15 min. UIC2 is a mouse monoclonal antibody that recognizes an extracellular epitope of human P-glycoprotein (Mechetner & Roninson, 1992). Uptake was terminated by the rapid removal of the medium containing rhodamine 123; thereafter, the cells were washed with 2 ml ice-cold Hanks' medium and 1.5 ml of 0.1% v/v Triton X-100 (dissolved in 5 mM HCl, pH 7.4) was added to each 2 cm^2 well, to solubilize the cells. Rhodamine 123 was measured by spectrophotometry at 499 nm. Triton X-100 (0.1 v/v) was found not to alter rhodamine 123 measurements.

Cell water content

Cell water content was simultaneously measured in parallel experiments with [^{14}C]-inulin as extracellular marker and tritiated water as total water marker. Intracellular water obtained by subtracting extracellular water from total water was expressed as μl of cell water mg^{-1} protein. Subsequently, the cells were solubilized by 0.1% v/v Triton X-100 (dissolved in 5 mM HCl, pH 7.4) and radioactivity was measured by liquid scintillation counting.

Protein assay

The protein content of monolayers of LLC-PK$_1$ cells was determined by the method of Bradford (1976), with human serum albumin as a standard.

Cell viability

CsA-treated and non-treated LLC-PK$_1$ cells were preincubated for 15 min at 37°C and then incubated in the absence or presence of L-DOPA for a further 15 min or 120 min. Subsequently the cells were incubated at 37°C for 2 min with trypan blue (0.2% w/v) in phosphate buffer. Incubation was stopped by rinsing the cells twice with Hank's medium and the cells were examined with a Leica microscope. Under these conditions, more than 95% of the cells excluded the dye.

Data analysis

The analysis of the time course of L-DOPA uptake in LLC-PK$_1$ cells was based on a one-compartment model. The parameters of the equation

$$C_i \; C_o \quad k_{in} \; k_{out} \quad 1 - e^{-k_{out}\, t}$$

were fitted to the experimental data by a non-linear regression analysis by means of a computed assisted method (Motulsky *et al.*, 1994). C_i and C_o represent the intracellular and extracellular concentration of the substrate, respectively, k_{in} is the rate constant for inward transport, k_{out} the rate constant for outward transport and t the incubation time. k_{in} and k_{out} are given in pmol mg^{-1} protein min^{-1}. A_{max} is defined as the factor of accumulation (C_i/C_o) at equilibrium ($t \rightarrow \infty$). K_m and V_{max} values for the uptake of L-DOPA and its decarboxylation to dopamine, as determined in saturation experiments, were calculated from non-linear regression analysis using the GraphPad Prism statistics software package (Motulsky *et al.*, 1994). Arithmetic means are given with s.e.mean. Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Student's *t* test for unpaired comparisons or the Newman-Keuls test for multiple comparisons. A *P* value less than 0.05 was assumed to denote a significant difference.

Results

To determine the characteristics of L-DOPA uptake, LLC-PK$_1$ cells were incubated, in the first series of experiments, with a non-saturating concentration (0.5 μM) of L-DOPA for increasing periods of time (1 to 120 min). In a second series of experiments, LLC-PK$_1$ cells were incubated with increasing concentrations of the substrate (10 to 500 μM) in conditions of initial rate of transfer (6 min). In both series of experiments, cells were cultured in collagen-treated plastic clusters, the substrate was applied from the apical cell border only and the incubation medium contained benserazide (50 μM) to inhibit L-DOPA decarboxylation. L-DOPA was rapidly accumulated in

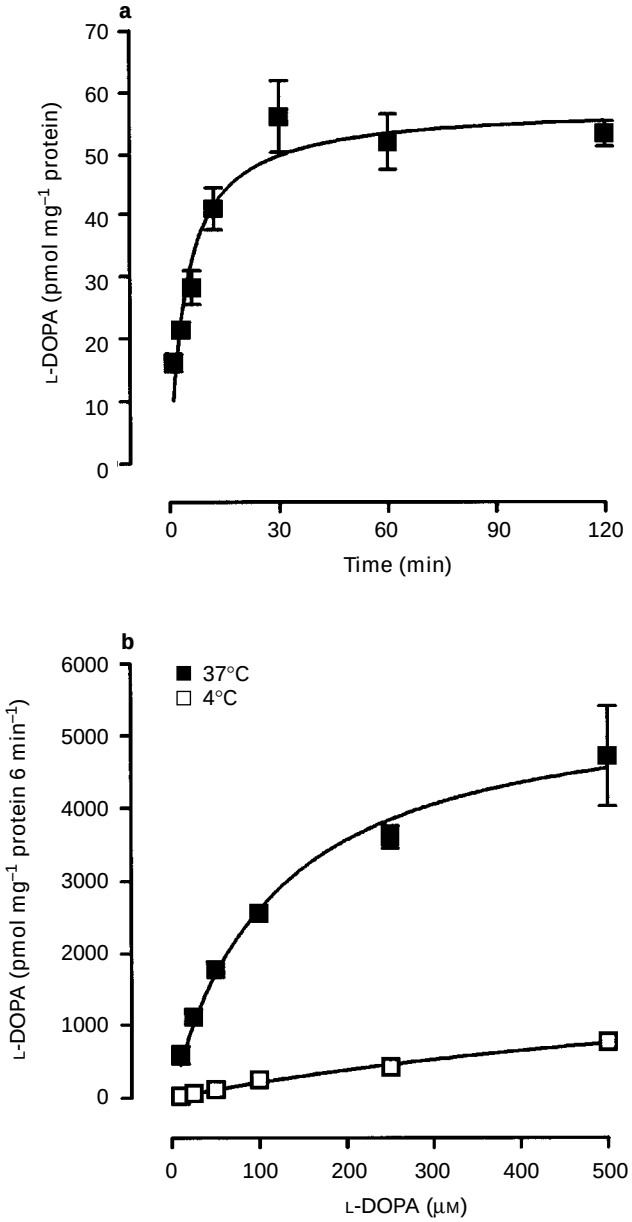

Figure 1 (a) Time-dependent accumulation of L-DOPA in LLC-PK$_1$ cells. Cells were incubated at 37°C with 0.5 μM L-DOPA in the presence of benserazide (50 μM) for increasing periods of time (1 to 120 min). (b) Concentration-dependent accumulation of L-DOPA in LLC-PK$_1$ cells. Cells were incubated for 6 min at 37°C or 4°C in the presence of benserazide (50 μM); increasing concentrations (10 to 500 μM) of the substrate were applied from the apical cell border. Symbols represent means of five experiments per group and vertical lines show s.e.mean.

LLC-PK$_1$ cells attaining equilibrium at 30 min of incubation (Figure 1a). From the initial rate of uptake, k_{in} (rate constant of total inward) and k_{out} (rate constant of total outward) were calculated. The analysis revealed a rate constant of inward transport (k_{in}) of 4.4 ± 0.2 pmol mg^{-1} protein min^{-1} and a rate constant of outward transport (k_{out}) of 1.3 ± 0.1 pmol mg^{-1} protein min^{-1} and an equilibrium factor (A_{max}) of 18.2 ± 0.5 ($n=4$). The intracellular water content of LLC-PK$_1$ cells was 7.1 ± 0.6 μl mg^{-1} protein ($n=12$). At equilibrium (30 min incubation), the intracellular L-DOPA concentration in LLC-PK$_1$ cells was 9.1 ± 0.3 μM at medium concentration of 0.5 μM. This represented a cell concentration of L-DOPA in LLC-PK$_1$ cells that was 18.7 ± 1.8 ($n=4$) times higher than the medium concentration. In saturation experiments, LLC-PK$_1$ cells were incubated for 6 min with increasing concentrations (10 to 500 μM) of the substrate. The accumulation of L-DOPA was found to be dependent on the concentration used and to be saturable at 250 μm (Figure 1b). Non-linear analysis of the saturation curves revealed a K_m of 113 ± 16 μM and a V_{max} of 5.6 ± 0.3 nmol mg^{-1} protein 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 non-saturable (Figure 1b).

The next set of experiments was performed in the absence of benserazide and LLC-PK$_1$ cells were found to convert rapidly the L-DOPA taken up to dopamine. In the first series of experiments, LLC-PK$_1$ cells were incubated with a non-saturating concentration of L-DOPA (0.5 μM) for increasing periods of time. As shown in Figure 2, the decarboxylation of the L-DOPA taken up was a time-dependent process and intracellular levels of L-DOPA and newly-formed dopamine attained equilibrium at 30 min incubation. By contrast, levels of dopamine in the incubation medium increased progressively up to 120 min. At 6 min incubation, the amount of newly-formed dopamine which had escaped to the incubation medium represented 27.5 ± 3.6% of the total amount of dopamine formed. In the second series of experiments, LLC-PK$_1$ cells were incubated with increasing concentrations of L-DOPA (10 to 500 μM) for 6 min. As shown in Figure 3a, the accumulation of newly-formed dopamine was found to be a saturable process reaching saturation at 100 μM L-DOPA. The apparent K_m and V_{max} values for intracellular decarboxylation of the L-DOPA taken up in LLC-PK$_1$ cells was 31 ± 6 μM and 1793 ± 91 pmol mg^{-1} protein 6 min^{-1}, respectively. Figure 3b shows the rate of outward transfer of newly-formed dopamine to the medium bathing the apical cell border as a function of intracellular levels of the amine (in μM); as shown in this figure,

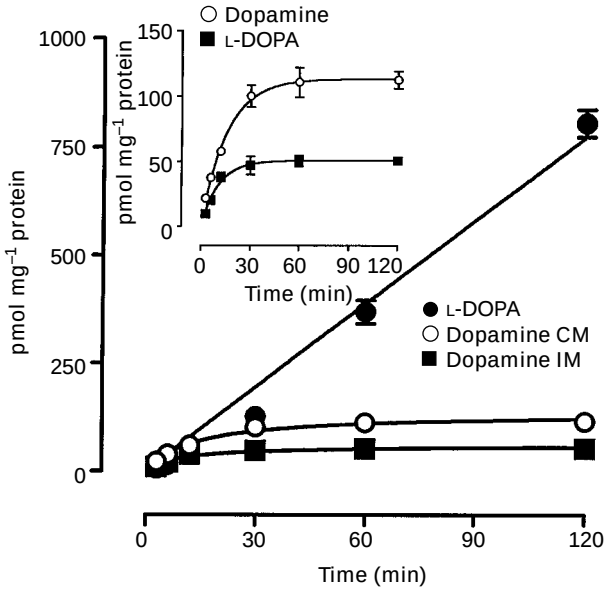

Figure 2 Time-dependent accumulation of L-DOPA and dopamine formation (cell monolayer, CM; incubation medium, IM) in LLC-PK$_1$ cells. The inset shows the intracellular levels of L-DOPA and newly-formed dopamine. Cells were incubated at 37°C with 0.5 μM L-DOPA in the absence of benserazide for increasing periods of time (1 to 120 min); the substrate was applied from the apical cell border only. Symbols represent means of five experiments per group and vertical lines show s.e.mean.

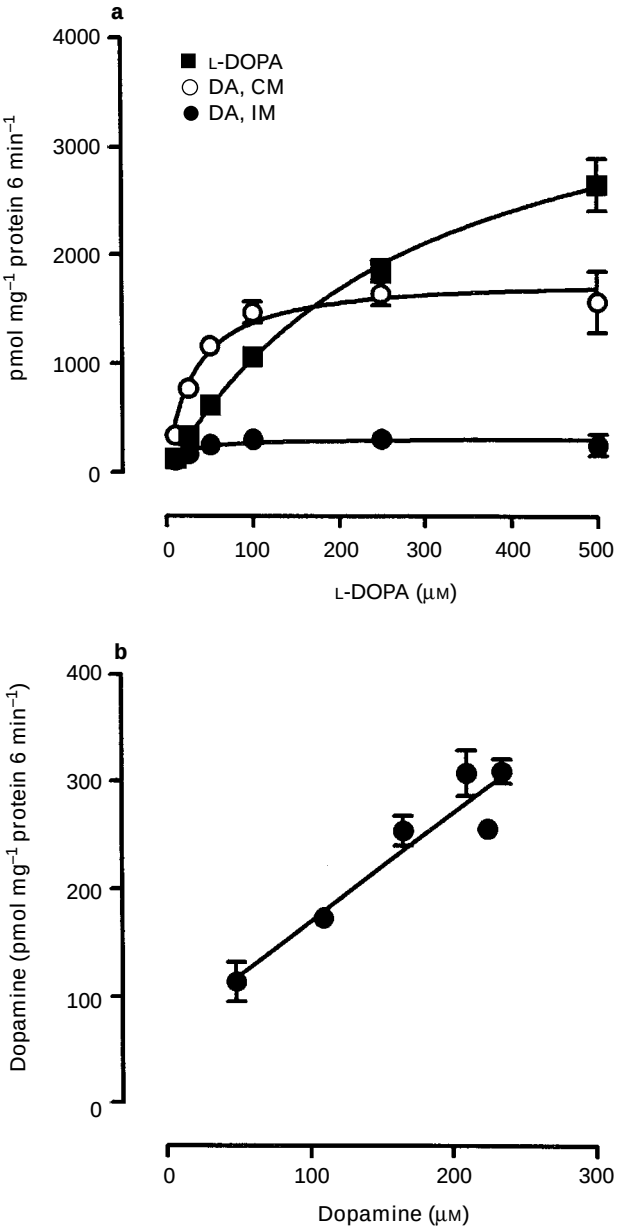

Figure 3 (a) Concentration-dependent accumulation of L-DOPA and dopamine (DA) formation (cell monolayer, CM; incubation medium, IM) in LLC-PK$_1$ cells. Cells were incubated for 6 min at 37°C in the absence of benserazide; increasing concentrations (10 to 500 μM) of the substrate were applied from the apical cell border only. (b) Reciprocal plot of intracellular dopamine (in μM) which has been formed in LLC-PK$_1$ cells loaded with increasing concentrations of L-DOPA (10 to 500 μM) and the amount of dopamine which has escaped to the incubation medium (in pmol mg^{-1} protein 6 min^{-1}) bathing the apical cell border. Linear coefficient values were $r^2=0.9118$, $n=30$. Symbols represent means of five experiments per group and vertical lines show s.e.mean.

the outward transfer of newly-formed dopamine was found to be non-saturable up to 250 μM intracellular dopamine.

In experiments designed to study the effects of CsA, cells were exposed to increasing concentrations (0.3, 1.0 and 3.0 μg ml^{-1}) of the immunosuppressant for 30 min or the vehicle (olive oil) and then incubated, in the absence of benserazide, with a non-saturating concentration (0.5 μM) of L-DOPA for 6 min. At the end of the incubation with L-DOPA, the parameters measured were the intracellular levels of L-DOPA and dopamine and the levels of dopamine in the incubation medium; the fractional outflow of dopamine was calculated by dividing the levels of dopamine in the incubation medium by the total amount of dopamine formed. As shown in Figure 4, CsA was found not to change the intracellular concentrations of newly-formed dopamine (Figure 4a). However, the levels of dopamine in the incubation medium (Figure 4b) and the total amount of dopamine formed (Figure 4c) were found to be increased (143% to 224% increase and 31% to 59% increase, respectively) in cells treated with CsA. As shown in Table 1, CsA did not change the decarboxylation of intracellular L-DOPA, despite the increases in total amount of dopamine formed; this indicates a parallel increase in intracellular L-DOPA. The increase in dopamine levels in the incubation is also evidenced by a significant ($P<0.01$) increase in the fractional outflow of newly-formed dopamine (Figure 4d). These effects of CsA on dopamine formation and dopamine outflow were concentration-dependent effects. CsA-treated cells excluded trypan blue as effectively as vehicle-treated cells, suggesting that CsA exposure for 30 min did not affect the viability of cells.

Table 1 Decarboxylation of intracellular L-DOPA

Treatment	*30 min exposure*	*14 h exposure*
Vehicle	61.4±3.9	88.9±1.0
CsA 0.03 μg ml^{-1}	–	81.7±3.8
CsA 0.10 μg ml^{-1}	–	88.0±1.2
CsA 0.30 μg ml^{-1}	77.6±4.1	81.3±4.0
CsA 1.00 μg ml^{-1}	76.2±3.6	84.5±2.3
CsA 3.00 μg ml^{-1}	79.1±3.9	81.7±2.2

Values represent % decarboxylation of intracellular L-DOPA in vehicle and cyclosporine (CsA)-treated LLC-PK$_1$ cells. Cells were preincubated in the absence of benserazide for 30 min and incubated for 6 min with 0.5 μM L-DOPA; the substrate was applied from the apical cell border. Results are means±s.e.mean of four to five experiments per group.

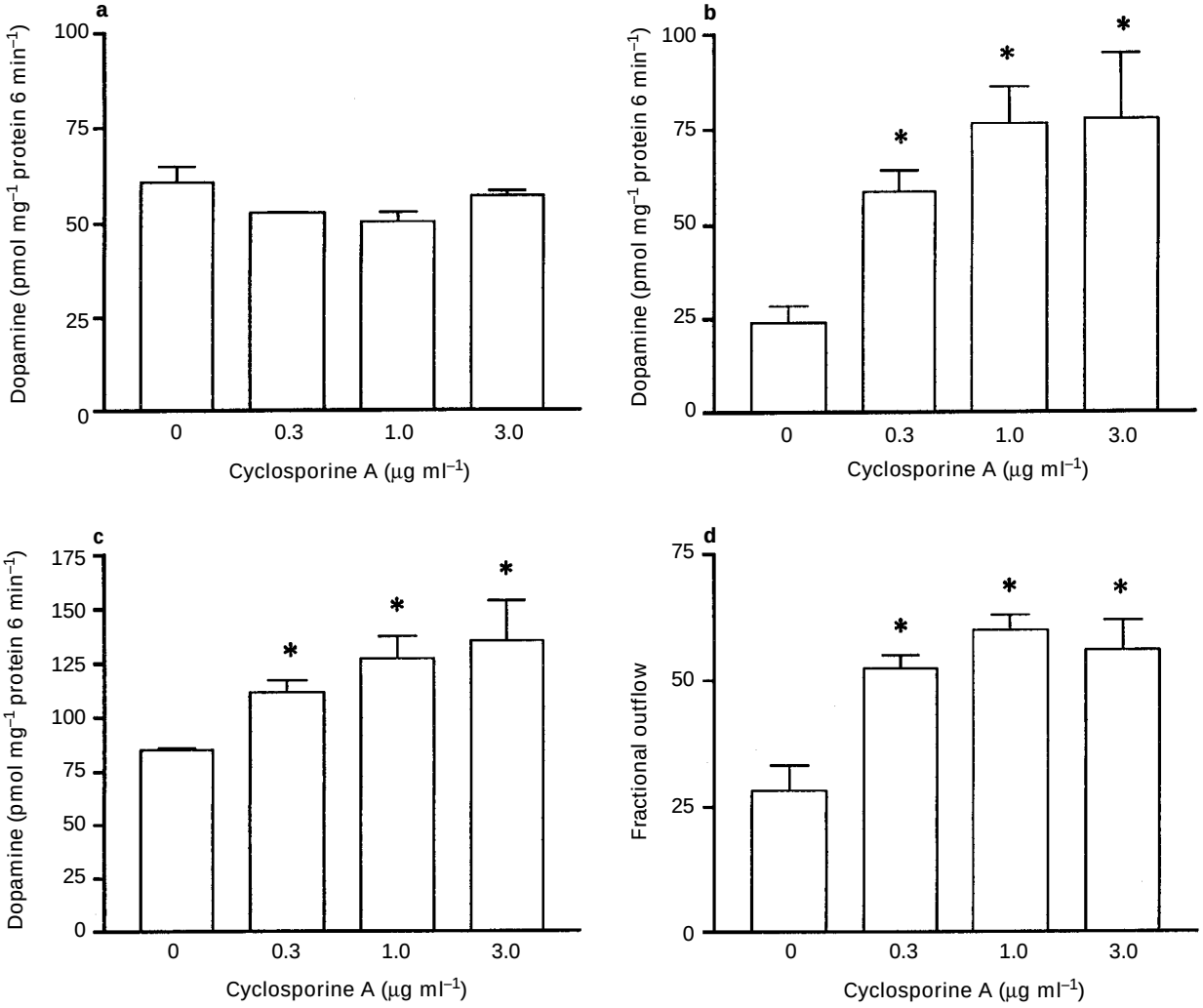

Figure 4 Levels of newly-formed dopamine in (a) cell monolayers, (b) the incubation medium and (c) cell monolayers plus incubation medium in LLC-PK1 cells exposed for 30 min to increasing concentrations of cyclosporine A or the vehicle (olive oil) and subsequently incubated for 6 min with 0.5 μM L-DOPA; the substrate was applied from the apical cell border only. The fractional outflow of newly-formed dopamine is shown in (d). Columns represent means of four to five experiments per group; vertical lines show s.e.mean. Significantly different from corresponding control values (*$P<0.05$) by use of Newman-Keuls test.

LLC-PK$_1$ cells exposed for 14 h to increasing concentrations of CsA (0.03 to 3.0 μg ml^{-1}) were found to form and accumulate less dopamine (15% to 39% reduction and 11% to 59% reduction, respectively) than vehicle-treated cells; this was a concentration-dependent effect (Figure 5a and c). Again, CsA treatment was found not to affect the decarboxylation of intracellular L-DOPA (Table 1). By contrast, the levels of dopamine in the incubation medium were found not to differ from those in vehicle-treated cells (Figure 5b). This resulted in a concentration-dependent increase in the fractional outflow of newly-formed dopamine (Figure 5d). Exposure of CsA for 14 h was found not to affect cell viability, as evidenced by the trypan blue exclusion method.

Figure 6 shows the uptake of L-DOPA in LLC-PK$_1$ cells cultured in polycarbonate filters which had not been exposed to either the vehicle or CsA. The substrate was applied from either the apical or basal cell borders and the accumulation of L-DOPA was found to be dependent on the concentration and saturable (Figure 6); non-linear analysis of saturation curves for apical and basal application revealed K_m (in μM) values of 63.8 ± 17.0 and 42.5 ± 9.6 and V_{max} values (in nmol mg^{-1} protein 6 min^{-1}) of 32.0 ± 5.8 and 26.2 ± 3.4, respectively. These data suggest that the L-DOPA transporter located in the apical cell border has the same kinetic characteristics of that in the basolateral cell border.

In the final series of experiments, cells were cultured on polycarbonate filters and exposed to CsA for 30 min or 14 h; CsA was applied from the apical cell border only. Thereafter, cells were incubated with 0.5 μM L-DOPA applied from the basolateral cell border; the incubation medium contained 50 μM benserazide in order to inhibit L-DOPA decarboxylation. At the end of the incubation, the levels of L-DOPA in LLC-PK$_1$ cells and the levels of L-DOPA which have escaped to the apical bathing fluid were measured. As shown in Figure 7a, the intracellular levels of L-DOPA in cells which had been treated for 30 min with CsA were similar to those in vehicle-treated cells. However, the levels of L-DOPA in cells treated with CsA for 14 h were significantly lower than in vehicle-treated cells and cells treated with CsA for 30 min. The flux of L-DOPA across the cell monolayer was significantly lower in cells treated for 30 min with CsA, whereas that in cells treated with CsA for 14 h was markedly higher, when compared with cells treated with CsA for 30 min (Figure 7b). Paracellular leakage measured by the fluxes of ^{3}H-sorbitol from either side

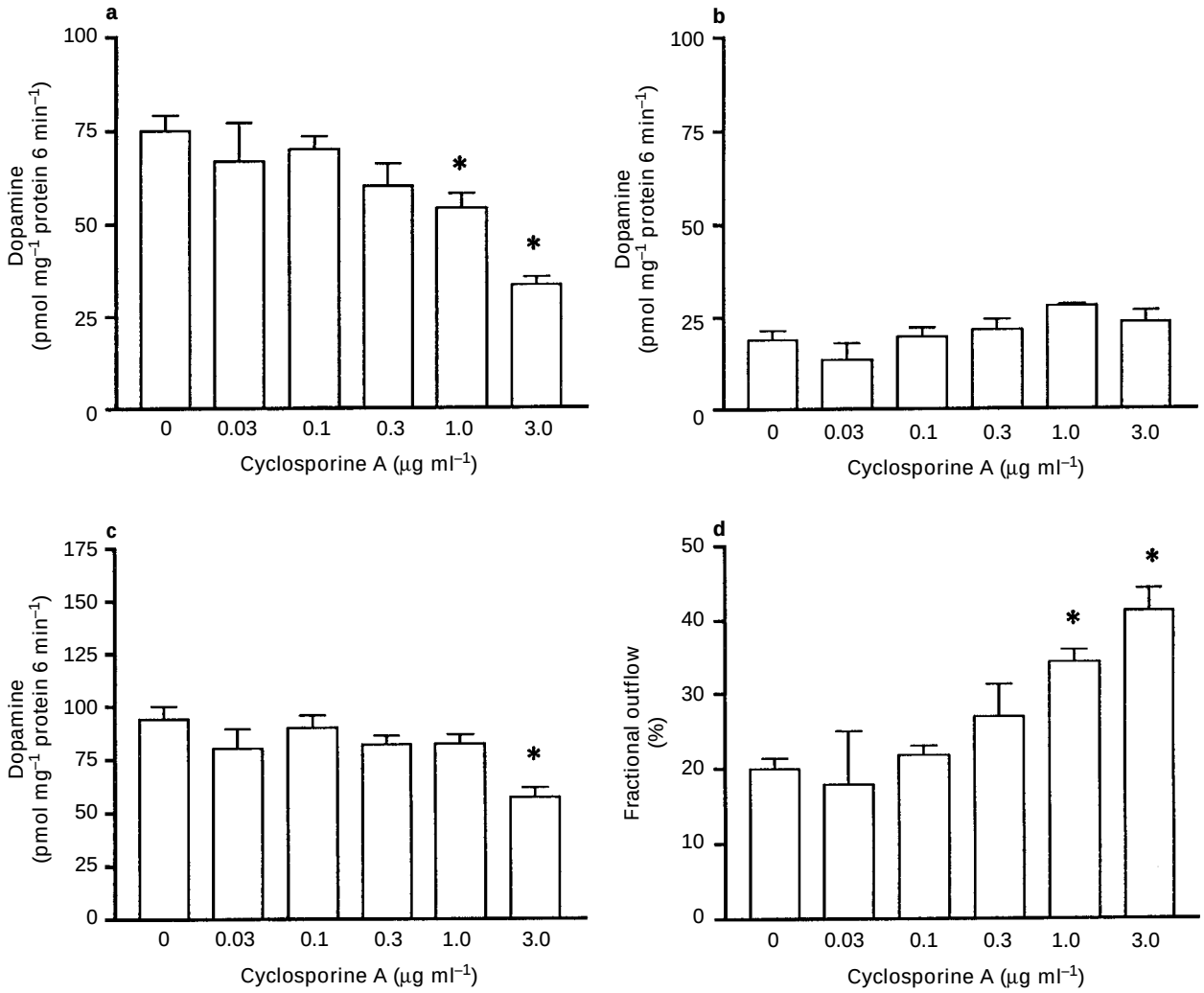

Figure 5 Levels of newly-formed dopamine in (a) cell monolayers, (b) the incubation medium and (c) cell monolayers plus incubation medium in LLC-PK$_1$ cells exposed for 14 h to increasing concentrations of cyclosporine A or the vehicle (olive oil) and subsequently incubated for 6 min with 0.5 μM L-DOPA; the substrate was applied from the apical cell border only. The fractional outflow of newly-formed dopamine is shown in (d). Columns represent means of four to five experiments per group; vertical lines show s.e.mean. Significantly different from corresponding control values (*$P<0.05$) by use of Newman-Keuls test.

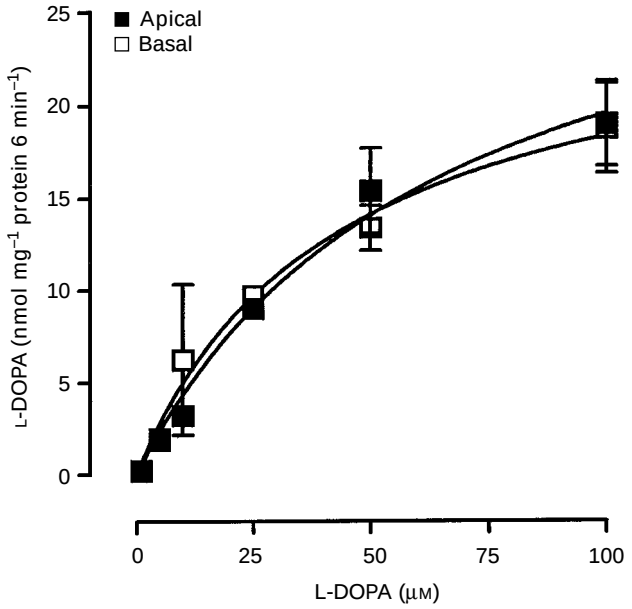

Figure 6 Accumulation of L-DOPA in LLC-PK$_1$ cells cultured in polycarbonate filters and incubated for 6 min at 37°C; the substrate was applied either from the apical or the basal cell border. Symbols represent means of four to five experiments per group and vertical lines show s.e.mean.

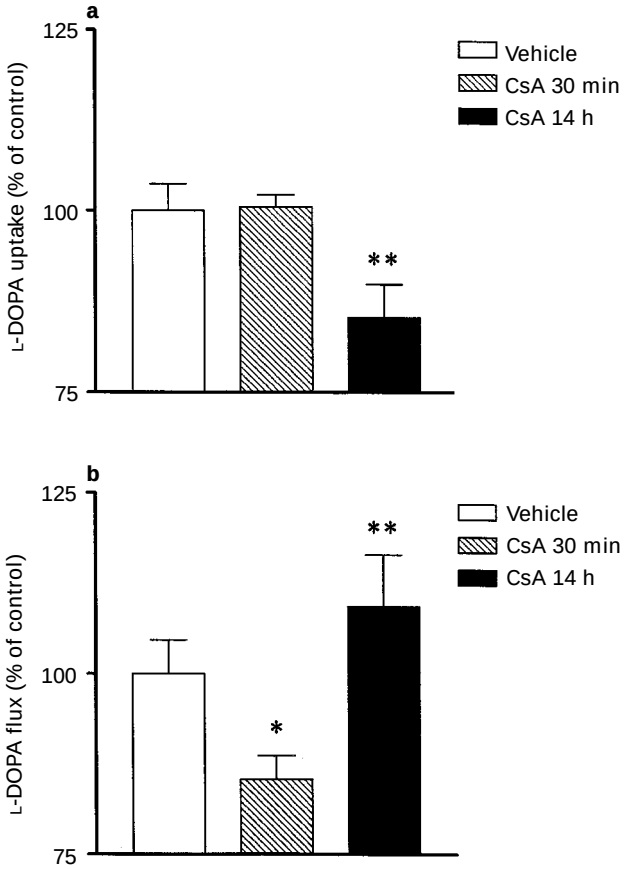

Figure 7 Levels of L-DOPA in (a) cell monolayers (uptake) and (b) the fluid bathing the apical cell border (flux) in LLC-PK$_1$ cells exposed for 30 min or 14 h to cyclosporine A (3.0 μg ml^{-1}) and olive oil and subsequently incubated for 6 min with 0.5 μM L-DOPA; the substrate was applied from the basolateral cell border only. The absolute levels of intracellular L-DOPA and in the fluid bathing the apical cell border in vehicle-treated cells were 0.13 ± 0.01 and 5.54 ± 0.18 nmol mg^{-1} protein 6 min^{-1}, respectively. Columns represent means of six experiments per group and vertical lines show s.e.mean. Significantly different ($P<0.05$) from corresponding values for vehicle-treated cells (*) and cells treated with cyclosporine A for 30 min (**), by use of Newman-Keuls test.

was minimal and represented 0.1% of the amount applied at the cell surface. CsA exposure (30 min and 14 h) did not affect the flux of ^{3}H-sorbitol from the basal to the apical cell side (data not shown).

The results shown in Figures 8 and 9 were obtained in experiments aimed at detecting the presence of P-glycoprotein activity in LLC-PK$_1$ cells and to look at the effects of CsA, respectively. The rationale for these experiments was based on the following evidence: (1) CsA acutely inhibits P-glycoprotein in human and rodent cells at doses in the range of 1 to 5 μg ml^{-1} (Twentyman, 1992); (2) chronic exposure to CsA stimulates P-glycoprotein and this is believed to represent a mechanism of cellular detoxification (Garcia del Moral *et al.*, 1995). Detection of P-glycoprotein activity was carried out by measuring verapamil- or UIC2-sensitive rhodamine 123 accumulation. Rhodamine 123 accumulation in LLC-PK$_1$ cells was found to be linear up to 300 μM (data not shown). As shown in Figure 8, both UIC2 (3 μg ml^{-1}) and verapamil (25 μM) significantly increased the accumulation of rhodamine 123 in LLC-PK$_1$ cells loaded with the dye (100 μM); the levels of rhodamine 123 in control conditions were found to be 8.1 ± 0.6 nmol mg^{-1} protein ($n=5$). As shown in Figure 9a, 30 min exposure to CsA was found not to affect the accumulation of rhodamine 123 in the presence of verapamil (25 μM). On the other hand, 14 h exposure to CsA was found to reduce the accumulation of rhodamine 123 (Figure 9b).

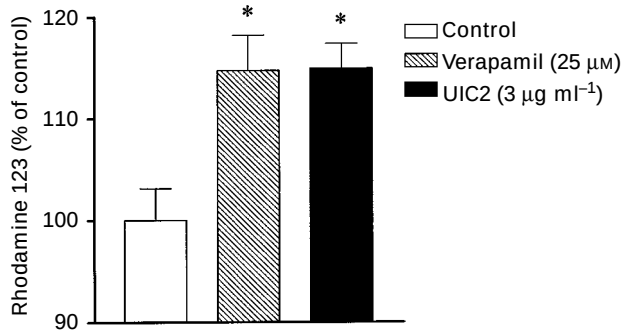

Figure 8 Accumulation of rhodamine 123 in monolayers of LLC-PK$_1$ cells treated with the vehicle, verapamil (25 μM) or the anti-P-glycoprotein monoclonal antibody UIC2 (3 μg ml^{-1}). The absolute levels of intracellular rhodamine 123 in vehicle-treated cells were 8.1 ± 0.6 nmol mg^{-1} protein 6 min^{-1} ($n=5$). Columns represent means of five experiments per group and vertical lines show s.e.mean. *Significantly different ($P<0.05$) from corresponding values for vehicle-treated cells, by use of Newman-Keuls test.

Discussion

Several findings demonstrate that the apical uptake of L-DOPA by LLC-PK$_1$ cells is a facilitated mechanism. Firstly, steady-state uptake of non-saturating concentrations of L-DOPA showed a curvilinear dependence on incubation time. Secondly, at initial rate of uptake (6 min incubation) the cellular transport of L-DOPA showed a curvilinear dependence on L-DOPA medium concentration with a K_m of 113 μM, suggesting that the uptake was saturable. Thirdly, this

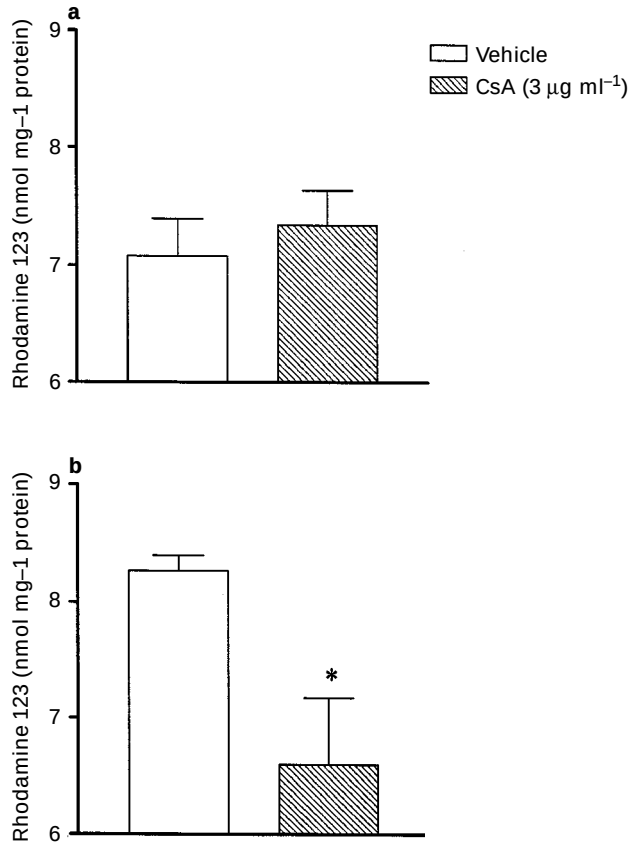

Figure 9 Effect of (a) 30 min or (b) 14 h exposure to cyclosporine A (CsA) on the accumulation of rhodamine 123 by monolayers of LLC-PK$_1$ cells in the presence of verapamil (25 μM). Columns represent means of five experiments per group and vertical lines show s.e.mean. *Significantly different ($P<0.05$) from corresponding values for vehicle-treated cells by use of Student's t test.

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-PK$_1$ cells can be also 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 would 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. Experiments carried out in cells cultured in polycarbonate filters showed that the apical uptake of L-DOPA obeys Michaelis-Menten kinetics similar to that observed when the substrate is applied from the basal cell border. However, it was interesting to observe that the apical uptake of L-DOPA in cells cultured in polycarbonate filters differed substantially from that observed in cells cultured in collagen-treated plastic, the main differences being a lower K_m value (63 ± 17 vs 113 ± 16 μM) and a higher V_{max} value (32.0 ± 4.4 vs 5.6 ± 0.3 nmol mg^{-1} protein 6 min^{-1}). It is possible that this may have been to do with the different environment of cells cultured in a more physiological system, such as in polycarbonate filters.

In the absence of benserazide, the L-DOPA taken up is rapidly converted to dopamine. When non-saturating concentrations (0.5 μM) of L-DOPA are used in time course experiments, the result is a rapid accumulation of the substrate followed by its fast decarboxylation to dopamine; equilibrium of intracellular concentrations of L-DOPA and newly-formed dopamine were reached at approximately 30 min of incubation. A considerable amount of newly-formed dopamine escaped to the incubation medium and this process was time-dependent. When LLC-PK$_1$ cells were incubated for 6 min with increasing concentrations of L-DOPA, the accumulation of newly-formed dopamine was found to be a saturable process reaching saturation at 100 μM L-DOPA with an apparent K_m value of 31 ± 6 μM. At 6 min incubation, the outward transfer of newly-formed dopamine was found to be non-saturable. However, the intracellular concentrations of dopamine generated from L-DOPA were not high enough to exclude the possibility that dopamine is extruded out of the cell by a low affinity transporter ($K_m<200$ μM). In fact, the K_m for the dopamine outward transfer in both rat and dog kidney cells was found to be greater than this (respectively, 319 ± 35 and 340 ± 41 nmol g^{-1}) (Soares-da-Silva, 1993a). Considering that 1 g of tissue contains approximately 1 ml of cell water, the K_m value for the rat and dog dopamine outward transfer would be higher than 300 μM.

In LLC-PK$_1$ cells exposed for 30 min to CsA the intracellular concentration of newly-formed dopamine was found to be similar to that in vehicle-treated cells. However, this was accompanied by a concentration-dependent increase in the amount of newly-formed dopamine in the incubation medium and the total amount of dopamine formed. As indicated in Figure 4, these effects of CsA were mainly the result of increases in (1) the fractional outflow of newly-formed dopamine and (2) the intracellular availability of L-DOPA, respectively. The effect of CsA on the formation of dopamine (an indirect measure of intracellular availability of L-DOPA) was clearly concentration-dependent. As shown here, the uptake of L-DOPA by LLC-PK$_1$ cells is a facilitated, saturable and energy-dependent process and CsA might have enhanced this uptake process. An alternative explanation for the increase in the intracellular availability of L-DOPA during short-term exposure to CsA could be a reduced extrusion of the substrate taken up. This would favour an increase in intracellular L-DOPA, which then undergoes rapid decarboxylation to dopamine.

Long-term exposure to CsA was found to produce opposite effects on the intracellular availability of L-DOPA in LLC-PK$_1$ cells. Cells exposed to CsA for 14 h were found to synthesize less dopamine than vehicle-treated cells. The intracellular concentrations of newly-formed dopamine were also found to be reduced, in a concentration-dependent manner, by CsA. The reason for this different behaviour does not appear to be related to a decrease in cell viability, since the number of CsA-treated cells excluding trypan blue was similar to vehicle-treated cells. Furthermore, the decarboxylation reaction in CsA-treated cells was not different from that in vehicle-treated cells (Table 1). As occurred during short-term exposure to CsA, cells exposed for 14 h to CsA were found to present an increase in the fractional outflow of newly-formed dopamine. A possible explanation for the decrease in the intracellular availability of L-DOPA during long-term CsA exposure might be the result of a reduced uptake of L-DOPA or an increased extrusion of the L-DOPA taken up.

To test the hypothesis that short- and long-term CsA exposure may modulate the cellular extrusion of the L-DOPA taken up, LLC-PK$_1$ cells were cultured in polycarbonate filters and L-DOPA was applied from the basal cell border; at the end of the incubation, the intracellular levels of L-DOPA and the levels of L-DOPA in the apical fluid were measured. LLC-PK$_1$ cells exposed to CsA for 30 min were found to extrude less L-DOPA than vehicle-treated cells. By contrast, cells exposed to CsA for 14 h were

found to extrude more L-DOPA than vehicle-treated cells and cells treated with CsA for 30 min. A possible explanation for these apparent contradictory effects of CsA on the cell extrusion of L-DOPA could be the involvement of P-glycoprotein as a transporter of intracellular L-DOPA. P-glycoprotein is a 170 kDa plasma membrane protein encoded by the mammalian multidrug resistant (MDR1) gene (Endicott & Ling, 1989), which functions as an adenosine 5′-triphosphate (ATP)-driven active efflux pump of a wide variety of drugs (Ford & Hait, 1990) and also acts as an efflux pump to expel hydrophobic substances from the cells (Rao, 1995). CsA is known to reverse acutely P-glycoprotein-mediated multidrug resistance (Keller *et al*., 1992) and to inhibit effectively P-glycoprotein mediated transfer mechanisms (Leveque & Jehl, 1995). On the other hand, chronic exposure to CsA stimulates P-glycoprotein and this is believed to represent a mechanism of cellular detoxification (Garcia del Moral *et al*., 1995). Thus, if L-DOPA apical outward transfer in LLC-PK$_1$ cells is going to be promoted by P-glycoprotein, short-term exposure to CsA should produce a reduction in the cellular extrusion of L-DOPA and increase its intracellular levels, whereas the long-term exposure to CsA should increase the cellular extrusion of L-DOPA and decrease its cellular availability. The finding that both the anti-P-glycoprotein monoclonal antibody, UIC2, and verapamil, a putative inhibitor of P-glycoprotein, increased rhodamine 123 accumulation agrees well with the suggestion that these cells contain P-glycoprotein activity; intracellular rhodamine 123 is extruded by P-glycoprotein, through UIC2- (Mechetner & Roninson, 1992) and verapamil-sensitive mechanisms (Fardel *et al*., 1996). On the other hand, the finding that cells submitted to a long-term exposure to CsA present a marked reduction in the accumulation of rhodamine 123 agrees well with the suggestion that chronic exposure to CsA enhances the activity of P-glycoprotein, which accords with an increased extrusion of intracellular rhodamine 123.

However, these mechanisms appear not to apply to newly-formed dopamine, since both short- and long-term exposure to CsA were found to increase the apical extrusion of the amine. In slices of dog renal cortex, the outward transfer of newly-formed dopamine was found to proceed through non-saturable and saturable mechanisms, the latter being a low affinity outward transfer (Soares-da-Silva, 1993a). The dopamine outward transfer in renal epithelial cells has been not characterized in detail, though it is known, at least in the dog, to be sensitive to activation of α_1- and α_2-adrenoceptor stimulation, a mechanism most probably resulting in activation of Na^+-H^+ exchange stimulation (Soares-da-Silva, 1993b). Intracellularly, CsA is known to bind to a cytosolic protein, cyclophilin, which has been shown to possess peptidyl-prolyl *cis*-trans isomerase activity (Twentyman, 1992); in addition, CsA may inhibit protein kinase C (Twentyman, 1992). It is possible this may be involved in the dopamine outward transfer promoting activity of the immunosuppressant, but further studies are needed to clarify this effect. However, this is an interesting observation since the increase in the outward transfer of dopamine by CsA may lead to a potentiation of the natriuretic effects of the amine in opposition to the antinatriuretic effects of CsA.

In conclusion the increase and the reduction in the formation of dopamine after short- and long-term exposure to CsA, respectively, correlate with the effects of the immunosuppressant on the apical cell extrusion of the L-DOPA taken up, suggesting the involvement of P-glycoprotein. The effects of CsA on the fractional outflow of newly-formed dopamine appear to be mediated by a different mechanism.

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