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Research Paper

Substrate Specificity and Mechanism of the Intestinal Clonidine Uptake by Caco-2 Cells

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Purpose. This study was performed to characterize the substrate specificity and mechanism of the intestinal clonidine transport.
Methods. Uptake of [^{3}H]clonidine into Caco-2 cells was investigated. Interaction with drugs was studied in competition assays.
Results. Uptake of [^{3}H]clonidine was linear for up to 2 min, Na^+-independent, and insensitive to changes in membrane potential, but strongly H^+-dependent. The uptake rate of clonidine was saturable with kinetic parameters of 0.5 ± 0.1 mM (K_t) and 16.6 ± 1.8 nmol/2 min per mg of protein (V_{max}) at an outside pH of 7.5. Many drugs such as clonidine, guanabenz, methamphetamine, imipramine, clomipramine, nortriptyline, quinine, xylazine, ephedrine, and diphenhydramine strongly inhibited the [^{3}H]clonidine uptake with K_i values between 0.15 and 1 mM.
Conclusions. Clonidine is transported by a carrier-mediated process. Substrate specificity and mechanism are very similar to the transport described in blood–brain barrier endothelial cells. The transport characteristics do not correspond to carriers for organic cations of the SLC22 family or the choline transporters CHT1 and CLT1. The system might be identical to the H^+/tertiary amine antiporter. It interacts with a large number of both hydrophilic and lipophilic cationic drugs, and also, interestingly, with opiates.

KEY WORDS: Caco-2 cells; clonidine-derived drugs; drug delivery; membrane transport.

INTRODUCTION

For 30 years clonidine has been used for treatment of hypertension (1), anesthesia, pain (2), and many other therapeutic applications. To the best of our knowledge, studies on the mechanism of the intestinal clonidine transport have not yet been performed. This is quite surprising considering the routine oral application of clonidine and the fact that clonidine is one of the most thoroughly studied drugs (>14,200 entries in medical databases) in the field. One reason for this serious gap of knowledge might be that clonidine was, and is, used by many as a marker for "lipophilic transcellular transport" as opposed to markers for "transporter-mediated transcellular transport" (3). In other words, clonidine is regarded by some groups as a compound for which no transporter exists. A similar view prevailed for many years regarding the clonidine transport at the blood–brain barrier. In 1997, however, Huwyler and coworkers (4) showed that clonidine is transported by a carrier. The authors studied the mechanism and kinetics of clonidine transport across microvessel endothelial cells isolated from porcine "tight" brain regions. Transport was sensitive to pH and saturable, with an apparent affinity constant (K_t) of 1.3 mM and a maximal velocity of transport (V_{max}) of 0.1 nmol/min per cm^2 (4). The same system might be expressed in the skin and placenta (5,6).

Because at pH 7.5 clonidine exists in aqueous solutions predominantly as cation (pK_a = 9.16), it could be hypothesized that it represents a substrate for organic cation transporters (OCT) such as OCT1, OCT2, OCT3 (EMT), OCTN1 (ETT), or OCTN2 (7,8). Indeed, clonidine is able to inhibit the uptake of [^{3}H]choline (9,10), a substrate for carriers for several organic cations (7,8). However, it soon became clear that unlabeled choline is not able to inhibit clonidine transport in blood–brain barrier endothelial cells (4), keratinocytes (5), or placenta cells (6). Moreover, clonidine uptake is not affected by the prototype OCT substrates tetraethylammonium (TEA) and *N*-methyl-4-phenylpyridinium (MPP^+) (5,6).

Because of the high oral bioavailability of clonidine (greater than 90%), we characterized the intestinal clonidine uptake using Caco-2 cells and studied the effect of a large selection of new, structurally related and unrelated, lipophilic and hydrophilic drugs to obtain insight into the transports' substrate specificity.

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MATERIALS AND METHODS

Cell Culture

Caco-2 cells obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) were routinely cultured (passages 8–20 and 79–102) in minimum essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acid solution, and gentamicin (45 μg/mL) (11,12). Cells grown to 80% confluence were released by trypsinization and subcultured in 35-mm disposable Petri dishes (Becton Dickinson, Oxford, UK). The medium was replaced every 2 days and the day before the uptake experiment. With a starting cell density of 0.8×10^6 cells/dish, the cultures reached confluence within 24 h. Uptake was measured 7 days after seeding. Caco-2 cells were also cultured on permeable TRANSWELL® cell culture inserts (diameter 24.5 mm, pore size 3 μm; Costar GmbH, Bodenheim, Germany) with a cell density of 0.2×10^6 cells/filter for 21 days as described (11,12).

Transport Measurements

Monolayers were rinsed once with buffer containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 25 mM MES/Tris (pH 6.0), 25 mM HEPES/Tris (pH 7.5) or 25 mM Tris/HEPES (pH 8.5). To initiate uptake, 1 mL buffer containing 1–3 nM [^{3}H]clonidine (specific activity 55.5 Ci/mmol; Amersham International, Freiburg, Germany) and drugs at increasing concentrations (purchased from Sigma, Deisenhofen, Germany; ICN, Eschwege, Germany; Solvay Pharmaceuticals GmbH, Hannover, Germany; or gifts from M.H. Zenk and P. Frohberg, Department of Pharmacy, Martin Luther University, Halle, Germany) were added to each dish for 1–10 min at room temperature. Uptake was stopped by washing the dishes four times with ice-cold buffer. Cells were solubilized and prepared for liquid scintillation counting. The protein content was measured according to the method described by Bradford.

NH_4Cl prepulse experiments for inner acidification of cells were routinely performed as follows: confluent monolayer cultures were treated for 15 min with either the control buffer (25 mM HEPES/Tris, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 140 mM choline chloride, pH 7.5) or the NH_4Cl-containing buffer (25 mM HEPES/Tris, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 115 mM choline chloride, 25 mM NH_4Cl, pH 7.5). After treatment, the monolayers were washed with the NH_4Cl-free uptake buffer and uptake of [^{3}H]clonidine was measured at pH 7.5 for 1 min.

Transepithelial flux of [^{3}H]clonidine across Caco-2 cell monolayers cultured on permeable filters was measured as previously described (11,12). Uptake was started by adding buffer (pH 7.5) containing [^{3}H]clonidine (3 nM) or [^{3}H]mannitol (28 nM) with or without 10 mM unlabeled clonidine to the apical compartment. Samples were taken from the receiver compartment. After 2 h, the filters were washed, cut out of the plastic insert, and prepared for liquid scintillation analysis.

Membrane Potential Assay

Caco-2 cells, subcultured as described above for 7 days but in 96-well plates at a starting density of 0.8×10^5 cells/well, were washed twice with uptake buffer (pH 7.5 or 6.0) and incubated with 50 μL red membrane potential (MP) dye (Molecular Devices Corp., Munich, Germany) per well for 60 min at 37°C. After that, compounds dissolved in 200 μL uptake buffer for a final concentration of 3 mM were added. Fluorescence was measured immediately at wavelengths of 530 nm (excitation) and 570 nm (emission) at a FLUOstar Galaxy (BMG Labtechnologies Offenburg, Germany) (13,14).

Data Analysis

In general, each data point was determined at least in triplicate for each experiment. Data are presented as mean ± SE. Statistical analyses were carried out via Mann–Whitney *U* test. The kinetic constants were calculated by nonlinear regression of the Michaelis–Menten plot and confirmed by linear regression of the Eadie–Hofstee plot. The calculated parameters are shown with their SE. Inhibition constants (K_i) were calculated from the IC_{50} values (i.e., the concentration of the unlabeled compound necessary to inhibit 50% of specific [^{3}H]clonidine uptake) using the K_t value of 0.5 mM obtained in this study.

RESULTS AND DISCUSSION

Uptake of [^{3}H]clonidine in Caco-2 cells was maximal at days 6–7 after seeding. Day 7 was chosen for further experiments. First, time, Na^+, K^+, and pH dependence of [^{3}H]clonidine uptake were determined. Uptake of [^{3}H]clonidine (3 nM) at pH 7.5 was linear for up to 2 min (Fig. 1). This period was chosen for substrate/inhibitor kinetic studies at pH 7.5. Next, the effect of extracellular Na^+ and K^+ was measured. Replacement of 140 mM NaCl by 140 mM

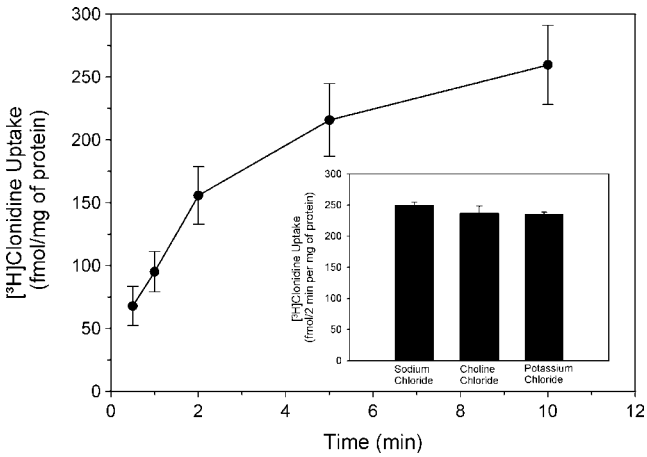

Fig. 1. Time-dependent [^{3}H]clonidine uptake in Caco-2 cells. Uptake of [^{3}H]clonidine (3 nM) was measured at pH 7.5 in cells cultured for 6 days post confluence. Values represent means ± SE, $n = 4$. Inset: Sodium chloride in the uptake buffer (pH 7.5) was replaced with choline chloride or with potassium chloride (140 mM), $n = 3$–6.

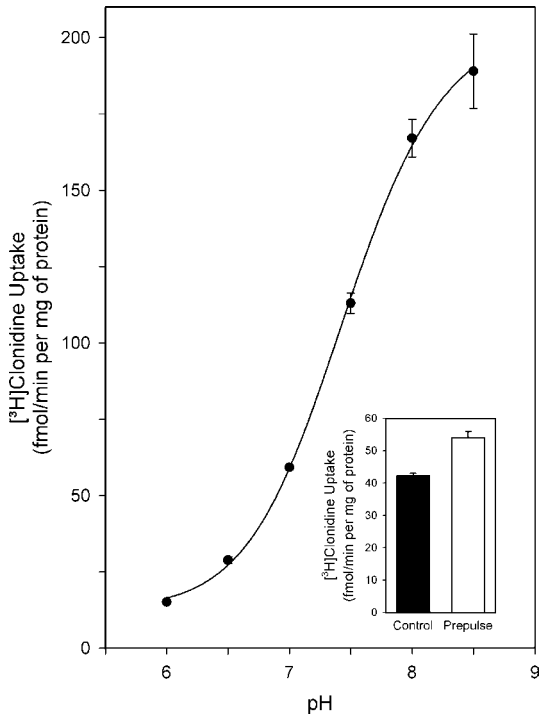

Fig. 2. pH dependency of [^{3}H]clonidine uptake in Caco-2 cells. Uptake of [^{3}H]clonidine (1 nM) was measured in the presence of sodium for 1 min. Inset: Confluent monolayer cultures of Caco-2 cells were treated for 15 min with either the control buffer (pH 7.5) or prepulse buffer (25 mM NH_4Cl, pH 7.5). After treatment, the monolayers were washed with the NH_4Cl-free uptake buffer and uptake of [^{3}H]clonidine was measured at pH 7.5 for 1 min. Values represent means ± SE, $n = 4$.

choline chloride had no effect on [^{3}H]clonidine uptake at pH 7.5 (Fig. 1, inset) nor at pH 8.5. This result clearly demonstrates the complete sodium independence of clonidine transport by Caco-2 cells. At the same time, the result unequivocally rules out choline as a potential substrate or inhibitor of this transport system. Hence, the system can not be identical to the Na^+-dependent choline transporter CHT1 or the Na^+-independent choline transporter CLT1 characterized recently (15). We then depolarized the membrane potential by increasing the concentration of K^+ in the uptake buffer (140 mM). Based on the result that K^+ had no effect on [^{3}H]clonidine uptake, we conclude that the system is insensitive to changes in membrane potential (Fig. 1, inset). The carrier is, however, strongly affected by a pH gradient; Fig. 2 illustrates the effect of outside pH on [^{3}H]clonidine uptake—uptake is increased 1.7-fold when the outside pH is increased from 7.5 to 8.5. Several possibilities exist to explain this marked pH sensitivity (4). First, the carrier might function as an H^+/clonidine antiporter energized by an outside directed pH gradient. In this case (and provided that the stoichiometry is 1:1), the transport might be electroneutral. Alternatively, this pH sensitivity could be explained by the inhibition of the carrier protein by extracellular protonation: For the vesicular acetylcholine transporter, it was shown very recently that certain extracellular sites must be unprotonated for transport (16). In this case, [^{3}H]clonidine uptake should be dependent on the extracellular pH *per se*, but independent of inside pH and the presence of a proton gradient. To test this possibility, we altered the intracellular pH via the NH_4Cl prepulse technique. The intracellular pH decreased due to the prepulse from 7.5 to 7.2 as determined by fluorescence spectroscopic measurement using the pH-sensitive marker BCECF. The result of this experiment is given in the inset of Fig. 2. Decreasing the intracellular pH when keeping the extracellular pH constant at 7.5 increases the [^{3}H]clonidine uptake significantly by 28%. Thus, it seems that the stimulation of [^{3}H]clonidine uptake caused by basic extracellular pH is the effect of an outwardly directed H^+ gradient rather than the basic extracellular pH *per se*. There is a further, less-probable mechanism. At pH 7.5 in aqueous solution, 84.9% of the clonidine molecules are in their positively charged form compared to only 36% at pH 8.5. At pH 6.0, 99% are protonated. For organic cation transporter 2 (OCT2), it was shown that for weak bases the degree of ionization plays a critical role in substrate binding (17). Cimetidine uptake decreased as pH values increased. The carrier seems to prefer the positively charged ionic subspecies. In our study, as in (4), however, [^{3}H]clonidine uptake increased with increasing pH. Hence, we conclude that all data are consistent with a Na^+-independent, membrane-potential independent H^+/clonidine antiport system preferring the positively charged ionic subspecies at a given pH. To di-rectly investigate the effect of clonidine and other drugs on the membrane potential, we applied a membrane potential assay established recently (13,14). After incubating the cells with red membrane potential dye for 60 min, drugs at a final concentration of 3 mM were added. Fluorescence units were recorded and converted into % of the buffer baseline (=100 ± 7%). As a positive control, taurine and L-proline (3 mM) were used. For both compounds, a strong increase in fluorescence signal was recorded (taurine: 305 ± 7%, L-proline: 314 ± 7%). This increase reflects the activity of the electrogenic H^+/amino acid symporter PAT1 (12,14). L-Phenylalanine and diphenhydramine had no effect. They did not elicit significant changes of 100% buffer baseline (127 ± 4 and 87 ± 7%, respectively). For clonidine (−146 ± 12%), imipramine (−8 ± 12%), quinine (−732 ± 11%), and quinidine (−743 ± 11%), negative signals below buffer baseline were recorded reflecting a significant hyperpolarization of the membrane potential. It has to be noted that these drugs themselves have strong and well-known effects on epithelial K^+ channels, Na^+ channels, and the Na^+/H^+ exchanger (18), respectively. With this assay, it is therefore impossible to differentiate between electrogenic transport and a transport-independent drug effect. In both cases a change in membrane potential should be observed.

In the next series of experiments, the transepithelial flux of [^{3}H]clonidine across Caco-2 cell monolayers was measured at pH 7.5 in cells cultured on permeable filters (Fig. 3). The flux of [^{3}H]clonidine of 243 ± 60 fmol/h per cm^2 (=5%/h per cm^2), calculated by linear regression, occurs at the same rate, e.g., as the flux of L-[^{3}H]proline that is mediated by the proton-coupled amino acid symporter hPAT1. It exceeds the flux of the standard space marker [^{3}H]mannitol by 70-fold (12). Both the transepithelial [^{3}H]clonidine flux across the cell monolayers and the [^{3}H]clonidine uptake during the 2-h incubation were significantly inhibited by unlabeled

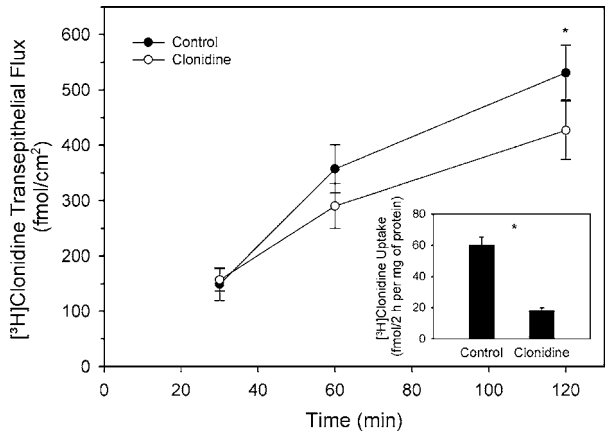

Fig. 3. Transepithelial flux of [^{3}H]clonidine across Caco-2 monolayers. Flux of [^{3}H]clonidine (3 nM) was measured in the absence (control) or presence of excess amount (10 mM) of unlabeled clonidine added to the apical compartment of TRANSWELL® chambers (pH 7.5, 37°C). Appearance of [^{3}H]clonidine in the basolateral compartment corrected for buffer replacement is plotted *vs.* time. Insert: Uptake of [^{3}H]clonidine into the cells on the filter within 2 h. Data are means ± SE, $n = 4$.

clonidine (10 mM) by 28 and 70%, respectively. That the degree of inhibition of flux is lower than the inhibition of uptake is, for many transporter substrates, a well-known phenomenon caused by the complex interplay of the efflux systems at both membranes (12). It is important to note that clonidine itself, even at a concentration of 10 mM, had absolutely no negative effect on monolayer integrity as measured using the reference flux marker [^{3}H]mannitol (28 nM).

To determine the kinetic parameters of the clonidine uptake process, the dependence of the clonidine uptake on the substrate concentration was measured at pH 7.5 in the range of 3 nM–3 mM (Fig. 4A). The nonmediated transport component, which might represent simple diffusion of the tracer plus adherent extracellular radioactivity at the apical cell membrane, was determined by measuring the uptake of [^{3}H]clonidine in the presence of excess amount (20 mM) of unlabeled clonidine. This component was 18.5% of the total [^{3}H]clonidine uptake at 3 nM. The relationship between carrier-mediated uptake rate and substrate concentration was found to be hyperbolic. When the results were expressed in the form of an Eadie–Hofstee plot (uptake rate/substrate concentration *vs.* uptake rate), a straight line was obtained. The maximal transport velocity (V_{max}) was 16.6 ± 1.8 nmol/2 min per mg of protein. The apparent Michaelis–Menten constant of clonidine transport (K_t) was 0.54 ± 0.09 mM, corresponding well with the K_t values at cultured brain microvessel endothelial cells, placental JEG-3 cells, or human keratinocytes (4–6). We also determined the kinetic parameters of clonidine transport at Caco-2 cells at pH 8.5, where total transport is higher compared to pH 7.5 but using an incubation time of 1 min. The V_{max} of clonidine uptake studied in the range of 1 nM–5 mM was 20.1 ± 0.7 nmol/1 min per mg of protein and K_t was 0.40 ± 0.05 mM (Fig. 4B). Again, Eadie–Hofstee transformation led to a straight line ($r^2 = 0.98$), suggesting that only one saturable system is involved in clonidine uptake. This result proves that pH affects only maximal velocity and not affinity of transport.

We next investigated in greater detail the substrate specificity of the system. This was done by measuring the ability of several physiologically and pharmacologically relevant drugs to inhibit the uptake of [^{3}H]clonidine (3 nM, pH 7.5) at a fixed concentration of 2 mM: [^{3}H]Clonidine uptake was inhibited by many therapeutically relevant drugs (Table I). Clonidine, guanabenz, methamphetamine, xylazine, imipramine, amitriptyline, desipramine, nortriptyline, doxepine, maprotiline, chlorpromazine, and others inhibited [^{3}H]-clonidine uptake by more than 50%. Particularly interesting for structure–affinity relationship conclusions is that (*S*)-reticuline, (*R*)-reticuline, ethylmorphine, cocaine, codeine, and diacetylmorphine, but not morphine, also strongly inhibit [^{3}H]clonidine uptake. Compounds such as *N*-butylscopolamine, isobutyl-methyl-xanthine, nicotinamide, L-phenylalanine, L-tyrosine, tyramine, dopamine, noradrenaline, L-tryptophan, L-histidine, metformine, and

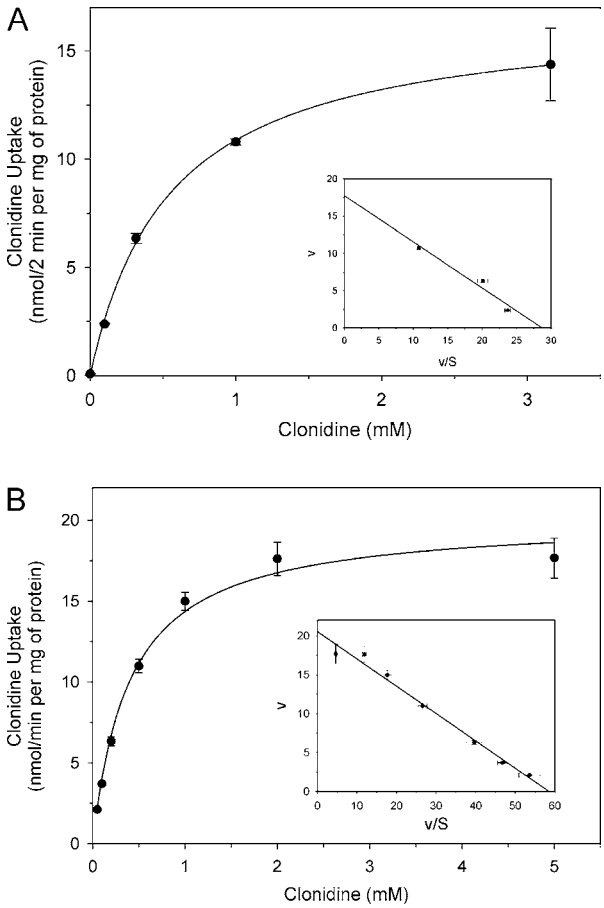

Fig. 4. Kinetics of carrier-mediated clonidine uptake in Caco-2 cells at pH 7.5 and 8.5. Nonmediated simple diffusion was determined by measuring the uptake of [^{3}H]clonidine in the presence of 20 mM unlabeled clonidine and subtracted from total uptake. (A) Uptake of [^{3}H]clonidine (3 nM) was measured at pH 7.5 for 2 min. Uptake buffer contained [^{3}H]clonidine and unlabeled clonidine (0–3 mM). Inset: Eadie–Hofstee transformation of the data. *v*, uptake rate in nmol/2 min per mg protein; *S*, clonidine concentration in mM, $n = 4$. (B) Uptake of [^{3}H]clonidine (3 nM) was measured at pH 8.5 for 1 min. Uptake buffer contained [^{3}H]clonidine and unlabeled clonidine (0–5 mM). Inset: Eadie–Hofstee transformation of the data. *v*, uptake rate in nmol/1 min per mg protein; *S*, clonidine concentration in mM, $n \geq 4$.

Table I. Inhibition of [^{3}H]Clonidine Uptake in Caco-2 Cells by Different Clonidine-Related and Unrelated Compounds (2 mM) at pH 7.5

Compound	[^{3}H]Clonidine uptake (%)
Control	100 ± 1
Clonidine	41 ± 3
Guanabenz	20 ± 1
Guanfacine	74 ± 3
Moxonidine	95 ± 1
Minoxidile	96 ± 5
Ephedrine	57 ± 2
Etilefrine	103 ± 2
Methamphetamine	32 ± 1
Xylometazoline	60 ± 2
Xylazine	38 ± 1
Naphazoline	76 ± 3
Tolazoline	99 ± 3
Dihydralazine	103 ± 3
Imipramine	17 ± 1
Amitriptyline	23 ± 2
Desipramine	18 ± 1
Clomipramine	33 ± 2
Nortriptyline	15 ± 1
Doxepine	20 ± 1
Maprotiline	16 ± 1
Sulpiride	85 ± 4
Chlorpromazine	30 ± 4
Pimozide	98 ± 4
Carbamazepine	111 ± 4
Procainamide	92 ± 3
Quinidine	34 ± 6
Quinine	35 ± 3
Antazoline	46 ± 1
Diphenhydramine	34 ± 1
Chlorphenamine	32 ± 1
Ranitidine	89 ± 1
Cimetidine	101 ± 1
Morphine	95 ± 5
(*S*)-Reticuline	46 ± 2
(*R*, *S*)-Reticuline	47 ± 2
(*R*)-Reticuline	48 ± 1
Ethylmorphine	44 ± 2
Codeine	57 ± 2
Diacetylmorphine	63 ± 1
Cocaine	39 ± 2
Atropine	78 ± 1
N-Butylscopolamine	118 ± 2
Guanidine	91 ± 1
Theophylline	94 ± 4
Isobutyl-methyl-xanthine	96 ± 3
Verapamil	30 ± 1
Nicotine	59 ± 2
Nicotinamide	99 ± 2
L-Phenylalanine	100 ± 2
Phenylethylamine	39 ± 1
L-Tyrosine	95 ± 1
Tyramine	96 ± 1
Dopamine	98 ± 3
Noradrenaline	103 ± 2
L-Tryptophan	103 ± 1
Tryptamine	68 ± 3
Serotonin	103 ± 2
L-Histidine	97 ± 4
Histamine	108 ± 6
L-Arginine	104 ± 3
Agmatine	100 ± 1
Metformine	103 ± 2
Thiamine	106 ± 1
Carnitine	92 ± 2
Choline	96 ± 5
N-Methyl-4-phenylpyridinium (MPP^+)	91 ± 1
Tetraethylammonium (TEA)	118 ± 4

Uptake of [^{3} H]clonidine (3 nM) at pH 7.5 was measured for 2 min in the absence or presence of 2 mM unlabeled compounds. Data are means ± SE, $n = 4$.

thiamine did not inhibit [^{3}H]clonidine uptake. Most importantly, [^{3}H]clonidine uptake was not affected by the prototype OCT substrates choline, MPP^+, and TEA.

Clonidine uptake was also not affected by agmatine and serotonin. Intestinal transport that is inhibited by clonidine has been reported for both drugs, (19,20). Because clonidine affects many transport systems for organic solutes or inorganic ions directly or indirectly, inhibition of such transporters by clonidine does not at all allow the conclusion of substrate inhibition. As shown here, the inability of agmatine and serotonin to inhibit clonidine uptake rules out a common transporter. We also measured the inhibition of [^{3}H]clonidine uptake by selected drugs at fixed concentrations of 1 or 10 mM at pH 8.5 (data not shown). The substrate specificity profile is virtually identical at both pH values. For selected compounds, we then performed detailed competition experiments using a broad concentration range (0–31.6 mM) to calculate apparent affinity constants. The inhibitory constants (K_i) ± SE obtained at both pH values shown in Table II were determined from data shown in Fig. 5 for pH 7.5 by nonlinear regression. Clonidine, guanabenz, methamphetamine, imipramine, clomipramine, nortriptyline, quinine, xylazine, ephedrine, desipramine, amitriptyline, quinidine, chlorphenamine, and diphenhydramine inhibited [^{3}H]clonidine uptake at pH 7.5 with K_i values between 0.15 and 1 mM. The highest apparent affinity was measured for nortriptyline, imipramine, desipramine, amitriptyline, and guanabenz. The K_i values of ligands determined at pH 8.5 were similar (Table II).

We then surveyed the structures and the affinity constants to generalize structural substrate requirements for the interaction of compounds with the clonidine transporting system. Surprisingly, many very lipophilic cationic drugs, such as quinine, quinidine, imipramine, amitriptyline, and chlorpromazine, display a high affinity to the clonidine transporting system. The system seems to prefer lipophilic cyclic structures with aromatic ring systems (exception: imipramine). Free ring standing hydroxyl groups dramatically reduce affinity, as shown by comparison between morphine and its derivatives and between tryptamine and serotonin. Basic side chains with tertiary amines are favorable, and the position can not be specified. Acidic groups in side chains decrease affinity (see nicotine and nicotinamide). Of interest are the following cutoffs: for all compounds that inhibit [^{3}H]clonidine uptake by more than 75%, the log P values are greater than 1.4. Compounds that inhibit uptake by more than 80% have log P values greater than 3.9. The pK_a values

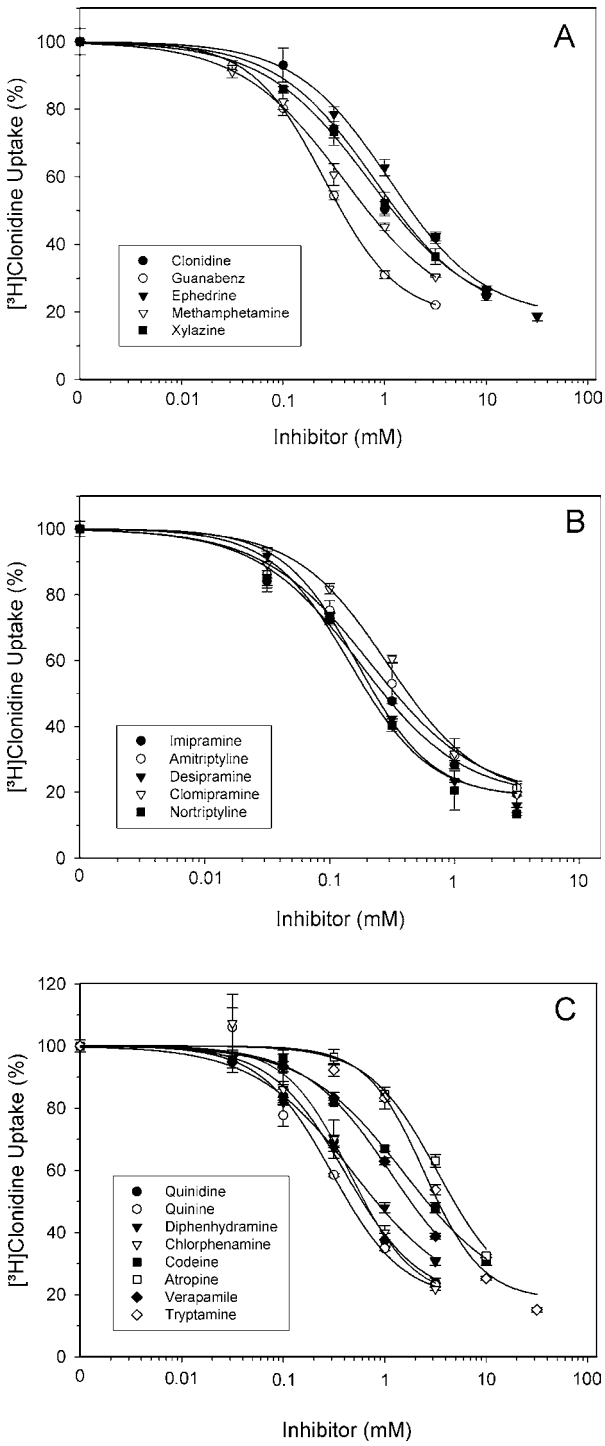

Fig. 5. Substrate specificity of [^{3}H]clonidine uptake in Caco-2 cells. Uptake of [^{3}H]clonidine (3 nM, 2 min, pH 7.5) was measured in the absence or presence of increasing concentrations of unlabeled compounds (A, B, C; 0–31.6 mM). Uptake of [^{3}H]clonidine measured in the absence of inhibitors (138 ± 4 fmol/2 min per mg protein) was taken as 100%. Data are means ± SE, $n = 4$.

of all compounds that inhibit [^{3}H]clonidine uptake by more than 75% are between 8.7 and 10.4.

Several transport systems for organic cations have been described at the intestinal epithelium, but those accept MPP^+ and TEA as substrates (7,8). Carriers for agmatine can also be ruled out (Table I). Very recently, Inazu *et al.* characterized the Na^+-independent choline transport system CLT1 on a molecular level (9,10,15). This carrier, originally cloned by O'Regan and coworkers (21), transports choline at astrocytes with an apparent Michaelis–Menten constant (K_m) of 36 μM. As with clonidine uptake in our study, choline uptake was significantly decreased by acidification of the extracellular medium. Among many other organic cations, TEA, MPP^+, and clonidine also inhibit choline uptake by CLT1 (15), but—as stated above—CLT1 can not be the system for clonidine in this study because it does not interact with choline (Fig. 1, inset).

The transport system described here is very likely identical or very similar to the system responsible for clonidine transport at the blood–brain barrier, JEG-3 placenta cells, or keratinocytes (4–6). Mizuuchi and coworkers (22,23) described the transport of diphenhydramine and chlorpheniramine at Caco-2 cells, and procainamide at rabbit brush-border membrane vesicles (24). Apical uptake was attributed to a H^+ antiport system for tertiary amines. Importantly, many functional parameters are very similar to the mechanism of clonidine transport described here. In addition, Han and coworkers (25) reported a novel membrane potential-independent verapamil transporter in human retinal pigment epithelial cells that is with regard to transport parameters and substrate specificity very similar to the H^+/tertiary amine transporter and the clonidine transporter described here. All three are Na^+-independent and stimulated by an outside-directed H^+ gradient. Verapamil uptake is not inhibited by cimetidine (25). In our study, clonidine uptake is strongly inhibited by verapamil, but not by cimetidine (Table I). With regard to the substrate specificity, diphenhydramine seems to be a common substrate of the system(s) in all three studies. We therefore determined the

Table II. Inhibition of [^{3}H]Clonidine Uptake in Caco-2 Cells by Clonidine-Related and Unrelated Compounds at pH 7.5 or 8.5

Compound	K_i (mM)	
	pH 7.5	pH 8.5
Clonidine	0.79 ± 0.10	0.36 ± 0.05
Guanabenz	0.26 ± 0.01	0.18 ± 0.01
Ephedrine	1.1 ± 0.1	–
Methamphetamine	0.39 ± 0.02	–
Xylazine	0.69 ± 0.02	–
Imipramine	0.17 ± 0.02	–
Amitriptyline	0.21 ± 0.02	–
Desipramine	0.17 ± 0.01	–
Clomipramine	0.29 ± 0.03	–
Nortriptyline	0.15 ± 0.02	–
Quinine	0.30 ± 0.05	0.75 ± 0.06
Quinidine	0.41 ± 0.03	–
Atropine	3.4 ± 0.4	6.9 ± 0.1
Diphenhydramine	0.38 ± 0.01	0.28 ± 0.01
Chlorphenamine	0.45 ± 0.07	–
Verapamil	1.1 ± 0.1	–
Codeine	1.6 ± 0.1	–
Tryptamine	2.5 ± 0.2	1.0 ± 0.1

Uptake of [^{3}H]clonidine (3 nM) at pH 7.5 or 8.5 was measured in the presence of increasing concentrations (0–31.6 mM) of unlabeled compounds as shown in Fig. 5. K_i values were calculated from competition curves by nonlinear regression. Data are means ± SE, $n = 4$, –: not determined.

effect of diphenhydramine on the kinetic clonidine transport parameters. The effect of 0.5 mM diphenhydramine (slightly above IC_{50}) was strictly competitive, only K_t was affected (control: $K_t = 0.8 \pm 0.1$ mM, diphenhydramine: 1.5 ± 0.5 mM, V_{max} unchanged). Moreover, the p value (Hill slope) of the competition curve shown in Fig. 5C is 0.9 ± 0.1, close to 1 as to be expected for a competitive type of inhibition. It is therefore very possible that these systems are identical and that this transporter is the carrier that predominantly contributes to the 90% bioavailability of clonidine after oral administration (26). It should be noted, however, that at cells where the extracellular pH is lower than the inside pH the maximum possible rate of the transport process is probably not achieved under physiological conditions. According to Daniel and coworkers (27), the rat intestinal luminal microclimate pH is about 6.7 in the upper parts of the villi and higher at their base. In the rat ileum the microclimate pH is about 7.3 (27). According to Fig. 1, uptake would be comparably low at a jejunal pH of 6.7. On the other hand, we do not yet know the distribution of the clonidine transport system along the human intestine.

In conclusion, clonidine is transported in Caco-2 cells by a carrier-mediated process. Substrate specificity and mechanism are very similar to the clonidine transport described at blood–brain barrier endothelial cells. The transport characteristics do not correspond to carriers for organic cations of the SLC22 family or the choline transporters CHT1 and CLT1. The system might be identical to the H^+/tertiary amine antiporter. It interacts with a large number of both hydrophilic and lipophilic cationic drugs, interestingly also with opiates.

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REFERENCES

1. G. A. Mansoor and W. H. Frishman. Comprehensive management of hypertensive emergencies and urgencies. *Heart Dis.* **4**:358–371 (2002).
2. P. M. Sanderson and R. Eltringham. The role of clonidine in anaesthesia. *Hosp. Med.* **59**:221–223 (1998).
3. J. N. Cogburn, M. G. Donovan, and C. S. Schasteen. A model of human small intestinal absorptive cells. 1. Transport barrier. *Pharm. Res.* **8**:210–216 (1991).
4. J. Huwyler, G. Fricker, M. Torok, M. Schneider, and J. Drewe. Transport of clonidine across cultured brain microvessel endothelial cells. *J. Pharmacol. Exp. Ther.* **282**:81–85 (1997).
5. F. Grafe, W. Wohlrab, R. Neubert, and M. Brandsch. Carrier-mediated transport of clonidine in human keratinocytes. *Eur. J. Pharm. Sci.* **21**:309–312 (2004).
6. J. Müller, R. Neubert, and M. Brandsch. Transport of clonidine at cultured epithelial cells (JEG-3) of the human placenta. *Pharm. Res.* **21**:692–694 (2004).
7. H. Koepsell and H. Endou. The SLC22 drug transporter family. *Pflugers Arch.* **447**:666–676 (2004).
8. H. Koepsell. Polyspecific organic cation transporters: their functions and interactions with drugs. *Trends Pharmacol. Sci.* **25**:375–381 (2004).
9. A. Friedrich, R. L. George, C. C. Bridges, P. D. Prasad, and V. Ganapathy. Transport of choline and its relationship to the expression of the organic cation transporters in a rat brain microvessel endothelial cell line (RBE4). *Biochim. Biophys. Acta* **1512**:299–307 (2001).
10. K. Hoffmann, F. Grafe, W. Wohlrab, R. H. Neubert, and M. Brandsch. Functional characterization of a high-affinity choline transport system in human keratinocytes. *J. Invest. Dermatol.* **119**:118–121 (2002).
11. B. Bretschneider, M. Brandsch, and R. Neubert. Intestinal transport of β-lactam antibiotics: analysis of the affinity at the H^+/peptide symporter (PEPT1), the uptake into Caco-2 cell monolayers and the transepithelial flux. *Pharm. Res.* **16**:55–61 (1999).
12. L. Metzner, J. Kalbitz, and M. Brandsch. Intestinal transport of pharmacologically relevant proline derivatives by the human proton-coupled amino acid transporter hPAT1. *J. Pharmacol. Exp. Ther.* **309**:28–35 (2004).
13. T. N. Faria, J. K. Timoszyk, T. R. Stouch, B. S. Vig, C. P. Landowski, G. L. Amidon, C. D. Weaver, D. A. Wall, and R. L. Smith. A novel high-throughput PepT1 transporter assay differentiates between substrates and antagonists. *Mol. Pharm.* **1**:67–76 (2004).
14. L. Metzner, G. Kottra, K. Neubert, H. Daniel, and M. Brandsch. L-Tryptophan, and tryptamine are effective inhibitors of the amino acid transport system PAT1. *FASEB J.* **19**:1468–1473 (2005).
15. M. Inazu, H. Takeda, and T. Matsumiya. Molecular and functional characterization of an Na^+-independent choline transporter in rat astrocytes. *J. Neurochem.* **94**:1427–1437 (2005).
16. D. T. Bravo, N. G. Kolmakova, and S. M. Parsons. Mutational and pH analysis of ionic residues in transmembrane domains of vesicular acetylcholine transporter. *Biochemistry* **44**:7955–7966 (2005).
17. W. M. Barendt and S. H. Wright. The human organic cation transporter (hOCT2) recognizes the degree of substrate ionization. *J. Biol. Chem.* **277**:22491–22496 (2002).
18. M. E. Ganapathy, F. H. Leibach, V. B. Mahesh, L. D. Devoe, and V. Ganapathy. Interaction of clonidine with human placental Na^+–H^+ exchanger. *Biochem. Pharmacol.* **35**:3989–3994 (1986).
19. G. J. Molderings, M. Bruss, H. Bonisch, and M. Gothert. Identification and pharmacological characterization of a specific agmatine transport system in human tumor cell lines. *Ann. N. Y. Acad. Sci.* **1009**:75–81 (2003).
20. E. Keating, C. Lemos, R. Monteiro, I. Azevedo, and F. Martel. The effect of a series of organic cations upon the plasmalemmal serotonin transporter, SERT. *Life Sci.* **76**:103–119 (2004).
21. S. O'Regan, E. Traiffort, M. Ruat, N. Cha, D. Compaore, and F. M. Meunier. An electric lobe suppressor for a yeast choline transport mutation belongs to a new family of transporter-like proteins. *Proc. Natl. Acad. Sci. USA.* **97**:1835–1840 (2000).
22. H. Mizuuchi, T. Katsura, H. Saitoh, Y. Hashimoto, and K.-I. Inui. Transport characteristics of diphenhydramine in human intestinal epithelial Caco-2 cells: contribution of pH-dependent transport system. *J. Pharmacol. Exp. Ther.* **290**:388–392 (1999).
23. H. Mizuuchi, T. Katsura, K. Ashida, Y. Hashimoto, and K.-I. Inui. Diphenhydramine transport by pH-dependent tertiary amine transport system in Caco-2 cells. *Am. J. Physiol. Gasterointest. Liver Physiol.* **278**:G563–G569 (2000).
24. T. Katsura, H. Mizuuchi, Y. Hashimoto, and K.-I. Inui. Transport of procainamide via H^+/tertiary amine antiport system in rabbit intestinal brush-border membrane. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **279**:G799–G805 (2000).
25. Y. H. Han, D. H. Sweet, D. N. Hu, and J. B. Pritchard. Characterization of a novel cationic drug transporter in human retinal pigment epithelial cells. *J. Pharmacol. Exp. Ther.* **296**:450–457 (2001).
26. M. Frisk-Holmberg, L. Paalzow, and P. O. Edlund. Clonidine kinetics in man—evidence for dose dependency and changed pharmacokinetics during chronic therapy. *Br. J. Clin. Pharmacol.* **12**:653–658 (1981).
27. H. Daniel, C. Fett, and A. Kratz. Demonstration and modification of intervillous pH profiles in rat small intestine *in vitro*. *Am. J. Physiol.* **257**:G489–G495 (1989).