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Research paper

Transport of phenylethylamine at intestinal epithelial (Caco-2) cells: Mechanism and substrate specificity

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ABSTRACT

This study was performed to characterize the intestinal transport of β-phenylethylamine (PEA). Uptake of [^{14}C]PEA into Caco-2 cells was Na^+-independent but strongly stimulated by an outside directed H^+ gradient. At extracellular pH 7.5, the concentration-dependent uptake of PEA was saturable with kinetic parameters of 2.6 mM (K_t) and 96.2 nmol/min per mg of protein (V_{max}). Several biogenic amines such as harmaline and *N*-methylphenylethylamine as well as cationic drugs such as phenelzine, tranylcypromine, D,L-amphetamine, methadone, chlorphenamine, diphenhydramine and promethazine strongly inhibited the [^{14}C]PEA uptake with K_i values around 1 mM. Tetraethylammonium, *N*-methyl-4-phenylpyridinium and choline had no effect. We also studied the bidirectional transepithelial transport of [^{14}C]PEA at cell monolayers cultured on permeable filters. Net transepithelial flux of [^{14}C]PEA from apical-to-basolateral side exceeded basolateral-to-apical flux 5-fold. We conclude that PEA is transported into Caco-2 cells by a highly active, saturable, H^+-dependent (antiport) process. The transport characteristics do not correspond to those of the known carriers for organic cations of the SLC22, SLC44, SLC47 and other families.

1. Introduction

β-Phenylethylamine (PEA) belongs to the group of so-called trace amines, a family of endogenous amines that is related to the classical monoamine neurotransmitters noradrenaline, dopamine and serotonin [1]. Physiologically, PEA is formed by decarboxylation of L-phenylalanine. It is metabolized by monoamine oxidase B (MAO-B), PEA being the prototype selective substrate for this enzyme [2]. Pharmacologically, PEA displays indirect sympathomimetic effects by modulating the activity of several monoamine neurotransmitters [1,3,4]. In the mammalian central nervous system, PEA shows a heterogeneous distribution with extracellular concentrations of 2–15 nM [1]. It exhibits a high turnover rate, a phenomenon that is believed to be related to the lack of vesicular storage mechanisms and to the high affinity to MAO-B [3,4]. In the brain, this so-called "endogenous amphetamine" [5] operates as neuromodulator, modifying the neuronal excitability of neurotransmitters at submicromolar levels [4,6]. Direct PEA effects occur by binding to G-protein-coupled trace amine-associated receptors and subsequent activation of cAMP biosynthesis [3,4,6–8].

PEA is involved in psychiatric disorders like depression, bipolar affective disorder or schizophrenia, in neurological disorders like attention-deficit hyperactivity disorder or Parkinson's disease, in eating disorders and in epilepsy [9]. There is evidence that these pathological effects are accompanied by alterations in PEA levels [10] either by disregulation in biosynthesis, catabolism or excretion or by uptake of exogenous PEA.

To the best of our knowledge, studies on the intestinal PEA uptake of exogenous PEA have yet not been performed. Membrane transporters for PEA have not been identified so far even though there is some evidence in the literature: ATP-stimulated [^{14}C]PEA uptake by isolated membranes of bovine adrenal chromaffin granules was shown in 1975 [11]. Powers and coworkers demonstrated cocaine-insensitive intracellular accumulation of [^{14}C]PEA in bovine adrenal chromaffin vesicles [12]. Uptake of PEA by a diffusional component and a saturable component with a K_m of 25 μM was shown in rat isolated lung [13]. Both lipid solubility-mediated simple diffusion and carrier-mediated facilitated transport utilizing a Na^+/K^+ gradient were suggested to be involved in PEA uptake in rabbit erythrocytes [10]. In pigeon red cells, PEA inhibited the Na^+-dependent and less potent also the Na^+-independent glycine entry as well as the Na^+ entry in a non-competitive and reversible manner [14]. Furthermore, the pH-dependent uptake of clonidine [15] and 3,4-methylendioxymethamphetamine (MDMA) [16] at Caco-2 cells was reduced by PEA suggesting competition during an uptake step.

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The intestinal uptake of PEA is of particular interest because many studies discuss psychostimulating and antidepressant effects of the "amphetamine" PEA present in chocolate or other processed food. Moreover, PEA is generated in the intestinal lumen in significant amounts by bacteria [17,18]. Because PEA exists in aqueous solution predominantly as cation (pK_a 9.73 [10]), it could be hypothesized that it is a substrate for intestinal organic cation transporters (OCT [19,20]). The current investigation at Caco-2 cells, a cell line used as a model for absorption in the small intestine, was performed to study the intestinal PEA uptake and transepithelial flux in order to elucidate the PEA transport mechanism and to obtain insight into the transports' pharmaceutical relevance.

2. Materials and methods

2.1. Materials

The Caco-2 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell culture reagents were purchased from PAA Laboratories GmbH (Cölbe, Germany) and Biochrom AG (Berlin, Germany). β-[Ethyl-1-^{14}C]phenylethylamine hydrochloride ([^{14}C]PEA, specific activity 55 μCi/mmol) was obtained from Biotrend Chemicals GmbH (Cologne, Germany) and [^{3}H]clonidine hydrochloride (specific activity 69 mCi/mmol) from NEN Biochemicals (Boston, USA). NaCl, MES, Hepes and Tris were from Carl Roth GmbH (Karlsruhe, Germany). All other chemicals were purchased from Sigma–Aldrich Chemical GmbH (Taufkirchen, Germany).

2.2. Cell culture

Caco-2 cells were routinely cultured in 75-cm^2 culture flasks (Greiner, Frickenhausen, Germany) with minimum essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acid solution and gentamicin (45 μg/ml) [15]. Cells grown to 80% confluence were released by trypsinization and subcultured in 35-mm disposable Petri dishes (Sarstedt AG & Co., Nümbrecht, Germany). Medium was replaced the day after seeding, then every 2 days and on 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 and were fully differentiated after 4–5 days. This technique was used to characterize apical uptake of PEA, to obtain the kinetic parameters of the transport process and to study the effect of competing compounds on PEA uptake. To study transepithelial net flux of PEA across monolayers, Caco-2 cells were cultured for 21–23 days on permeable polycarbonate Transwell® culture inserts (diameter 24 mm, pore size 3 μm, Corning Life Sciences, Schiphol-Rijk, The Netherlands) with a starting cell density of 0.2×10^6 cells/filter as described [21]. At this stage, the Caco-2 monolayers used in this study displayed a transepithelial electrical resistance of 942 ± 15 Ω cm^2. Medium was replaced every 2 days and the day before flux experiment.

2.3. Transport measurements

Caco-2 cell monolayers were rinsed one time 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). For Na^+-free buffer, NaCl was substituted with 140 mM choline chloride or LiCl, respectively. For Cl^--free buffer, NaCl, $CaCl_2$ and KCl were replaced by the respective gluconate salts, for buffer free of Na^+ and Cl^- 280 mM mannitol was used instead of NaCl and $CaCl_2$ and KCl were substituted by the gluconate salts. For K^+-, Ca^{2+}- or Mg^{2+}-free incubation medium, KCl, $CaCl_2$ or $MgSO_4$ were replaced by equimolar NaCl. K^+-rich buffer contained 5.4 mM NaCl and 140 mM KCl.

To initiate uptake, 0.6 ml incubation buffer containing [^{14}C]PEA (1.1 μM) or 1 ml buffer containing [^{3}H]clonidine (3 nM) and unlabeled compounds at increasing concentrations (0–50 mM) were added to each dish at room temperature for the desired time (0–10 min). 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 of Bradford.

NH_4Cl prepulse experiments for lowering the intracellular pH (pH_i) were routinely performed as follows: Caco-2 cell monolayers were pretreated for 15 min at 37 °C with either Na^+-free control buffer (140 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 25 mM Hepes/Tris, pH 7.5) or with NH_4Cl-containing Na^+-free buffer (25 mM NH_4Cl, 115 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 25 mM Hepes/Tris, pH 7.5). After treatment, the monolayers were washed with NH_4Cl-free buffer, and uptake of [^{14}C]PEA (1.1 μM) was measured at pH 7.5 for 10 min.

Transepithelial flux of [^{14}C]PEA in both directions across Caco-2 cell monolayers cultured on permeable filters was investigated at 37 °C and pH 7.5 at the apical (1.5 ml) and at the basolateral compartment (2.6 ml) [15,21]. After measuring transepithelial electrical resistance, monolayers were rinsed two times with buffer. Uptake was started by adding incubation medium containing [^{14}C]PEA (1.1 μM) to the donor compartment. At given times, samples were taken from the receiver compartment and replaced with fresh buffer. After finishing the experiment after 2 h, samples were taken also from the donor compartment.

2.4. Data analysis

Each data point was determined at least in triplicate for each experiment. Data are presented as mean ± SE. Statistical analyses were done with the *U*-test by Mann and Whitney. The kinetic constants were calculated by non-linear regression of the Michaelis–Menten plot and confirmed by linear regression of the Eadie–Hofstee plot. The calculated parameters are shown with their SE. IC_{50} values (i.e. the concentration of the unlabeled compound necessary to inhibit 50% of specific [^{14}C]PEA or [^{3}H]clonidine uptake) were determined by non-linear regression using the logistical equation for an asymmetric sigmoid (allosteric Hill kinetics) [22]. Inhibition constants (K_i) were calculated from the IC_{50} values using the K_t value of 2.6 mM for PEA uptake obtained in this study and 0.5 mM for clonidine uptake, respectively [15]. Flux data were calculated after correction for the amount taken out by linear regression of appearance in the receiver well vs. time [23].

3. Results

3.1. Dependence of [^{14}C]PEA accumulation in Caco-2 cells on ion gradients

We first investigated the influence of ion gradients on [^{14}C]PEA uptake (1.1 μM, 10 min) into Caco-2 cells. Fig. 1A illustrates the strong effect of outside pH (pH_o) on [^{14}C]PEA uptake. Decreasing the extracellular H^+ concentration from pH 6.0 to pH 7.5 caused a 5-fold increase in [^{14}C]PEA uptake to 161 pmol per 10 min per mg of protein. Increasing the pH from 7.5 to 8.5 resulted in a further 1.7-fold increase in [^{14}C]PEA uptake. Fig. 1A also shows that the uptake of [^{14}C]PEA is highly saturable: adding 10 mM of unlabeled PEA decreased tracer uptake by 79% (pH 7.5) and 83% (pH 8.5), respectively. To determine whether a low extracellular H^+ concentration *per se* or an outside directed H^+ gradient stimulates

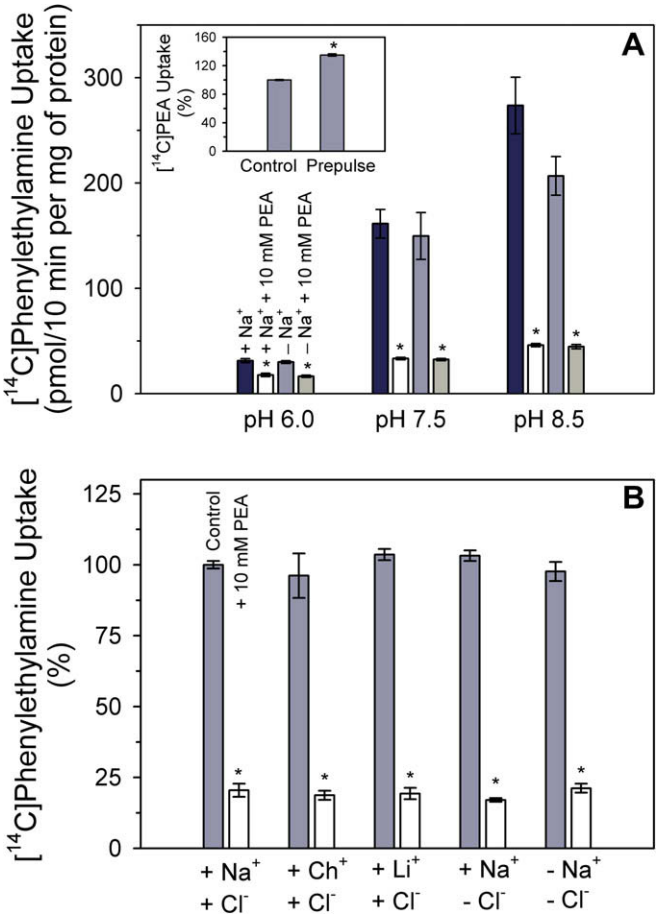


Fig. 1. Dependence of [^{14}C]PEA uptake in Caco-2 cells on ion gradients. (A) Uptake of [^{14}C]PEA (1.1 μM) was measured for 10 min at room temperature in the absence or presence of Na^+ and 10 mM unlabeled PEA at different pH. Sodium chloride was iso-osmotically replaced by choline chloride. Inset: uptake of [^{14}C]PEA (1.1 μM, pH 7.5) in Caco-2 monolayers was measured for 10 min in Na^+-free buffer after cell cultures were pretreated for 15 min with either the Na^+-free control buffer (without NH_4Cl) or Na^+-free prepulse buffer (25 mM NH_4Cl) at pH 7.5 at 37 °C. Values represent means ± SE ($n \geqslant 3$). (B) Uptake of [^{14}C]PEA (1.1 μM) was measured for 10 min at pH 7.5 at room temperature. Sodium chloride in the uptake buffer was replaced equimolar with choline chloride (Ch^+), with lithium chloride (Li^+) or with 280 mM mannitol (free of Na^+ and Cl^-). Potassium chloride and calcium chloride were substituted by the respective gluconate salts. Values represent means ± SE, $n = 4$. *Significantly different from control with $p \leqslant 0.05$.

the uptake of [^{14}C]PEA uptake, the NH_4Cl prepulse technique was applied. Lowering the pH_i in Caco-2 cells while keeping pH_o at 7.5 increased the [^{14}C]PEA uptake by 26% (Fig. 1A, inset). This result suggests that the outside directed proton gradient stimulates [^{14}C]PEA uptake. We then studied the effect of the protonophore *p*-trifluoromethoxyphenylhydrazone (FCCP), a weak organic acid that only transports protons across membranes in its neutral acidic form and crosses back in its anionic form [24]. Adding FCCP (50 μM) reduced the uptake of [^{14}C]PEA (1.1 μM, 10 min, pH 8.5), again confirming that the outside directed H^+ gradient is the driving force for PEA transport in Caco-2 cells. Pretreatment of the cell monolayers with the inhibitor of ATP synthesis 2,4-dinitrophenole (DNP; 1 mM, pH 7.5) had no effect on [^{14}C]PEA uptake (1.1 μM, 1 min) (data not shown). [^{14}C]PEA uptake does not depend on extracellular Na^+ (Fig. 1). Uptake was also not affected by removal of Cl^- (Fig. 1B). Uptake was independent of extracellular K^+, Ca^{2+}, Mg^{2+} ions or glucose and was not affected by increasing extracellular K^+ to 140 mM (data not shown). With regard to uptake time dependence, we observed that [^{14}C]PEA uptake (1.1 μM, pH 7.5) saturated rapidly and reached a plateau at 172 pmol/mg of protein after 5 min (Fig. 2). Taking the y_0 value representing unspecific binding of [^{14}C]PEA into account, the [^{14}C]PEA uptake was linear for up to 1 min. With regard to culture-time dependence, uptake of [^{14}C]PEA (1.1 μM, pH 7.5, 10 min) into Caco-2 cells was constant after 7 days (Fig. 2, inset). Hence, a culture period of 7 days and an uptake time of 1 min were chosen for further experiments.

3.2. Substrate saturation kinetics of PEA uptake in Caco-2 cells

To determine the kinetic parameters of PEA uptake, Caco-2 monolayers were incubated for 1 min at pH 7.5 with buffer containing [^{14}C]PEA (1.1 μM) and increasing concentrations of unlabeled PEA (0–50 mM). Increasing the concentration of unlabeled PEA strongly inhibited [^{14}C]PEA uptake (Fig. 3, main figure). The linear, non-saturable transport component representing simple diffusion and unspecific binding of the tracer was determined by

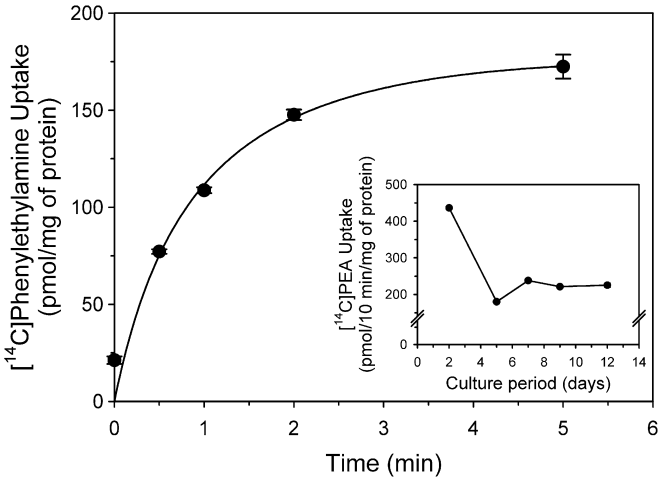


Fig. 2. Time dependence of [^{14}C]PEA uptake in Caco-2 cells. Uptake of [^{14}C]PEA (1.1 μM) was measured at pH 7.5 on day 7 after seeding. Inset: culture-time dependence of [^{14}C]PEA uptake (1.1 μM, 10 min, pH 7.5). Values represent means ± SE, $n = 4$.

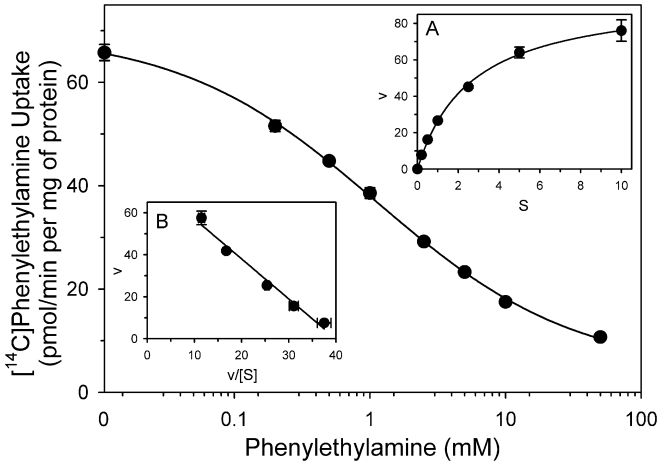


Fig. 3. Substrate concentration kinetics of PEA uptake in Caco-2 cells. Uptake of [^{14}C]PEA (1.1 μM) was measured at pH 7.5 for 1 min at increasing concentrations of PEA (main figure). For insets, non-saturable tracer transport and binding were determined by measuring the uptake of [^{14}C]PEA in the presence of an excess amount of unlabeled compound (50 mM) and subtracted from total uptake rates. Inset A: transformation of data into Michaelis–Menten plot. Inset B: Eadie–Hofstee transformation of the data. *v*, uptake rate in nmol/min per mg of protein; *S*, concentration of PEA in mM, $n = 4$.

measuring [^{14}C]PEA uptake in the presence of 50 mM unlabeled PEA. This component (14.5% of [^{14}C]PEA uptake at 1.1 μM), which, interestingly, corresponds very well with PEA uptake at pH_o 6.0 (Fig. 1), was subtracted from total [^{14}C]PEA uptake. Fig. 3 also shows the Michaelis–Menten plot (inset A) and the Eadie–Hofstee plot (inset B) of the data. The relationship between PEA uptake rate and PEA concentration was found to be hyperbolic. The maximal velocity of transport (V_{max}) was 96.2 ± 2.1 nmol/min per mg of protein. The apparent Michaelis constant (K_t) was 2.6 ± 0.2 mM.

3.3. Specificity of [^{14}C]PEA accumulation in Caco-2 cells

In the next series of experiments, we investigated the substrate specificity of the PEA uptake process. Uptake of [^{14}C]PEA (1.1 μM, 1 min, pH 7.5) into Caco-2 cells was measured in the presence of 2 mM of various endogenous compounds and structurally related drugs. Not only PEA itself but also its metabolite, the trace amine *N*-methylphenylethylamine (MPEA), the MAO-B inhibitor tranylcypromine, D,L-amphetamine, guanabenz, methadone and promethazine as well as the cyclic antidepressants imipramine, doxepine and maprotiline strongly reduced [^{14}C]PEA uptake by >55% (Table 1). Less potent but still very effective inhibitors were the MAO-B inhibitors phenelzine, selegiline and harmaline, the imidazolines clonidine and idazoxan, the opiate fentanyl, rivastigmine, quinine, quinidine as well as the antihistaminics chlorphenamine and diphenhydramine. Tryptamine, cocaine, naloxone and verapamil were found to be weak but significant inhibitors (by <20%). Interestingly, for none of the amino acids, the biogenic amines – even the trace amines tyramine and *p*-octopamine – or the neurotransmitters such as serotonin and noradrenaline, a significant effect on [^{14}C]PEA uptake was found. Similarly, prototype substrates of known membrane transporters for organic cations such as *N*-methyl-4-phenylpyridinium (MPP^+), tetraethylammonium (TEA), carnitine and choline did not affect the uptake of [^{14}C]PEA (Table 1). This result clearly shows that organic cation transporters such as CHT1, CTL1 or MATE or members of the OCT and OCTN family are not involved in intestinal PEA uptake.

For the most effective inhibitors, we then performed detailed dose–response experiments using a broad concentration range (0–31.6 mM) to calculate the apparent affinity constants for [^{14}C]PEA uptake inhibition. The inhibitory constants (K_i) ± SE shown in Table 2 were determined by non-linear regression from data shown in Fig. 4. These inhibitory constants were in the range from 0.66 mM (tranylcypromine) to 5.5 mM (ethylmorphine). Interestingly, clonidine was found to be a very potent inhibitor of [^{14}C]PEA uptake. PEA, on the other hand, inhibits clonidine uptake at Caco-2 cells [15]. To compare the inhibitor specificity spectra, we also determined the K_i values of potent [^{14}C]PEA uptake inhibitors for the inhibition of [^{3}H]clonidine uptake (3 nM, 2 min, pH 7.5). The K_i values of the drugs tested for [^{3}H]clonidine uptake inhibition were in the range of 0.42 mM (harmaline) to 2.2 mM (ethylmorphine) and are also shown in Table 2.

To analyze whether the degree of [^{14}C]PEA uptake inhibition depends on the ionization grade of the inhibitors, the K_i values of the inhibitors were plotted vs. their amount of positively charged molecules at pH 7.5 (Table 2). No correlation between the extend of ionization and the inhibitory constants was found (linear correlation coefficient $r = 0.0699$, $r^2 = 0.0049$, $p = 0.78$).

3.4. Bidirectional transepithelial flux of [^{14}C]PEA across Caco-2 cell monolayers

We then studied the bidirectional transepithelial flux of [^{14}C]PEA across Caco-2 cell monolayers cultured on permeable filters for 21–23 days. The net transepithelial flux of [^{14}C]PEA (1.1 μM) was measured in apical-to-basolateral (J_{a-b}) and in basolateral-to-apical direction (J_{b-a}) at pH 7.5 at 37 °C. Apical-to-basolateral flux was linear for 30 min and reached a plateau after 1 h when equilibrium between donor and acceptor occurred (Fig. 5).

Table 1
Substrate specificity of [^{14}C]phenylethylamine uptake in Caco-2 cells.

Compound	[^{14}C]PEA uptake (%)
Control	100 ± 1
β-Phenylethylamine (PEA)	41.4 ± 0.4[a]
Phenelzine	56.8 ± 3.2[a]
Tranylcypromine	44.7 ± 1.5[a]
Selegiline	55.4 ± 3.8[a]
Harmaline	50.4 ± 5.9[a]
Harmine	82.6 ± 0.9[a]
p-Octopamine	93.1 ± 2.0
N-methylphenylethylamine (MPEA)	42.2 ± 2.7[a]
D,L-Amphetamine	40.0 ± 2.7[a]
Cocaine	74.6 ± 3.8[a]
Clonidine	63.1 ± 5.6[a]
Guanabenz	31.2 ± 3.4[a]
Idazoxan	61.4 ± 2.6[a]
Oxymetazoline	110 ± 5
Codeine	88.1 ± 3.7
Methadone	41.1 ± 1.1[a]
Fentanyl	62.6 ± 3.2[a]
Ethylmorphine	81.0 ± 3.9
Naloxone	71.5 ± 2.3[a]
Agmatine	104 ± 4
Spermidine	110 ± 5
Putrescine	109 ± 2[a]
Guanidine	106 ± 1[a]
Carnitine	99.9 ± 3.8
Creatine	96.0 ± 3.1
Tetraethylammonium (TEA)	95.4 ± 2.5
N-methyl-4-phenylpyridinium (MPP^+)	97.3± 5.5
Choline	116± 2[a]
Benzyltrimethylammonium	96.5 ± 3.4
Rivastigmine	60.4 ± 1.9[a]
Procainamide	100 ± 3
Phentolamine	101 ± 2
Atropine	91.5 ± 4.1
Verapamil	74.5 ± 6.3[a]
N-butylscopolamine	114 ± 4[a]
Ephedrine	80.5 ± 4.3[a]
Quinine	63.1 ± 0.4[a]
Quinidine	60.7 ± 1.5[a]
Chlorphenamine	48.0 ± 1.9[a]
Diphenhydramine	46.2 ± 3.0[a]
Promethazine	32.3 ± 1.0[a]
Imipramine	40.6 ± 1.2[a]
Doxepin	38.6 ± 1.2[a]
Maprotiline	38.3 ± 0.7[a]
L-Alanine	102 ± 2
L-Arginine	98.8 ± 1.9
L-Cysteine	108 ± 3
Glycine	111 ± 9
L-Histidine	98.8 ± 2.9
L-Lysine	98.0 ± 4.5
L-Phenylalanine	92.8 ± 2.0
L-Proline	99.0 ± 4.7
L-Serine	103 ± 2
L-Tryptophan	92.5 ± 3.9
L-Tyrosine (1 mM)	102 ± 2
Tyramine	94.1 ± 1.8
Tryptamine	74.6 ± 1.8[a]
Serotonin	98.4 ± 1.5
Noradrenaline	104 ± 2
Dopamine	94.9 ± 2.3
γ-Aminobutyric acid (GABA)	95.7 ± 1.8
Taurine	96.8 ± 3.2
Betaine	100 ± 4

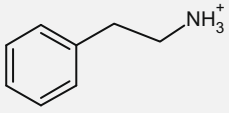
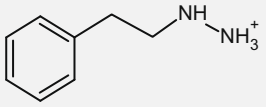
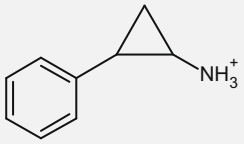
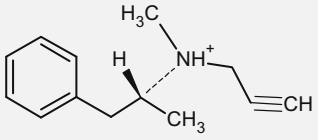
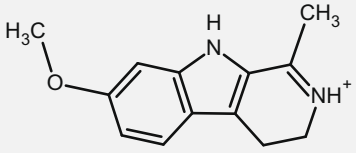
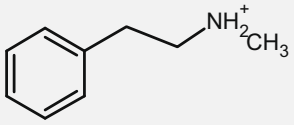
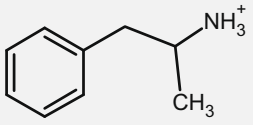
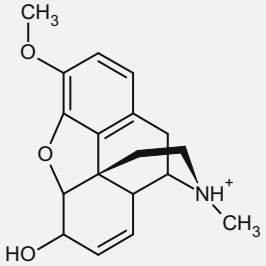
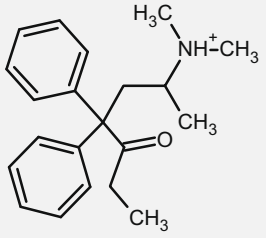
Uptake of [^{14}C]PEA (1.1 μM) into Caco-2 monolayers was measured at pH 7.5 for 1 min in the absence (control) or presence of unlabeled compounds (2 mM or as indicated). Data are means ± SE, $n = 4$.
[a] Significantly different from control with $p \leqslant 0.05$.

The total transepithelial transport from apical-to-basolateral direction J_{a-b} was 7.2%/30 min/cm^2 (114.0 pmol/30 min/cm^2) and exceeded the flux of the non-transported hydrophilic space marker [^{14}C]mannitol by a factor of 100 (0.14%/h/cm^2, [21]). Basolateral-to-apical flux (J_{b-a}) was 1.4%/30 min/cm^2 (41.3 pmol/30 min/cm^2), i.e. 5-fold lower than transport in the absorptive direction.

4. Discussion

The results of the present investigation show that the uptake of [^{14}C]PEA into Caco-2 cell monolayers proceeds independent on extracellular Na^+, K^+, Ca^{2+}, Mg^{2+} or Cl^- gradients. [^{14}C]PEA uptake is, however, strongly stimulated by an increase in the pH_o. The ef-

Table 2
Inhibition constants (K_i) of different compounds for the inhibition of [^{14}C]phenylethylamine and [^{3}H]clonidine uptake at Caco-2 cells.

Compound	Structure	K_i (mM)	
		[^{14}C]PEA uptake	[^{3}H]Clonidine uptake
β-Phenylethylamine (pK_a 9.73[a])		0.82 ± 0.15	0.83 ± 0.10
Phenelzine (pK_a 8.14[b])		1.3 ± 0.2	1.7 ± 0.1
Tranylcypromine (pK_a 8.78[b])		0.66 ± 0.06	0.70 ± 0.06
Selegiline (pK_a 7.53[b])		3.6 ± 0.3	–
Harmaline (pK_{a1} 7.24[b], pK_{a2} 16.60[b])		1.2 ± 0.1	0.42 ± 0.03
N-Methylphenylethylamine (pK_a 8.14[b])		1.6 ± 0.1	–
D,L-Amphetamine (pK_a 9.94[b])		1.0 ± 0.1	0.89 ± 0.07
Codeine (pK_{a1} 8.25[b], pK_{a2} 13.41[b])		4.6 ± 0.4	1.6 ± 0.1[c]
Methadone (pK_a 9.05[b])		0.70 ± 0.03	0.46 ± 0.03

(*continued on next page*)

Table 2 (*continued*)

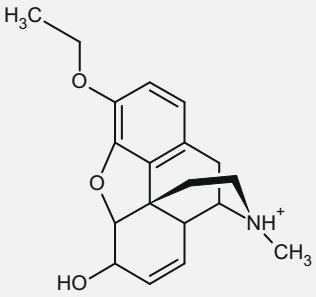
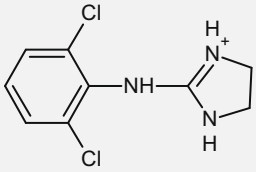
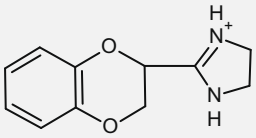
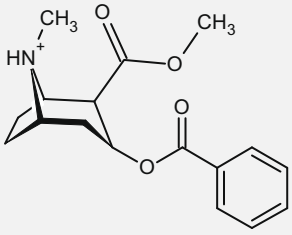
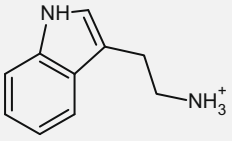
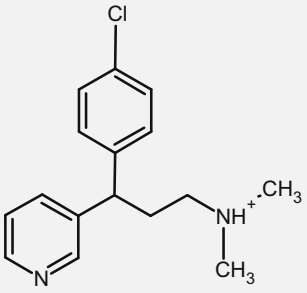
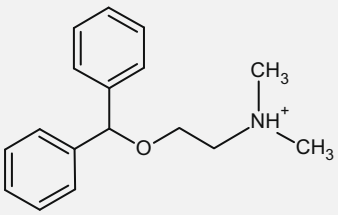
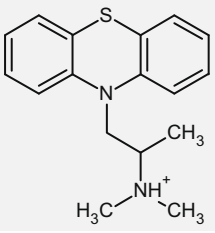
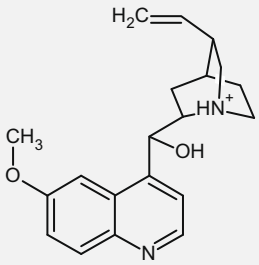
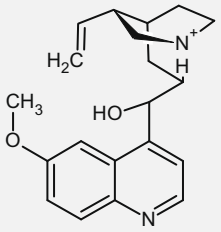
Compound	Structure	K_i (mM)	
		[^{14}C]PEA uptake	[^{3}H]Clonidine uptake
Ethylmorphine (pK_{a1} 8.25[b], pK_{a2} 13.41[b])		5.5 ± 0.2	2.2 ± 0.4
Clonidine (pK_a 8.18[d])		3.4 ± 0.3	0.79 ± 0.10[c]
Idazoxan (pK_a 9.51[b])		2.5 ± 0.2	0.86 ± 0.10
Cocaine (pK_a 8.97[b])		4.5 ± 0.7	2.0 ± 0.3
Tryptamine (pK_{a1} 10.27[b], pK_{a2} 17.21[b])		3.9 ± 0.8	2.5 ± 0.2[c]
Chlorphenamine (pK_a 9.33[b])		1.4 ± 0.1	0.45 ± 0.07[c]
Diphenhydramine (pK_a 8.76[b])		0.98 ± 0.01	0.38 ± 0.01[c]
Promethazine (pK_a 8.98[b])		1.1 ± 0.1	–

Table 2 (*continued*)

Compound	Structure	K_i (mM)	
		[^{14}C]PEA uptake	[^{3}H]Clonidine uptake
Quinine (pK_{a1} 9.28[b], pK_{a2} 12.80[b])		3.2 ± 0.8	0.30 ± 0.05[c]
Quinidine (pK_{a1} 9.28[b], pK_{a2} 12.80[b])		2.8 ± 0.1	0.41 ± 0.03[c]

Uptake of [^{14}C]PEA (1.1 μM, 1 min) and [^{3}H]clonidine (3 nM, 2 min) into Caco-2 monolayers was measured at pH 7.5 in the presence of unlabeled inhibitors (0–31.6 mM). Parameters are shown ± SE, $n = 4$.

[a] Value from [10].
[b] Calculated data from https://scifinder.cas.org (06/2009).
[c] Data from [15].
[d] Value from [37].

fect of FCCP and the effect of lowering pH_i show that the outwardly directed H^+ gradient itself is the driving force. It is less likely that PEA is transported by passive diffusion in its neutral unprotonated form according to the pH partition theory to any significant extent. Although the extent of ionization of PEA differs depending on pH (99.98%, 99.41% or 94.44% of PEA molecules positively charged at pH 6.0, 7.5 or 8.5), there is no correlation between uptake rates and charge. Moreover, PEA uptake into the cells is strongly saturable. The transepithelial transport of [^{14}C]PEA is mainly an absorptive process. We conclude that a saturable, pH-dependent transport component and low simple diffusion contribute to PEA uptake into Caco-2 cells.

PEA is present in processed foods in amounts of up to 1 mg per 100 g. Luminal concentrations in the intestine in the lower micromolar range are conceivable. In some cases, after consuming PEA-enriched diet supplements, concentrations in the higher micromolar or even lower millimolar range are possible. In addition, PEA might also be biosynthesized in the intestinal lumen to some extent by bacteria [17,18]. So far, no evidence was found in the literature for significant enzymatic degradation of luminal PEA by intestinal bacteria. Within the human organism, PEA is metabolized by MAO-B, by flavon-containing monooxygenase 3 (FOM3) and others [25].

Our results show that the mechanism underlying the saturable PEA accumulation is not identical to the ATP-stimulated [^{14}C]PEA uptake found at isolated membranes of bovine adrenal chromaffin granules [11], because in our investigations, ATP synthesis inhibition by DNP did not alter PEA uptake. The mechanism is also not identical to the intracellular accumulation of [^{14}C]PEA in bovine adrenal chromaffin vesicles [12], which was insensitive of cocaine in contrast to PEA uptake in Caco-2 cells, where cocaine was a potent inhibitor with $K_i = 4.5$ mM. Na^+-dependent transporters that were suggested to be involved in PEA uptake in rabbit erythrocytes [10] can also be excluded. Mason and coworkers described the Na^+/K^+ gradient as the driving force responsible for operation of this carrier mechanism, yet in our investigations, PEA uptake was independent of extracellular Na^+ and K^+ concentration. Similarly, the Na^+-dependent PEA uptake system in rat isolated lung [13] can also not be identical to the mechanism at Caco-2 cells. The uptake process described here is in some aspects similar to the Na^+-independent, H^+-coupled electroneutral MDMA transport process in Caco-2 cells [16]. MDMA uptake is mediated by a saturable process with a similar Michaelis constant (K_m) of 1.1 mM. Uptake was found, however, to be bidirectional, depending on the magnitude of the proton gradient and the substrate concentration gradient. The transporter might serve as a proton-coupled efflux pump, when the inside directed proton gradient exceeds the inside directed substrate concentration gradient. Under reversed conditions, it might function as proton-moving facilitated transporter [16]. There are, however, significant differences between PEA and MDMA transepithelial transport. In our investigation, the total net apical-to-basolateral flux of [^{14}C]PEA at apical pH 7.5 exceeded the basolateral-to-apical flux 5-fold. Apical-to-basolateral MDMA flux, however, is lower than basolateral-to-apical flux [16]. There are also some similarities between our data on PEA flux and the transepithelial transport of quinidine, a potent PEA uptake inhibitor in our study. Quinidine flux from apical-to-basolateral side exceeded the basolateral-to-apical flux significantly [26].

All three compounds, PEA, MDMA and quinidine, are transported across the intestinal epithelial cells in a pH-dependent manner. Moreover, the mechanism and substrate specificity of the reversible H^+/antiport system responsible for MDMA uptake in Caco-2 cells [16] and the PEA uptake (this study) are comparable to those of the clonidine uptake at several cell types [15,27–28]. The best approach to obtain more relevant insight into the different uptake processes is a thorough investigation of the substrate specificity. In our study, various organic cations – biogenic compounds and drugs – interact with the PEA uptake system at Caco-2 cells, namely, the MAO-B inhibitors phenelzine, tranylcypromine, selegiline, and harmaline, the PEA metabolite MPEA, D,L-amphetamine, guanabenz, methadone, the antihistaminics chlorphenamine, diphenhydramine, and promethazine and the cyclic

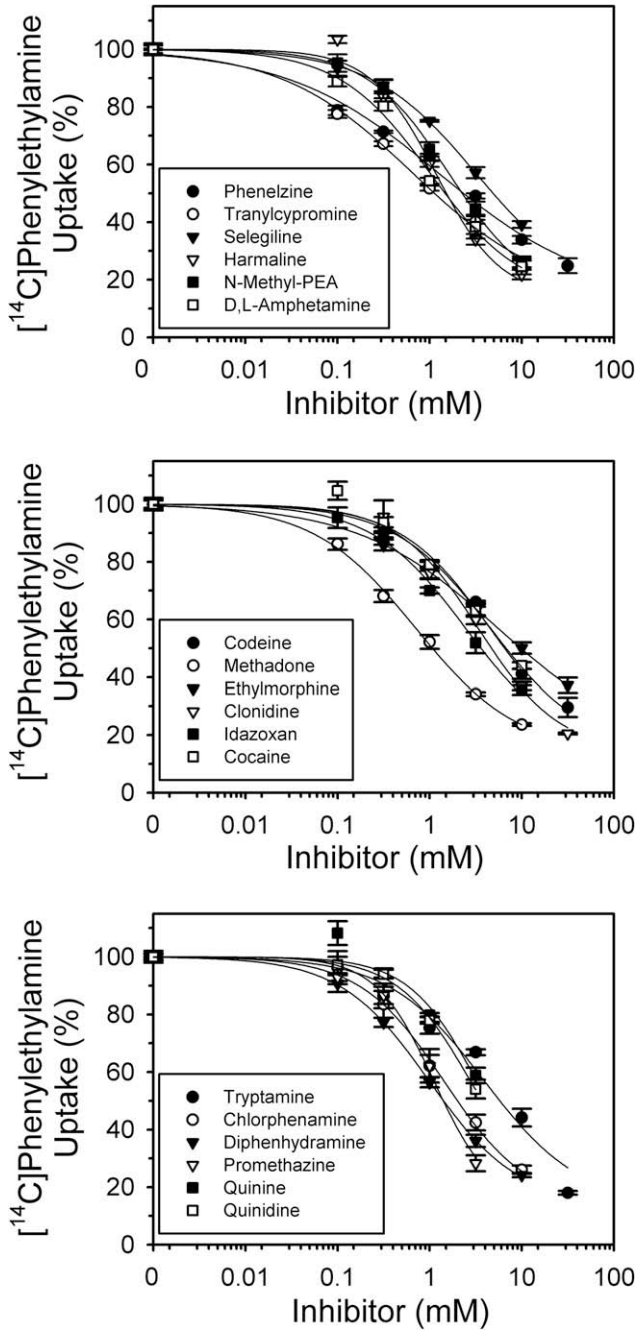


Fig. 4. Substrate specificity of [^{14}C]PEA uptake in Caco-2 cells. Uptake of [^{14}C]PEA (1.1 μM, 1 min, pH 7.5) was measured in the presence of increasing concentrations of unlabeled compounds (0–31.6 mM). Values represent means ± SE, $n = 4$.

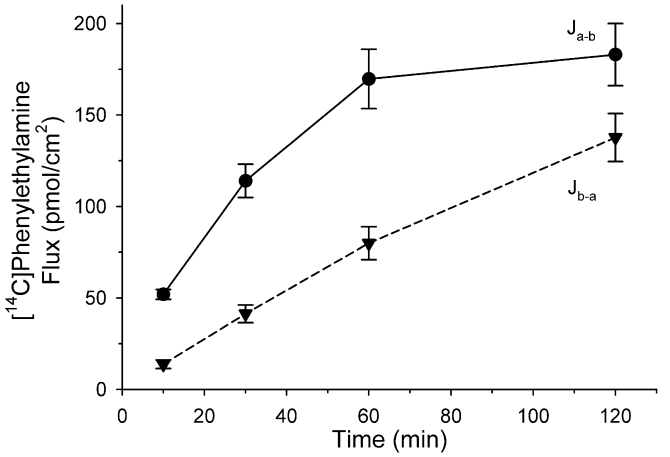


Fig. 5. Transepithelial transport of [^{14}C]PEA across Caco-2 monolayers. Flux of [^{14}C]PEA (1.1 μM) was measured at 37 °C at pH 7.5 in the apical and the basolateral compartment of Transwell® systems. [^{14}C]PEA appearance corrected for buffer replacement is plotted vs. time (transepithelial flux in apical-to-basolateral J_{a-b} or in opposite direction J_{b-a}). Values represent means ± SE, $n = 3$.

antidepressants imipramine, doxepin and maprotiline. Quaternary amines such as TEA, MPP$^+$ or choline had no inhibitory effects. So far, the substrate specificity of PEA uptake seemed comparable to that of clonidine uptake. Therefore, and to obtain more precise data, the K_i values of crucial compounds were determined both for [^{14}C]PEA and [^{3}H]clonidine uptake inhibition. For PEA, phenelzine, tranylcypromine and D,L-amphetamine apparent affinity constants were similar. For other compounds, the K_i values for the inhibition of clonidine uptake were significantly lower than for the inhibition of PEA uptake. The strongest differences were found for quinine (factor 10) and quinidine (factor 7).

To compare the specificity of PEA uptake with earlier reports on transport of related drugs, we also studied the effect of diphenhydramine, chlorphenamine, procainamide and verapamil: The MDMA uptake system mentioned earlier recognizes methamphetamine, diphenhydramine ($K_i = 2$ mM) and clonidine as potent inhibitors [16]. At Caco-2 cells, a pH-dependent tertiary amine transport system has been described to be involved in diphenhydramine and chlorphenamine transport ($K_t = 0.9$ mM) [29,30]. Katsura and coworkers described the transport of procainamide at rabbit brush-border membrane vesicles [31]. Han and coworkers reported a membrane potential-independent verapamil transporter in human retinal pigment epithelial cells [32]. All these systems show similar functional characteristics such as Na^+ independence and stimulation by an outside directed H^+ gradient and also some similarities in substrate specificity. They all seem to prefer lipophilic aromatic structures with aliphatic amino moieties. They all exclude TEA, MPP$^+$ and choline. In the present study, procainamide did not affect [^{14}C]PEA uptake. Therefore, the transport mechanisms seem to be different even though the term H^+/tertiary amine antiport might apply for all these pathways.

In the human plasma, Huebert and coworkers determined PEA concentrations in the range of 127–1002 pg/ml (mean 335 pg/ml) [33]. This amount corresponds to an average concentration of 3 nM (range 1–10 nM). In the mammalian brain, PEA concentrations between 2 and 15 nM were measured [1,33]. Considering the amount of PEA in the diet, plasma levels in the low micromolar range can be reached. In the brain, PEA operates as neuromodulator in submicromolar levels [4,6].

In conclusion, prototype substrates of known membrane transporters for amino acids, biogenic amines, neurotransmitters or organic cations did not affect the uptake of PEA. We must conclude that neither organic cation transporters such as CHT1, CTL1 or MATE1 and 2 [34–36] or members of the OCT and OCTN groups of SLC family 22 [19,20], nor transport systems for monoamines, neurotransmitters or amino acids are involved in intestinal PEA uptake. Our findings indicate that a highly active, saturable and absorptive process is involved in PEA transport at Caco-2 cells. We do not postulate that this system is a specific PEA transporter. PEA uptake is affected by a large number of pharmaceutically relevant cationic drugs. Further investigations of orphan transporters are required to identify the apparent epithelial H^+/antiport of secondary and tertiary amines on a molecular level.

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