Biochimica et Biophysica Acta, 1028 (1990) 25–30
Elsevier

BBAMEM 74972

Transport of a large neutral amino acid (phenylalanine) in a human intestinal epithelial cell line: Caco-2

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(Received 15 November 1989)
(Revised manuscript received 14 June 1990)

Key words: Caco-2; Amino acid transport; Phenylalanine; Transcellular transport

We have characterized the transcellular transport of a large neutral amino acid (LNAA) in Caco-2 cell monolayers. The apical (AP) to basolateral (BL) transport of phenylalanine (Phe) was approximately 10-times faster than BL-to-AP transport. The evidence for the carrier-mediated AP-to-BL transport of Phe include: (a) temperature dependence and saturability, (b) Phe transport was not affected by a reverse gradient, (c) the activation energy for transport was 12.0 kcal/mol, and (d) an excess amount of unlabeled Phe caused a 75% reduction in transport rate and a delay (lag time) in the appearance of Phe in the BL side. The V_m and K_m for Phe transport were 572.4 pmol · mg protein^{-1} · min^{-1} and 0.56 mM, respectively. Phe transport was decreased in the absence of glucose and in the presence of sodium azide or ouabain. The carrier interacted with LNAAs and with cationic amino acids but not with small neutral or anionic amino acids.

Introduction

Numerous techniques have been utilized in investigating the transport of nutrients across the intestinal mucosa. These techniques include: everted intestinal sacs, everted intestinal rings, isolated mucosal cells, brush border and basolateral membrane preparations [1–3]. All these preparations have been useful in the characterization of the uptake of nutrients and drugs by the intestinal mucosa. However, the limitations (short viability, lack of cell/tissue polarity, difficulty of access to the BL side, the presence of numerous tissue layers, lack of cellular metabolism) associated with some of the previous systems [4] may limit their usefulness in the study of epithelial transport at the cellular level.

Since attempts to culture isolated mucosal cells and to establish cell lines derived from the small intestinal epithelium have not been successful [5,6], the utilization of alternative model systems appears justified. The ability of some human colon adenocarcinoma cell lines (e.g., Caco-2, HT-29, SW1116, LS174T [7–10]) to undergo enterocytic differentiation in culture makes them attractive as potential model systems of polarized small intestinal epithelium. In an earlier study, we characterized Caco-2 cell monolayers, grown on a microporous membrane, as a transport model system for the intestinal epithelium [11].

In this study we decided to determine whether Caco-2 cells might be a useful in vitro cell culture model system in the investigation of intestinal epithelial transport of amino acids. Accordingly, the transcellular transport of the large neutral amino acid phenylalanine (Phe) was assessed in Caco-2 cell monolayers. In addition to determining the polarity of Phe transport, we investigated the temperature-, time- and concentration-dependence of transport and some biochemical requirements for amino acid transport. Structural requirements for interactions with the carrier were examined by measuring the effect of charge, bulkiness and stereospecificity on Phe transport. We conclude that the similarities between this system and the human intestinal epithelium will probably permit modest extrapolations regarding the uptake and transcellular transport of amino acids in the small intestine.

Portions of this work were presented at the Annual Meeting of the American Association of Pharmaceutical Scientists, 2 November 1988, and published in abstract form in Pharmaceutical Research 5, S-110 (1988).

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Materials and Methods

(A) Materials

The Caco-2 cell line was obtained from American Type Culture Collection, Rockville, MD. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAA) and L-glutamine were obtained from Hazleton Research, Lenexa, KS. Penicillin and streptomycin were obtained as a mixture from Hazleton Research. Transwell™ clusters, polyvinyl-pyrrolidone-free, 24.5 mm diameter and 3.0 μm pore size were purchased from Costar, Cambridge, MA. Rat tail collagen (Type I) was from Collaborative Research, Bedford, MA. D-[1-^{14}C]Mannitol (spec. act., 55.6 mCi/mmol), L-[2,6-^{3}H]phenylalanine ([^{3}H]Phe, spec. act., 53 mCi/mmol) and L-[U-^{14}C] phenylalanine ([^{14}C]Phe, spec. act., 55.7 mCi/mmol) were from Amersham, Arlington Heights, IL. Hank's balanced salt solutions (HBSS), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes), ouabain, sodium azide, 2-deoxyglucose (2-DG), L-phenylalanine (Phe), D-phenylalanine (D-Phe), L-leucine (Leu), L-tyrosine (Tyr), L-lysine (Lys), L-histidine (His), L-glutamic acid (Glu), L-aspartic acid (Asp), L-glycine (Gly), L-tryptophan (Trp), L-Dopa and α-methylDopa (α-MD) were from Sigma Chemical Co., St Louis, MO. All other chemicals were reagent grade.

(B) Cell monolayers

Caco-2 cells were plated at a density of 63000 cells/cm^2 on Transwell™ polycarbonate membranes which had been coated with collagen. The culture medium consisted of DMEM, 10% FBS, 1% NEAA, 100 U/ml penicillin and 100 μg/ml streptomycin. After confluence (6 days) the culture medium was replaced (1.5 ml inside and 2.6 ml outside) daily. Cells were maintained at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. All cells used in this study were between passages 59 and 70.

(C) Transport studies

The culture medium was replaced with HBSS, containing 25 mM glucose and 10 mM HEPES buffer (pH 7.35) (transport buffer). After incubation at 37°C for 30 min, transepithelial electrical resistance (TEER) values were measured, using the Epithelial Voltohmmeter, World Precision Instruments, New Haven, CT. Prior to transport studies, Caco-2 cell monolayers were equilibrated at the appropriate temperature for 30 min. Sampling was performed as described previously [11]. The entire volume of the receiver compartment was withdrawn at the end of each time interval and replaced with fresh transport buffer. The milligrams of protein in the monolayers were measured using the method of Lowry et al. [12].

(i) Inhibition of transport by experimental conditions. We determined the amounts of Phe that appeared in the BL side after the administration of a 10 μM [^{3}H]Phe (0.1 μCi/μmol) solution to the AP side of monolayers which were incubated in transport buffer alone (control) or in the presence of an inhibitor of Na^+,K^+-ATPase (ouabain) or an inhibitor of oxidative phosphorylation (sodium azide). The Na^+-dependence of Phe transport was further assessed by measuring the transport of Phe in the absence of Na^+ or glucose under the specific conditions described in the appropriate figure or table legends.

(ii) Inhibition of transport by other amino acids. To gain some insight into the structural properties favorable for interaction with this amino acid carrier system, a [^{14}C]Phe solution was administered on the AP surface alone (control) and in the presence of 1 mM concentrations of several amino acids and amino acid-type drugs. Finally, the effect of increasing concentrations of Lys and unlabeled Phe on the transport of 10 and 100 μM [^{14}C]Phe were compared.

(iii) Effect of time in culture on transport. Both Phe and mannitol diffused approximately 12%/cm^2 per h across cell-free membranes (not shown), indicating that the membrane alone does not constitute a barrier to the movement of these compounds.

The AP-to-BL rates of transport of 10 μM Phe were 4632 (±926), 1429 (±268), 1125 (±282), 989 (±183) pmol · mg protein^{-1} · h^{-1} at days 8, 13, 18 and 25, respectively, and the corresponding transport ratios in the opposite direction (i.e., BL-to-AP) were 1103 (±271), 452 (±184), 305 (±170) and 109 (±83) pmol · mg protein^{-1} · h^{-1}. After 15 days the leakage of the membrane-impermeant compound mannitol was less than 0.5% per h. Because neither Phe transport nor mannitol leakage changed significantly ($P > 0.05$) after day 15, all monolayers were used between days 18 and 22.

(iv) Monolayer integrity. To further assess the integrity of the monolayers, we measured the transepithelial electrical resistances (TEER) of the monolayers prior to using them in transport studies. This was done for two reasons: first, TEER values of Caco-2 cell monolayers have been shown to be comparable to those of the small intestine and colon [11,13]; and second, as much as 85% of the electrical current applied to leaky epithelia, such as the small intestine, flows between the cells [13]. Thus, a monolayer with extremely low TEER was assumed to exhibit excessive leakage, through imperfect occluding junctions or 'holes' in the monolayer, to be useful in transcellular transport studies. All the monolayers used in this study exhibited TEER values ranging from 250 to 330 Ω · cm^2 and [^{14}C]mannitol fluxes of less than 0.5%/h.

Results

At day 18 the AP-to-BL transport of Phe was greater than 10-times faster than that in the BL-to-AP direction

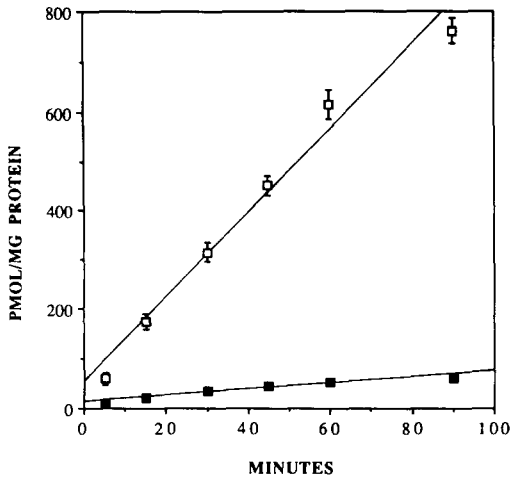

Fig. 1. AP-to-BL and BL-to-AP transport of Phe across Caco-2 cell monolayers. Transwell™-grown Caco-2 cell monolayers (18 days old) were rinsed three times with transport medium pre-equilibrated at 37°C. After applying 0.01 mM [^{3}H]Phe (70000 dpm/ml) in 1.5 ml transport medium to the AP side (AP-to-BL) (□) or 2.5 ml to the BL side (BL-to-AP) (■), monolayers were incubated at 37°C under 5% CO_2 and at a 90% relative humidity. Samples were withdrawn, from the side opposite to that on which [^{3}H]Phe was applied, at the times indicated. S.D. values ($N = 4$) were negligible.

(Fig. 1). At this time the protein content was approximately 2.0 mg per monolayer.

Of that Phe taken up by the AP membranes in 30 min, 60% was transported across the monolayer and released in the BL side and 40% was incorporated into trichloroacetic acid precipitable proteins (data not shown).

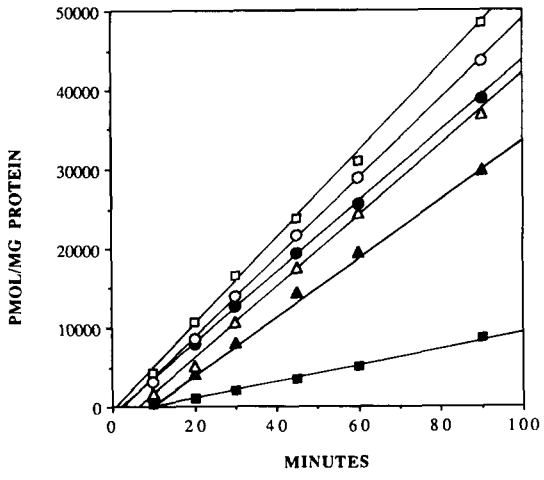

Fig. 2. Time- and concentration-dependence of Phe transport. Caco-2 cell monolayers were rinsed with warm transport medium and then incubated with 1.5 transport medium (AP side) containing 0.1 (■), 0.5 (▲), 1.0 (△), 2.5(●), 5.0 (○) or 10 (□) mM [^{14}C]Phe. The incubation conditions were identical to those described in Fig. 1. Samples were taken from the BL side at the times shown. S.D. values are not indicated for clarity of presentation. However, the values shown represent the mean of four monolayers and the variability in each group was less than 15%.

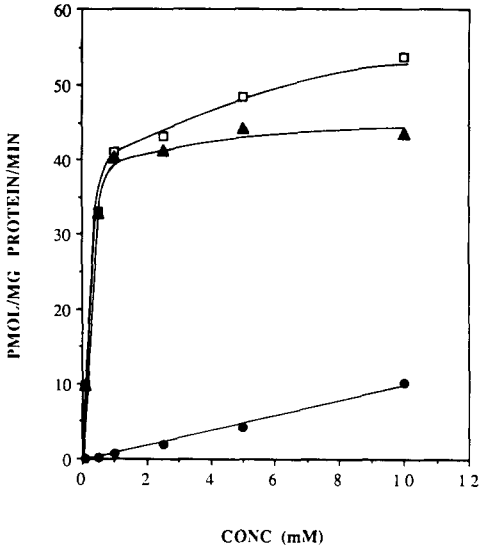

Fig. 3. Temperature- and concentration-dependence of Phe transport. Caco-2 monolayers were rinsed with transport medium pre-equilibrated at 37°C (warm) or 4°C (cold). 1.5 ml of transport medium (warm or cold) that contained 0.1, 0.5, 1.0, 2.5, 5.0 or 10.0 mM [^{14}C]Phe were added to the AP side of four monolayers, which were then incubated at 37°C (□) or on ice (●) for 60 min. Samples were taken from the BL side at 5, 10, 15, 30, 45 and 60 min. The rates of transport were determined in four monolayers and expressed per mg of protein. To obtain the temperature-dependent component (▲), the transport at 4°C was subtracted from the rate of transport obtained at 37°C.

AP-to-BL transport of Phe was time-, concentration- and temperature-dependent (Figs. 2, 3). At concentrations below 0.1 mM transcellular transport at 4°C was negligible (Fig. 3). At higher concentrations the effect of temperature became less pronounced, indicating that there are two transport pathways and that only one of them is temperature-dependent. The temperature-dependent component of Phe transport was isolated by subtracting the transport obtained at 4°C from that obtained at 37°C (Fig. 3). Saturation of the temperature-dependent process was observed above 0.5 mM (Fig. 3). Linear least-squares regression analysis on the double-reciprocal plot of the rate of transport vs. concentration data (Table I) provided a good correlation ($R^2 = 0.991$). The slope ($= K_m/V_{max}$) and intercept ($= 1/V_{max}$) of the regression equation were $9.83 \cdot 10^{-4}$ and $1.75 \cdot 10^{-3}$, respectively. The transport parameters, V_m

TABLE I

Double-reciprocal transformation of data on rate of transport as a function of concentration [a]

V_m and K_m were obtained by linear regression analysis ($R^2 = 0.991$; slope $= 9.83 \cdot 10^{-4}$; intercept $= 1.75 \cdot 10^{-3}$) on these data.

Rate^{-1}	12[b]	3.1	2.5	2.4	2.2	2.1
Concn.$^{-1}$	10.0	2.0	1.0	0.4	0.2	0.1

[a] Rate, pmol·mg protein^{-1}·min^{-1}; Concn., mM.
[b] These values have been multiplied by 10^3.

TABLE II

Transport of [^{3}H]Phe against a concentration gradient

Caco-2 monolayers were rinsed with warm transport medium and then received 1.5 ml transport medium containing 10 μM [^{3}H]Phe on the AP side. In the control group the BL side contained transport medium alone and in the test group the BL side contained 100 μM unlabeled Phe. $N = 4$. S.D. values were omitted because the two groups are identical.

Time (min)	Transport (dpm/mg protein)				
	5	10	30	45	60
Control	1750	3460	9150	14360	19570
Gradient	1670	3310	8620	13610	18610

and K_m, calculated from the regression parameters, were 570 pmol · mg protein^{-1} · min^{-1} and 0.56 mM, respectively.

To further characterize the carrier, we selected Phe concentrations below the K_m value. The transport of 10 μM Phe against a steep concentration gradient (100 μM) was indistinguishable from that in the absence of such gradient (control) (Table II). A linear least-squares regression analysis on the Arrhenius plot of the transport rates at different temperatures showed an excellent correlation between log rate and $1/T$ ($R^2 = 0.999$). The activation energy (E_a) for the transcellular transport of Phe, calculated from the slope of the regression line, was 12.0 kcal/mol.

This amino acid carrier system showed reduced transport in the presence of 0.1 mM ouabain ($P < 0.01$) or 1 mM sodium azide + 50 mM 2-deoxyglucose ($P < 0.05$) and in the absence of glucose ($P < 0.05$, Table III).

Amino acids differed in their ability to alter the transport of Phe. The cationic amino acids Lys and His and the neutral amino acids Leu and Tyr reduced significantly ($P < 0.05$) Phe transport (Table IV). On the other hand, the anionic amino acids Asp and Glu had no affinity for the carrier as indicated by a lack of statistically significant effect ($P > 0.05$) on the transport of Phe (Table IV).

The catechol amino acid L-Dopa was the most potent inhibitor of Phe transport. Indeed, the effect of L-Dopa was similar to that of unlabeled L-Phe (Table IV). The antihypertensive drug α-MD caused a significant ($P < 0.05$) inhibition of Phe transport.

Unlabeled Phe reduced the transport of 10 μM and 100 μM [^{14}C]Phe in a concentration-dependent fashion (Table V). At 100 μM [^{14}C]Phe concentrations, the

TABLE III

Effect of experimental conditions on [^{3}H]Phe transport

Caco-2 cell monolayers ($N = 4$) were preincubated (30 min) with either transport buffer alone (control), 0.1 mM ouabain (+ouabain), 1 mM sodium azide plus 50 mM 2-deoxyglucose (+azide), or transport buffer that did not contain glucose (−glucose). Subsequently, the monolayers were incubated for an additional 30 min with transport buffer containing the appropriate inhibitor plus 10 μM [^{3}H]Phe.

Conditions	Transport (pmol/mg protein per h)			
	control	+ouabain	+azide	−glucose
Transport [a]	480	320 *	410 *	380 *
S.D.	18	50	42	30

* $P < 0.05$.

TABLE IV

Effect of amino acids and amino acid analogues on [^{14}C]Phe transport across Caco-2 cell monolayers

[^{14}C]Phe was applied at 10 μM concentration (dissolved in transport buffer) to the AP side of 18-day-old monolayers alone (control) or in the presence of 1 mM concentration of each amino acid listed. The [^{14}C]Phe transported in the presence of each inhibitor was expressed as percent of the [^{14}C]Phe transported in the control group (i.e., 423 ± 75 dpm/mg protein per min).

Inhibitor	% of control (S.D.)
Control	
None	100 (18)
Acidic	
Asp	106 (11)
Glu	86 (7)
Basic	
Lys	50 (8) *
His	67 (6) *
LNAA	
Trp	64 (9) *
Tyr	55 (3) *
Leu	45 (5) *
Small AA	
Gly	102 (12)
Stereoisomers	
L-Phe	43 (9) *
D-Phe	89 (5)
Drugs	
L-Dopa	39 (5) *
α-MD	72 (3) *

* $P < 0.05$; $N = 4$.

TABLE V

Rate of [^{14}C]Phe transport in the presence of increasing concentrations of unlabeled Phe

Groups of four monolayers were incubated with 10 or 100 μM [^{14}C]Phe in the presence of the indicated concentrations of unlabeled Phe. In parentheses the percentages of the rates relative to the rate without unlabeled Phe are given.

[^{14}C]Phe	Rate (dpm/mg protein per h); concn. of unlabeled Phe (mM)						
	0	0.1	0.5	1.0	2.5	5.0	10.0
10 μM	13550	8130	3672	3662	1622	2908	2181
	(100)	(60)	(27)	(27)	(12)	(21)	(16)
100 μM	3958	3072	1891	1271	794	618	542
	(100)	(78)	(48)	(32)	(20)	(16)	(14)

TABLE VI

Concentration-dependent inhibition of [^{14}C]Phe transport by lysine

Groups of four monolayers were incubated with 10 or 100 μM [^{14}C]Phe in the presence of the indicated concentrations of Lys. In parentheses the percentages of the rates relative to the rate without Lys are given.

[^{14}C]Phe	Rate (dpm/mg protein per h); concn. of Lys (mM)						
	0	0.1	0.5	1.0	2.5	5.0	10.0
10 μM	630	530	490	400	340	300	210
	(100)	(84)	(78)	(64)	(54)	(48)	(33)
100 μM	520	490	320	310	230	220	210
	(100)	(93)	(62)	(60)	(44)	(42)	(40)

inhibition of transport caused by unlabeled Phe was similar to that achieved when 10 μM [^{14}C]Phe was utilized (Table V). However, the effect of Lys (Table VI) was not as dramatic as that caused by comparable concentrations of unlabeled Phe (Table V). Moreover, the inhibitory effect of Lys was less pronounced at 100 μM [^{14}C]Phe than at 10 μM (Table VI).

Discussion

The Caco-2 cell monolayer system utilized in this study was previously characterized with respect to paracellular leakage using membrane impermeant, hydrophilic compounds with molecular weights ranging from 457 to 70000 [11]. In that study, Caco-2 cell monolayers achieved functional polarity, as judged by apical (but not basolateral) localization of the brush border marker enzyme alkaline phosphatase, by day 6 and complete morphological differentiation by day 15 in culture [11]. Noticeably, the specific activities of the brush border marker enzymes alkaline phosphatase, sucrase-isomaltase and aminopeptidase were found to increase even after, by morphological criteria, brush borders were fully developed [7,11]. The transport of Phe across 18-day-old monolayers was not different from that across older monolayers, suggesting the presence of an amino acid transport system, which, unlike those for some enzymes [7] and bile acids (Hidalgo and Borchardt, unpublished results), was not dependent on time in culture. Similarly, the leakage of mannitol did not change significantly in monolayers older than 18 days, attesting to the stability of the barrier properties of the monolayers in long culture times.

The fact that Phe was transported much faster from the AP to the BL side than in the opposite direction is indicative of substantial polarity of the transport system. Since the AP-to-BL polarity was strongly positive, additional experiments were carried out only in the AP-to-BL direction. Although the radioactivity in the BL buffer was considered solely due to [^{14}C]Phe, the possibility that some of the radioactivity appearing in the BL buffer may be due to some metabolite of [^{14}C]Phe cannot be ruled out entirely. However, the possibility that the BL radioactivity is associated with radioactive protein is somewhat lessened by the fact that 100 μg/ml cycloheximide did not affect the transport of Phe (not shown).

The results from several experiments provided evidence that the transport of Phe was carrier-mediated: (a) A 10-fold reverse gradient had no effect on Phe transport. This observation cannot be explained by dilution of the labeled Phe along the concentration gradient because the presence of excess unlabeled Phe reduced the transport of Phe by approx. 70%; (b) The activation energy, E_a, for transcellular transport of Phe was 12.0 kcal/mol. This E_a value further supports the involvement of a carrier system because the normal range for E_a values associated with enzymatic reactions or carrier-mediated processes lies between 7 and 25 kcal/mol, while E_a values associated with simple diffusion processes are less than 4.0 kcal/mol [14]; (c) The transport of Phe was significantly reduced by sodium azide, indicating the energy-dependence of the transport process; (d) The lower transport of Phe in the absence of glucose ($P < 0.05$). Although the exact mechanism responsible for such decrease is not yet clear, the most likely explanation may be the role that glucose plays in providing a source of energy to maintain cellular metabolism and function. An alternative explanation is that glucose may stimulate the paracellular transport of Phe by solvent drag [15,16]; and (e) The significant reduction of Phe transport by ouabain is in keeping with the well known observation that uphill transepithelial transport of amino acids is Na^+-dependent and susceptible to inhibition by digitalis glycosides [17,18].

A rigorous comparison of our transport kinetic parameters with those obtained in previous studies with small intestinal preparations may not be warranted. First, most studies of the intestinal mucosal absorption of amino acids at the cellular and subcellular level have focused on uptake and not on transepithelial transport. And second, this study constitutes the first description of the transport of an amino acid across a single layer of viable and polarized intestinal epithelial cells. However, an examination of the transport parameters obtained in our study in light of previous uptake parameters may be of some value. For example, our K_m values for transcellular transport are smaller than the mean value for uptake K_m values (0.56 mM vs. 3.63 mM) (For a review of K_m values of amino acid uptake in different animal species see Ref. 19). But our K_m value falls within the range of uptake K_m values (0.23–8.8 mM) obtained with intestinal preparations from several species [19]. It is not yet clear whether the difference in K_m values between Caco-2 and normal enterocytes reflects a true difference between the affinities of the

LNAA carrier of Caco-2 cells and that of intestinal cells, or is just the result of an inappropriate comparison (i.e., uptake vs. transcellular transport). A meaningful comparison will be possible only when uptake (as opposed to transcellular transport) data in Caco-2 cells become available.

To gain additional insight into the structural requirements of this carrier, the effect of selected amino acids on Phe transport was investigated. The effect of Leu on Phe transport in Caco-2 cell monolayers indicates that, as in the small intestine [1], Phe is transported by the same neutral amino acid carrier responsible for the transport of Leu. The lack of effect of Gly most likely indicates that this amino acid carrier system is reactive with bulky amino acids (a suggestion supported by the effect of Leu) but not by small neutral amino acids. Our data are in agreement with a recent study (in guinea pig brush border membrane vesicles) which showed that the uptake of Phe was inhibited by Leu and Lys but not by Asp [20]. The reason for the approx. 30% inhibition of Phe uptake by Gly found in that study is not clear as Phe failed to inhibit the uptake of Gly [20]. The pronounced effect of the aromatic amino acids Trp and Tyr further emphasized the importance of side chain bulkiness and agrees with an earlier study showing that Trp and Met inhibited Phe transport by 34% ($P < 0.01$) and 55% ($P < 0.01$), respectively [21].

That the cationic amino acids Lys and His inhibited Phe transport by 49.6% ($P < 0.01$) and 67.4% ($P < 0.01$) most likely indicates that, as in the small intestine, neutral amino acids are also transported by the cationic amino acid carrier system [22]. The concentration-dependent inhibition of [^{14}C]Phe transport by unlabeled Phe and Lys may indicate that in Caco-2 cells cationic amino acids and large neutral amino acids may share the same carrier.

As expected, unlabeled Phe was a more efficient substrate for the carrier than Lys. Since the p$K_a$values of Lys are 8.95 and 10.53, this compound is almost totally in the cationic form at the pH of the experiment (i.e., 7.35). Thus the difference in the inhibitory effect of Lys cannot be ascribed to the ability of the unionized fraction of the amino acid to interact with the LNAA carrier but most likely reflects true interaction of the ionized species with this carrier. The concentration of Phe that caused a 50% ($P < 0.01$) inhibition of [^{14}C]Phe transport (IC_{50}) was between 0.1 and 0.5 mM at both [^{14}C]Phe concentrations used. These values are very similar to the K_m for Phe transport. The fact that the IC_{50} for Lys appeared to be slightly higher suggests that Phe is a preferred substrate for the carrier. The inhibition of Phe transport by the L-isomer and the lack of effect of the D-isomer is consistent with the stereospecificity of the carrier. Although both α-MD and L-Dopa reduced Phe transport, L-Dopa was a more potent inhibitor. More work will be needed to determine whether the substitution of a methyl group in the α-position may lower the affinity for the carrier.

Finally, these data suggests that, as in the intestinal epithelium, the transcellular transport of Phe in Caco-2 monolayers is mediated by a carrier system. This carrier system which is responsible for the polarized transport of Phe from the apical to the basolateral side requires Na^+, seems to be shared by large neutral amino acids and cationic amino acids, and exhibits stereospecificity towards L-isomers.

Acknowledgements

This work was supported by a grant from The Upjohn Company.

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