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International Journal of Pharmaceutics 275 (2004) 189–200
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Identification and functional characterization of a Na^+-independent large neutral amino acid transporter (LAT2) on ARPE-19 cells

Mohit D. Gandhi, Dhananjay Pal, Ashim K. Mitra*

Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri Kansas City, 5005 Rockhill Road, Kansas City, MO 64110-2499, USA

Received 11 September 2003; received in revised form 27 January 2004; accepted 27 January 2004

Abstract

The objective of this study was to investigate the presence of a large neutral amino acid transporter on the ARPE-19 cell line. ARPE-19 cells were grown on 24-well plates for uptake studies. Uptake characteristics of [^{3}H]L-phenylalanine (L-Phe) were determined at various concentrations and pH at 37 °C. Inhibition studies were conducted in presence of L- and D-amino acids, metabolic inhibitors, like ouabain, sodium azide, and in presence of sodium-free medium, to delineate the mechanism of uptake. RT–PCR was carried out on total RNA isolated from the ARPE-19 cells. Presence of Na^+-free buffer did reduce the uptake rate. Hence, all experiments were carried out in Na^+-free medium to delineate the sodium-independent uptake mechanism. Uptake of L-Phe on ARPE cells was found to be saturable with a $K_m = 89.35 \pm 14\,\mu M$, $V_{max} = 58.9 \pm 2.5\,pmol\,min^{-1}\,mg\,protein^{-1}$, and $K_d = 0.108 \pm 0.04\,\mu l\,min^{-1}\,mg\,protein^{-1}$. Dose-dependent inhibition was observed with increasing concentrations of unlabeled L-Phe. Uptake also was found to be energy independent. Significant inhibition of [^{3}H]L-Phe was observed with large neutral aromatic and aliphatic amino acids as well as small neutral amino acids. System L-specific inhibitor BCH produced partial inhibition of uptake. Neither acidic nor basic amino acids altered the uptake rate. Results obtained were predominantly characteristic of LAT2, particularly with respect to substrate selectivity and pH dependence. Bands for LAT2 were detected by RT–PCR in the ARPE cell line. This study provides biochemical evidence of the presence of a Na^+-independent, facilitative transport system, LAT2, on the ARPE-19 cells.

Keywords: ARPE-19; Amino acid transporter; Phenylalanine; LNAA; LAT2

1. Introduction

The retinal pigment epithelium (RPE) is a single cell layer cells lying below the photoreceptors of the vertebrate eye. In between the neural retina and choroidal blood supply, the polarized RPE cells mediate the transport of endogenous and exogenous compounds, metabolites, ions, and fluid (Joseph and Miller, 1991). The RPE forms the outer blood–retinal barrier and supports the function of photoreceptors (Ban et al., 1991). It possesses tight junctions, and can act as a barrier for entry of small drug molecules into the retina from choroidal blood supply. Different transporters are reportedly expressed in RPE cells of various species, such as monocarboxylic acids (Gerhart et al., 1999), taurine (Vinnakota et al., 1997),

* Corresponding author. Tel.: +1-816-235-1615; fax: +1-816-235-5190.
E-mail address: mitraa@umkc.edu (A.K. Mitra).

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doi:10.1016/j.ijpharm.2004.01.035

peptide/histidine (Yamashita et al., 1997), glucose (Kumagai et al., 1994), and folic acid (Huang et al., 1997). Presence of such amino acid transporters have also been reported on human retina (Pow, 2001; Gu et al., 2001). These transporters are required for supply of nutrients and other substances across the cell membranes.

Transporters, including organic cation transporter, glutamate transporter, glucose, taurine transporter, and folic acid, have been identified in RPE cells (Huang et al., 1997; Harada et al., 1998). Biochemical expression of monocarboxylate transporters, MCT1 on the apical side and MCT3 on the basolateral side of the RPE, have already been reported (Yoon et al., 1997). It may be possible to utilize the transporters present on the retinal pigmented epithelium in order to enhance the ocular bioavailability of systemically administered compounds.

Both Na^+-dependent and Na^+-independent transport systems are known to mediate the transport of neutral amino acids. To identify these transport systems, molecular cloning approaches have been utilized. Characterization at the molecular level have been carried out extensively for systems L, y + L, A, Asc, asc, b0+, B0+, Gly, N, and T (Christensen et al., 1994; Kekuda et al., 1996; Palacin et al., 1998; Pfeiffer et al., 1999; Torrents et al., 1998; Wagner et al., 2001). System L is known to mediate Na^+-independent transport of large neutral amino acids with branched aromatic side chains in almost all types of cells. Originally identified in Ehrlich ascites carcinoma cells, system L is also known to be essential for cellular nutrition and transport of neutral amino acids across blood–tissue barriers and epithelia (Pineda et al., 1999; Segawa et al., 1999). This system has also been reported to be involved in the transport of amino acid-related compounds, such as L-dopa, a therapeutic drug for Parkinsonism (Gomes and Soares-da-Silva, 2002). Its two isoforms, LAT1 and LAT2, though identified mainly by their substrate affinities, differ in terms of their substrate specificity, tissue distribution, pH dependence, and their interaction with D-amino acids (Rajan et al., 2000).

LAT1 which is expressed in the brain, spleen, thymus, testis, skin, liver, placenta, skeletal muscle, and stomach was isolated by expression cloning in Xenopus oocytes (Naggar et al., 2002). LAT1 has a high affinity for neutral amino acids with K_m values in the micromolar range. Phe is reportedly a good substrate for this isoform. LAT2 cDNA is highly expressed in polarized epithelia (Segawa et al., 1999). In addition to large neutral amino acids, small neutral amino acids, such as L-cysteine (Cyst), L-glycine, L-alanine (Ala), and L-serine (Ser), are transported by LAT2 and serve as poor substrates for LAT1 (Wagner et al., 2001). This isoform is reported to have a variable affinity with K_m values ranging from micromolar to millimolar range (Pineda et al., 1999). Studies are carried out specifically with Phe which also acts as a substrate for both the isoforms.

The ARPE-19 cell line is of human origin and is used as an in vitro representation of the RPE layer. Various studies have shown that this cell line represents the RPE with respect to the polarization and expression of transporters and RPE-specific markers (Aukunuru et al., 2001; Dunn et al., 1996; Han et al., 2001; Holtkamp et al., 1998; Naggar et al., 2002). All of our experiments have been designed using this cell line to investigate for the presence of large neutral amino acid transporter in RPE cells.

2. Materials and methods

2.1. Materials

L-Phe [2,3,4,5,6-^{3}H] having a specific activity of 120 Ci/mmol, was purchased from Amersham Pharmacia Biotech (Massachusetts, USA). The unlabeled amino acids, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH), *N*-methyl aminoisobutyric acid (NMeAIB), choline chloride, Triton X-100, HEPES, D-glucose, sodium azide, ouabain, and all other chemicals were purchased from Sigma Chemical Co (St. Louis, MO, USA). All the chemicals were products of special reagent grade and used without further purification.

2.2. Cell culture

ARPE-19 cells, obtained from American Type Culture Collections (ATCC), were cultured in DMEM-F12 (Dulbecco's minimum essential medium; Gibco BRL, Grand Island, NY, USA) supplemented with 10% non-heat-inactivated fetal bovine serum (JRH Life Sciences, Lenexa, KS, USA), HEPES,

sodium bicarbonate, penicillin (100 units/ml), and streptomycin (100 μg/ml) and were maintained at 37 °C, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. The cells (passages # 21–30) were plated at a density of 125,000 cells/well on 24-well culture plates for uptake studies. The medium, was replaced every alternate day and uptake studies were carried out on 14- to 21-day old cultures.

2.3. Uptake experiments

2.3.1. Na^+ dependence of uptake

The effect of Na^+ on uptake of amino acids was studied by using equimolar quantities of choline chloride and potassium phosphate dibasic (K_2HPO_4) to substitute NaCl and sodium phosphate monobasic (Na_2HPO_4), respectively, in the Dulbecco's phosphate-buffered saline, i.e. DPBS, pH 7.4, containing 130 mM NaCl, 0.03 mM KCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 5 mM glucose. All further experiments were carried out in the sodium-free buffer.

2.3.2. Concentration dependence

Uptake studies were performed based on the method of Surendran et al. (1999), with slight modifications. At 14–21 days postseeding, the medium was aspirated and cells washed twice with DPBS. Different concentrations (0.01–2 mM) of unlabeled L-Phe solutions were prepared in sodium-free DPBS, pH 7.4, and then mixed with fixed amount of [^{3}H]L-Phe (0.25 μCi/ml). For uptake studies, the cells were incubated with 0.5 ml of each solution for a suitable time period at 37 °C. At the end of each experiment, the solution was aspirated off and cells washed with 2 × 0.5 ml of ice-cold stop solution (210 mM KCl, 2 mM HEPES), pH 7.4, to terminate the cellular uptake. The cells were then solubilized in 0.5 ml of 0.1% Triton-X solution in 0.3% NaOH and transferred to scintillation vials containing 5 ml of scintillation cocktail. Radioactivity associated with the cells was quantified using a scintillation counter (Beckman Instruments Inc., Model LS-9000) and the protein content of each sample measured by the method of Bradford (1976) using bovine serum albumin as the standard (Bio-Rad protein estimation kit, Hercules, CA, USA). Non-specific binding was assessed by performing zero time uptake at 4 °C and subtracted from total uptake values obtained.

2.3.3. pH dependence of uptake

The pH of the buffer was adjusted to 5.5, 6.0, 7.4, and 8.5 for pH dependence studies. Uptake of [^{3}H]L-Phe (0.25 μCi/ml) was carried out as described above at each of these four pH values.

2.3.4. Competitive inhibition studies

Uptake experiments based on the method of Hidalgo and Borchardt (1990), were carried out with slight modifications using competitive inhibitors for the respective substrates. These experiments were carried out to elucidate the structural properties favorable for interaction with the carrier system. The unlabeled inhibitor was simultaneously incubated with the respective radiolabeled substrate and uptake carried out as described earlier. The unlabeled amino acids were used at a concentration of 2mM. Both L- and D-amino acids were used as inhibitors to determine the stereoselectivity of the amino acid transporter system.

2.3.5. Energy dependence

Here, the cells were preincubated with 1mM concentrations of metabolic inhibitors, like ouabain (an inhibitor of Na^+/K^+-ATPase), or sodium azide (an inhibitor of oxidative phosphorylation), for 30 min. Uptake was then carried out as described previously at pH 7.4 containing [^{3}H]L-Phe (0.25 μCi/ml).

2.4. RT–PCR and sequencing

Reverse transcription–PCR was done based on the method of Sugawara et al. (2000), using 1 μg of total RNA isolated from ARPE-19 cells by acid guanidinium thiocyanate–phenol–chloroform extraction method of Chomczynski and Sacchi (1987).

The forward primer for human LAT2 was 5′-TAG CCC TGA AGA AAG AGA TCG G-3′, and the reverse primer was 5′-GCG ACA TTG GCA AAG ACA TAC A-3′. These primers correspond to the nucleotide positions 101–123 and 836–858 in hLAT2 cDNA, respectively, giving a 757-base pair (bp) product. Reverse transcription–PCR was done using the GenAmp RNA PCR kit (Roche Applied Biosystems). The conditions for reverse transcription were as follows: denaturation of the template RNA for 10 min at 70 °C;

reverse transcription for 60 min at 42 °C. The conditions for PCR amplification were as follows: denaturation for 1 min at 94 °C; annealing for 1 min at 58 °C, and extension for 1 min at 72 °C; 37 cycles; final extension for 10 min at 72 °C. The resultant product (757 bp) was sequenced from both directions by SeqWright using an automated Perkin-Elmer Applied Biosystems 3730xl Prism™ DNA sequencer to establish its molecular identity.

2.5. Computer analysis

Nucleotide sequence homology searching was performed using basic local alignment tool (BLAST) via on-line connection to the National Center of Biotechnology Information (NCBI). Multiple nucleotide sequence comparisons were done using CLUSTAL W (1.81) multiple sequence alignment tool from SwissProt.

2.6. Data treatment

The uptake data was fitted to a modified Michaelis–Menten equation denoted by Eq. (1). This equation takes into account the carrier-mediated process and the non-saturable process.

$$V = \frac{V_{max}[C]}{K_m + [C]} + K_d[C] \quad (1)$$

where V is the total rate of uptake, V_{max} is the maximum uptake rate for the carrier-mediated process, K_m is the concentration at half-saturation (Michaelis–Menten constant), and K_d is the rate constant for the non-saturable diffusion component.

Data was fitted to this equation using a nonlinear least square regression analysis program (Kaleida Graph Version 3.09).

Dose-dependent inhibition data were fitted to a dose–response relationship calculated by Eq. (2)

$$y = \min + \frac{\max - \min}{1 + 10^{(\log IC_{50} - x)H}} \quad (2)$$

where IC_{50} is the inhibitor concentration at twice the rate of uptake and H is the Hill constant. Data were fitted to Eq. (2) with a transformed nonlinear regression curve analysis program (Prism, version 3.03; GraphPad, San Diego, CA, USA).

2.7. Statistical analysis

All experiments were conducted at least in triplicate and results are expressed as mean ± S.D. Michaelis–Menten parameters K_m and V_{max} are expressed as mean ± S.E. Unpaired Student's t-test was used to calculate statistical significance. A difference between mean values was considered significant if the P-value was equal to 0.05.

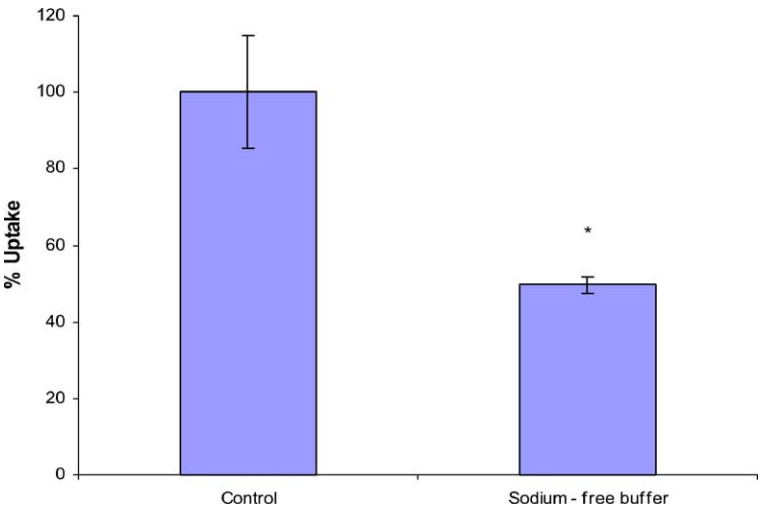

Fig. 1. Uptake of L-Phe in presence and absence of sodium in DPBS buffer. Here, uptake is expressed as percentage of control. Each value represents the mean ± S.D. ($n = 3$–4). Asterisk (∗) represents significant difference from control ($P < 0.05$).

3. Results

3.1. Na^+ dependence of uptake

The effect of Na^+ on uptake was studied to determine the presence of a Na-dependent carrier. In Fig. 1, partial inhibition (more than 50%) of L-Phe uptake was seen with Na^+-free medium. Hence, to delineate the characteristics of the sodium-independent carrier, all further experiments were performed using sodium-free buffer.

3.2. Concentration dependence

Uptake of [^{3}H]L-Phe consisted of a minor carrier-mediated saturable component and a major non-saturable component (Fig. 2). The data were fitted to Michaelis–Menten equation as per Eq. (1) and kinetic parameters of L-Phe uptake were determined by nonlinear regression analysis of the data. V_{max} and K_m values were calculated to be 58.9 ± 2.5 pmol min^{-1} mg protein^{-1} (mean ± S.D.) and 89.35±14 μM (mean±S.D.), respectively. K_d was

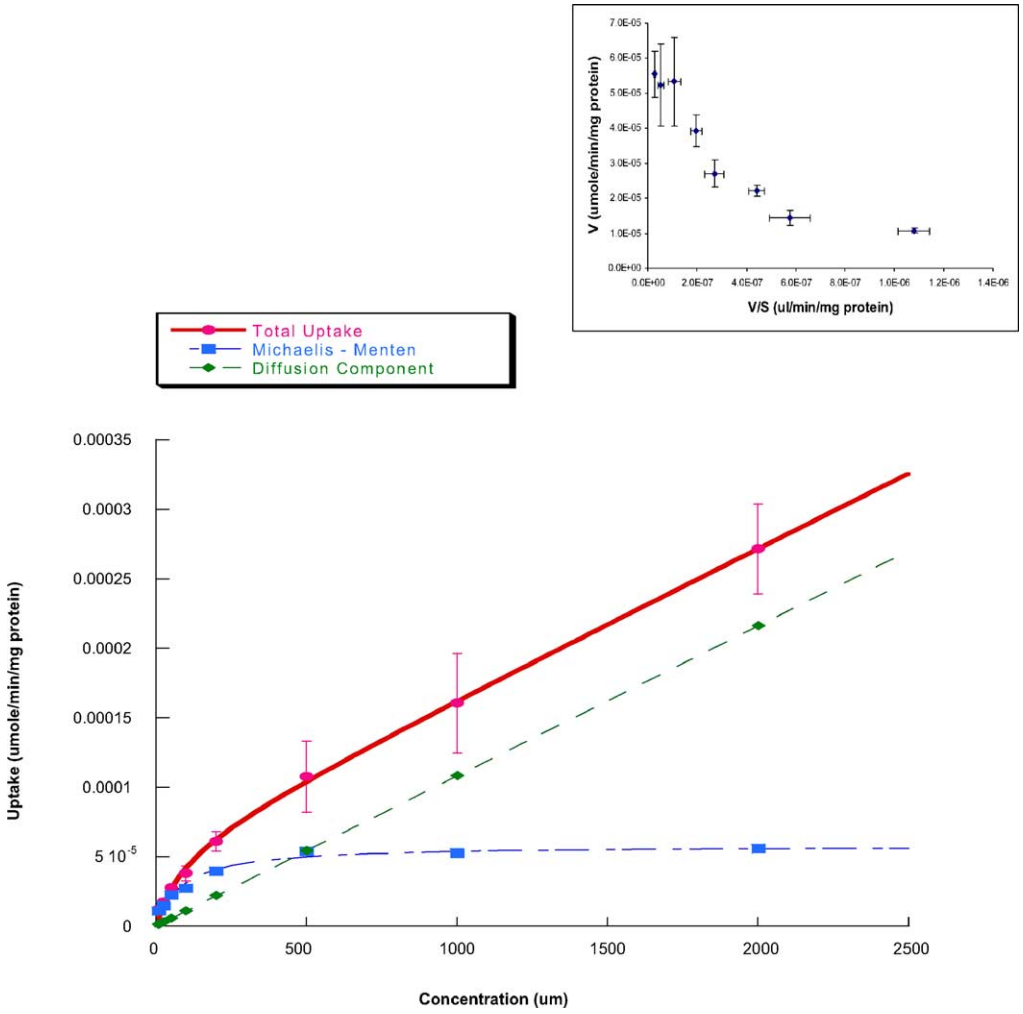

Fig. 2. Concentration-dependent L-Phe uptake in ARPE-19 cells at 37 °C, pH 7.4. (●)—total uptake, (◆)—linear, non-saturable component, K_d = 0.108 ± 0.04 μl min^{-1} mg protein^{-1} (mean ± S.E.). (■)—Michaelis–Menten component K_m = 89.35 ± 14 μM (mean ± S.E.), V_{max} = 58.9 ± 2.5 pmol min^{-1} mg protein^{-1} (mean ± S.E.). Inset shows Eadie–Hofstee transformation of the data. Each value represents the mean ± S.D. (n = 3–4).

estimated to be $0.108 \pm 0.04\,\mu l\,min^{-1}\,mg\,protein^{-1}$ (mean ± S.D.). At concentrations from 0 to 250 μM, the saturable component of uptake accounted for the bulk of L-Phe entry in to the RPE cells (Fig. 2). The non-saturable uptake was greater at higher concentrations. Eadie–Hofstee transformation of the results was nonlinear and showed presence of more than one carrier system (inset, Fig. 2). Inhibition studies using cold L-Phe were also carried out at concentrations ranging from 10 μM to 10 mM. Dose-dependent inhibition was observed with an IC_{50} value of 346.8 μM (Fig. 3).

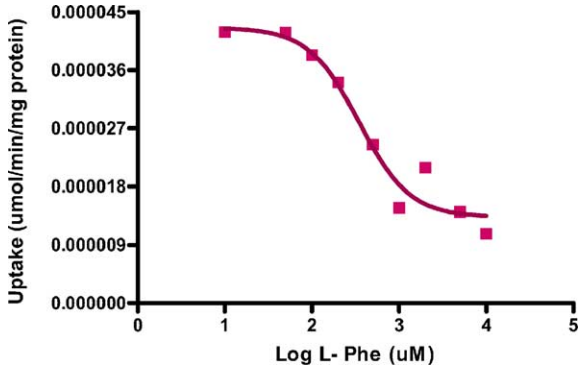

Fig. 3. Uptake of [^{3}H]L-Phe (0.25 μCi/ml) in ARPE-19 cells in and presence of increasing concentrations of unlabeled L-Phe. Dose-dependent inhibition was observed. Each value represents the mean ($n = 3$–4).

3.3. Substrate specificity

Various amino acids, amino acid-type drugs (L-dopa), and dipeptides, like glycyl sarcosine (Gly-Sar), were used as inhibitors during the uptake of [^{3}H]L-Phe to determine substrate specificity. All the inhibitors were tested at a concentration of 2 mM. Inhibition with large neutral amino acids (e.g. Phe, leucine (Leu)), small neutral amino acids (e.g. Cyst, Ala), basic (e.g. lysine (Lys), arginine (Arg)), and acidic amino acids (e.g. glutamic acid (Glu), aspartic acid (L-Asp)) were examined. Fig. 4 shows that the large neutral amino acids (LNAAs), such as the L-isomers of Phe, tryptophan (Trypto), tyrosine (Tyro), and both the isomers of valine (Val) and Leu were able to limit the uptake of L-Phe. Unlabeled L-Phe showed the maximum inhibition among the L-amino acids followed by L-Val, L-Trypto, L-Leu, and L-Tyro. The stereoeselectivity of this carrier was

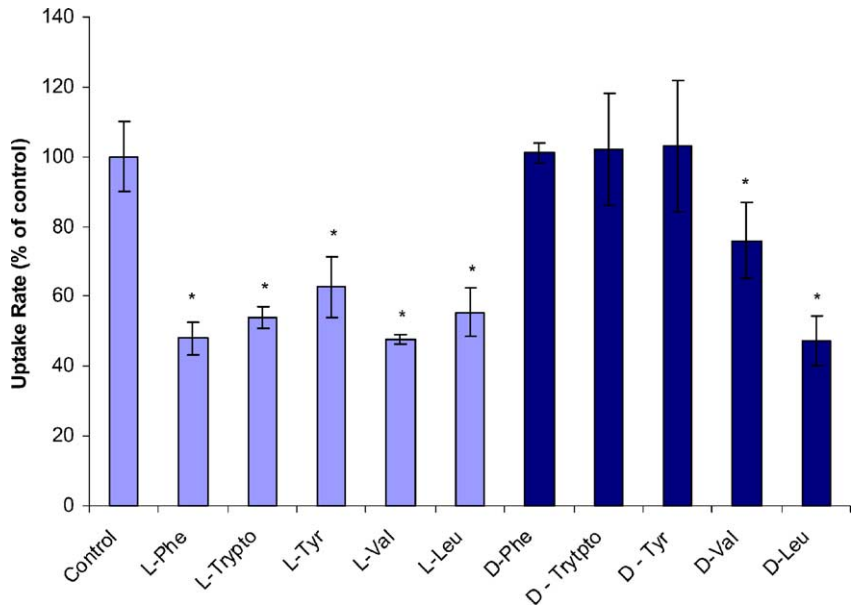

Fig. 4. Competitive inhibition of [^{3}H]L-Phe (0.25 μCi/ml) uptake in ARPE-19 cells by specific inhibitors, L- and D-isomers of different amino acids and (1 mM), indicating the substrate specificity and stereoselectivity of the transport system. Here, uptake is expressed as percentage of control. Each value represents the mean ± S.D. ($n = 3$–4). Asterisk (∗) represents significant difference from control ($P < 0.05$).

evident as variable inhibition was seen with both L- and D-isomers of the LNAAs. There was no significant decrease in the uptake of [^{3}H]L-Phe with the L-isomers of Phe, Tyro, and Trypto when compared with their L counterparts. However, reduced accumulation of L-Phe in the ARPE-19 cells was evident with D-isomers of Leu and Val. D-Leu, showed up to 50% inhibition when compared with the control. This particular dextro- and levorotatory isomer specificity observed may be attributed to LAT2.

Basic amino acids, L-Lys and L-Arg, were ineffective as inhibitors of L-Phe uptake. Acidic amino acids, such as Glu, asparagine (Asn), and Asp, also did not have any appreciable effect on the [^{3}H]L-Phe uptake. An affinity toward the small neutral amino acids was observed with a slight but significant decrease using L-Ala. Cyst showed a maximal decrease in uptake rate of up to threefold compared to other amino acids studied. L-Phe influx was also decreased in presence of large aromatic amino acid histidine (His).

The possibility of any overlapping substrate specificities between this LNAA transporter and the peptide transporter was also examined. L-Phe uptake was performed in the presence of dipeptide Gly-Sar. The contribution of the peptide carrier system was ruled out as the dipeptide did not have any effect (Fig. 5).

We further investigated the possible involvement of multiple transport systems in this uptake process. The effect of 1 mM NMeAIB, a specific inhibitor of Ala preferring neutral amino acid transport system A, and BCH, a specific inhibitor of the Leu preferring L-type amino acid transport system, was determined. Uptake of L-Phe was sensitive to the presence of BCH while NMeAIB did not have any significant effect on the uptake (Fig. 6). This indicates that uptake is predominantly mediated by the Leu preferring, L-type neutral amino acid transport system.

However, though BCH did show partial reduction in uptake, it was unable to completely inhibit L-Phe. This raises the possibility of the involvement of another Na^+-independent transporter system.

3.4. Effects of pH and metabolic inhibitors

L-Phe uptake was significantly altered with change in buffer pH. A sharp increase in uptake rate was

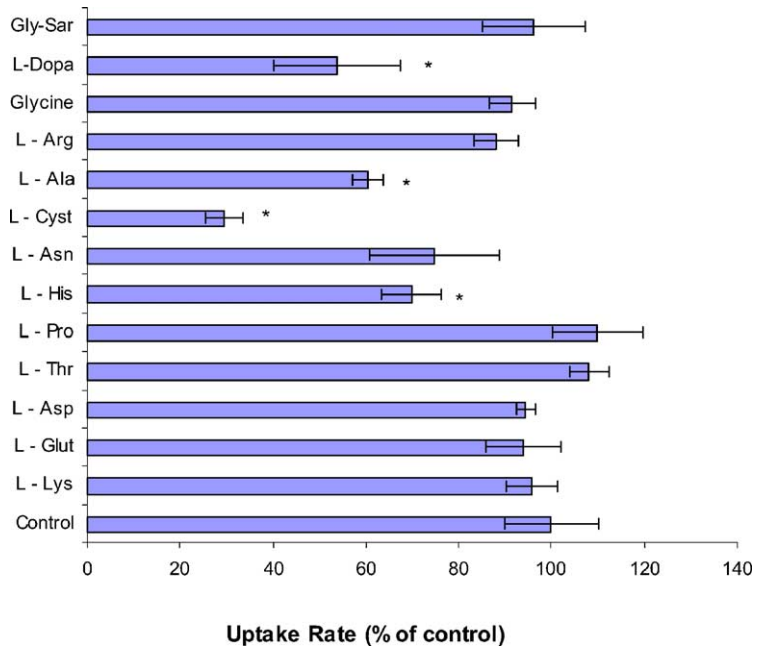

Fig. 5. Inhibition of [^{3}H]L-Phe (0.25 μCi/ml) uptake in ARPE-19 cells in presence of different amino acids, dipeptide glycyl sarcosine (Gly-Sar), and amino acid-type drug L-dopa (2 mM), in sodium-free buffer. Here, uptake is expressed as percentage of control. Each value represents the mean ± S.D. ($n = 3$–4). Asterisk (∗) represents significant difference from control ($P < 0.05$).

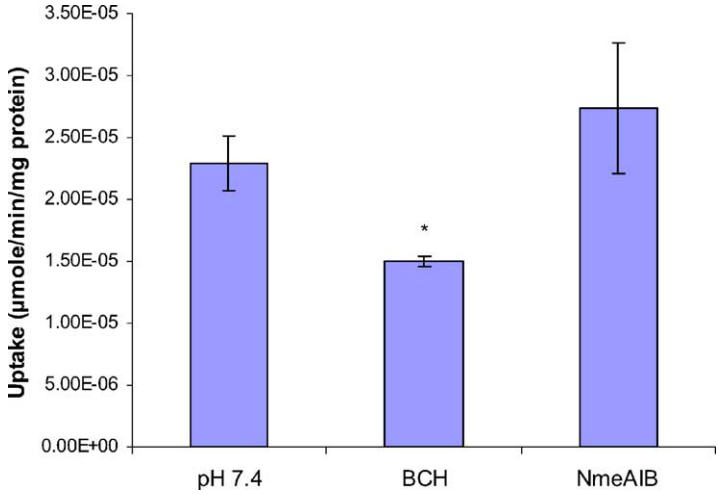

Fig. 6. Competitive inhibition of [^{3}H]L-Phe (0.25 μCi/ml) uptake in ARPE-19 cells by specific system L and system A inhibitors, BCH and *N*-methyl aminoisobutyric acid (NMeAIB) (1 mM). Each value represents the mean ± S.D. ($n = 3$–4). Asterisk (∗) represents significant difference from control ($P < 0.05$).

observed with lower pH values, 5.5 and 6 compared to 7.4 and 8.5. The cell viability remained unaltered under these conditions. The accumulation of L-Phe was highest at pH 5.5 and gradually decreased as the buffer pH increased from 5.5 to 8.5. This shows that the transporter favors uptake at acidic pH. This observation is characteristic of LAT2 (Fig. 7). We further studied the effect of metabolic inhibitors on the uptake of L-Phe. Na^+/K^+-ATPase inhibitor ouabain, and oxidative phosphorylation inhibitor sodium azide were used as metabolic inhibitors. The purpose of these inhibitors was to delineate the involvement of an energy-dependent uptake process. No inhibitory effect of ouabain or sodium azide was observed on the uptake of L-Phe in ARPE-19 cells (Fig. 8).

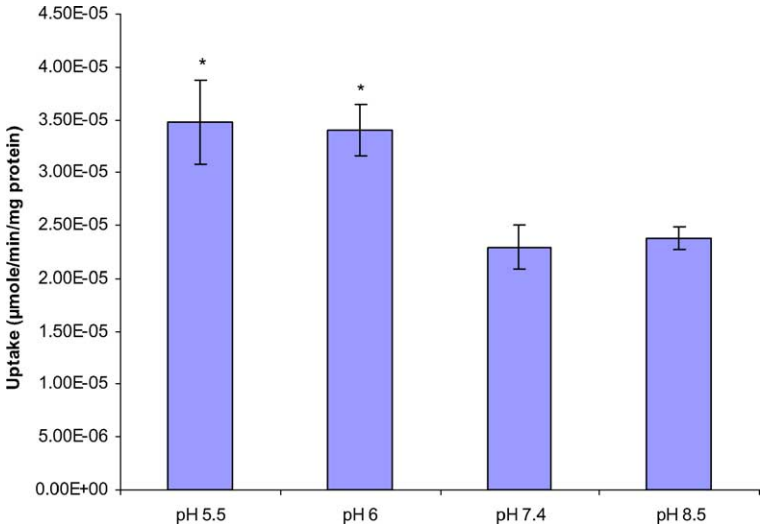

Fig. 7. Uptake of [^{3}H]L-Phe (0.25 μCi/ml) in ARPE-19 cells as a function of pH. Uptake remained altered and therefore pH dependent. Each value represents the mean ± S.D. ($n = 3$–4). Asterisk (∗) represents significant difference from control ($P < 0.05$).

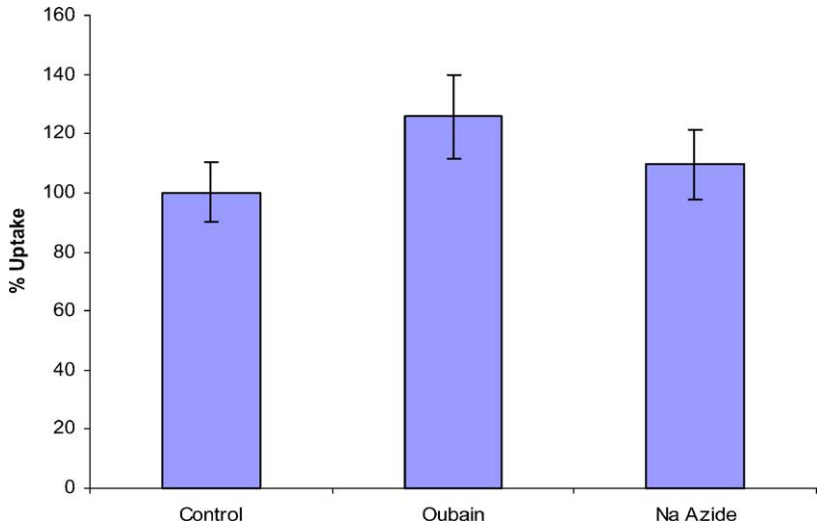

Fig. 8. Uptake of [^{3}H]L-Phe (0.25 μCi/ml) in ARPE-19 cells in absence and presence of metabolic inhibitors ouabain and sodium azide (1 mM) in sodium-free buffer. Uptake remained unaltered and therefore energy independent. Here, uptake is expressed as percentage of control. Each value represents the mean ± S.D. ($n = 3$–4).

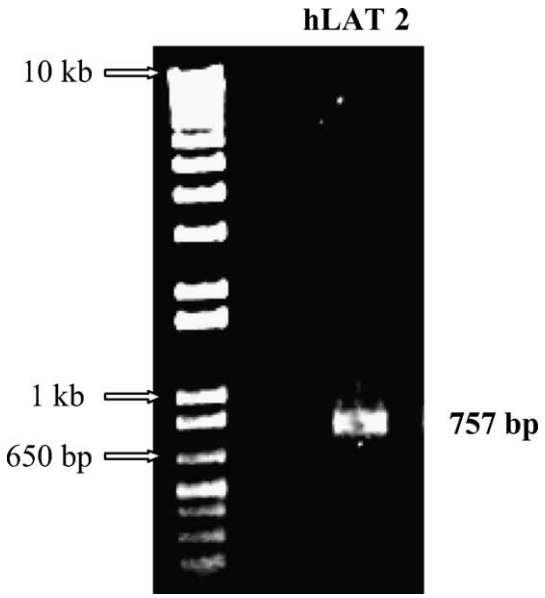

Fig. 9. LAT2 cDNA was generated by reverse transcription–PCR amplification of total RNA from ARPE-19. Aliquots of PCR products were analyzed by gel electrophoresis on 4% agarose and ethidium bromide staining of gel showed an ∼757-bp band corresponding to the amplified human LAT2 cDNA. The sizes (kb) of the DNA standards are shown on the left.

3.5. RT–PCR and sequencing

PCR products were analyzed by gel electrophoresis on 4% agarose. cDNA generated from total RNA isolated from ARPE-19 cells, was PCR amplified using the primers specific for human LAT2 sequence. A 757-bp product was obtained (Fig. 9) in ARPE-19 cells using primers specific for human LAT2. This fragment was sequenced in both directions. The sequence obtained showed maximum homology to hLAT2 using the BLAST search program (NCBI). This molecular evidence establishes the presence of LAT2, a large neutral amino acid transporter, in ARPE-19 cells.

4. Discussion

Peptide and amino acid transporters have been known to play an important role in tissue nutrition and regulation of endogenous and exogenous substances. The transporters have been classified into various families, depending on their numerous functions. These groups have been differentiated in terms of their transport mechanisms, ion coupling, and substrate specificities. In the present study, we have explored the possibility of the presence of these

carrier systems and have attempted to establish their existence by investigating their functional expression.

Presence of sodium-free buffer produced significant, but not complete inhibition. These results suggest the possibility of both, sodium-dependent and sodium-independent carrier system. In this study, an effort has been made to characterize the sodium-independent system by performing all experiments using sodium-free buffer.

Large neutral amino acid transporter was not previously reported on the ARPE cell line. The uptake of L-Phe was observed to be saturable in a concentration-dependent manner (Fig. 2). The saturation K_m of $89.35 \pm 14\,\mu M$ and V_{max} of $58.9 \pm 2.5\,pmol\,min^{-1}\,mg\,protein^{-1}$ were indicative of a high affinity low capacity transporter. L-Phe exhibited dose-dependent inhibition with $IC_{50} = 346.8\,\mu M$ (Fig. 3). This value also suggests high affinity of L-Phe for this transporter.

This K_m value for L-Phe is comparable with the lower K_m values (30–150 μM) that have been reported for LAT1 and LAT2 (Pineda et al., 1999; Segawa et al., 1999; Mastroberardino et al., 1998). Eadie–Hofstee transformation supports the possible presence of more than one carrier system. In addition to system L, it is unclear as to what other carrier system may be involved. This carrier system(s) did not involve much energy expenditure as metabolic inhibitors, ouabain and sodium azide, did not alter the L-Phe uptake (Fig. 8).

System L is a sodium-dependent transporter. The LAT2 isoform of system L exhibits broad substrate selectivity. LAT1, in contrast is more specific for large neutral amino acids with branched or aromatic side chains. This narrow substrate specificity has been associated with a higher affinity to neutral amino acids when compared with LAT2. Both LAT1 and LAT2 show sensitivity to L-type inhibitor BCH. The system A inhibitor NMeAIB, was not effective but we see that BCH caused partial reduction in uptake rate of [^{3}H]L-Phe. This confirms the presence of system L. This partial uptake can be inferred as contribution from a non-BCH sensitive carrier system. PCR studies (Fig. 9) and sequencing confirmed molecular evidence of LAT2. In this study, the functional evidence is more characteristic of LAT2. The presence of this BCH sensitive system on the ARPE-19 cell line cannot be ruled out.

Inhibition with L- and D-isomers of LNAAs was studied. A unique characteristic of LAT1 is its ability to interact with D-amino acids (Kanai et al., 1998). D-isomers of Leu and Phe are recognized as substrates by LAT1, while D-isomers of Val, His, Tyro, and Trypto are not (Yanagida et al., 2001). No stereospecificity was observed, as seen in Fig. 4 except in the case of Val and typical LAT2 substrate Leu, where inhibition was seen with both isomers. The most significant decrease in uptake was observed with L-Phe, L-Val, and D-Leu followed by L-Trypto, L-Leu, L-Tyr, and D-Val. A substrate for both L-isoforms, D-Leu, caused the highest inhibition compared with the other LNAAs. The D-isomers of Phe, Tyro, and Trypto did not produce any inhibition. These results, indicating a preference for D-Val and a lack of affinity for D-Phe, are in contrast with the observations reported previously for LAT1.

In Fig. 5, we see that neither basic amino acids like L-Lys nor acidic amino acids L-Glu and L-Asp have a significant effect on L-Phe uptake. Similar results were seen with glycine. Other small neutral amino acids like L-Ala showed a slight but significant difference. Maximum inhibition was observed with small neutral amino acid, Cyst, compared to all amino acids studied. The amino acid-type drug L-dopa which is known to be transported by system L (Gomes and Soares-da-Silva, 2002), also reduced influx of L-Phe. L-His, an aromatic amino acid, produced marginal inhibition of uptake. This observation was in accordance to previous report (Pineda et al., 1999), where L-His did limit LAT2-mediated transport. The results obtained so far indicates that the carrier(s) is more or less exclusive for large neutral and aromatic amino acids, as well as small neutral amino acids, like Cyst and Ala.

LAT1-mediated uptake is not supposed to be influenced by change in pH where as LAT2 is reported to accelerate uptake rate at lower pH. The uptake activity of L-Phe is highly affected between pH ranges 5.5 and 6 (Fig. 7). The transporter preferred uptake at acidic pH compared to neutral (7.4) and alkaline pH (8.5). This adds further evidence to the presence of LAT2.

Large neutral amino acid and the peptide transporters have been reported to have overlapping substrate specificities (Surendran et al., 1999). L-Phe uptake across ARPE-19 was investigated to check for any such occurrence. The presence of dipeptide Gly-Sar (Fig. 4) did not affect the rate of uptake. This

indicated a lack of affinity for dipeptides and a high degree of substrate selectivity of the transporter for amino acids.

In conclusion, this study demonstrates functional evidence of a high affinity, Na-independent Phe carrier system on ARPE-19 cell line. The Na-independent carrier predominantly has characteristics similar to that of LAT2. Further investigation is required to characterize the secondary non-BCH sensitive and the sodium-dependent component. The ARPE-19 cell line can thus be used as a valuable in vitro screening tool to determine affinities of different amino acid-type drugs for the amino acid transporters. The study provides useful information on the substrate specificity of these carrier systems. As RPE forms the outer blood–retinal barrier, the presence of these transporters on the RPE will offer newer strategies for the design of transporter-targeted prodrugs having enhanced permeability.

Acknowledgements

The research is been supported by National Eye Institute Grants R01 EY 10659-05 and R01 EY 09171-08.

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