Preliminary Investigation into the Expression of Proton-Coupled Oligopeptide Transporters in Neural Retina and Retinal Pigment Epithelium (RPE): Lack of Functional Activity in RPE Plasma Membranes

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Received February 18, 2003; accepted April 11, 2003

Purpose. To determine the expression and functional activity of proton-coupled oligopeptide transporters (POT) in retinal pigment epithelial (RPE) cells.

Methods. RT-PCR was used to probe the presence of POT mRNA in freshly isolated bovine RPE (BRPE) and human RPE (HRPE) cells, a human RPE cell line (ARPE-19), and human and bovine neural retina. [^{14}C]GlySar uptake was used to characterize POT activity in cultured ARPE-19 cells and freshly isolated BRPE cell sheet suspensions.

Results. PHT1 mRNA was expressed in BRPE, HRPE, ARPE-19, and bovine and human neural retina. In contrast, PEPT2 and PHT2 were expressed only in bovine and human retina, and PEPT1 could not be detected. GlySar exhibited a linear uptake over 6 h at pH values of 6.0 and 7.4, with greater uptake at pH 7.4 ($p < 0.01$). GlySar uptake did not exhibit saturability (5–2000 μM) and was unchanged when studied in the presence of 1 mM L-histidine. In contrast, GlySar uptake was significantly decreased when studied at 4°C or in the presence of endocytic inhibitors at 37°C ($p < 0.01$). Studies in BRPE cell sheet suspensions validated the results obtained in ARPE-19 cells and strongly suggested the absence of POT on the apical and basolateral membranes of RPE.

Conclusions. PHT1 mRNA is present in native bovine and human RPE and a human RPE cell line. However, the data argue against PHT1 being expressed on plasma membranes of RPE. Overall, GlySar appears to be taken up by RPE cells via a low-affinity, endocytic process.

KEY WORDS: PEPT1; PEPT2; PHT1; PHT2; RPE.

INTRODUCTION

The retinal pigment epithelium (RPE) is a polarized epithelial monolayer that separates the retina from the underlying choroid and plays a key role in regulating the microenvironment surrounding the photoreceptor outer segments (1,2). The RPE cells and their linking tight junctions form the outer blood-retinal barrier that limits the diffusion of small polar molecules between blood and retina. However, specific carrier systems at the apical and basolateral membrane surfaces allow the RPE to selectively transport nutrients, metabolites, and xenobiotics between the choroidal capillaries and cells of the distal retina (1,3–6).

In mammals, the POT family currently consists of four members (i.e., PEPT1, PEPT2, PHT1, PHT2) and is responsible for the symport of small peptides/mimetics across biologic membranes via an inwardly directed proton gradient and negative membrane potential. PEPT1 was first cloned from a rabbit intestinal cDNA library (7) and shown to be of high capacity and low affinity for di- and tripeptides (8,9). It is found primarily in the epithelia of intestine and, to a lesser extent, in kidney (10). PEPT2 was subsequently cloned from a human kidney cDNA library (11) and, in contrast to PEPT1, was found to be of low capacity and high affinity (12). It is the predominant POT in kidney. Together, PEPT1 and PEPT2 work in concert to efficiently absorb and conserve protein digestive products arising in the intestine and kidney. More recently, PEPT2 transcripts (13), protein (14,15), and functional activity (16,17) have been reported in choroid plexus, and, as a result, this transporter is believed to play a role in neuropeptide homeostasis and the efflux of peptides/mimetics from cerebrospinal fluid. PEPT2 mRNA is also expressed by glial cells in the retina (13); however, its subcellular distribution and functional activity have not been investigated.

Two peptide/histidine transporters, PHT1 (18) and PHT2 (19), were subsequently cloned from a rat brain cDNA library. The transporters are distinct from PEPT1 and PEPT2 in that they transport the amino acid L-histidine as well as di- and tripeptides. PHT1 and PHT2 share 49% amino acid identity, but homology to either rat PEPT1 or PEPT2 is less than 25%. Unfortunately, information on the tissue distribution, cellular localization, and transport properties of these two peptide/histidine transporters is limited. In this regard, it was reported that PHT1 transcripts are abundantly expressed in the brain and eye and that cloned PHT1 expressed in *Xenopus laevis* oocytes transports L-histidine with high affinity (K_m of 17 μM) (18). In contrast, PHT2 transcripts are abundant in the lung, spleen, and thymus, have been detected faintly in the brain, but have not been investigated in the eye (19). A proton-dependent uptake of L-histidine and histidyl-leucine was observed when rat PHT2 protein was reconstituted into liposomes, although the transport kinetics were not determined. Thus, the physiologic role of specific POT family members in the retina and RPE is unclear.

In the current study, we investigated which POT members are present in RPE and retina and the potential functional role of POT transporters in the RPE. Specifically, we used RT-PCR to determine the presence of specific POT mRNA in freshly isolated bovine RPE (BRPE) and human RPE (HRPE) cells, a human RPE cell line (ARPE-19), as well as bovine and human neural retina. The functional activity of POT was characterized in ARPE-19 cells by uptake assays with the model dipeptide GlySar and validated in freshly isolated BRPE cell sheet suspensions.

MATERIALS AND METHODS

All animal procedures were approved by the University of Michigan's Unit for Laboratory Animal Medicine and adhered to the "Principles of Laboratory Animal Care" (NIH

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0724-8741/03/0900-1364/0 © 2003 Plenum Publishing Corporation

publication 85-23, revised 1985). The isolation of human tissues adhered to the tenets of the Declaration of Helsinki promulgated in 1964 with informed consent being obtained.

Materials

[^{14}C]Glycylsarcosine (GlySar; 106 mCi/mmol) was purchased from Amersham Biotechnologies (Chicago, IL) and [^{14}C] D-mannitol (50 mCi/mmol) from Moravek Biochemicals (Brea, CA). Dispase-II enzyme was purchased from Roche Molecular Diagnostics (Indianapolis, IN), and Chee's Essential Media (CEM) from Kemp Biotechnologies (Frederick, MD). Primers for the RT-PCR analyses were synthesized by Invitrogen Life Technologies (Carlsbad, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Cultures

ARPE-19 cells were obtained from ATCC (Rockville, MD). Cells were grown in 75 cm^2 cell culture flasks (BD Biosciences, San Jose, CA) and were cultured in 1:1 DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 56 mM sodium bicarbonate, 2 mM L-glutamine, 15 mM HEPES buffer, 10% fetal bovine serum (Invitrogen), and 1% penicillin G (100 IU/ml)/streptomycin (100 mg/ml) solution (Invitrogen). ARPE-19 cell monolayers were given fresh complete medium every 3 to 4 days and were allowed to grow until they reached approximately 80–90% confluency, as determined by visualization with a light microscope. Subconfluent cultures were passaged by dissociation in 0.05% trypsin and 0.53 mM EDTA in Ca^{2+}- and Mg^{2+}-free Hanks buffered saline solution and subsequently plated on to 24-well cell culture clusters (Costar) at a seeding density of 1.66×10^5 cells/cm^2. ARPE-19 cells were used for experiments between passages 21 and 30 and 7 to 8 days after initial seeding.

RT-PCR Analysis of POT mRNA Expression

RT-PCR was performed on ARPE-19 cells (grown in standard propagation media or CEM), and freshly isolated RPE and neural retina samples from bovine and human sources. In brief, mRNA expression of PEPT1, PEPT2, PHT1, and PHT2 was evaluated using the GeneAmp PCR System® (Perkin Elmer, Boston, MA). Total RNA was isolated from ARPE-19 cells and from freshly isolated human and bovine samples with TRI Reagent® (Molecular Research Center, Cincinnati, OH), according to the manufacturer's protocol. RNA was reverse-transcribed in a 20-μl reaction mixture using 200 U of SuperScript II® reverse transcriptase and oligodT as a primer. cDNA amplification was performed using specific 5′ and 3′ primers for the four oligopeptide transporters. The primers (listed in Table I) were designed using a multiple-sequence alignment program (Clustal method with weighted residue weight table) and the known DNA sequences from human, rat, mouse, and rabbit PEPT1 and PEPT2, and the known sequences from human and rat PHT1 and PHT2. PCR was performed in a standard 50-μl reaction mixture containing 5 U of *Taq* DNA polymerase, 10 pmol each of the 5′ and 3′ primers for each POT, 10 mM deoxytriphosphate nucleotide mixture, and 1.5 mM $MgCl_2$. PCR was also conducted on all cDNA sample preparations using 10 pmol of the 5′ and 3′ primers for human β-actin (i.e., internal control). The positive controls for each peptide transporter were mouse small intestine (PEPT1), mouse kidney (PEPT2), rat choroid plexus (PHT1), and rat lung (PHT2). The negative controls, lacking sample cDNA, were run in an identical manner. The PCR cycles consisted of denaturing at 94°C for 30 s, annealing of primers at 48°C for 30 s, primer extension at 74°C for 1 min, and were repeated 40 times. The amplified products were separated on a 1.2% agarose gel and visualized with ethidium bromide.

Time Course of GlySar Uptake

GlySar uptake was measured in ARPE-19 cells using transport buffer that contained 122 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 0.4 mM $K_2HPO_4 \bullet 3H_2O$, 1.4 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.4) or 117 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.78 mM NaH_2PO_4, 1.80 mM $CaCl_2$, 5.55 mM glucose, and 15 mM MES (pH 6.0). ARPE-19 cell monolayers were preincubated with 1.0 ml of transport buffer (pH 7.4 or 6.0) for 10 min at 37°C and 5% CO_2. The buffer was then removed, and the time course study (up to 24 h) was initiated by the addition of 1.0 ml of the appropriate transport buffer containing 0.1 μCi

Table I. Primers for RT-PCR Analysis

	PEPT1
Sense	5′-CAC TGG CTG GAC TGG GCT AA-3′
Antisense	5′-GGA AGA GTT TTA TCG ATT TCC ACC TGC A-3′
	PEPT2
Sense	5′-CCA TCA ATG CAG GGA GCT TGA TTT C-3′
Antisense	5′-ACC TGC ATC TGA TCC GGC TGG AA-3′
	PHT1
Sense	5′-AGC CTC CTG ATG GCA GTG CCT T-3′
Antisense	5′-GCA TTT GCA AAT ACA CTG TCC AGT AAG-3′
	PHT2
Sense	5′-TTC CAG ATG CAG TCC ACC TAT GT-3′
Antisense	5′-AGG CAG AAG AAG AAG ATG CCC ATG AT-3′
	β-Actin
Sense	5′-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3′
Antisense	5′-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3′

of [^{14}C]GlySar (final concentration 10 μM). To measure intracellular accumulation of GlySar, the buffer was aspirated at the end of the incubation period, and the cells were rapidly washed three times with ice-cold buffer. The cells were then solubilized with 1 ml of 1% SDS/0.2 M NaOH and transferred to scintillation vials. EcoLite(+) (5 ml) (ICN, Irvine, CA) was added to all the samples, which were counted on a dual-channel liquid scintillation counter (Beckman LS 3801, Fullerton, CA). The nonspecific component (i.e., nonspecific binding) of GlySar uptake was determined by measuring the amount of radiolabel uptake in the presence of an excess amount (50 mM) of unlabeled substrate. This value was subtracted from the overall uptake to calculate the specific uptake for GlySar. As a control, similar studies were performed with 0.1 μCi of [^{14}C] D-mannitol (10 μM), a nontransported compound.

Concentration-Dependent Studies with GlySar

The potential for concentration-dependent uptake of GlySar and mannitol was investigated in ARPE-19 monolayers over a concentration range of 5–2000 μM, at pH 6.0 and 7.4. The amount of radiolabeled GlySar and mannitol remained constant at 0.1 μCi/well for all concentrations of substrate studied. As described previously, cells were preincubated with 1.0 ml of the respective uptake buffer for 10 min at 37°C and 5% CO_2. The experiment was then initiated by aspirating the buffer and adding transport buffer containing the specific substrate concentration for 3 h (uptake for GlySar and mannitol was linear for 6 h) at 37°C and 4°C. The intracellular accumulation for GlySar and mannitol was determined in the absence and presence of excess (50 mM) unlabeled substrate.

Effect of L-Histidine on GlySar Uptake

Transport buffer (pH 6.0 and 7.4) containing unlabeled 1 mM L-histidine and 10 μM radiolabeled GlySar was added to ARPE-19 monolayers grown on cell culture clusters and incubated for 3 h at 37°C and 5% CO_2. This experiment was performed in order to determine if L-His could inhibit the uptake of GlySar and, as a result, provide functional evidence for the presence of either PHT1 or PHT2 on the apical membrane of ARPE-19 cells.

Effect of Endocytic Inhibitors on GlySar Uptake

Stock solutions were prepared by dissolving cytochalasin B (CCB) in ethanol and phenylarsine oxide (PAO) in dimethyl sulfoxide at concentrations of 5 mM and 50 mM, respectively. Appropriate volumes of each stock solution were added to the corresponding uptake buffer (pH 6.0 and 7.4) to achieve a final concentration of 10 μM for each inhibitor. ARPE-19 monolayers were preincubated for 30 min at 37°C and 5% CO_2 with transport buffer containing either CCB or PAO. After the preincubation period, buffer was aspirated, and the experiment initiated by the addition of transport buffer containing radiolabeled and cold GlySar (i.e., 5–2000 μM) and either endocytic inhibitor. The cells were then incubated for 3 h at 37°C and 5% CO_2. Each endocytic inhibitor was studied at 10 μM because this concentration can fully inhibit endocytosis in other experimental cell culture systems (20,21).

Time Course of GlySar Uptake in BRPE Cell Sheet Suspensions

BRPE cell sheet suspensions were prepared, as described previously (22). In brief, 10 adult bovine eyes were obtained from a local slaughterhouse, and the eyes were enucleated within 1 h of death and placed on ice. After removal of the anterior segment, lens, vitreous, and neural retina, RPE cells were dissociated from the eye by treatment with a 1% Dispase-II solution at 37°C and 5% CO_2 for 1 h. The enzyme solution was then removed, and a HEPES-buffered Ringer solution consisting of 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 1.8 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (titrated to pH 7.4 with NaOH) was added to the eye cups. The RPE cell sheets were removed from Bruch's membrane by gentle pipetting with a Pasteur pipette. The BRPE cell sheets were quickly washed and then aliquoted into preweighed plastic microcentrifuge vials and centrifuged at 1000 rpm for 1 min. The resulting supernatant was removed, and the wet tissue weight of the cell sheets was determined. To each vial, 0.95 ml of pH 7.4 or pH 6.0 transport buffer (described previously) was quickly added and incubated at 37°C and 5% CO_2 for 10 min. After the 10-min preincubation, 50 μl of a concentrated transport buffer solution (200 μM of [^{14}C]GlySar and unlabeled GlySar) was added to each vial to initiate the uptake experiment and achieve a final GlySar concentration of 10 μM. The uptake experiment was terminated by filtering the cell suspensions on a 0.2-μm nylon filter and washing the filter three times with ice-cold buffer. The cells attached to the filter were then transferred to scintillation vials and solubilized with 1 ml of 1% SDS/0.2 M NaOH for 1 h. Ecolite(+) was added, and the radioactivity associated with the filter was determined by liquid scintillation counting. The nonspecific binding of GlySar was determined in the presence of 50 mM unlabeled substrate and was subtracted from the overall uptake value. A correction was also made for filter binding in which 1 ml of transport buffer (containing 10 μM GlySar ± 50 mM unlabeled substrate) was filtered in the absence of BRPE cells and then washed three times with ice-cold buffer.

Protein Assay

The protein content of the solubilized ARPE-19 cell monolayers was determined after each experiment by the method of Bradford (23) using the DC Bio-Rad protein assay kit, with bovine γ-globulin as the standard. Protein standards and samples were run in triplicate.

Data Analysis

Data are reported as mean ± SE of four independent experiments, with each experiment being run in triplicate. The observed uptake of GlySar and mannitol were standardized for total cellular protein (mg) in ARPE-19 cells and for wet tissue weight (mg) of BRPE cell sheets. Statistical analyses of two unpaired sample populations were performed using analysis of variance (ANOVA), and the slopes of linear regression analyses were compared using analysis of covariance [SPSS for Windows, v9.0.1, Chicago, IL]. A probability of $p \leq 0.05$ was considered statistically significant.

RESULTS

RT-PCR Analysis of POT Expression in the RPE and Retina

Total RNA isolated from bovine and human RPE, ARPE-19 cells, and bovine and human retina was examined for PEPT1, PEPT2, PHT1, and PHT2 mRNA expression (Fig. 1, Table II). The results demonstrate that PEPT1 transcripts (Fig. 1A) were lacking in bovine and human samples of RPE and neural retina. Although PEPT2 (Fig. 1A) and PHT2 mRNA (Fig. 1B) were absent from bovine and human RPE, both transporter transcripts were present in the neural retina of both species. In contrast, PHT1 mRNA (Fig. 1B) was expressed in bovine and human RPE, ARPE-19 cells, and bovine and human neural retina. ARPE-19 cells grown in CEM had the same PCR products as ARPE-19 cells grown in standard media (gel not shown). β-Actin internal controls were run for all cDNA sample preparations (Fig. 1C), and negative controls were included on the gels (not shown).

Functional Characterization of Dipeptide Uptake in ARPE-19 Cells

GlySar, a hydrolysis- and peptidase-resistant model dipeptide, was used to probe POT activity in ARPE-19 cells. This dipeptide exhibited a linear cellular uptake for at least 6 h, with accumulation continuing for up to 24 h (Fig. 2A). Intracellular accumulation was significantly greater at pH 7.4 than at pH 6.0 ($p < 0.01$; Fig. 2B), a finding opposite to that expected for a proton-coupled oligopeptide transporter. In contrast, mannitol uptake was pH-independent and significantly less than that of GlySar at both pH values ($p < 0.01$; Fig. 2B), indicating that the observed uptake of GlySar was not mediated by a simple diffusional process. Time course studies were also performed with 10 μM GlySar (at pH 6.0 and 7.4) on ARPE-19 cells grown in CEM, a defined medium that has been reported to support the polarized expression of several transport proteins on the apical membrane in RPE cell cultures (24). The results demonstrated the same pH dependence and uptake profile for GlySar over 24 h, with values statistically no different from cells grown in standard propagation media (data not shown). Therefore, ARPE-19 cells, cultured in standard propagation media, were used for all functional activity studies.

There was no evidence for a saturable uptake of GlySar or mannitol over the concentration range of 5–2000 μM at either pH 7.4 or 6.0 (Fig. 3). However, the cellular accumulation of GlySar but not mannitol decreased by 78% (pH 7.4) or 62% (pH 6.0) when the temperature was reduced to 4°C ($p < 0.01$). Thus, GlySar appears to be actively transported at 37°C, which is dramatically limited at the lower temperature of 4°C.

As a final test for functional PHT1 in the plasma membrane, we measured the GlySar uptake in the presence of 1 mM L-histidine. Because PHT1 transports histidine with high affinity (18), this concentration should be more than sufficient to compete with GlySar for cellular uptake if PHT1 were indeed present. The observed uptake values of GlySar, at pH 7.4 or 6.0, were not statistically different when studied in the absence and presence of excess concentrations of L-histidine (Fig. 4). This finding suggests that PHT1 is not present on the apical membrane.

Mechanism for GlySar Uptake in ARPE-19 Cells

Because the uptake of GlySar in ARPE-19 cells was neither enhanced by low pH nor saturable, it is highly unlikely that a POT is involved in the uptake process. Moreover, PHT1 and PHT2 are not involved in GlySar uptake, given the lack of inhibition by histidine. Yet, the temperature-dependent uptake of GlySar and its significantly greater accumulation over mannitol suggest a specific mechanism for uptake. Therefore, the potential role of endocytosis was examined using two different endocytic inhibitors, cytochalasin B (CCB) (20) and phenylarsine oxide (PAO) (21). The addition of either 10 μM CCB or 10 μM PAO to the transport buffer significantly reduced the uptake of GlySar at 37°C (at pH 7.4 and 6.0) over the concentration range 5–2000 μM ($p < 0.01$; Fig. 5). In fact, GlySar uptake at 4°C was essentially the same as that obtained for GlySar at 37°C when in the pres-

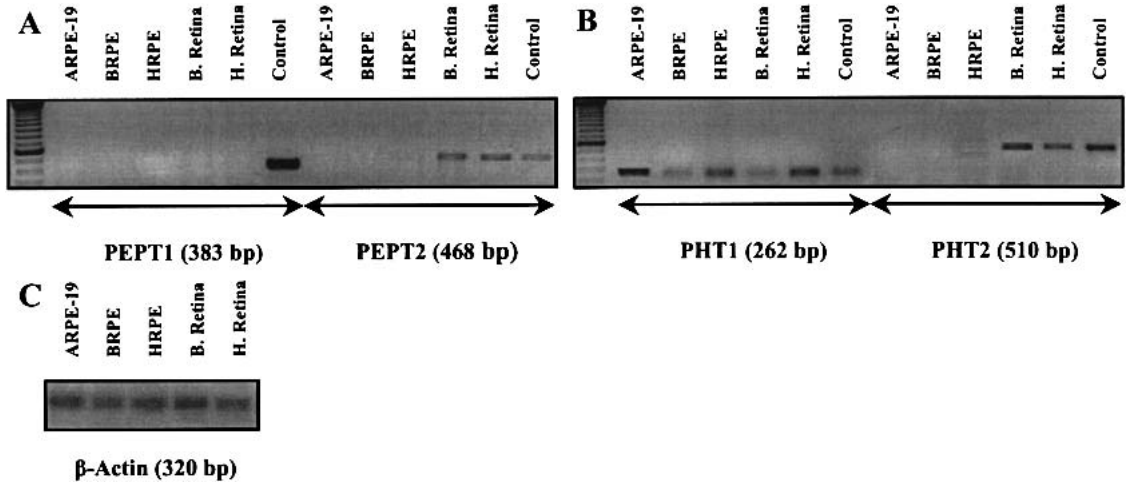

Fig. 1. RT-PCR analysis of POT mRNA in RPE and neural retina (3 μg total RNA). Samples were run on a 1.2% agarose gel and visualized with ethidium bromide. A, Screening for PEPT1 and PEPT2 transcripts. B, Screening for PHT1 and PHT2 transcripts. C, β-Actin controls for ARPE-19, BRPE, HRPE, B. (bovine) retina and H. (human) retina cDNA samples. Not shown are ARPE-19 cells grown in CEM, which exhibited the same products observed for ARPE-19 cells grown in standard propagation media.

Table II. RT-PCR Screening for POT mRNA in the RPE and Retina

Sample	PEPT1	PEPT2	PHT1	PHT2
BRPE			X[a]	
HRPE			X	
ARPE-19			X	
ARPE-19 (CEM)[b]			X	
Bovine retina		X	X	X
Human retina		X	X	X

[a] X indicates the presence of a PCR product.
[b] ARPE-19 cells grown in Chee's Essential Media (CEM).

ence of these two inhibitors. A pH-dependent uptake of GlySar was not observed in the presence of either CCB or PAO, unlike the results obtained with GlySar in the absence of endocytic inhibitors at 37°C (Figs. 2 and 3). Thus, it appears that a low-affinity endocytic process is primarily responsible for GlySar uptake in the RPE, one that is both pH- and temperature-dependent (i.e., less endocytosis at lower pH and temperature).

Time Course of GlySar Uptake in BRPE Cell Sheet Suspensions

The above studies focused on the apical membrane of ARPE-19 cells because previous studies have shown that oligopeptide transporters (namely PEPT1 and PEPT2) are present in apical membranes of epithelial cell lines (Caco-2 and SKPT) (25) and choroid plexus epithelium (15). However, the functional data from ARPE-19 cells argue against PHT1 being present in the apical membrane. As a result, we examined the potential localization of PHT1 on basolateral membranes. As shown in Fig. 6A, the time course profiles of GlySar uptake in BRPE cell sheet suspensions (at pH 7.4 and 6.0) were very similar to the profiles observed in ARPE-19 cells (Fig. 2A). In this regard, GlySar uptake appeared linear over the time range studied, with a statistically greater amount of uptake occurring at pH 7.4 than at pH 6.0 ($p < 0.05$; Fig. 6B). Thus, in freshly isolated BRPE cells with an exposed basolateral membrane, the same pH-dependent uptake of GlySar to that found in ARPE-19 cells was observed. Again, this result is contrary to what would be expected if a POT were responsible for uptake on either the apical or basolateral membrane of the cell.

DISCUSSION

Among the many diverse functions of the RPE is the vectorial transport of nutrients and metabolites between the choroidal circulation and the neural retina. This is accomplished by a wide array of specialized transporters located on distinct apical and basolateral membranes of the cell. Previous studies have reported the presence of PEPT2 and PHT1 mRNA in retina (13) and eye (18), respectively. However, it is unclear whether or not these same transporters or, perhaps, other members of the POT family are expressed in RPE. Moreover, their functional role has not been investigated in this tissue. In theory, oligopeptide transporters could have a profound impact on the trafficking of small peptides and peptide-like drugs from systemic blood to retina or, conversely, from retina to blood. For example, valganciclovir, which is used for the treatment of cytomegalovirus retinitis, is also a substrate for PEPT1 and PEPT2 (26). Thus, identifying which POT members are present and active in RPE could provide a new target to exploit for drug delivery purposes.

In the present study, we demonstrated that although PEPT2, PHT1, and PHT2 transcripts are expressed in bovine and human retina, PHT1 is the only known POT present in the RPE of both species. However, results from GlySar uptake studies in ARPE-19 cells and BRPE cell sheet suspensions provide strong evidence that RPE cells do not express any oligopeptide transporter, including PHT1, on either the apical or basolateral membrane. If a POT were expressed on the cell membrane, one would expect to see saturation in GlySar uptake along with a significant increase in uptake at pH 6.0 as compared to pH 7.4. Instead, there was no evidence of capacity-limited (i.e., saturable) kinetics, and a greater uptake was observed for GlySar at pH 7.4. The fact that GlySar uptake was not inhibited by an excess concentration of L-histidine provides additional evidence for PHT1 being absent on the apical membrane of RPE. Although GlySar was not specifically studied by Yamashita *et al.* (18), they reported that L-histidine and carnosine were transported by *Xenopus* oocytes expressing PHT1. Moreover, L-histidine uptake was significantly inhibited by several dipeptides, including glycyl-

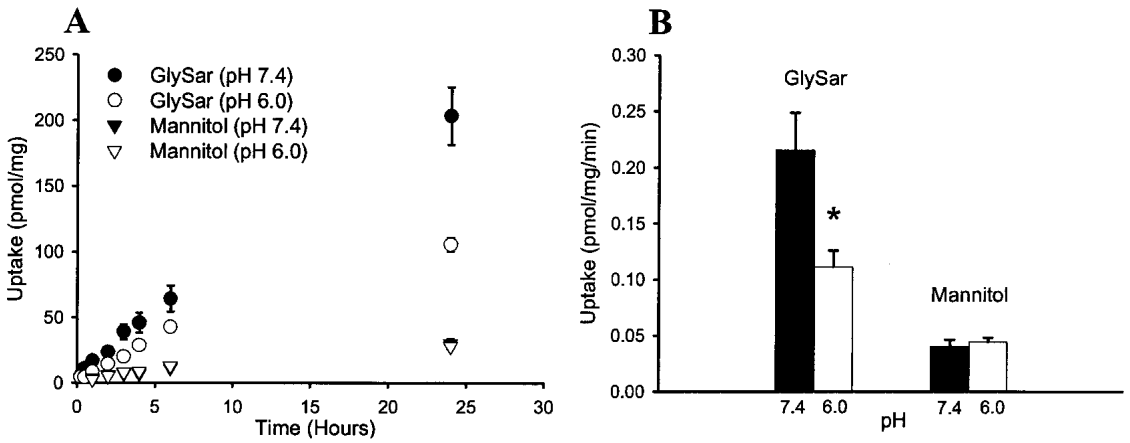

Fig. 2. A, Time course for the apical uptake of GlySar (10 μM) and mannitol (10 μM) in ARPE-19 cell monolayers at 37°C. B, Effect of pH on the 3-h apical uptake of GlySar and mannitol. *$p < 0.01$, for pH 6.0 vs. pH 7.4. Data reported as mean ± SE (n = 4).

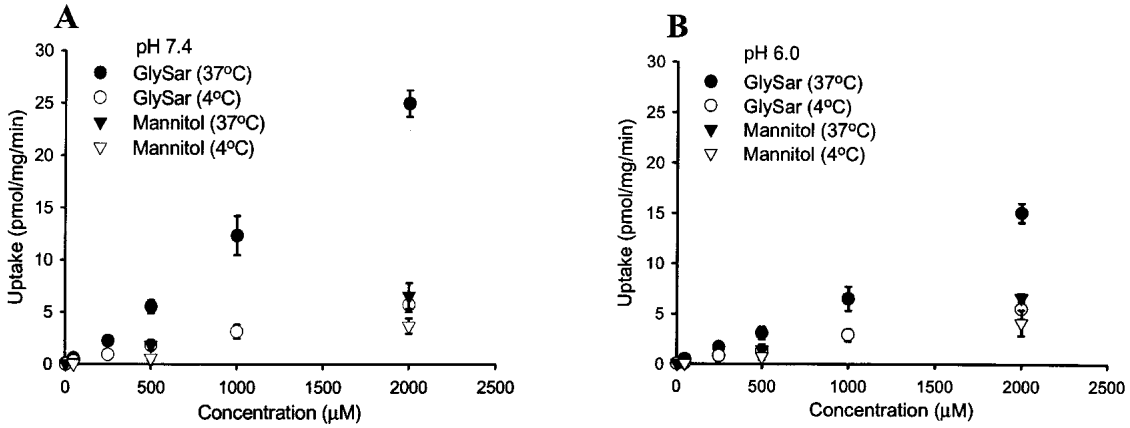

Fig. 3. Effect of concentration and temperature on the 3-h apical uptake of GlySar and mannitol in ARPE-19 cell monolayers at (A) pH 7.4 and (B) pH 6.0. GlySar uptake was significantly reduced by lower temperature at pH 7.4 and pH 6.0 ($p < 0.01$). No temperature differences were observed for mannitol uptake at either pH. Data reported as mean ± SE (n = 4).

glycine (GlyGly). Given these findings, it is reasonable to assume that GlySar would also be transported by PHT1, if present.

Several slice variants (A, A′, and B) of human PHT1 were described by Botka *et al.* (27), and the nucleotide sequence data of a peptide/histidine transporter variant in brain (i.e., human PTR4) was recently deposited in GenBank (accession number AY038999). Although our PHT1 primers would have potential cross-reactivity with variants A and A′ of PHT1, they would not cross-react with variant B. These primers would also have potential cross-reactivity with PTR4. Notwithstanding this uncertainty, the PHT1 variants (including PTR4) are putative transporters at present because they have not been cloned and/or tested for functional activity. Our PEPT1 primers would not cross-react with the human PEPT1 splice variant, PEPT1-RF (28). This variant is a pH-sensing regulatory factor that modulates PEPT1 activity but, when expressed alone, lacks transport function.

The decreased uptake of GlySar at lower pH or temperature values in ARPE-19 cells, along with its greater uptake over mannitol, suggested the presence of a specific (i.e., non-diffusional) transport process. In this regard, investigators had reported an endocytic mechanism that was less efficient at acidic pH for horseradish peroxidase uptake (29), and at reduced temperature for liposomal uptake (30) into cells. To test our hypothesis that endocytosis might be involved in the uptake of GlySar in RPE, we chose two inhibitors with different mechanisms of action for study. CCB, an inhibitor of microfilament-dependent processes (20), significantly decreased the uptake of GlySar by the ARPE-19 cell line. Likewise, PAO, an inhibitor of clathrin-dependent processes (21), reduced the uptake of GlySar to values obtained in the absence of endocytic inhibitors at 4°C. Taken together, these results suggest strongly that the majority (if not all) of GlySar's intracellular accumulation in ARPE-19 cells occurs by a low-affinity, nonsaturable endocytic process. It should be appreciated that the rate of GlySar uptake by this endocytic mechanism is much less than that expected by a POT-mediated mechanism. At physiologic pH, GlySar (10 μM) was transported at about 0.2 pmol/mg/min in ARPE-19 cells (this study) compared to about 8 pmol/mg/min when present at 2 μM in rat choroid plexus epithelial cells in primary culture (15). Corrected for concentration differences, GlySar uptake is about 200× lower in RPE than in choroid plexus. It is of interest to note this difference in transport considering that both tissues are derived from neuroepithelium. Of course, species differences (human vs. rat) and methodologic conditions (cell line vs. primary cell culture) may also influence this comparison.

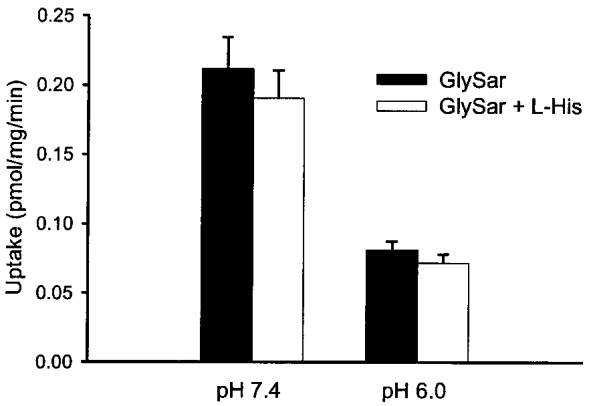

Fig. 4. Effect of 1 mM L-histidine on the 3-h apical uptake of GlySar (10 μM) in ARPE-19 cell monolayers at pH 7.4 and pH 6.0 (37°C). No significant differences were observed at either pH. Data reported as mean ± SE (n = 4).

ARPE-19 cells have been reported to have structural and functional properties that are characteristic of RPE cells *in vivo* (31). Additionally, ARPE-19 cells exhibit a polarized distribution of cell surface markers and, thus, have been proposed as a suitable *in vitro* model for HRPE cells (32). Despite these favorable characteristics, there is always some concern that the observed lack of functional uptake for GlySar via PHT1 may be limited to this cell line. Experiments were, therefore, also performed in freshly isolated BRPE cell sheet suspensions. Qualitatively, the results were very similar to those obtained in ARPE-19 cells. These experiments also demonstrated that PHT1 was not involved in GlySar uptake across the basolateral membrane, a transport site that would have been inaccessible in the ARPE-19 studies.

At this point, the role of PHT1 in the RPE is uncertain. Although PHT1 mRNA is expressed in RPE, our functional

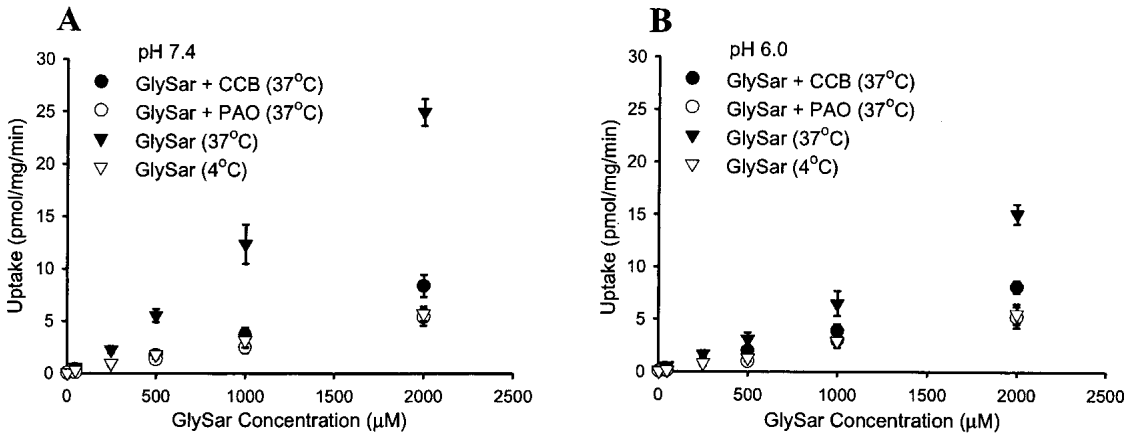

Fig. 5. Effect of cytochalasin B (CCB; 10 μM) and phenylarsine oxide (PAO; 10 μM) on the 3-h apical uptake of GlySar (5–2000 μM) in ARPE-19 cell monolayers (37°C) at (A) pH 7.4 and (B) pH 6.0. GlySar uptake was significantly reduced by endocytic inhibitors (CCB or PAO) at pH 7.4 and pH 6.0 ($p < 0.01$). Data reported as mean ± SE (n = 4).

studies with GlySar in ARPE-19 cell cultures and native bovine RPE sheets do not support its presence at either the apical or basolateral membrane. Given these two findings, we speculate that PHT1 is located on the membrane of an intracellular organelle such as a lysosome and is involved in the intracellular trafficking of small peptides. Such a hypothesis is supported by the lack of functional activity for peptide/histidine transporters (in contrast to PEPT2) in the uptake of GlySar in freshly isolated rat choroid plexus (16) and primary cell cultures of rat choroid plexus epithelium (15). Additional support is provided by the observed lysosomal localization of rPHT2 protein in transfected HEK-293T and BHK cells (19). Still, other studies have reported the existence of a low-affinity dipeptide transporter in rat liver and kidney lysosomal membranes that appears to belong to the POT family (33,34). These results suggest an extended role of oligopeptide transporters, where they not only transport small peptides and peptidomimetic compounds from the extracellular environment across the plasma membrane but may also be involved in trafficking and transport within the cell.

The known cellular characteristics of RPE cells lend credence to the need for PHT1 in the intracellular milieu. RPE cells are rich in smooth endoplasmic reticulum and lysosomes, reflecting the high metabolic rate of this tissue (35). RPE cells function in the internalization and subsequent degradation of rod outer segment fragments, which are shed continuously from the ends of photoreceptors (36). With the relatively large amount of protein taken in by the RPE, and the corresponding large amount of protein degradation that is occurring, there may be a situation created that requires an oligopeptide transport system that can efficiently translocate the end products of protein degradation from inside the lysosome to the cytosol. This would help to prevent osmotically-induced swelling and rupture of lysosomes.

In conclusion, this study is the first to demonstrate that several proton-coupled oligopeptide transporters are present in the RPE and neural retina. Although PEPT2, PHT1, and PHT2 mRNA are expressed in bovine and human retina, PHT1 is uniquely found in RPE. However, a POT-mediated uptake of the dipeptide GlySar was conspicuously absent in ARPE-19 cells and bovine RPE cell sheet suspensions. Instead, it appears that GlySar enters RPE cells by a low-affinity, endocytic mechanism. Moreover, the lack of functional activity at the apical and/or basolateral membrane of RPE cells suggests an intracellular localization of PHT1, perhaps in lysosomes. Based on the molecular and functional results in ARPE-19 cells, this cell line seems to be a suitable *in vitro* model for subsequent studies of PHT1 in human RPE.

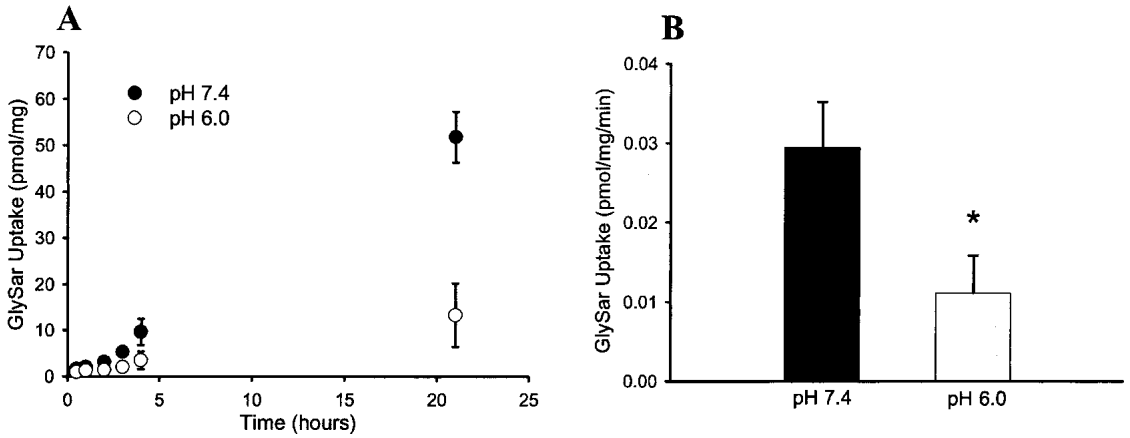

Fig. 6. A, Time course for the uptake of GlySar (10 μM) in BRPE cell sheet suspensions (37°C) at pH 7.4 and pH 6.0. B, Effect of pH on the 3-h uptake of GlySar. *$p < 0.05$ for pH 6.0 vs. pH 7.4. Data reported as mean ± SE (n = 4).

In particular, the preparation of lysosomal membrane vesicles will allow one to test whether or not PHT1 is located intracellularly and is responsible for exporting histidine, di- and tripeptides, and peptidomimetic compounds from the highly acidic environment of the lysosome to the more physiologic pH of the cytosol.

ACKNOWLEDGMENTS

This work was supported in part by grants R01GM035498 (D.E.S.), R01NS034709 and P01HL018575 (R.F.K.), and R01EY008850 and P30EY007003 (B.A.H.) from the NIH and the Foundation Fighting Blindness (B.A.H.). Scott M. Ocheltree was supported by an AFPE Pre-Doctoral Fellowship and the Pharmacological Sciences Training Program of the National Institutes of Health (GM07767).

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