ABSTRACT

PEPT2 expression has been established in brain and, in particular, mRNA transcripts and PEPT2 protein have been identified in choroid plexus tissue. However, there is little evidence for the functional presence of this peptide transporter in choroid plexus tissue. In this study, we examined the in vitro uptake of a model dipeptide, glycylsarcosine (GlySar), with whole tissue rat choroid plexus in artificial cerebrospinal fluid. Our findings are consistent with the known transport properties of PEPT2, including its proton dependence, lack of sodium effect, specificity, and high substrate affinity for dipeptides. Kinetic analysis showed saturable transport of GlySar with a Michaelis constant ($K_m$) of $129 \pm 32 \mu M$ and a maximum velocity ($V_{\text{max}}$) of $52.8 \pm 3.6 \text{ pmol/mg/min}$. GlySar uptake ($1.88 \mu M$) was not inhibited by 1.0 mM concentrations of amino acids (glycine, sarcosine, l-histidine), organic acids and bases (4-acetamido-4'-isothiocyanatoestilbene-2,2'-disulfonic acid, tetraethylammonium), non-$\alpha$-amino cephalosporins (cephaloridine, cephalothin), or non-$\alpha$-amino cephalosporins (cefadroxil, cephealin) inhibited the uptake of GlySar by 85 to 90% at 1.0 mM. These findings indicate that PEPT2 is functionally active in choroid plexus and that it might play a role in neuropeptide homeostasis of cerebrospinal fluid. The ability of PEPT2 to transport drugs at the choroid plexus also may be important for future drug design, delivery, and tissue-targeting considerations.

The peptide transporters PEPT1 and PEPT2 mediate the transport of small peptides, peptide fragments, and peptidomimetics across biological membranes. Initial findings in rat kidney and intestine indicate that these transporters are located on the brush-border membrane (Shen et al., 1999) and that they facilitate the movement of small peptides and peptide-like compounds from the lumen to the cytosol (Daniel, 1996). These peptide transporters are proton-coupled (Ganapathy and Leibach, 1983; Daniel, 1996; Chen et al., 1999) and are not sodium dependent (Ganapathy and Leibach, 1983). PEPT1 is found primarily in the small intestine and, at low levels, in the proximal tubule of the kidney (Terada et al., 1997; Shen et al., 1999). It is characterized as a low-affinity, high-capacity transporter. In contrast, PEPT2 is characterized as a high-affinity, low-capacity transporter and is located primarily and abundantly in the kidney (Shen et al., 1999).

PEPT2 has been cloned from rat brain homogenate (Wang et al., 1998), whereas in situ hybridization analysis of rat brain has placed PEPT2 mRNA transcripts in the astrocytes, ependymal cells, and choroid plexus epithelial cells (Berger and Hediger, 1999). In contrast, PEPT1 mRNA appears to be absent in rat cerebellum (Fujita et al., 1999), and rat (Saito et al., 1995) and rabbit brain (Döring et al., 1998). Recently, the presence of PEPT2 and absence of PEPT1 proteins were confirmed in rat choroid plexus by Western blotting (Novotny et al., 2000). A third peptide transporter, peptide/histidine transporter (PHT1), transports histidine and small peptides with high affinity and in a proton gradient-dependent manner (Yamashita et al., 1997). Although expressed in the brain and eye, PHT1 is not found in the intestine or kidney and shows little homology to PEPT1 or PEPT2 (<20% amino acid identity). Its physiological role is yet to be determined. Likewise, no studies have, as yet, analyzed PEPT2 in brain tissue to establish the presence of a functional transporter or to evaluate its importance.

Peptide transport at the choroid plexus was initially demonstrated by Huang (1981, 1982) in his analysis of Tyr-o-Ala-Gly (TAG). In Huang (1981), TAG accumulation was inhibited by some metabolic inhibitors and peptides, but not by

ABBREVIATIONS: PHT1, peptide/histidine transporter; TAG, Tyr-o-Ala-Gly; CSF, cerebrospinal fluid; GlySar, glycylsarcosine; GlyGlyHis, glycyglycylhistidine; SITS, 4-acetamido-4'-isothiocyanatoestilbene-2,2'-disulfonic acid; TEA, tetraethylammonium; aCSF, artificial CSF; MES, 2-(N-morpholino)ethanesulfonic acid.
amino acids. Accumulation was unaffected by sodium ions, suggesting the presence of a sodium-independent transport system for peptides in the choroid plexus. In Huang (1982), it was shown that TAG accumulation occurred at the apical membrane of the choroidal epithelium. Further analyses have been reported regarding the saturable uptake of small peptides across the blood-brain and blood-cerebrospinal fluid (CSF) barriers (Banks and Kastin, 1987; Zlokovic et al., 1988, 1991), however, no specific transporters have been directly identified.

The presence of PEPT2 transporter in the choroid plexus is of considerable interest. The choroid plexus acts as the barrier between the blood and CSF, and it also is involved in CSF production (Bradbury, 1979). PEPT2 expression in choroid plexus could serve three distinct purposes. First, it could be a transport system to supply the CSF and brain with peptides for building neuropeptides. Second, it could facilitate the clearance of peptides from the CSF. Third, PEPT2 could be involved in peptide uptake for use in the choroid plexus itself. Any of these transport purposes could affect the disposition of drugs that are substrates for the PEPT2 transporter.

Thus, the functional properties of PEPT2-mediated transport were evaluated in rat with isolated choroid plexuses and a model dipeptide, glyylsarcosine (GlySar). Our findings are novel in demonstrating a pH but not sodium-dependent uptake of dipeptide, and a transport process that was saturable and specific for known substrates of the high-affinity, low-capacity symporter PEPT2. Therefore, the functional presence of PEPT2 has been established for the first time in whole tissue rat choroid plexus.

**Experimental Procedures**

**Materials.** [14C]GlySar (119 mC/mmol) was purchased from Amersham (Chicago, IL) and [3H]mannitol (19.9 Ci/mmol) from NEN Life Science Products (Boston, MA). Amino acids (glycine, sarcosine, l-histidine, cephalosporins (cephadroxil, cephalaxin, cephaloridine, cephalothin), peptides [GlySar, glyyl proline, glycyglycylhistidine (GlyGlyHis), carnosine], and organic acids and bases [4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) and tetraethylammonium (TEA), respectively] were purchased from Sigma (St. Louis, MO). Nitex nylon monofilament screening fabric (118-μm mesh opening) was purchased from Sefar America Inc. (Kansas City, MO). Other chemicals were obtained from standard sources and were of the highest quality available.

**Buffers.** Experiments were performed in bicarbonate artificial CSF (aCSF) or Tris-2-(N-morpholino)ethanesulfonic acid (MES) buffers. The bicarbonate aCSF buffers (~310 mM osmol/kg) were continuously bubbled with 5% CO2, 95% O2 and contained 127 mM NaCl, 20 mM NaHCO3, 2.4 mM KCl, 0.5 mM KH2PO4, 1.1 mM CaCl2, 0.5 mM Na2SO4, and 5.0 mM glucose (pH 7.3). In experiments where a low sodium buffer was used, NaCl and NaHCO3 were replaced by choline chloride and choline bicarbonate, respectively, producing a 1.0 mM sodium solution due to the presence of Na2SO4. The Tris-MES buffers (~320 mM osmol/kg) were bubbled with 100% O2 and contained 147 mM NaCl, 2.4 mM KCl, 0.5 mM KH2PO4, 1.1 mM CaCl2, 0.85 mM MgCl2, 0.5 mM Na2SO4, 5.0 mM glucose, and 10 mM Tris and/or MES. The pH of these solutions was varied from 5.5 to 8.0 with different combinations of Tris and MES, with osmolality being held constant. Choline chloride was used to replace NaCl in the low sodium (1.0 mM) Tris-MES buffers.

**GlySar Uptake.** Lateral ventricle choroid plexuses were isolated from anesthetized (pentobarbital; 65 mg/kg i.p.) male Sprague-Dawley rats aged 30 to 50 days. Detailed methods are described in Keep and Xiang (1995) for glutamine uptake. The lateral ventricle plexuses were weighed and transferred to bicarbonate aCSF buffer at 37°C. A 5-min recovery period was allowed before the beginning of an experiment. After the recovery period, the plexuses were transferred to 0.95 ml of buffer with or without drug for 0.5 min. Uptake was initiated by addition of 0.05 ml of buffer with approximately 0.1 μCi of [14C]GlySar and 0.2 μCi of [3H]mannitol (an extracellular marker). Unless stated, the uptake was terminated after 3 min by transferring the plexus to ice-cold aCSF buffer and filtering under reduced pressure. The filters (118-μm mesh) were washed three times with the same buffer. The filters and choroid plexuses were then soaked in 0.33 ml of 1 M hyamine hydroxide (a tissue solubilizer) for 30 min before the addition of scintillation cocktail (Cytoscient) and counting with a dual-channel liquid scintillation counter (Beckman LS 3801; Fullerton, CA).

The uptake of radiolabeled GlySar into choroid plexus, in microliters per milligram of wet tissue weight, was calculated according to the following equation (Keep and Xiang, 1995):

\[
\text{GlySar uptake} = \frac{St - Sf - [(Mt - Mf)] \cdot \text{ratio}}{Smedia}
\]

where \(St\) is the total substrate (GlySar) concentration in the plexus plus filter, \(Sf\) is the filter binding of substrate, and \(Smedia\) is the concentration of substrate in the external media. The term \((Mt - Mf) \cdot \text{ratio}\) is a correction for extracellular space, where \(Mt\) is the total mannitol concentration in the plexus plus filter and \(Mf\) is the filter binding of mannitol. Multiplying the difference between these two parameters by the ratio of [14C]GlySar to [3H]mannitol in the external medium provides an estimate of the extracellular content of GlySar. The unidirectional influx rate \((V)\) can then be calculated by multiplying GlySar uptake by Smedia and dividing by the duration of the experiment.

The rats were not exsanguinated before choroid plexus removal, raising the question of whether erythrocytes trapped in the choroid plexus might take up GlySar. However, measurement of erythrocyte uptake in vitro indicated that GlySar's rate of transport was about 1% of that by the isolated choroid plexus. In addition, choroid plexus uptake where rats were transeptally perfused to remove erythrocytes was not significantly different from that of nonexsanguinated rats (data not shown).

**Data Analysis.** For kinetic studies, the concentration-dependent uptake of GlySar was fit to the Michaelis-Menten relationship:

\[
V = \frac{V_{\text{max}} \cdot C}{K_m + C}
\]

where \(V_{\text{max}}\) is the maximal rate of GlySar uptake, \(K_m\) is the Michaelis constant, and \(C\) is the substrate (GlySar) concentration. To evaluate if more than one class of transporters was operational for GlySar, a Woolf-Augustinsson-Hofstee transformation was performed:

\[
V = \frac{V_{\text{max}} - K_m}{C}
\]

Statistical comparisons were performed with ANOVA (SYSTAT, version 8.0; SPSS Inc., Chicago, IL) and pairwise comparisons were made with Tukey’s test. A probability of \(P \leq .05\) was considered statistically significant. Linear and nonlinear regression analyses were performed with SCIENTIST (version 2.01; MicroMath Scientific Software, Salt Lake City, UT) and a weighting factor of unity. The quality of fit was determined by evaluating the coefficient of determination \((r^2)\), the standard error of parameter estimates, and by visual inspection of the residuals. Data are reported as mean ± S.E.
Results

Time Course and Buffer Analysis. The time-dependent uptake of GlySar is shown in Fig. 1. GlySar uptake was linear for about 5 min. The y-intercept being no different from zero ($P = .911$) indicates negligible nonspecific binding. Based on these results, an uptake time of 3 min was used for subsequent experiments to maximize radiotracer uptake while remaining within the linear region of uptake. GlySar uptake reached a plateau value of approximately 9.5 pmol/mg at 20 to 30 min into the experiment. Because the medium concentration of GlySar was 0.94 μM, this represents a tissue/medium ratio of 10:1. Thus, isotope was accumulated in the choroid plexus far above that in the medium, indicating an active uptake process. GlySar uptake values in sodium-containing and low sodium bicarbonate aCSF buffers suggested that there may be both Na$^+$-dependent and Na$^+$-independent GlySar transport (Fig. 2). Both sodium-containing and low sodium Tris-MES CSF buffers showed uptake values equivalent to that of low sodium aCSF. Further examination of the Tris-MES CSF buffer showed that Tris-MES is a selective inhibitor of the Na$^+$-dependent portion of GlySar uptake (Fig. 3). Because PEPT2 is an Na$^+$-independent transporter, low sodium Tris-MES CSF buffer was used for all subsequent experiments. This eliminated the potentially confounding effects of the sodium-dependent portion of GlySar uptake.

Characterization of Sodium-Independent Transport. The Na$^+$-independent transport of GlySar was pH dependent with maximal uptake being observed from pH 5.5 to 6.5. As the pH increased from 6.5 to 8.0 there was a steady decrease in GlySar uptake (Fig. 4). This pH dependence is similar to findings in other PEPT2 systems (Ganapathy and Leibach, 1983; Daniel, 1996; Chen et al., 1999). As a result, the concentration-dependent and inhibition experiments were performed at pH 6.5 to maximize GlySar transport while maintaining a stable tissue preparation. As shown in Fig. 5, the choroid plexus showed saturable transport of GlySar with a Michaelis constant ($K_m$) of 129 ± 32 μM and maximum velocity ($V_{max}$) of 52.8 ± 3.6 pmol/mg/min. Furthermore, a Woolf-Augustinsson-Hofstee transformation of the data shows a single slope linear relationship indicating that, under low sodium conditions, only one transport system is involved in GlySar uptake (Fig. 5, insert).

Inhibitor Analysis. Four groups of potential inhibitors were used against GlySar uptake, namely, amino acids, di- and tripeptides, cephalosporins, and organic anions and cations. Glycine, sarcosine, and L-histidine showed no inhibition of GlySar uptake relative to the control (Fig. 6A). The di- and tripeptides GlySar, glycylylproline, GlyGlyHis, and carnosine
all showed significant reductions of GlySar uptake. In fact, an 85 to 90% reduction in GlySar uptake was observed for all di- and tripeptide inhibitors that were tested (Fig. 6B). The cephalosporins were divided into two groups. Cefadroxil and cephalaxin contain α-amino carbons, whereas cephalexin and cephalothin do not. Both cefadroxil and cephalaxin showed an 85% reduction in GlySar uptake, whereas there was no significant difference between cephalexin or cephalothin and the control uptake values for GlySar (Fig. 6C). Finally, the organic anion SITS and the organic cation TEA showed no significant reduction in GlySar uptake (Fig. 6D).

**Discussion**

It is apparent from our studies that PEPT2 mediates the concentration-dependent uptake of GlySar in the rat choroid plexus. A portion of the choroid plexus GlySar uptake is sodium independent but proton dependent, both characteristic of the PEPT2 transporter (Ganapathy and Leibach, 1983). The GlySar uptake is saturable with a Michaelis constant of about 100 to 150 μM. Furthermore, inhibitor analysis indicates that the specificity of the PEPT2 in choroid plexus is identical with the high-affinity transporter, PEPT2, in rabbit renal brush-border membrane vesicles (Akarawut et al., 1998). A PEPT1-mediated uptake of GlySar is remote because micromolar and not millimolar affinities were observed. In addition, several investigators were unable to detect PEPT1 mRNA in rat (Saito et al., 1995; Fujita et al., 1999) and rabbit brain (Döring et al., 1998) after amplification by reverse transcription-polymerase chain reaction, as well as PEPT1 protein in rat choroid plexus with immunoblot analyses (Novotny et al., 2000).

The pH dependence of GlySar is evident by a sharp decrease in uptake as the extracellular pH rises from 6.5 to 8.0. The peak uptake from pH 5.5 to 6.5 is similar to the values reported in other studies of PEPT2 (Ganapathy and Leibach, 1983; Daniel, 1996; Chen et al., 1999). To be consistent with these previous studies, an acidic pH of 6.5 was used to maximize the uptake of GlySar by PEPT2. This study did not measure intracellular pH of choroid plexus epithelial cells. Although it is known that the normal pH is about 7.0 (Johanson, 1978) and that the choroid plexus does pH regulate, the degree of regulation is compromised in vitro (Johanson, 1984; Johanson et al., 1985). This study alone, therefore, cannot eliminate the possibility that the effects of altered extracellular pH on GlySar uptake are not mediated by a change in intracellular pH. However, studies of PEPT2-mediated transport in other tissues and vesicles indicate that PEPT2 is modulated by extracellular pH (Ganapathy and Leibach, 1983; Daniel et al., 1991).

The extracellular-to-intracellular pH gradient that drives...
PEPT2 in other tissues is normally generated by Na\(^+\)/H\(^+\) exchange (Daniel, 1996). There is evidence for an Na\(^+\)/H\(^+\) exchanger in choroid plexus from whole tissue experiments (Johanson et al., 1985), cultured choroid plexus epithelial cell transport studies (Hakvoort et al., 1998), and Northern blotting (Kalaria et al., 1998), but whether such transport is limited to the apical or basolateral membranes is uncertain.

Analysis of the kinetics of GlySar uptake in the choroid plexus shows that the estimated transport parameters are similar to findings in other systems. The Michaelis constant of the GlySar transport in rat choroid plexus (K\(_m\) = 129 \(\mu\)M) is comparable to the values reported for PEPT2 in rabbit renal brush-border membrane vesicles (K\(_m\) = 155 \(\mu\)M; Akarawut et al., 1998), Xenopus oocytes expressing rat PEPT2 (K\(_m\) = 22.3 \(\mu\)M; Zhu et al., 2000), and culture of SHR kidney proximal tubule cells (K\(_m\) = 67 \(\mu\)M; Brandsch et al., 1995). The maximum uptake rate (V\(_{max}\)) in the renal brush-border membrane vesicles and SHR kidney proximal tubule cells was 2.5 and 7.2 nmol/mg of protein/min, respectively (Brandsch et al., 1995; Akarawut et al., 1998). The choroid plexus has about 200 \(\mu\)g of protein/mg of wet weight and, thus, has a V\(_{max}\) of 0.25 nmol/mg of protein/min. This value is less than that of the other two preparations, but considering that the choroid plexus is a tissue preparation and the kidney preparation is enriched with brush-border membranes, the quantity of functional PEPT2 in the choroid plexus may be comparable to that found in the kidney. Interestingly, almost identical findings to ours in rat choroid plexus were recently reported (Fujita et al., 1999) in synaptosomes prepared from rat cerebellum (K\(_m\) = 0.13 \(\mu\)M; V\(_{max}\) = 0.056 nmol/mg of protein/min).

The inhibitor analysis provides information about the specificity of the transporter. The amino acids glycine, sarcosine, and \(\alpha\)-histidine did not significantly inhibit the uptake of GlySar. The lack of inhibition by glycine and sarcosine indicates that degradation of GlySar is highly unlikely, and that choroid plexus uptake involved only intact GlySar substrate. PHT1 is an Na\(^+\)-independent, proton-coupled transporter that transports peptides as well as \(\alpha\)-histidine (K\(_m\) = 17 \(\mu\)M). The mRNA for PHT1 is present in the choroid plexus (Yamashita et al., 1997). The lack of inhibition of GlySar uptake by saturating concentrations of \(\alpha\)-histidine, however, suggests the PHT1 transporter protein may have low abundance in the choroid plexus relative to PEPT2. It is possible that PHT1 is inhibited by Tris-MES, but the Na\(^+\)-independent GlySar uptake was not significantly different between Tris-MES and aCSF buffers. The organic anion SITS and cation TEA had no significant effect on GlySar uptake. Thus, it appears that organic anion (Kusuhara et al., 1999) and cation (Miller et al., 1999) transporters do not mediate Na\(^+\)-independent GlySar uptake in the choroid plexus. The inhibition profile of the cephalosporins is consistent with that from previous experiments with PEPT2 (Akarawut et al., 1998). The cephalosporins with \(\alpha\)-amino carbons (cefoxidril, cephalolin) completely inhibited GlySar uptake in the choroid plexus, whereas those without \(\alpha\)-amino carbons (cephaloridine, cephalothin) had no effect on GlySar uptake. The ability of PEPT2 in choroid plexus to mediate drug uptake may be important for future drug design as well as for drug delivery and targeting to select tissues. The level to which PEPT2 controls drug distribution between the CSF and the blood also may affect the pharmacological effect.

Glycylsarcosine is resistant to enzymatic hydrolysis and, as a result, is used as a model substrate for small peptide transport in the kidney and intestine. Daniel et al. (1992) have shown that virtually any di- or tripeptide can inhibit GlySar uptake via the peptide transporters PEPT1 and PEPT2. All of the di- and tripeptides tested in our study inhibit GlySar uptake almost completely at 1.0 mM. Among these peptides are two neuropeptides, carnosine and GlyGlyHis. Carnosine is present in the brain under physiological conditions (Babizhayev, 1989) and is thought to be a neuropeptide (Hipkiss et al., 1995) involved in antioxidation. GlyGlyHis, a zinc(II)- and copper(II)-binding tripeptide, is present in the plasma (Halman et al., 1971) and is indicated in Zn\(^{2+}\) cotransport via the intestinal peptide transporter PEPT1 (Tacnet et al., 1993). That both of these compounds have an affinity for PEPT2 in the choroid plexus suggests that PEPT2 could be involved in neuropeptide homeostasis or the cotransport of metal ions and their peptide chelators. This is supported by a study with rat brain PEPT2 clones expressed in Xenopus oocytes in which the neuropeptide N-acetylaspartylglutamate exhibits saturable uptake via the PEPT2 transporter (Wang et al., 1998). Establishing the affinity of other neuropeptides as well as the cellular location of PEPT2 in the choroid plexus might help in understanding how peptide homeostasis occurs in the brain.

In summary, our studies have characterized, for the first time, the functional properties of GlySar transport in whole tissue rat choroid plexus. The pH dependence, kinetic profile, and specificity of choroid plexus PEPT2 are similar to that previously described in renal models. A definitive determination of the membrane location of PEPT2 and the vectorial transport of peptide substrates would help evaluate its purpose in the choroid plexus. Isolated choroid plexus studies cannot determine these characteristics of the transporter. Future studies will be directed at determining transporter location and directionality with primary cell culture and/or stable choroid plexus cell lines.

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References


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