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# Characterization of the glycine transport system GLYT 1 in human placental choriocarcinoma cells (JAR)

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**Abstract**

Transport of glycine in confluent monolayer cultures of JAR human placental choriocarcinoma cells was investigated. Glycine uptake in these cells was made up of two components, one being $Na^+$-dependent with no requirement for $Cl^-$ and the other being dependent on $Na^+$ as well as $Cl^-$. Substrate specificity studies indicated that distinct transport systems were responsible for these two components. Alanine inhibited the $Na^+$-dependent glycine uptake preferentially and the $Na^+$- and $Cl^-$-dependent glycine uptake represented > 95% of total uptake in the presence of 5 mM alanine. Competition experiments revealed that the $Na^+$- and $Cl^-$-dependent transport system exhibited a very narrow substrate specificity with affinity toward only glycine and its derivatives such as sarcosine, glycine methyl ester and glycine ethyl ester. These characteristics identify the transport system as GLYT 1. This system showed high affinity for glycine, with a Michaelis-Menten constant of 15 $\mu$M. The $Na^+$:$Cl^-$: glycine stoichiometry appeared to be 2:1:1. Treatment of JAR cells with calmodulin antagonists resulted in the inhibition of the transport function of GLYT 1 and this inhibition was solely due to a decrease in the maximal velocity of the system with no change in the substrate affinity. It is concluded that the placental choriocarcinoma cell line JAR expresses robust activity of the glycine transporter GLYT 1 and that the activity of this transporter is under the regulation of calmodulin-dependent cellular processes.

*Key words:* Glycine transport; Sodium chloride dependence; Stoichiometry; Sarcosine; GLYT 1; Calmodulin antagonist; (Choriocarcinoma cell)

## 1. Introduction

Glycine is an important amino acid which is a precurser not only for proteins but also for purines and pyrimidines involved in nucleic acid biosynthesis and for glutathione, a component of cellular protective machinery against oxidative damage. Transport systems which participate in the cellular uptake of this amino acid have been investigated in several tissues and it has become apparent that multiple amino acid transport systems have the ability to mediate glycine uptake. Among these glycine transport systems, system GLY is unique for several reasons. This system is very selective for glycine and has no or little affinity for other amino acids involved in protein synthesis. Furthermore, this system is highly concentrative, and its activity is obligatorily dependent on $Na^+$ as well as $Cl^-$. Interestingly, expression of this transport system is restricted to only a limited number of tissues which include erythrocytes [1–3], brain [4], liver [5], and unfertilized and fertilized eggs [6,7].

Transport of amino acids from mother to fetus across the human placenta plays an important role in the growth and development of the fetus. This tissue expresses several amino acid transport systems [8,9]. Transport of glycine across the placenta has been investigated with purified maternal-facing brush border

Abbreviations: MeAIB, methylaminoisobutyric acid; GABA, $\gamma$-aminobutyric acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; CGS 9343 B, 1,3-dihydro-1[1-((4-methyl-4*H*, 6*H*-pyrrolo[1,2-*a*] [4,1]-benzoxapin-4-yl)-methyl)-4-piperindinyl]-2*H*-benzimidazol-2-one; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide; SDS, sodium dodecyl sulfate.

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membrane vesicles [10,11] and available evidence indicates that at least part of glycine transport in these vesicles occurs via system GLY [10]. In our laboratory, we have used human placental choriocarcinoma cells as a model to study placental amino acid transport [12–15]. These cell lines are very useful not only to investigate the operational mechanisms of a transporter but also to delineate the cellular pathways involved in the regulation of the transporter function. The present study was initiated to characterize glycine transport in JAR human placental choriocarcinoma cells. The results of the study demonstrate that these cells express robust activity of system GLY which is responsible for 75–80% of glycine transport and that the transport function of system GLY is under the regulation of calmodulin-dependent processes.

## 2. Materials and methods

### 2.1. Materials

[2-$^3$H]Glycine (spec. radioactivity, 35.1 Ci/mmol) and L-[4,5-$^3$H]leucine (spec. radioactivity, 60.0 Ci/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA, USA). The JAR human placental choriocarcinoma cell line was obtained from the American Type Culture Collection. RPMI 1640 medium and penicillin-streptomycin were from Life Technologies (Gaithersburg, MD, USA). Fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA, USA). Calmidazolium, W-7, and amino acids were from Sigma (St. Louis, MO, USA). CGS 9343 B (CGS) was a gift from Ciba-Geigy (Summit, NJ, USA). All other chemicals were of analytical grade. Disposable plasticware for cell culture was purchased from Fisher (Pittsburgh, PA, USA).

### 2.2. Cell culture

The JAR human placental choriocarcinoma cells were cultured as described previously [12–15] using RPMI 1640 medium and 10% fetal bovine serum in the presence of penicillin (100 units/ml) and streptomycin (100 $\mu$g/ml). Confluent stock cultures were treated with trypsin and the released cells were seeded in 35 mm Petri dishes at a density of $1.5 \cdot 10^6$ cell/dish. The medium was replaced by fresh culture medium 24 h after starting of the subculture and the cells were used for uptake measurements the following day. The cells grew as a monolayer and cultures were confluent on the day of uptake measurement. When the effects of calmodulin antagonists on uptake were tested, fresh culture medium which was used to feed the cells 24 h after starting of the subculture contained these agents at desired concentrations. The time between the addition of these agents to the cell cultures and the measurement of uptake was approximately 16 h.

### 2.3. Uptake measurements

The medium was removed by aspiration from monolayer cultures and replaced with the uptake buffer containing radiolabeled glycine or leucine. The composition of the uptake buffer in most cases was 25 mM Hepes-Tris (pH 7.5), 5.4 mM potassium gluconate, 1.8 mM calcium gluconate, 0.8 mM $MgSO_4$, 140 mM NaCl, and 5 mM glucose. After incubation at room temperature for a desired time, the buffer was removed and the cells were quickly washed three times with the uptake buffer. After washing, 1 ml of 1% SDS/0.2 M NaOH was added to each dish and left for 1 h. The contents from the dish were then transferred to a scintillation vial for determination of radioactivity.

### 2.4. Protein determination

Protein content of the cells was determined in each experiment by using monolayer cultures which were grown under conditions identical to those employed for culturing the cells for uptake measurements. The culture medium was removed from the dish and 1 ml of water was added to lyse the cells. Lysis was facilitated by freezing and thawing after which the contents of the dish were passed through a 25 gauge needle several times to prepare the homogenate. Protein concentration of the homogenate was determined by the Lowry method [16].

### 2.5. Statistics

Experiments were routinely performed in duplicate or triplicate and each experiment was repeated two or three times. Results are expressed as means $\pm$ S.E. for replicate determinations. Statistical and kinetic analyses were done using a commercially available computer software called *Fig. P* version 6.0 (Biosoft, Cambridge, UK).

## 3. Results

### 3.1. Time-course of glycine uptake in the presence of $Na^+$ or $Na^+$ plus $Cl^-$

Uptake of glycine (0.75 $\mu$M) into monolayer cultures of JAR cells was measured from uptake media containing $Na^+$ plus $Cl^-$ (NaCl), $Cl^-$ but no $Na^+$ (choline chloride) or $Na^+$ but no $Cl^-$ (sodium gluconate). Results given in Fig. 1 show that when both $Na^+$ and $Cl^-$ were present in the uptake medium, glycine uptake was maximal, the uptake rate under

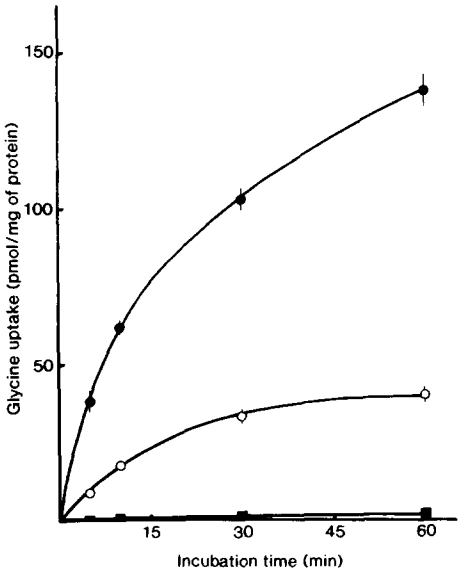


Fig. 1. Time-course of glycine uptake in JAR placental choriocarcinoma cells. Uptake of glycine (0.75 $\mu$M) in monolayer cultures of JAR cells was measured from uptake media containing NaCl (●), sodium gluconate (○) or choline chloride (■). Composition of the uptake medium was (in mM) 25 Hepes-Tris (pH 7.5), 5.4 potassium gluconate, 1.8 calcium gluconate, 0.8 $MgSO_4$, 5 glucose, and 140 NaCl, sodium gluconate or choline chloride. Values are means ± S.E. for four determinations.

these conditions being 37.68 ± 2.35 pmol(mg of protein)$^{-1}$(5 min)$^{-1}$. In contrast, uptake was negligible in the absence of $Na^+$. However, uptake increased considerably when $Cl^-$ was omitted and $Na^+$ was added to the medium. Uptake rates measured in the presence of choline chloride and sodium gluconate were 0.73 ± 0.02 and 9.14 ± 0.26 pmol(mg of protein)$^{-1}$(5 min)$^{-1}$, respectively. Thus, glycine uptake measured in the presence of $Na^+$ and $Cl^-$ was about four times the uptake measured in the presence of $Na^+$ alone. Obviously, two components, one dependent on $Na^+$ alone and the other dependent on $Na^+$ as well as $Cl^-$, were responsible for the observed glycine uptake in these cells.

### *3.2. Sensitivity of $Na^+$-dependent and NaCl-dependent glycine uptake to inhibition by other amino acids*

To determine whether the $Na^+$-dependent and the NaCl-dependent glycine uptake components represent the function of a single transport system or two different transport systems, we studied the potency of various amino acids to inhibit these two components (Table 1). Among the amino acids studied, the $Na^+$-dependent uptake of [$^3$H]glycine was inhibited markedly by alanine and serine. The inhibition caused by glycine and MeAIB was intermediate while proline and sarcosine caused only a slight inhibition. In contrast, the inhibitory pattern of the NaCl-dependent uptake of [$^3$H]glycine was quite different. The most potent inhibitors were glycine and sarcosine, followed by proline, alanine, serine and MeAIB. These results suggest that the $Na^+$-dependent and the NaCl-dependent glycine uptake components do not represent the function of a single carrier system and provide evidence for the presence in JAR cells of a distinct glycine transport system whose catalytic function is dependent on $Na^+$ as well as $Cl^-$.

### *3.3. Influence of alanine on the ratio of NaCl-dependent glycine uptake to $Na^+$-dependent glycine uptake*

Since alanine was found to be the most potent inhibitor of $Na^+$-dependent glycine uptake but only a weak inhibitor of NaCl-dependent glycine uptake, we studied the effect of increasing concentrations of alanine on these two uptake components. The purpose of the experiment was to determine the concentration of alanine which would preferentially eliminate the $Na^+$-dependent component of glycine uptake with little or no effect on the $Na^+$ plus $Cl^-$-dependent component of glycine uptake. This would enable us to characterize the $Na^+$ plus $Cl^-$-dependent glycine transport system without the interference of the $Na^+$-dependent glycine transport system. The results given in Fig. 2A show that increasing concentrations of alanine inhibited the uptake of glycine measured both in the presence of $Na^+$ alone and in the presence of $Na^+$ and $Cl^-$. However, the degree of inhibition was different in both cases which was clearly evident when the ratio of $Na^+$- and $Cl^-$-dependent glycine uptake to $Na^+$-dependent glycine uptake was calculated at different concentrations of alanine (Fig. 2B). In the absence of alanine, the ratio was 4.3 ± 0.4, meaning that about 20–25% of

Table 1
Distinction between $Na^+$-dependent and NaCl-dependent transport systems for glycine in JAR placental choriocarcinoma cells

| Unlabeled amino acid | [$^3$H]Glycine uptake (pmol(mg of protein)$^{-1}$(10 min)$^{-1}$) | |
|---|---|---|
| | sodium gluconate | NaCl |
| None | 24.4 ± 2.4 (100) | 74.1 ± 2.6 (100) |
| Alanine | 5.3 ± 0.4 (22) | 53.5 ± 2.1 (72) |
| Serine | 8.6 ± 0.5 (35) | 64.7 ± 1.9 (87) |
| Proline | 17.6 ± 1.3 (72) | 52.6 ± 2.0 (71) |
| Sarcosine | 19.3 ± 1.6 (79) | 20.9 ± 1.2 (28) |
| Glycine | 14.4 ± 0.3 (59) | 14.6 ± 1.4 (20) |
| MeAIB | 14.3 ± 1.2 (59) | 65.7 ± 4.8 (89) |

Values are means ± S.E. for 4–6 determinations. Uptake of [$^3$H]glycine into monolayer cultures of JAR cells was measured with a 10 min incubation from an uptake medium containing either sodium gluconate or NaCl. Composition of the uptake medium was (in mM) 25 Hepes-Tris (pH 7.5), 5.4 potassium gluconate, 1.8 calcium gluconate, 0.8 $MgSO_4$, 5 glucose, and 140 sodium gluconate or NaCl. Concentration of radiolabeled glycine was 0.75 $\mu$M and that of unlabeled amino acids was 1 mM. Values given in parantheses are percent of respective control uptake.

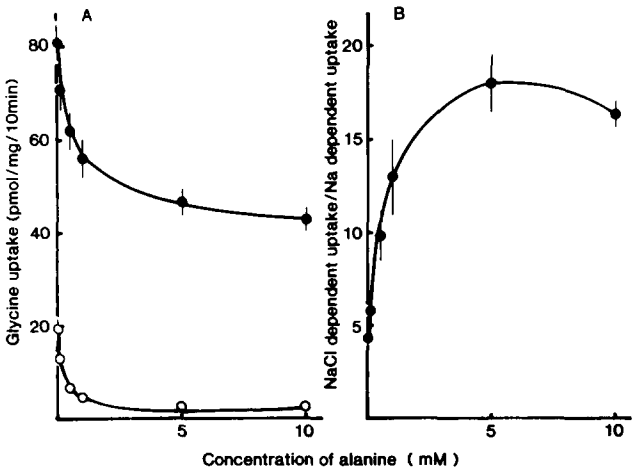


Fig. 2. Sensitivity of $Na^+$-dependent and NaCl-dependent glycine uptake to inhibition by alanine. (A) Uptake of glycine (0.75 $\mu$M) in JAR cells was measured with a 10 min incubation from uptake media containing NaCl, sodium gluconate, or choline chloride. Composition of the uptake medium was (in mM) 25 Hepes-Tris (pH 7.5), 5.4 potassium gluconate, 1.8 calcium gluconate, 0.8 $MgSO_4$, 5 glucose and 140 NaCl, sodium gluconate or choline chloride. Concentration of alanine was varied over the range of 0.1–10 mM. Uptake measured in the presence of choline chloride was subtracted from the uptake values measured in the presence of sodium gluconate and NaCl to determine $Na^+$-dependent (•) and NaCl-dependent (○) uptake, respectively. Values are means ± S.E. for three determinations. (B) Influence of alanine on the ratio of NaCl-dependent glycine uptake/$Na^+$-dependent glycine uptake.

the total uptake measured in the presence of NaCl was contributed by the $Na^+$-dependent uptake component and the remaining uptake was contributed by the $Na^+$- and $Cl^-$-dependent uptake component. As the concentration of alanine was increased, this ratio increased considerably, indicating preferential elimination of the $Na^+$-dependent uptake component with little effect on the $Na^+$- and $Cl^-$-dependent component. The ratio was maximal (18.0 ± 1.5) in the presence of 5 mM alanine. This means that almost 95% of the total uptake of glycine measured in the presence of NaCl under these conditions was due to the $Na^+$- and $Cl^-$-dependent uptake component and that the contribution to the uptake by the $Na^+$-dependent component was only about 5%. Subsequent experiments designed to characterize the $Na^+$- and $Cl^-$-dependent glycine transport system were done by measuring glycine uptake from NaCl-containing medium in the presence of 5 mM alanine.

### *3.4. Cation and anion specificity of the $Na^+$- and $Cl^-$-dependent glycine transport system*

The cation specificity of the transport system was studied by measuring glycine uptake in the presence of chloride salts of different cations. Alanine (5 mM) was present in all experiments. Replacement of $Na^+$ with $K^+$, $Li^+$ and choline totally abolished the uptake (Table 2), demonstrating the absolute requirement for $Na^+$ for the transport function of the system. The anion specificity was studied by measuring glycine uptake in the presence of sodium salts of different anions (Table 2). Uptake was maximal in the presence of $Cl^-$. Uptake was minimal when $Cl^-$ was replaced by gluconate or $F^-$. $I^-$ and $NO_3^-$ could substitute for $Cl^-$ to an appreciable extent. Among the anions tested, $SCN^-$ was the most effective substituent for $Cl^-$, the uptake in the presence of NaSCN being about 60% of the uptake in the presence of NaCl. The specific anion requirement for the transport function was not due to the diffusion potentials induced by these anions because, if this were to be the case, $SCN^-$ being more permeant than $Cl^-$ would have supported the uptake more effectively than $Cl^-$. Therefore, it appears that the stimulation of glycine uptake by $Na^+$ and $Cl^-$ was due to a direct effect of these ions on the transport system.

### *3.5. Substrate specificity of the $Na^+$- and $Cl^-$-dependent glycine transport system*

To determine the substrate specificity of the $Na^+$- and $Cl^-$-dependent transport system that was responsible for glycine uptake, we conducted competition experiments in which the potency of various amino acids to inhibit the uptake of [$^3$H]glycine was evaluated (Table 3). Among the compounds tested, glycine and sarcosine were the most potent inhibitors of [$^3$H]glycine uptake. Glycine methyl ester and glycine ethyl ester also caused marked inhibition. Pipecolic acid produced

Table 2
Influence of monovalent cations and anions on alanine-insensitive glycine uptake in JAR placental choriocarcinoma cells

| Inorganic salt | Glycine uptake | |
|---|---|---|
| | pmol(mg of protein)$^{-1}$(5 min)$^{-1}$ | % |
| Influence of cations | | |
| NaCl | 22.1 ± 0.4 | 100 |
| KCl | 0.4 ± 0.0 | 2 |
| LiCl | 0.6 ± 0.0 | 2 |
| Choline chloride | 0.4 ± 0.0 | 2 |
| Influence of anions | | |
| NaCl | 22.1 ± 0.4 | 100 |
| NaI | 6.8 ± 0.1 | 31 |
| NaF | 3.8 ± 0.1 | 17 |
| $NaNO_3$ | 6.3 ± 0.1 | 28 |
| NaSCN | 13.1 ± 0.2 | 59 |
| Sodium gluconate | 1.4 ± 0.1 | 6 |

Values are means ± S.E. for three determinations. Uptake of [$^3$H]glycine in monolayer cultures of JAR cells was measured with a 5 min incubation in the presence of 5 mM alanine. Composition of the uptake medium was (in mM) 25 Hepes-Tris (pH 7.5), 5.4 potassium gluconate, 1.8 calcium gluconate, 0.8 $MgSO_4$, 5 glucose, and 140 indicated inorganic salt. Concentration of radiolabeled glycine was 0.5 $\mu$M.

Table 3
Substrate specificity of the $Na^+$- and $Cl^-$-dependent glycine transport system in JAR placental choriocarcinoma cells

| Unlabeled amino acid | [$^3$H]Glycine uptake | |
|---|---|---|
| | pmol(mg of protein)$^{-1}$(5 min)$^{-1}$ | % |
| None | 32.2±1.1 | 100 |
| Glycine | 1.7±0.3 | 5 |
| Sarcosine | 2.2±0.1 | 7 |
| Glycine methyl ester | 9.6±0.6 | 30 |
| Glycine ethyl ester | 16.9±0.3 | 52 |
| Pipecolic acid | 24.2±0.7 | 75 |
| Proline | 29.2±1.4 | 91 |
| Betaine | 31.2±1.1 | 97 |
| Taurine | 31.4±0.8 | 98 |
| GABA | 32.5±1.4 | 101 |
| Leucine | 30.1±1.5 | 93 |
| MeAIB | 32.6±0.3 | 101 |
| Serine | 39.9±1.0 | 124 |

Values are means ± S.E. for 3–5 determinations. Uptake of [$^3$H]glycine in monolayer of JAR cells was measured with a 5 min incubation in the presence of 5 mM alanine. Composition of the uptake medium was (in mM) 25 Hepes-Tris (pH 7.5), 5.4 potassium gluconate, 1.8 calcium gluconate, 0.8 $MgSO_4$, 140 NaCl, and 5 glucose. Concentration of radiolabeled glycine was 1 μM and that of unlabeled amino acids was 500 μM.

a small, but significant ($P < 0.05$) inhibition. Amino acids which had no effect on [$^3$H]glycine uptake included proline, betaine, GABA, taurine, leucine, and MeAIB. [$^3$H]Glycine uptake was consistently higher in the presence of serine than in its absence, but the mechanisms underlying this apparent stimulation were not investigated further.

These competition experiments lead to the identification of the $Na^+$- and $Cl^-$-dependent transport system which catalyzes glycine uptake in JAR cells. Recently, two different glycine transport systems have been cloned from neuronal tissue [17–19]. Both systems are specific for glycine and depend on $Na^+$ as well as $Cl^-$ for their transport function. However, these two systems can be distinguished by their differential affinity toward sarcosine, *N*-methylglycine. One system has high affinity for sarcosine and uptake of glycine via this system is strongly inhibited by this glycine derivative. The second system exhibits no or little affinity for sarcosine and thus glycine uptake via this system is totally insensitive to sarcosine. The sarcosine-sensitive glycine transport system has been designated as GLYT 1 and the sarcosine-insensitive glycine transport system as GLYT 2. Since the $Na^+$- and $Cl^-$-dependent glycine uptake in JAR cells is almost totally inhibited by sarcosine, the system responsible for this uptake is GLYT 1. There is no evidence for expression of GLYT 2 in these cells because of the absence of sarcosine-resistant glycine uptake.

### *3.6. Dependence of the uptake rate via GLYT 1 on the concentration of glycine*

Fig. 3 describes the dependence of the uptake rate on glycine concentration. The uptake rate was measured with a 5 min incubation from an uptake medium containing 140 mM NaCl and 5 mM alanine. Under these conditions, glycine uptake occurred predominantly via GLYT 1. Glycine concentration was varied over the range of 1–60 μM. The plot of uptake rate versus glycine concentration was hyperbolic and the experimental data obeyed Michaelis-Menten kinetics describing a single saturable transport system. Analysis of the data gave a value of 14.8 ± 1.6 μM for the Michaelis-Menten constant ($K_t$) and 423 ± 17 pmol(mg of protein)$^{-1}$(5 min)$^{-1}$ for the maximal velocity ($V_{max}$).

### *3.7. $Na^+$ and $Cl^-$ kinetics of GLYT 1*

We investigated the dependence of the catalytic function of GLYT 1 on the concentration of $Na^+$. In these experiments, uptake of 1 μM glycine was measured with a 5 min incubation in the presence of varying concentrations of $Na^+$ (0–120 mM). The concentration of $Cl^-$ was kept at 140 mM. Alanine was present at a concentration of 5 mM in all experiments. The results are given in Fig. 4. The dependence of the uptake rate on the concentration of $Na^+$ was found to be sigmoidal, indicating a $Na^+$/glycine coupling ratio

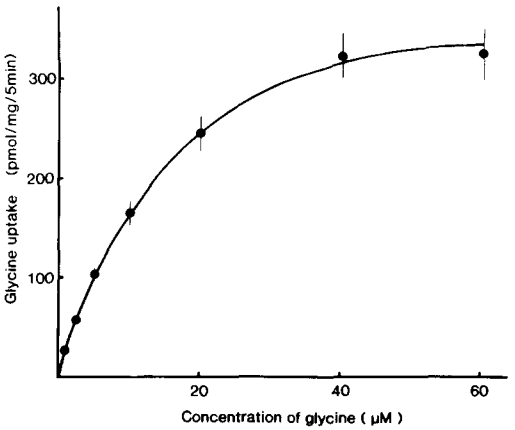


Fig. 3. Kinetics of NaCl-dependent glycine transport system. Uptake of glycine in JAR cells was determined with a 5 min incubation in the presence of NaCl and over the glycine concentration range of 1–60 μM. All uptake measurements were made in the presence of 5 mM alanine. Concentration of [$^3$H]glycine was maintained at 1 μM at all glycine concentrations. Uptake of radioactivity determined under identical conditions but in the presence of 1 mM unlabeled glycine was used to compute nonmediated uptake. Mediated uptake was calculated by subtracting nonmediated uptake from total uptake. Values given represent only mediated uptake and are means ± S.E. for four determinations.

of > 1. The experimental data were analyzed according to the Hill-type equation

$$v = \frac{V_M \cdot [Na^+]^n}{K^n_{(Na^+)} + [Na^+]^n}$$

where $v$ is the uptake rate, $V_M$ is the maximal glycine uptake, $[Na^+]$ is the concentration of $Na^+$, $K_{(Na^+)}$, is the concentration of $Na^+$ necessary to elicit 50% of the maximal effect, and $n$ is the number of $Na^+$ ions involved in the transport of one glycine molecule. This analysis gave a value of $72 \pm 6$ mM for $K_{(Na^+)}$ and $1.44 \pm 0.10$ for $n$. Assignment of a value of 1 or 2 for $n$ rendered the Hill-type plots curvilinear (Fig. 4B).

In contrast, similar experiments on the dependence of glycine uptake rate on the concentration of $Cl^-$ done in the presence of a fixed concentration of $Na^+$ revealed that the relationship in the case of $Cl^-$ was not sigmoidal but instead hyperbolic (Fig. 5). These results suggest that the coupling ratio of $Cl^-$/glycine is 1. Analysis of the data using the Hill-type equation as described earlier for $Na^+$ gave a value of $1.0 \pm 0.3$ for $n$ (the number of $Cl^-$ ions involved in the transport of one glycine molecule) and $36 \pm 8$ mM for $K_{(Cl^-)}$ (the concentration of $Cl^-$ necessary to produce 50% of the maximal effect). The linearity ($r = -0.98$) of the Hill-type plot (Fig. 5, inset) with $n = 1$, i.e., $v$ vs. $v/[Cl^-]$, supports the $Cl^-$/glycine coupling ratio of 1.

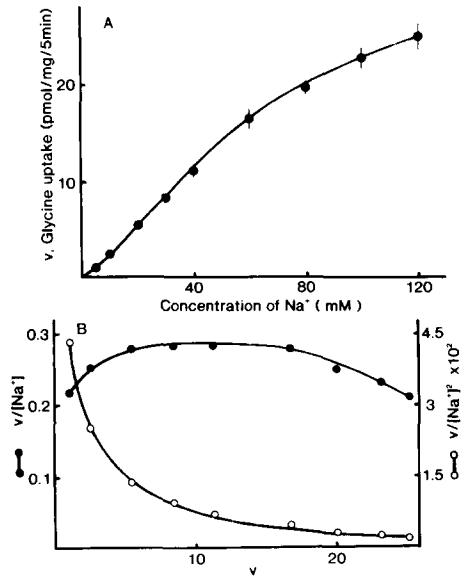


Fig. 4. Dependence of NaCl-dependent glycine transport system on the concentration of $Na^+$. Uptake of glycine (1 $\mu$M) was determined in JAR cells with a 5 min incubation in the presence of 5 mM alanine. Concentration of $Na^+$ in the uptake medium was varied over the range of 5–120 mM while maintaining the concentration of $Cl^-$ at 140 mM. Uptake media were prepared by appropriately mixing two uptake buffers containing 25 mM Hepes-Tris (pH 7.5), 5.4 mM potassium gluconate, 1.8 mM calcium gluconate, 0.8 mM $MgSO_4$, 5 mM glucose, and 140 mM NaCl or LiCl. Uptake measured in the absence of $Na^+$ was subtracted from total uptake to determine $Na^+$-dependent uptake. Values are means ± S.E. for four determinations. (A) $Na^+$-dependent glycine uptake ($v$) versus $Na^+$ concentration. (B) Hill-type plot: $v$ versus $v/[Na^+]$ (●); $v$ versus $v/[Na^+]^2$ (○).

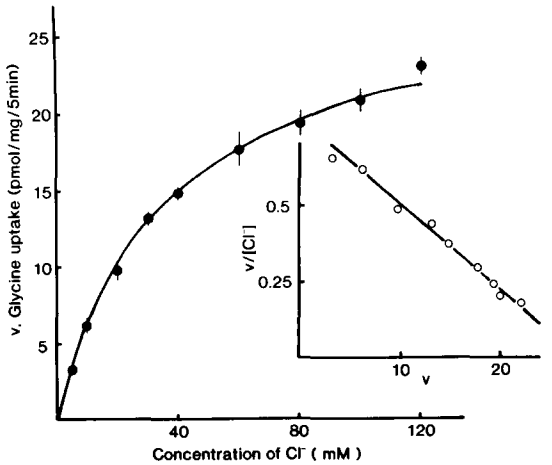


Fig. 5. Dependence of NaCl-dependent glycine transport system on the concentration of $Cl^-$. Uptake of glycine (1 $\mu$M) was determined in JAR cells with a 5 min incubation in the presence of 5 mM alanine. Concentration of $Cl^-$ was varied over the range of 5–120 mM, while maintaining the concentration of $Na^+$ at 140 mM. Uptake media were prepared by appropriately mixing two uptake buffers containing 25 mM Hepes-Tris (pH 7.5), 5.4 mM potassium gluconate, 1.8 mM calcium gluconate, 0.8 mM $MgSO_4$, 5 mM glucose, and 140 mM NaCl or sodium gluconate. Uptake measured in the absence of $Cl^-$ was subtracted from total uptake to determine $Cl^-$-dependent uptake. Values are means ± S.E. for four determinations. (Inset) Hill-type plot; $Cl^-$-dependent glycine uptake ($v$) versus $Cl^-$-dependent glycine uptake/$Cl^-$ concentration.

### *3.8. Influence of calmodulin antagonists on the activity of GLYT 1*

Our previous studies have shown that several transport systems in JAR cells are regulated by calmodulin-dependent cellular processes [14,15]. These experiments were done with CGS, a highly specific calmodulin antagonist [20]. In the present study, we investigated whether the activity of GLYT 1 in these cells is also regulated by calmodulin-dependent processes. The cells were treated with different concentrations of CGS (0–60 $\mu$M) for 16 h at 37°C, following which the activity of GLYT 1 (i.e., the NaCl-dependent glycine uptake in the presence of 5 mM alanine) was measured. As shown in Fig. 6, treatment of the cells with the calmodulin antagonist caused marked inhibition of GLYT 1 activity. At a CGS concentration of 60 $\mu$M, the inhibition was about 80%. Under identical conditions, the uptake of leucine catalyzed by the amino acid transport system L was stimulated by CGS. This suggests that the inhibitory effect of CGS treatment on the activity of GLYT 1 was not without specificity. The participation of calmodulin in the regulation of GLYT 1 activity was further confirmed by using two other widely used calmodulin antagonists, W-7 and calmida-

zolium. Treatment of JAR cells with 40 $\mu$M W-7 and 5 $\mu$M calmidazolium for 16 h at 37°C caused $26 \pm 4\%$ and $22 \pm 2\%$ inhibition of GLYT 1 activity, respectively.

We also studied the influence of CGS treatment on the kinetic parameters of GLYT 1. JAR cells were treated with or without 40 $\mu$M CGS for 16 h at 37°C and the dependence of glycine uptake rate on the concentration of glycine was studied in both groups. In control as well as CGS-treated cells, the plot of uptake rate vs. glycine concentration was hyperbolic (not shown). The Eadie-Hofstee plots (uptake rate/glycine concentration vs. uptake rate) in both cases were linear (Fig. 7). The kinetic parameters ($K_t$ and $V_{max}$) were calculated by linear regression analysis of the data. In control cells, the values for $K_t$ and $V_{max}$ were $13.9 \pm 1.0$ $\mu$M and $376 \pm 14$ pmol(mg of protein)$^{-1}$(5 min)$^{-1}$, respectively. The corresponding values in CGS-treated cells were $15.2 \pm 0.4$ $\mu$M and $178 \pm 3$ pmol(mg of protein)$^{-1}$(5 min)$^{-1}$, respectively. These results show that CGS-induced inhibition of GLYT 1 activity in JAR cells is solely due to a decrease in the $V_{max}$. The affinity of GLYT 1 for glycine is similar in control cells and in CGS-treated cells.

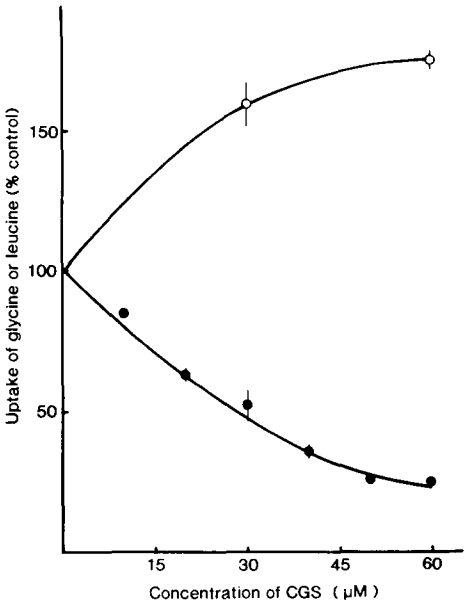


Fig. 6. Influence of CGS on leucine uptake and NaCl-dependent glycine uptake. Monolayer cultures of JAR cells were treated for 16 h at 37°C in the absence or presence of CGS at indicated concentrations. Following this treatment, uptake of glycine (1 $\mu$M) was measured with a 5 min incubation in the presence of 5 mM alanine (●). When uptake of leucine (10 nM) was determined, alanine was not present and incubation time was 1 min instead of 5 min (○). Composition of the uptake medium was (in mM) 25 Hepes-Tris (pH 7.5), 5.4 potassium gluconate, 1.8 calcium gluconate, 0.8 $MgSO_4$, 5 glucose, and 140 NaCl. In control cells which were not treated with CGS, values for glycine uptake and leucine uptake were $31.5 \pm 1.3$ pmol/mg per 5 min and $0.46 \pm 0.03$ pmol/mg per min, respectively. These values were taken as 100 percent in respective experiments. Values are means ± S.E. for four determinations.

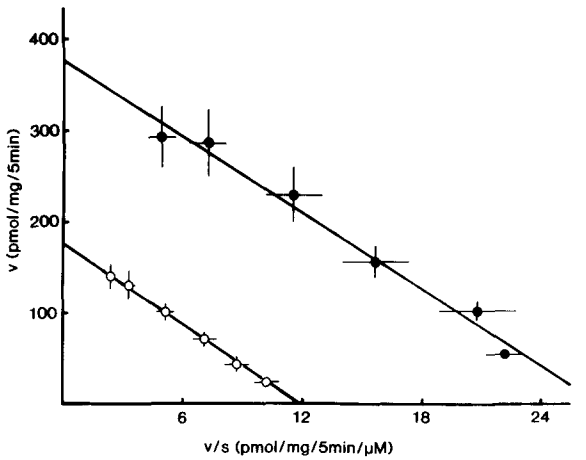


Fig. 7. Influence of CGS on the kinetics of NaCl-dependent glycine transport system. Monolayer cultures of JAR cells were treated for 16 h at 37°C in the absence (●) or presence (○) of 40 $\mu$M CGS. Following this treatment, uptake of glycine was determined with a 5 min incubation in the presence of NaCl and over a glycine concentration range of 1–60 $\mu$M. All uptake measurements were made in the presence of 5 mM alanine. Concentration of [$^3$H]glycine was maintained at 1 $\mu$M at all glycine concentrations. Uptake of radioactivity determined under identical conditions but in the presence of 1 mM unlabeled glycine was used to compute nonmediated uptake. Mediated uptake was calculated by subtracting nonmediated uptake from total uptake. Results are given for mediated uptake in the form of Eadie-Hofstee plots. Values are means ± S.E. for four determinations.

## 4. Discussion

Amino acid transport in mammalian cells is a very complex phenomenon, involving at least 15 distinct transport systems [21,22]. Glycine is a substrate for several of these systems. However one particular system, called GLY, has been shown to be specific for glycine and its methyl derivative sarcosine [1–7]. Recent studies [17–19] have demonstrated that there are at least two subtypes of system GLY based on substrate specificity. GLYT 1 accepts glycine as well as sarcosine as substrates whereas GLYT 2 accepts only glycine as the substrate. GLYT 1 is expressed in various regions of the brain [4,18,23] and in nonneuronal tissues such as erythrocytes, liver and unfertilized and fertilized eggs [1–3,5–7]. In contrast, expression of GLYT 2 is strictly restricted to neuronal tissue, in particular, brain stem and spinal cord [17]. GLYT 1 exhibits two isoforms [17,18,23] both resulting from alternative splicing.

There is evidence for the presence of GLYT 1 in the human placental brush border membrane [10] where it is expected to participate in the transfer of glycine from maternal blood into placental trophoblast. De-

tailed characterization of this system was however not possible because transport via GLYT 1 represented only about 20% of total glycine transport in brush border membrane vesicles. We initiated the present studies to determine whether the human placental choriocarcinoma cell line JAR expresses GLYT 1 with the aim to identify a cellular model to characterize GLYT 1 in the placenta. To our knowledge, GLYT 1 has not been previously described in a cultured cell line of human origin and identification of a human cell line expressing GLYT 1 would certainly facilitate studies on the regulation of this interesting transport system.

We demonstrate in the present study that JAR cells exhibit robust activity of GLYT 1. The identity of the transport system was established by its requirement for $Na^+$ as well $Cl^-$, narrow substrate specificity and acceptance of sarcosine as a high affinity substrate. Transport of glycine in JAR cells via GLYT 1 represents about 75% of total glycine transport, the remainder being most likely carried out by System A. It is interesting to note that contribution to total glycine transport by System A is much greater than by GLYT 1 in isolated placental brush border membrane vesicles [10]. It is difficult to assign any significance at this time to this difference in the relative contribution of System A and GLYT 1 to glycine transport in JAR cells and in brush border membrane vesicles. However, it is known that the activity of GLYT 1 is developmentally regulated in fertilized eggs [6,7]. Transport of glycine occurs predominantly via GLYT 1 in fertilized eggs and in two-cell and eight-cell conceptuses. At later stages of development, amino acid transport systems other than GLYT 1 become primarily responsible for glycine transport. Functional relevance of such a developmental pattern of GLYT 1 expression in fertilized eggs is evident from the findings that glycine is the most predominant amino acid in unfertilized and fertilized eggs and that the glycine content decreases gradually as the fertilized egg develops through two-cell and eight-cell stages into blastocyst [24]. It is very likely that expression of various amino acid transport systems and their relative activities change in the placenta during its development depending upon the metabolic needs of the tissue for particular amino acids as has been shown in early stage conceptuses. Therefore, the relative activities of different glycine transport systems in JAR cells may not represent the corresponding activities in the brush border membrane of the term placenta.

The JAR human placental choriocarcinoma cell offers an excellent experimental tool to characterize the transport system GLYT 1. In these cells, GLYT 1 mediates as much as 75% of glycine transport. The contribution of GLYT 1 to glycine transport can be increased further to 95% if uptake measurements are done in the presence of 5 mM alanine. Moreover, glycine transport measured in the presence of alanine is almost completely inhibitable by sarcosine, indicating that there is no measurable GLYT 2 activity in these cells. Our studies show that GLYT 1 in JAR cells is dependent on $Na^+$ as well as $Cl^-$ and is specific only for glycine and its derivatives such as sarcosine, glycine methyl ester and glycine ethyl ester. There are other $Na^+$- and $Cl^-$-coupled transport systems which mediate the transport of different amino acids and related compounds such as proline, betaine, taurine and GABA [25]. But, these compounds do not interact with GLYT 1. Most of the $Na^+$- and $Cl^-$-coupled transporters which mediate the transport of zwitterionic substrates exhibit a $Na^+/Cl^-$/substrate stoichiometry of 2:1:1. In the present study, we have determined the apparent $Na^+$/glycine and $Cl^-$/glycine coupling ratios for GLYT 1 in JAR cells. The $Na^+$/glycine coupling ratio has been shown to be 2 for GLYT 1 in other cell types [3,26–28]. The corresponding value in JAR cells is 1.44. The most likely reason for this value to be significantly less than 2 is the presence of residual activity of System A. Even though the coupling ratio was determined in the presence of alanine which preferentially eliminated System A activity, there was still some residual activity of this system contributing to glycine transport. The contribution of System A to glycine transport in the presence of 140 mM $Na^+$ is about 5%. However, the experiments dealing with the $Na^+$/glycine coupling ratio were done over a range of 0–120 mM $Na^+$. Since the dependence of System A activity on $Na^+$ concentration is known to be hyperbolic [27], the contribution of System A to glycine transport is likely to be much greater than 5% when transport measurements are done at $Na^+$ concentrations $< 140$ mM, especially at very low concentrations of $Na^+$. This would mask to a significant extent the sigmoidal relationship between glycine transport rate and $Na^+$ concentration that is expected for GLYT 1. For these reasons, it is concluded that the apparent $Na^+$/glycine coupling ratio for GLYT 1 in JAR cells is 2 as has been shown in other systems. The value of 1 for the apparent $Cl^-$/glycine coupling ratio obtained in the present study for GLYT 1 is probably correct because these experiments were done at 140 mM $Na^+$ with varying concentrations of $Cl^-$. Since System A activity is not dependent on $Cl^-$, the contribution of System A to glycine transport in experiments dealing with the determination of $Cl^-$/glycine coupling ratio of GLYT 1 was minimal at all concentrations of $Cl^-$.

To our knowledge, there is no information available on the regulation GLYT 1 in any tissue. The JAR cell line provides a convenient system to investigate the regulatory aspects of this amino acid transport system. In the present study, we have investigated the role of calmodulin-dependent processes in the modulation of

GLYT 1 activity in JAR cells. Experiments reported here were primarily done with the calmodulin antagonist CGS, and the results were confirmed with other widely used calmodulin antagonists W-7 and calmidazolium. Most calmodulin antagonists that are currently in use have questionable selectivity toward calmodulin and thus are of only limited potential in unequivocally establishing the involvement of calmodulin in any biochemical process. Neuroleptic drugs such as trifluoperazine, chlorpromazine, and pimozide which are widely used as calmodulin antagonists in fact utilize dopamine receptors as primary targets to elicit their pharmacological effects. Furthermore, these drugs also act as inhibitors of protein kinase C [29]. Similarly, W-7 is also an inhibitor of protein kinase C [30]. In contrast to these compounds, the selectivity of CGS toward calmodulin has been clearly established [20]. The present study shows that treatment of JAR cells with this selective calmodulin antagonist leads to a marked decrease in the activity of GLYT 1. The effect of CGS on GLYT 1 is not without specificity because the activity of the amino acid transport System L is stimulated under identical conditions. The inhibitory effect of CGS on GLYT 1 is reproducible with W-7 and calmidazolium. These results suggest the involvement of calmodulin-dependent cellular processes in the regulation of GLYT 1 activity in JAR cells. Since the activity is decreased by calmodulin antagonists, it can be inferred that calmodulin enhances the activity of this transport system in JAR cells. The decrease in the activity of GLYT 1 following treatment of the cells with CGS is primarily due to a decrease in the maximal velocity of the system. The affinity of the system for glycine is not affected by the treatment. The exact cellular mechanism by which the calmodulin antagonists inhibit the activity of GLYT 1 in JAR cells remains to be established.

In conclusion, the JAR human placental choriocarcinoma cells express high activity of the $Na^+$- and $Cl^-$-dependent and sarcosine-inhibitable glycine transport system GLYT 1. Since there is evidence for the occurrence of this transport system in normal human placenta, this cell line offers an excellent model to investigate various aspects of this transport system including its role in the amino acid nutrition of the placenta and the developing fetus and regulation of its activity by maternal and fetal hormones.

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