0031-3998/03/5306-1025
PEDIATRIC RESEARCH

Vol. 53, No. 6, 2003
Printed in U.S.A.

Glutamate Transport by Rcho-1 Cells Derived From Rat Placenta

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ABSTRACT

Marginal giant cells within the rodent placenta are important sources of androgens, critical to maintenance of pregnancy. Androgen synthesis requires NADPH, a by-product of glutamate oxidation. We examined the uptake of glutamate into rat choriocarcinoma cells, which have been shown to maintain many of the characteristics of marginal giant cells in culture. Na^+-dependent, D-aspartate inhibitable uptake consistent with System X_{AG}^- mediated transport was present, as were proteins capable of System X_{AG}^- activity, EAAC1, GLAST1, and GLT1. Glutamate uptake in rat choriocarcinoma cells was up-regulated by amino acid deprivation—a response that was not reversed by the addition of glutamate to the media. Inhibition data suggested up-regulation of transport activity mediated by either EAAC1 or GLAST1 at 6 h, whereas at 24 and 48 h, up-regulation of GLT1 plays an increasing role. Levels of EAAC1 immunoreactive protein increased with time under amino acid depleted conditions, whereas those of GLAST1 and GLT1 remained stable or declined but not significantly. **(*Pediatr Res* 53: 1025–1029, 2003)**

Abbreviations

MeAIB, (methylamino)isobutyric acid
DHK, dihydrokainate
KRP, Krebs-Ringer phosphate

The rat placenta is an anatomically heterogeneous organ. Nutrient transfer from maternal to fetal circulations is generally assumed to occur in the labyrinth region. Maternal blood, however, also directly bathes cells within the junctional zone, including spongiotrophoblast basophilic cells, glycogen cells, and marginal giant cells (1). Each of these cell types is thought to have specific and unique functions. Perhaps the best described of these is the production of steroid and polypeptide hormones by marginal giant cells (2–5). Specifically, these cells produce androstenedione, important in the maintenance of pregnancy (2, 6–8). The synthesis of androstenedione is an NADPH-dependent process (8). Maintenance of adequate cellular NADPH, therefore, is important. NADPH is derived as a by-product of glutamate oxidation. In fact, the metabolism of glutamate, shown in the human placenta to occur primarily through the transferase and deamination pathways, with subsequent incorporation into the tricarboxylic acid cycle (9), produces a variety of important energetic intermediates, including NADPH, NADH, reduced flavin adenine dinucleotide, and guanosine triphosphate. Given the relatively low activity of pentose phosphate shunt enzymes in human and rat placenta, the NADPH produced from glutamate by cytoplasmic glutamate dehydrogenase has been hypothesized to be important in placental steroidogenesis and, to a limited degree, in lipogenesis (10–14). In fact, the production of progesterone, also an NADPH-dependent process, in mitochondria isolated from human placenta is increased in the presence of supplemental glutamate (7, 14). Hence, characterization of glutamate transfer into marginal giant cells within the rat placenta is of importance.

Rat choriocarcinoma (Rcho-1) cells, in the differentiated state, possess a secretory profile that is consistent with that of the trophoblast giant cell, producing both androstenedione and progesterone (7, 15, 16). The synthesis of both hormones involves the NADPH-dependent cytochrome P450 enzyme system (7). Given that NADPH is a product of glutamate metabolism, we examined glutamate transport activity and substrate-dependent regulation in differentiated Rcho-1 cells.

METHODS

Chemicals. [^{3}H]glutamic acid was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade or of the highest grade commercially available. The EAAC1 antibody was made as previously described (17). GLT1 and GLAST1 antibodies were the gift of Jeffrey Rothstein (Johns Hopkins University, Baltimore, MD, U.S.A.).

Cell culture. Rcho-1 cells were the gift of and were grown as described by Soares and colleagues (18, 19). For inducing

Received December 18, 2001; accepted November 18, 2002.
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This work was supported by grant HD-29934 from the National Institutes of Health.

DOI: 10.1203/01.PDR.0000061542.06874.2A

hormonal and morphologic differentiation, Rcho-1 cells were grown to confluence in 175-mm flasks in NCTC-135 media with 20% fetal bovine serum, passed into 24-well trays, and then switched to media containing 5% horse serum but no fetal bovine serum. Cells were plated at 80% confluence unless otherwise noted. Amino acid depletion experiments were initiated by changing media to Selectamine (GIBCO BRL) media (no added amino acids; 5% dialyzed horse serum) with addition of the designated amino acid in a concentration of 1 mM for 6, 24, or 48 h.

Uptake. Whole-cell uptake measurements were performed as previously described using cells seeded into 24-well dishes as described above (20). Uptake was initiated by replacing depletion (amino acid free) buffer (2 × 15 min) with Na^+-containing Krebs-Ringer phosphate (KRP) or Na^+-free (choline KRP) buffers that contained the appropriate amount of radiolabeled (10 μCi/mL) and unlabeled L-glutamate and, when indicated, inhibitors. After the appropriate time interval, which was typically <60 s, uptake was terminated by four rinses of 4°C choline-KRP (2 mL/well). After air-drying, cellular protein was precipitated with 10% trichloroacetic acid, and the supernatant radioactivity was quantified by liquid scintillation counting. Subsequently, the precipitated proteins were solubilized in 0.2 N sodium hydroxide/0.2% SDS and analyzed for total cellular protein. Uptake velocities (uptake · mg^{-1} protein · min^{-1}) are reported as the mean ± SE unless otherwise noted; derived (*i.e.* Na^+-dependent, starvation-induced) velocities were obtained by subtracting uptakes in the absence of Na^+/presence of inhibitor from that in the presence of Na^+ or absence of inhibitor.

Western analysis. Protein aliquots (50 μg/lane) were electrophoresed on 7.5% SDS-polyacrylamide by the method of Laemmli (21). Proteins were electrotransferred to a 0.45-μm nitrocellulose membrane, and blots were probed with the specified amount/dilution of antibody using previously described conditions (17). For the detection of EAAC1 protein, blots were probed with 43 ng IgG/mL of the EAAC1 polyclonal antibody or preimmune sera in blocking solution [1% nonfat dry milk and 2% casein hydrolysate in 10 mM Tris-HCl (pH 7.5) and 300 mM NaCl] for 1 h at room temperature with agitation. For the detection of GLAST1 and GLT1 proteins, blots were probed with rat GLAST1 (320 ng IgG/mL) or rat GLT1 (68 ng IgG/mL) antibodies as described previously (22). Immunoreactive bands were detected with Protein A conjugated to horseradish peroxidase or horseradish peroxidase–conjugated secondary antibody (1:1000), as appropriate. Visualization was performed with an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, U.S.A.). These antibodies have been previously validated and found to be specific through the use of preincubation of primary antibody with the appropriate peptide or fusion protein against which each antibody was made, with subsequent disappearance of visible bands. The presence of multiple bands on immunoblotting of these transport proteins has been noted previously, by both ourselves and others (23). We have previously shown that the bands visualized compete with specific peptide (17). Thus, all visible bands were included in the analysis. Densitometry was performed using National Institutes of Health software.

Data analysis. Differences between groups were evaluated using *t* test or by the test of differences of means (in the case of derived values, such as Na^+-dependent uptakes). Regression lines were calculated with SigmaPlot (SPSS, Inc., Chicago, IL, U.S.A.)

RESULTS

Glutamate uptake into apical and basal membrane vesicles derived from rat placental labyrinth has previously been demonstrated to occur *via* System X_{AG}^- (17). For determining whether Rcho-1 cells were capable of glutamate transport, the uptake of 1 μM glutamate was evaluated in the presence and absence of Na^+ (Fig. 1). Glutamate uptake was, for the most part, Na^+-dependent and linear through at least 5 min. A significant portion of uptake was inhibited by D-aspartate (Fig. 2); this uptake was attributed to System X_{AG}^-.

System X_{AG}^- activity is up-regulated by substrate deprivation in HRP.1 cells derived from midgestation rat placenta, as well as in a renal epithelial cell line (24, 25). Accordingly, the influence of amino acid deprivation on glutamate uptake by Rcho-1 cells was evaluated (Fig. 3). Compared with the nondepleted (control) treatment, amino acid depletion resulted in System X_{AG}^-–mediated glutamate uptakes that were significantly increased by at least 4-fold at 6, 24, and 48 h. As contrasted to previous studies performed in HRP.1 cells (derived from rat placental labyrinth), the addition of 1 mM glutamate did not abrogate this stimulatory effect (17, 24). Although no tested amino acid, by itself, entirely abolished the amino acid deprivation–induced increase in activity at each time point, glutamine, leucine, and α-(methylamino)isobutyric acid (MeAIB), a nonmetabolizable amino acid analogue substrate of System A, had intermediate effects.

Five amino acid transporters capable of glutamate transport consistent with System X_{AG}^- activity have been cloned. We

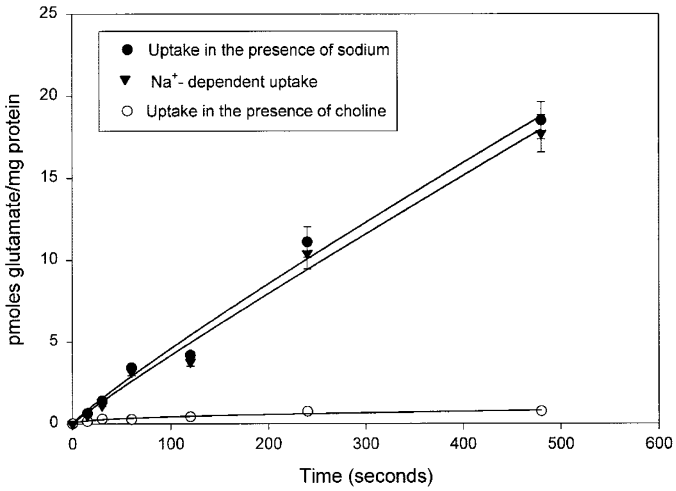

Figure 1. Time course of glutamate uptake into Rcho-1 cells. The transport of 1 μM [^{3}H]glutamate into Rcho-1 cells at 80% confluence is depicted. Conditions are as depicted; uptake was performed in either the presence of 100 mM Na^+ or choline gradients. Na^+-dependent uptake was calculated by subtracting uptake in the presence of choline from that in the presence of Na^+. All data points represent mean ± SE of pooled data points from replicate experiments. Lines are best-fit regressions calculated from the data points.

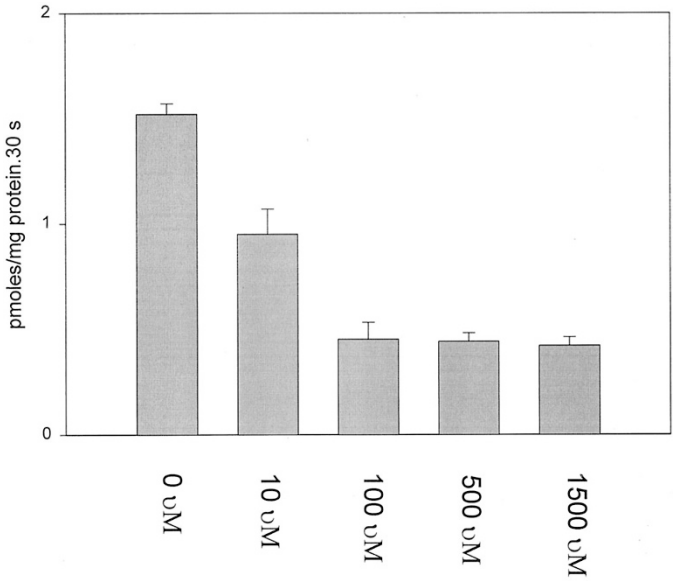

Figure 2. Inhibition of [^{3}H]glutamate uptake into Rcho-1 cells by varied concentrations of D-aspartate. The Na^+-dependent transport of 1 μM [^{3}H]glutamate into Rcho-1 cells at 80% confluence is depicted. Points shown are mean ± SE of pooled 30-s observations from replicate experiments.

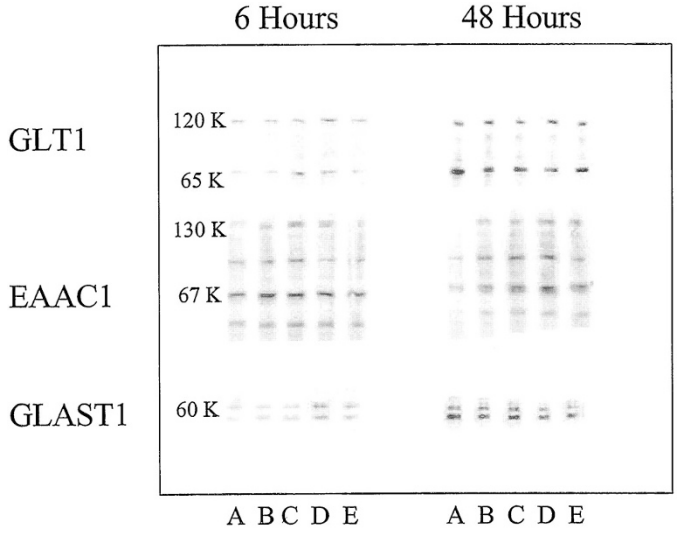

Figure 4. Immunoblot analysis of System X_{AG}^- transport proteins in Rcho-1 homogenates. Images shown are representative of immunoblots performed on cellular proteins derived from at least three replicate experiments. Equal protein loading was ensured by fast green staining before immunoblot analysis. A, + amino acids; B, minus amino acids; C, + 1 mM glutamate; D, + 1 mM glutamine; E, + 1 mM 2- MeAIB.

have previously demonstrated the presence of three of these, EAAC1, GLT1, and GLAST1, in rat placenta, as well as in HRP.1 cells (17, 24). To explore the mechanism by which glutamate uptake was enhanced by amino acid depletion, we used previously characterized antibodies directed against EAAC1, GLT1, and GLAST1 to perform immunoblotting of Rcho-1 cell homogenates in the presence/absence of amino acids. As depicted in Figures 4 and 5, GLT1 immunoreactive protein was unaffected at 6 h by any study condition. In contrast, GLT1 content was diminished by approximately 50%

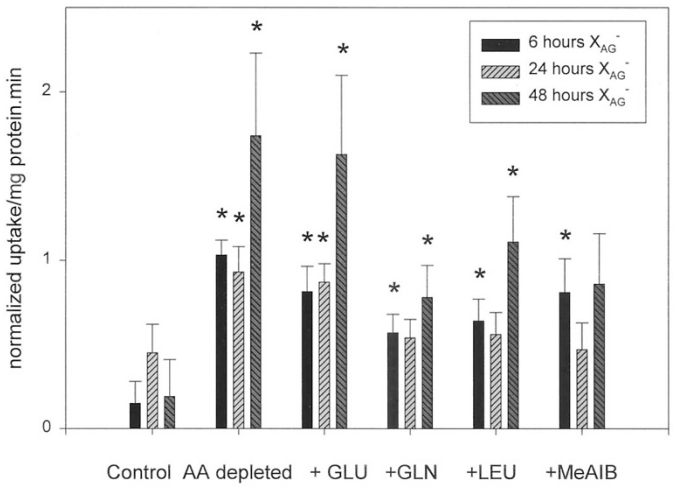

Figure 3. Influence of amino acid depletion upon System X_{AG}^-–mediated uptake in Rcho-1 cells. Experiments were performed as detailed in "Methods." Each point represents the mean ± SE (n = 6) of 1-min uptakes normalized (to compensate for interexperiment variability, n = 3) to total uptake of 1 μM [^{3}H]glutamate in the presence of amino acids. Control, normal complement of amino acids in Selectamine media; AA-depleted, lack of amino acids in media; GLU, glutamate; GLN, glutamine; LEU, leucine. Significance of differences between groups was determined by using the test of differences of means (*$p \leq 0.05$).

after 48 h in culture in the presence of 1 mM glutamine. All other experimental conditions were similar to amino acid–replete controls. EAAC1 immunoreactive protein was increased in Rcho-1 cell homogenates at 6 h in amino acid–depleted cells and glutamate-supplemented cells. No increase in EAAC1 content compared with amino acid–replete control was seen when either glutamine or MeAIB was added to the media. After 48 h, EAAC1 content in amino acid–depleted cells was increased approximately 2.5-fold compared with amino acid–replete control. Significant increases were also noted in the presence of glutamine and glutamate; although EAAC1 content was also increased in the presence of MeAIB, this difference did not reach significance ($p \leq 0.08$). Immunoreactive GLAST1 was unaffected at 6 h by amino acid depletion compared with control. This effect persisted despite the addition of either glutamate to the media, whereas the addition of glutamine produced a significant increase in protein content. At 48 h, immunoreactive protein contents were not different from control in any group.

We have previously shown in HRP.1 cells that the enhancement in glutamate uptake brought about by amino acid deprivation could be accounted for, to a significant degree, by an increase in dihydrokainate (DHK)-inhibitable activity (24). In the present studies, at 6 h, transport activity induced by amino acid deprivation was inhibited by D-aspartate, an inhibitor of GLT1, GLAST1, and EAAC1, to a significantly greater degree than by DHK, an inhibitor of GLT1 but not EAAC1 or GLAST1 (Fig. 6) (26). This observation indicates that EAAC1 and/or GLAST1 activity was primarily responsible for the observed System X_{AG}^-–mediated glutamate uptake. In contrast, after 24 and 48 h in culture, GLT-1 seemed to be responsible for the increase in System X_{AG}^-–mediated glutamate uptake induced by amino acid deprivation as the amount of D-aspartate–inhibitable and DHK-inhibitable uptake did not

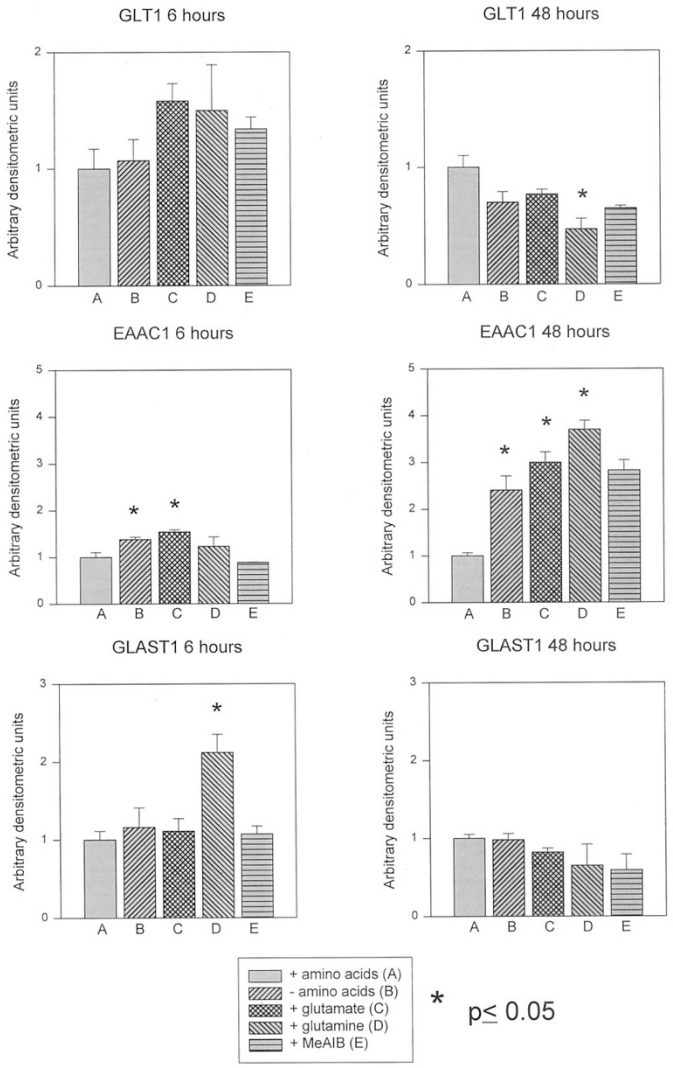

Figure 5. Densitometric analysis of protein expression in Rcho-1 cells. Densitometry was performed as described in "Methods" and normalized within experiments to that in the presence of amino acids. Values depicted are mean ± SE. Significant differences were determined through the application of two-tailed *t* test (*$p \leq 0.05$).

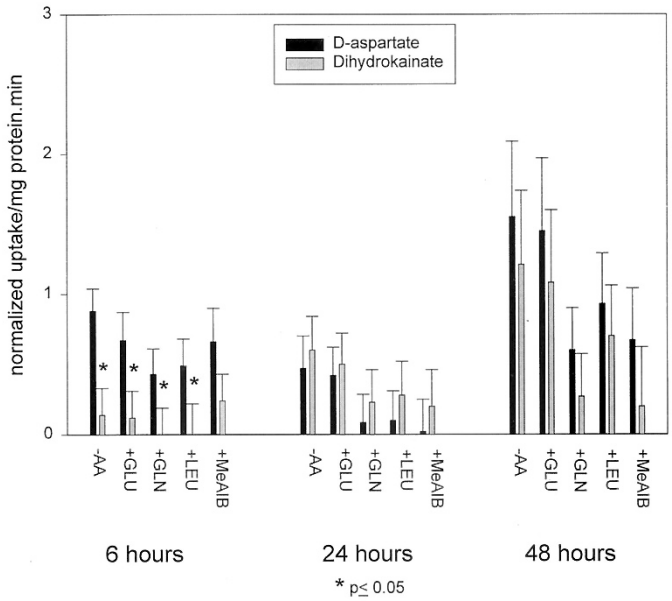

Figure 6. Inhibition of amino acid depletion–induced glutamate uptake by DHK and D-aspartate. All uptakes (1 min) were performed with 1 μM [^{3}H]glutamate in the presence of a 100-mM inwardly directed Na^+ gradient with and without 1 mM DHK or 500 μM D-aspartate. Shown for each depicted medium is the difference between uptake in the presence or absence of inhibitor (either D-aspartate or DHK), minus the same value obtained in amino acid–replete media. Each point represents the mean ± SE of six individual observations normalized (to compensate for interexperiment variability) to total uptake of 1 μM [^{3}H]glutamate in the presence of amino acids from three temporally separate experiments. Potential differences were determined through use of the test of means of differences (induced uptake; *$p \leq 0.05$).

differ. These data suggest that glutamate transfer attributable to GLT1 assumes increasing importance with longer periods of amino acid depletion.

DISCUSSION

The maternal/fetal "glutamine-glutamate cycle" has been carefully defined in the ovine model (12, 13, 27–29). Maternal glutamine is transferred across the placenta to the fetal circulation, extracted by the fetal liver, and then deaminated for nitrogen utilization and release of glutamate. Glutamate thus derived is then returned to the placenta, where the majority is oxidized to CO_2 and H_2O. We previously have demonstrated, through immunohistochemical studies, the presence of three proteins known to mediate Na^+-dependent, D-aspartate–inhibitable anionic amino acid transport [GLAST1 (30), GLT1 (31), EAAC1 (32)], previously defined as System X_{AG}^-, in rat placenta (17). We have subsequently documented the presence of these proteins in a cell line derived from rat trophoblast (HRP.1 cells) and delineated their regulation by amino acid availability (24). The current studies examine anionic amino acid uptake and its regulation in a cell line that models placental marginal giant cells.

The findings of this study contrast with those referred to above in HRP.1 cells. Whereas System X_{AG}^-–mediated glutamate uptake is present in both cell lines, that in Rcho-1 cells is approximately 1 order of magnitude lower than that in HRP.1 cells at comparable time points (24). Although data obtained from the *in vitro* study of cell lines must always be compared with caution to that present *in vivo*, these data suggest differing capacities for substrate passage into these cell types. In contrast to HRP.1 cells, uptake of glutamate into Rcho-1 cells does not seem to be regulated by glutamate sufficiency. This suggests the need for a constant supply of this amino acid, regardless of extracellular concentrations. Conflicting data exist regarding variations in maternal serum glutamate levels in pregnancies complicated by intrauterine growth retardation, having been reported both as diminished or unchanged (33, 34). Fetal serum levels seem unchanged in both groups. We have reported similar findings in animals fed a protein-restricted diet (35). Limited available evidence suggests that placental tissue concentrations rise in pregnancies complicated by growth retardation (36). Even in the normal situation, however, *in vivo* placental tissue glutamate concentrations are among the highest of any of the amino acids (37). *In vitro*, however, intracellular glutamate metabolism may be sufficiently rapid to prevent the rise of intracellular concentra-

tions, with subsequent down-regulation of transfer. Alternatively, transfer may be regulated by an amino acid not tested in this study.

We have demonstrated the presence of three anionic amino acid transfer proteins, GLAST1, GLT1, and EAAC1, in Rcho-1 cells. This observation correlates with that previously made in marginal giant cells *in situ* (17). As in HRP.1 cells, GLT1-mediated glutamate uptake assumes increased importance under conditions of amino acid depletion, despite the overall decrement in cellular GLT1 content. We hypothesize that this discrepancy may be explained by an increase in the surface expression of this transport protein, as we have shown in HRP.1 cells (24).

In a seeming paradox, EAAC1 content increased with amino acid deprivation at 48 h, whereas DHK-insensitive System X_{AG}^-–mediated glutamate uptake did not. Possible explanations for this phenomenon include a concurrent decrease in glutamate transfer mediated by GLAST1 or the possibility that whole-cell EAAC1 content does not correlate directly with EAAC1-mediated glutamate transfer. This possibility is consistent with the recent identification of a distinct protein (GTRAP3-18) involved in the regulation of EAAC1 (38). Other potential regulators of the anionic amino acid transfer proteins include the insulin-like growth factors, epidermal growth factor, fibroblast growth factor, protein kinase C, and phosphatidyl inositol 3 kinase (39–41).

CONCLUSION

In summary, System X_{AG}^-–mediated glutamate uptake and transport proteins associated with that activity are present in Rcho-1 cells derived from rat placenta. Uptake is stimulated by amino acid deprivation but is not suppressed by glutamate sufficiency. These data, when considered in the context of that obtained both *in situ* and in a cell line derived from an anatomically and functionally distinct placental cell type, confirm that glutamate transfer within the placenta is heterogenous, in both its magnitude and its regulation.

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