Induction of High Affinity Glutamate Transport Activity by Amino Acid Deprivation in Renal Epithelial Cells Does Not Involve an Increase in the Amount of Transporter Protein*

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In renal epithelial cells amino acid deprivation induces an increase in L-Asp transport with a doubling of the V_{max} and no change in K_{m} (4.5 μM) in a cycloheximide-sensitive process. The induction of sodium-dependent L-aspartate transport was inhibited by single amino acids that are metabolized to produce glutamate but not by those that do not produce glutamate. The transaminase inhibitor aminooxycetate in glutamine-free medium caused a decrease in cell glutamate content and an induction of glutamate transport. In complete medium aminooxycetate neither decreased cell glutamate nor increased transport activity. These results are consistent with a triggering of induction of transport by low intracellular glutamate concentrations. High affinity glutamate transport in these cells is mediated by the excitatory amino acid carrier 1 (EAAC1) gene product. Western blotting using antibodies to the C-terminal region of EAAC1 showed that there is no increase in the amount of EAAC1 protein on prolonged incubation in amino acid-free medium. Conversely, the induction of high affinity glutamate transport by hyperosmotic shock was accompanied by an increase in EAAC1 protein. It is proposed that low glutamate levels lead to the induction of a putative protein that activates the EAAC1 transporter. A model illustrating such a mechanism is described.

In mammalian cells intracellular glutamate concentrations are maintained at a high level by the presence of active transport systems for glutamate in the plasma membrane. A number of different glutamate transporters have been kinetically characterized in various cell types including the Na^{+}-independent transporter System x_{c}, and the high affinity (K_{m} 2–50 μM) sodium-dependent glutamate transporter X_{AG}. System X_{AG} (1), which is the major glutamate transporter in nonneuronal cell types, is characterized by its specificity for L-glutamate but nonselectivity for aspartate as both the D- and L-isomers are transported. The transport process is electrogenic, and it is suggested that two sodium ions and one glutamate are transported into the cell in exchange for one potassium and one bicarbonate ion (2).

In 1992 three high affinity Na^{+}-dependent glutamate transporters were cloned. These are termed EAAC1 (1) (the human homologue is excitatory amino acid transporter 3, EAAT3) (3), GLT-1 (EAAT2) (4), and GLAST-1 (EAAT1) (5). The distribution of these EAATs has been characterized by immunohistochemistry. EAAT2 is confined to specific parts of the brain, central nervous system, and placenta (6); EAAT1 is confined to the brain (7), retina (8), heart, and skeletal muscle (6). EAAC1 is found in brain but also occurs in other tissues particularly in kidney and gut (3, 6). More recently a further transporter EAAT4 has been identified in the cerebellum and placenta (9). The renal bovine epithelial cell line (NBL-1) has been used in our laboratory as a model system to study the regulation of amino acid transport (10–12). These cells, which are probably of distal tubule origin, express high activities of Na^{+}-dependent glutamate transport which have properties similar to System X_{AG} as studied in other cell types (1). In these cells glutamate transport is highly regulated. When NBL-1 cells are starved of amino acids, there is an increase in X_{AG} transport activity with a doubling of V_{max} and no change in the K_{m} after 10 h (13). Incubation of NBL-1 cells in 200 mM sucrose also induced glutamate transport with a 3-fold increase in V_{max} and no change in K_{m} (14). System X_{AG} in NBL-1 cells has the same kinetic properties as glutamate transport induced in Xenopus oocytes by injection of EAAC1 cDNA; since EAAC1 is known to be expressed in kidney while the other transporters are mainly restricted to brain, it is likely that EAAC1 encodes the NBL-1 cell glutamate transporter. We have shown that induction of System X_{AG} activity by hyperosmotic shock in NBL-1 cells is accompanied by a 3-fold increase in EAACL-specific mRNA (14). Conversely, there is no such increase in EAAC1 mRNA during induction of transport activity by amino acid deprivation (13). In this paper the induction of System X_{AG} by amino acid deprivation is further characterized, and sequence information is exploited to raise an antibody to show changes in the protein level during the induction of transport. We have shown that the internal glutamate level is the major determinant of the induction of System X_{AG} (13); furthermore, there is no change in the amount of transport protein during the increase in transport activity.

EXPERIMENTAL PROCEDURES

Materials—[U-^{14}C]-Aspartate, 3^{5}S-dATP, the Sequenase kit, and ECL reagents were from Amersham International (Amersham, UK). Sulfo-SMCC was obtained from Pierce (Chester, UK); pBluescript and pGEX2T were from Stratagene (Cambridge, UK) and Pharmacia (St. Albans, UK), respectively. Restriction enzymes were from Boehringer Mannheim (Lewes, UK). The synthetic peptide and oligonucleotides AOA, aminooxacetic acid; EAAT, excitatory amino acid transporter; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GRP78, 78-kDa glucose-regulated protein; GBP, glutamate-binding protein; Sulfo-SMCC, sulfoconimidyl 4-[N-maleimidomethyl]-cylohexane-1-carboxylate.

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The abbreviations used are: EAAC1, excitatory amino acid carrier 1;
were synthesized in the Department of Biochemistry. Tissue culture reagents were purchased from Life Technologies, Inc. (Paisley, UK) except the dialyzed newborn calf serum which was from Sigma.

Cell Culture—Unless indicated otherwise the NBL-1 cells were seeded at a density of 6 x 10^4 cells/ml into 35-mm Petri dishes in Ham’s F-12 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamin, and antibiotics as described previously (11). The cells were fed every other day until used in experiments, typically after 4 days. The amino acid-free medium used contained the inorganic salts of Ham’s F-12 medium together with 10 mM glucose and 0.1% bovine serum albumin (11). The glutamine-free medium contained all the ingredients of Ham’s F-12 except glutamine and was supplemented with 10% (v/v) dialyzed newborn calf serum.

Transport Measurements—System X_{AG} activity was measured as the initial rate of sodium-dependent uptake of 50 μM [U-14C]-aspartate into NBL-1 cells over 5 min at 20 °C in the presence of 0.5 mM aminoxyacetic acid to inhibit aspartate metabolism (13). In this system Na+-dependent L-aspartate transport is linear with time over 10 min (13). Sodium-independent transport was measured in medium where NaCl was replaced by an equal concentration of choline chloride. At 50 μM L-Asp the Na+-independent rate was less than 15% of the sodium-dependent rate and did not change under any of the conditions used.

Cell protein was measured as described (15).

Anti-peptide Antibody—The anti-peptide antibody was raised against a synthetic C-terminal peptide CAVDKDST15F QFTQSFQ (amino acids 520-524 of the EAAC1 sequence) with an cysteine at the N-terminal end. The peptide was linked to keyhole limpet hemocyanin via the terminal cysteine using sulfo-SMCC. The antibody was purified by passing down a column of the synthetic peptide cross-linked to CNBr-activated Sepharose 4B beads.

Cloning of the Hydrophilic Loop Region of EAAC1 and Preparation of an Anti-Fusion Protein Antibody—The cDNA encoding the putative extracellular loop between helices 3 and 4 (amino acids 117-215) was first amplified from NBL-1 cell cDNA using nested PCR. The flanking primers were 5’-GCT/CT CCC GGA/C GAG ATT/C CGT/GT IC ATG and 5’-TCA/G TAAG/C AGA/G GCG GTT/G CCA/G TCC AT. The product of the first reaction was used as template for second round PCR. The nested primers were 5’-T TTG GAA CCT CAG/C GTG and 5’-TTG CCT CCA CCG/G ACC/AAG. GCAT-TCA and GCAT-GCG were used as sequencing primers for sequencing pBluescript. A specific primer GCAT-GCAGT was used for sequencing across the pGEX2T multiple cloning site.

The PCR product was sequenced and shown to have 83.75% identity with the rabbit intestine EAAC1 DNA sequence and 78.5% identity at the protein level. Because problems were encountered in direct ligation into the prokaryotic expression vector pGEX2T, the DNA was blunted and cloned into EcoRV-cut pBluescript, which had been pre-treated with calf intestinal alkaline phosphatase. The construct was transformed into Ca2+-competent Escherichia coli XL-1 by heat shock. Using the BamHI sites engineered into the primers, the insert was excised from pBluescript and ligated into pGEX2T, which was used to transform XL-1 as before. The construct was sequenced across the insertion site to ensure that no frame shifts had occurred. Once the absorption of the transformed cells at 600 nm reached 0.3, glutathione-S-transferase-loop construct expression was induced for 1/2 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. 10 out of 20 transformants expressed the glutathione-S-transferase-loop construct in the correct orientation. The bacterial proteins were separated on SDS-PAGE, and the fusion protein was introduced overnight at 50 mA.

Western Blots—Cell extracts were prepared as follows. Cell cultures were washed twice in ice-cold phosphate-buffered saline (PBS). The cells were scraped off into ice-cold PBS in the presence of protease inhibitors. 25 mM detergent MEGA-10 to a final concentration of 1% in the presence of 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and centrifuged. The PBS was removed, and the cells were resuspended in 20 mM Tris-HCl, pH 7.4, before dissolving in the neutral detergent MEGA-10 to a final concentration of 1% in the presence of protease inhibitors. 25 μg of the samples were then separated on SDS-PAGE under reducing conditions. The proteins were transferred into the absence of SDS using a semi-dry blotting apparatus, 1 h, 10 V.

The blots were blocked for 20 min in PBS/0.02% Tween containing 5% dried milk powder. The anti-peptide antibody was added for 1 h in the same buffer, and the blots were then washed three times in PBS/0.02% Tween before anti-rabbit horseradish peroxidase conjugate was added at 1μg/ml in 5% powdered milk/PBS/0.02% Tween for 45 min.

Characterization of the Induction of System X_{AG} Activity—Activity of Amino Acid Deprivation in NBL-1 Cells—Total amino acid deprivation causes an increase in the V_{max} of Na+-dependent L-aspartate transport in NBL-1 cells. This process is sensitive to cycloheximide and is maximal after 10 h (13). On repeating this work it was found that the magnitude of the stimulation depended on the degree of confluency of the cells (Fig. 1). There was a 300% induction by amino acid starvation 3 days after seeding the cells (confluency approximately 80%), but the degree of induction decreased as the cells became more confluent and was relatively low in confluent cells. Subsequent experiments were performed under conditions where the induction was maximal, as indicated in the figure legends.

Addition of certain single amino acids to the amino acid-free medium reduced the induction of System X_{AG} activity, and this is further characterized in Table I. All the substrates of System X_{AG} when added at 1 mM concentration at zero time caused 50% inhibition when transport was measured 24 h later. In addition to the substrates of System X, the amino acids L-Asn, L-Ala, and L-Gln, which can be converted to glutamate by transamination, reduced the induction of transport activity. Conversely, D-Gln, L-Cys, and L-Leu, which cannot produce intracellular glutamate in these cells, did not influence the induction of transport. D-Glu, which is a poor substrate for System X_{AG}, also inhibited the induction in accordance with previous work (13).

In order to confirm that the effect of added amino acids on the apparent induction of transport was not in fact due to trans-inhibition of uptake by differential internal concentrations of glutamate, cells were incubated with various single amino acids (1 mM) in amino acid-free medium for 1 h allowing intracellular glutamate accumulation but not allowing enough time for protein synthesis to occur. In all cases there was no increase above the rate in amino acid-free medium (not shown).
It was previously shown, and is confirmed here, that the induction of transport activity by amino acid-free medium was completely inhibited by cycloheximide, confirming that the effect was due to protein synthesis.

The results in Table I suggest that the intracellular glutamate concentration may determine the level of expression of System XAG activity. An independent way of manipulating intracellular glutamate levels is by the use of aminooxyacetic acid (AOA), which is a specific inhibitor of transaminases. AOA prevents the formation of glutamate from other amino acids. Fig. 2 shows an experiment in which cells were incubated in the presence and absence of AOA in either normal medium or glutamine-free medium. Transport was measured after 24 h. AOA caused an induction of transport when present in the glutamine-free medium but not in normal medium. In the absence of AOA, omission of glutamine produced only a small induction. In the absence of glutamine and the presence of AOA glutamate can be produced neither by glutamine hydrolysis nor by transamination of other amino acids, and intracellular glutamate levels would be predicted to fall. The fact that AOA did not induce transport activity in the presence of glutamine indicates that AOA per se was not responsible for the induction. Under these conditions intracellular glutamate is predicted to be high since glutamate produced by glutaminase cannot be transaminated. Since the rates of Na\(^+\)-dependent L-Asp transport were not markedly reduced by AOA under any conditions, it is unlikely that AOA was significantly damaging cell function.

In order to confirm this interpretation, the cell glutamate content was determined enzymatically. Fig. 3 shows that on switching cells to amino acid-free medium the cellular glutamate content fell from 30 nmol/mg protein to 13 nmol/mg protein over a period of 5 h. In cells transferred to fresh normal medium at zero time, the cellular glutamate concentration fell much more slowly. Under the conditions corresponding to the experiment shown in Fig. 2, the internal glutamate contents at 1 h were determined. In glutamine-free media + 0.5 mM AOA the cell glutamate content fell to 15.0 ± 0.6 nmol/mg protein, while in normal media in the presence of 0.5 mM AOA the cell glutamate content was 29.2 ± 1.0 nmol/mg protein, which is similar to the control values. These results suggest that the induction of System XAG is triggered when the glutamate level falls to about 15 nmol/mg protein. This occurs within an hour in the presence of AOA and the absence of glutamine or in amino acid-free cells after 2–3 h. Glutamate levels did not reach this value in cells cultured in normal medium.

Induction of System XAG Activity by Tunicamycin—Tunicamycin is an inhibitor of protein glycosylation. Incubation of cells with 0.1 μg/ml tunicamycin in amino acid-free medium for 24 h apparently reduced the amino acid starvation-dependent induction of aspartate transport, suggesting that glycosylation of a protein is a necessary step in this process (Fig. 4). However, the addition of 0.1 μg/ml tunicamycin to cells in normal medium for 24 h itself induced transport activity. The induction was cycloheximide-sensitive (Fig. 4). This effect was characterized by an increase in the V\(_{\text{max}}\) from 110 to 180 pmol/mg/min whereas the K\(_{\text{m}}\) for L-aspartate was unchanged at 4.6 μM (results not shown). The half-maximal effect was at 0.055 μg/ml tunicamycin, which is a concentration at which the effect of this compound on protein glycosylation should be specific.
PCR products were in close agreement with the published using NBL-1 cell cDNA as a template; partial sequences of the rabbit intestine produced PCR products of the expected size (3). Primers designed to two internal EAAC1 sequences from those of the EAAC1 gene product expressed in Xenopus oocytes were produced. One antibody was raised to the C-terminal amino acid deprivation, two specific antibodies to the protein measure changes in the level of the EAAC1 protein during high affinity glutamate transport in NBL-1 cells. In order to show that the EAAC1 gene product is responsible for activity by exposure of NBL-1 cells to hyperosmotic media was accompanied by a 3-fold increase in EAAC1 mRNA levels (14). The purified anti-peptide C-terminal antibody recognized a major band at 64 kDa in NBL-1 cells, together with some minor bands (Fig. 5). This is reasonably consistent with the molecular mass of the EAAC1 protein from rabbit intestine which is predicted to be 57 kDa. The molecule contains four potential glycosylation sites, but the extent of glycosylation is not known. Fig. 5 also shows that the anti-loop antibody recognized a major band of the same size as that recognized by the C-terminal anti-peptide antibody. The fact that antibodies raised to different regions of the EAAC1 protein recognize the same protein is good evidence that this protein is the EAAC1 gene product. Western blots (not shown) indicate that the C-terminal antibody recognized a protein of the same molecular weight in rat brain. This is also consistent with the molecular weight of the protein recognized in rat brain by another antibody raised to the C-terminal of EAAC1 (7).

Fig. 5. Duplicate Western blots showing that both the anti-peptide (lane 1) and anti-fusion protein (lane 2) antibody recognize a band of the same size. Three days after seeding, the mean ± S.E. of initial rates of Na+-dependent aspartate transport obtained from three dishes of cells in each case.

Fig. 6 shows Western blots of NBL-1 cells incubated for 24 h in normal medium (lane 1), amino acid-free medium (lane 2), normal medium + 200 mM sucrose (lane 3), and normal medium + 0.1 μg/ml tunicamycin (lane 4). Three days after seeding, the medium was changed to that indicated. 24 h later whole cell extracts were prepared as described under “Experimental Procedures,” separated by SDS-PAGE, and transferred to nitrocellulose. Duplicate 25-μg samples were probed with either the anti-peptide antibody or anti-fusion protein antibody.

DISCUSSION

The results presented above indicate that the necessary condition for the induction of glutamate transport by amino acid deprivation in NBL-1 cells is the presence of a cellular glutamate content of less than about 15 nmol/mg. Since the intracellular volume in these cells is about 6.2 μl/mg protein (10), this would correspond to an intracellular concentration of 2.5 mM. When glutamate is depleted from 30 to about 15 nmol/mg the transport activity) showed a very significant increase in the amount of protein detected by the anti-peptide antibody. Table I quantifies the changes in protein over a number of different experiments.

No change in the protein level was observed between normal and amino acid-starved cells, although an increase in transport under these conditions has been shown to occur. However, cells exposed to sucrose or tunicamycin (other conditions that induce transport activity) showed a very significant increase in the amount of protein detected by the anti-peptide antibody. Table II also reports the results of a similar experiment where transport was induced by the addition of 0.5 mM AOA and in the absence of 1 mM L-Gln. There was no change in the amount of protein even though an increase in L-Asp transport occurred under these conditions (see Fig. 2).
of glutamine reduces glutamate levels to 15 nmol/mg and causes the induction of transport. (iv) AOA in the presence of glutamine neither reduces glutamate levels nor induces transport. This is likely to be a physiologically important mechanism for maintaining glutamate levels.

Western blots have shown that there is no change in the EAAC1 protein level during amino acid deprivation even though there is an increase in transport. Under other conditions where transport has been shown to be induced, i.e. exposure to hyperosmotic medium or tunicamycin, a clear increase in the amount of protein detected by the C-terminal antibody is observed. The increase in transport activity is dependent on protein synthesis and is not due to differential transinhibition as a result of different internal glutamate concentrations. Also it has been previously shown in this laboratory that EAAC1 mRNA levels do not increase during amino acid deprivation, although these mRNA levels do increase as a result of hyperosmotic shock (14) and tunicamycin treatment. Since the induction of transport activity requires protein synthesis but the amount of EAAC1 protein itself does not increase, we postulate that the induction of a putative EAAC1-activating protein is responsible for the increase in the rate of aspartate transport. As the Km is unchanged on incubating the cells in amino acid-free medium (13), it is unlikely that this is due to the induction of a different glutamate transporter. Since the activation is reduced by tunicamycin, this suggests the putative activating protein may well be a glycoprotein.

Tunicamycin itself in normal medium caused increases in EAAC1 mRNA and protein. The mechanism of this effect is not clear but is likely to be related to a stress effect possibly triggered by the presence of unfolded proteins as has been suggested for GRP78 (18). As there is a 4-fold increase in EAAC1 protein, but only a doubling in the rate of transport, it appears that some of EAAC1 protein is not reaching the plasma membrane due to mistargeting in the absence of glycosylation. The question arises as to how the cell is able to detect changes in the cellular glutamate level in the range of 30 to 15 nmol/mg. There are a number of proteins that regulate amino acid transport in bacteria such as the leucine-responsive regulatory protein in E. coli (19) and the glutamate uptake regulatory protein in Zymomonas mobilis. This protein has a helix-turn-helix motif that is typical of a transcription factor and has been shown by gel retardation assays to bind the regulatory region of the E. coli gene gltP, which encodes a proton symporter for glutamate and aspartate (20). The results in this paper are consistent with the presence of a low affinity glutamate binding protein (GBP) that can act in one of two ways to switch on the synthesis of a protein that activates System XAG2 (Fig. 7). One possibility is that GBP is a transcription factor that binds L-glutamate with low affinity. Alternatively, the GBP could be an mRNA-stabilizing factor or an mRNA-binding protein affecting mRNA translation. In either case GBP-glutamate complex is assumed to be inactive. Relatively more of the active form of the protein would be present at low glutamate concentrations. Since a-glutamate also inhibits the amino acid deprivation-induced increase in System XAG activity, GBP must also be assumed to bind a-glutamate.

In NBL-1 cells System A is also induced by amino acid deprivation in a process that is protein synthesis-dependent, sensitive to tunicamycin, and reversed or prevented by the addition of single amino acids (11); these results are consistent with earlier work on hepatocytes (21). Since System A has not yet been cloned, no definitive mechanism for this effect has been established. There are indications that the induction of System A involves the synthesis of a hypothetical transport activating protein (21) rather than the System A transport protein itself. This conclusion has been reinforced by studies of Chinese hamster ovary cell mutants that do not induce System A activity on amino acid deprivation (Ref. 22; for review see Ref. 23). It is possible that amino acid deprivation leads to the

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**TABLE II**

Quantification of the relative amount of EAAC1 protein in NBL-1 cells incubated for 24 h in various conditions

| Medium                                      | Relative amounts of EAAC1 protein | mean ± S.E. (number of tracks scanned) |
|---------------------------------------------|----------------------------------|----------------------------------------|
| Complete medium                             | 1.0 ± 0.1 (6)                    |                                        |
| Amino acid deprivation medium               | 1.1 ± 0.3 (3)                    |                                        |
| Complete medium + 200 mM sucrose            | 2.5 ± 0.3* (3)                   |                                        |
| Complete medium + 1 μg/ml tunicamycin       | 4.2 ± 0.8* (3)                   |                                        |
| Glutamine-free medium                       | 1.0 ± 0.1 (3)                    |                                        |
| Glutamine-free + 0.5 mM AOA                 | 1.0 ± 0.1 (6)                    |                                        |
| Complete medium + 0.5 mM AOA                | 1.0 ± 0.1 (6)                    |                                        |

* p < 0.005 versus cells in complete medium.
  b p < 0.05 versus cells in complete medium.

2 B. Nicholson, unpublished results.
synthesis of one or more glycoproteins that act as activators of amino acid transport proteins in cell membranes, thus assisting in maintenance of the intracellular amino acid pool under these conditions.

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