Induction of the high-affinity Na⁺-dependent glutamate transport system X_{AG}⁻ by hypertonic stress in the renal epithelial cell line NBL-1

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The high-affinity Na⁺-dependent glutamate transport system X_{AG}⁻ is induced (threefold increase in $V_{\max}$ with no change in K_m) by hypertonicity in the renal epithelial cell line NBL-1. This effect is dependent on protein synthesis and glycosylation and is accompanied by an increase in EAAC1 mRNA levels. Other Na⁺-dependent transport systems in this cell line do not respond to hypertonic stress. In contrast to recent findings [Ruiz-

Montasell, Gomez-Angelats, Casado, Felipe, McGivan and Pastor-Anglada (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 9569–9573] showing that increased system A activity after hyperosmotic shock results from induction of a regulatory protein, this is the first demonstration that hypertonicity may increase the expression of the gene for an amino acid transport protein itself.

INTRODUCTION

The Na⁺-dependent uptake of various substrates is induced by hyperosmotic stress in a variety of mammalian cell types. In particular, the induction of the transport systems for the osmolytes betaine and *myo*-inositol in renal medulla cells in response to prolonged exposure to hypertonic medium is associated with increased levels of their respective mRNAs [1–3]. This response may have a particular physiological significance in renal cells.

System A activity for neutral amino acid transport is similarly induced in a variety of cell types (for a review see [4]). In the bovine renal epithelial cell line NBL-1, system A activity is induced by hypertonic shock only after derepression by amino acid starvation [5]. Recent evidence suggests that the inductions mediated by starvation and by medium hypertonicity are the result of separate mechanisms, probably involving the synthesis of different proteins [6]. There is no clear evidence that the system A protein is itself induced by hypertonic stress.

In order to determine whether any amino acid transport system may be directly induced by hypertonic stress we have studied the high-affinity glutamate transport system X_{AG}⁻ in NBL-1 cells. Previous work has shown that a transport system kinetically identical to X_{AG}⁻, as defined in other cell types, is present in NBL-1 cells and is induced by amino acid starvation [7]. Evidence has been presented [7] that system X_{AG}⁻ in this cell line is encoded by the EAAC1 cDNA-related gene cloned by Kanai and Hediger [8].

This paper shows that hypertonic stress increases the $V_{\max}$ of the X_{AG}⁻ transport system in a glycoprotein synthesis-dependent manner and this induction of transport activity is associated with an increase in the level of EAAC1 mRNA.

EXPERIMENTAL

Cell line and culture conditions

The bovine renal epithelial cell line NBL-1 was obtained from Flow Laboratories (McLean, VA, U.S.A.). Cells were routinely

grown in Ham's F-12 medium supplemented with 10% (v/v) newborn-calf serum, 2 mM glutamine and antibiotics as described previously [9]. For hypertonicity experiments confluent cells were cultured in the same medium containing various concentrations of sucrose.

Transport measurements

Cell cultures were washed extensively in amino acid- and serum-free medium prior to transport measurements. System X_{AG}⁻ activity was routinely measured as the uptake of 5 μ M L-[¹⁴C]aspartate (Amersham, U.K.) in the presence of 0.5 mM amino-oxyacetate to inhibit aspartate metabolism. In this system Na⁺-dependent aspartate uptake is linear over 20 min. Aspartate uptake in the absence of Na⁺ (choline chloride medium) was negligible. Previous studies [7] have demonstrated that Na⁺-dependent aspartate uptake in these cells is mediated exclusively by a transport system with properties identical to those of system X_{AG}⁻ as defined in other cell systems. Details of the procedures used for measurement of transport have been described elsewhere [7].

RNA extraction and Northern blot analysis

Total RNA was extracted from confluent cell cultures using the guanidinium thiocyanate method [10], separated on a 1% agarose gel under denaturing conditions, transferred overnight to nylon membranes (Hybond N, Amersham, U.K.) in 20× SSC (SSC: 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) and cross-linked by UV exposure. The blot was prehybridized overnight at 42 °C in 45% formamide, 10% dextran sulphate, 4× standard sodium phosphate-EDTA (0.6 M NaCl, 40 mM NaH₂PO₄, 4 mM EDTA, pH 7.4), 5× Denhardt's solution (1 g/l BSA, 1 g/l polyvinylpyrrolidone 40, 1 g/l Ficoll), 0.1 mg/ml sonicated salmon sperm DNA and 0.1% SDS. Blots were hybridized for 24 h at 42 °C in the same solution with the ³²P-labelled EAAC1 probe (10⁶ c.p.m./ml). Filters were washed twice for 20 min at 55 °C with 1× SSC and 0.1% SDS, and twice with 0.2× SSC and 1% SDS before autoradiography.

The EAAC1 probe (nucleotides 986–1716, see [8]) was generated using PCR and primers to the published sequence. Details of this probe are given elsewhere [7]. The probe was labelled by random priming using the megaprime kit (Amersham, U.K.).

RESULTS

Effect of hyperosmotic medium on system X_{AG}^- activity

Figure 1 shows that system X_{AG}^- activity increases more than threefold in cells cultured in hypertonic medium (200 mM sucrose added to the normal medium). The increase occurred between 12 and 24 h after exposure of the cells to sucrose-containing medium. Similar results were obtained when mannitol was used instead of sucrose (results not shown). The activity in the absence of Na^+ was negligible and was not affected by the hyperosmotic medium. The increase in the rate of transport after 18 h was due to an approximately threefold increase in V_{max} , from 0.31 ± 0.1 to 0.86 ± 0.04 pmol/mg per 3 min, with no significant change in the K_m for L-aspartate (control K_m 4.0 ± 0.81 μ M, hyperosmotic medium K_m 5.2 ± 1.1 μ M) as shown in Figure 2. The effect of sucrose after 18 h was dose-dependent with little effect below 50 mM and a half-maximal effect at approximately 120 mM (Figure 3).

Effect of inhibitors on the induction of system X_{AG}^- activity

Table 1 shows the effect of various inhibitors on the induction of system X_{AG}^- activity after an 18 h exposure to medium supplemented with 200 mM sucrose in the continuous presence of the

Figure 1 Time course of the induction of system X_{AG}^- activity by exposure of cells to hyperosmotic medium

The initial rate of aspartate uptake (5 μ M aspartate) was measured over a 3 min time period. (●) Normal culture medium was replaced by the same medium supplemented with 200 mM sucrose at zero time; (○) cells cultured in normal medium throughout. Each point is the mean $\pm$ S.E.M. of 4–8 individual culture dishes (four different experiments). (■, □) Corresponding experiments in choline chloride medium.

inhibitors. The protein synthesis inhibitor cycloheximide and protein glycosylation inhibitor tunicamycin blocked the induction when used at concentrations which did not affect the basal

Figure 2 Concentration dependence of aspartate transport in NBL-1 cells cultured in normal or hypertonic medium

Confluent cultures of NBL-1 cells were incubated in (●) normal medium or (○) medium supplemented with 200 mM sucrose for 18 h. The initial rate of Na^+ -dependent aspartate transport was measured over a 3 min time period using a range of aspartate concentrations as shown. Kinetic parameters were evaluated by non-linear regression to the Michaelis–Menton equation. The results shown are the means $\pm$ S.E.M. of measurements from three individual culture dishes for each point, in a single experiment.

Figure 3 Sucrose concentration dependence of the induction of aspartate transport

Confluent cultures of NBL-1 cells were incubated in normal medium containing added sucrose as indicated for 18 h; aspartate transport (5 μ M aspartate) was then measured over a 3 min time period. Each point is the mean $\pm$ S.E.M. of 3–6 individual culture dishes (three different experiments).

Table 1 Effect of different inhibitors on Na^+ -dependent L-aspartate transport in NBL-1 cells

Confluent cultures of cells were incubated in normal medium or medium supplemented with 200 mM sucrose plus further additions as shown for 18 h. The uptake of 5 μ M aspartate was measured over 3 min. Results are the means $\pm$ S.E.M. of 4–8 individual culture dishes in four separate experiments.

	Control	Sucrose
No further additions	118 $\pm$ 4	408 $\pm$ 8
Cycloheximide (1 μ g/ml)	111 $\pm$ 2	102 $\pm$ 9
Tunicamycin (0.1 μ g/ml)	106 $\pm$ 10	129 $\pm$ 4
Colcemide (10 μ g/ml)	111 $\pm$ 5	389 $\pm$ 12

Figure 4 Northern blot of EAAC1-hybridizing RNA in NBL-1 cells exposed to hypertonic medium for various times

Details of the RNA extraction, transfer and hybridization with EAAC1 cDNA are given in the Experimental section. Results shown are: a representative Northern blot, the ethidium bromide-stained ribosomal bands and the quantification of the specific hybridization to the 3.5 kb EAAC1 RNA transcript of three independent blots (means $\pm$ S.E.M.) NBL-1 cells were cultured with 200 mM sucrose for the time indicated in hours: 0, RNA from control cells (unchanged over 9 h incubation, results not shown); 3, 6, 9, RNA from cells incubated for 3, 6 and 9 h respectively. The percentage increase is shown as the induction over control values for each time.

rate of Na^+ -dependent aspartate transport. Colcemide, an inhibitor of microtubule function, when used at a concentration previously shown to inhibit induction of system A in NBL-1 cells [5] did not block the induction of system X_{AG-} .

Effect of hypertonic stress on the levels of EAAC1 mRNA

As shown previously [7], the EAAC1 cDNA probe hybridized to a single 3.5 kb transcript from NBL-1 cell RNA (Figure 4). Exposure of NBL-1 cells to medium supplemented with 200 mM sucrose for 9 h resulted in an approximately threefold increase in the amount of EAAC1 transcript. The specific hybridization of the EAAC1 probe to the filters was quantified by means of an AMBIS apparatus (Ambis Inc.) The result shown is a representative Northern blot. The quantification of three independent experiments is shown as means $\pm$ S.E.M. in the histogram. We have eliminated the possibility of differential loading by the measurement of the ethidium bromide-stained ribosomal

bands. Common loading markers, such as β -actin, may not be suitable for this purpose because they are induced at the transcriptional level by anisotonicity [11,12]. The densitometric analysis of the bands was evaluated by the program Whole Band Analyser (BioImage Applications, Millipore). Differences between the lanes were less than 10%. Thus, no correction was made.

DISCUSSION

This paper shows that exposure of NBL-1 cells to hyperosmotic medium causes an increase in Na^+ -dependent aspartate transport activity. The characteristics of the increased activity (increase in V_{max} , no change in K_m), its blocking by selected inhibitors and the evidence that it occurs after the increase in the amount of EAAC1 mRNA are consistent with the induction of system X_{AG-} by a protein synthesis-dependent mechanism which presumably results in the synthesis and insertion of new transporters in the plasma membrane. The time-course of this effect is similar to the induction of *myo*-inositol and betaine transport described in MDCK cells [3,13] and to the induction of system A in NBL-1 cells by hyperosmotic stress [5,6]. In NBL-1 cells this effect does not reflect a general induction of Na^+ -dependent transporters since the broad-specificity amino acid transport system B⁰ [5] and Na^+ -dependent phosphate transport activity are not affected under these conditions (A. Ferrer-Martinez, A. Felipe, B. Nicholson, J. Casado, M. Pastor-Anglada and J. McGivan, unpublished work).

Inhibition of the induction by tunicamycin suggests that glycosylation of the transporter is important either for its insertion into the membrane or its activity. Similar results have been reported previously for the induction of system A [9]. The deduced amino acid sequence of the EAAC1 transporter contains four putative extracellular N-glycosylation sites [8], but nothing is known at present about the glycosylation state of the transport protein.

To date two amino acid transport systems, system A and system X_{AG-} , have been shown to be under regulation by medium hypertonicity in NBL-1 cells. However, the mechanisms involved in each case are likely to be different. The increase of system A activity requires a prior derepression by amino acid starvation; it is insensitive to tunicamycin addition but is highly sensitive to inhibition by colcemide [5]. These observations, together with other findings, have led us to suggest that the induction of a regulatory protein is responsible for this increase in system A activity [6]. Up-regulation of system X_{AG-} resembles the induction of betaine- and *myo*-inositol-transport systems, where increased activity is accompanied by higher levels of the respective mRNA species [1–3].

This is the first report showing a direct effect of hypertonicity on the expression of an amino-acid-transport system. The physiological significance of this finding is obscure, since it is unlikely that glutamate represents a significant osmolyte in renal cells. It may be that a number of Na^+ -dependent substrate transporters in various cell types are coordinately regulated by a common mechanism in response to hypertonic stress. Recently, a tonicity-sensitive element has been identified in the 5'-flanking region of the betaine transporter gene [14]. Similarly, a tonicity response element has been postulated for the aldose reductase gene [15]. Although this has still to be determined, the X_{AG-} gene may have the same osmotic response elements in its promoter.

The identification of osmotic-sensitive plasma membrane transport systems and their comparison with those which are insensitive to osmotic stress will provide a basis for future studies

on the molecular mechanisms involved in the osmotic regulation of gene expression.

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