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Long-term osmotic regulation of amino acid transport systems in mammalian cells

Review Article

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Summary. Mammalian cells accumulate organic osmolytes, either to adapt to permanent osmotic changes or to mediate cell volume increase in cell cycle progression. Amino acids may serve as osmolytes in a great variety of cells. System A, a transport system for neutral amino acids, is induced after hypertonic shock by a mechanism which requires protein synthesis and gene transcription. Indirect evidence supports the view that system A activity increases due to the interaction of pre-existing A carriers with putative activating proteins. The intracellular accumulation of most neutral amino acids after hypertonic shock depends, exclusively, on the increase in system A activity. Long-term activation of system A is dependent on the integrity of cytoskeletal structures, but in a different way depending on whether cells are polarized or not.

Keywords: Amino acids – Transport – System A – Osmotic regulation

Introductory remarks

Most of the regulatory and molecular aspects of amino acid transport systems in mammalian cells have recently been reviewed from different perspectives (Kanner, 1993; Kilberg et al., 1993; Bertrán et al., 1994; Christensen et al., 1994; Kanai et al., 1994; MacLeod et al., 1994; McGivan and Pastor-Anglada, 1994; Palacín, 1994; Mailliard et al., 1995). Extensive progress has been made during this decade in the understanding of the molecular structures involved in amino acid uptake, but some major transporters of mammalian cells, such as system A, have not yet been characterized at the molecular level. Moreover, although some features of the modulation of system A are well known and have been extensively described before, the exact mechanisms involved in

such responses are not well understood and a new series of physiological stimuli able to modulate this and other amino acid transport activities have been added to others better characterized. In recent years, the osmotic regulation of amino acid transport systems as a way to modulate cell volume in response to an isotonic shock has attracted increasing interest. In particular, selected transport systems seem to be responsive to an isotonic shock by mechanisms which from now on will be referred to as "long-term" mediated. A long-term effect implies that the activity of the transport system will be modified after the osmotic stimulus by a mechanism which will require gene transcription and protein synthesis and, consequently, will take a few hours to develop and show maximal biological responses. The evidence that some non-protein amino acids and derivatives, such as taurine and betaine, can play a role in the long-term osmotic regulation of renal epithelial cells by being concentrated inside the cell by specific transporters and acting as organic osmolytes (Garcia- Perez and Burg, 1991; Burg, 1995), raised the question of how cells which do not express these transport activities adapt (if they do so) to changes in medium tonicity. Considering that amino acids are highly concentrated in the cytosol, it is not surprising that they can contribute to the osmotic adaptation of the cell to an isotonic environment. Consequently, the transport systems involved in amino acid uptake in mammalian cells may be a target of the osmotic regulation and may contribute to the modulation of the intracellular concentration of potential osmolytes.

In summary, what we will discuss in this article is a particular feature of amino acid transport regulation which has not been reviewed in detail before, in an attempt to link two aspects of cell physiology, the transporters which are responsible for the uptake of protein amino acids (substrates which are not metabolically inert) and their potential role in long-term adaptation of the cell to anisotonic, essentially hypertonic, conditions.

A need for organic osmolytes in mammalian cells

Organic osmolytes are present in all phyla from Man to prokaryotes (Kinne, 1993). This observation by itself may justify the requirement of these compounds for cell survival and can be interpreted on the basis that the biochemical processes underlying the control of osmolyte concentration inside a cell have never been counterselected through evolution, although they have probably specialized. Excellent reviews on this topic have been published and thus we will merely summarize the main ideas which will help us to understand why amino acids may behave as organic osmolytes in most mammalian cells.

The idea of compatible osmolytes arise from the evidence that high concentrations of ions can disturb protein structure and function while selected organic solutes do not. This is the case of polyols and amino acids. Alternatively, some osmolytes accumulate as a way to compensate for the perturbing action of solutes like urea. This is the case of methylamines, which efficiently counteract the perturbing effect of urea. In summary, although ionic move-

ment through the plasma membrane may be a suitable mechanism to counterbalance changes in medium tonicity during short periods of time, they may not be convenient for long-term adaptations, which probably require the stable accumulation of osmolytes compatible with protein stability and biological activity. Nevertheless, the short-term mediated regulatory-volume responses may also depend on amino acid movements through the plasma membrane, which are even mediated by ion channels (Banderali and Roy, 1992; Roy and Banderali, 1994). This appears to be the case for different cell types including erythrocytes and renal cells.

Adaptation of a cell to hypertonicity involves the immediate and subsequent induction of short-term and long-term mediated effects oriented towards cell volume recovery after rapid shrinking. A less well known aspect of this response is the crowding and confinement of macromolecules which takes place during shrinking and does not trigger exclusively a transient change in salt concentration, but strikingly affects protein function. A recent and excellent review by Garner and Burg (1994) emphasizes the highly significant role of molecular crowding in determining changes in the biological activity not only of the molecules which are concentrated but also of that of other macromolecules sharing a common cellular space. While crowding may induce the formation of globular species, confinement seems to promote the formation of linear aggregates such as actin filaments. In vitro approaches clearly demonstrated that macromolecular crowding modifies a variety of parameters such as affinity constants for enzymes or association of selected proteins with the cytoskeletal network. In some instances, this interaction may be relevant in regulatory terms. A variety of cellular events which follow hypertonic shock and which are not necessarily involved, at least apparently, in the volume regulatory response may be explained on the basis of these intriguing effects, which have not yet been clearly characterized in the complex eukaryotic cell. Obviously, it is also likely that the crowding/confinement effect contributes to the triggering of those changes which are directly responsible for long-term adaptation to anisotony, such as the ones discussed below, but this requires demonstration. Accumulation of compatible osmolytes will start mechanisms characterized by altered macromolecular structure and behavior and will drive cell physiology back to the basal state prior to hypertonic challenge.

Selected groups of scientists have dealt with basic questions regarding the physiology of organic cell osmolytes: i) how is the concentration of these compounds regulated inside the cell?; and ii) to what extent do the plasma membrane transporters involved in their uptake contribute to that purpose?

How is the concentration of organic osmolytes regulated in mammalian cells?

As recently reviewed (Burg, 1995), there are essentially three ways of regulating the intracellular concentration of organic osmolytes (Fig. 1). The first is the induction of the expression of a gene which codes for an enzyme which

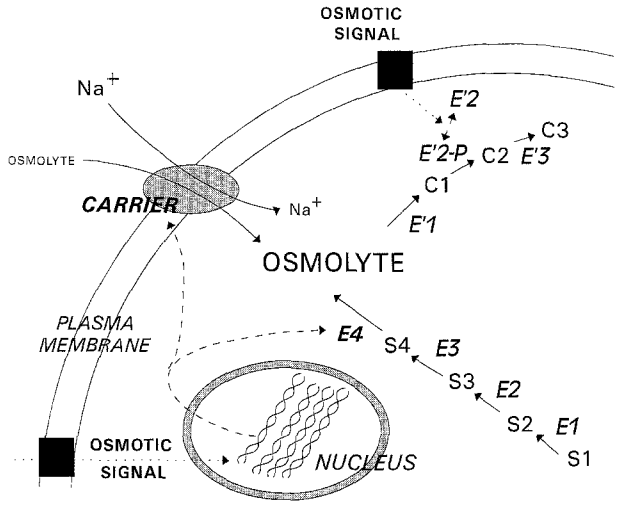

Fig. 1. Mechanisms responsible for the accumulation of organic osmolytes in mammalian cells. Scheme of the suggested mechanisms involved in the intracellular accumulation of organic osmolytes. Transcriptional activation of selected genes triggered by an unknown osmotic signal is responsible for the synthesis of two types of proteins, plasma membrane transporters involved in the uptake of osmolytes from the extracellular medium and enzymes involved in the pathway leading to de novo synthesis of osmolytes. A third mechanism deals with post-translational inhibition of an enzyme involved in osmolyte catabolism. This scheme is an idealization of these three mechanisms, which in fact never converge in a single osmolyte. As indicated in the text, every mechanism modulates the intracellular concentration of a specific organic osmolyte. *E* enzyme; *S* metabolite of the anabolic pathway; *C* catabolite; *E-P* phosphorylated enzyme

bears a high control coefficient in the metabolic pathway leading to *de novo* synthesis of an organic osmolyte. This is represented in Figure 1 as a hypothetical biosynthetic pathway which leads from the original substrate (S1) to the end-product, the organic osmolyte. This is regulated by the activity of E4, which catalyzes the rate-limiting step. An example of this type of regulation is the transcription up-regulation of aldose reductase leading to increased synthesis of sorbitol. The second way of regulating the intracellular concentration of an organic osmolyte involves the inhibitory control of its catabolism. This is represented in Fig. 1 as a hypothetical catabolic pathway where the conversion of catabolite C1 into C2 is modulated by the phosphorylation state of the enzyme E'2. This model of regulation fits with what has been reported for glycerophosphocholine (GPC) and the enzyme GPC: choline phosphodiesterase, which can be inhibited after hypertonic treatment by a mechanism which, in principle, is consistent with post-translational processing of the protein. The third mechanism of response to changes in extracellular tonicity involves the selective induction of those plasma membrane transport systems which are responsible for the uptake of organic osmolytes. This is also represented in Fig. 1 as a concentrative, Na^+-dependent carrier, which is transcriptionally regulated by hypertonic treatment of the cells. This is the case of betaine, taurine and myo-inositol, and, as already indicated, of

many amino acids which can be metabolically less inert, like most neutral amino acids which are highly concentrated inside the cell. The rest of this review will concentrate on this aspect of cell adaptation to hypertonic shock.

Amino acids as organic osmolytes

The sum of individual amino acid concentrations in a mammalian cell can easily reach 0.1 M or more (Roy and Sauvé, 1987). Interestingly, the amino acids which show the highest concentrations inside the cell are also the substrates of concentrative Na^+-dependent amino acid transport systems. Indeed, in our cell models, selected amino acids are much more concentrated inside the cells when these are grown in hypertonic medium (Gómez-Angelats et al., in preparation). Although the exact relevance of these changes in vivo is still to be determined, it is interesting to point out that in a model which involves an osmotic challenge, the hyponatremic rat, plasma osmolarity and sodium concentration decrease and significant changes in osmolyte levels occur in brain. Strong correlation is reported between sodium plasma levels and the total amount of osmolytes. It has been recognized that the brain may also accumulate specific osmolytes, and indeed the amounts of myo-inositol and taurine are significantly decreased in the hyponatremic rat brain. Moreover, marked changes involving amino acids such as glutamine, glutamate and aspartate have also been described in this animal model (Verbalis and Gullans, 1991; Sterns et al., 1993).

The physiological reason for any mammalian cell to accumulate amino acids or deplete their pools in response to changes in medium osmolarity may be somehow obscure when not dealing with cell types exposed to a changing environment, but it is clear that the mechanism exists in a great variety of cells, even non-epithelial ones. Besides some pathophysiological situations in which the isosmotic equilibrium may be challenged, we should not rule out the possibility that these transport systems mediate the uptake of organic osmolytes in isotonic conditions to exert selective effects on other aspects of cell physiology. Thus, we should probably analyze this adaptation the other way around. Let us consider that selected transport systems for those amino acids which are highly concentrated inside the cell contribute to the accumulation of organic osmolytes to trigger increases in cell volume, in a way which couples this process to specific requirements, like those of mitosis, when osmolytes may be necessary for cells to swell without major ionic disturbance (Dall'Asta et al., 1996). In these situations, the activity of transport could be enhanced in isotonic conditions by other factors, probably involved in cell proliferation, like growth factors or pancreatic hormones.

System A for neutral amino acid transport in mammalian cells

Almost all the amino acids that are highly concentrated inside mammalian cells and show intracellular pools highly sensitive to medium tonicity are

substrates for system A (Yamauchi et al., 1994, Gómez-Angelats et al., in preparation). The only exception is glutamate, which is taken up by another transport activity for anionic amino acids, usually ascribed to what is known as system X_{AG^-}. System A is thus a putative target of osmotic regulation.

System A activity for neutral amino acid transport is still a kinetic identity, which is considered to correspond either to the Na^+-dependent component of MeAIB (Methyl-α-aminoisobutyric acid) uptake or to the fraction of Na^+-dependent neutral amino acid transport inhibitable by a saturating excess of MeAIB. Undoubtedly, this is the major transport system responsible for neutral amino acid uptake in most cell types (it is almost a ubiquitous system) and it is the one which is regulated the most by a great variety of factors including substrates, hormones, growth factors and osmotic challenges (McGivan and Pastor-Anglada, 1994). A good example of this complex regulation comes from evidence generated in our laboratory showing that many physiological and pathophysiological situations associated with altered nutritional and hormonal status are characterized by marked changes in hepatic system A activity (Martínez-Mas et al., 1993a; Martínez-Mas et al., 1993b; Ferrer-Martínez et al., 1994; Gómez-Angelats et al., 1995; Felipe et al., 1995). The nutritional regulation of this transporter involves a direct effect of amino acid availability on system A activity. This means that cells cultured in an amino acid-free medium up-regulate this transport system in a manner which is dependent on gene transcription, protein synthesis and N-glycosylation (McGivan and Pastor-Anglada, 1994). This response is not a typical stress-mediated effect because individual amino acids selectively block it. In this context from now on these amino acids will be called repressors. The pattern of repression does not vary significantly from one cell to the other and it has been used as a way to approach the molecular identity of the transporter itself (Kilberg et al., 1994). System A activity induced after amino acid starvation is rapidly inactivated by selected amino acids, which are generally system A substrates (Moffett and Englesberg, 1986). This inactivation is a characteristic feature of the derepressed activity, since basal system A is obviously not inactivated by its substrates and shows classical hyperbolic Michaelis-Menten kinetics.

Cells from absorptive epithelia, like those from kidney and intestine, appear to express concentrative Na^+-dependent amino acid transport activity showing broad substrate specificity and likely to be attributable to system B^0 (see McGivan et al., 1996). System A does not seem to be significantly expressed in kidney epithelial cells, although extensive study on system A regulation has been done using the MDCK cell line, which is a classical and widely used cell model for absorptive epithelia (Saier et al., 1988). Other cell lines, like the bovine renal epithelial cell line NBL-1 used in our laboratory, do not clearly show a typical system A activity.

The use of this cell line to study transport processes was initially introduced by John McGivan and coworkers (Doyle and McGivan, 1992). When grown in a regular medium these cells show a broad specifity Na^+-dependent amino acid transport system which resembles that described in brush border

membrane vesicles from bovine kidney, classified as B^0. If these cells are grown in an amino acid free medium, then system A activity emerges (Felipe et al., 1992). Whether this is the consequence of de novo synthesis of plasma membrane transporters or it simply means that, in basal conditions, system A activity is extremely low and below the sensitivity of the uptake measurements, remains to be determined.

Osmotic regulation of system A

The first observations showing that system A activity could be modulated by hypertonicity in the renal epithelial cell line NBL-1 came from a series of experiments in which we showed that the activity of this transport system could be enhanced even more than by derepression alone if the amino acid-free medium was made hypertonic (Soler et al., 1993). The lag-time for this effect was rather long (12–24 h for the maximal effect), which was consistent with a requirement on protein synthesis for the induction to occur. Indeed, the increased activity triggered by hypertonic treatment was blocked by the addition of inhibitors of protein and mRNA synthesis. The addition of 100–150 mM sucrose, which results in an increase in medium tonicity of about 100–125 mosmoles, was enough to enhance system A transport activity after a 24 h incubation. A maximal response was achieved at 200 mM sucrose. Other osmolytes, like mannitol, triggered a similar physiological response. Interestingly, hypertonicity by itself could not make system A emerge in the renal epithelial cell line NBL-1. This means that the activity was osmotically modulated only if it had been previously induced by amino acid starvation. At that stage, it was assumed that the osmotic regulation of system A activity could be mediated by de novo synthesis of carrier proteins, by a mechanism which depended on the previous derepression of the transport protein gene, suggesting a link between both processes and a sort of hierarchy in the transduction signal pathway leading to enhanced system A gene expression. Further evidence led us to different conclusions.

A second set of experiments was designed to examine the possible relationship between system A derepression and the osmotic control of the transport activity (Ruiz-Montasell et al., 1994). NBL-1 cells were cultured for 12 h in isotonic amino acid-free medium and cells were then exposed for a few more hours (4–6 h) to hypertonic shock. This relatively short time is enough to exert maximal stimulation, in contrast to the time course for the derepression control alone. Unless both stimuli, amino acid starvation and hypertonic shock, were triggering signals with very different lag-times, this apparent discrepancy could be otherwise explained on the basis that the synthesized proteins involved in both biological responses would have different turnover rates. In this context, the evidence that the derepression effect could be blocked by tunicamycin but the osmotic-mediated effect could not, made us suspect that indeed different proteins were involved in these physiological responses.

Indirect evidence supports the view that the system A protein(s) must

contain glycosylated domains which may be essential for function. System A activity can be fully reconstituted from the glycosylated fraction of protein extracts obtained through con-A columns, and, so far, up-regulation of system A activity triggered by a great variety of stimuli has always been blocked by adding an inhibitor of N-glycosylation, tunicamycin, to the culture medium (McGivan and Pastor-Anglada, 1994). The activity induced after hypertonic treatment cannot be blocked by this inhibitor and, consequently, the protein responsible for the increased activity is either non glycosylated or does not require glycosylation for correct biological function. Interestingly, the hypertonicity-induced activity could be selectively inactivated by individual amino acids, in a pattern which was identical to the one found for derepressed system A activity. This inactivation was also independent of protein synthesis and insensitive to the osmolarity of the medium. The idea that the derepressed activity could be further enhanced by a second protein or group of proteins without altering the inactivation properties of selected amino acids is consistent with these results (Ruiz-Montasell et al., 1994).

Somatic cell genetics was useful at this stage to further demonstrate that the derepression response was not related to the osmotic control of system A (Gómez-Angelats et al., 1994; Ruiz-Montasell et al., 1994). Non-epithelial cells, like fibroblasts, also increase system A activity after hypertonic treatment. Indeed, CHO-K1 cells may be considered even more sensitive to changes in medium tonicity than NBL-1 cells. An increase of 50mosmols is enough for a low but significant increase in system A activity. 100 mosmols doubles the basal transport activity. Furthermore, CHO-K1 cells, which express system A activity in basal conditions may be a better model to study regulatory processes, because they do not need to be derepressed by amino acid starvation prior to the osmotic challenge. This is more physiological and less stressing to the cells. An alanine-resistant mutant, CHO-K1 alar4, derived from the wild type CHO-K1, which is auxotroph for proline, was selected on the basis of its resistance to a high [alanine]/[proline] ratio. Its ability to grow in these conditions arose from its phenotype which constitutively expressed derepressed system A activity (Moffett and Englesberg, 1986; Qian et al., 1991). This means that this mutant had lost its ability to respond to amino acid starvation. Interestingly the alar4 mutant was still able to respond to hypertonic treatment, reaching system A activity values which corresponded to the ones found in the wild type when grown under hypertonic amino acid-free medium (Gómez-Angelats et al., 1994; Ruiz-Montasell et al., 1994). Indeed, both stimuli induced additive responses in the wild type. As previously shown in epithelial cells (NBL-1), the hypertonicity-mediated induction of system A activity was not sensitive to tunicamycin, while the derepression response was. The complementary evidence that the hypertonic effect resulted in a mixed kinetic change, inducing higher Vmax and lower Km values, further supported our working hypothesis which is based on the existence of putative system A activating protein(s) (SAAPs) able to further induce pre-existing system A carrier proteins (see model in Fig. 3). It may be argued that hypertonicity selectively promotes the synthesis of another type of system A protein, which may not require glycosylation for function and

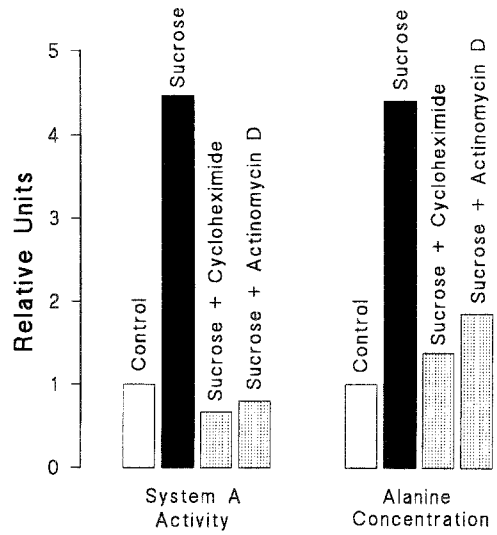

Fig. 2. Induction of system A activity after amino acid starvation and/or hypertonic shock in CHO-K1 cells and an alanine resistant mutant. Relationship between system A up-regulation after hypertonic treatment and intracellular L-alanine concentrations. CHO-K1 cells show a strong relationship between the induction of system A activity after hypertonic stress and the steady state concentrations of system A substrates inside the cell. Inhibition of system A up-regulation by cycloheximide and actinomycin D equally affect the ability of the cells to concentrate amino acids. The results are expressed in relative units assuming that 1 is the value for control conditions. Basal system A activity was 1.5 nmol/2 min/mg protein. Basal L-alanine concentration inside the cell was 40 nmol/mg protein. In all the experiments system A activity was measured as the MeAIB inhibitable fraction of Na^+-dependent 0.1 mM L-alanine uptake

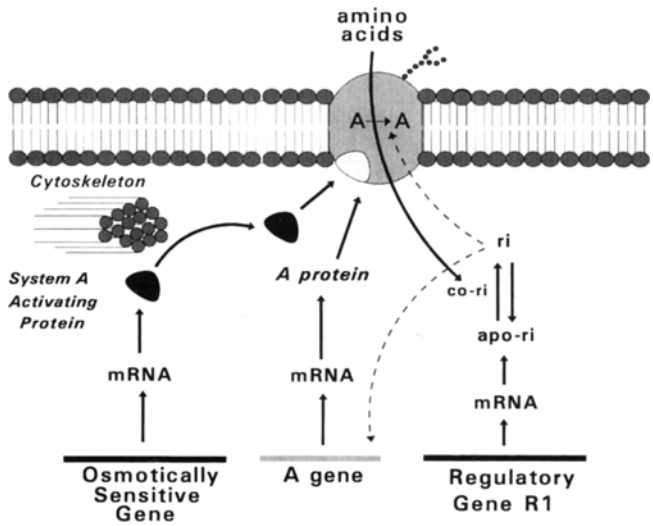

Fig. 3. Suggested model for system A regulation by hypertonic shock and its relationship with the derepression response. System A activity is regulated by amino acid starvation and hypertonicity by two different mechanisms. Amino acids induce the activation of a putative repressor (apo-ri) which blocks system A gene expression in basal conditions. ri would be the active form of the repressor protein which may also show the ability to inactivate system A carriers by an unknown mechanism. The regulatory mutant alar4 would present a mutation in the regulatory gene R1 that would make it loose its adaptive regulation ability. However, alar4 cells would still be osmotically responsive as a consequence of having a normal system A activating protein (SAAP). The putative SAAP would require an intact cytoskeleton network to trigger the osmotic response. Adapted from Qian et al. (1991) and Ruiz-Montasell et al. (1994)

even show different kinetic behavior. Nevertheless, this possibility seems unlikely according to the requirement for previous expression of system A in NBL-1 cells for further response to hypertonic treatment.

The most direct evidence that induction of system A really contributes to the generation of a higher amino acid concentration gradient across the plasma membrane comes from recent experiments (Gómez-Angelats et al., in preparation), where it is shown that the intracellular concentration of system A substrates is much higher in CHO-K1 cells grown under hypertonic conditions than in those cells which were grown in isotonic medium. Prevention of system A induction by the addition of inhibitors like cycloheximide and actinomycin D prevents the increase in intracellular amino acid levels (Fig. 2).

A role for the cytoskeleton in system A osmotic regulation

There is increasing evidence that plasma membrane transporters and pumps may be structurally linked to cytoskeletal elements. In some cases, the integrity of these structures may be essential for the regulation of their activity, as for example the cAMP-mediated activation of $Na^+/K^+/Cl^-$ cotransport in intestinal epithelial cells, which can be blocked by agents which stabilize actin filaments (Matthews et al., 1992). Furthermore, the osmotic treatment induces dramatic changes in cell volume that should be accompanied by marked modifications of the cytoskeletal network.

Recent work from our laboratory (Gómez-Angelats et al., in preparation) has addressed this specific aspect of system A induction after hypertonic treatment of both NBL-1 and CHO-K1 cells. Colcemide, an inhibitor of the microtubular network, is not able to block the derepression response of system A (amino acid starvation) in NBL-1 cells, but it inhibits the further enhancement of system A activity which develops when derepressed cells are cultured in hypertonic medium. Similar results are found when using inhibitors of microfilament function, like cytokalasin B. This drug is unable to inhibit the derepression response but it can block the osmotic response of system A. Interestingly, this pattern of inhibition is different to the one found in fibroblasts. The osmotic response of system A activity in CHO-K1 cells can be blocked by inhibitors of the microfilament but not the microtubule network. Basal system A activity in this fibroblast cell line is not sensitive to cytochalasin B, which may be interpreted on the basis that the increase in system A transport activity triggered by hypertonic shock requires the integrity of the microfilament network. There is, however, a chance that these drugs modify the ability of the cells to adapt to a changing environment, which could disrupt the classical shrinking/swelling response of any mammalian cell to a hypertonic shock. In that case, the ability or inability of an inhibitor to block the osmotic-mediated response could rely upon the generation of the osmotic signal itself, instead of modifying the last step in system A activation, which may involve the intracellular processing of putative activating proteins. Experiments designed to address this point fully support the view that the cytoskeletal network is needed mainly for the second part of the activation

process, without altering the generation of the osmotic signal (Gómez-Angelats et al., in preparation).

Although an unaltered cytoskeletal network seems essential for system A activation after hypertonic treatment of the cells the elucidation of the exact role of microtuble and microfilament structures in this physiological response will require more experimental work.

Other transport systems may be osmotically regulated

The osmotic modulation of solute transport systems in NBL-1 cells is not specific to system A (Fig. 4). Although major concentrative Na^+-dependent

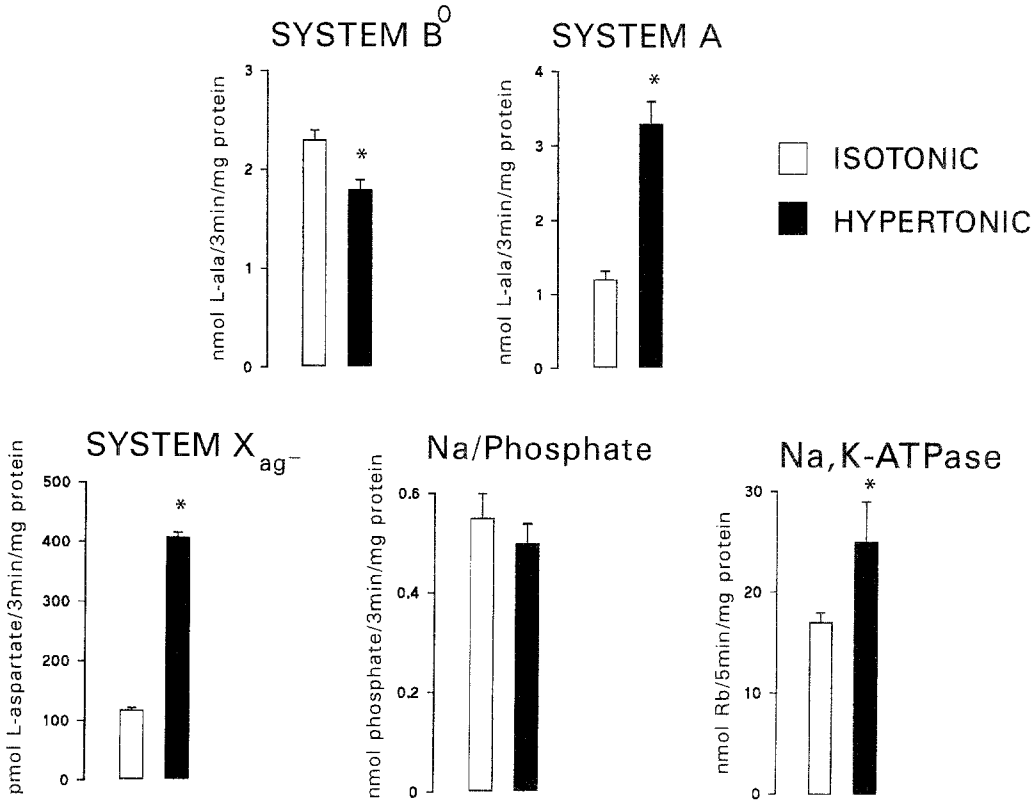

Fig. 4. Modulation of Na^+-dependent transport systems in NBL-1 cells under hypertonic shock. The activity of selected transport systems of NBL-1 cells is shown in cells grown either in isotonic conditions or in a hypertonic medium for 10–12 hours. Selective inductions of systems A, X_{AG^-} and the sodium pump were found. System B^0 activity was even decreased after hypertonic treatment of the cells. As indicated in the text, system A activity was up-regulated in NBL-1 cells after hypertonic treatment only if previous derepression of this transport activity was allowed in an amino acid-free medium. System B^0 and A activities were measured as the MeAIB-insensitive and -sensitive fractions of Na^+-dependent 0.1 mM L-alanine uptake. System Xag^- activity was determined by measuring Na^+-dependent 5 μM L-aspartate uptake. The activity of the Na^+-dependent phosphate carrier was measured using 100 μM Pi as substrate. Na,K-ATPase activity was measured as the ouabain-sensitive fraction of 5.4 mM Rb uptake. Ouabain was used at a concentration of 1.2 mM. These data have been adapted from Soler et al. (1993); Ruiz-Montasell et al. (1994); Ferrer-Martínez et al. (1995 and unpublished observations)

amino acid transport activities, like B^0, are not up-regulated after hypertonic shock and their activity even decreases (Soler et al., 1993), others are clearly up-regulated after hypertonic treatment of the cells. This is the case of the high-affinity Na^+-dependent glutamate transport system X_{AG^-} (Ferrer-Martínez et al., 1995). When NBL-1 cells are grown for at least 12 hours in a hypertonic medium, the Na^+-dependent fraction of 5μM L-aspartate uptake, which is a good estimate of X_{AG^-} transport activity in this cell line, dramatically increases up to three fold above basal values (isotonic medium). This induction is due to a change in Vmax (0.9 vs 0.3 pmol/3 min/mg protein) without modifications of Km values and is blocked by cycloheximide. Thus, protein synthesis is required for the biological effect to occur. Since the induction of transport activity follows a previous increase of nearly three fold above basal of the amount of EAAC1 transcripts, which is already significant 6 hours after hypertonic treatment, it is rather likely that the induction of system X_{AG^-} activity is the result of transcriptional activation of the carrier gene. If this is the case, the finding that tunicamycin completely blocks the hypertonicity-mediated increase in transport activity, supports the view that the putative N-glycosylation sites of the carrier protein, deduced from the nucleotide sequence, may be relevant for biological function. Interestingly, the induction of this transporter in NBL-1 cells is completely insensitive to the inhibitor of microtubular function colcemide, under the same experimental conditions where system A up-regulation is. This evidence supports the view that either there is a different pathway for protein processing depending on the carrier activity which is being activated or, alternatively, there is merely a different polarity in the insertion of the proteins synthesized de novo after hypertonic shock. This possibility is now under research.

Other concentrative transport systems which do not recognize amino acids as substrates, despite being concentrative and Na^+-dependent, like the Na^+/Pi cotransporter, are not sensitive to osmotic changes in NBL-1 cells. Nevertheless, the increase in activities of systems A and X_{AG^-} clearly corresponds with the reported changes in the intracellular levels of individual amino acids reported in renal cells after hypertonic shock. Furthermore, their induction may also be relevant enough in terms of disturbance of the Na^+ transmembrane gradient, to trigger a compensating increase in the activity of the Na^+,K^+-ATPase. Indeed, NBL-1 cells, when grown in hypertonic treatment, show increased sodium pump activity (Ferrer-Martínez et al., in press). However, this induction appears to be mediated, basically, by changes in electrochemical transmembrane gradients. Indeed, the induction can be mimicked and even abolished by the addition of Na^+ ionophores, like monensin. Nevertheless, the amount of α1 subunit mRNA dramatically increases after hypertonic shock (nearly four fold above basal values), by a mechanism which is assumed to be transcriptional in origin. This is based upon the observation that the half-life of the α1 subunit mRNA is not affected by hypertonic treatment of the cells. Another group has reported an increase in both subunit mRNAs in another epithelial renal cell line grown under hyperosmotic conditions, which is partially consistent with our observations (Yordy and Bowen,

1993). The discrepancy between our results showing that only the α1 subunit gene is under osmotic control in NBL-1 cells and these results, reporting that both subunit genes are osmotically regulated, may be explained on the basis of the respective origin and differentiation state of the two cell types. It is evident that basal Na^+,K^+-ATPase activity and its hormone responsiveness are clearly zonated along the nephron (Doucet et al., 1989).

Although coordinate expression of system A and the α1 subunit Na^+,K^+-ATPase gene has been shown in derepressed CHO-K1 cells (Qian et al., 1991), it is clear from the observations detailed above that the activities of these two transport systems, system A and the sodium pump, are increased after hypertonic treatment of the cells by different mechanisms which precludes co-regulation at the gene level.

Conclusions and future developments

Work from the authors' laboratory has shown that the major neutral amino acid transport system of mammalian cells, system A, besides being very well regulated in a complex way by both nutritional and endocrine factors, is also modulated by changes in medium tonicity. This may have physiological relevance because those amino acids which are recognized as substrates by this transport system are markedly concentrated inside the cell and their concentration seems to adapt to the osmolarity of the extracellular medium. This response is not unique to epithelial cell lines, and even fibroblasts, which theoretically may face more constant osmolarities in vivo, retain the ability to respond to hypertonic treatment by increasing the activity of system A. Nevertheless, this physiological response, depending on the cell type, may have different requirements in terms of the likely role of the cytoskeleton network in triggering up-regulation of system A. Indirect evidence supports the view that it is not system A itself that is being synthesized but rather a putative activating protein is acting on pre-existing system A carriers. Obviously, this is a working hypothesis which will await demonstration until the required molecular probes are available.

The osmotic modulation of system A is unlikely to be a classical stress response. In a recent report from Maurice Burg's laboratory, it is clearly shown that, although some heat shock proteins are osmotically regulated, the osmotic sensitive transporters like the betaine one is not a heat shock protein itself (Sheikh-Hamad et al., 1994). It has not yet been elucidated whether this also applies to the transport systems we have just summarized in this review. However, it is interesting to point out that the CHO-K1 mutant we have obtained which is unable to up-regulate system A activity after hypertonic treatment overexpresses a P1-like heat shock protein (Jones et al., 1994).

Evidence in favor of the specificity of the osmotic response comes from the preliminary screening of the osmotically-sensitive transport agencies present in NBL-1 cells. Indeed only system X_{AG}^- was found to be responsive to anisotony, by a mechanism which, conversely to system A, seems consistent

with transcriptional activation of the carrier gene and precludes a putative activating protein.

The identification of osmotic-sensitive plasma membrane transport systems and their comparison with those that are insensitive to osmotic stress may provide a tool for the detailed study of the cellular events which modulate the osmotic sensitivity of gene expression. More specifically, the detailed study of the regulatory events involved in the changes in system A activity in hypertonic conditions may also provide new tools to undertake the molecular identification of either the carrier protein itself or the putative regulatory protein(s). So far, it has been suggested that two osmotically sensitive genes, the aldose reductase and the betaine transporter, have osmotic response elements in their promotors (Ferraris et al., 1994; Takenaka et al., 1994). The one at the betaine carrier gene has recently been identified by Joseph Handler's group (Takenaka et al., 1994). Nevertheless, these genes code for proteins which may have, essentially, a single physiological role: osmotic regulation. It is necessary to determine whether or not the same osmotic response elements are present in transporter genes that code for carrier proteins involved in many other key physiological processes which are not related to osmotic regulation. More interesting perhaps, will be the elucidation of the hierarchical processes which may promote the transcriptional activation of these genes when a variety of stimuli act simultaneously on the cell, which is the classical physiological situation where, for instance, system A activity is up-regulated by changes in the endocrine context, which determines an altered substrate concentration pattern and, probably, medium anisotony.

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