Regulation of Na⁺,K⁺-ATPase and the Na⁺/K⁺/Cl[−] co-transporter in the renal epithelial cell line NBL-1 under osmotic stress

Andreu FERRER-MARTINEZ, F. Javier CASADO, Antonio FELIPE and Marçal PASTOR-ANGLADA*

Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain

The long-term adaptation of the Na⁺,K⁺-ATPase to hypertonicity was studied using the bovine renal epithelial cell line NBL-1. Na⁺,K⁺-ATPase activity measured in intact cells as the ouabain-sensitive fraction of Rb⁺ uptake was stimulated (40 % above controls) after incubating the cells in hypertonic medium. This stimulation was not correlated with significant changes in the amount of Na⁺,K⁺-ATPase α 1 subunit protein. Nevertheless, the amount of α 1 but not β 1 subunit mRNA progressively increased after hypertonic shock (3–4-fold above basal values). These results suggest that the α 1 subunit gene is modulated by medium osmolarity, although this does not necessarily involve enhanced translation of the mRNA into active α 1 protein. Indeed, the increase in the biological activity of the Na⁺,K⁺-ATPase is abolished when the electrochemical Na⁺ transmembrane gradient is depleted by monensin, which is consistent with a post-

translational effect on the activity of the sodium pump. A furosemide-sensitive component of Rb⁺ uptake, attributable to Na⁺/K⁺/Cl[−] co-transporter activity, was very low when cells were cultured in a regular medium, but was greatly induced after hypertonic shock. This induction could not be blocked by cycloheximide. Colcemide addition slightly reduced the absolute increase in Na⁺/K⁺/Cl[−] co-transporter activity, while cytochalasin B significantly potentiated the effect triggered by hypertonic shock. It is concluded: (i) that in NBL-1 cells the α 1 but not the β 1 subunit of the Na⁺,K⁺-ATPase is encoded by an osmotically sensitive gene, and (ii) that the Na⁺/K⁺/Cl[−] co-transporter, although an osmotically sensitive carrier, is induced by a mechanism that is independent of protein synthesis but may rely, in an undetermined manner, on the structure of the cytoskeletal network.

INTRODUCTION

The role of osmolytes in the adaptation of cells to anisotonic stress has been recognized from prokaryotes to humans [1,2]. The accumulation of inert organic solutes inside the cell may be a suitable way of compensating for extracellular osmotic changes. Most osmolytes are accumulated by increasing their uptake from the outside of the cell (for a recent review, see [2]). This process is related to the selective, stable, induction of plasma membrane transport systems responsible for the uptake of osmolytes such as betaine, taurine and *myo*-inositol. These transport systems share the property of being concentrative and Na⁺-dependent. Increasing evidence suggests that this phenomenon may also apply to other compounds such as amino acids, which may be osmotically compatible but less inert from a metabolic point of view. Amino acids are highly concentrated inside the cell and are good candidates for the osmotic regulation of cell volume (for a review, see [3]).

In our laboratory we have used the renal epithelial cell line NBL-1 (MDBK) to study the modulation of amino acid transport systems. In this cell line, as well as in fibroblasts, the activity of system A for neutral amino acid transport increases when cells are exposed to a hypertonic milieu [4–6]. This adaptive response has been well characterized, and has been suggested to be carried out by a system A-activating protein or group of proteins (putative SAAPs) which may be induced after hypertonic stress by a mechanism that involves gene transcription and protein synthesis [6]. In a preliminary screening of several other amino acid transport activities, we have observed that neither system B⁰ in NBL-1 cells nor system ASC in fibroblasts is affected by hypertonic shock [5,6]. On the other hand, system X_{AG}[−] activity is up-regulated in NBL-1 cells cultured in hypertonic conditions by a mechanism that is consistent with *de novo* synthesis of carrier proteins. This is based on the evidence that the induction

is blocked by tunicamycin and cycloheximide, and is apparent just after a significant increase in the mRNA transcripts of EAAC1, the putative X_{AG}[−] gene [7]. Most of the characteristics of the long-term osmotic regulation of amino acid transport systems in mammalian cells have recently been reviewed [8].

Physiological adaptations leading to increased activity of Na⁺-coupled transport systems are usually counterbalanced by a stimulation of the sodium pump. To test whether this applies to the osmotic stimulus, we have analysed the long-term effects of hypertonicity on Na⁺,K⁺-ATPase expression in NBL-1 cells. The unexpected finding that most of the Rb⁺ transport activity induced after hypertonic shock is furosemide-sensitive, rather than ouabain-sensitive, indicates that the Na⁺/K⁺/Cl[−] co-transporter may be under osmotic regulation in NBL-1 cells.

MATERIALS AND METHODS

Cell line and culture conditions

The bovine epithelial cell line NBL-1 (MDBK) was originally obtained from Flow Laboratories (McLean, VA, U.S.A.) and was routinely cultured in Ham's F12 medium supplemented with 10 % (v/v) newborn calf serum, 2 mM L-glutamine and a mixture of antibiotics as previously reported [9]. Cells were grown in 75 cm² flasks for continuous culture and in 35 mm-diameter multiwell plates for transport experiments. Cells used for Western and Northern blot analysis were grown in 100 mm-diameter Petri dishes. Cells were fed every 2 days until confluence, when they were used for experiments.

Hypertonicity studies

To study the effect of hyperosmolarity on the biochemical parameters indicated below, we routinely used sucrose at a

* To whom correspondence should be addressed.

concentration, 200 mM, known to exert maximal stimulation of amino acid transport activity. Since Na^+/K^+ -ATPase activity and expression seems somehow to be regulated by serum itself in other cell types, such as the rat liver cell line Clone 9 [10], we decided to culture the cells before every experiment for 24 h in fresh serum-free Ham's F12 medium supplemented with 0.1 % (w/v) BSA. This was done to magnify the likely effects induced by hypertonic shock. If necessary, the medium was supplemented with inhibitors as indicated in the Results section.

Na^+/K^+ -ATPase activity and ion content in NBL-1 cells

The biological activity of the pump was measured as the ouabain-sensitive fraction of total Rb^+ uptake into NBL-1 cells. The method was adapted from that reported in [11], which has the advantage of using non-radioactive Rb^+ . This technique is safer and less expensive than the classical methods involving ^{86}Rb as a tracer. The method was proved to be reliable when used in adherent cells, which is the case here. Confluent monolayers were washed once in 1 ml of Tris (26 mM, pH 7.4)-buffered Earle's balanced salt solution containing 116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 1 mM NaH_2PO_4 , 0.8 mM MgSO_4 and 5.5 mM D-glucose, supplemented with 1 % (w/v) BSA. Then the cells were incubated in 1 ml of the same fresh medium for 30 min. Next the medium was rapidly aspirated and replaced by 1 ml of incubation medium (as described above, but with 5.4 mM RbCl instead of KCl). The reaction was stopped at the desired times (routinely 5 min) by rapidly aspirating the medium, followed by two washings in 2.5 ml of ice-cold 0.1 M MgCl_2 . Cells were then dried and 0.2 ml of ethanol was added and allowed to evaporate. Plates were usually frozen and Rb^+ contents were later measured by atomic absorption. To measure Na^+/K^+ -ATPase activity, cells were preincubated (30 min) and incubated either in the absence or in the presence of 1.2 mM ouabain. These conditions were shown to exert maximal inhibition of ouabain-sensitive Rb^+ transport.

Measurement of the biological activity of the pump required the determination of intracellular Rb^+ content under the conditions indicated above. To this end, dried cell extracts were dissolved in 4 ml of a 20 mM KCl solution, and the intracellular Rb^+ concentration was determined by atomic absorption spectrophotometry (Philips PU9200X instrument) using a wavelength of 780 nm and a slit of 1 nm. Experiments run in parallel were designed to monitor the changes in intracellular Na^+ concentration, which was measured using the same technique at 589 nm with a slit of 0.2 nm.

Measurement of cell volume

Cell volume was measured using the [^{14}C]urea distribution space, as previously described [5]. Briefly, cells were loaded for 30 min with [^{14}C]urea (1 $\mu\text{Ci}/\text{ml}$) and processed as indicated above, at the times indicated in the Results section. Contamination by extracellular medium was negligible, as determined either by monitoring contamination using [^3H]inulin or by calculating the amount of [^{14}C]urea retained in blank assays, in which the cells were exposed to ice-cold medium for a few seconds and then treated as indicated above.

Na^+/K^+ -ATPase $\alpha 1$ subunit protein

The amount of Na^+/K^+ -ATPase $\alpha 1$ subunit protein in NBL-1 cells cultured either in basal conditions or in hypertonic medium was monitored by Western blotting using an $\alpha 1$ -specific polyclonal antibody kindly provided by Dr. Käthi Geering (Institute of Pharmacology, University of Lausanne, Switzerland). Briefly,

cells were cultured until confluence on 100 mm plates and kept either in the absence or in the presence of sucrose for 24 h. At the indicated times, cell monolayers were washed once with 1 ml of 10 mM Tris (pH 7.4), then scraped into 1 ml of the same medium. The total extract was then added to 9 ml of the same buffer and centrifuged for 10 min at 10000 g. The pellet was resuspended in 10 ml of 10 mM Tris and centrifuged again in the same conditions. The pellet to be used for electrophoresis was then resuspended in 0.1 ml of 0.5 % Triton X-100. This crude membrane extract was first analysed for protein concentration according to [12]. Extracts (8 μg of protein), supplemented with β -mercaptoethanol (5 % final concentration), were routinely run on a 0.1 % SDS/polyacrylamide gel. Proteins were transferred to hydrophobic filters (Immobilon-P; Millipore, Bedford, MA, U.S.A.), blocked in 5 % milk and 0.2 % Tween-20/PBS and immediately used for immunoreaction.

Na^+/K^+ -ATPase subunit mRNAs

Total RNA was extracted from confluent cell cultures using the guanidinium thiocyanate method [13], separated on a 1 % agarose gel under denaturing conditions, transferred overnight to nylon membranes (Hybond N; Amersham) in $20\times\text{SSC}$ (3 M NaCl, 300 mM sodium citrate, pH 7.0) and cross-linked under UV exposure. The blot was prehybridized for 6 h at 42 °C in 45 % formamide, 10 % dextran sulphate, $1\times\text{Denhart's}$ solution (1 g/l BSA, 1 g/l polyvinylpyrrolidone 40, 1 g/l Ficoll), $4\times\text{SSC}$, 100 mM sodium phosphate buffer (pH 7), 0.1 % sodium pyrophosphate, 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 ^{32}P -labelled $\alpha 1$ subunit cDNA probe (10^6 c.p.m./ml). Filters were washed twice for 20 min at 55 °C in $1\times\text{SSC}/0.1\%$ SDS, and twice in $0.2\times\text{SSC}/1\%$ SDS before autoradiography. The $\alpha 1$ subunit cDNA was excised from the *Eco*R1 site of plasmid pRB 5.1, kindly provided by Robert Levenson (Yale University School of Medicine). Densitometric analyses of the Northern blots were performed by means of Biomage (Millipore) and normalized to the ethidium bromide staining of the ribosomal bands, which were also quantified using the same system. Other common loading markers such as β -actin mRNA could not be used because these genes are also under osmotic control [14].

RESULTS

Time course of Rb^+ uptake into NBL-1 cells

The time course of Rb^+ uptake into NBL-1 cells cultured in basal conditions is shown in Figure 1. Total uptake was linear throughout the incubation time tested (10 min). Most of the Rb^+ uptake in basal conditions was ouabain-sensitive (1.2 mM ouabain), which suggests that, in NBL-1 cells grown in a regular medium, most of the Rb^+ uptake is mediated by the Na^+/K^+ -ATPase. Nevertheless, a small fraction of uptake was not inhibited when NBL-1 cells were preincubated with this agent; however, when we preincubated the cells for 30 min in the presence of 1.2 mM furosemide, significant inhibition of Rb^+ uptake was found. This suggests that NBL-1 cells express $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter activity. When both inhibitors were added simultaneously, Rb^+ uptake was negligible. All of the following experiments were then performed after a 5 min incubation, close to initial-velocity conditions.

Time course of the effect of hypertonic shock on Rb^+ uptake in NBL-1 cells

In order to check whether Na^+/K^+ -ATPase activity is under osmotic regulation in NBL-1 cells, we incubated confluent

Figure 1 Time course of Rb^+ uptake into NBL-1 cells

Confluent monolayers were incubated for the indicated times, as described in the Materials and methods section, in the presence of 5.4 mM Rb^+ , either in the absence of inhibitors (■) or after preincubation of the cells for 30 min with 1.2 mM ouabain (▲), 1.2 mM furosemide (●) or both inhibitors added simultaneously (★). The results are the means of triplicate measurements made on a representative cell preparation.

Figure 2 Rb^+ uptake into NBL-1 cells cultured under hypertonic conditions

Confluent monolayers were incubated for the indicated times either in an isotonic medium (□, ○) or in the presence of 200 mM sucrose (■, ●). Using the appropriate inhibitors, total Rb^+ uptake was subdivided essentially into two fractions: the ouabain-sensitive fraction (□, ■), corresponding to Na^+ , K^+ -ATPase activity, and the furosemide-sensitive fraction (○, ●), corresponding to a $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter activity. The results are means $\pm$ S.E.M. from eight independent preparations.

monolayers for the times indicated in Figure 2, either in a regular medium or in a medium supplemented with 200 mM sucrose. Other transport systems, such as system A and X_{AGT} , are up-regulated in this cell type when this or other osmolytes are added to the medium [5–7]. The sucrose concentration used (200 mM) is high enough to exert maximal stimulation and gives a medium osmolality of 480 mosM, as measured with an osmometer. Since serum by itself seems to exert significant induction of the basal expression of Na^+ , K^+ -ATPase [10], cells were routinely grown in the absence of serum for 24 h prior to the hypertonicity experiments. In these conditions serum was replaced by 0.1 % BSA. Transport inhibitors (ouabain or furosemide) were routinely preincubated for 30 min in the absence of sucrose, even if cells

Figure 3 Time course of cell-volume recovery

NBL-1 cells incubated for 21 h either in an isotonic medium (lower panel) or in a hypertonic medium (upper panel) were used for cell volume determination, as described in the Materials and methods section. Hypertonicity-treated cells were placed in the isotonic medium used for the preincubation in the presence of inhibitors, and the time course of the cell-volume recovery was monitored. This experiment was performed either in the absence (●) or in the presence of the Rb^+ uptake inhibitors ouabain (■) and furosemide (▲) added at 1.2 mM. The results are means $\pm$ S.E.M. from two independent cell preparations.

had been exposed to a hypertonic medium. The aim of this protocol was to measure Rb^+ uptake rates in isotonic conditions, because we were interested exclusively in the long-term effects of the anisotonic shock on the transport activity and we aimed to measure uptake rates in similar conditions of tonicity. Nevertheless, in order to avoid the interference of a probable regulatory volume response when replacing the high-sucrose medium, we monitored cell volume changes during the transition of the cells from a hypertonic to an isotonic medium. Figure 3 shows the time course of cell volume recovery in sucrose-treated cells. The ability to recover cell volume was not affected by the addition of the inhibitors. Moreover, inhibitors did not modify cell volume in cells maintained in the control medium (isotonic). In summary, Rb^+ transport activity in hypertonicity-treated cells was always measured in isotonic medium, once the cells had recovered a constant volume after the regulatory volume response.

During the incubation in isotonic conditions, the furosemide-sensitive Rb^+ transport component was responsible for less than 20 % of the total Rb^+ uptake measured in NBL-1 cells. Exposure to hypertonic medium significantly enhanced the ouabain-sensitive component of Rb^+ transport, but the effect was relatively low (about 40 % above control values after 21 h of sucrose treatment) (Figure 2). This induction required at least 9 h of incubation in the presence of the osmolyte. Interestingly, most of the increase in Rb^+ uptake after hypertonic shock was mediated by the furosemide-sensitive component, which was dramatically stimulated above control values (Figure 2).

Effects of hypertonic shock and monensin treatment on ouabain- and furosemide-sensitive Rb^+ uptake and intracellular Na^+ content

Since Na^+ , K^+ -ATPase activity is repressed *in vivo* by low intracellular Na^+ concentrations, we monitored the activity of the sodium pump either in the absence or in the presence of a Na^+ ionophore (monensin), to determine whether the induction of Na^+ , K^+ -ATPase activity after hypertonic shock could be some-

Table 1 Effects of monensin and hypertonicity on ouabain-sensitive Rb^+ uptake and intracellular content of Na^+

Ouabain-sensitive Rb^+ uptake was measured in basal conditions (isotonic medium) or after a 21 h incubation in the presence of 200 mM sucrose (hypertonic medium), either in the absence or in the presence of 50 μM monensin, as indicated in the Materials and methods section. Results are the mean $\pm$ S.E.M. from five independent cell preparations. Intracellular Na^+ content was measured, as indicated in the Materials and methods section, either under basal conditions or after a 21 h incubation in the presence of 200 mM sucrose, with or without 50 μM monensin. Na^+ levels were referred to cell volumes shown in Figure 3 to allow conversion into units of mM. The results are means $\pm$ S.E.M. of four independent cell preparations. The significance of differences was determined by the Student's *t* test ($*P < 0.05$).

	Isotonic medium	Hypertonic medium
Ouabain-sensitive Rb^+ uptake (nmol/5 min per mg)		
No ionophore	16.0 $\pm$ 1.1	23.8 $\pm$ 2.1*
50 μM Monensin	32.2 $\pm$ 2.6	31.9 $\pm$ 5.8
Na^+ (nmol/mg of protein)		
No ionophore	26.6 $\pm$ 1.8	34.2 $\pm$ 2.7*
50 μM Monensin	85.4 $\pm$ 6.9	130.5 $\pm$ 22.2
Na^+ (mM)		
No ionophore	5.4 $\pm$ 0.4	5.5 $\pm$ 0.6
50 μM Monensin	17.3 $\pm$ 1.4	19.3 $\pm$ 3.3

how related to changes in the intracellular sodium concentration. Table 1 shows the effect of monensin on the hypertonicity-triggered increase in ouabain-sensitive Rb^+ uptake in NBL-1 cells after 21 h of exposure to 200 mM sucrose. Treatment of cells for 5 min with 50 μM monensin induced a 2-fold increase in ouabain-sensitive Rb^+ uptake. The hypertonicity-mediated increase in this transport activity disappeared when cells that had been incubated in hypertonic medium were exposed to the ionophore prior to Rb^+ uptake measurements. The furosemide-sensitive component of Rb^+ uptake that was induced after hypertonic treatment of the cells was not affected by the addition of monensin (results not shown).

The intracellular Na^+ concentrations after hypertonic shock in NBL-1 cells that had been exposed to monensin and in cells that had not been treated with the ionophore are also shown in Table 1. Results correspond to the concentrations after the 30 min recovery period, prior to Rb^+ uptake measurements. Obviously, the intracellular Na^+ concentration of the cells was dramatically increased after inhibition by ouabain (results not shown). When results were expressed per mg of protein, hypertonicity seemed to induce a slight increase in intracellular Na^+ content, but this apparent difference disappeared when results were referred to cell volume. As expected, monensin treatment induced a marked increase in intracellular Na^+ concentration. Under these conditions, no differences were found between cells that had been exposed to hypertonic medium and those cultured in isotonic conditions.

Effect of hypertonic shock on Na^+ , K^+ -ATPase $\alpha 1$ subunit protein and mRNA expression in NBL-1 cells

Figure 4 shows the amount of Na^+ , K^+ -ATPase $\alpha 1$ subunit protein and mRNA after incubating confluent monolayers of NBL-1 cells for the indicated times either in isotonic conditions or in hypertonic medium. The amount of $\alpha 1$ subunit protein was not modified after hypertonic shock at any time tested, even when the biological activity of the pump was already enhanced. In contrast, the amount of $\alpha 1$ subunit mRNA increased progressively after incubating the cells in hypertonic medium. The accumulation of mRNA was already significant 6 h after the

Figure 4 Na^+ , K^+ -ATPase $\alpha 1$ subunit protein and mRNA levels in NBL-1 cells after hypertonic shock

(A) Na^+ , K^+ -ATPase $\alpha 1$ subunit Northern blot of total RNA extracted from NBL-1 cells either maintained in isotonic conditions or incubated for the indicated times in the presence of 200 mM sucrose. Basal mRNA levels (time 0) were not altered throughout the incubation in isotonic medium (results not shown). (B) Ethidium bromide staining of the representative Northern blot shown in (A). (C) Densitometric analysis of four independent Northern blots after normalization of the mRNA amounts according to the ethidium bromide staining of the gels. The results are means $\pm$ S.E.M. (D) Na^+ , K^+ -ATPase $\alpha 1$ subunit Western blot of NBL-1 cells cultured either in basal conditions or in the presence of 200 mM sucrose for the indicated times. Basal $\alpha 1$ subunit protein levels were not modified during the incubation in isotonic conditions, and a representative result (time 0) is shown. This observation was repeated in three independent experiments.

addition of sucrose. All of these experiments were performed in the same conditions used for the studies aimed at the determination of Rb^+ uptake. Interestingly, $\beta 1$ subunit mRNA levels, measured with the corresponding $\beta 1$ subunit cDNA probe (kindly donated by Dr. Robert Levenson), were not significantly modified after hypertonic treatment of the cells. $\beta 1$ subunit specific antibodies, either obtained commercially or kindly donated by Dr. Käthi Geering, were checked for immunoreactivity in our cell system but no signal was obtained; thus no data are available on the effects of medium tonicity on $\beta 1$ subunit protein content.

Effects of cycloheximide, colcemide and cytochalasin B on the increase in Na^+ / K^+ / Cl^- co-transporter activity triggered by hypertonicity

An independent set of experiments was designed to determine whether the increase in Na^+ / K^+ / Cl^- co-transporter activity, which occurs after hypertonic treatment of the cells, could be blocked either by inhibitors of protein synthesis or by inhibitors of the cytoskeletal network. Results are shown in Table 2. As shown above (Figure 2), basal uptake rates, prior to hypertonic treatment, were very low and variable, since most of the Rb^+ uptake under these experimental conditions occurs via the ouabain-sensitive component of transport. Cycloheximide at 1 $\mu\text{g}/\text{ml}$ did not block the induction of the furosemide-sensitive Rb^+ uptake triggered by sucrose treatment. Colcemide at

Table 2 Effects of cycloheximide, colcemide and cytochalasin B on the induction of the furosemide-sensitive component of Rb⁺ uptake after hypertonic shock

Confluent monolayers of NBL-1 cells were incubated for 9 h either in isotonic or in hypertonic (200 mM sucrose) medium. Then, uptake was measured with 5.4 mM Rb⁺, as indicated in the Materials and methods section, either in the absence or in the presence of 1.2 mM furosemide, to allow the calculation of the furosemide-sensitive component of transport. Three independent sets of experiments were performed. Results from experiment 1 are means $\pm$ S.E.M. from four independent cell preparations. Results from experiments 2 and 3 are mean $\pm$ S.E.M. from three independent cell preparations. The statistical significance of the changes induced by hypertonic treatment of the cells was assessed by the paired *t* test (***P* < 0.01; ****P* < 0.001). The statistical significance of the changes induced by the inhibitors on the increment in the furosemide-sensitive Rb⁺-transport activity after hypertonic treatment was also assessed by the paired *t* test ($\dagger P$ < 0.01).

Experiment	Inhibitor	Furosemide-sensitive Rb ⁺ uptake (nmol/5 min per mg of protein)		
		Isotonic	Hypertonic	Increment
1	None	4.8 $\pm$ 1.2	10.4 $\pm$ 0.9**	5.6 $\pm$ 0.9
	Cycloheximide (1 μ g/ml)	3.2 $\pm$ 0.9	9.0 $\pm$ 0.7**	5.8 $\pm$ 1.5
2	None	6.2 $\pm$ 0.5	10.6 $\pm$ 1.0**	4.4 $\pm$ 0.7
	Colcemide (10 μ g/ml)	7.8 $\pm$ 1.0	10.5 $\pm$ 0.8	2.7 $\pm$ 0.4
3	None	4.2 $\pm$ 1.2	9.6 $\pm$ 1.0**	5.4 $\pm$ 0.8
	Cytochalasin B (1 μ M)	3.4 $\pm$ 1.7	13.1 $\pm$ 1.8***	9.7 $\pm$ 0.4 $\dagger$

10 μ g/ml seemed to induce partial inhibition, although this effect was mostly due to a slight but non-significant increase in the basal Na⁺/K⁺/Cl⁻ co-transporter activity triggered by the drug. In contrast, 1 μ M cytochalasin B significantly potentiated (nearly 2-fold) the effect triggered by the hypertonic treatment, with slight alterations of the basal Na⁺/K⁺/Cl⁻ co-transporter activity.

DISCUSSION

This study shows that, in the bovine renal epithelial cell line NBL-1, both the ouabain- and furosemide-sensitive components of Rb⁺ uptake (Na⁺,K⁺-ATPase and Na⁺/K⁺/Cl⁻ co-transporter activities respectively) are under osmotic control.

The long-term modulation of Na⁺,K⁺-ATPase activity by anisotonic shock has been reported previously. In human lens epithelial and human retinal pigment epithelial cells, the activity of the pump is significantly increased (up to double the basal values) after 24 h of hypertonicity, triggered by either NaCl or cellobiose [15]. In these cell types this induction was related to the simultaneous activation of the Na⁺-dependent transport systems involved in the uptake of osmolytes such as taurine and myo-inositol. The molecular basis for Na⁺,K⁺-ATPase induction in human lens epithelial and human retinal pigment epithelial cells was not studied. Nevertheless, NaCl may enhance α 1-subunit expression by itself, since the promoter of this gene may contain putative Na⁺-sensitive responsive element(s) [16]. However, increases in α 1-subunit mRNA levels associated with low intracellular concentrations of Na⁺ have also been reported in CHO-K1 cells [17], which suggests that the transcriptional regulation of the α 1 gene may rely upon heterogeneous factors.

Bowen [18], using the renal epithelial cell line MDCK, also reported an increase in Na⁺,K⁺-ATPase activity after hypertonic shock. Although that study suggested that the stimulation of Na⁺,K⁺-ATPase activity, linked to enhanced subunit gene expression, could represent a long-term mechanism to allow the cells to adapt to a hypertonic environment, this view is not in agreement with our findings, since the induction of the activity of the pump is somehow deleterious, easily masked when using a Na⁺ ionophore and, apparently, not related to a significant change in α 1-subunit protein amounts. The possibility that renal cells may show a different pattern of Na⁺,K⁺-ATPase sensitivity to potential modulators depending on their particular origin in the nephron cannot be ruled out. Indeed, aldosterone modulation of the activity of the pump is clearly zonated along the nephron,

although in this case the differential regulation is clearly explained by the distribution of mineralocorticoid receptors [19,20].

Transcriptional and/or pre-translational modulation of the Na⁺,K⁺-ATPase subunit genes, leading to accumulation of their respective mRNAs but resulting in no significant change in Na⁺,K⁺-ATPase activity and/or the amount of the two subunit proteins, has been extensively reported. For instance, in CHO-K1 cells, the α 1 subunit mRNA accumulates when cells are incubated in an amino-acid-free medium, although ouabain-sensitive Rb⁺ uptake is unchanged [9]. Similarly, in the rat liver cell line Clone 9, dexamethasone treatment induces mRNA levels to 2-fold and 40-fold above basal levels for α 1 and β 1 subunits respectively, without significantly altering the activity of the enzyme [21]. In contrast, thyroid regulation of renal Na⁺,K⁺-ATPase expression *in vivo* depends on both subunit protein synthesis and accumulation of the respective mRNAs [22,23]. Nevertheless, renal Na⁺,K⁺-ATPase is also under complex regulation by post-translational events (reviewed in [24]). In general, a number of factors are believed to modulate the activity of the Na⁺ pump. Phosphorylation by either protein kinase A or protein kinase C and dephosphorylation by protein phosphatases 1, 2a and 2b have been postulated in renal tissues on the basis of indirect evidence. More interestingly, Na⁺,K⁺-ATPase is linked to actin-binding proteins in renal epithelial cells, and short actin filaments seem to stimulate the activity of the pump via an increase in its affinity for intracellular sodium, which may explain the presence of different catalytic activities even if the sodium content is kept constant, as in the present study. The stimulation of the sodium pump triggered by hypertonicity may be less evident or even negligible when Na⁺,K⁺-ATPase activity is measured at high intracellular sodium concentrations, such as those reached after monensin treatment. The exact role of the cytoskeletal rearrangement associated with anisotonic shock in the modulation of the sodium pump remains to be elucidated.

Hypertonic treatment of MDCK cells did not significantly affect what Bowen [18] called the ouabain-resistant fraction of Rb⁺ uptake. This is in contrast with the present findings, where it is clearly shown that the furosemide-sensitive (ouabain-resistant) Rb⁺ uptake is markedly induced by hypertonic shock. In contrast to the ouabain-sensitive Rb⁺ uptake, the induction of the furosemide-sensitive component after hypertonic treatment of the cells was not dependent on the transmembrane Na⁺ gradient.

The Na⁺/K⁺/Cl⁻ co-transporter activity of absorptive epithelia

is activated by cAMP in a manner that depends on the integrity of the microfilament network [25,26]. Indirect evidence supports the view that the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter may be functionally linked to the cytoskeleton. Our results using inhibitors of the microtubule and microfilament networks (colcemide and cytochalasin B respectively) support this view and suggest that the activity of the co-transporter in NBL-1 cells under hypertonic treatment relies, in a different manner, upon the integrity of these elements. Nevertheless, the exact role of the structural interaction of this carrier and the cytoskeleton in the hypertonicity-mediated increase in $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter activity remains to be elucidated.

In summary, this study has demonstrated (i) that the $\text{Na}^+/\text{K}^+/\text{ATPase } \alpha 1$ subunit gene may be a hypertonicity-sensitive gene, although complex translational regulation may modulate *de novo* synthesis of pumps and their insertion into the plasma membrane; and (ii) that the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter, although osmotically sensitive, may not belong to the increasing family of osmotic-sensitive genes. Long-term modulation of $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter activity may rely, in an undetermined manner, on the cytoskeletal network structure.

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REFERENCES

- Kinne, R. K. H. (1993) *J. Exp. Zool.* **265**, 346–355
- Burg, M. B. (1995) *Am. J. Physiol.* **37**, F983–F996
- McGivan, J. D. and Pastor-Anglada, M. (1994) *Biochem. J.* **299**, 321–334
- Gazzola, G. C., Dall'Asta, V., Nucci, F. A., Rossi, P. A., Bussolati, O., Hoffmann, E. K. and Guidotti, G. G. (1991) *Cell. Physiol. Biochem.* **1**, 131–142
- Soler, C., Felipe, A., Casado, F. J., McGivan, J. D. and Pastor-Anglada, M. (1993) *Biochem. J.* **289**, 653–658
- Ruiz-Montasell, B., Gómez-Angelats, M., Casado, F. J., Felipe, A., McGivan, J. D. and Pastor-Anglada, M. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9569–9573
- Ferrer-Martínez, A., Felipe, A., Nicholson, B., Casado, J., Pastor-Anglada, M. and McGivan, J. D. (1995) *Biochem. J.* **310**, 689–692
- Pastor-Anglada, M., Felipe, A., Casado, J., Ferrer-Martínez, A. and Gómez-Angelats, M. (1996) *Amino Acids*, in the press
- Felipe, A., Soler, C. and McGivan, J. D. (1992) *Biochem. J.* **284**, 577–582
- Bhutada, A. and Ismail-Beigi, F. (1991) *Am. J. Physiol.* **261**, C699–C707
- Longo, N., Griffin, L. D. and Elsas, L. J. (1991) *Am. J. Physiol.* **260**, C1341–C1346
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Theodoropoulos, P. A., Stournaras, C., Stoll, B., Markogiannakis, E., Lang, F., Gravanis, A. and Häussinger, D. (1992) *FEBS Lett.* **311**, 241–245
- Yokoyama, T., Lin, L.-R., Chakrapani, B. and Reddy, V. N. (1993) *Invest. Ophthalmol. Vis. Sci.* **34**, 2512–2517
- Yamamoto, K., Ikeda, U., Seino, Y., Tsuruya, Y., Oguchi, A., Okada, K., Ishikawa, S., Saito, T., Kawakami, K., Hara, Y. and Shimada, K. (1993) *J. Clin. Invest.* **92**, 1889–1895
- Qian, N. X., Pastor-Anglada, M. and Englesberg, E. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3416–3420
- Bowen, J. W. (1992) *Am. J. Physiol.* **262**, C845–C853
- Doucet, A. and Barlet-Bas, C. (1989) *Curr. Top. Membr. Transp.* **34**, 185–208
- Todd-Turla, K. M., Schnermann, J., Fejes-Tóth, G., Naray-Fejes-Tóth, A., Smart, A., Killen, P. D. and Briggs, J. P. (1993) *Am. J. Physiol.* **264**, F781–F791
- Bhutada, A., Wassinger, W. W. and Ismail-Beigi, F. (1991) *J. Biol. Chem.* **266**, 10859–10866
- McDonough, A. A., Brown, T. A., Horowitz, B., Chiu, R., Schlatterbeck, J., Bowen, J. and Schmitt, C. A. (1988) *Am. J. Physiol.* **254**, C323–C329
- Horowitz, B., Hensley, C. B., Quintero, M., Azuma, K. K., Putnam, D. and McDonough, A. A. (1990) *J. Biol. Chem.* **265**, 14308–14313
- Bertorello, A. M. and Katz, A. I. (1993) *Am. J. Physiol.* **265**, F743–F755
- Shapiro, M., Matthews, J., Hecht, G., Delp, C. and Madara, J. L. (1991) *J. Clin. Invest.* **87**, 1903–1909
- Matthews, J. B., Awtrey, C. S. and Madara, J. L. (1992) *J. Clin. Invest.* **90**, 1608–1613