Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1712 (2005) 71 – 80
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Membrane insertion of betaine/GABA transporter during hypertonic stress correlates with nuclear accumulation of TonEBP

Stephen A. Kempson[a,*], Jeffrey A. Beck[a], Philip E. Lammers[a], J. Scott Gens[a], Marshall H. Montrose[b]

[a]Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN 46202-5120, USA
[b]Department of Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA

Received 4 January 2005; received in revised form 15 March 2005; accepted 17 March 2005
Available online 7 April 2005

Abstract

MDCK cells stably transfected with betaine/GABA transporter tagged with EGFP (EGFP–BGT) were used to study plasma membrane insertion of EGFP–BGT. Adaptive response to hypertonicity requires nuclear migration of TonEBP. Confocal microscopy showed that after 6 h hypertonicity, the nuclear/cytoplasmic ratio of TonEBP fluorescence was increased to 2.4 compared to 1.4 in isotonic controls ($P<0.001$). The ratio in hypertonic cells was reduced by the proteasome inhibitor MG-132 in a dose-dependent way. Inhibition was 50% at 3 μM. After 6 h, hypertonicity expressed EGFP–BGT was localized in the plasma membrane, but there was no change in total EGFP–BGT abundance compared to isotonic controls. In contrast, EGFP–BGT remained mostly intracellular when 3 μM MG-132 was included in the hypertonic medium. The transport function of EGFP–BGT was studied as Na^+-dependent uptake of [^{3}H]GABA. This was not changed by MG-132 in isotonic controls, but MG-132 produced dose-dependent inhibition of hypertonic upregulation of Na^+/GABA cotransport. Inhibition was 80% at 3 μM MG-132. Transport likely reflects membrane insertion of EGFP–BGT and there was a positive correlation ($P<0.05$) between Na^+/GABA cotransport and the N/C ratio of TonEBP. Results are consistent with a role for TonEBP-mediated transcription in synthesis of additional proteins required for membrane insertion of EGFP–BGT protein.

Keywords: MDCK cells; Betaine; Kidney; Stable transfections; MG-132

1. Introduction

Cells regulate cytosolic volume by adjusting solute content to produce an osmotically driven gain or loss of water. Cell solute content is altered by a variety of mechanisms including ion transport, formation, or degradation of macromolecules, and accumulation of organic osmolytes [1,2]. Osmolytes are especially important for long-term volume adjustments because they do not perturb cellular function [3,4]. Betaine is an important osmolyte in the kidney, and the renal betaine/GABA transporter (BGT1) protects cells in the hypertonic inner medulla by mediating cell uptake and accumulation of betaine [5]. There is prolonged hypertonicity in this region of the kidney during the normal operation of the urinary concentrating mechanism. In addition to functioning as an osmolyte, betaine also may act as a chaperone to stabilize protein structure under stressful conditions [6].

Transcriptional activation of the *BGT1* gene in MDCK cells during hypertonic stress [7] is mediated by TonEBP, a member of the NFAT family of transcription factors [8,9]. TonEBP abundance in the nucleus is increased by hypertonicity, a step that begins within 60 min [10] and occurs prior to the increase in BGT1 mRNA [11,12]. Failure of this adaptive mechanism in the kidney leads to severe atrophy and loss of medullary tissue [13,14].

Previous work in MDCK cells [15] utilized BGT1 tagged at the N-terminus with enhanced green fluorescent protein (EGFP). In transiently transfected cells, normal transport

* Corresponding author. Medical Sciences Room 309,635 Barnhill Drive, Indianapolis, IN 46202-5120, USA. Tel.: +1 317 274 1444; fax: +1 317 274 3318.
E-mail address: skempson@iupui.edu (S.A. Kempson).

0005-2736/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2005.03.006

function of the fusion protein (EGFP–BGT) was not altered, as determined by ion dependence, inhibitor sensitivity, and kinetics. The EGFP–BGT protein also trafficked normally to the plasma membrane during hypertonic stress. Confocal imaging of EGFP–BGT distribution in live cells detected EGFP–BGT fluorescence in the plasma membrane within 2 h after switching the cells to hypertonic (500 mosM) growth medium. While this model was adequate for initial characterization of EGFP–BGT trafficking, the low transfection efficiency was problematic for studying cell populations.

In the present study, MDCK cells stably expressing the EGFP–BGT fusion protein have been used to further investigate the plasma membrane insertion of EGFP–BGT during early onset of osmotic stress. The advantage of this system compared to native MDCK cells is that overexpression provides the cells with an abundance of EGFP–BGT protein, which allows a focus on the membrane insertion step independent of de novo synthesis. Furthermore, in stable transfectants the membrane insertion of EGFP–BGT can be quantitated both in single cells by confocal microscopy and in cell populations by biotinylation and measurement of transport function. Using these approaches, the time course of plasma membrane insertion of EGFP–BGT in MDCK cells has been characterized. By use of a proteasomal inhibitor to block nuclear translocation of TonEBP, we have determined also if membrane insertion is dependent on transcriptional activation by TonEBP.

2. Methods

Culture of MDCK cells, measurements of GABA transport, Western blotting, and surface biotinylation procedures

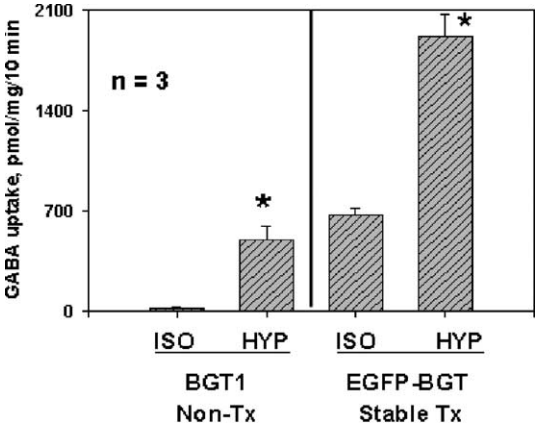

Fig. 1. Na^+-dependent GABA uptake by monolayers of native non-transfected (Non-Tx) MDCK cells and MDCK cells stably transfected (Tx) with BGT1 tagged with EGFP (EGFP–BGT). Uptake was determined after 24 h incubation in normal isotonic (ISO) or hypertonic (HYP) growth medium. Data are mean±S.D. from three separate experiments and have been corrected by subtraction of Na^+-independent uptake which was not changed by hypertonic stress or transfection. *Significantly different from respective isotonic group (P<0.02).

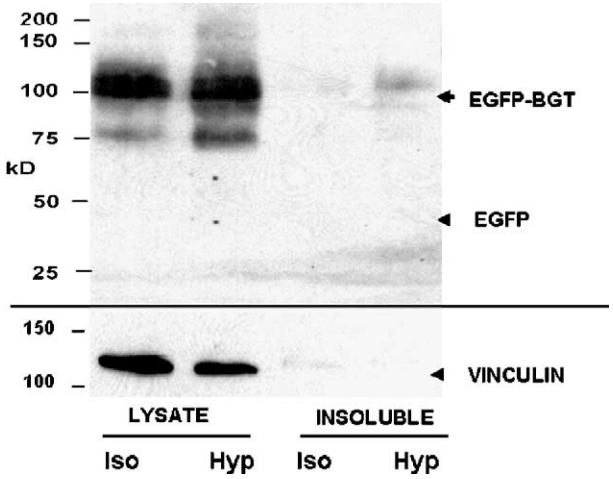

Fig. 2. Abundance of EGFP–BGT protein in whole cell lysates of stable transfectants determined by Western blotting. After 24 h incubation in isotonic (Iso) or hypertonic (Hyp) growth medium, cells were lysed in 1% Triton X-100 solution containing protease inhibitors (16) and centrifuged. Supernatant (lysate) was removed and pellets (insoluble) were dissolved by heating in 1% SDS (in 150 mM NaCl, 50 mM Tris, pH 7.4) for 10 min at 90 °C. Protein samples were separated on 10% polyacrylamide/SDS gels, transferred to nitrocellulose and blotted with antibodies to GFP (16). Blots were stripped and reprobed with anti-vinculin antibodies.

were described previously in detail [15,16]. All transport data have been corrected by subtraction of values for Na^+-independent uptake, determined in the same experiment. MDCK cells stably expressing EGFP-tagged BGT1 were obtained by transfection, as described previously [15], followed by selection in complete growth medium containing geneticin (G418,400 μg/ml). The maintenance dose was 200 μg/ml and was removed prior to experiments. Expression of EGFP-tagged BGT1 was verified by fluorescence microscopy and Western blotting with affinity purified rabbit polyclonal antibody to GFP (Abcam, Cambridge, UK) [15].

Affinity-purified polyclonal antibodies to TonEBP were generously provided by Dr. H.M. Kwon, University of Maryland, and diluted 1:2000 for westerns. Antibodies to E-cadherin and vinculin were obtained from Dr. J Marrs and Dr. F Pavalko, Indiana University School of Medicine, and used at 1:10,000 and 1:5000 dilutions, respectively. Mouse monoclonal antibody to β-actin from Sigma-Aldrich (St. Louis, MO) was used at 1:1000 dilution. MG-132 was from Calbiochem (San Diego, CA) and a stock solution was prepared in dimethylsulfoxide at 2000-fold working concentration and stored at −20 °C. All test compounds were added to cells in isotonic medium for 1 h to allow uptake and accumulation prior to switching to hypertonic medium for 24 h. Cell culture medium was made hypertonic (500 mosmol/kg) by addition of sucrose [15,16].

Cell viability was determined by crystal violet staining of DNA of adherent cells following various treatments in hypertonic medium for 24 h [17,18]. Cells that detach in hypertonic medium were shown previously to be apoptotic and non-viable [17]. Each well of a 6-well plate was seeded

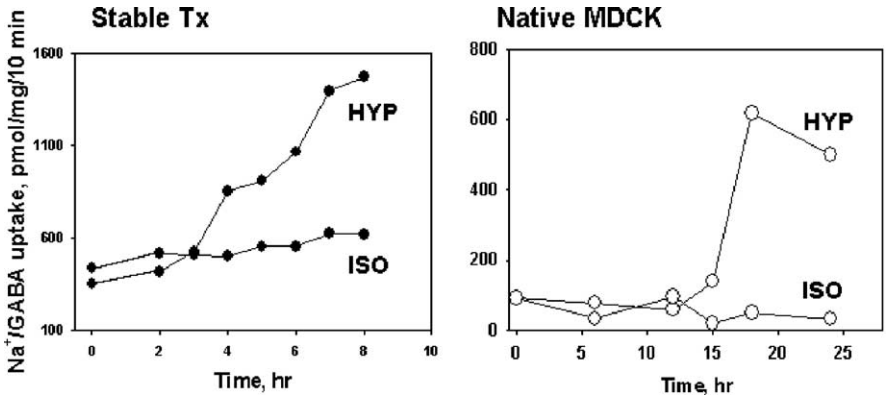

Fig. 3. Comparison of time course of hypertonic (HYP) activation of Na^+-dependent GABA transport in native MDCK (right) and stably transfected (Tx, left) MDCK cells. Transport in isotonic (ISO) controls remained unchanged. Mean data from a typical experiment (analyzed in triplicate) are shown. Bars for S.D. are small and are obscured by the data symbols.

with 2×10^5 cells, and experiments were begun 48 h later when the cells were just prior to confluence. After the 24-h treatments, the cells were washed four times in PBS of matching tonicity, and stained by addition of 0.75% crystal violet in 30% acetic acid for 10 min. The cells were washed five times in normal PBS, extracted with methanol, and absorbance at 630 nm was determined. Percent viability of treated cells was expressed as the absorbance relative to isotonic control cells.

The proportion of EGFP–BGT that reached the plasma membrane after 6 h hypertonic stress was quantitated by confocal morphometric analysis of live cells on glass coverslips. The red lipophilic FM 4-64 dye was applied extracellularly to specifically stain the plasma membrane and to form a mask overlapping EGFP–BGT localized in the plasma membrane, as described elsewhere [19–23]. MDCK cells were cooled to 4 °C to block endocytosis and incubated in 10 μM FM 4-64 in PBS for 2 min immediately prior to visualization. There was no improvement in labeling intensity at concentrations above 10 μM. Images were collected using a Zeiss LSM 510 confocal microscope equipped with a Zeiss C-Apo x40 water immersion lens, and controlled by LSM 510 software version 3.0 SP3. FM 4-64 was excited by 488 nm with emission collected between 620 and 680 nm. EGFP was excited by 488 nm and emission was collected at 500–550 nm. Optical sections at 1-μm intervals were obtained to produce a three-dimensional image of each cell, and cells in 3–4 different fields were sampled. The instrument gain, offset, and other settings were the same for all samples. Fluorescent images were examined with LSM software (Carl Zeiss, Thornwood, NY) and Metamorph software (Universal Imaging, Downingtown, PA) was used for analysis.

MDCK cells were grown on glass coverslips for visualization of TonEBP distribution. Following incubation in isotonic or hypertonic test medium, the cells were washed four times in isotonic or hypertonic PBS and fixed for 5 min in 4% paraformaldehyde/PBS. The cells were washed in PBS, permeabilized in 0.2% Triton X-100/PBS for 5 min, rinsed again, and incubated in PBS containing 2% gelatin for 30 min at 37 °C to block non-specific binding. The cells were next incubated for 1 h at 37 °C in 1% gelatin/PBS containing TonEBP antibody at 1:1,000 dilution. The primary antibody was detected by incubating cells for 45 min in 1% gelatin/PBS containing affinity-purified goat anti-rabbit IgG conjugated to FITC (Jackson Immuno-Research, West Grove, PA) at 1:100 dilution. Cells were washed and counterstained for 10 min in propidium iodide (2 μg/ml) in PBS. After final washes, the cells were mounted with fluoromount-G (Southern Biotechnology, Birmingham, AL) and examined by confocal microscopy, as described previously [16,24]. For detection of FITC fluorescence, the excitation was at 488 nm and emission was collected at 500–530 nm. Propidium iodide was detected in the same image by exciting at 488 nm and collecting emission at 620–680 nm. A single optical plane was chosen that intersected all the nuclei (20–30) in the field of view. Images were stored and Metamorph software was used for analysis. The periphery of each nucleus was traced on the propidium iodide image. A second larger region was traced outside the first to include the cytoplasm immediately adjacent to each nucleus. Nuclei were chosen randomly to include different staining intensities. The marked regions were transferred to the TonEBP image.

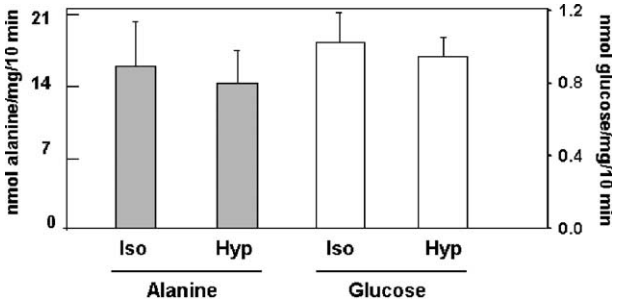

Fig. 4. Na^+-dependent uptakes of L-alanine and D-glucose in stably transfected MDCK cells after 6 h incubation in isotonic (Iso) or hypertonic (Hyp) growth medium. Data are mean±S.D. from three separate experiments.

The average fluorescence intensity of each region was determined and thresholded by subtracting the value for a region that showed no TonEBP staining. For each cell, the average fluorescence intensity of the nucleus was divided by the average intensity of the adjacent cytoplasm to obtain the nuclear/cytoplasmic (N/C) ratio. The procedure was repeated in a second field of view to determine the average ratio for a total of 30–40 cells chosen randomly.

Data are expressed as mean ± S.D. Analysis of variance was used to evaluate differences between multiple groups, and individual groups were compared by the Tukey test [25] using Instat v. 3.06 (GraphPad Software, San Diego, CA). A probability of $P<0.05$ was considered statistically significant.

3. Results

Na^+-dependent uptake of GABA by whole cell monolayers was activated by hypertonic stress (500 mosM) for 24 h in both native MDCK and stable transfectants (Fig. 1), but the overall uptake was almost three times greater in

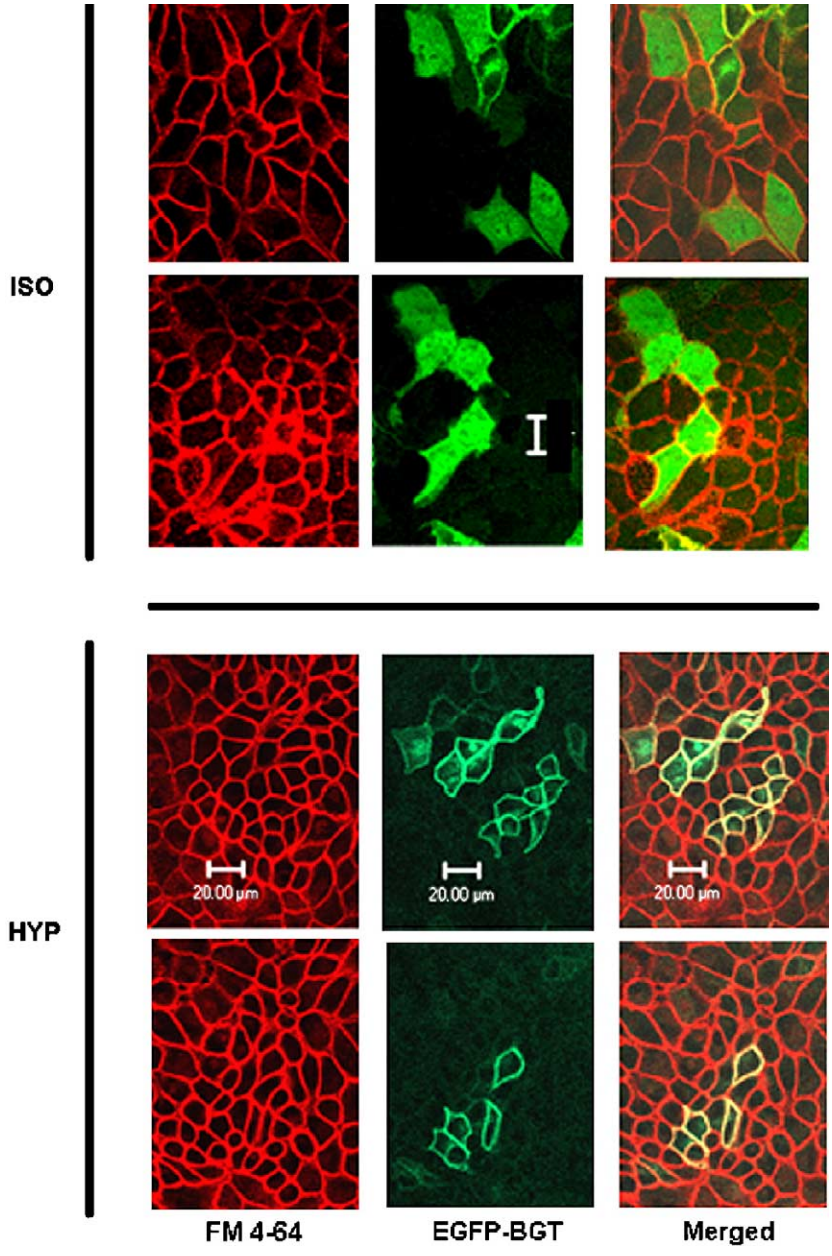

Fig. 5. Confocal micrographs comparing distribution of EGFP-tagged BGT1 (EGFP–BGT, center panels) in stable transfectants after 6 h incubation in isotonic (ISO) or hypertonic (HYP) growth medium. Plasma membranes in the same field of view were labeled with FM 4-64 (left panels). Two separate experiments are depicted for both ISO (upper section) and HYP (lower section) groups. Instrument settings were the same for all samples. In each cell the proportion of total cellular EGFP–BGT that localized to the plasma membrane was determined by use of Metamorph software, as described under Methods. Scale bar is 20 μm.

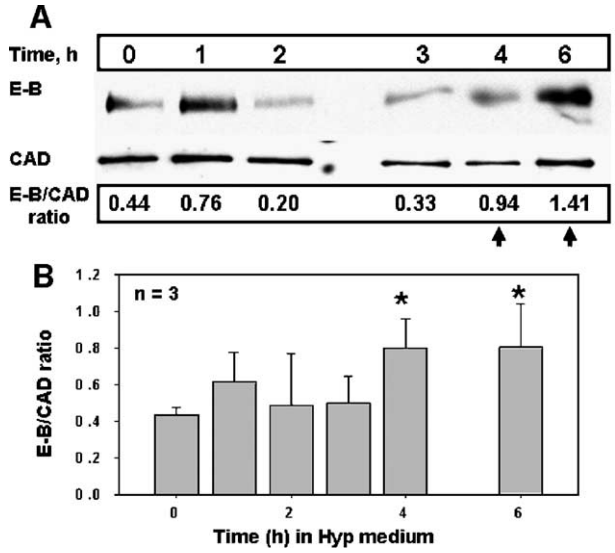

Fig. 6. Time course of surface expression of EGFP-tagged BGT1 (E–B) in stable transfectants during hypertonic stress for 0–6 h, as determined by surface biotinylation. (A) Surface abundance of E–B relative to E-cadherin (CAD) increased at 4 and 6 h. (B) Values for E–B/CAD ratios obtained from three separate experiments (mean±S.D.). Significantly different (*P<0.02) compared to ratio at time zero.

transfectants compared to native cells. The data have been corrected for Na^+-independent uptake of GABA which was unchanged by hypertonic stress and did not differ between native and transfected cells.

The increased transport activity in stable transfectants during hypertonic stress is probably due to a change in intracellular distribution because the total amount of expressed EGFP–BGT was unchanged, based on Western blots of whole cell lysates. All the expressed EGFP–BGT was recovered in the Triton-soluble fraction and the abundance after 24 h hypertonicity was very similar to the abundance in isotonic controls (Fig. 2).

We next tested the time course of hypertonic adaptation in stable transfectants to determine if EGFP–BGT overexpression allowed a faster adaptive response. Upregulation of Na^+/GABA cotransport in native non-transfected cells was not detected until after 15–18 h of hypertonic treatment, as reported previously [26,27]. In marked contrast, hypertonic

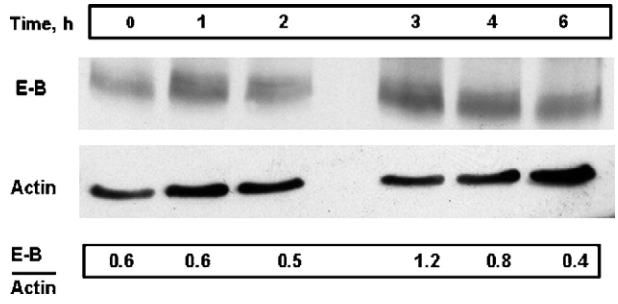

Fig. 7. Western blot comparing relative abundance of expressed EGFP–BGT (E–B) and actin proteins in whole cell lysates of stable transfectants during 0–6 h hypertonic stress. The E–B/actin ratio was unchanged throughout.

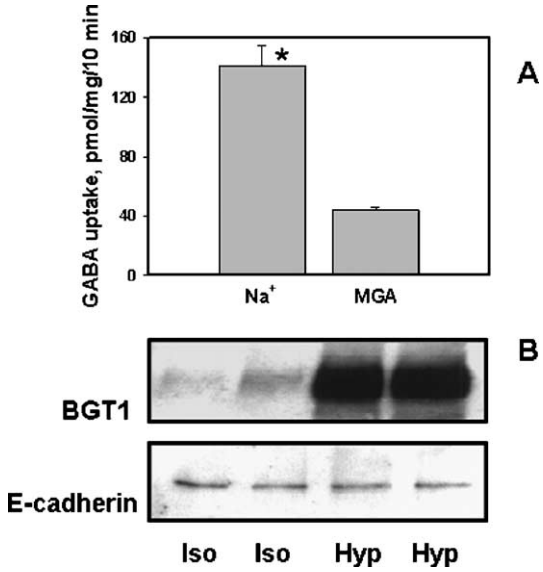

Fig. 8. Surface expression of BGT1 in non-transfected MDCK cells under isotonic (Iso) conditions. (A) Detection as Na^+-dependent GABA transport in cell monolayers. Data are mean±S.D. from three experiments. *Significantly different (P<0.02) compared to GABA uptake when Na^+ in the uptake solution was replaced by methyl-D-glucamine (MGA) (16). (B) Detection by surface biotinylation under isotonic conditions and after 24 h hypertonic (Hyp) stress.

upregulation was detected within 4 h in the stable transfectants (Fig. 3) and was significantly different after 5–8 h hypertonic stress. Absolute values after 6 h were 853±102 in the hypertonic group compared to 502±96 pmol/mg/10 min (n=3, P<0.05) in isotonic controls. The Na^+/GABA uptake continued to increase as hypertonic stress was maintained and reached a peak at 15–24 h (not shown) that was almost double the uptake after 7–8 h.

During hypertonic stress, native MDCK cells also upregulate the Na^+-dependent transport of alanine much sooner (after 5–6 h) than Na^+/GABA transport [26]. Alanine transport was determined in the stable transfectants

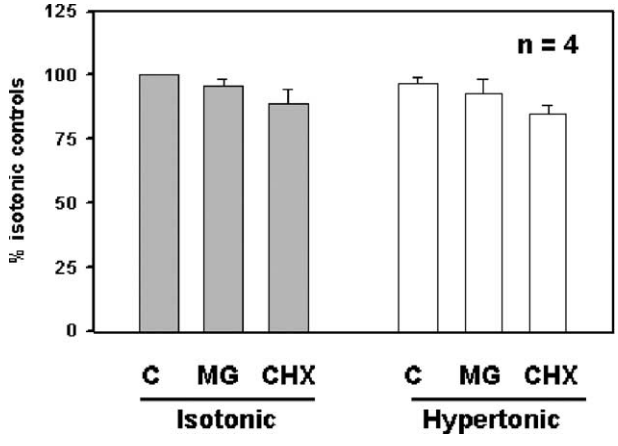

Fig. 9. Viability of stably transfected MDCK cells determined by crystal violet staining after incubation for 6 h in either isotonic or hypertonic growth medium. Medium contained either no additions (C), or 1 μM MG-132 (MG) or 3 μM cycloheximide (CHX). Data are expressed as % of isotonic controls, and are mean±S.D. from four experiments.

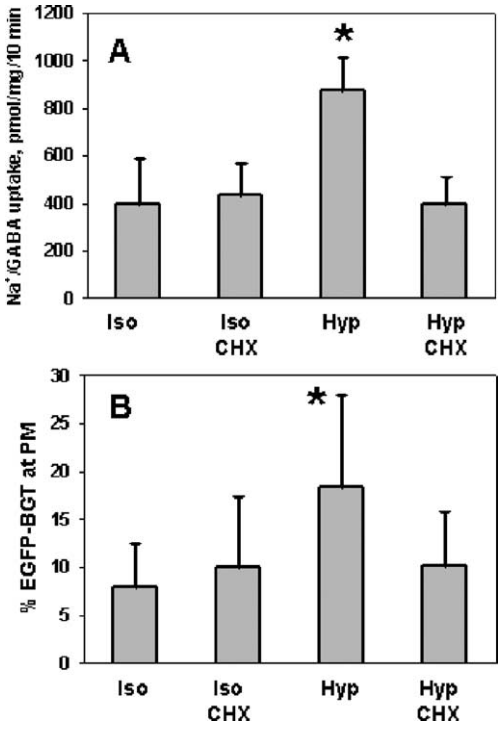

Fig. 10. Blockade by 3 μM cycloheximide (CHX) of the surface expression of EGFP–BGT during adaptation to 6 h hypertonic (Hyp) stress. (A) Na^+/GABA cotransport by MDCK cells stably transfected with EGFP-tagged BGT1 (EGFP–BGT). (B) Proportion of EGFP–BGT localized to the plasma membrane (PM) expressed as % total cellular expression, as determined by the morphometric analysis. CHX had no effect under isotonic (Iso) conditions. Data are mean±S.D. from three experiments (A) and 18–22 cells per group (B). Significantly different (*P<0.05) compared to untreated isotonic control group.

after 6 h hypertonicity, and D-glucose was used as an additional control. There was no change in either Na^+/alanine or Na^+/glucose cotransport (Fig. 4) at this time, suggesting that the much earlier upregulation of BGT1 after 4–6 h (Fig. 2) was adequate for providing intracellular osmolytes in these cells. Similar experiments after 1 and 2 h hypertonicity (not shown) revealed no change in Na^+/alanine transport, indicating that the normal upregulation at 5–6 h was truly absent and not just shifted to earlier time points.

When stably transfected cells maintained in isotonic medium were analyzed by confocal morphometric analysis, expressed EGFP–BGT was detected in the plasma membrane and the proportion accounted for 7.2±4.7% (mean±S.D., n=8) of total cellular fluorescence. This finding helps to explain the increased transport in this group under isotonic conditions (Fig. 1). In contrast, after 6 h hypertonic stress, there was 21.4+6.4% (n=12, P<0.001) of total EGFP–BGT in the plasma membrane, representing a 3-fold increase (Fig. 5).

Surface biotinylation studies on stable transfectants detected increased abundance of EGFP–BGT protein at the plasma membrane, relative to E-cadherin, after 4–6 h hypertonicity (Fig. 6A). Data from three experiments were quantitated by densitometry [15] and show that the ratio almost doubled after 4 h and this increase was maintained at 6 h. The increases were significantly different compared to the ratio at time zero (Fig. 6B). In contrast, whole cell lysates from the same experimental groups showed no change in total EGFP–BGT abundance (Fig. 7). The EGFP–BGT/actin ratios were determined by densitometry. The ratio at time zero was 0.82±0.32 (mean±S.D., n=3) and did not change significantly during hypertonic stress for 1–6 h, confirming that the increased amount at the plasma membrane was due primarily to redistribution.

Biotinylation of transfected cells also confirmed the presence of EGFP–BGT in the plasma membrane of isotonic controls (time 0, Fig. 6A), consistent with the findings of the confocal studies (Fig. 5) and morphometric analysis. This may be due in part to the saturation of membrane trafficking pathways by over-expressed EGFP–BGT protein. However, when native non-transfected MDCK cells were re-investigated under isotonic conditions, a small but significant amount of Na^+/GABA cotransport was detected (Fig. 8A). Biotinylation also detected a small amount of BGT1 protein in the plasma membrane that showed a large increase in abundance after 24 h hypertonic stress (Fig. 8B). This strongly suggests that the increased GABA transport function and the detection of membrane localization of EGFP–BGT in isotonic transfectants (Figs. 1 and 5) are to be expected, based on BGT1 behavior in native cells.

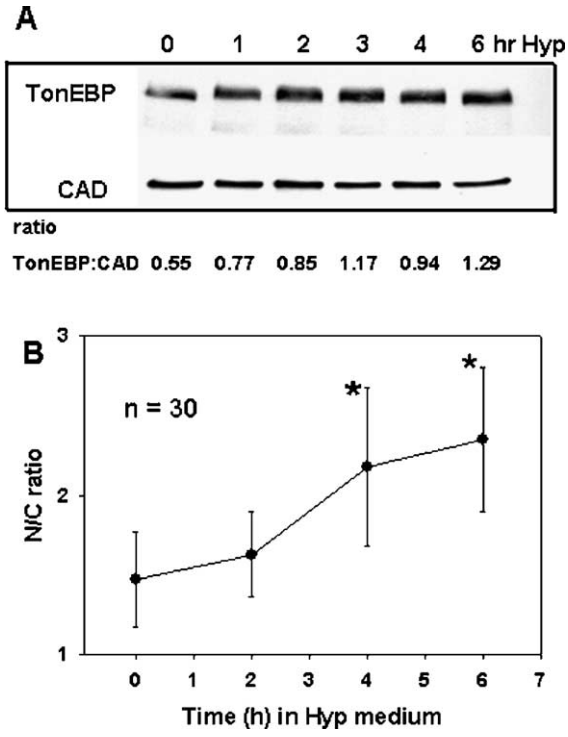

Fig. 11. Time-dependent increases in (A) total cellular abundance of TonEBP relative to E-cadherin (CAD), determined by Western blotting, and (B) nuclear localization of TonEBP, expressed as the nuclear/cytoplasmic (N/C) ratio of TonEBP fluorescence. Data obtained after subjecting stably transfected MDCK cell monolayers to hypertonic (Hyp) stress for 0–6 h. Data in B are mean±S.D. from 30 cells at each time point. *Significantly different (P<0.001) compared to N/C ratio at time.

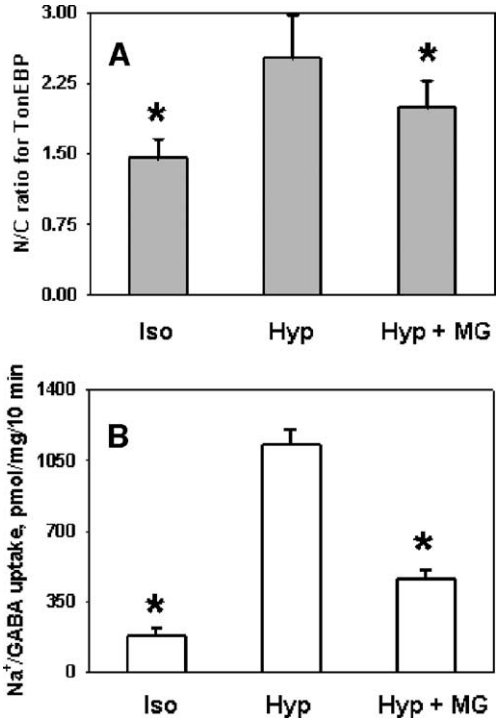

Fig. 12. Blockade by 1 μM MG-132 (MG) of the adaptation to 6 h hypertonic (Hyp) stress in stable transfectants. (A) Nuclear localization of TonEBP, determined as N/C ratio. (B) Surface expression of EGFP–BGT determined as Na^+/GABA cotransport. Data are mean±S.D. from 40 cells in each group (A) or three experiments (B). *Significantly different ($P<0.001$) compared to hypertonic group.

Previous confocal studies on transiently transfected MDCK cells showed that cycloheximide blocked membrane insertion of EGFP–BGT during hypertonic stress for 14 h. We tested the action of cycloheximide (3 μM) in stable transfectants during a 6-h hypertonic period. This time and concentration had little effect on cell viability, based on assay with crystal violet [17,18]. Viability of hypertonic cells exposed to cycloheximide was 89% compared to hypertonicity alone (Fig. 9). Cycloheximide had no effect on transport in isotonic controls, but the increase in Na^+/GABA cotransport after 6 h hypertonicity was blocked completely (Fig. 10A). Cycloheximide also blocked localization of EGFP–BGT to the plasma membrane, as determined by confocal morphometric analysis. The proportion of EGFP–BGT localized to the plasma membrane in isotonic controls was not changed by treatment with 3 μM cycloheximide for 6 h. As shown previously (Fig. 5), there was a significant increase in plasma membrane localization of EGFP–BGT after 6 h hypertonicity. However, this increase was blocked completely when cycloheximide was present, and the membrane localization of EGFP–BGT was not different from isotonic controls (Fig. 10B).

Both the overall cellular abundance of TonEBP, which was expressed relative to E-cadherin content, and the proportion of TonEBP present in the nucleus, expressed as the nuclear/cytoplasmic (N/C) fluorescence ratio [28], were increased in the stably transfected MDCK cells during hypertonic stress for 4–6 h (Fig 11). These responses are very similar to those reported previously for native non-transfected MDCK cells [10,29]. During 6-h hypertonicity, the proteasome inhibitor MG-132 (1 μM) blocked the normal increase in the nuclear/cytoplasmic (N/C) ratio of TonEBP by 40% (Fig. 12A), as expected [30]. MG-132 treatment also blocked the hypertonic stimulation of Na^+/GABA cotransport by 70% (Fig. 12B). There was no significant effect of MG-132 on cell viability under these conditions. Viability of hypertonic cells treated with 1 μM MG-132 was 96% compared to hypertonicity alone (Fig. 9). Thus, in spite of over-expression of EGFP–BGT, the trafficking and membrane insertion steps of this protein are still sensitive to a proteasomal inhibitor that also blocked nuclear accumulation of TonEBP. The inhibitory actions of MG-132 on the N/C ratio of TonEBP and Na^+/GABA transport were dose-dependent at concentrations of 0, 0.3, 1.0, 3.0, and 10.0 μM, and there was a significant correlation between the N/C ratio and transport (Fig. 13). Confocal microscopy confirmed that 3 μM MG-132 inhibited membrane insertion of EGFP–BGT during 6-h hypertonicity (Fig. 14).

4. Discussion

The overall BGT1 transport activity in the stable transfectants exceeded that in native MDCK cells, under both isotonic and hypertonic conditions (Fig. 1), and the difference is likely due to the additional activity of expressed EGFP–BGT fusion protein in the transfected cells. The adaptation to 24 h hypertonic stress was not accompanied by an increase in abundance of EGFP–BGT (Fig. 2), indicating that the mechanism involved redistribution rather than increased synthesis of EGFP–BGT. Similar findings were reported for MDCK cells transiently transfected with EGFP–BGT [15]. Quantitation by morphometric analysis

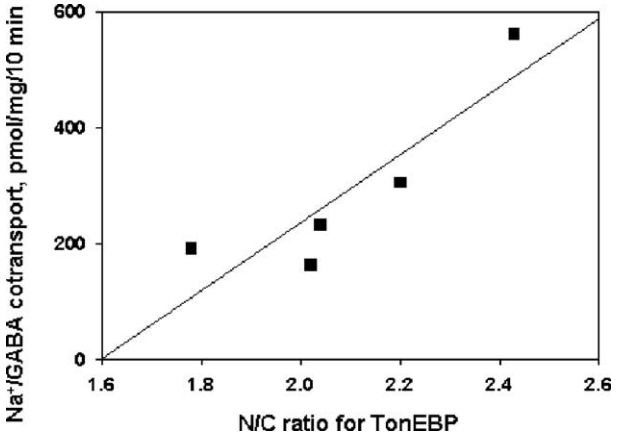

Fig. 13. Regression analysis revealed a significant ($P<0.05$) correlation between Na^+/GABA cotransport and nuclear localization (N/C ratio) of TonEBP. The r value was 0.86. Data derived from use of MG-132 at 0, 0.3, 1.0, 3.0, and 10.0 μM. Other details as in Fig. 12.

showed that just 7.2% of cellular EGFP–BGT was present in the plasma membrane under isotonic conditions, and this increased 3-fold after 6 h hypertonic stress. Although the increased transport under isotonic conditions appears to reflect abnormal trafficking, a detailed study of non-transfected MDCK cells maintained in isotonic medium detected a small amount of Na^+-dependent GABA transport as well as surface expression of BGT1 protein (Fig. 8). Thus, the behavior of EGFP–BGT protein under both isotonic and hypertonic conditions is identical in all respects to native BGT1 protein.

The increased plasma membrane content of EGFP–BGT after just 6 h hypertonicity prompted examination of the time course of the adaptation in stable transfectants. This revealed a significant increase in transport activity after just 4 h hypertonicity, much sooner than the 18-h period required to activate transport activity in native MDCK cells (Fig. 3). This time difference in upregulation indicates that measurements of Na^+/GABA cotransport after 6 h hypertonicity likely reflect only the activity of transfected EGFP–BGT rather than the native BGT1 that is also expressed in the same cells. The early increase in transport activity apparently obviated the requirement for upregulation of alanine transport (Fig. 4) that normally occurs in native MDCK cells [26]. This process involves increased expression of system ATA2 [31] and is likely an intermediate step in osmolyte accumulation. The finding of unchanged Na^+/alanine and Na^+/glucose cotransport also illustrates the specificity of the increase in Na^+/GABA cotransport at 6h hypertonicity.

Independent analysis via surface biotinylation revealed a 2-fold increase in surface expression of EGFP–BGT, relative to E-cadherin expression, after 4–6 h hypertonic stress (Fig. 6). In the same experiments, there was no change in EGFP–BGT abundance in whole cell lysates after 4–6 h hypertonicity (Fig. 7). This confirms the results after 24 h hypertonicity (Fig. 2) and indicates that the increased surface expression was due to a shift from cytoplasm to plasma membrane. We conclude that the overexpression of EGFP–BGT protein bypasses the need for de novo protein synthesis and allows more rapid insertion in the plasma membrane when hypertonic stress is encountered. Although there is abundant EGFP–BGT protein in the cells, there still remains a 3- to 4-h delay in the adaptive response measured in a population of cells (Figs. 3 and 6). Previous confocal studies on individual transfected cells revealed at least a 2-h delay [15]. The delay period is consistent with the need to synthesize accessory proteins, for example, Munc and other SNARE proteins [32], that may be required for fusion and membrane insertion of EGFP–BGT.

Inclusion of 3 μM cycloheximide, a non-specific inhibitor of protein synthesis, in hypertonic medium eliminated the normal adaptation to 6 h of hypertonic stress.

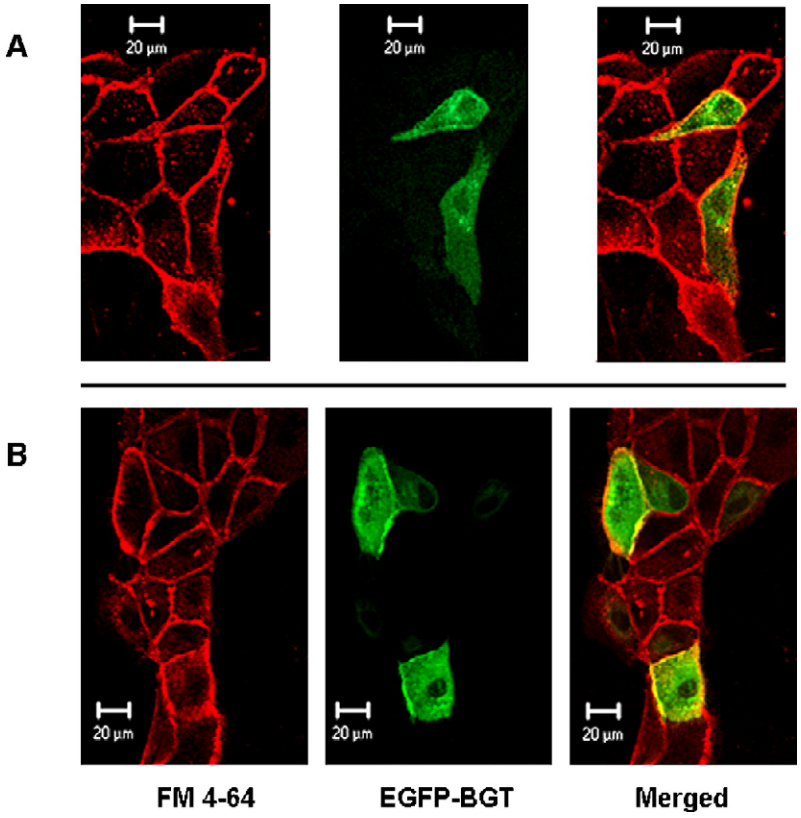

Fig. 14. Distribution of EGFP–BGT (center panels) is primarily cytoplasmic after 6 h incubation in hypertonic medium containing 3 μM MG-132. Panels A and B are from two different experiments. Plasma membranes in the same field of view were labeled with FM 4-64 (left panels). Instrument settings were the same for all samples. Scale bar is 20 μm.

Both the increase in Na^+/GABA cotransport and the increase in plasma membrane localization of EGFP–BGT were blocked (Fig. 10). This provides indirect support for the concept that additional proteins must be synthesized to facilitate membrane insertion of EGFP–BGT protein, even when the latter is present in excess. Based on these observations, it appears that hypertonic upregulation of betaine transport involves at least a two-step process, starting with synthesis of BGT1 and accessory proteins, and ending with trafficking and plasma membrane insertion of BGT1 protein [33].

Finally, the possible role of TonEBP in the adaptive mechanism in stable transfectants was examined. The expected increases in TonEBP abundance and nuclear accumulation were present after 4–6 h hypertonic stress (Fig. 11). The proteasome inhibitor MG-132 (1 μM) significantly inhibited both the increase in nuclear accumulation and the increase in Na^+/GABA cotransport (Fig. 12). The transport measured at 6 h hypertonicity primarily reflects membrane insertion of EGFP–BGT. When these measurements were made using MG-132 over a range of concentrations, a positive correlation emerged between Na^+/GABA cotransport and the N/C ratio of TonEBP (Fig. 13). These findings should be interpreted with caution because the correlation does not prove a causal relationship and there may be other aspects of proteasome inhibition (or MG-132 action) that contribute to the blockade of transport. For example, both membrane insertion of EGFP–BGT and nuclear translocation of TonEBP may be influenced by unknown factors that are present due to proteasomal inhibition (Fig. 14). However, it is known that TonEBP stimulates transcription of a number of target genes [34]. Thus, blockade of nuclear accumulation of TonEBP may interfere with membrane localization of EGFP–BGT because TonEBP also regulates expression of the putative accessory proteins required for membrane insertion of EGFP–BGT. Additional work will be required to define these new features of BGT1 protein regulation.

Acknowledgements

Published in part in abstract form (J Am Soc Nephrol 15: 89A, 2004). Supported by grants from the American Heart Association Greater Midwest Affiliate (SAK), the National Institutes of Health (MHM), NIH F32 DK61178 (JSG), and the Student Research Program in Academic Medicine at Indiana University School of Medicine (JAB). We thank Lixuan Tackett for expert technical assistance.

References

[1] F. Lang, G.L. Busch, M. Ritter, H. Volkl, S. Waldegger, E. Gulbins, D. Haussinger, Functional significance of cell volume regulatory mechanisms, Physiol. Rev. 78 (1998) 247–306.

[2] F. Wehner, H. Olsen, H. Tinel, E. Kinne-Saffran, R.K. Kinne, Cell volume regulation: osmolytes, osmolyte transport and signal transduction, Rev. Physiol., Biochem. Pharmacol. 148 (2003) 1–80.

[3] M. Brigotti, P.G. Petronini, D. Carnicelli, R.R. Alfieri, M.A. Bonelli, A.F. Borghetti, K.P. Wheeler, Effects of osmolarity, ions and compatible osmolytes on cell-free protein synthesis, Biochem. J. 369 (2003) 369–374.

[4] M.B. Burg, H.M. Kwon, D. Kultz, Osmotic regulation of gene expression, FASEB J. 10 (1996) 1598–1606.

[5] A. Yamauchi, S. Uchida, H.M. Kwon, A.S. Preston, R.B. Robey, A. Garcia-Perez, M.B. Burg, J.S. Handler, Cloning of a Na+- and Cl-dependent betaine transporter that is regulated by hypertonicity, J. Biol. Chem. 267 (1992) 649–652.

[6] D. Sheikh-Hamad, A. Garcia-Perez, J.D. Ferraris, E.M. Peters, M.B. Burg, Induction of gene expression by heat shock versus osmotic stress, Am. J. Physiol. 267 (1994) F28–F34.

[7] S. Uchida, A. Yamauchi, A.S. Preston, H.M. Kwon, J.S. Handler, Medium tonicity regulates expression of the Na+- and Cl-dependent betaine transporter in MDCK cells by increasing transcription of the transporter gene., J. Clin. Invest. 91 (1993) 1604–1607.

[8] J.S. Handler, H.M. Kwon, Transcriptional regulation by changes in tonicity, Kidney Int. 60 (2001) 408–411.

[9] S.N. Ho, The role of NFAT5/TonEBP in establishing an optimal intracellular environment, Arch. Biochem. Biophys. 413 (2003) 151–157.

[10] S.C. Dahl, J.S. Handler, H.M. Kwon, Hypertonicity-induced phosphorylation and nuclear localization of the transcription factor TonEBP, Am. J. Physiol., Cell Physiol. 280 (2001) C248–C253.

[11] H. Miyakawa, S.K. Woo, S.C. Dahl, J.S. Handler, H.M. Kwon, Tonicity-responsive enhancer binding protein, a Rel-like protein that stimulates transcription in response to hypertonicity., Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 2538–2542.

[12] S. Woo, S.C. Dahl, J.S. Handler, H.M. Kwon, Bidirectional regulation of tonicity-responsive enhancer binding protein in response to changes in tonicity, Am. J. Physiol. 278 (2000) F1006–F1012.

[13] C. Lopez-Rodriguez, C.L. Antos, J.M. Shelton, J.A. Richardson, F. Lin, T.I. Novobrantseva, R.T. Bronson, P. Igarashi, A. Rao, E.N. Olson, Loss of NFAT5 results in renal atrophy and lack of tonicity-responsive gene expression., Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 2392–2397.

[14] A.K. Lam, B.C. Ko, S. Tam, R. Morris, J.Y. Yang, S.K. Chung, S.S. Chung, Osmotic response element binding protein (OREBP) is an essential regulator of urine concentrating mechanism, J. Biol. Chem. 279 (2004) 48048–48054.

[15] S.A. Kempson, V. Parikh, L. Xi, S. Chu, M. Montrose, Subcellular distribution of the renal betaine transporter during hypertonic stress, Am. J. Physiol., Cell Physiol. 285 (2003) C1091–C1100.

[16] J.L. Bricker, S. Chu, S.A. Kempson, Disruption of F-actin stimulates hypertonic activation of the BGT1 transporter in MDCK cells, Am. J. Physiol. Renal. Physiol. 284 (2003) F930–F937.

[17] B.C. Santos, A. Chevaile, M.J. Hebert, J. Zagajeski, S.R. Gullans, A combination of NaCl and urea enhances survival of IMCD cells to hyperosmolality, Am. J. Physiol. 274 (1998) F1167–F1173.

[18] P. Mehlen, K. Schulze-Osthoff, A.P. Arrigo, Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death, J. Biol. Chem. 271 (1996) 16510–16514.

[19] R.S. Hoover, E. Poch, A. Monroy, N. Vazquez, T. Nishio, G. Gamba, S.C. Hebert, N-Glycosylation at two sites critically alters thiazide binding and activity of the rat thiazide-sensitive Na(+):Cl(−) cotransporter, J. Am. Soc. Nephrol. 14 (2003) 271–282.

[20] P. Meade, R.S. Hoover, C. Plata, N. Vazquez, N.A. Bobadilla, G. Gamba, S.C. Hebert, cAMP-dependent activation of the renal-specific Na+-K+-2Cl-cotransporter is mediated by regulation of cotransporter trafficking, Am. J. Physiol., Renal. Physiol. 284 (2003) F1145–F1154.

[21] J.S. Marchant, V.S. Subramanian, I. Parker, H.M. Said, Intracellular trafficking and membrane targeting mechanisms of the human reduced

folate carrier in mammalian epithelial cells, J. Biol. Chem. 277 (2002) 33325–33333.
[22] V.S. Subramanian, J.S. Marchant, I. Parker, H.M. Said, Cell biology of the human thiamine transporter-1 (hTHTR1). Intracellular trafficking and membrane targeting mechanisms, J. Biol. Chem. 278 (2003) 3976–3984.
[23] J.S. Gens, S. Chu, M. Montrose, Na/H exchanger isoforms NHE2 and NHE3 both translocate to the plasma membrane in response to a cellular acid load., Exp. Biol. (2003) (San Diego, CA, Abstract #797.6).
[24] J.C. Basham, A. Chabrerie, S.A. Kempson, Hypertonic activation of the renal betaine/GABA transporter is microtubule dependent, Kidney Int. 59 (2001) 2182–2191.
[25] K. Godfrey, Statistics in practice. Comparing the means of several groups, N. Engl. J. Med. 313 (1985) 1450–1456.
[26] S.A. Kempson, Differential activation of system A and betaine/GABA transport in MDCK cell membranes by hypertonic stress, Biochim. Biophys. Acta 1372 (1998) 117–123.
[27] A. Yamauchi, H.M. Kwon, S. Uchida, A.S. Preston, J. Handler, Myo-inositol and betaine transporters regulated by tonicity are basolateral in MDCK cells, Am. J. Physiol. 261 (1991) F197–F202.
[28] J.A. Beck, P.E. Lammers, M.H. Montrose, S.A. Kempson, Membrane insertion of EGFP-tagged BGT1 protein during hypertonic stress is TonEBP-dependent, J. Am. Soc. Nephrol. 15 (2004) 89.
[29] S.K. Woo, S.D. Lee, H.M. Kwon, TonEBP transcriptional activator in the cellular response to increased osmolality, Pflugers Arch. 444 (2002) 579–585.
[30] S.K. Woo, D. Maouyo, J.S. Handler, H.M. Kwon, Nuclear redistribution of tonicity-responsive enhancer binding protein requires proteasome activity, Am. J. Physiol., Cell Physiol. 278 (2000) C323–C330.
[31] R.R. Alfieri, P.-G. Petronini, M.A. Bonelli, A.E. Caccamo, A. Cavazzoni, A.F. Borghetti, K.P. Wheeler, Osmotic regulation of ATA2 mRNA expression and amino acid transport System A activity, Biochem. Biophys. Res. Commun. 283 (2001) 174–178.
[32] J.A. Nicoletta, J.J. Ross, G. Li, Q. Cheng, J. Schwartz, E.A. Alexander, J.H. Schwartz, Munc-18-2 regulates exocytosis of H+-ATPase in rat inner medullary collecting duct cells, Am. J. Physiol., Cell Physiol. 287 (2004) C1366–C1374.
[33] S.A. Kempson, M.H. Montrose, Osmotic regulation of renal betaine transport: transcription and beyond, Pflugers Arch. 449 (2004) 227–234.
[34] K.H. Han, S.K. Woo, W.Y. Kim, S.H. Park, J.H. Cha, J. Kim, H.M. Kwon, Maturation of TonEBP expression in developing rat kidney, Am. J. Physiol., Renal. Physiol. 287 (2004) F878–F885.