Stress-induced Expression of the γ Subunit (FXYD2) Modulates Na,K-ATPase Activity and Cell Growth*

Randall K. Wetzel, Jennifer L. Pascoa, and Elena Arystarkhova‡

From the Laboratory of Membrane Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

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In kidney, the Na,K-ATPase is associated with a single span protein, the γ subunit (FXYD2). Two splice variants are differentially expressed along the nephron and have a differential influence on Na,K-ATPase when stably expressed in mammalian cells in culture. Here we used a combination of gene induction and gene silencing techniques to test the functional impact of γ by means other than transfection. NRK-52E cells (of proximal tubule origin) do not express γ as a protein under regular tissue culture conditions. However, when they were exposed to hyperosmotic medium, induction of only the γα splice variant was observed, which was accompanied by a reduction in the rate of cell division. Kinetic analysis of stable enzyme properties from control (α1β1) and hypertonicity-treated cultures (α1β1γa) revealed a significant induction (up to 60%) of Na,K-ATPase activity measured under Vmax conditions with little or no change in the amounts of α1β1. This effect as well as the reduction in cell growth rate was practically abolished when γ expression was knocked down using specific small interfering RNA duplexes. Surprisingly, a similar induction of endogenous γα because of hypertonicity was seen in rat cell lines of other than renal origin: C6 (glioma), PC12 (pheochromocytoma), and L6 (myoblasts). Furthermore, exposure of NRK-52E cells to other stress inducers such as heat shock, exogenous oxidation, and chemical stress also resulted in a selective induction of γα. Taken together, the data imply that induction of γα may have adaptive value by being a part of a general cellular response to genotoxic stress.

Na,K-ATPase is a ubiquitous enzyme of mammalian cells that maintains the non-equilibrium distribution of Na+ and K+ ions against their electrochemical gradients across the plasma membrane. The ability of cells to control Na,K-ATPase activity (in concert with other membrane components) is critical for responding to fluctuations in such factors as plasma ion concentrations, diet, water load, glomerular filtration rate, hormones that affect ion homeostasis (insulin, aldosterone, vasopressin, parathyroid hormone, and atrial natriuretic peptide), and even diurnal cycles in urine production.

The Na,K-ATPase has two obligatory subunits, a catalytic α subunit and a glycoprotein β (for review, see Ref. 1). Regulation of the pump occurs at several levels: gene expression (long-term regulation), recruitment/internalization of the active pumps to/from the plasma membrane, and modulation of ATPase activity (short term regulation) (for review, see Ref. 2). In the last few years evidence for a completely new mechanism of regulation of Na,K-ATPase has emerged; the intrinsic properties of Na,K-ATPase can be modulated by association with a small single span membrane protein belonging to the “FXYD” family (3). Expression of the FXYD proteins is abundant in tissues that are involved in fluid and solute transport or that are electrically excitable (4, 5).

To date, five members of the family have been found to associate with the Na,K-ATPase in a cell- and tissue-specific manner: phospholemman (PLM) (FXYD1) (6, 7), the γ subunit, which occurs in at least two splice variants, γα and γβ (FXYD2) (8, 9), corticosteroid hormone-induced factor (CHIF) (FXYD4) (10), phospholemman-like protein from shark (PLMS) (11), and the FXYD7 protein (12). Three of them have now been identified as constitutively expressed in kidney. γ colocalizes with the Na,K-ATPase in proximal tubules, distal convoluted tubules, medullary thick ascending limb, and deep inner medulla (13–15). CHIF colocalizes with the pump in the collecting duct (10). Phospholemman is expressed in extraglomerular mesangial cells of the juxtaglomerular apparatus and in the afferent arteriole (16). Segment-specific distribution of these proteins predicts distinct functional consequences for their association with the Na,K-ATPase. Indeed, as shown from experiments with different expression systems, association of Na,K-ATPase with CHIF resulted in a significant increase in the affinity for intracellular Na+ and a decrease in the affinity for K+ (10, 17), whereas phospholemman significantly decreased the affinity for Na+ and, although to a lesser extent, for K+ (18). As for the γ subunit, a consensus has been reached that both splice variants modify enzyme properties notably by decreasing the affinity for Na+ (18, 19), although we saw differential modulation of affinities for Na+ and K+ in stably transfected NRK-52E cells that was dependent on post-translational modification of γα and γβ (8, 20).

Interestingly, expression of splice variants of γ is also segment-specific (14, 19). Both γα and γβ are found in medullary thick ascending limb and in inner medulla (i.e. in the segments that are exposed to very different osmotic environments and fluctuations). On the contrary, γα and γβ are individually expressed in cortical proximal tubules and distal convoluted tubules, respectively, which do not experience significant osmolarity shifts. Whether the expression profile reflects any specific needs for modulation of the ATPase activity remains to be determined.

Although γ is abundantly expressed in kidney, expression of γ as a protein is absent in any renal derived established cell line.
line (MDCK, mIMCD-3, LLC-PK1, HEK 293, and NRK-52E) under regular culture conditions (20–22), thus facilitating the use of transfection as a primary tool to assess the functional role of γ and its splice forms (20, 23). Nevertheless, as reported by Capasso et al. (22), both γα and γb can be induced in mIMCD-3 cells (a cell line derived from inner medullary collecting duct) with hypertonicity. Because both splice forms are found in inner medulla, where hypertonic conditions are the norm, this would suggest a potential tubule-specific control of γ gene expression.

However, protein instability and DNA damage because of hypertonicity result in activation of a cellular stress response program, which includes cell growth arrest, initiation of DNA repair events, induction and/or activation of molecular chaperones, activation of the proteasome degradation pathways, and initiation of apoptosis. To assess whether induction of γ with hypertonicity is a part of a cellular response to stress, we used the NRK-52E cell line, originally derived from proximal tubule cells. NRK-52E cells were shown to have characteristics of energy metabolism similar to fresh isolated proximal tubule cells and primary cultures except with a lower content of brush border membranes (24). What we found is that one of the splice variants, namely γα, indeed represents a stress-responsive protein, the induction of which is apparently required for down-regulation of Na,K-ATPase activity in cellular adaptation to environmental changes, including osmotic stress, thermal stress, heavy metal stress, and oxidative stress. In addition the evidence is presented that changes in Na,K-ATPase activity in response to hypertonicity caused by association with the γ subunit are important for reduction in cell growth. Preliminary reports of this work have been presented previously (25, 26).

EXPERIMENTAL PROCEDURES

Cell Culture and Membrane Preparations—Normal rat kidney epithelial cells (NRK-52E) were grown to 85–90% confluence in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (300 mosM). The medium was then replaced with either control or hyperosmotic medium supplemented with NaCl, urea, or sucrose. Other stress treatments were performed as specified in the figure legends. Flasks were harvested at various times, washed with Dulbecco’s phosphate-buffered saline with Ca2+ and Mg2+, and frozen at −80 °C.

Crude membranes from the scraped cells were obtained by homogenization and differential centrifugation (20). Isolation of membranes from rat kidney outer medulla was performed as described elsewhere (27). Final purification of Na,K-ATPase was with SDS extraction, which leaves the Na,K-ATPase (including the γ subunit) in the lipid bilayer but removes a majority of contaminating proteins. Typical specific activity of Na,K-ATPase was about 50–150 μmol P/mg of protein/h in preparations from NRK-52E cells.

C6 glioma, L6 myoblasts, and PC12 pheochromocytoma cells (all purchased from the ATCC) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum, whereas RCT-G1 (described above) or its monoclonal analog 11H11 was used for γ subunit detection, whereas RCT-G1 (described above) or its monoclonal analog 11H11 was used to probe the γ subunit. The secondary antibodies were either Cy3-conjugated goat anti-mouse IgG (1:300; Accurate, Westbury, NY) or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:100; Jackson ImmunoResearch, West Grove, PA). Slides were examined with a Nikon TE300 fluorescence microscope equipped with a Bio-Rad MRC 1024 scanning laser confocal system (version 3.2).

RESULTS

Induction of γ by Hypertonicity—Fig. 1A shows that expression of γ indeed can be turned on in NRK-52E cells (which do not express the γ subunit under normal conditions) after 24 h of incubation in hyperosmotic medium supplemented with either sucrose or NaCl. Crude membrane preparations were tested on Western blots with antibodies specific for α1 and γ subunits. Although only small changes were detected in the amount of α1 (typical variations were within 14 ± 7% as compared with control), the level of γ expression was clearly dependent on osmolality and the type of osmolyte in the culture medium. Induction was much more pronounced in a medium supplemented with sucrose than with NaCl, and the level of induction was higher with 500 mosM than with 400 mosM medium in both cases. A higher level of osmolality (600–700 mosM) either with high sucrose or high NaCl was deleterious for cell survival, and no induction of γ was detected (not shown). Similarly, no γ was detected when osmolality of the medium was reduced (150 mosM) (Fig. 1B).

Notably, the induced γ subunit appeared as a doublet on SDS-Tricine gels (Fig. 1B, top and middle) similar to the doublet usually seen with the kidney preparations. However, no γb stain was detected with the γb-specific antibodies (Fig. 1B, bottom). Therefore, the doublet comprised only γa, which apparently undergoes some posttranslational modification in NRK-52E cells. The data are reminiscent of what we have seen.
before with the \( H^+ \) stable transfectants, where post-translational modification of \( H^+ \) was observed in most of the clones (20).

Induction of \( H^+ \) in NRK-52E cells in response to osmotic stress was time-dependent. The level of \( H^+ \) expression relative to \( \nu \) was the highest after 48 h in both sucrose- and NaCl-supplemented media, and in both cases it declined somewhat after 5 days in hyperosmotic medium (Fig. 1C).

When hyperosmolality in the culture medium was raised up to 500 mosM with urea, no induction of either \( H^+ \) or \( H^+ \) was detected (Fig. 1D), which is in agreement with the data on mIMCD-3 cells (22). Because urea is a membrane permeant whereas \( Na^+ \) and sucrose represent impermeant osmolytes, the data suggest the involvement of specific hypertonicity-dependent signaling pathways in up-regulation of \( H^+ \) expression in cultured NRK-52E cells.

**Newly Synthesized \( \gamma \) Colocalizes with the Na,K-ATPase**—To determine the subcellular location of the newly synthesized \( \gamma \) subunit, immunofluorescence analysis of NRK-52E cells grown under isotonic or hypertonic conditions was performed. Bright \( \alpha \) stain (Fig. 2, left) was always localized at the plasma membrane, regardless of the osmolality in the medium. NRK-52E cells grown in isosmotic conditions had no detectable \( \gamma \) stain (Fig. 2, center). However, after 24 h of exposure to 500 mosM medium supplemented with sucrose (or with NaCl (not shown)), a uniform honeycomb staining (similar to the \( \alpha \) stain) was detected with the \( \gamma \)-specific antibodies (Fig. 2, right) suggesting plasma membrane localization of the endogenously induced \( \gamma \). Lack of staining with secondary anti-mouse Cy3-conjugated antibodies alone of either hypertonicity-treated or untreated cells confirmed the specificity of immunofluorescence (not shown).

**Induction of \( \gamma \) in Response to Hypertonicity Is Not Tissue-specific**—Kidney is the tissue that is exposed to large varia-

![Fig. 1. Expression of \( \gamma \) splice variants is selectively controlled by hypertonicity in NRK-52E cells. A, 24-h treatment with control (300 mosM) or hypertonic medium (400 and 500 mosM) supplemented with either NaCl (400 Na\(^+\) and 500 Na\(^+\)) or with sucrose (400 Su and 500 Su). The blots were stained with K1 (\( \alpha \)) or RCT-G1 (\( \gamma \)) antisera. B, 24- or 48-h treatment with control (300 mosM), hypertonic (150 mosM), or hypertonic (500 mosM) medium supplemented with either NaCl (500 Na\(^+\)) or sucrose (500 Su24 and 500 Su48). Blots were stained with RCT-G1 (\( \gamma \)) and RNGB (\( \nu \)) antisera. RK, rat kidney microsomes. C, densitometric analysis of \( \gamma \) expression in cells exposed to hypertonicity (NaCl or sucrose) for 6, 12, 24, 48, and 120 h. 50 \( \mu \)g of crude membrane fractions was run on SDS-Tricine gel and transferred to nitrocellulose, and the blots were stained for the \( \alpha \) (K1) and \( \gamma \) (RCT-G1) subunits and scanned. The relative ratio between \( \gamma \) and \( \alpha \) in arbitrary units was taken as a percent of maximum as detected in 1 \( \mu \)g of rat kidney microsomes. The graph represents a summary of three independent experiments and demonstrates relative changes in expression of \( \gamma \) with time. D, cells were exposed for 48 h to either control (300 mosM) or hyperosmotic medium (500 mosM) supplemented with either NaCl (+Na\(^+\)) or urea.
tions in hyperosmolality, but fluctuations occur mostly in the medullary region with the highest impact in the inner medullary zone, whereas the cortex environment is mostly isotonic. The fact that we observed induction of γ by hypertonicity in renal cells of proximal tubule origin apparently indicates a general cell response via a signaling process linked to the primary osmotic insult. In agreement, we observed similar induction of γ by hypertonicity (with NaCl) in different rat cell lines of other than renal origin: rat C6 glioma (Fig. 3A), rat pheochromocytoma PC12 (non-differentiated) (Fig. 3B), and rat L6 myoblasts (not shown). The data are especially interesting because expression of γ as a protein was shown to be almost limited to kidney (30), although reverse transcriptase-PCR analysis revealed some other mouse tissues to express γ (31).

**Induction of γ Is Not Limited to Hypertonicity**—It is well established that hypertonicity elicits a genotoxic stress that is accompanied by cell growth arrest (delay) and concurrent DNA repair events (32, 33). To test whether expression of γ is a part of a general adaptive response, NRK-52E cells were subjected to other stresses known to have effects similar to hypertonicity, namely heat shock, oxidative stress, and chemical stress. As shown in Fig. 4A, incubation of NRK-52E either at 43 °C for 1 h or 24 h of treatment with hydrogen peroxide (1 mM) or heavy metals (30 μM HgCl$_2$) resulted in an induction of the γ subunit, although the level of γ expression was lower than in hypertonicity-treated cells. Similar to what was observed under hypertonic conditions, no γ was detected with specific antibodies (not shown). Accumulation of γ was apparently dose-dependent. More γ protein, for instance, was detected when cells were treated with 50 μM CdCl$_2$ versus 1 μM CdCl$_2$ (Fig. 4B). Conversely, practically no difference in the level of induced γ was detected in crude membranes from NRK-52E that had been heated at 43 °C for 30 min as opposed to those cells exposed to 45 min of heat shock (Fig. 4C).

**Functional Consequences of γ Induction**—To assess whether the newly synthesized γ was associated with the sodium pump and to determine the functional impact of γ expression, partial purification of the Na,K-ATPase from control or hypertonic cultures was performed using mild SDS treatment. The procedure results in extraction of most of the membrane-bound proteins while leaving the active sodium pump within the lipid bilayer (20, 27). After the SDS treatment, the membrane fragments containing Na,K-ATPase were isolated on sucrose density gradients and were tested on SDS-Tricine gels. The pilot experiments showed parallel co-distributions of α1 and γ along the gradient. The peak-containing fractions containing either α1β1 or α1β1γ complexes were collected and used for the subsequent kinetic assays to monitor the effect of γ on the stable properties of Na,K-ATPase, isolated from potential direct effects of hypertonicity.

Dose-dependent accumulation of γ was obtained with NaCl as an osmolyte in culture medium (Fig. 5, A and B). As before, no stain was seen with the yb-specific antibodies, thus suggesting that the γ doublet apparently comprised the different structural variants of γ. No significant difference was noticed in the levels of the α1 subunit in the purified preparations. Further, we did not observe any significant difference in the phosphorylation level of α1 at the protein kinase C site (Ser-18) (34, 35). Staining with McK1 antibody, which recognizes only non-phosphorylated α1, was practically identical in preparations from control and hypertonicity-treated cultures (500 mosm, NaCl) (Fig. 5C, bottom panel). Similarly, no hypertonicity-induced changes were seen in the level of the β1 subunit, the only known isoform of the β subunit expressed in NRK-52E cells (2).

Different expression systems have been employed before to characterize the functional impact of γ on Na,K-ATPase activity. Based on the previous data from transfection studies (18–20), the major effect of γ on Na,K-ATPase was a reduction of the apparent affinity of the enzyme for Na$^+$. Thus, sodium dependence was tested here in SDS-purified preparations from control and high Na$^+$-treated cultures (Fig. 5, B and D). Spe-

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**Fig. 3.** Induction of γ by hypertonicity is not limited to renal cells. C6 (rat glioma) (A) or PC12 (rat pheochromocytoma) (B) cells were exposed for 48 h to either control (300 mosm) or hypertonic medium (500 mosm) supplemented with NaCl. Crude membranes were tested in Western blots with the antibodies against α1 (K1) and γ (RCT-G1).

**Fig. 4.** Induction of γ in response to genotoxic stress. NRK-52E cells were exposed to different stressful conditions. Crude membranes were tested on Western blots with the antibodies against the α (K1) and γ subunit (RCT-G1). A, heat shock treatment was for 1 h at 43 °C followed by a 24-h recovery period at 37 °C. Hypertonic stress (500 mosm with NaCl), oxidative stress (1 mM hydrogen peroxide), or heavy metal treatment (30 μM HgCl$_2$) was for 24 h. RK, rat kidney microsomes. B, NRK-52E cells were treated for 24 h with different concentrations of CdCl$_2$ (1–100 μM). C, heat shock was performed for different periods of time (0, 30, and 45 min) followed by a recovery period of 24 h at 37 °C.

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2 E. Arystarkhova and K. J. Sweadner, unpublished observation.

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- **A**
  - RK
  - NRK-52E
  - 500 mosm NaCl
  - 43°C
  - H$_2$O$_2$
  - HgCl$_2$

- **B**
  - RK
  - NRK-52E
  - C$_{\text{Cdt}}$H$_2$
  - 1 μM
  - 0
  - 5
  - 10
  - 50
  - 100

- **C**
  - γ
  - 45°, min
  - 0
  - 30
  - 45

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**Notes:**
- **A**: Analysis of γ induction in response to different stressful conditions.
- **B**: Effect of CdCl$_2$ concentration on γ induction.
- **C**: Time course of γ induction following heat shock.

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**Figures:**
- **Fig. 3**: Western blots showing γ induction in C6 and PC12 cells under hypertonic conditions.
- **Fig. 4**: Impact of different stresses on γ expression.

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**Diagrams:**
- **Fig. 4A**: Heat shock protocol.
- **Fig. 4B**: Hypertonicity protocol.
- **Fig. 4C**: Sodium dependence analysis.

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**References:**
- 32, 33 (cell growth arrest and DNA repair).
- 500 mosm NaCl for stress.
- 1 μM CdCl$_2$ for heavy metal treatment.
- 45°C for heat shock.
- Analysis of γ expression using RCT-G1 and McK1 antibodies.

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**Conclusion:**
- γ expression is induced by hypertonicity and other stresses.
- Functional consequences include reduced Na,K-ATPase activity.
- Sodium dependence is altered upon γ induction.

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**Additional Information:**
- Use of SDS-Tricine gel for protein analysis.
- Reverse transcriptase-PCR for γ expression in various tissues.
- Kinetic studies to understand γ's role in Na,K-ATPase regulation.
Fig. 5. Na,K-ATPase from high Na\textsuperscript{+}-treated NRK-52E cells is associated with γ and exhibits significantly lower enzymatic activity. NRK-52E cells were exposed for 24 h either to control medium (300 mosM) or hypertonic medium supplemented with NaCl (400 and 500 mosM). Analysis of γ induction (purified preparations of Na,K-ATPase) was on Western blots (A and C) with the antibodies to the C terminus of γ (RCT-G1) and the N terminus of β (RNGB). Polyclonal antiserum K3 raised against rat kidney enzyme was used to detect the α1 and β1 subunits (29). The protein kinase C phosphorylation level of α1 (C, α1\textsuperscript{ATPase}) at the Ser-18 was monitored with Mc1 antibody (34). Na,K-ATPase activity was determined as a function of Na\textsuperscript{+} (B and D), and the curves were normalized to the amount of α as detected on blots. Data are the summary of three or four independent experiments. RK, rat kidney microsomes.

Contrary to what we expected, only small (although statistically significant, p < 0.001) changes were observed in apparent affinity for Na\textsuperscript{+} between control and high Na\textsuperscript{+}-treated (500 mosM) cultures, with $K_{0.5}$ Na\textsuperscript{+} = 4.4 ± 0.3 (n = 3) versus 5.7 ± 0.4 (n = 4). However, kinetic analysis revealed a decrease of the pump $V_{max}$. Increasing hypertonicity up to 400 mosM resulted in a slight reduction in $V_{max}$ (up to 15%) (n = 3) (Fig. 5B), whereas up to 60% inhibition (n = 4) was observed in preparations purified from cells grown in 500 mosM medium (Fig. 5D). The kinetic changes in the isolated Na,K-ATPase were fully reversible when cells were put back to isosmotic medium (not shown). Notably, γ expression went away under these conditions in agreement with Capasso et al. (22). Thus the data indicate the adaptive nature of γ induction in response to hypertonicity.

This is obviously different from what we saw when γ was expressed by transfection; affinity for Na\textsuperscript{+} was significantly reduced in γ stable transfectants ($K_{0.5}$ Na\textsuperscript{+} = 7.8 ± 0.3) compared with mock-transfected γ-deficient cells ($K_{0.5}$ Na\textsuperscript{+} = 5.3 ± 0.2) (8, 20). However, the smaller shift in the apparent affinity for Na\textsuperscript{+} as detected here might be attributed to the lower yield of γ overexpression and therefore the γ/α1 ratio in hypertonicity-induced cells as compared with stable transfectants (Fig. 6A). In support of this, Fig. 6B demonstrates an apparent correlation between the level of overexpression of γ in two different clones of γ stable transfectants (8) and relative changes in apparent affinity for Na\textsuperscript{+}.

Similarly, we found significant inhibition of Na,K-ATPase (30–35%, n = 3) in preparations from high sucrose-treated cultures (24 h, 500 mosM, n = 3) compared with control. Based on non-linear regression analysis, the changes were in $V_{max}$ with only a small shift in $K_{0.5}$ for Na\textsuperscript{+} (not shown).

Strikingly, similar exposure to hypertonicity (500 mosM with NaCl, 24–48 h) of NRK-52E cells grown in the presence of 0.5% fetal bovine serum led to activation of Na,K-ATPase (202 ± 16% compared with control, n = 5) and correlated with up-regulation of the catalytic α subunit (180 ± 28%, n = 5). No induction of γ was detected by Western blot. The data are in agreement with several previous reports on regulation of Na,K-ATPase activity under hypertonic conditions (36–41) and suggest functional interference of different signaling pathways involved in cellular response to hypertonicity.

γ is Directly Involved in Inhibition of Na,K-ATPase by Hypertonicity—Hypertonicity is a complex signal and affects multiple signaling pathways (42, 43). Although all the ATPase assays were performed on partially purified preparations of the Na,K-ATPase, a possibility still exists that a factor other than the γ subunit is involved in modulation of the pump activity. To dissect out the effect of the γ subunit from other unknown effects of hypertonicity that might affect Na,K-ATPase, we set up experiments with siRNAs to selectively knock down γ in the presence of hypertonicity.

NRK-52E cells were transfected with either nonspecific or γ-specific (γ) siRNA duplexes (2 nmol/60-cm\textsuperscript{2} plate) followed by exposure to either isosmotic (300 mosM) or hypertonic (500 mosM with NaCl) conditions. The typical efficiency of γ knockdown was at least 75–85%, whereas the expression level of the α1
Significant reduction in the $V_{\text{max}}$ of the pump under hypertonicity was apparently caused by association of the $\alpha_1\beta_1$ complex with the de novo synthesized $\gamma$. Induction of $\gamma$ Correlated with a Reduction in Cell Growth—

Strikingly, when $\gamma$-specific siRNA duplexes ($\gamma$) were added prior to exposure to hypertonicity, the effect on NRK-52E cell growth delay (Fig. 8B) was practically abolished. In parallel, cells treated with either LipofectAMINE alone or in combination with control siRNA duplexes (NS) in hypertonic medium (500 mosM with NaCl) displayed significant delay in the rate of cell proliferation. Thus selective silencing of $\gamma$ induction resulted in escape from cell growth arrest even under non-permissive hypertonic conditions. Because $\gamma$ is a negative regulator of the Na,K-ATPase, this interesting result suggests that...
the activity of the Na,K-ATPase (or, the resulting concentrations of Na\(^+\) or K\(^+\)) has an important role in determining the rate of proliferation upstream of cell cycle control.

**DISCUSSION**

**Na,K-ATPase and Hypertonicity**—The evidence is presented here that regulation of Na,K-ATPase in response to acute application of hypertonicity requires association with the auxiliary protein, the γ subunit. The novelty of this work is that association of the α1β1 complexes with the de novo synthesized γα results in a significant inhibition of the Na,K-ATPase activity at the V\(_{\text{max}}\) level/unit of α, measured with enriched enzyme preparation.

It should be stressed that the prior work on hypertonicity revealed stimulation (rather than inhibition) of Na,K-ATPase activity in a wide variety of cells: MDCK (canine distal tubule cells) (36), bovine renal epithelial cells (41), primary cultures of human proximal tubule cells (37), rat vascular smooth muscle cells (38), as well as primary cultures of inner medullary collecting duct (IMCD) cells (39). In all the cases there was accumulation of α1β1 or only α1 mRNA(s), and higher enzymatic activity (measured either by Rb\(^+\) uptake or in cell homogenates) was detected in hypertonicity-treated cells compared with control. Although the results presented here may sound contradictory to those reported previously (36–38, 41), there was a significant, but often overlooked, difference in experimental design. Acute treatment with hypertonicity in all the cases mentioned above was performed on a background of serum starvation, whereas here 10% fetal bovine heat-inactivated serum was present at all times. Thus a potential cross-talk between trophic factor-dependent pathways and the hypertonicity-activated program might account for differences in the responses of the cells. Our own data on up-regulation of the α subunit in NRK-52E cells exposed to hypertonic stress in the presence of 0.5% fetal bovine serum with a consequent increase in Na,K-ATPase activity strongly support this hypothesis.

Serum is vital for cell growth and proliferation. In general, addition of serum stimulates cell growth by enhancing RNA, DNA, and protein synthesis with an accompanying increase in Na,K-ATPase activity (37, 44). In parallel, serum withdrawal is known to trigger apoptosis with ensuing down-regulation of the pump activity (45). Application of hypertonicity significantly augmented apoptosis in MDCK (46) and in rat vascular smooth muscle cells (45) grown in serum-depleted medium as judged by DNA fragmentation. Whether up-regulation of Na,K-ATPase under similar conditions (36, 38) represents a protective mechanism allowing cells to sustain the dual apoptotic insult requires further investigation. Interestingly, similar hypertonicity treatment (extra 200 mosm with mannitol) of rat vascular smooth muscle cells in serum-containing medium caused only slight progression in apoptosis (47).

Serum may also have a significant value in mediating the cellular response to extracellular stimuli. For instance, the effect of low K\(^+\) on the Na,K-ATPase had been repeatedly demonstrated in various cell models. Up-regulation of the Na,K-ATPase was observed in MDCK cells (48), ARL-15 (49), and LLC-PK1 (50) cells grown in serum-supplied medium. However, as demonstrated recently by Yin et al. (51), withdrawal of serum (for a period longer than 16 h) from MDCK cultures abolished the effect of low K\(^+\), and up-regulation of Na,K-ATPase was partially mimicked by addition of transferrin to the cell culture medium. In contrast, serum factors were not required for induction of Na,K-ATPase by low K\(^+\) in ARL-15 cells (52).

The hypertonicity-induced increase in Na,K-ATPase activity seen in previous studies was argued to be required for restoration of the intracellular ionic milieu as a complex cellular adaptation process following osmotic stress. However, from a different point of view, hypertonicity is a genotoxic stress resulting in DNA damage and apoptosis (32, 53, 54). It also affects mitochondrial metabolism (55), with consequent inhibition of substrate oxidation, reduction in respiration, and decrease in the cellular ATP/ADP ratio. In this case, activation of Na,K-ATPase (the major energy consumer in a cell) would exacerbate an energy crisis and would be threatening for cell survival, whereas inhibition of the pump activity would be beneficial for overcoming the apoptotic insult (56). Interestingly, inhibition of Na,K-ATPase by ouabain is known to delay the development of apoptosis (57, 58). Our data showing that other types of genotoxic stress such as exogenous oxidation, heat shock, and treatment with heavy metals led to induction of the endogenous inhibitor of Na,K-ATPase, the γ subunit, would substantiate this hypothesis.

Induction of γα by hypertonicity even in cells of non-renal origin, such as C6 glioma, PC12 pheochromocytoma, and L6 myoblasts, would also favor the adaptive nature of γ in regulation of Na,K-ATPase under stress conditions. Because the expression of γ as a protein was not reported previously in tissues other than kidney from healthy animals, similar responses to osmotic (genotoxic) stress may underlie important physiological and pathophysiological consequences throughout the body.

In line with our findings, the γ subunit was identified recently by DNA microarray analysis in the mouse hippocampus after chemical injury induced with trimethyltin, the drug causing mitochondrial dysfunction (59). Although no information is available about which splice form of γ was expressed, we hypothesize that induction of γα and the subsequent inhibition of the Na,K-ATPase activity might favor neuronal cell survival during energy deficiency and would be adaptive to sustain apoptotic insult.

**Functional Impact of γ**—The previous experiments with transfected cells (8, 18–20) revealed that γ slows down the Na,K-ATPase activity by lowering its apparent affinity for Na\(^+\). What we found here is that modulation of Na,K-ATPase activity by γα occurs mostly at the V\(_{\text{max}}\) level. However, this does not necessarily contradict the previous observations. First of all, variable levels of γ expression in stable transfectants made it hard to be quantitative about the V\(_{\text{max}}\) (8, 18–20). In addition, different clonal cell lines as well as individual oocytes might have different levels of basal activity for unrelated reasons. This would make a quantitative analysis of absolute changes in ATPase activity at the V\(_{\text{max}}\) level difficult. The experimental approach employed here allowed differences in kinetic properties of Na,K-ATPase to be detected in an individual clone starting from a constant base line. On the other hand, the smaller changes in the apparent affinity for Na\(^+\) seen in hypertonicity-induced cells might reflect a much lower yield of γα expression compared with the transfected cells (20).

Exposure of cells to hypertonicity inevitably activates multiple pathways controlling changes in gene expression of osmoprotective/osmosensitive proteins, which in turn may mediate changes in ATPase activity. In addition, expression of other FXYD proteins (3, 4) and subsequently Na,K-ATPase activity can be altered with hypertonicity as well. However, specific knockdown of hypertonicity-induced γ using RNA interference methodology allowed us to prove that down-regulation of Na,K-ATPase activity (mostly at the V\(_{\text{max}}\) level) ultimately involves the γα splice variant.

**γ and Cell Growth**—Recent findings revealed that Na,K-ATPase, besides being a major player in maintenance of ion homeostasis in the cell, may also represent a part of a signaling pathway that ultimately leads to changes in cell growth and
difference. As detected in cardiac myocytes, partial inhibition of the Na,K-ATPase by ouabain produces a hypertrophy signal that is transmitted from the plasma membrane to the nucleus via intracellular signaling cascades (60).

Several lines of experimental evidence suggest that changes in Na,K-ATPase activity caused by association with the γ subunit are important as part of a signaling pathway that leads to changes in cell growth. 1) Stable transfection of γ in NRK-52E cells, which do not express γ under normal conditions, results in a significant delay of cell growth compared with non-transfected cells (8, 20). 2) When being exclusively expressed in kidney, expression of γ is normally absent in renal cells in culture (20, 21) but can be induced with genotoxic stress (hypertonicity (22, 25), heat shock, oxidative stress, and treatment with heavy metals). As we showed here, induction of endogenous γ in NRK-52E treated with hypertonicity correlated with significant reduction in the V\textsubscript{max} of the pump and was accompanied by a reduction in the rate of cell division. 3) Strikingly, when γ-specific siRNA duplexes were added prior to exposure to hypertonicity, practically no change in the cell growth rate was observed after addition of NaCl to the culture medium. In parallel, the presence of the siRNA duplexes prevented any γ-dependent growth arrest. Taken together, the data imply that expression of γ is negatively correlated with the proliferative status of the cell and may have adaptive value being a part of a general cellular mechanism of regulation of cell growth. Interestingly, down-regulation of γ has been already demonstrated in Wilms tumor (61) (a kidney tumor in children).

The future challenge would be identifying signaling pathways involved in suppression of cell growth via the γ subunit. The working hypothesis is that changes in intracellular ion concentration associated with modulation of Na,K-ATPase via FXYD2 might affect expression of cell cycle regulators, such as cyclins, cyclin-dependent kinases or cyclin-dependent kinase inhibitors, or proteins involved in DNA synthesis and regulation. A different potential pathway is that elevation of intracellular Na\textsuperscript{+} (because of decreased Na,K-ATPase activity) would affect the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and therefore would increase free [Ca\textsuperscript{2+}], which is known to serve as a second messenger in several signal transduction pathways. Alternatively, association with γ may trigger conformational changes in Na,K-ATPase favoring its assembly with protein partners in signaling modules. A recent report on activation of the Src-epidermal growth factor receptor-extracellular signal-regulated kinase (Src-EGFR-EKR) signaling pathway in response to chronic ouabain infusion (62) and identification of γ within a signaling complex in renal caveolae membranes makes this hypothesis credible.

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Randall K. Wetzel, Jennifer L. Pascoa and Elena Arystarkhova

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