The expression of the γ subunit of Na-K-ATPase is regulated by osmolality via C-terminal Jun kinase and phosphatidylinositol 3-kinase-dependent mechanisms

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The α and β subunits of Na-K-ATPase are up-regulated by hypertonicity in inner-medullary collecting duct cells adapted to survive in hypertonic conditions. We examined the regulation of the γ subunit by hypertonicity. Although cultured inner-medullary collecting duct cells lacked the γ subunits, both variants γ_a and γ_b were expressed in cells adapted to 600 and 900 mosmol/KgH_2O. This expression was reversible with a half-time of 17.2 ± 0.5 h. The message of the γ subunit was absent in isotonic conditions and increased with higher tonicity in adapted cells. In acute experiments the appearance of the γ subunit was found to be both time-dependent (≥24 h) and osmolality-dependent (≥500 mosmol/KgH_2O). No induction was noted with urea and only minimal induction with mannitol. Increasing concentrations of the phosphatidylinositol 3-kinase inhibitor LY294002 resulted in a dose-dependent decrement in the expression of the γ subunit with total abolition at 10 μM. This was associated with a decrease in cell viability as <20% survived the treatment with 10 μM of LY294002. Neither inhibition of extracellular response kinase nor p38 mitogen-activated protein kinase inhibited osmotic induction of the γ subunit. In contrast, cells transfected with a dominant negative c-Jun N-terminal kinase 2-APF construct displayed complete inhibition of the γ subunit. Such cells have accelerated loss of viability in hypertonic conditions. This study describes the regulation of the γ subunit of Na-K-ATPase by hypertonicity. This regulation is transcriptionally regulated and involves signaling mediated by phosphatidylinositol 3-kinase and c-Jun N-terminal kinase 2 pathways.

The survival of the renal cells in the hypertonic inner medulla of the mammalian nephron is critical to the function of the concentrating mechanism. This survival is made possible by the early activation of ion-transport systems and subsequently by the accumulation of organic osmolytes that are metabolically inert (1, 2). The exposure of renal cells to a high osmolality environment elicits the activation of a number of signaling pathways, primarily in the mitogen-activated protein (MAP) kinase family (3–5) and the up-regulation of an increasing number of proteins. It is very likely that the stress imposed on renal cells by hypertonicity brings about a coordinated response in which a number of cellular process are activated, many of which are critical to cell viability. Among the proteins that seem important to this process are the heat shock proteins (6), cycloxygenase 2 (7), and, as we recently reported, Na-K-ATPase (8). This study examined the up-regulation of the α and β subunits of the protein. Renal Na-K-ATPase has an additional γ subunit. Partial sequence information obtained from the γ subunit purified from sheep kidney (9) led to its cloning from other species including human (10, 11). Specificity for a renal distribution was provided by Therien *et al.* (12), who found this protein in the medulla, but not the glomeruli of the rat, pig, and dog, or in any other tissues. Furthermore, it was entirely absent in a variety of cultured renal cells. The function of the γ subunit is quite complex. There is evidence that it raises the affinity for ATP (13) by stabilizing the E_1 conformation of the Na-K-ATPase (12, 13), and also that it reduces the enzyme's affinity for Na^+ at cytoplasmic sites (14) by increasing the affinity of cytoplasmic K^+ as a competitor for cytoplasmic Na^+ (15). The γ subunit also affects the affinity for extracellualr K^+ ions in a voltage-sensitive fashion and can induce nonselective cation channel activity (16). The γ subunit may be a tissue-specific regulator, and some of the functions described above may serve cells well in their defense against a hypertonic environment. In such a setting the transport of NaCl must be enhanced and energy consumption is greatly increased. In particular, an increment in affinity to ATP and lowering of cytoplasmic Na^+ affinity may permit optimal rate of active transport in such an environment.

Regulation of the expression of the γ subunit in various physiologic conditions has not been previously described, nor has the role of the γ subunit in osmoregulation been explored. We undertook the present study to determine whether hypertonicity alters the expression of the γ subunit of Na-K-ATPase in cultured renal cells as well as renal tissue and to establish the signaling pathways that may be involved in this expression.

Materials and Methods

Materials. The antisera used were purchased from Upstate Biotechnology (Lake Placid, NY) and Cell Signaling Technology (Beverly, MA). Anti-γ and anti-γ_b-specific antibodies were generously provided by Steven Karlish (The Weizmann Institute of Science, Rehovot, Israel). Signaling pathway inhibitors were obtained from Calbiochem.

Cell Culture. Cells were propagated as described (8). JNK1-APF and JNK2-APF stable transfectants (17) were propagated in the presence of 500 μg/ml of G418. Cell survival was measured by using the CellTiter cell proliferation assay (Promega).

Treatment with Inhibitors. Confluent cell cultures were preincubated for 24 h in low serum medium (0.5% FBS) after which the appropriate concentration of inhibitors was added. After 2 h the osmolality was increased to 550 mosmol/kgH_2O by the addition of sterile 5 M NaCl solution. After a further 24-h incubation, the cells were harvested as described below.

Hydration State in Mice. Mice were housed in metabolic cages with food and water ad libitum for at least 24 h before the start of the

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Abbreviations: MAP, mitogen-activated protein; IMCD, inner-medullary collecting duct; PI3-kinase, phosphatidylinositol 3-kinase.

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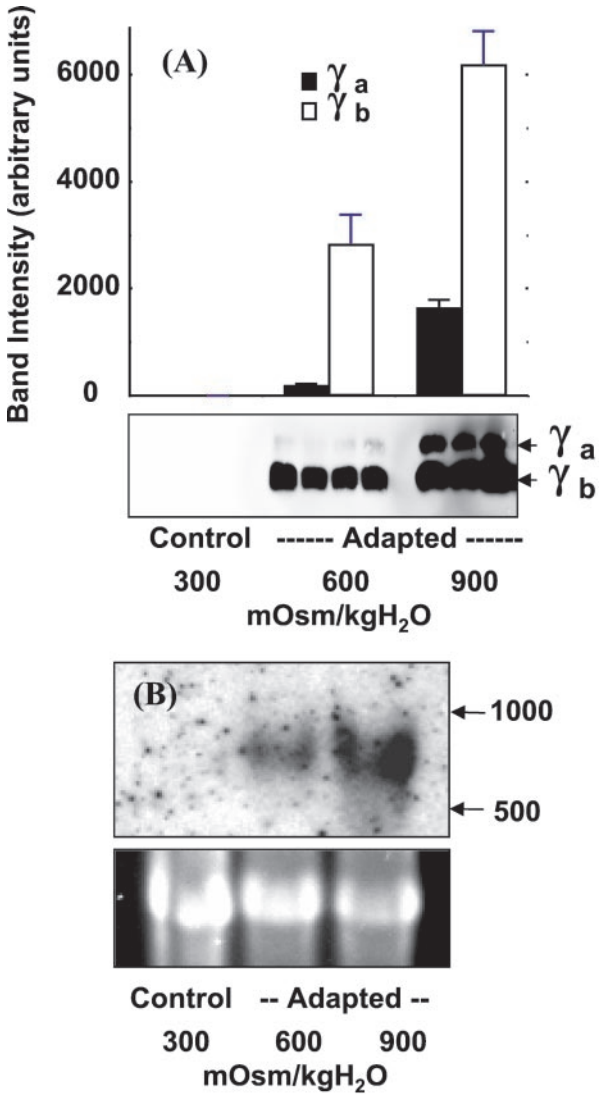

Fig. 1. (*A*) Cell lysates (100 μg of protein per line) from IMCD3 control and adapted cells were analyzed by Western blot using γ subunit ATPase antibody. The identity of γ_b was corroborated with a specific antibody. (*Upper*) The mean and SEM from four different experiments at 300 and 600 mosmol/kgH_2O and three at 900 mosmol/kgH_2O. (*Lower*) A representative Western blot. (*B*) Cytosolic RNA was isolated from IMCD3 control and adapted cells as described. Aliquots of RNA (15 μg) were run in duplicate agarose gels, transferred to a nylon membrane, and probed with ^{32}P-labeled oligonucleotide mapping to the middle of γ ATPase mRNA (*Upper*) or stained with ethidium bromide reflecting equal loading (*Lower*). Arrows indicate M_r markers.

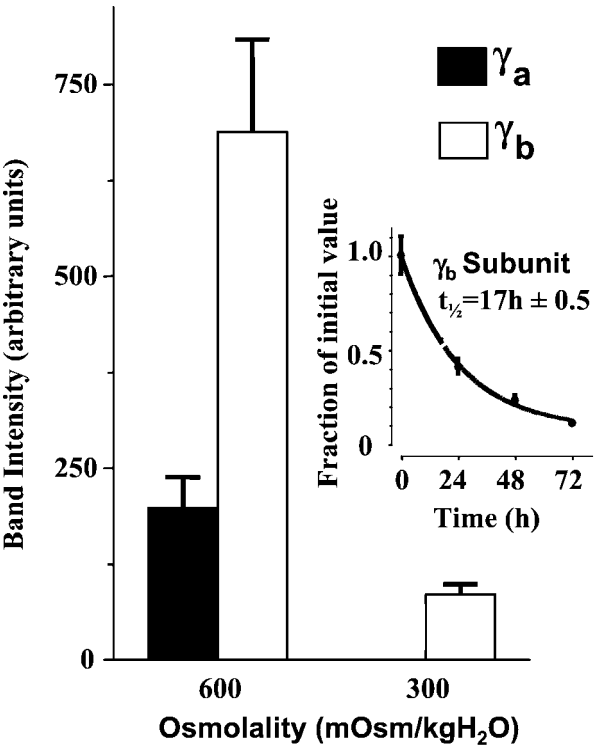

Fig. 2. IMCD3 cells adapted to 600 mosmol/kgH_2O were transferred to 300 mosmol/kgH_2O medium, harvested at different time intervals, and analyzed by Western blot. Mean and SEM of γ variant band intensity at initial time and after 3 days under isotonicity ($n = 3$). (*Inset*) The γ_b band intensity data were subjected to best-fit analysis and determined to match one-component exponential decay kinetics with $k = 0.0408$ h^{-1}.

experiment. At that time, water was replaced with a 5% solution of dextrose in the D5W group. Urine samples were collected at various times. After 1 wk, mice were killed by cervical dislocation, kidneys were removed, and the inner medulla and cortex were dissected in ice-cold saline.

Western Analysis. Cell lysates and tissue homogenates were obtained as described (8). Electrophoresis in tricine-Tris-SDS buffer, electroblot to poly(vinylidene difluoride) membrane, and immundetection were as described (8, 18).

Northern Analysis. Cytosolic RNA was isolated from inner-medullary collecting duct 3 (IMCD3) cells, separated by agarose gel electrophoresis, and transferred to nylon membrane as described (8).

The γ-specific oligonucleotide (5′-CATTGACCTGCCTATGTTTCTTACCGCC-3′) (GenBank accession no. X70060) was used as probe.

Statistics. Results were analyzed by using the INSTAT software package (GraphPad, San Diego). A value of $P < 0.05$ was considered significant.

Results

Expression of the γ Subunits in IMCD Cells Adapted to Hypertonicity. Fig. 1*A* depicts a representative Western blot and the corresponding densitometric measurements of the γ subunit of Na-K-ATPase in control cells (at 300 mosmol/kgH_2O) and in cells adapted to either 600 mosmol/kgH_2O or 900 mosmol/kgH_2O ($n = 4$). As has been reported in other renal cells studied in culture, we found no detectable γ subunit in IMCD3 cells kept at 300 mosmol/kgH_2O. In contrast, the protein was clearly present in the cells adapted to 600 mosmol/kgH_2O and even more at 900 mosmol/kgH_2O. Our data, obtained with both γ- and γ_b-specific antibodies, reveals that as has been previously described in other species (12, 19), there are also two variants (γ_a and γ_b) in mouse cells. By using renal Na-K-ATPase as a reference and assuming equimolar expression of γ and α subunits in kidney (19), we estimate that in the IMCD3 cells the abundance of the γ in relation to the α subunit is ≈15–20% at 600 mosmol/kgH_2O and ≈40–50% at 900 mosmol/kgH_2O. Both variants are increased in the adapted cells but the expression of γ_b is greater than that of γ_a.

Fig. 1*B* depicts a Northern blot for the ATPase γ subunit message in cells adapted to 600 and 900 mosmol/kgH_2O. The message for this protein is entirely absent in cells at 300 mosmol/kgH_2O but is increasingly expressed in cells adapted to

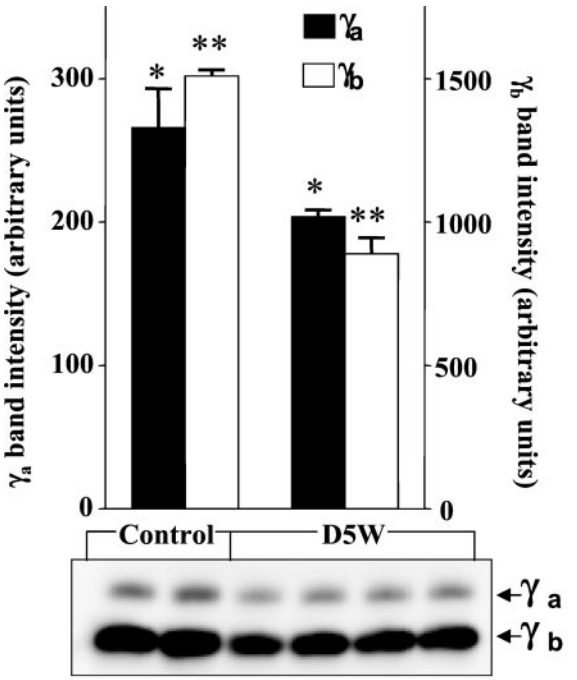

Fig. 3. Effects of water hydration in mice on γ_a (solid bars) and γ_b (open bars) variants in kidney inner medulla. Note the different scales for the two variants. Mice tissues were harvested after 1 wk of treatment and homogenates analyzed by Western blot ($n = 4$). The expression of γ_a (*, $P < 0.02$) and γ_b (**, $P < 0.002$) Na-K-ATPase subunits were significantly decreased in the inner medulla of water diuresis mice drinking D5W (5% dextrose in water). A representative Western blot of the inner medulla γ Na-K-ATPase obtained from two control and four D5W groups of mice are shown.

the hypertonic conditions. The implication is that the observed increment of the γ subunit protein results from the transcription of new message and suggests that this occurs, at least in part, after transcriptional regulation by hypertonicity. A decrease in the rate of mRNA degradation could not be ruled out. To determine whether the observed up-regulation of the protein is reversible, we returned the cells to isotonic conditions and the levels of γ ATPase were measured at 1, 2, and 3 days. This was associated with a loss of γ ATPase expression. Fig. 2 depicts the densitrometric analysis of the γ_a and γ_b subunit at 600 mosmol/kgH_2O and after 72 h in isotonic conditions $n = 3$. At the latter time, the γ_a subunit is again not detectable and only a minimal expression of γ_b is still evident. Fig. 2 *Inset* depicts the exponential down-regulation of the variants, with a calculated half-time of 17.2 ± 0.5 h.

Expression of γ Subunits in Mice Under Various States of Hydration. To determine whether the expression of the γ subunit of Na-K-ATPase is altered when the osmolality of the inner medulla is changed *in vivo*, we studied the content of this protein in mice on ad libitum water intake, compared to that of water-loaded mice drinking dextrose solution for 1 wk. This maneuver lowers urinary osmolality from a mean of 3066 ± 238 to a mean of 499 ± 72 mosmol/kgH_2O.

Fig. 3 shows a representative Western blot and the corresponding denisitometric analysis of γ subunit level in four groups of mice. The decrement in urinary osmolality was associated with a significant decrease in the expression of both variants of the γ subunit, γ_a by 25% ($P < 0.02$) and γ_b by 41% ($P < 0.002$), when mice on ad lib water intake were compared to those drinking dextrose in water for 1 wk. The changes were limited to the inner medulla as the levels in the cortex (data not shown) were unaltered by water intake.

Osmolality, Time, and Solute Dependence of γ Subunit Induction. We examined the time course of γ subunit induction in IMCD3 cells after acute exposure to hypertonic conditions and found the process to be time- and osmolality-dependent. No detectable protein was found at either 24 or 48 h in cells exposed to 400 mosmol/kgH_2O. At 500 mosmol/kgH_2O (Fig. 4*A*), a small, but detectable, expression of the subunit was found at 24 h and a further increment at 48 h. Cells adapted chronically to 500 mosmol/kgH_2O displayed a further increase in γ_b subunit of the enzyme. At 550 mosmol/kgH_2O (Fig. 4*B*), the expression at 24 h remained minimal but was clearly observed at 48 h. It is of particular note that the cells adapted to live at 550 mosmol/kgH_2O displayed a much higher level of γ_b expression. We used

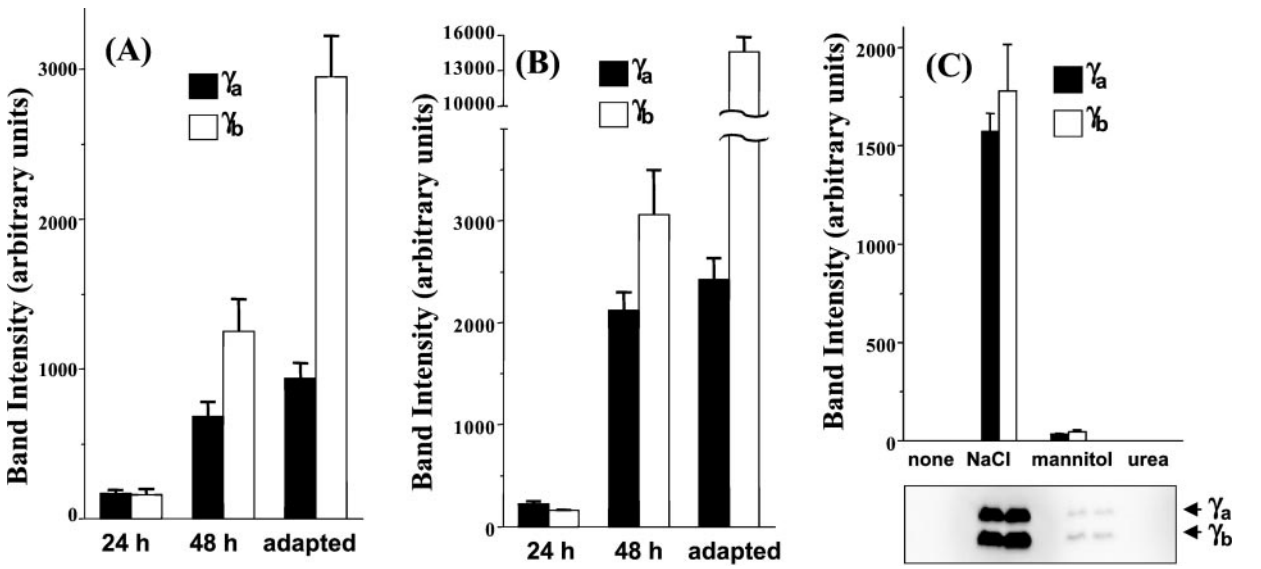

Fig. 4. IMCD3 cells were challenged with 200 mosmol/kgH_2O of NaCl (*A*) or 250 mosmol/kgH_2O of NaCl (*B*) for 24 h, 48 h, or adapted to such conditions. Cell lysates were analyzed by Western blot ($n = 4$). Notice that in *B* there is a break in the scale for γ_b in the adapted cells. (*C*) Cells were challenged with 250 mosmol/kgH_2O, mannitol, or urea for 48 h ($n = 4$) and analyzed as above. A representative blot shown of two of these experiments is shown.

these osmolalities (500 and 550 mosmol/kgH_2O) because we and others have shown low viability when cells are exposed to osmolalities ≥600 mosmol/kgH_2O for more than 24 h.

We determined whether the above-observed increment in the γ subunit is a function of effective tonicity or whether it is sodium-dependent, by examining the effects of urea (an ineffective solute) and mannitol. To do so we exposed the cells to 550 mosmol/kgH_2O media for 48 h. As the representative Western blot and the densitometric analysis of four experiments depicted in Fig. 4*C*, neither urea nor mannitol significantly up-regulate the expression of γ_a or γ_b.

Studies on the Signaling Mechanism Involve the Up-Regulation of the γ Subunit by Osmolality. The role of the phosphatidylinositol 3-kinases (PI3-kinases)and MAP kinase in the regulation of the γ subunit was examined by a combination of pharmacological maneuvers and in transfected cells.

Effects of Inhibition of PI3-Kinase. The PI3-kinase inhibitor LY294002 was used in various doses and the expression of the γ subunit as well as the α_1 subunit of Na-K-ATPase was examined. A representative Western blot and the mean of densitometric measurements at 48 h is shown in Fig. 5*A*. As expected, the α_1 subunit of Na-K-ATPase is present in the basal conditions at 300 mosmol/kgH_2O and is increased several-fold at 550 mosmol/kgH_2O. Inhibition of PI3-kinase did not significantly change the expression of the α subunit ($P > 0.25$). In contrast the PI3-kinase inhibitor markedly altered osmolality stimulated γ subunit expression in a dose- and time-dependent manner. At 24 h there was no decrement with 2 μM, a 75% decrement at 5 μM and abolition at 10 μM LY294002. At 48 h the 5 μM dose decreased γ subunit expression by 95% ($P < 0.01$), and it was entirely abolished at 10 μM LY294002. This failure to generate the γ subunit was accompanied by loss of cell viability (Fig. 5*B*). IMCD3 cells remain viable at 550 mosmol/kgH_2O. However in the presence of 10 μM LY294002 cell viability is rapidly lost. This loss of cell viability is not a nonspecific consequence of the PI3-kinase inhibition because the cells in isotonic conditions remain fully viable in the presence of the drug.

Effect of Inhibition of MAP Kinase Pathways. The role of the extracellular response kinase (ERK) pathway was examined with the ERK kinase inhibitor PD93085 (7), whereas the p38 MAP kinase pathway was inhibited with SB203580 (7). As depicted in Fig. 6*A*, neither of these drugs given in concentrations that are known to completely inhibit their respective pathways, significantly altered the osmotically stimulated increase in the levels of the α_1 subunit or of either variant of the γ subunit, shown in the representative Western blot. In contrast, in cells that express a dominant negative JNK2 construct (JNK2-APF) the expression of both γ_a and γ_b were markedly decreased, and in fact showed no expression at all at either 24 or 48 h (Fig. 6*B*). The results of one clone are shown, and the same effect was observed in two other independent clones (not shown). To ensure that this finding is not a consequence of the transfection itself, we studied cells transfected with a dominant negative JNK1 mutant (JNK1-APF). These cells displayed a modest blunting in the expression of the γ subunit variants but these were still clearly up-regulated in a time-dependent manner, under hypertonic conditions. Likewise the α_1 subunit was also increased by hypertonicity, but was unaffected by either JNK1 or JNK2 dominant negative cells. We have previously shown that JNK2- APF transfected cells have decrease viability under hypertonic conditions (17).

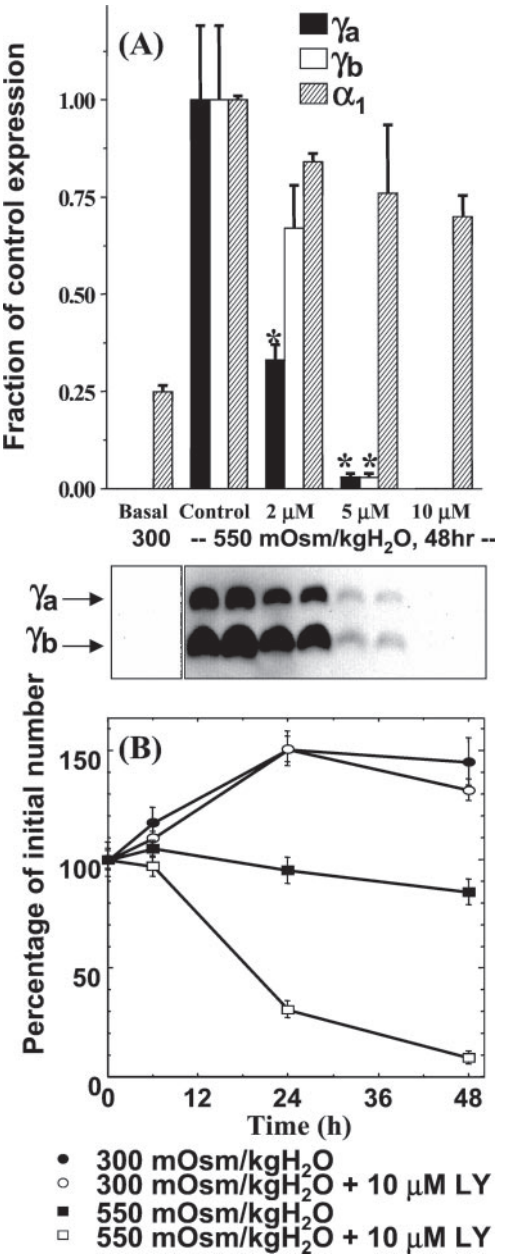

Fig. 5. (*A*) Confluent IMCD3 cell cultures were kept for 24 h in low serum medium, incubated for 2 h with different LY294002 concentrations, and challenged with 250 mosmol/kgH_2O of NaCl for 48 h. Cell lysates were prepared and analyzed by Western blot analysis ($n = 4$). A representative blot of two of these experiments is shown. *, $P < 0.01$ when compared to controls without LY294002. (*B*) IMCD3 cells were grown in 24-well plates to confluence and treated as above with or without 10 μM LY294002 and 250 mosmol/kgH_2O NaCl. Cell survival was measured by the formation of formazan as described in *Materials and Methods*. Results are the mean and SEM of six determinations.

Discussion

The survival of renal cells in the rapidly changing hypertonic environment of the inner medulla brings forth a number of cellular responses that have been well characterized over the last years. A critical component of this response is the generation of a number of inert osmolytes that allow for the maintenance of cell volume in this environment. Many of these osmolytes, including inositol and betaine, enter the cells by Na^+ or Na^+ and Cl^--coupled transporters (20, 21). Therefore the basolateral exit of sodium is critical to maintenance of the transmembrane electrochemical gradient that drives these transporters. Furthermore, the basolateral exit of Na has to occur against a higher concentration gradient because the sodium concentration in the

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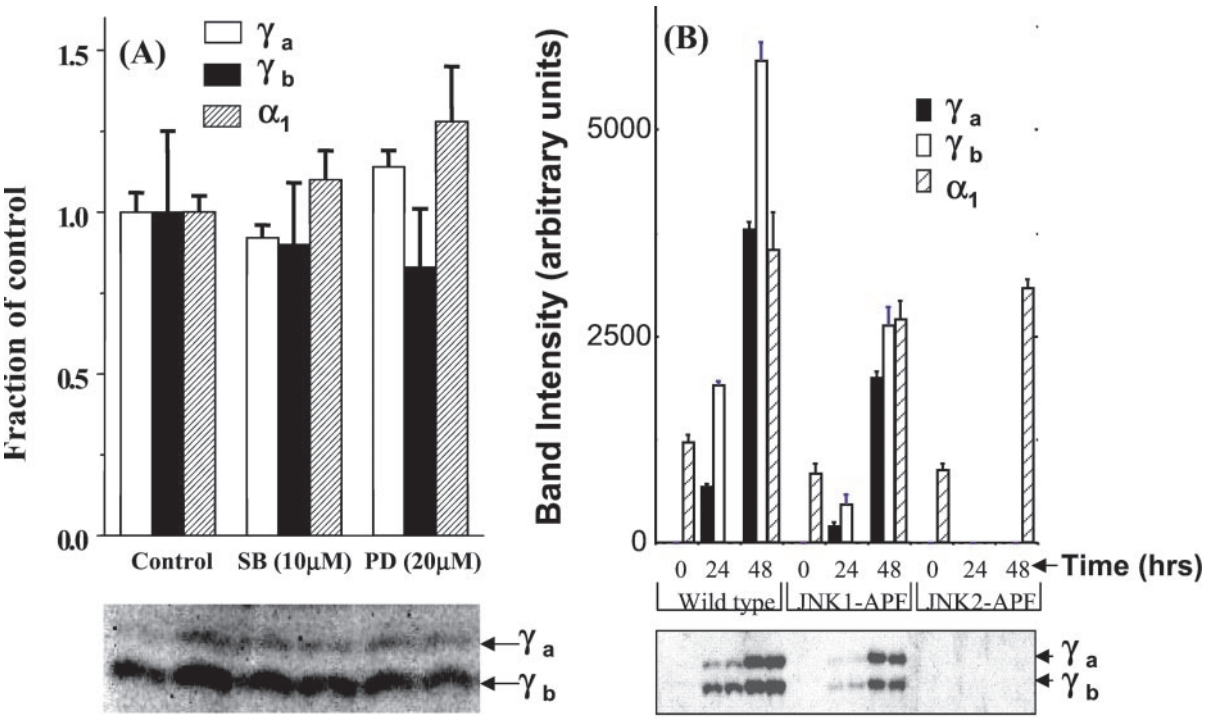

Fig. 6. (*A*) Confluent IMCD3 cell cultures were kept for 24 h in low serum medium, incubated for 2 h with either SB203580 (10 μM) or PD98059 (20 μM), and challenged with 250 mosmol/kgH_2O of NaCl for 48 h. Cell lysates were prepared and analyzed by Western blot analysis ($n = 4$). A representative blot of two experiments is shown. (*B*) Wild-type IMCD3 cells and the dominant negative transfected JNK1-APF clone no. 5 and JNK2-APF clone no. 10 were challenged with 250 mosmol/kgH_2O NaCl for 24 and 48 h before being analyzed by Western blot ($n = 4$). A representative blot of two experiments is shown.

inner medulla is much higher than 150 mM. We have reported recently (8) that inner medullary collecting duct cells, both *in vitro* and *in vivo*, up-regulate the expression, and display enhanced activity of Na-K-ATPase, which may subserve this function at least to some extent. However, the present results reveal that the adaptive response to hypertonicity also involves the γ subunit in a major way. We describe here the regulation of the γ subunit by changes in tonicity and suggest that this regulation may be of great importance to renal cells in their adaptation to hypertonicity. The cultured IMCD3 cells, which are devoid of the γ subunit in the basal state, express the protein when adapted to live at 600 and 900 mosmol/kgH_2O. After adaptation to 900 mOsmol/kgH_2O, the stoichiometric ratio of γ to α subunits of Na-K-ATPase is calculated to be close to 0.5. It is of note that, in the rat, the γ subunit has been localized in the thick ascending limb (15) whereas CHIF, another member of the FXYD family, has been found in the inner medulla.[†] We also found the induction of γ_b to be greater than that of γ_a. Because the two have similar functions (15), the significance of the asymmetry in the γ variants expression is unknown. However, we have detected the same difference in the mouse kidney (Fig. 3). The observation that the message for the protein is absent in the basal state but clearly present in adapted cells suggests that the regulation is at least in part transcriptionally mediated. Furthermore, the restoration to isotonic conditions is associated with rapid down-regulation of the protein (with a half-time of $\approx$17 h), suggesting that in this isotonic conditions no further synthesis occurs. It is of interest that regulation of the γ subunit is not merely an *in vitro* observation because modulation by alteration in water balance *in vivo* is also observable. The subunit is expressed in both the cortex and inner medulla of mice but when these rodents are water loaded, and the urinary osmolality is reduced from 3,000 to 500 mosmol/kgH_2O, a significant decrement in the γ subunit expression is observed. This is reminiscent of the change we described for the α subunit of mice treated in this way (8). However, our acute studies reveal a critical difference between the osmotic stimulation of the α and β when compared to the γ subunit. The exposure of renal cells to hypertonicity causes an up-regulation of the α and β that is maximal at 48 h. This up-regulation allows for cell viability at osmolalities of 600 mosmol/kgH_2O over this time period but ultimately does not prevent cell death in these conditions. Although we observed some up-regulation of the γ under these acute conditions, it is evident that cells that survive the hypertonic stress continue to synthesize the protein as its expression is many-fold higher in the adapted cell lines. We surmise that this ongoing synthesis may be critical to the adaptive process because it distinguishes cells that are fully viable at very high tonicities (900 mosmol/kgH_2O) from those cells acutely exposed to osmolalities in excess of 550 mosmol/kgH_2O, which fail to survive despite full expression of enhanced osmolyte uptake (8, 22), heat shock proteins (6, 8), and the α and β subunits of Na-K-ATPase. It is also of interest that we observed the increase in the γ subunit only in cells exposed to NaCl and not to mannitol or urea substances that were also incompatible with the development of stably adapted cell lines (8).

In addition to describing the osmotic regulation of the γ subunit, our experiments also explored the cellular mechanisms that may be involved in the process. Because the activation of the MAP kinases by hypertoniciy has been well described (3), we investigated their role. Neither the inhibition of p38 MAP kinases nor the inhibition of extracellular response kinases was associated with a significant decrease in the expression of the protein. We have previously shown that these inhibitors do not alter cell viability in hypertonic conditions

[†]Garty, H., Cluzeaud, F., Farman, N., Goldshleger, R. & Karlish, S. J. D. (2001) *Biophys. J.* **80,** 501a (abstr).

(17). In contrast, cells transfected with a dominant negative terminal c-Jun kinase (JNK2-APF) displayed essentially no expression of the γ subunit. This observation was not caused by the transfection itself because JNK1-APF mutants had somewhat blunted, but yet significant, up-regulation of the protein. As we have observed before (17), JNK2-APF cells display accelerated cell loss when placed in hypertonic conditions, paralleling the inability to up-regulate the γ subunit shown in the present study. We observed a similar parallelism when the γ subunit expression was suppressed by inhibition of PI3-kinase. When PI3-kinase was inhibited with 10 μM LY294002 the protein was absent and the cells rapidly lost viability. This was not a consequence of a nonspecific toxic or metabolic effect of the drug itself because it was well tolerated by cells in isotonic conditions (Fig. 5*B*). At the used concentration ($<$20 μM), LY294002 is highly selective and does not affect other protein kinases (23). It is of interest, however, that the PI3-kinase has been implicated in vesicular trafficking of membrane proteins including endocytosis of the Na-K-ATPase, a process that appears to involve the α subunit (24). A role for PI3-kinase has not been fully explored particularly as it pertains to hypertonic stress. An increase in PI3-kinase activity has been described in hepatocytes (25) as well as in the inner strips of the outer medulla of the kidney (26) in hypotonic conditions. In addition, inhibition of the kinase delays volume recovery in hepatic cells exposed to hypotonicity (27) and block hypoosmotic stimulation of bicarbonate absorption in the medullary thick ascending limbs (26). The effects of the inhibition of PI3-kinase on hypertonic stress in renal cells has not been reported previously. The mechanism whereby such inhibition decreases cell viability will require further investigation, but our data suggest that the failure to synthesize the γ subunit of the Na-K-ATPase may play a critical role.

In summary, our study demonstrates the presence of the γ subunit of the Na-K-ATPase in cultured renal cells, but this presence is limited to cells exposed to hypertonic conditions. The osmotic regulation of the γ subunit is also seen in the renal inner medulla in association with changes in hydration. An increment to at least 500 mosmol/kgH_2O is necessary for the induction of the synthesis of the protein, and this synthesis and accumulation persist well beyond 48 h of hypertonicity. The γ subunit is induced solely when NaCl is the added solute. Signaling pathways involving both the c-Jun terminal kinase and PI3-kinase are involved in the regulation of the γ subunit expression, and failure to synthesize the γ subunit of Na-K-ATPase is associated with ultimate loss of cell viability. Whether the effect of the inhibition of JNK2 kinase and PI3-kinase on cell viability is caused by its effects on the γ subunit expression remains to be established. We postulate that the increased demand for luminal sodium uptake on which the maintenance of cell volume critically depends leads to enhanced Na-K-ATPase activity at the basolateral membrane to allow the exit of sodium from the cell. This enhancement is made possible by the additional expression of the γ subunit of the protein.

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