Hypotonic induction of SGK1 and Na\(^+\) transport in A6 cells

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Rozansky, David J., Jian Wang, Ninh Doan, Timothy Purdy, Tonya Faulk, Aditi Bhargava, Kevin Dawson, and David Pearce. Hypotonic induction of SGK1 and Na\(^+\) transport in A6 cells. Am J Physiol Renal Physiol 283: F105–F113, 2002. First published February 19, 2002; 10.1152/ajprenal.00176.2001.—Serum and glucocorticoid-regulated kinase-1 (SGK1) is a serine-threonine kinase that is regulated at the transcriptional level by numerous regulatory inputs, including mineralocorticoids, glucocorticoids, follicle-stimulating hormone, and osmotic stress. In the distal nephron, SGK1 is induced by aldosterone and regulates epithelial Na\(^+\) channel-mediated transepithelial Na\(^+\) transport. In other tissues, including liver and shark rectal gland, SGK1 is regulated by hypertonic stress and is thought to modulate epithelial Na\(^+\) channel- and Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter-mediated Na\(^+\) transport. In this report, we examined the regulation of SGK1 mRNA and protein expression and Na\(^+\) currents in response to osmotic stress in A6 cells, a cultured cell line derived from Xenopus laevis distal nephron. We found that in contrast to hepatocytes and rectal gland cells, hypotonic conditions stimulated SGK1 expression and Na\(^+\) transport in A6 cells. Moreover, a correlation was found between SGK1 induction and the later phase of activation of Na\(^+\) transport in response to hypotonic treatment. When A6 cells were pretreated with an inhibitor of phosphatidylinositol 3-kinase (PI3K), Na\(^+\) transport was blunted and only inactive forms of SGK1 were expressed. Surprisingly, these results demonstrate that both hypertonic and hypotonic stimuli can induce SGK1 gene expression in a cell type-dependent fashion. Moreover, these data lend support to the view that SGK1 contributes to the defense of extracellular fluid volume and tonicity in amphibia by mediating a component of the hypotonic induction of distal nephron Na\(^+\) transport.

serum and glucocorticoid-regulated kinase; osmoregulation; sodium transport; phosphatidylinositol-3-kinase; epithelial sodium channel

THE DISTAL NEPHRON (DN) OF the kidney plays a central role in maintaining extracellular fluid (ECF) volume and tonicity in terrestrial and freshwater vertebrates. It performs this function by mediating transepithelial Na\(^+\) and water transport in response to regulatory factors such as aldosterone, insulin, atrial-natriuretic peptide, and osmotic stress (2, 17–19). The adaptation of the DN to changes in an organism’s surrounding environment is thought to have played a critical role in vertebrate evolution and the survival of euryhaline species (e.g., salmon and tilapia) during migration between saltwater and brackish water or freshwater (3, 7). Moreover, renal mechanisms coordinating conservation of Na\(^+\) and water were critical to the evolution and survival of amphibia and higher vertebrates on land. In humans, disruption of the DN pathways promoting Na\(^+\) and water reabsorption has been associated with several genetic disorders, including Liddle’s syndrome (20), apparent mineralocorticoid excess (55), pseudohypoaldosteronism (10), and nephrogenic diabetes insipidus (37).

Na\(^+\) transport also plays a critical role in maintaining the volume of individual cells. In contrast to regulatory actions on the DN from hormonal influences, Na\(^+\) transport in individual cells is altered in response to changes in intracellular volume (9). When intracellular volume is decreased in cells exposed to hypertonic conditions, cells restore appropriate volume by initiating a cascade of events that includes Na\(^+\) influx from the extracellular into the intracellular space (32). An example of this phenomenon is illustrated by hepatocytes, which respond to osmotic loads within the portal circulation by increasing Na\(^+\) influx from the extracellular space (32). The cellular mechanisms involved in these responses are complex, utilizing several Na\(^+\), K\(^+\), Cl\(^-\) channels and/or transporters in conjunction with water channels (6).

The cellular mechanisms that control the volume of individual cells are potentially in conflict with mechanisms that modulate ECF volume and tonicity. For example, Na\(^+\) influx serves as a component of the regulatory volume increase in cells exposed to hypertonic conditions (6, 51); however, Na\(^+\) entry into DN epithelial cells is an essential step in the defense of ECF volume and tonicity in response to hypotonic
conditions (29, 53). Recently, serum and glucocorticoid-regulated kinase-1 (SGK1) (50), a serine-threonine kinase, has been independently isolated by several laboratories as an early-response gene that is induced by nonhormonal and hormonal factors involved in stress responses. Hypertonic stress has been shown to increase SGK1 mRNA or protein levels in nonrenal epithelia such as human hepatocytes (47), acinar cells of the pancreas (25), mouse mammary tumor cells (4), and the shark rectal gland (48). SGK1 mRNA and/or protein expression also is induced by aldosterone in the DN of several species (11, 31) as well as in the mammalian colon (39).

SGK1 affects ion transport in pathways mediated by the amiloride-sensitive epithelial Na⁺ channel (ENaC) and furosemide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter (11, 25). Several recent reports have begun to elucidate the mechanisms by which SGK1 mediates the effects of mineralocorticoids on ENaC-mediated Na⁺ transport. Aldosterone increases DN SGK1 mRNA and protein expression substantially within 15–30 min (5, 35, 43). This precedes and correlates with the so-called “early phase” of aldosterone action (30 min–3 h), during which the augmentation of transepithelial Na⁺ transport in the DN occurs almost exclusively by increasing the activity of apical ENaC and does not occur via other effectors of Na⁺ transport, such as Na⁺-K⁺-ATPase (22, 33). SGK1, in turn, has been shown to increase ENaC-mediated Na⁺ transport as well as ENaC localization to the plasma membrane in an oocyte coexpression system (1, 27). Moreover, recent evidence establishing that SGK1 knockout mice are unable to conserve Na⁺ further substantiates the importance of SGK1 in mediating mineralocorticoid effects (56).

These same time frames have been observed with renal tubular A6 cells, a well-established cell line for studying ENaC-mediated Na⁺ transport of Xenopus laevis DN origin (38). In A6 cells, the regulation of SGK1 and its correlation with aldosterone-induced Na⁺ transport depend on two features: 1) abundance of the enzyme is transcriptionally regulated by corticosteroids and 2) activation of the kinase requires phosphorylation of specific threonine and serine residues by the phosphoinositide-dependent kinases (23, 49). These same regulatory principles of SGK1 appear to be essential in other cell types, such as Hep G2, HEK-239, and mammary tumor cells, but with hypertonic stress as the condition inducing SGK1 gene expression (34, 46, 51).

We were interested in studying the effect of osmotic stress on SGK1 expression in A6 cells. However, because hypertonic stress induces amiloride-sensitive Na⁺ transport in these cells, we postulated that if SGK1 were to play a role in mediating effects on Na⁺ transport, its expression would correspondingly be induced by this treatment, in contrast to other cell types. We further sought to characterize potential second-messenger systems involved in osmotic control of SGK1 expression and Na⁺ transport in this cell type.

**METHODS**

**Cell culture.** A6 cells were obtained from the American Tissue Culture Collection (Manassas, VA). Cells were grown as previously described in DMEM with 5% fetal bovine serum (FBS) at ~250 mosM, an osmolarity suitable for growth of these amphibian cells. Maintenance growth conditions included humidified air at 30°C with 1% CO₂.

**Na⁺ transport measurements.** A6 cells were plated onto 24-mm-diameter Transwell plates (Costar, Corning, NY) with 0.4-μm permeabilized membranes coated with collagen type VI (Sigma, St. Louis, MO). Cells were plated at a concentration of 1.0 × 10⁶ cells/cm², grown for 7–10 days until formation of tight epithelia with an additional 2–3 days of growth in A6 media with 5% FBS stripped of endogenous steroids, and prepared by a previously described charcoal treatment (12). By using a voltage meter (Millicell-ERS, Millipore, Bedford, MA) to measure potential difference and resistance across the layer of A6 epithelia, equivalent current [potential difference/resistance (PD/R)] was calculated to estimate Na⁺ transport. Amiloride was used to verify that currents were ENaC dependent.

**Osmotic studies.** A6 cells were treated with hypotonic, isotonic, and hypertonic media. All media contained 5% FBS stripped of endogenous steroids. Hypotonic media were set at ~150 or 200 mosM as follows: 150 mosM media included (in mM) 56.7 NaCl, 7.7 Na HCO3, and 5.3 KCl as the major osmolar components, in addition to other components previously described (12); and 200 mosM media included 86 mM NaCl with no other differences compared with 150 mosM media. Isotonic media were prepared in two ways, standard A6 media at ~250 mosM and 150 mosM media with a supplement of 100 mM sorbitol. Solutions of 300 and 350 mosM were prepared as standard A6 media supplemented with 50 and 100 mM sorbitol, respectively.

**Pharmacological agents.** Reagents used to study Na⁺ transport and SGK1 expression are referenced here according to supplier, preparative concentration, and experimental concentration, respectively: dexamethasone, Sigma, 100 μM in ethanol, 100 mM; LY-294002, Calbiochem (La Jolla, CA), 50 mM in DMSO, 50 μM; amiloride, Sigma, 50 mM in ethanol, 50 μM; cycloheximide, Sigma, 20 mM in ethanol, 20 μM; and actinomycin D, Sigma, 5 mg/ml in water, 5 μg/ml.

**Detection and quantitation of SGK1 mRNA.** Total RNA was isolated from A6 cells by using RNA Stat-60 (Tel-Test, Friendswood, TX) according to the manufacturer’s instruction. RNA was electrophoretically separated on a 0.95% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane (Hybond-NX, Amersham-Pharmacia, Piscataway, NJ), and cross-linked to the membrane by ultraviolet cross-linking (Strategene UV Crosslinker, Stratagene, La Jolla, CA). 32P-radiolabeled probes of X. laevis SGK1 and β-actin were produced by using a random primer method of incorporation [α-32P]dCTP into complementary strands of the respective cDNA (Prime-a-Gene, Promega, Madison, WI). After 3–6 h of incubation at 42°C in a standard prehybridization solution supplemented with 100 μg/ml denatured salmon testes DNA, membranes were exposed overnight to 2 × 10⁶ counts-min⁻¹·ml⁻¹ radiolabeled probe in a 50% formamide standard hybridization solution at 42°C. Membranes were rinsed and washed twice in 2× SSC-0.1% SDS at 50°C before undergoing autoradiography.

**Detection of phosphorylated and unphosphorylated forms of SGK1.** Protein from A6 cells grown on Transwell filters was isolated by using a lysis buffer and protocol described previously (12). By using 70 μg of protein from a given sample, protein was separated by 7.5% SDS-PAGE and elec-
Hypotonic stress induced an increase in PD/R within 5 min, whereas a delay of 30–45 min was seen after dexamethasone treatment, as previously described (see Ref. 43 and references therein). After the rapid initial induction of PD/R by osmotic stress, a slower rise toward a plateau occurred and was reached at ~90–120 min; the later phase of the time course was similar to that observed in dexamethasone-treated cells. Amiloride treatment at 0, 30, and 180 min after osmotic treatment decreased PD/R to <20% of baseline (or pretreatment) values and blocked responses to both dexamethasone and osmotic stress, suggesting that the induction of current by either stimulus was ENaC dependent (Fig. 1B and data not shown). It is apparent from Fig. 1B that the induction by osmotic stress was more rapid and achieved a greater maximum than that induced by a saturating dose of dexamethasone.

**Effect of hypotonic stress on SGK1 mRNA and protein expression.** SGK1 is strongly stimulated by hypertonic stress in a variety of cell types, as well as by aldosterone and dexamethasone in A6 cells (4, 11, 45, 46). In all of the cases that have been examined, the induction of SGK1 paralleled the induction of Na+ transport. With this in mind, we next examined the effect of osmotic stress on SGK1 expression in A6 cells. A6 cells were grown to high resistance on permeable supports, exposed to hypotonic (150 mosM), isotonic (250 mosM), or hypertonic (350 mosM) conditions, and were blotted to nitrocellulose membranes (Micron Separations, Westborough, MA). After nonspecific binding was reduced by 3 h preincubation in 5% dry milk dissolved in a PBS-0.1% Tween 20 (PBS-T) solution, the membranes were exposed overnight to a 1:500 rabbit polyclonal anti-rat SGK1 antibody (courtesy of Dr. Gary Firestone, University of California, Berkeley, CA). The membranes were then washed with PBS-T solution and exposed to rabbit Ig-horseradish peroxidase-linked whole antibody (Amersham-Pharmacia) at 1:5,000 in 7.5% dry milk in PBS-T solution for 1 h at room temperature. After another wash with PBS-T solution, bound antibody was detected by a chemiluminescence protocol (ECL; Amersham-Pharmacia). Phosphorylated and unphosphorylated forms of SGK1 were distinguished by size because of their different migrations during electrophoresis (12, 49).

Statistical analysis. Significance was determined by using a two-tailed Student’s t-test to compare test samples with control samples. A P value of <0.01 was considered significant.

**RESULTS**

**Effect of hypotonic and hypertonic stress on Na+ transport in A6 cells.** When grown on permeable supports, A6 cells form a high-resistance monolayer that is useful in the study of transepithelial Na+ transport. Previous work demonstrated that a variety of physiologically relevant factors, including corticosteroids, vasopressin, insulin, and osmotic stress, stimulate amiloride-sensitive transepithelial Na+ transport (15, 38).

However, unlike other cell types, hypotonic conditions, rather than hypertonic conditions, stimulated A6 Na+ transport (52).

We initially compared the effects of osmotic stress and corticosteroids on the electrical properties of A6 monolayers by using PD/R as a surrogate for short-circuit current (SCC). To establish optimal osmotic conditions to stimulate Na+ current, we first performed a dose-response experiment, varying the medium osmolarity from 150 (hypotonic) to 350 mosM (hypertonic) by altering the NaCl concentration; an osmolarity of 200 mosM was considered isotonic and equal to the tonicity of the growth medium. As shown in Fig. 1A, exposing the basolateral membrane to hypotonic conditions stimulated PD/R, whereas hypertonic conditions decreased it. PD/R was significantly increased by osmolarity of 200 mosM and further increased by osmolarity of 150 mosM. In contrast, osmotic changes to the medium bathing the apical surface of the monolayers had no apparent effect on current, as previously demonstrated (14, 36, 52). Moreover, PD/R was not appreciably induced over basal when medium NaCl concentration was reduced while osmolarity was maintained at 250 mosM by addition of sorbitol (data not shown). This latter result confirms that Na+ current was induced by changes in osmolarity, not by changes in Na+ concentration per se (52).

We next compared the induction of PD/R by hypertonic (150 mosM) stress and dexamethasone (100 nM). As shown in Fig. 1B, hypertonic stress induced an increase in PD/R within 5 min, whereas a delay of 30–45 min was seen after dexamethasone treatment, as previously described (see Ref. 43 and references therein).
RNA and protein were harvested at the time points shown in Fig. 2. In contrast to other cell types, but consistent with its effect on \( \text{Na}^+ \) current, hypotonic stress induced SGK1 expression in A6 cells. Hypertonic stress, on the other hand, appeared to reduce SGK1 mRNA, although the background was too high to conclude this with certainty (Fig. 2 and data not shown). As with induction of the \( \text{Na}^+ \) current, no effect was seen when the osmotic gradient was applied to the apical membrane. As shown in Fig. 2B, SGK1 mRNA was induced within 15 min and peaked around 45 min after hypotonic treatment, a time course similar to that observed for dexamethasone induction of SGK1 mRNA in these same cells (11).

We next examined SGK1 protein induction under the same conditions with Western blot analysis. Hypotonic stress increased SGK1 protein expression, as it did SGK1 mRNA (Fig. 2C). SGK1 protein runs in SDS-PAGE as a doublet that can be resolved into two distinct bands representing the phosphorylated and unphosphorylated forms of the protein (34, 49). Previous studies have demonstrated that the phosphorylated protein is the active form and that preventing phosphorylation prevents SGK1 activity (23, 34, 49). Notably, both forms of SGK1 were increased in response to hypotonic conditions (Fig. 2, C and D). Also, note that hypotonic stress induced SGK1 protein expression to a comparable extent or slightly more strongly than dexamethasone and that further induction was achieved when the two treatments were combined (Fig. 2C). Moreover, the time course of SGK1 protein induction by hypotonic stress was similar to that of dexamethasone (Fig. 2D and data not shown; also see Ref. 11).

**Fig. 2.** Regulation of serum and glucocorticoid-regulated kinase-1 (SGK1) mRNA and protein expression in A6 cells by media osmolarity. A: SGK1 mRNA expression is altered by changes in basolateral, but not apical, osmolarity. Representative Northern blot analysis (n = 3) shows SGK1 mRNA expression relative to \( \beta \)-actin mRNA expression 1 h after treatment with differing osmotic conditions to apical and basolateral surfaces. I, isotonic media (250 mosM); ↓, hypotonic media (150 mosM); ↑, hypertonic media (350 mosM). Far right lane: SGK1 mRNA induction after exposure to 100 nM dexamethasone. B: 1-h time course of the increase in SGK1 mRNA expression after exposure to 150 mosM media is depicted. Far right lane: SGK1 mRNA induction 1 h after exposure of cells to 100 nM dexamethasone. Expression of SGK1 mRNA is compared with \( \beta \)-actin mRNA. C: response of SGK1 protein to 1 h of 150 mosM hypertonic media ± 100 nM dexamethasone or 250 mosM isotonic media ± 100 nM dexamethasone. D: time course of SGK1 immunoreactive protein after hypotonic stress. Far left lane: SGK1 immunoreactivity in cells under isotonic conditions treated with 100 nM DEX and harvested at 60 min (positive control). SGK1 expression at 0, 30, 60, and 180 min after hypotonic treatment is also shown. phosph, Phosphorylated; unphosph, unphosphorylated.

Effect of inhibition of transcription or protein synthesis on hypotonic induction of \( \text{Na}^+ \) transport and SGK1 expression. The initial rise in PD/R in response to hypotonic treatment occurred within 5 min and preceded the induction of either SGK1 mRNA or protein (Figs. 1B and 2, B and D). PD/R during the later phase after osmotic treatment (>30 min) paralleled the response to dexamethasone treatment, a treatment in which induction of PD/R is known to be dependent on changes in gene transcription. In light of the strong induction of SGK1 protein by osmotic stress and the evidence supporting a role for SGK1 in stimulating ENaC-mediated \( \text{Na}^+ \) transport (11, 27, 31, 39), we wanted to examine the possibility that transcriptional induction of SGK1 might play a role in the second phase of hypotonic induction of PD/R (between 30 and 120 min).

We therefore examined the effect of inhibiting gene transcription on hypotonic induction of PD/R and SGK1 expression. Thirty minutes before the addition of hypotonic media, A6 monolayers were treated with actinomycin D (5 \( \mu \)g/ml) or vehicle, and the time course of PD/R was followed. As shown in Fig. 3, during the first 30 min after hypotonic treatment, the rise in PD/R appeared similar in monolayers treated without or with actinomycin D, suggesting that the rise in current during this time period does not require transcriptional events. However, after 30 min, the two curves began to diverge, with the actinomycin D-treated monolayers demonstrating a more flattened response compared with cells not treated with the inhibitor. Figure 3 also shows that inhibition of transcription with actinomycin D did not affect PD/R under isotonic conditions, supporting the conclusion that the cells
remained healthy with intact Na\(^+\) translocation mechanisms during the time course of the experiment. Figure 3 also reveals the strong dependence of steroid induction of PD/R on changes in gene transcription.

To confirm that the differences observed in Fig. 3 were not artifacts or peculiar to actinomycin D inhibition, we tested the effect of inhibiting protein synthesis with cycloheximide on hypotonic activation of SCC. As shown in Fig. 4A, the responses to hypotonic treatment were similar to those obtained when transcription was curtailed (Fig. 3). Notably, the response to hypotonic stress was blunted in those monolayers in which protein synthesis was inhibited. That cycloheximide inhibited SGK1 protein but not RNA synthesis was confirmed by Northern and Western blot analyses (not shown).

Comparison of the osmotic induction of PD/R in the presence and absence of inhibitor (either actinomycin D or cycloheximide) suggests that the later phase of the response (30–120 min) requires changes in gene transcription and, ultimately, in protein synthesis (Figs. 3 and 4). To more precisely quantitate the contribution of new protein synthesis to the overall response to osmotic stress, the PD/R measured in monolayers treated with cycloheximide was subtracted from the corresponding level in untreated monolayers. These derived data points were then graphed and are shown in Fig. 4B for both hypotonic and dexamethasone-treated cells. Notably, the component of the PD/R attributable to new protein synthesis is comparable for monolayers treated with hypotonic media or dexamethasone. In addition, a similar time course is observed, with a 30- to 45-min latent period emerging for the protein synthesis-dependent component of the osmotic effect. Taken together, these observations support the view that a correlation exists between SGK1 gene expression and induction of PD/R during the later (30–120 min) phase of the osmotic effect.

Phosphatidylinositol 3-kinase inhibition blunts and delays hypotonic induction of Na\(^+\) transport. Because dexamethasone induction of SGK1 activity and Na\(^+\) transport has been shown to be phosphatidylinositol 3-kinase (PI3K) dependent, we investigated whether the correlation between SGK1 and Na\(^+\) transport persisted under hypotonic conditions. A6 monolayers were pretreated with the PI3K inhibitor LY-294002 (44) or vehicle 30 min before altering of medium tonicity. As shown in Fig. 5, PI3K inhibition reduced baseline SCC by ~60% and completely prevented the early increase (<30 min) of PD/R in response to hypotonic media (Fig. 5A). Moreover, the later sustained rise in PD/R (after 30 min) in response to hypotonic conditions was also markedly blunted by LY-294002, to an extent equal to
or greater than the inhibition of the dexamethasone-induced current (Fig. 5, A and B). Cells harvested for protein 1 h after addition of stimulus in the presence or absence of LY-294002 were then probed in immunoblots with SGK1-specific antibody (Fig. 5C). Although hypotonic media and dexamethasone each increased the abundance of SGK1, it is notable that in the presence of PI3K inhibitor, only the unphosphorylated form of the SGK1 was expressed.

**DISCUSSION**

Hypotonic induction of SGK1 in A6 cells correlates with increased Na\(^+\) transport. Similar to previous investigations, the present study found that SGK1 gene expression is osmotically regulated. However, in marked contrast to previous reports in which hypertonic conditions in the shark rectal gland and hepatocytes induced SGK1 mRNA and protein levels, our results demonstrate that SGK1 expression is increased under hypotonic conditions in renal tubular cells of amphibian origin. The study further provided evidence that augmented SGK1 expression correlates with activation of Na\(^+\) transport, a finding that may have evolutionary significance.

The osmotic induction of SGK1 mRNA and protein in A6 cells occurred rapidly and paralleled the induction seen by corticosteroids (Fig. 2). SGK1 mRNA began to increase within 15 min and protein within 30 min of exposure to hypotonic conditions. The data (Fig. 1) also confirm previous reports that hypotonicity induced a rapid and marked rise in amiloride-sensitive Na\(^+\) current. The current increased within 5 min, and its initial rise within the first 30 min was not blocked by inhibitors of mRNA or protein synthesis. The later phase of hypotonic-induced Na\(^+\) current (after 30 min and up to 120 min), by contrast, was blunted by inhibition of gene transcription or protein synthesis (Figs. 3 and 4A). Indeed, as revealed in Fig. 4B, the component of PD/R induced by hypotonicity that is dependent on new protein synthesis is similar in its magnitude and time course to the increase observed after exposure to corticosteroids. The data therefore lend support to
the hypothesis that transcriptional induction of SGK1 plays a role in the sustained increase in Na\(^+\) current across A6 monolayers 30 min after exposure to corticosteroids or hypotonic conditions.

Although it is important to emphasize that the present observations between increased SGK1 expression and increased Na\(^+\) transport are not causal but rather only correlative, the parallel between these two observations has remained consistent across cell types despite the contrasting stimuli such as hypertonicity and hypotonicity. Moreover, this consistency remains functionally appropriate for the homeostatic needs of the organism. In amphibia and other freshwater or euryhaline vertebrates, the ability of the DN to reabsorb Na\(^+\) and generate dilute urine is essential to the adaptation of decreases in environmental tonicity. This is particularly true for the euryhalines, which migrate through an Sp1-like site in the SGK1 5' flanking region (48). Initial inhibitor experiments (not shown) suggest that hypotonic stress does not require p38 MAPK, because it (unlike the effect of hypertonic stress on SGK1 expression in mammmary epithelial cells) is not inhibited by MAPK antagonists SB-202190 or SB-203580. Regardless of the physiological role of SGK1 in mediating osmotic effects on Na\(^+\) transport, it appears that SGK1 gene transcription can be oppositely regulated by osmotic stress in a cell type-dependent fashion. This is, to our knowledge, the first description of such paradoxical regulation by a single stimulus of an endogenous gene's transcription. It will be of mechanistic interest to determine how these opposing signals are channeled into a common effect on the SGK1 gene.

**Role of PI3K in hypotonic induction of Na\(^+\) transport.** In contrast to gene transcription, which appears to be implicated only in the later phase of the hypotonic induction of Na\(^+\) current in A6 cells, PI3K activity is required for both the initial and the later phases of the response. Because both the kinase activity and the phosphorylation state of SGK1 protein appear to be PI3K dependent (Fig. 5) (23, 34), it is tempting to speculate that SGK1 is implicated in the early, transcription-independent phase of osmotic induction. However, initial levels of SGK1 are low and might not be adequate to mediate the early effect. Moreover, there are several other PI3K-dependent kinases, most notably SGK2, SGK3, and protein kinase B/Akt, that could be implicated (24). Indeed, recent evidence suggests that SGK2 and SGK3, but not protein kinase B/Akt, stimulate ENaC-mediated Na\(^+\) transport (Lang F, personal communication). Additional studies will be needed to examine the roles of these and other kinases in mediating the effects under hypotonic conditions. Whichever the mechanism, our data suggest that two distinct, convergent pathways are required for the full range of Na\(^+\) transport regulation: 1) a PI3K-dependent pathway that promotes the active form of SGK1 and/or other related kinases and 2) a transcriptional pathway that increases the abundance of SGK1 (and possibly other transport regulators).

**Role of differential osmotic regulation of SGK1 in evolution.** Although A6 cells are the first cell line in which hypotonic treatment has been shown to induce SGK1, the role of hypotonic activation of Na\(^+\) transport has been known for years in amphibia and other freshwater vertebrates as they adapt to osmotic changes in their environments. Indeed, Ussing originally proposed a link between increased Na\(^+\) transport and hypotonic stress in frog skin nearly 40 years ago (28, 42), with subsequent reports of this linkage made in the frog kidney tubule (30), toad bladder (26), and larval salamander kidney (41). It is thus not surprising that A6 cells, derived from the DN of the freshwater African claw-toed frog (52), show similar behavior. We have examined whether osmotic stress stimulates Na\(^+\) transport or SGK1 expression in renal tubular cells of mammals. Interestingly, neither hypotonic nor hypertonic conditions had much effect on Na\(^+\) transport and, importantly, on SGK1 expression (Rozansky DJ, unpublished observations).

SGK homologues have been identified in all eukaryotic species examined, including *Saccharomyces cerevisiae* (8), *Caenorhabditis elegans* (54), and a variety
of vertebrates (11, 31, 46, 47, 50). Although SGK1 regulation in invertebrates and yeast has not been extensively studied, it is known that yeast has two SGK homologues, YPK1 and YKR2, which appear to be at least partially redundant. Yeast deficient in both these genes is nonviable, although the cause of the lethal phenotype remains unknown. Interestingly, one study found that the YPK1/YKR2-deficient yeast can be rescued by expression of rat SGK1 (8). Taken together with our present data, these observations suggest the possibility that SGK1 has served throughout eukaryotic evolution as a facilitator of volume homeostasis, initially of individual cells and subsequently of ECF. Perhaps in amphibia, toxicity is the primary regulator of DN SGK1 expression through its direct effects on DN cells and through the renin-angiotensin-aldosterone system, which appears to be regulated by toxicity in amphibia (53). In mammals, the regulation of toxicity and ECF volume is independent, and toxicity per se appears to have little effect on either DN Na⁺ transport or SGK1 expression. Rather, toxicity is regulated primarily by vasopressin (which affects mainly water transport), whereas ECF volume is regulated primarily by the renin-angiotensin-aldosterone system through its toxicity-independent effects on DN ion transport.

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REFERENCES

HYPOTONIC ACTIVATION OF SGK1

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