Intracellular calcium plays a role as the second messenger of hypotonic stress in gene regulation of SGK1 and ENaC in renal epithelial A6 cells

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Submitted 29 May 2007; accepted in final form 16 October 2007

THE DISTAL NEPHRON of the kidney plays an important role in homeostasis of extracellular fluid (ECF) volume and ECF osmolality by regulating the amount of renal NaCl excreted into urine. Fine tuning of renal NaCl excretion in the distal nephron is achieved by amiloride-sensitive epithelial Na+ channel (ENaC)-mediated transepithelial Na+ reabsorption. The ENaC consists of three homologous subunits, α, β, and γ, which share 35% homology in amino acid sequence (8, 13), and is expressed in various types of epithelia, such as kidney, colon, sweat glands, and airways. Renal NaCl is reabsorbed by transcellular Na+ transport through the apical membrane-located ENaC followed by Cl− transport via the paracellular pathway. Thus ENaC plays the key role in homeostasis of ECF volume and osmolality. Furthermore, the importance of the ENaC is actually indicated by loss-of-function mutations of ENaC subunits or the mineralocorticoid receptor stimulating transcription of ENaC characterized as pseudohyopaldosteronism type 1 (9, 36). Conversely, gain-of-function mutations of ENaC (Liddle’s syndrome) or the mineralocorticoid receptor (14) lead to severe hypertension.

Serum- and glucocorticoid-inducible kinase 1 (SGK1) is thought to play a critical role in the regulation of NaCl trafficking, activity, and transcription (2, 7, 20, 25, 30). For instance, SGK1 is required for basal and insulin-stimulated Na+ transport in renal epithelial cells (12). Furthermore, SGK1 plays a crucial role in stimulation of Na+ transport by various hormones, including aldosterone (12). At the trafficking level, SGK1 phosphorylates and, thereby, inactivates the ubiquitin-ligase Nedd4-2 (11, 35). Consequently, SGK1 may retard ubiquitination and subsequent degradation of ENaC (11, 35), resulting in the increase in ENaC protein abundance in the apical membrane. However, whether SGK1 is essentially required for ENaC insertion into the apical membrane remains controversial. Blazer-Yost and colleagues reported that insulin stimulates ENaC insertion into the apical membrane (6) and that the activity of SGK1 is essentially required for insulin to show its stimulatory action on the amiloride-sensitive Na+ transport (12). These in vitro studies by Blazer-Yost and colleagues strongly suggest the involvement of SGK1 in ENaC insertion. The in vivo study by Wulf et al. (42) suggests that SGK1 is not involved in ENaC insertion. At the activity level, expression of a constitutively active mutant of SGK1 induces the increase of ENaC open probability in the A6 Xenopus laevis renal epithelial cell line (2). At the transcriptional level, in mouse cortical collecting duct (CCD) cells stably expressing full-length SGK1, the expression level of α- and β-subunit mRNA, but not γ-subunit mRNA, of ENaC is higher than in mouse CCD cells expressing a kinase-dead dominant-negative SGK1 (7). SGK1 was originally found in mammary epithelial cells (41) and subsequently identified as an aldosterone-induced gene product in renal epithelial cells, where SGK1 is involved in the aldosterone-induced stimulation of Na+ reabsorption (10). Interestingly, SGK1 has previously been shown to respond to osmotic stimuli in a variety of cell types (5, 33, 39), suggesting participation of SGK1 in the hormone-independent direct regulation of ECF osmolality, particularly in the kidney.

In A6 cell monolayers, hypotonic stress induces Na+ transport immediately (within 10 min) after application of the stress (18, 24, 26, 27, 29, 37). Several studies revealed that hypotonic stress greatly increased the activity of SGK1 by 10.220.33.2 on June 25, 2017 http://ajprenal.physiology.org/ Downloaded from http://ajprenal.org

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stress has a genomic and a nongenomic impact on Na\(^+\) reabsorption (26, 29, 33, 37). We previously reported that the rapid, nongenomic effect of hypotonic stress on Na\(^+\) reabsorption is mediated through ligand-independent activation of receptor-type tyrosine kinases (RTKs), such as epidermal growth factor receptor and platelet-derived growth factor receptor (37). With regard to the genomic effect, Rozansky et al. (33) reported that hypotonic stress stimulates the mRNA and protein expression of SGK1 and that the hypertonicity-induced SGK1 is involved in induction of Na\(^+\) transport at the delayed phase (>30 min), although the mechanism of hypertonic induction of SGK1 remains unknown. Moreover, our previous studies (17, 26, 28) documented that chronic (24 h) application of hypotonic stress increased mRNA expression of all three ENaC subunits, resulting in stimulation of Na\(^+\) transport, although we did not clarify the time courses or the detailed mechanisms of hypertonic induction of ENaC subunits.

Polarized A6 epithelia have been reported to immediately respond to hypotonic stress, showing a biphasic rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (19). However, we have no information on the role of the hypertonicity-induced biphasic rise in [Ca\(^{2+}\)]\(_i\), in the genomic action of hypotonicity; specifically, no reports clarify the role of intracellular Ca\(^{2+}\) in transcriptional regulation of SGK1. The purpose of the present study was to understand how the hypertonicity-induced rise in [Ca\(^{2+}\)]\(_i\) contributes to the genomic action of hypotonicity on Na\(^+\) reabsorption by investigating the regulatory mechanism of gene expression of three ENaC subunits by hypotonic stress and the mechanism of hypertonicity-induced expression of SGK1 mRNA and protein. We hypothesized that hypertonic stress induces SGK1 expression via the increase in [Ca\(^{2+}\)]\(_i\), and that the expression of ENaC subunits is also induced via Ca\(^{2+}\)-dependent signaling cascades. The present study shows for the first time that Ca\(^{2+}\)-related signal transduction pathways are involved in the hypotonic stimulation of SGK1 and ENaC subunit transcription.

**MATERIALS AND METHODS**

**Chemicals and materials.** BAPTA-AM, W7, ML-7, KN-93, cyclosporin A, and SB-203580 were obtained from Calbiochem (San Diego, CA); fetal bovine serum from Cambrex Bio Science (Walkersville, MD); permeable tissue culture supports (tissue culture inserts) from Nunc (Roskilde, Denmark); and NCTC-109 medium, dexamethasone, and benzamil from Sigma (St. Louis, MO).

**Cell culture.** Renal epithelial A6 cells derived from the kidney of *X. laevis* were purchased from American Type Culture Collection (Rockville, MD) at passage 68. A6 cells (passages 76–84) were grown on plastic flasks at 27°C in a humidified incubator with 1.0% CO\(_2\)-in-air in a culture medium (the isotonic medium) containing 75% (vol/vol) NCTC-109, 15% (vol/vol) distilled water, and 10% (vol/vol) fetal bovine serum. The medium was made hypotonic by reducing NaCl from 120 mM in the isotonic medium to 60 mM. Cells were seeded onto tissue culture inserts (Nunc) for Western blotting and quantitative RT-PCR (qRT-PCR) or onto tissue culture-treated Transwell filter cups (Costar, Cambridge, MA) for electrophysiological measurements at a density of 5 × 10\(^4\) cells/well and cultured for 11–15 days.

**Electrophysiological measurements.** Short-circuit current (I\(_sc\)) and transepithelial conductance (G\(_t\)) were measured as previously described (37). A6 epithelia were bathed in a serum-free culture medium containing 75% (vol/vol) NCTC-109 and 15% (vol/vol) distilled water in 1.0% CO\(_2\)-99% O\(_2\). The benzamil-sensitive I\(_sc\) and the benzamil-sensitive G\(_t\) were used as indicators of transepithelial Na\(^+\) transport and ENaC activity (conductance), respectively.

**Western blotting.** Western blotting was performed on whole cell lysates of A6 cells as previously described (37). To detect *Xenopus* SGK1 (xSGK1) protein, we used a rabbit polyclonal anti-SGK antibody (Cell Signaling Technology, Beverly, MA). The original peptide used in the creation of this polyclonal antibody is a region in the COOH terminus of human SGK1. The similarity between the hSGK1 antigen peptide used in creating this antibody and xSGK1 is 88% in this region; therefore, this antibody may detect xSGK1 as well. Furthermore, as shown in the present study, the response of the band detected with this antibody in Western blots was consistent with the results from qRT-PCR in which sense and antisense primers were designed for xSGK1. For an active form of SGK1, we used a goat polyclonal anti-phosphorylated SGK1 (Ser\(^{422}\)) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). We aligned the xSGK1 protein sequence with the human SGK1 protein sequence and reviewed specifically the antigenic region around Ser\(^{422}\), xSGK1 also has Ser\(^{425}\), and the two proteins are conserved in this region with high similarity. Since the band detected with this antibody showed a synchronous response to total xSGK1 protein, we concluded that this antibody detects xSGK1 phosphorylated at Ser\(^{425}\).

**Real-time qRT-PCR.** To compare the relative amounts of ENaC subunit mRNA and SGK1 mRNA, real-time RT-PCR was performed on total RNA obtained from A6 cells using TaqMan probe (Applied Biosystems, Rotkreuz, Switzerland), a real-time PCR Master Mix (Toyobo), and a real-time PCR system (model 7300, Applied Biosystems). Total RNA was isolated from cells using the RNAeasy Mini Kit (Qiagen, Valencia, CA), and then contaminated genome DNA was digested with RNase-free DNase (Qiagen) according to the manufacturer’s protocol. The SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) was used to synthesize first-strand cDNA in a volume of 21 µl containing 1 µg of total RNA, calculated from the absorbance measured at 260 nm. Sense and antisense primers and TaqMan probes were specifically designed for three *Xenopus* ENaC subunits, xSGK1, and *Xenopus* GAPDH (Table 1) using Primer Express Software version 3.0 (Applied Biosystems). After denaturation at 95°C for 10 min, PCR was performed in triplicate for 40 cycles, with 1 µl of first-strand cDNA (20:1 dilution) used as a template. Each of the 40 PCR cycles was consisted of 15 s at 95°C and 1 min at 60°C. For quantification of the amount of specific mRNA in the samples, standard curves were generated for

<table>
<thead>
<tr>
<th>Protein (Gene)</th>
<th>GenBank Accession</th>
<th>Forward Primer (5’-3’)</th>
<th>TaqMan Probe (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
<th>Amplicon, bp</th>
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<td>TGCCTGAAAGATCAGCTCTGTTTACCC</td>
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<tr>
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<tr>
<td>γ-ENaC</td>
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<td>TGGGAGATGCTGTGTGCTGCTGCTG</td>
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<td>77</td>
</tr>
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ENaC, epithelial Na\(^+\) channel; SGK1, serum- and glucocorticoid-regulated kinase 1.
ENaC subunits and SGK1. In addition, a standard curve generated for GAPDH enabled us to standardize the initial RNA content of a sample relative to the amount of GAPDH.

Temperature. All experiments for electrophysiological and other measurements were performed at 27–28°C unless otherwise indicated.

Data presentation. Values are means ± SE. Where SE bars are not visible, they are smaller than the symbol size. Student’s t-test and ANOVA were used for statistical analysis as appropriate, and P < 0.05 was considered significant.

RESULTS

Hypotonic stress induces expression of SGK1 mRNA and protein. We initially investigated the time course of hypotonicity-induced expression of SGK1 mRNA and protein. A6 cells were cultured in a 10% serum-containing growth medium on permeable supports to form a polarized high-resistance monolayer. Then A6 cell monolayers were exposed to a serum-free hypotonic medium for 0–120 min, and proteins were harvested for subsequent Western blotting. Our inability to detect SGK1 protein expression under the basal (i.e., isotonic) condition (Fig. 1A) suggests that the level of SGK1 protein expression is very low and correlates with an in vivo study reporting no or very low SGK1 protein expression under the basal condition in glomeruli, proximal tubules, or medullary collecting ducts, including the papilla, in the rat kidney (1). In this way, hypotonic stress-induced expression of SGK1 protein became detectable over the time course of 30–60 min after exposure to hypotonic stress, and a phosphorylated (i.e., active form) SGK1 protein was also induced. At the same time, expression of phosphorylated glucocorticoid-inducible kinase 1 (SGK1) mRNA and protein. A single ~56-kDa band indicates SGK1 protein.

Hypotonic stress rapidly (within 30 min) stimulated SGK1 mRNA expression, which reached the maximum level, i.e., a 15-fold; increase, at 3 h. The time course of the increase in SGK1 mRNA expression was faster than that of the induction of SGK1 protein. This delay is presumably due to the time required for translation from SGK1 mRNA to SGK1 protein.

Involvement of Ca2+/calmodulin-dependent signaling cascades in the hypotonic induction of SGK1. As described above, earlier work documented that polarized A6 epithelia respond to hypotonic stress, inducing a biphasic rise in [Ca2+]i, (19). Therefore, we examined a possibility that the increase in [Ca2+]i, following the hypotonicity-induced cell swelling activates a Ca2+/calmodulin (CaM) cascade, which, in turn, participates in SGK1 expression. A6 epithelia were bilaterally pretreated with 20 μM BAPTA-AM, an intracellular Ca2+ chelator, or 50 μM W7, a CaM antagonist, for 30 min and then exposed to a serum-free hypotonic medium. Protein and total RNA were harvested 2 h after the hypotonic treatment for Western blotting and qRT-PCR. As shown in Fig. 2, A and B, BAPTA-AM and W7 abolished the hypotonic induction of SGK1 protein. Also, BAPTA-AM and W7 diminished the hypotonic induction of SGK1 mRNA (Fig. 2C). There is a discrepancy between protein and mRNA levels relative to the effectiveness of Ca2+/CaM cascade inhibition. This may simply mean that the partial inhibition of SGK1 mRNA expression is enough for the complete inhibition of SGK1 protein induction. In other words, a 10-fold increase in SGK1 mRNA in cells treated with BAPTA-AM or W7 is not enough for detectable induction of SGK1 protein. However, the hypothesis worth mentioning is that the Ca2+/CaM cascade makes a much greater contribution to SGK1 translation than to SGK1 transcription. These observations suggest that the Ca2+/CaM-dependent signaling cascade plays a major role in the hypotonic stimulation of SGK1 expression at mRNA and protein levels.

Fig. 1. Hypotonic stress-induced expression of serum- and glucocorticoid-inducible kinase 1 (SGK1) mRNA and protein. A and B: time course of hypotonic stress-induced active form of SGK1 protein (phospho SGK1) and total SGK1 protein. A single ~56-kDa band indicates SGK1 protein. Within 60 min, hypotonic stress stimulated expression of SGK1 protein. At the same time, expression of phosphorylated (i.e., active form) SGK1 protein was also induced. Values are means ± SE (n = 3). C and D: time course of hypotonic stress-induced expression of SGK1 mRNA. Hypotonic stress induced a rapid (within 30 min), transient expression of SGK1 mRNA, with its peak at 3 h. Values are means ± SE (n = 4). *P < 0.05 vs. control (isotonic) cells.
Ionomycin induces SGK1 expression under an isotonic condition. Next, we tested the effect of ionomycin on SGK1 induction under an isotonic condition to determine whether an increase in \([\text{Ca}^{2+}]\) is actually a stimulus of SGK1 expression. In an isotonic serum-free medium, A6 epithelia were treated bilaterally with 1 \(\mu\)M ionomycin (a \(\text{Ca}^{2+}\) ionophore), hypotonic stress, or 1 \(\mu\)M dexamethasone for 1–3 h. Total RNA and protein were then harvested. As shown in Fig. 3, A and B, ionomycin alone elevated the amount of the phosphorylated form of SGK1 protein under an isotonic condition by the same amount as dexamethasone. The level of SGK1 protein induced by ionomycin or dexamethasone was significantly smaller than that induced by hypotonic stress. These observations suggest that an increase in \([\text{Ca}^{2+}]\) would play an important role in the hypotonic induction of SGK1 protein, but whether an increase in \([\text{Ca}^{2+}]\) is a sufficient signal for the hypotonic stress to show its action remains unclear. Ionomycin stimulated SGK1 transcription under an isotonic condition (Fig. 3C). The level of SGK1 mRNA expression rapidly rose within 1 h after application of ionomycin, reached the maximum level at 3 h, and dropped toward the baseline over the ensuing 12 h. Thus the time course of the effect of ionomycin on SGK1 transcription was remarkably similar to that of the hypotonic action on SGK1 mRNA expression. Furthermore, we studied the dose-dependent effects of ionomycin on the expression of SGK1 mRNA. Ionomycin stimulated SGK1 mRNA expression dose dependently, with the half-maximum effect at \(\sim 500\) nM (Fig. 3D). These data clearly indicate that an increase in \([\text{Ca}^{2+}]\), can stimulate the expression of SGK1 mRNA and protein under an isotonic condition.

Time courses of hypotonicity-induced expression of three ENaC subunits. We demonstrated the involvement of \(\text{Ca}^{2+}\)/CaM-dependent signaling cascades in the hypotonicity-induced expression of SGK1. To our knowledge, the mRNA expression of all three ENaC subunits, \(\alpha\), \(\beta\), and \(\gamma\), is also stimulated by the reduction of osmolality in an ECF for 24 h (17, 26, 28), but the time courses of hypotonicity-induced expression of ENaC subunits have not been investigated. Therefore, we performed qRT-PCR on mRNA of all three ENaC subunits. As shown in Fig. 4, the patterns of \(\alpha\)- and \(\beta\)-ENaC mRNA stimulation over the time course were identical. Expression of \(\alpha\)- and \(\beta\)-ENaC mRNA began to rise within 3 h after application of the hypotonic stress in a serum-free medium and was still increasing at 12 h. On the other hand, expression of \(\gamma\)-ENaC mRNA began to increase between 1 and 3 h, reached its maximum level at 3 h, and remained at this maximum level without further increases over the ensuing 12 h.

\(\text{Ca}^{2+}\)-dependent transcriptional regulation of ENaC subunits under hypotonic stress. Next, we tried to address whether the hypotonicity-induced stimulation of ENaC mRNA expression is mediated via \(\text{Ca}^{2+}\)/CaM-dependent signaling systems. We tested the effects of BAPTA-AM and W7 on the hypotonic stimulation of mRNA expression of all three ENaC subunits under the conditions described in Fig. 2. A6 epithelia were bilaterally pretreated with 20 \(\mu\)M BAPTA-AM or 50 \(\mu\)M W7 for 30 min and subsequently exposed to a serum-free hypotonic medium. Total RNA was harvested 3 h after application of the hypotonic stress. The hypotonicity-induced stimulation of \(\alpha\)- and \(\beta\)-ENaC mRNA expression was abolished by 20 \(\mu\)M BAPTA-AM (Fig. 5, A and B), whereas 50 \(\mu\)M W7 did not affect \(\alpha\)- or \(\beta\)-ENaC mRNA expression under the basal or hypotonic condition (Fig. 5, A and B). Under the isotonic condition, chelation of intracellular \(\text{Ca}^{2+}\) slightly but significantly inhibited mRNA expression of \(\alpha\)- but not \(\beta\)-ENaC, suggesting that the basal expression of \(\alpha\)-ENaC mRNA requires the basal level of \([\text{Ca}^{2+}]\). These observations suggest that a \(\text{Ca}^{2+}\)-dependent, but CaM-independent, pathway is essentially required for the signal transduction of the hypotonic stress to stimulate \(\alpha\)- and \(\beta\)-ENaC transcription. BAPTA-AM and W7 decreased the basal level of \(\gamma\)-ENaC mRNA expression by 90% and 70%, respectively. This observation suggests that \(\text{Ca}^{2+}\) has a role in the basal expression of ENaC subunits.
that the mRNA expression of γ-ENaC at the basal level is dependent on Ca\textsuperscript{2+}/CaM-dependent signaling pathways. Under the hypotonic condition, BAPTA-AM and W7 significantly diminished the hypotonicity-induced expression of γ-ENaC mRNA, although the hypotonic stress showed its stimulatory action on the mRNA expression of γ-ENaC, even in the presence of BAPTA-AM or W7 (Fig. 5C). These observations suggest that Ca\textsuperscript{2+}/CaM-dependent signaling pathways play important key roles in the hypotonic stress-induced expression of γ-ENaC mRNA.

Fig. 4. Effects of hypotonic stress on transcription of α-, β-, and γ-subunits of epithelial Na\textsuperscript{+} channel (ENaC). A: hypotonic stress stimulated α-ENaC transcription within 3 h, and hypotonic stress-induced high level of α-ENaC mRNA expression was maintained over 12 h. B: hypotonic stress significantly increased β-ENaC transcription within 3 h, and hypotonic stress-induced high level of β-ENaC mRNA expression was sustained over 12 h. C: in contrast to α- and β-ENaC, transcription of γ-ENaC was rapidly increased by hypotonic stress, reaching its maximum level at ~3 h, and hypotonic stress-induced high level of γ-ENaC mRNA expression was maintained over ensuing 12 h. Values are means ± SE [n = 6 (A and C) and 5 (B)]. *P < 0.05 vs. control under isotonic condition.

Fig. 3. Effect of ionomycin on SGK1 protein and mRNA expression. A: induction of phosphorylated SGK1 protein by 1 μM ionomycin, hypotonic stress, or 1 μM dexamethasone. Arrow shows single ~56-kDa band indicating SGK1. Each blot is representative of results from 4 different experiments. B: relative intensity of phosphorylated SGK1 protein induced by 1 μM ionomycin, hypotonic stress, or 1 μM dexamethasone. Ionomycin alone stimulated SGK1 protein expression at the same level as dexamethasone alone. Induction of SGK1 protein by hypotonic stress was significantly larger than that by ionomycin or dexamethasone. Values are means ± SE [n = 4]. *P < 0.05 vs. control under isotonic condition. #P < 0.05 vs. 3 h of hypotonic stress. C: time course of ionomycin action on SGK1 mRNA expression. Under isotonic condition, 1 μM ionomycin stimulated transcription of SGK1. Values are means ± SE [n = 4]. *P < 0.05 vs. control under isotonic condition. D: dose-dependent effects of ionomycin on SGK1 mRNA expression. Total RNA was harvested 3 h after application of ionomycin. Ionomycin stimulated SGK1 transcription in a dose-dependent manner, with half-maximal effect at ~500 nM. Values are means ± SE [n = 4].
Effects of BAPTA-AM on hypotonicity-provoked Na\(^+\) current and conductance. We have demonstrated that Ca\(^{2+}\)-dependent signaling cascades participate in the gene regulation of SGK1 and ENaC subunits. To determine whether Ca\(^{2+}\)-dependent signal transduction systems functionally play critical roles in hypotonicity-induced transepithelial Na\(^+\) reabsorption, we tested the effect of BAPTA-AM on hypotonicity-induced transepithelial Na\(^+\) reabsorption. As reported previously (33, 37), in A6 epithelia the \(I_{sc}\) observed under the hypotonic condition consists of the transepithelial Na\(^+\) transport characterized as the amiloride-sensitive \(I_{sc}\). A6 epithelia mounted on modified Ussing chambers were preincubated bilaterally with 20 \(\mu\)M BAPTA-AM or vehicle for 30 min in a serum-free medium and subsequently exposed to a serum-free hypotonic medium containing 20 \(\mu\)M BAPTA-AM or vehicle. Benzamil (10 \(\mu\)M, an amiloride derivative and a more potent, specific blocker of ENaC) was added to the apical side 180 min after application of hypotonic stress to measure transepithelial Na\(^+\) transport. As shown in Fig. 6A, the initial increases in \(I_{sc}\) up to 50 min after application of hypotonic stress were not significantly affected by BAPTA-AM, but \(I_{sc}\) was significantly diminished by BAPTA-AM 60 min after the hypertonic treatment. In Fig. 6B, we show the time course of the BAPTA-AM-sensitive \(I_{sc}\) and the BAPTA-AM-insensitive \(I_{sc}\) under the hypotonic stress condition. The BAPTA-AM-sensitive phase slowly appeared 30–60 min after exposure of the cell to hypotonicity and then increased with time up to 3 h. On the other hand, the BAPTA-AM-insensitive phase rapidly appeared within 10 min, showing its peak at \(~90\) min, and then gradually decreased. The time course of the BAPTA-AM-sensitive phase (Fig. 6B) was identical to that of active SGK1 protein expression (Fig. 1). At 180 min after application of hypotonic stress, the benzamil-sensitive \(I_{sc}\) and \(G_{t}\) were significantly smaller in cells treated with BAPTA-AM than in control cells (Fig. 6, C and D). BAPTA-AM did not affect the benzamil-sensitive \(I_{sc}\) or \(G_{t}\) under the isotonic condition (Fig. 6, C and D). Since W7 gradually increased \(G_{t}\) to levels that did not allow us to accurately measure electrophysiological parameters (i.e., the monolayers became leaky), it was impossible to obtain reliable \(I_{sc}\) and \(G_{t}\) in the presence of W7 for sufficient time to clarify the role of Ca\(^{2+}\)/CaM-dependent signaling pathways in the hypotonicity-induced Na\(^+\) transport. The leakiness of the monolayers caused by W7 might be due to cell death. Therefore, we used trypan blue to clarify cell viability and found that \(>97\%\) of W7-treated cells were not stained; i.e., most of the cells treated with W7 were intact. Nevertheless, these results confirm that Ca\(^{2+}\)/related signal transduction pathways actually function in the delayed phase of hypotonicity-provoked Na\(^+\) reabsorption via ENaC activity.

**DISCUSSION**

Hypotonic stress stimulates mRNA expression of SGK1 and ENaC subunits. As shown in Figs. 1 and 4, hypotonic stress stimulated transcription of Na\(^+\) transport-related genes, SGK1 and three subunits of ENaC. To our knowledge, this is the first report documenting the time courses of hypotonicity-stimulated mRNA expression of ENaC subunits. The stimulated pattern of \(\beta\)-ENaC mRNA expression by hypotonic stress was identical to that of \(\alpha\)-ENaC mRNA. Stimulation of \(\alpha\)- and \(\beta\)-ENaC mRNA expression was first observed within 3 h after application of hypotonic stress in a serum-free medium and was still increasing at 12 h, whereas the expression level of \(\gamma\)-ENaC mRNA began to increase more rapidly, reaching its maximum level \(~3\) h after application of hypotonic stress, and was sustained at around the maximum level over the ensuing 12 h (Fig. 4). The difference between the patterns of hypotonicity-induced stimulation of mRNA expression of ENaC subunits indicates that hypotonic stress regulates the genes of ENaC subunits via individual pathways. Indeed, glucocorticoid stimulates \(\alpha\)-ENaC, but not \(\beta\)- or \(\gamma\)-ENaC, in the CCD (3, 7, 23). A glucocorticoid response element in the \(\alpha\)-ENaC promoter participates in this gene regulation (34). Similarly, the promoter of each ENaC subunit may have an individual set of sites where hypotonicity-activated transcription factors can bind for stimulation of a downstream gene.

The time course of the hypertonic stress induction of SGK reported by Bell et al. (5) and Waldegger et al. (39) is similar to or even slower than that reported in our present study (1 h...
after hypotonic stress), although the mechanism of hypertonic induction of SGK (p38-dependent mechanism) is totally different from that in hypotonic induction of SGK (intracellular Ca\(^{2+}\)-dependent mechanism). The induction of SGK by hypertonic stress in A6 cells has also been reported by Rozansky et al. (33), who describe somewhat faster (30 min after the start of hypertonic stimulation) hypotonic induction of SGK protein than in our present study (1 h after hypotonic stress). The NaCl concentration in the culture medium used in the study of Rozansky et al. was much lower than that used in our present study. The lower NaCl concentration in the culture medium (33) might cause the faster time course in the hypotonic stress induction of SGK protein by affecting the process of transcription and translation of SGK.

**Ca\(^{2+}\)/CaM-dependent SGK1 gene regulation under hypotonic stress.** SGK1 has been originally identified as a “serum- and glucocorticoid-inducible kinase” in a rat mammary tumor cell line (41). In addition to corticosteroids, SGK1 gene transcription has been shown to be stimulated in a tissue-specific manner by ischemic injury in the brain (15), changes in cell volume (22, 40), and hypertonic stress (5). Recently, Rozansky et al. (33) reported that, in renal epithelial A6 cells, hypertonic, rather than hypotonic, stress stimulates SGK1 transcription through an unknown mechanism. Since the MKK3/MKK6-p38/MAPK stress-signaling cascade has been clarified to be involved in hypertonic stress-induced stimulation of SGK1 transcription, we tested the effect of the p38/MAPK cascade inhibitor SB-203580 on the hypertonic stimulation of SGK1 mRNA. However, SB-203580 did not affect the hypertonic action on SGK1 induction (data not shown). Thus the signaling cascade involved in hypotonic stimulation of SGK1 transcription is distinct from the MKK3/MKK6-p38/MAPK cascade. Although polarized A6 epithelia show a biphasic rise in [Ca\(^{2+}\)], in response to hypotonic stress (19), no reports indicate whether the rise in [Ca\(^{2+}\)], caused by hypotonic stress plays a physiological role in regulation of gene expression. In particular, we have no information on the role of intracellular Ca\(^{2+}\) in transcriptional regulation of SGK1 via the Ca\(^{2+}\)/CaM cascade. Our observations in Fig. 2 suggest that the Ca\(^{2+}\)/CaM cascade plays an important role in the hypotonicity-induced SGK1 transcription. To confirm the role of [Ca\(^{2+}\)], in SGK1 induction, we investigated the effect of ionomycin on the expression of SGK1 mRNA and protein under an isotonic condition. As shown in Fig. 3, the increase in [Ca\(^{2+}\)], can stimulate SGK1 transcription followed by SGK1 protein induction under an isotonic condition. These data show for the first time that hypotonic stress increases SGK1 expression through the Ca\(^{2+}\)/CaM-dependent signaling cascade.

Next, we tried to determine the stimulants for transcription factor(s) activating SGK1 transcription downstream from the Ca\(^{2+}\)/CaM cascade. Calmodulin is known to regulate three signaling pathways (21): I) protein phosphatase 2B, 2) Ca\(^{2+}\)/CaM-dependent protein kinase II, and 3) myosin light chain kinase. Pretreatments with cyclosporin A (an inhibitor of protein phosphatase 2B), KN93 (an inhibitor of Ca\(^{2+}\)/CaM-dependent protein kinase II), or ML-7 (an inhibitor of myosin light chain kinase) did not influence the hypotonic action on SGK1 transcription (data not shown). Besides these three pathways, CaM has many downstream signaling pathways. We have not yet identified the signaling pathway downstream from CaM that stimulates SGK1 transcription.

**Gene regulation of ENaC under hypotonic stress via Ca\(^{2+}\)-dependent mechanisms.** The results obtained with BAPTA-AM and W7 in Fig. 5 suggest that stimulation of α- and β-ENaC expression by hypotonic stress is mediated through a Ca\(^{2+}\)-dependent, CaM-independent pathway, whereas a Ca\(^{2+}\)/CaM-dependent pathway is required for stimulation of γ-ENaC.
transcription by hypotonicity. Since SGK1 stimulates α- and β-ENaC subunit transcription via the activation of unidentified transcription factors in Madin-Darby canine kidney cells (7), there is a possibility that hypotonic stress increases in α- and β-ENaC transcription by activating SGK1 protein. However, the present study indicates that the hypotonic stress-induced stimulation of SGK1 protein expression required a Ca\textsuperscript{2+}/CaM-dependent pathway, but the Ca\textsuperscript{2+}/CaM-dependent pathway was not required for the mRNA expression of α- or β-ENaC. These observations do not support this possibility. On the other hand, γ-ENaC mRNA expression was sensitive to BAPTA-AM and W7 under the basal and hypotonic conditions, and the rapid induction of γ-ENaC mRNA by hypotonicity was similar in its time course to that of SGK1 mRNA by hypotonicity and ionomycin, although the hypotonicity-induced high level of γ-ENaC mRNA expression was maintained for up to 12 h. So, a part of the hypotonic stress-induced stimulation of the ENaC-mediated epithelial Na\textsuperscript{+} transport would be mediated via an increase in ENaC function due to elevation of γ-ENaC expression caused by SGK1 expression mediated by a Ca\textsuperscript{2+}/CaM-dependent pathway.

Role of intracellular Ca\textsuperscript{2+} as the second messenger of hypotonic stress in hypotonicity-induced Na\textsuperscript{+} transport. Because of a number of conflicting findings regarding the role of [Ca\textsuperscript{2+}], in Na\textsuperscript{+} transport, it is difficult to reach a general agreement. Awayda et al. (4) reported that a decrease of extracellular pH causes an increase in the number of ENaC in the apical membrane and that chelation of [Ca\textsuperscript{2+}] by BAPTA abolished the effect of pH on the number of ENaC. On the other hand, Ishikawa et al. (16) reported an inhibitory effect of intracellular Ca\textsuperscript{2+} on Na\textsuperscript{+} transport in Madin-Darby canine kidney cells expressing rat ENaC. They observed a downregulation of the activity of ENaC by increasing [Ca\textsuperscript{2+}], by the use of ionomycin. Recently, Jans et al. (19) studied the effect of [Ca\textsuperscript{2+}], on the stimulation of Na\textsuperscript{+} transport in renal epithelial A6 cells. They conclude that extracellular, but not intracellular, Ca\textsuperscript{2+} is essentially required for the initial trigger for hypotonicity-induced stimulation of Na\textsuperscript{+} transport through activation of a putative Ca\textsuperscript{2+}-sensing receptor and that the [Ca\textsuperscript{2+}], changes under the lowered osmotic conditions are not required for stimulation of Na\textsuperscript{+} transport. On the other hand, our observations suggest that the increase in [Ca\textsuperscript{2+}], is involved in the delayed phase (>60 min) of hypotonicity-induced Na\textsuperscript{+} transport through transcriptional stimulation of SGK1 and ENaC subunit mRNA, although the hypotonicity-provoked Na\textsuperscript{+} transport in the early phase (<60 min) does not require a change in [Ca\textsuperscript{2+}]. The difference in conclusions between our group and Jans et al. would be due to the difference in the experimental conditions. In contrast to the study of Jans et al., compositions of the bathing solutions in all experiments of the present study were sufficient to maintain cellular functions, including protein synthesis (Fig. 1C), and $I_{\text{sc}}$ was measured over a period (180 min) that was long enough to detect the influence of the hypotonic stress-induced increase in [Ca\textsuperscript{2+}], on gene expression (Fig. 6). On the basis of these studies, we conclude that, under a lowered osmotic condition, the intracellular Ca\textsuperscript{2+} plays a role as the second messenger of hypotonic stress in the stimulation of Na\textsuperscript{+} transport in the delayed phase (>60 min), but not in the early phase (<60 min). These complicated roles of [Ca\textsuperscript{2+}], in Na\textsuperscript{+} transport reported by several groups may implicate the delicate mechanism modulating the rate of renal Na\textsuperscript{+} transport; however, further studies are required to clarify the mechanism in detail.

**Two-step model of hypotonicity-induced Na\textsuperscript{+} transport.** The present study shows that hypotonicity stimulates transcription of SGK1 and all three ENaC subunits and that the Ca\textsuperscript{2+}- dependent signals are involved in the regulation of ENaC and SGK1 transcription for stimulation of renal transepithelial Na\textsuperscript{+} reabsorption. We propose a two-step model for ENaC regulation by hypotonic stress (Fig. 7). In the early phase of the hypotonic stress-induced signaling pathway, several types of RTKs are activated by hypotonic stress without ligand binding; then phosphatidylinositol 3-kinase, activated via the RTK/JNK cascade, upregulates preexisting transport machinery involved in an increase of the ENaC protein in the apical membrane or increases the channel activity of an individual ENaC (29, 31). In the delayed phase of the hypotonic stress-induced signaling pathway, the Ca\textsuperscript{2+}-dependent signal transduction system activated by the hypotonicity-induced rise in [Ca\textsuperscript{2+}], (19) stimulates SGK1 and ENaC transcription. The newly synthesized active SGK1 protein is involved in translocation of the preexisting ENaC in the cytosolic space to the apical membrane (2, 7, 20, 25, 30), whereas the stimulation of ENaC transcription contributes to the increase in the total amount of ENaC, although unidentified mechanisms are maintained for stimulation of γ-ENaC transcription.

![Fig. 7. Model of hypotonic stress-induced transepithelial Na\textsuperscript{+} transport. Hypotonic stress-induced transepithelial Na\textsuperscript{+} transport consists of 2 phases. During the early phase (nongenomic action), hypotonic stress transactivates the receptor-type tyrosine kinase (RTK/JNK cascade to activate phosphatidylinositol 3-kinase, increasing channel activity of preexisting ENaC and stimulating translocation of preexisting ENaC into the apical membrane from the cytosolic space. During the delayed phase (genomic action), intracellular Ca\textsuperscript{2+} plays a role as second messenger of hypotonic stress by stimulating transcription of SGK1 and 3 ENaC subunits. SGK1 phosphorylates proteins downstream, elevating ENaC function via activation and translocation of preexisting ENaC. De novo synthesis of ENaC is also involved in hypotonic stress-induced stimulation of Na\textsuperscript{+} transport.](http://ajprenal.physiology.org/)
Ca\(^{2+}\) REGULATES SGK1 AND ENaC TRANSCRIPTION

GRANTS

This work was supported by Japan Society for the Promotion of Science Grants-in-Aid 17390057, 17590191, 18659056, and 19590212, the Fuji Foundation for Protein Research, Salt Science Research Foundation Grant 0736, and a Leading Project for Biosimulation from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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