Role of SGK in hormonal regulation of epithelial sodium channel in A6 cells

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Role of SGK in hormonal regulation of epithelial sodium channel in A6 cells. Am J Physiol Cell Physiol 284: C404–C414, 2003. First published October 16, 2002; 10.1152/ajpcell.00398.2002.—The purpose of this study was to examine the role of the serum- and glucocorticoid-induced kinase (SGK) in the activation of the epithelial sodium channel (ENaC) by aldosterone, arginine vasopressin (AVP), and insulin. We used a tetracycline-inducible sodium channel (ENaC) by aldosterone, arginine vasopressin-induced kinase (SGK) in the activation of the epithelial membrane.

Aldosterone-induced protein in mammalian and amphibian renal cells (5, 17), and, most significantly, SGK activates ENaC when the two proteins are expressed together in Xenopus oocytes (5, 17) or in renal epithelial cells (8). Activation of SGK is controlled by the phosphoinositide 3-kinase (PI3-kinase) pathway, which promotes the phosphorylation of SGK at serine 422 (human SGK), which in turn increases the phosphorylation of threonine 256 by phosphatidylinositol-dependent kinase (PDK) 1. Phosphorylation of these two residues renders the kinase active (13, 19). There is indirect evidence linking aldosterone to the activation of SGK by PI3-kinase. First, inhibition of PI3-kinase by the specific and reversible blocker LY-294002 decreases the basal levels of ENaC activity and abolishes the aldosterone effects (4, 20). Second, aldosterone increases one of the products of PI3-kinase, phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3], in A6 cells (4).

Hormones other than aldosterone, such as insulin and arginine vasopressin (AVP), also modulate the activity of ENaC in the kidney. It has been suggested that the actions of these hormones may also require SGK. In muscle and adipose tissues PI3-kinase plays a central role in insulin signaling, making possible the participation of SGK in the insulin-mediated regulation of ENaC (21). Recently, Faletti et al. (8) found that expression of one kinase-inactive SGK mutant (SGKp222A), but not expression of a different inactive mutant (SGKS422A), abrogated the insulin response in A6 cells.

Involvement of SGK in the AVP response has been proposed in light of the finding that cAMP analogs can activate SGK (22), although this result was not confirmed by another group (26). AVP binding to V2 receptors in principal cells of the rat cortical collecting duct increases the intracellular levels of cAMP, which in turn activates amiloride-sensitive sodium permeability (25). Faletti et al. (8) found that transfection of wild-type SGK in A6 cells increased the AVP effect, whereas SGKp222A abolished the response and another kinase-inactive mutant, SGKS422A, did not show any effect. However, a caveat in this latter study is that the authors could not detect expression of the transfected SGK proteins.
In this study, we investigated the role of SGK in the modulation of ENaC activity by hormones in renal epithelial cells. We used as a model the A6 cell line derived from frog kidney, which expresses endogenous ENaC and SGK (5) and responds to aldosterone, insulin, and AVP by increasing amiloride-sensitive transepithelial sodium transport. The experiments indicate that SGK targets a mechanism that is common to the insulin and AVP signaling pathways but does not seem to be shared by the aldosterone response.

MATERIALS AND METHODS

Plasmid constructs. Full-length Xenopus SGK cDNA was amplified by RT-PCR from aldosterone-treated A6 cells. Specific primers were designed according to the published sequence (5), and restriction sites were added to the 5'- and 3'-ends to facilitate cloning. The amplified fragment was cloned in pcDNA4/TO (Invitrogen, Carlsbad, CA). Point mutations were introduced by PCR with the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). All constructs were sequenced at the Keck Facility at Yale University. Plasmid pcDNA6TR, containing the coding sequence for the tetracycline repressor, was obtained from Invitrogen.

Antibody generation. A polyclonal anti-SGK antibody was generated in rabbits. Animals received subcutaneous injections of a gluthathione S-transferase (GST) protein fused to a segment of the mouse SGK comprised of amino acids 301 to 405. The GST-SGK fusion protein was expressed in Escherichia coli and affinity purified with a gluthathione-agarose column.

Cell culture, transfection, and cell line generation. Experiments were performed on A6-S2 cells (11), a clone obtained by limiting dilution of A6 cells derived from the kidney of Xenopus laevis and selected for high transepithelial resistance (R_T) and responsiveness to hormones. These cells were kindly provided by Dr. John Hayslett, Yale University. Cells were maintained in amphibian medium (0.75× DMEM, 10% FBS, buffered with sodium bicarbonate) in an incubator set at 27°C and 1.5% CO_2. Cells expanded in plastic dishes were seeded on Transwell permeable supports (Corning, Corning, NY) for biochemical experiments (4.7-cm² filters) or for electrical measurements (0.33-cm² filters). After 10–14 days in culture, cells were washed twice in serum-free medium and maintained without serum for two more days before experiments were performed. Aldosterone (Sigma, St. Louis, MO) was added to a final concentration of 100 nM. Tetracycline (Invitrogen) was added to a final concentration of 1 μg/ml. LY-294002 was obtained from Calbiochem (La Jolla, CA) and was used at a final concentration of 50 μM. Insulin and AVP were obtained from Sigma and used at the final concentrations of 100 nM and 1 μM, respectively. When ethanol or DMSO was the solvent of stock solutions, the final dilution was 1:500 or 1:1,000, depending on the experiment. Control experiments included ethanol or DMSO at the same dilution and showed no effect.

Transfection of A6 cells grown on plastic was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. A 5:1 mixture of pcDNA6TR and pcDNA4/TO-SGK plasmids was used in every transfection. Stable cell lines expressing the tetracycline repressor and pcDNA4/TO-SGK constructs were obtained by growth on selective media containing 500 μg/ml zeocin and 10 μg/ml blasticidin. Clones were tested for SGK expression on induction with 1 μg/ml tetracycline and development of high R_T when grown on filters. CHO cells were obtained from the American Type Culture Collection and transiently transfected with Lipofectamine 2000 (Invitrogen).

RT-PCR. Total RNA was extracted from A6 cell lines grown on filters with the RNeasy kit from Qiagen (Valencia, CA) following the manufacturer’s instructions. RNA concentration was measured by absorption spectroscopy. One microgram of RNA was used for first-strand cDNA synthesis with the SuperScript system (Invitrogen). Five percent of each RT reaction was used as a template for amplification of full-length heterologous SGK by PCR with a forward primer specific for Xenopus SGK and a reverse primer specific for the pcDNA4/TO-poly-linker region. Full-length Xenopus β-actin was amplified as a control for each sample with specific primers designed by using the published sequence (GenBank accession number AF079161).

Western blotting. A6 cells grown on plastic or filters were washed twice with ice-cold PBS, scraped in the same buffer, recovered by centrifugation, and lysed with SDS-PAGE loading buffer. Samples were separated by electrophoresis in 10% SDS-PAGE gels and transferred to Immobilon-P (Millipore, Bedford, MA). After blocking with 5% dry milk, the membranes were probed with anti-SGK antibody at 1:5,000 dilution. Secondary anti-rabbit IgG labeled with peroxidase (Sigma) was used at 1:10,000. The signal was developed with ECL + (Amersham), and blots were exposed to BioMax MR film (Eastman Kodak, New Haven, CT). In some experiments the intensity of the SGK signal was quantified by densitometric analysis with a GS-800 densitometer (Bio-Rad Laboratories, Hercules, CA).

Equivalent short-circuit current measurement. Transepithelial voltage (V_T) and equivalent short-circuit current (I_E) across A6 monolayers were measured as previously described (1). R_T was calculated from V_T and I_E by Ohm’s law.

Statistical analysis. Data points represent the means ± SE of n independent experiments. Differences between groups were evaluated by nonpaired t-test. P and n values are given in the text or Figs. 1–10 for each experiment.

RESULTS

Tetracycline-inducible expression of SGK in A6 cells. By using a tetracycline-regulated expression system we could control the expression of SGK independently from aldosterone and other extracellular stimuli. Thus we generated stable A6 cell lines coexpressing the tetracycline repressor protein (TetR) together with various forms of SGK (SGK^7). Transcription of both cDNAs was controlled by a cytomegalovirus (CMV) promoter, but SGK^7 had in addition two copies of the tetracycline operator (TetO) inserted in tandem downstream of the CMV promoter (Fig. 1A). Constitutively expressed TetR binds to the TetO, repressing the transcription of SGK^7. Addition of tetracycline releases the TetR from the TetO, and the CMV promoter drives expression of the kinase (Fig. 1A).

Tetracycline-induced SGK^7 expression was examined by RT-PCR with a pair of primers specific for the transfected SGK to differentiate it from the endogenous transcript. The tetracycline effect was rapid because a transcript could be detected by RT-PCR in as short as 30 min after the addition of the drug (Fig. 1B).
The repression system was remarkably tight, because no transcript could be detected in the absence of tetracycline even with RT-PCR. Actin was amplified in every condition as a control for the integrity and amount of the RNA added to the reaction (Fig. 1B).

To detect SGKT protein, we raised an anti-SGK polyclonal antibody by immunizing rabbits with the carboxy terminus of mouse SGK fused to GST. The immune serum specifically detected Xenopus SGK in transiently transfected CHO cells examined by Western blotting (Fig. 1C). No signal was detected in nontransfected cells or when the cognate peptide was added to the reaction. The preimmune serum also did not produce a signal (Fig. 1C). It is worth noting that we consistently observed slower than predicted SGK migration on SDS-PAGE. SGK molecular mass deduced from the sequence is 49.1 kDa, and it migrates at around 56 kDa. Another group, using an unrelated antibody, also noted abnormal electrophoretic migration of SGK (5). The newly developed antibody detected the endogenous SGK protein induced by 100 nM aldosterone added to serum-starved A6 cells (Fig. 1D).

The tetracycline-inducible system was used to generate A6 cell lines conditionally expressing wild-type SGK (SGKwt; clones 1, 2, and 11) and SGK with point mutations S425D (SGKS425D; clone 1) or K130M (SGKK130M; clones 3 and 10). Substitution of serine 425 (equivalent to serine 422 in human SGK) for a negatively charged amino acid mimics the addition of a phosphate group, promoting phosphorylation by PDK1 and making the kinase constitutively active independent of PI3-kinase activity (13, 19). Mutation K130M replaces a lysine in the ATP-binding cassette of the protein (equivalent to lysine 127 in human SGK), abolishing kinase activity (13, 14, 19).
All cell lines exhibited similar kinetics of SGK induction. In Western blots, SGK was first detected 1–3 h after addition of tetracycline and peaked at 6–24 h (Fig. 1D).

Effects of SGK expression on transepithelial sodium transport and aldosterone action. To examine the effects of SGK expression on ENaC function we measured transepithelial sodium transport in the parental A6 cell line and in A6 cells transfected with SGKwt or mutants. Cells were seeded on permeable supports and cultured for 10–14 days. Cell monolayers displayed high $R_T$, on the order of 5–6 kΩ•cm². Cells were then maintained in serum-free medium for 2 days before experiments were performed. Control experiments showed that tetracycline does not affect $I_{sc}$, $V_T$, or $R_T$ in the A6 parental cell line or in A6 cells transfected with an empty vector and grown in the presence of the selective antibiotics (not shown).

Four independent clones were examined for SGKwt protein expression and electrical properties. Clone 2 was selected for further experiments because it exhibited basal $I_{sc}$ of a magnitude similar to that of the parental cell line. After 2 days in the absence of serum, the $I_{sc}$ value was stable, ranging from 4.0 ± 0.2 to 5.4 ± 0.3 μA/cm² over the 24 h of the experiment (Fig. 2). One hundred nanomolar aldosterone stimulated sodium transport in a time-dependent manner, reaching a fourfold increase after 24 h of treatment (from 4.0 ± 0.2 to 17.8 ± 1.1 μA/cm²). These values are consistent with our previously described (1) values in nontransfected A6 cells. Tetracycline-induced expression of SGKwt significantly increased $I_{sc}$ after 6 h of induction, reaching a maximum at 24 h (8.7 ± 0.6 μA/cm²), a 2.2-fold increase over basal values (Fig. 2A). The effects of aldosterone and SGKwt expression on $I_{sc}$ were additive, giving a 6.7-fold increase after 24 h of treatment (26.9 ± 2.2 μA/cm²). The statistical significance of the described differences was $P < 0.01$ in each case (n = 34–36 for each condition). Addition of 50 μM amiloride to the apical chamber completely inhibited both basal and stimulated $I_{sc}$, consistent with the notion that $I_{sc}$ reflects sodium flow through ENaC. A comparison of the time course of SGKwt protein induction (Fig. 1D) and $I_{sc}$ in the same clone (Fig. 2A) shows a significant delay in the activation of ENaC.

Increases in $I_{sc}$ induced by aldosterone or SGKwt were paralleled by hyperpolarization of the epithelium and a decrease in $R_T$ (Fig. 2, B and C, and Table 1).

In addition to clone 2, three other independent clones (1, 8, and 11) expressing SGKwt were studied. These clones exhibited different $I_{sc}$ under basal conditions: clone 1, 11.2 ± 2.9 μA/cm²; clone 8, 5.0 ± 1.8 μA/cm²; clone 11, 21.1 ± 0.7 μA/cm² (n = 6). Expression of SGKwt for 24 h increased $I_{sc}$: clone 1, 41.7 ± 4.2 μA/cm² (3.7-fold); clone 8, 7.2 ± 1.0 μA/cm² (1.4-fold); clone 11, 50.0 ± 1.7 μA/cm² (2.4-fold) (n = 6). For each of the four clones, $I_{sc}$ after 24 h of treatment was normalized to the corresponding control. The means of these values are represented in Fig. 3. Aldosterone alone induced an ∼3.5-fold increase in $I_{sc}$. SGKwt increased it by ∼2.5-fold, and the combination of both stimuli produced a 5.5-fold increase (Fig. 3).

As a control for the specificity of SGK effects, we used one of the stable cell lines expressing the inactive mutant SGKK130M (clone 3; Fig. 1D). Cells were treated
overnight with 1 µg/ml tetracycline to maximize the levels of expression of SGK<sup>T</sup> K<sub>130M</sub> (Fig. 1D). The presence of the mutant kinase did not affect the basal levels of current in A6 cells (Fig. 4). Addition of 100 nM aldosterone increased <i>I</i><sub>sc</sub> by threefold in 6 h (from 11.7 to 35.6 µA/cm<sup>2</sup>), a change identical to that in the parental cell line.

We also determined the effects of expressing a constitutively active mutant of SGK, SGK<sup>T</sup> S<sub>425D</sub>, on ENaC activity. Three different clones were tested for SGK<sup>T</sup> S<sub>425D</sub> expression and electrical properties. Clone 1 was selected for further experiments because it exhibits <i>I</i><sub>sc</sub> similar to the parental cell line. Under basal conditions clone 1 had <i>I</i><sub>sc</sub> of 4.8 ± 0.4 µA/cm<sup>2</sup>, and aldosterone induced a fourfold increase by 24 h (19 ± 0.9 µA/cm<sup>2</sup>) (Fig. 5A). Induction of expression of SGK<sup>T</sup> S<sub>425D</sub> with tetracycline progressively increased <i>I</i><sub>sc</sub>. A small but significant change over the control value was observed 1 h after induction (from 4.9 ± 0.4 to 7.4 ± 0.8 µA/cm<sup>2</sup>, <i>n</i> = 32–40; *<i>P</i> < 0.02). After 24 h <i>I</i><sub>sc</sub> increased by 8.3-fold (39.7 ± 2.1 µA/cm<sup>2</sup>). A combination of aldosterone and tetracycline gave a 14.5-fold increase over basal conditions (69.4 ± 2.6 µA/cm<sup>2</sup>). This experiment shows that the correlation of SGK<sup>T</sup> S<sub>425D</sub> protein levels and the increase in <i>I</i><sub>sc</sub> is very good in the A6-SGK<sup>T</sup> S<sub>425D</sub> cell line and is much better than in the A6-SGK<sup>T</sup> cell line.

The increase in <i>I</i><sub>sc</sub> was accompanied by hyperpolarization of the epithelium and a decrease in <i>R</i><sub>T</sub>, respectively. The electrical parameters of this A6-SGK<sup>T</sup> S<sub>425D</sub> cell clone are summarized in Table 1.

In addition to clone 1, two other independent clones expressing SGK<sup>T</sup> S<sub>425D</sub> were studied (clones 7 and 22). On average, aldosterone increased <i>I</i><sub>sc</sub> by 3.6-fold, whereas expression of SGK<sup>T</sup> S<sub>425D</sub> produced a 5.1-fold increase. Simultaneous addition of aldosterone and tetracycline produced additive effects, increasing <i>I</i><sub>sc</sub> by ninefold (Fig. 6A). The amount of SGK<sup>T</sup> S<sub>425D</sub> protein was different in the various clones, as shown in the Western blot in Fig. 6B. The values of <i>I</i><sub>sc</sub> in these clones varied in direct relation with the amount of expressed SGK<sup>T</sup> S<sub>425D</sub> (Fig. 6, C and D). The data also show that the effect of

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Table 1. Electrical parameters of A6 cell lines expressing SGK<sup>T</sup> wt and SGK<sup>T</sup> S<sub>425D</sub>

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<th>Control</th>
<th>Aldosterone</th>
<th>Tetracycline</th>
<th>Aldosterone + Tetracycline</th>
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<tr>
<td>&lt;i&gt;I&lt;/i&gt;&lt;sub&gt;sc&lt;/sub&gt;, µA/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.0 ± 0.2</td>
<td>17.8 ± 1</td>
<td>8.7 ± 0.6</td>
<td>26.9 ± 2.2</td>
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<tr>
<td>&lt;i&gt;V&lt;/i&gt;&lt;sub&gt;r&lt;/sub&gt;, mV</td>
<td>-24.1 ± 1.7</td>
<td>-57.8 ± 1.6</td>
<td>-42.7 ± 2.5</td>
<td>-72 ± 3.2</td>
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<tr>
<td>&lt;i&gt;R&lt;/i&gt;&lt;sub&gt;T&lt;/sub&gt;, kΩ·cm&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>5.4 ± 0.3</td>
<td>3.7 ± 0.3</td>
<td>4.5 ± 0.2</td>
<td>2.6 ± 0.2</td>
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Values are means ± SE. SGK, serum- and glucocorticoid-dependent kinase; A6-SGK<sup>T</sup> wt, A6 cells expressing tetracycline repressor protein (TetR); and wild-type SGK AG-SGK<sub>S425D</sub>, A6 cells expressing TetR and SGK with S425D mutation; <i>I</i><sub>sc</sub>, short-circuit current; <i>V</i><sub>r</sub>, transepithelial voltage; <i>R</i><sub>T</sub>, transepithelial resistance.

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Fig. 3. The effects of aldosterone, SGK<sup>T</sup> wt expression, or a combination of both on <i>I</i><sub>sc</sub> after 24 h of treatment were studied in 4 independent A6-SGK<sup>T</sup> wt clones grown on filters. For each independent clone, peak <i>I</i><sub>sc</sub> was normalized to its own control. Bars represent the means ± SE of the 4 normalized values. The differences between each treatment and control condition are statistically significant (*<i>P</i> < 0.05, <i>n</i> = 4).

Fig. 4. Effects of kinase-inactive mutant SGK<sup>T</sup> K<sub>130M</sub> expression on transepithelial sodium transport. Cells were grown on permeable supports treated overnight with 1 µg/ml tetracyline to induce expression of SGK<sup>T</sup> K<sub>130M</sub> (clone 3). Aldosterone (100 nM) was added to the basolateral side, and <i>I</i><sub>sc</sub> was monitored after 1, 3, 6, and 24 h. Each data point represents the mean ± SE value of 26–30 measurements.
ton I_sc is proportional to the abundance of the active kinase.

To test further the effect of aldosterone and SGK on the stimulation of ENaC, we sequentially added tetracycline and aldosterone to A6-SGK<sup>S425D</sup> cells (clone 1). The order of addition did not modify the final I_sc value, which was equal to that induced by both stimuli added together (Fig. 7, A and B).

**PI3-kinase role in SGK-stimulated sodium transport.**

We took advantage of the A6 cell line expressing the constitutively active mutant SGK<sup>S425D</sup> to study the role of PI3-kinase on sodium transport. SGK depends on PI3-kinase for its activation by PDK1 and PDK2, but the mutant SGK<sup>S425D</sup> is independent of PI3-kinase (13). It was demonstrated previously that SGK<sup>S425D</sup> on I_sc is proportional to the abundance of the active kinase.

Fig. 5. Effects of SGK<sub>S425D</sub> expression on transepithelial sodium transport. A6-SGK<sub>S425D</sub> cells (clone 1) were grown on permeable supports. After 2 days in serum-free medium, cells were stimulated with 100 nM aldosterone, 1 μg/ml tetracycline, or a combination of both. I<sub>sc</sub> (A), V<sub>T</sub> (B), and R<sub>T</sub> (C) were measured at the indicated times. Each data point is the mean ± SE of 32–40 independent measurements. All data points after the start of the treatments are significantly different from control values (P < 0.02 at 1 h time point, P < 0.01 at later time points).

Fig. 6. SGK<sub>S425D</sub> levels of expression correlate with magnitude of I<sub>sc</sub> from independent clones. A: I<sub>sc</sub> was measured in 3 independent A6-SGK<sub>S425D</sub> clones after 24 h of induction with 1 μg/ml tetracycline. I<sub>sc</sub> values from each clone were normalized to their own controls. Values represent means ± SE for the 3 clones. The differences between each treatment and control condition are statistically significant (*P < 0.05, **P < 0.01; n = 4). B: Western blot showing SGK<sub>S425D</sub> levels of expression in clones 1, 7, and 22 after addition of tetracycline (Tet). C: quantification of SGK<sub>S425D</sub> by Western blotting (shown in B) expressed as amount of protein relative to the highest-expressing clone (clone 1). D: normalized I<sub>sc</sub> of clones 1 (n = 32–40; P < 0.01 vs. control), 7 (n = 6; P < 0.01), and 22 (n = 6; P < 0.01) after 24 h of SGK<sub>S425D</sub> expression, represented as means ± SE.
inhibition of PI3-kinase with LY-294002 decreases basal $I_{sc}$ and abolishes the aldosterone response (4, 20). If the PI3-kinase effect is entirely due to SGK activation, inhibitors of PI3-kinase should not have an effect on SGK$^{S425D}$-induced $I_{sc}$. A6-SGK$^{S425D}$ cells were grown on permeable supports for 14 days and serum starved for another 2 days before the experiment. Six different conditions were studied over a time period of 24 h (Fig. 8). 1) Control cells showed stable $I_{sc}$ over the entire experiment ($6.7 \pm 0.0 \, \mu A/cm^2$). 2) Aldosterone-treated cells reached $I_{sc}$ values of $20.8 \pm 0.8 \, \mu A/cm^2$ after 24 h, a 3.1-fold increase over control conditions (Fig. 8). 3) As previously described (4), 50 $\mu M$ LY-294002 rapidly decreased $I_{sc}$, inhibiting it completely after 18 h of treatment. 4) When the PI3-kinase inhibitor was added in combination with aldosterone it produced a biphasic effect, as noted previously by Wang et al. (33). $I_{sc}$ initially decreased but recovered after 3 h of treatment, maintaining steady, although low, levels (Fig. 8). 5) SGK$^{S425D}$ induced an increase in $I_{sc}$, reaching a peak of $54.2 \pm 2.5 \, \mu A/cm^2$ after 24 h of treatment. 6) This effect was significantly inhibited by LY-294002. After an initial small decrease, SGK$^{S425D}$ together with LY-294002 induced a rise in $I_{sc}$ that reached a peak of only $18.3 \pm 1.0 \, \mu A/cm^2$ after 6 h of treatment (Fig. 8). These results indicate that PI3-kinase participates in pathways that are necessary for the maintenance of transepithelial sodium transport and its modulation by aldosterone and SGK.

**SGK effects on insulin response.** We also studied the effects of SGK on insulin-mediated ENaC upregulation in A6 cells. Cells grown on filters and placed in serum-free medium for 2 days were treated with aldosterone, tetracycline, or a combination of both for 24 h. Insulin was then added to the basolateral side to a final concentration of 100 nM. $I_{sc}$ was measured after 30 min of incubation, 50 $\mu M$ amiloride was added to the apical chamber, and $I_{sc}$ was measured again.

The addition of insulin to A6-SGK$^{wt}$ cells (clone 2) grown on filters increased $I_{sc}$ from $3.3 \pm 0.0$ to $11.1 \pm 0.6 \, \mu A/cm^2$ (Fig. 9A). Pretreatment of cells with aldosterone for 24 h potentiated the insulin response, increasing the $I_{sc}$ values from $13 \pm 1.1$ to $33 \pm 2.2 \, \mu A/cm^2$, consistent with previous reports.
REGULATION OF ENaC BY SGK AND HORMONES

Fig. 9. SGK effects on insulin response. A6 cell lines expressing the different SGK proteins (SGK\textsubscript{wt}, SGK\textsubscript{S425D}, or SGK\textsubscript{K130M}) under the control of tetracycline were grown on filters and treated overnight with 100 nM aldosterone, 1 \(\mu\)g/ml tetracycline, or a combination of these agents. Inserts of each group were then divided into 2 groups. One group remained as a control, and the other group was treated with 100 nM insulin for 30 min before \(I_{sc}\) was measured. After addition of 50 \(\mu\)M amiloride \(I_{sc}\) was measured again, resulting in complete inhibition of the current in every condition. Amiloride-sensitive \(I_{sc}\) is represented. A: \(I_{sc}\) measurements in A6-SGK\textsubscript{wt} cells \((n = 9–11; *P < 0.01)\). B: \(I_{sc}\) values obtained from A6-SGK\textsubscript{S425D} cells \((n = 9; *P < 0.01)\). C: \(I_{sc}\) from A6-SGK\textsubscript{K130M} cells \((n = 9; *P < 0.05)\).

(23). When SGK\textsuperscript{wt} expression was induced for 24 h, insulin increased \(I_{sc}\) from 12.5 ± 1.2 to 19.6 ± 1.8 \(\mu\)A/cm\(^2\), but no potentiation was observed. Finally, insulin also increased \(I_{sc}\) in cells expressing SGK\textsuperscript{wt} and pretreated with aldosterone (31.7 ± 1.2 to 46.0 ± 1.8 \(\mu\)A/cm\(^2\)). Table 2 summarizes the increase in \(I_{sc}\) \((\Delta I_{sc})\) observed in each condition. Statistical analysis showed that the differences between control and insulin-treated cells in each group were significant in every case \((P < 0.01; n = 9–11)\). Addition of 50 \(\mu\)M amiloride to the apical chamber completely inhibited \(I_{sc}\), indicating that all the response induced by insulin is mediated by ENaC.

Insulin was also able to activate ENaC in A6-SGK\textsubscript{S425D} cells (clone 1), both in the absence and in the presence of aldosterone (Fig. 9B; \(P < 0.01\), \(n = 9\)). Without pretreatment the increase in \(I_{sc}\) was equal to that detected in the A6 cell line transfected with SGK\textsuperscript{wt} (Table 2). When expression of SGK\textsubscript{S425D} was induced, the insulin response was abolished (Fig. 9B).

The effects of SGK\textsubscript{K130M} expression were also tested (clone 3). In this cell line the insulin response was larger than in the parental line and other transfected clones. Insulin increased \(I_{sc}\) from 8.1 ± 1.7 to 25 ± 4.8 \(\mu\)A/cm\(^2\) in control cells (Fig. 9C), and in cells pretreated with aldosterone \(I_{sc}\) increased from 23.6 ± 4.3 to 37.0 ± 5.7 \(\mu\)A/cm\(^2\). This cell line, aldosterone did not potentiate insulin action (Table 2). However, it is significant that expression of SGK\textsubscript{K130M} did not affect the insulin response, either in the absence or in the presence of aldosterone (Fig. 9C, Table 2).

**SGK effects on AVP response.** The effects of SGK on AVP-mediated upregulation of ENaC activity were examined in the transfected A6 cell lines (Fig. 10). We followed the experimental protocol described above for the insulin response experiments. Briefly, control cells or cells previously stimulated with aldosterone, tetracycline, or both were exposed to 1 \(\mu\)M AVP. After 30 min, total and amiloride-sensitive \(I_{sc}\) were measured. The treatment with AVP induced a significant amiloride-insensitive \(I_{sc}\) because of the cAMP-dependent activation of an apical chloride conductance, as previously described (30).

Under control conditions, addition of AVP to A6-SGK\textsubscript{wt} (clone 2) increased amiloride-sensitive \(I_{sc}\) from a resting value of 3.3 ± 0.0 \(\mu\)A/cm\(^2\) to 12.8 ± 0.8 \(\mu\)A/cm\(^2\) (Fig. 10A). In cells pretreated with aldosterone, \(I_{sc}\) increased from 14.7 ± 0.8 to 47.3 ± 1.2 \(\mu\)A/cm\(^2\), confirming the synergistic effect between aldosterone and AVP. In cells in which expression of SGK\textsubscript{wt} was induced with tetracycline, AVP treatment increased \(I_{sc}\) from 10 ± 0.0 to 19.3 ± 0.7 \(\mu\)A/cm\(^2\). Thus SGK\textsubscript{wt} did not potentiate the AVP response (Table 2). When cells expressing SGK\textsubscript{S425D} and pretreated with aldosterone were exposed to AVP, \(I_{sc}\) increased from 35.3 ± 2.3 to 58.7 ± 1.3 \(\mu\)A/cm\(^2\). The differences described between control and AVP-treated groups were all statistically significant \((P < 0.01; n = 5;\) Fig. 10A).

The action of AVP on A6-SGK\textsubscript{S425D} cells in control conditions or pretreated with aldosterone was comparable to that obtained with A6-SGK\textsubscript{wt} cells or the parental cell line (Fig. 9B; see also Table 2 for \(\Delta I_{sc}\))
values). When SGK<sup>T</sup><sub>S425D</sub> expression was induced the response to AVP, in the presence or absence of aldosterone, was abolished (Fig. 10B).

AVP stimulation of A6-SGK<sup>T</sup><sub>K130M</sub> produced a very robust response. In control cells $I_{sc}$ increased 4.8-fold, changing from $11.1 \pm 1.1$ to $53.3 \pm 3.3 \mu A/cm^2$ (Fig. 10C). In cells pretreated with aldosterone $I_{sc}$ increased from $32.2 \pm 0.7$ to $91.1 \pm 3.3 \mu A/cm^2$. The results show a synergistic effect between aldosterone and AVP (Table 2).

The expression of SGK<sup>T</sup><sub>K130M</sub> induced overnight with tetracycline did not affect the AVP response in control cells or in cells pretreated with aldosterone (Fig. 10C, Table 2).

**DISCUSSION**

In this study we sought to further examine the contribution of SGK to ENaC regulation on renal epithelial cells. With the tetracycline-inducible system in the A6 cell line we were able to control the expression of SGK independently of hormonal stimulation. This experimental maneuver allowed us to study the interactions between SGK and the regulatory pathways involved in activation of ENaC by aldosterone, insulin, and AVP. The importance of a system in which each clone serves as its own control is illustrated by the large variability in basal $I_{sc}$ among clones transfected with the same DNA construct. For instance, basal $I_{sc}$ values in four different clones of A6 cells transfected with SGK<sub>wt</sub> measured 3 days after removal of serum were $11.2 \pm 2.9$, $4.0 \pm 0.2$, $5.0 \pm 1.8$, and $21.1 \pm 0.7 \mu A/cm^2$. The response to hormones also varied among clones, as illustrated by one clone transfected with SGK<sup>T</sup><sub>K130M</sub>, which exhibited a larger than average response to AVP (Table 2, Fig. 10). These differences in basal and hormonally stimulated $I_{sc}$ among the generated cell lines are inherent to the cloning procedure of A6 cells, and they do not reflect expression levels of SGK. We avoided this pitfall with the tetracycline-inducible system. Thus our results represent the effects induced by the expression of SGK<sub>wt</sub> and SGK<sup>T</sup> mutants and not the effects of cloning bias.

It has been proposed that induction and activation of SGK are responsible for the early increase in ENaC activity induced by aldosterone. The mechanism of induction has been well documented, and it represents enhanced transcription of the SGK gene by steroids (5, 17). Activation depends on increased levels of PtdIns(3,4,5)<sub>3</sub>; however, the mechanism(s) by which aldosterone may increase PtdIns(3,4,5)<sub>3</sub> levels is not known.

Our results showed that expression of SGK<sup>T</sup> induced by tetracycline increased $I_{sc}$ in a time-dependent manner. There was a significant delay between the onset of expression of SGK<sup>T</sup><sub>wt</sub>, detected by Western blotting at 1 h after initiation of induction (Fig. 1D), and the beginning of the increase in $I_{sc}$, which was apparent after 6 h of induction (Fig. 2). In contrast, the correlation of protein level and $I_{sc}$, both in magnitude and time course, was much better in the cell lines expressing SGK<sup>S425D</sup>, demonstrating that the increase in ENaC activity correlates directly with the abundance of active SGK. Additionally, these results indicate that the delay observed in cells expressing SGK<sup>S425D</sup> represents the time required for activation of SGK, which is a slow process in A6 cells maintained without serum or hormones.

Another issue is whether or not aldosterone promotes activation of SGK. As indicated by the results shown in Fig. 2, addition of aldosterone to cells expressing high levels of exogenous SGK<sub>wt</sub> increased $I_{sc}$, but not to the level expected by the abundance of the protein or to the level reached with the constitutively active SGK<sup>S425D</sup>. Thus aldosterone does not significantly activate SGK<sub>wt</sub> in A6 cells. As we indicated above, in light of a finding by Blazer-Yost et al. (4) that aldosterone increases the abundance of PtdIns(3,4,5)<sub>3</sub>, measured by Western blotting at 1 h after induction, it is possible that blockage of any of those processes may disrupt the maintenance and/or regulation of the $I_{sc}$.

### Table 2. Insulin- and AVP-induced increase in $I_{sc}$ in A6 cell lines expressing SGK<sup>T</sup>

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>AVP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SGK&lt;sup&gt;T&lt;/sup&gt;&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>SGK&lt;sup&gt;S425D&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>7.8</td>
<td>7.4</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>20.0</td>
<td>23.3</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>7.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Aldosterone + tetracycline</td>
<td>14.3</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Values are expressed in $\mu A/cm^2$. AVP, vasopressin; SGK<sup>T</sup><sub>K130M</sub>, A6 cells expressing TetR and SGK with K130M mutation; n.s., not significantly different from control.
In our experiments, SGK<sub>K130M</sub> expression did not affect basal or hormone-stimulated $I_{sc}$. It has been proposed that SGK<sub>K130M</sub> acts as a dominant-negative mutant (15, 32), perhaps by displacing the wild-type kinase from its cellular targets (21). Accordingly, to detect a dominant-negative effect it would be necessary to have a large ratio of mutant to wild-type kinase. It is possible that in our system the levels of expression of SGK<sub>K130M</sub> were not high enough to compete with the endogenous SGK.

Another important finding in our study is that SGK<sub>S425D</sub> completely abrogates the insulin and AVP responses, indicating that these hormones share a common pathway that saturates with high levels of SGK activity. There is increasing evidence that insulin and AVP control the number of ENaC channels in the plasma membrane rather than their open probability (3, 7, 16, 24). SGK has also been shown to increase ENaC density in the plasma membrane of <i>Xenopus</i> oocytes (2, 6, 15), although a recent report suggested that SGK might in addition increase the open probability of ENaC (31).

An increase in channel density at the plasma membrane could result from increased delivery, slower retrieval, or a combination of both processes. Our results cannot be used to distinguish which of those processes is mediated by SGK. It has been proposed that ENaC abundance in the plasma membrane is controlled by the ubiquitin-ligase Nedd4, possibly by regulating endocytosis through interaction with the PY motifs of the carboxy terminal of ENaC subunits (12, 28). Recent publications have described in vitro phosphorylation of Nedd4-2 by SGK and subsequent downregulation of the ubiquitin-ligase activity (6, 27), although the requirement of ENaC PY motifs for SGK action is controversial (reviewed in Ref. 12). On the other hand, both insulin and AVP induce mobilization of intracellular pools of vesicles containing GLUT-4 in fat and muscle cells (9) or aquaporin (AQP)-2 in principal cells of the distal tubule (18). The fast time course of insulin and AVP action, which increases $I_{sc}$ by twofold in 10–15 min (3, 11), argues in favor of the insertion hypothesis. It is difficult to explain how $I_{sc}$ can increase so rapidly with a decrease or block of ENaC endocytosis, even if channels have a short half-life in the membrane (1 h in MDCK cells, ~15 min in A6 cells) (1, 10).

It is important to emphasize that insulin and AVP are able to activate ENaC in the absence of any detectable SGK. We cannot exclude that a small amount of SGK, not detectable by Western blotting, is sufficient to mediate the response to these hormones; however, the dose-dependent nature of ENaC stimulation by active SGK does not support this interpretation.

In conclusion, we show that the effects of SGK in the aldosterone response are modest, indicating that most of the stimulation of ENaC induced by aldosterone is mediated by a mechanism(s) distinct from SGK. In contrast, the responses to AVP and insulin share a common step, which is saturated by the activity of SGK.

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