SGK integrates insulin and mineralocorticoid regulation of epithelial sodium transport

JIAN WANG,1* PASCAL BARBRY,1* ANITA C. MAIYAR,2 DAVID J. ROZANSKY,1 ADITI BHARGAVA,1 MEREDITH LEONG,2 GARY L. FIRESTONE,2 AND DAVID PEARCE1

1Division of Nephrology, Department of Medicine, and 2Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco 94143; and 2Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

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Wang, Jian, Pascal Barbry, Anita C. Maiyar, David J. Rozansky, Aditi Bhargava, Meredith Leong, Gary L. Firestone, and David Pearce. SGK integrates insulin and mineralocorticoid regulation of epithelial sodium transport. Am J Physiol Renal Physiol 280: F303–F313, 2001.—The epithelial Na+ channel (ENaC) constitutes the rate-limiting step for Na+ transport across tight epithelia and is the principal target of hormonal regulation, particularly by insulin and mineralocorticoids. Recently, the serine-threonine kinase (SGK) was identified as a rapidly mineralocorticoid-responsive gene, the product of which stimulates ENaC-kinase (SGK) was identified as a rapidly mineralocorticoid-regulated gene, the product of which strongly stimulates ENaC-mediated Na+ transport. SGK mRNA is rapidly increased in response to mineralocorticoids in A6 (frog kidney) cells and rat kidney collecting duct (8), as well as in primary cultures of rabbit collecting duct (26) and a variety of other cells and tissues. Furthermore, SGK protein levels are potently and rapidly increased by mineralocorticoids and, when coexpressed in Xenopus laevis oocytes, SGK strongly stimulates ENaC-mediated Na+ currents (8, 26). SGK is highly conserved with >90% identity between mammalian and amphibian peptide sequences (30).

SGK was originally identified in rat mammary epithelial cells as a serum and glucocorticoid-regulated gene, the closest relative of which was protein kinase B (PKB; also called Akt) (Fig. 1), an integral component of the insulin signaling pathway (45). Like PKB/Akt, SGK activity, as assessed by its ability to phosphorylate an oligopeptide target in vitro, is controlled by phosphatidylinositol 3-kinase (PI3K) (20, 28), a lipid kinase that is essential for a variety of receptor tyrosine kinase actions, most notably those of the insulin receptor. Interestingly, PI3K appears to be required for most of the events triggered by insulin, including stimulation of glucose transport via GLUT-4 and Na+ transport via ENaC (34, 39). Moreover, insulin has recently been shown to activate SGK kinase activity in human embryonic kidney fibroblasts in a PI3K-dependent manner (28). PI3K is activated by recruitment to the tyrosine phosphorylated insulin receptor in complex with insulin receptor substrate-1 (see Fig. 9 for schematic). Once localized to the plasma membrane, PI3K catalyzes the production of 3-phosphorylated inositol lipids, particularly phosphatidylinositol 3,4,5-triphosphate (PIP3), the principal mediator of PI3K effects (39). The immediate upstream regulators of both PKB/Akt and SGK, 3-phosphoinositide-dependent kinase-1 and 2 (PDK1 and PDK2, respectively), are

epithelial sodium channel; phosphatidylinositol 3-kinase; serine-threonine kinase

THE EFFECTS OF MINERALOCORTICOIDS ON Na+ TRANSPORT IN TIGHT EPITHELIA ARE MEDIATED BY INTRACELLULAR RECEPTORS THAT MODULATE THE ACTIVITY OF SPECIFIC GENES (2, 29, 42). AFTER A LATENT PERIOD OF ~30–60 MIN, THE EARLIEST DETECTABLE EFFECT ELICITED BY MINERALOCORTICOIDS IS STIMULATION OF APICAL MEMBRANE Na+ TRANSPORT MEDIANED BY

*J. Wang and P. Barbry contributed equally to this work.

Address for reprint requests and other correspondence: D. Pearce, Div. of Nephrology, Dept. of Medicine and Dept. of Cellular and Molecular Pharmacology, Box 0532, Univ. of California, San Francisco, San Francisco, CA 94143 (E-mail: pearced@medicine.ucsf.edu).

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strongly activated by direct physical association with
PIP3 through their pleckstrin homology (PH) domains
(11). PDK1 and PDK2 activate PKB/Akt and SGK
through serine phosphorylation in a PIP3-dependent
manner (20, 28). Taken together, these observations
suggested that PI3K activity would be required
for SGK-dependent activation of ENaC and thus for
mineralocorticoid-stimulated Na+ transport. Furthermore,
the central role that PI3K plays in mediating insulin
signaling suggested the possibility that SGK might be
regulated by insulin as well. We therefore examined
the dependence of ENaC activity, mineralocorticoid-
stimulated Na+ transport and SGK phosphorylation
on PI3K, as well as the role of SGK in mineralocorti-
roid-insulin synergy.

METHODS

A6 cell culture and electrical measurements. A6 cells, orig-
inally obtained from the American Type Culture Collection,
were maintained at 30°C in a humidified incubator with 1% CO2
in culture medium containing 5% fetal bovine serum, as
described (11). For electrical measurements, cells were
seeded on type VI collagen (Sigma)-coated filter inserts
(Costar) at a density of 106 cells/cm2. After epithelia had
initially obtained from the American Type Culture Collection,
were maintained at 30°C in a humidified incubator with 1% CO2
in culture medium containing 5% fetal bovine serum, as
described (11). For electrical measurements, cells were
seeded on type VI collagen (Sigma)-coated filter inserts
(Costar) at a density of 106 cells/cm2. After epithelia had
initially obtained from the American Type Culture Collection,
Hemmings (Friedrich Miescher-Institut, Basel, Switzerland) and have been described previously (10, 32).

Glutathione S-transferase (GST)-ENaC fusion proteins were engineered and expressed by using the GST purification system (Amersham Pharmacia Biotech) as follows: the COOH-terminal cytoplasmic domains of the X. laevis α-ENaC (542–633 aa) and β-ENaC (551–647 aa) were sub-cloned from the respective full-length X. laevis ENaCs (gifts of Dr. Jim Stockand) into the GST containing prokaryotic-expression vector pGEX-4T3 (Amersham Pharmacia Biotech) by using PCR. Primers for α-subunit amplification were 5’-CACACGGATCCCTGCTACATCGATTACTAC-3’ (sense) and 5’-CACACCTGAGTCAGTTCCTACTCTCATCTCTC-3’ (antisense). Primers for β-subunit amplification were 5’-CACACGGATCCGGACACCCGGAGC-3’ (sense) and 5’-CACACCTGAATAGCTTCTTACTAATGATATTACTAC-3’ (antisense). The PCR fragments were digested and ligated into the BamH I/Xho I multiple cloning site of pGEX-4T3, yielding ENaC COOH-terminal tails fused to the COOH terminus of GST, as confirmed by nucleotide sequence analysis. The plasmids were then transformed into BL-21 Escherichia coli for high-level expression followed by purification on a glutathione-Sepharose column according to the manufacturer’s protocol. GST alone, by using pGEX-4T3, was also purified in this manner and served as a negative control.

Binding experiments were performed by using 10 μg of GST-β-ENaC immobilized on glutathione-Sepharose beads incubated with 5 μl of 35S-SGK translation product in 180 μl of binding buffer (in mM: 20 HEPES-KOH, pH 7.9, 50 KCl, 2.5 MgCl2, 1 DTT, 1.5 PMSF, 10% glycerol, 0.2% NP-40, and 3 μl of normal goat serum/180 μl binding buffer). The slurry was incubated overnight at 4°C on a nutator, following which the beads were washed five times in wash buffer (200 mM NaCl, 0.2% Tween 20, 10 mM Tris, pH 7.5, and 0.5% nonfat dry milk). The pellet was resuspended in 25 μl of 2× SDS sample buffer, boiled for 5 min, and retained proteins were resolved by SDS-PAGE. Gels were dried at 60°C followed by autoradiography. Band intensity was compared with 10% of the 35S-methionine-labeled input.

RESULTS

PI3K is necessary for mineralocorticoid-induced Na+ current and phosphorylation of SGK in A6 cells. We first examined the effect of PI3K inhibition on mineralocorticoid-stimulated Na+ transport. A6 cells were grown to high resistance on permeable supports and incubated in serum-free medium as described in METHODS. Cells were treated with the potent highly specific PI3K inhibitor LY-294002 (LY) for 0.5 h before addition of DEX. Potential difference (PD) (A) and resistance (R) (B) were measured at times shown. The derived parameter, equivalent current (PD/R) is shown in (C). Error bars, SD (n = 4). Where not shown, error bars were smaller than data symbols. PD/R were inhibited >90% by 10 μM amiloride, indicating that the current is largely epithelial Na+ channel (ENaC) mediated (not shown). The experiment was performed a total of 5 times on different days with similar results.

We next examined by immunoblot the abundance and phosphorylation state of SGK. Depending on gel composition and duration of electrophoresis, immunoblots reveal two or three closely spaced bands around 58 kDa, specifically recognized by affinity-purified

Fig. 2. Phosphatidylinositol 3-kinase (PI3K) inhibition blocks the dexamethasone (DEX)-induced early increase in equivalent current in A6 cells. Cells were treated with the specific PI3K inhibitor, LY-294002 (LY), for 0.5 h before addition of DEX. Potential difference (PD) (A) and resistance (R) (B) were measured at times shown. The derived parameter, equivalent current (PD/R) is shown in (C). Error bars, SD (n = 4). Where not shown, error bars were smaller than data symbols. PD/R were inhibited >90% by 10 μM amiloride, indicating that the current is largely epithelial Na+ channel (ENaC) mediated (not shown). The experiment was performed a total of 5 times on different days with similar results.

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Fig. 3. PI3K inhibition blocks SGK phosphorylation. After measurement of PD and resistance (Fig. 2, A and B), A6 cells were harvested at times shown and immunoblots were prepared and probed as described in METHODS. Bottom band, the nonphosphorylated form of SGK; top band(s), the phosphorylated forms. Representative blots are shown. The experiment shown was performed a total of 4 times (with the exception of the 24-h time point that was performed 3 times) with similar results. Data from all experiments were quantitated by densitometry, and ratio of phosphorylated to nonphosphorylated (Table 1) and degree of stimulation (Table 2) were determined.

SGK antibody (see Figs. 3 and 7 and Ref. 45). The top band(s) corresponds to the phosphorylated forms, and the bottom band to the nonphosphorylated form of SGK (28). As shown in Fig. 3, dexamethasone markedly induced SGK protein expression in both the presence and absence of LY-294002. The PI3K inhibitor, however, markedly reduced formation of the top band at all time points (Fig. 3 and Table 1), indicating that phosphorylation was prevented. LY-294002 also appeared to have a modest effect on the kinetics and extent of induction of SGK protein levels; however, this effect did not reach statistical significance (Table 2). These data strongly suggest that although dexamethasone increases SGK abundance in a largely PI3K-independent fashion, PI3K is required for SGK’s subsequent phosphorylation.

Effects on Na⁺ transport are not due to toxicity of PI3K inhibitors. The observation that the cells maintained their high electrical resistance in the presence of LY-294002 for at least 24 h suggested that they were healthy and that effects on Na⁺ transport were not due to generalized toxicity. To obtain additional evidence of cell viability, we examined the effect of withdrawing LY-294002 on the electrical properties of A6 cells in the presence and absence of dexamethasone. LY-294002 inhibition of PI3K, unlike that of wortmannin, was shown previously to be reversible (43). LY-294002 was applied to cells for 24 h in the presence or absence of dexamethasone, and then medium was replaced with LY-294002-free medium without altering of the dexamethasone concentration. As shown in Fig. 4, within 1 h of removal of LY-294002, equivalent current (PD/R)

Table 1. PI3K inhibition reduces the ratio of phosphorylated to nonphosphorylated SGK

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>2 h</th>
<th>6 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td>No LY</td>
<td>0.77 ± 0.090</td>
<td>0.86 ± 0.08</td>
<td>0.93 ± 0.14</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td>LY</td>
<td>0.33 ± 0.047</td>
<td>0.20 ± 0.05</td>
<td>0.20 ± 0.06</td>
<td>0.55 ± 0.17</td>
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Values are means ± SE. Ratios of phosphorylated to nonphosphorylated serine-threonine kinase (SGK) were determined by densitometry as described in METHODS. PI3K, phosphatidylinositol 3-kinase; LY, LY-294002. At 0, 2, and 6 h, the ratio of phosphorylated to nonphosphorylated SGK was significantly lower in the presence of LY-294002 (P < 0.02 by 2-tailed unpaired Student’s t-test). At 24 h, this difference did not reach significance (P = 0.72).

Table 2. The principal effect of PI3K inhibition with LY-294002 is to block formation of the phosphorylated form of SGK

<table>
<thead>
<tr>
<th></th>
<th>2 h</th>
<th>6 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td>No LY</td>
<td>39.7 ± 7.99</td>
<td>58.1 ± 18.52</td>
<td>8.7 ± 3.84</td>
</tr>
<tr>
<td>LY</td>
<td>41.1 ± 13.46</td>
<td>51.9 ± 18.16</td>
<td>10.77 ± 3.97</td>
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Data represent degree of increases in total immunoreactive SGK (±SE) relative to levels at t = 0 (arbitrarily set to 1) averaged from 4 blots (except 24-h time point that is averaged from 3 blots). See METHODS and legend of Fig. 3 for details.

increased in the dexamethasone-treated monolayers, approaching that of cells treated with dexamethasone in the absence of LY-294002 (compare LY withdrawal + Dex with Dex alone in Fig. 4). The recovery of cells untreated with dexamethasone was slower, but, by 6 h after removal of LY-294002, electrical parameters were nearly back to control values (LY withdrawal, no Dex). Equivalent current remained unchanged over the same time period in cells maintained in LY-294002 (LY, no Dex). Resistance remained stable for up to 28 h in LY-294002; however, cells maintained in the inhibitor in the absence of dexamethasone for >28 h began to lose resistance (not shown).

These results suggest that LY-294002 does not have a generalized toxic effect on the cells for at least 24 h and that its effects are rapidly reversible, particularly in the presence of dexamethasone. In further support of the conclusion that the early LY-294002 effect on Na⁺ transport was not due to toxicity, we also found that electrical properties returned to baseline values when the inhibitor was removed after only 1 h of treatment (not shown). However, after ~28 h of LY-294002 treatment toxic effects begin to appear.

SGK stimulation of ENaC activity is PI3K dependent in X. laevis oocytes. The above observations are consistent with the idea that PI3K-dependent SGK activity is required for basal and mineralocorticoid-stimulated ENaC activity. To more directly examine the role of PI3K in SGK-stimulated ENaC activity, a X. laevis oocyte coexpression system was used. Oocytes, which have endogenous PI3K activity (25), have been used extensively to study the activity and regulation of ion transport proteins (6, 8, 15, 17). Oocytes were injected with cRNAs for the ENaC subunits and SGK and, after 2–4 days incubation, amiloride-sensitive currents were determined. As shown in Fig. 5, LY-294002 rapidly inhibited SGK-stimulated ENaC activity in a manner similar to its effect on equivalent current in A6 cells (Figs. 2 and 3). This effect was reversible in that equivalent current began to increase within 45 min after removal of LY-294002 and had returned to baseline by 7 h (Fig. 5). Also consistent with its effect on Na⁺ transport in A6 cells, LY-294002 inhibited ENaC-mediated Na⁺ transport in both the presence and absence of SGK, although the effect in the presence of SGK was substantially greater (Fig. 6).

Insulin stimulates Na⁺ transport and increases SGK phosphorylation. PI3K is required for both mineralocorticoid and insulin stimulation of Na⁺ transport [see
Fig. 2 (4, 34)] as well as for phosphorylation of SGK [Fig. 3 (28)]. Together with previous reports that insulin and mineralocorticoids stimulate Na⁺ transport synergistically (14, 19), these observations suggested that the insulin- and mineralocorticoid-signaling pathways converge at SGK (34). With these observations in mind, we examined the effects of insulin and dexamethasone on Na⁺ transport, and SGK abundance and phosphorylation in the absence of serum. As shown in Fig. 7, both mineralocorticoids and insulin stimulated Na⁺ transport (PD/\(R\)) in A6 cells grown in serum-free medium. The change in equivalent current induced by insulin and dexamethasone together was ~1.5-fold the sum of the change induced by each separately, indicating a moderate level of synergy. As shown in Fig. 3, dexamethasone increased both the phosphorylated and nonphosphorylated forms of SGK. In support of the idea that the insulin- and mineralocorticoid-signaling pathways converge at SGK, SGK phosphorylation was further increased by insulin (compare lanes 2 and 4, Fig. 7, bottom). In some experiments, insulin appeared to further increase SGK protein level as well, although this effect was inconsistent and insulin by itself did not stimulate SGK expression. Due to the low signal-to-noise ratio in the absence of hormone, the various forms of SGK could not be separately quantitated and compiled under those conditions. However, ratios of

![Graph showing equivalent current over time](image1)

**Fig. 5.** Time course of LY inhibition of ENaC-mediated Na⁺ current in *Xenopus laevis* oocytes. Oocytes were injected with RNA encoding ENaC subunits with or without SGK, as shown. After 40 h, electrodes were placed and a stable baseline was determined. Oocytes were then incubated in buffer containing 100 μM LY (t = 0), where t is time. ENaC-mediated Na⁺ current was determined by a 2-electrode voltage clamp (see METHODS). LY was washed out at t = 32 min, as shown. At t = 62 min, current was undetectable. A representative experiment is shown. The experiment was performed 3 times with similar results. In the absence of LY, the baseline remained stable over the course of the experiment. Statistical analysis of pooled data is shown in Fig. 6.

![Graph showing current over time](image2)

**Fig. 6.** Inhibition of ENaC-mediated Na⁺ transport by LY in *X. laevis* oocytes. *X. laevis* oocytes were injected with ENaC cRNA without (A) or with (B) SGK mRNAs. After 40 h, oocytes were incubated in LY or buffer for 2 h, and amiloride-sensitive current was determined as in Fig. 5. LY inhibition of Na⁺ transport in the presence of SGK was greater than in its absence (81% in the presence and 52% in the absence of SGK; \(P = 0.02\) by Student’s unpaired two-tailed t-test, n = 6). Data points represent average amiloride-inhibitable currents (means ± SE).
unaffected by Dex.

presence of insulin (t0.01 without Dex or insulin; t0.001 by unpaired Student's t-test). The experiment was performed a total of 4 times on different days with similar results. Western blots were performed on 4 independent monolayers. Bands were quantitated as described in METHODS, and ratios of phosphorylated to nonphosphorylated SGK were determined. Average values (±SE) were 0.8 ± 0.01 without Dex or insulin; 0.6 ± 0.03 with Dex, without insulin; 1.5 ± 0.14 without Dex, with insulin; 1.3 ± 0.19 with both Dex and insulin. Ratio of phosphorylated to nonphosphorylated SGK was significantly higher in the presence of insulin (P < 0.01 by unpaired Student’s t-test) but was unaffected by Dex.

phosphorylated to nonphosphorylated forms of SGK could be determined and are shown in the legend to Fig. 7.

SGK physically interacts with ENaC subunits. As a first step toward determining the mechanistic basis of SGK stimulation of ENaC-mediated Na+ transport, we examined whether SGK physically interacts with the COOH-terminal tails of ENaC subunits. This region interacts with other putative regulatory proteins such as Nedd4 (41), and its deletion can result in increased or decreased Na+ transport depending on which subunit is affected and the extent of the deletion (2). These observations point to the COOH-terminal tails of all of the ENaC subunits as important sites of regulation that might directly interact with SGK. This possibility was tested for α-ENaC and β-ENaC, by using GST-pulldown assays of [35S]-SGK. GST-α-ENaC and GST-β-ENaC fusion proteins were expressed in E. coli, bound to glutathione-Sepharose beads, and incubated with the [35S]methionine-labeled in vitro translation product of full-length SGK. As shown in Fig. 8A, SGK bound to both GST-α-ENaC and GST-β-ENaC (lanes 4 and 5), whereas it displayed negligible binding to GST alone (lane 3). The γ-ENaC COOH-terminal tail was not successfully expressed and hence has not yet been tested. The experiments shown were performed with the phosphorylated form of SGK. Similar results were obtained with nonphosphorylated SGK (not shown), indicating that both the active and the inactive forms of the kinase can interact with ENaC subunits (see DISCUSSION).

To ascertain the specificity of interaction with SGK, the GST-ENaC fusion proteins were incubated with [35S]methionine-labeled JNK or PDK1 (both SGKs) and binding compared with that of [35S]methionine-labeled SGK. As shown in Fig. 8B, no binding was detectable between either GST-β-ENaC and JNK or PDK1, whereas under the same conditions, specific interaction between GST-β-ENaC and SGK was readily apparent (compare lanes 2, 4, and 6). None of the in vitro translated products bound GST alone (data not shown). Similar results were obtained by using GST-α-ENaC (not shown).

The specific regions within SGK involved in binding to β-ENaC were further characterized by incubating GST-β-ENaC with various SGK truncations followed by SDS-PAGE as in Fig. 8, A and B. The different SGK fragments included full-length WT-SGK (1–431 aa), KD-SGK (K127M), NH2-terminal deletion mutant of SGK that lacks the first 60 aa (ΔN-SGK, 60–431 aa), COOH-terminal deletion mutant of SGK that is devoid of 76 aa at the COOH end (ΔC-SGK, 1–355 aa), and SGK sequences encompassing the catalytic domain

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**Fig. 7.** Insulin increases phosphorylation of SGK and synergizes with Dex in stimulating equivalent current. A6 cells were cultured and treated as in Fig. 2. Cells were treated with Dex alone (or vehicle) for 2 h followed by addition of insulin (or vehicle) for an additional 0.5 h, as shown. PD and resistance were measured and cells were harvested and immunoblotted for SGK, as described in METHODS. Shown are equivalent currents (±SD, n = 5). Changes in equivalent current relative to no Dex, no insulin were (in μA/cm2) 1.3 ± 0.60.6 ± 0.6 SE) were 0.8 ± 0.01 without Dex or insulin; 0.6 ± 0.03 with Dex, without insulin; 1.5 ± 0.14 without Dex, with insulin; 1.3 ± 0.19 with both Dex and insulin. Ratio of phosphorylated to nonphosphorylated SGK was significantly higher in the presence of insulin (P < 0.01 by unpaired Student’s t-test) but was unaffected by Dex.

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**Fig. 8.** α-ENaC and β-ENaC physically interact with SGK in vitro. A: [35S]methionine-labeled in vitro translation (IVT) product for full-length wild-type SGK was incubated with glutathione S-transferase (GST) alone (lane 3), GST-α-ENaC (lane 4), or GST-β-ENaC (lane 5), as indicated. After recovery of fusion protein on glutathione-Sepharose beads, the bound fraction was analyzed by SDS-PAGE and visualized by autoradiography. The unprogrammed lysate did not contain any IVT product (data not shown), and 10% of the IVT product (input) is denoted 10% of the labeled proteins used in the GST pulldown assays. B: in vitro synthesized product for full-length SGK, Jun NH2-terminal kinase (JNK), or PDK1 was incubated with GST-β-ENaC (lanes 2, 4, and 6), and proteins bound to the beads were resolved by SDS-PAGE (A). The IVT designated as input in lanes 1, 3, and 5 denotes 10% of the labeled proteins used in the GST pulldown assays. C: various fragments of SGK composed of wild-type full-length SGK [WT-SGK, 1–431 amino acids (aa)], kinase-dead SGK (KD-SGK, K127M), NH2-terminal (term.) deleted SGK (ΔN-SGK, 60–431 aa), COOH-terminal deleted SGK (ΔC-SGK, 1–355 aa), and catalytic domain only SGK (cat-SGK, 60–355 aa) were synthesized as [35S]methionine-labeled products (A), and incubated with either GST alone (lanes 6, 8, 10, 12, and 14) or GST-β-ENaC (lanes 7, 9, 11, 13, and 15), and samples were processed (A). Lanes 1–5 depict 10% of the IVT products included in the binding reactions. Predicted molecular weights (MW) for each of the SGK products were WT-SGK and point mutant K127M-47.8 kDa; ΔN-41.2 kDa; ΔC-39.4 kDa; and cat-32.8 kDa. MW markers are shown (far left) in A, B, and C. Discrepancies between predicted and actual MW are likely due to posttranslational modification (28, 45). Similar results were obtained in 3 separate experiments.
Specific binding between GST-β-ENaC and SGK was observed in all the fragments tested in preference to GST alone (Fig. 8C; compare lanes 7, 9, 11, 13, and 14 with 6, 8, 10, 12, and 15), thereby defining the catalytic domain of SGK as a region mediating interaction with β-ENaC. Figure 8C (middle) shows 10% of the in vitro translated products of the SGK deletions used as input for the binding assays. Taken together, these data demonstrate direct specific association between the catalytic domain of SGK and β-ENaC, in vitro.

**DISCUSSION**

Mineralocorticoid effects on ENaC-mediated Na+ transport are PI3K dependent. SGK is a corticosteroid-regulated gene, the product of which activates the ENaC (8). Its mRNA levels are stimulated by ligands...
PI3K activity is not shown (see text for details). ROMK2 potassium channel. Also, the unknown activator of basal transporters are not shown, for example, the Na/K-ATPase and the bidirectional transport emphasizing the dual regulation of SGK. According to this view, mineralocorticoid-dependent stimulation of SGK gene expression is a necessary, but not sufficient, event in the early activation of Na+/K+ transport. Additional activation of SGK is required for the downstream events to occur.

Although this model explains many of the observed characteristics of mineralocorticoid-stimulated Na+ transport, several important points remain unaddressed: 1) PI3K inhibition with LY-294002 disrupts both basal and hormone-stimulated Na+ transport; 2) although insulin increases SGK phosphorylation and synergizes with dexamethasone, SGK is phosphorylated in the absence of insulin and the extent of synergy is modest; 3) LY-294002 strongly inhibits SGK phosphorylation but also has modest effects on the kinetics of SGK induction by dexamethasone; and 4) PI3K inhibition greatly reduces the early response to dexamethasone (Figs. 2 and 4) but only blunts the later response. Some of these caveats are interpretable in light of the present data, whereas others will require further clarification.

First, the PI3K dependence of basal Na+ current is consistent with the interpretation shown in Fig. 9, if basal levels of SGK are not negligible. Western blot data are difficult to interpret in the absence of mineralocorticoids due to the low signal-to-noise ratio; however, SGK mRNA and protein have been detected in the basal state (8). Basal expression of SGK not withstanding, the important caveat is raised that the dependence of basal current on PI3K could be due to the activity of another PI3K-dependent kinase such as SGK2, SGK3, or PKB/Akt (21). Similarly, the efficacy of insulin in the absence of mineralocorticoids could be due to basal levels of SGK (more appropriately referred to as SGK1) or to one of the alternative PI3K-dependent kinases. It is interesting to speculate that the ratio of active to inactive SGK is as important a determinant of ENaC activity as the absolute level of the active form (see below). Further studies are needed to determine whether SGK1 sustains basal ENaC activity and mediates insulin effects in the absence of mineralocorticoid. In particular, anti-SGK antibodies with improved sensitivity and specificity are essential.

Finally, one must address the observation that PI3K inhibition only blunted the later effect of dexamethasone, consistent with the idea that the later phase of mineralocorticoid action is, at least in part, non-PI3K dependent. In this regard, it is interesting to note that mineralocorticoid effects have long been divided into four distinct phases: latent, early, middle, and late (reviewed in Ref. 42). The latent phase of mineralocorticoid action (≈30–60 min) appears to be due largely to its dependence on changes in gene transcription, whereas the early phase (≈0.5–3 h) primarily reflects changes in ENaC-mediated apical Na+ transport. In contrast, the middle and late phases (3–24 h) reflect changes in Na-K-ATPase activity, the metabolic rate of cells, and alterations in membrane surface area (42). It is interesting to suggest that these later events are PI3K independent; however, further investigation will be required to address these issues.

SGK is an integrator of mineralocorticoid and insulin effects. It is notable that PI3K, a key mediator of insulin signaling, is also essential for rapid mineralocorticoid regulation of Na+ transport. Insulin strongly stimulates Na+ transport in the renal tubule including the collecting duct (13), and synergy between insulin and mineralocorticoids has been reported (14, 19), consistent with our present findings (Fig. 7). Figure 9 presents a schematic view of SGK as an integrator of insulin and aldosterone actions that account for their synergistic activation of Na+ transport. According to this view, SGK abundance is regulated by aldosterone through changes in gene transcription while its activ-
ity is controlled by PI3K through phosphorylation; insulin is a key regulator of PI3K.

It is interesting to note that slightly less than additive (i.e., nonsynergistic) effects of aldosterone and insulin were found in at least one report (35). The basis for the discrepancy between that report and the data referred to above is unknown but could be explained by variability in basal levels and activities of SGK and PI3K (the latter determining the ratio of phosphorylated to nonphosphorylated SGK). For example, if SGK levels and PI3K activity were low in the absence of mineralocorticoids and insulin, respectively, then mineralocorticoid-insulin synergy would be high. Conversely, if either were high, then synergy would be low.

It is also possible that other PI3K-dependent kinases are implicated in insulin-regulated Na\(^+\) transport. SGK2 and SGK3 levels are not regulated by corticosteroids (21), and either or both of these may contribute to insulin regulation of Na\(^+\) transport in the absence of mineralocorticoids. It seems less likely, but also plausible, that insulin might act through PKB/Akt, which has overlapping substrate specificities with SGK (20). In either case, synergy would be high when basal levels of SGK (or related kinase) are low and, conversely, synergy would be low when basal levels are high. The roles of SGK, its novel isoforms, and PKB/Akt in mediating the effects of insulin in the absence of corticosteroids will require further study. It is interesting to note that our data also suggest that *X. laevis* oocytes have significant levels of SGK or a related PI3K-dependent kinase (Figs. 4 and 5) that is required for basal ENaC-mediated Na\(^+\) transport. Western blots in our hands have not been sufficiently sensitive to determine the abundance of oocyte SGK. The role of this PI3K-dependent kinase in oocyte physiology is uncertain at this time.

**Constitutive PI3K activity in A6 cells is high.** In most cells PI3K activity depends on receptor tyrosine kinases (such as the insulin receptor or platelet-derived growth factor receptor), and basal activity is low (39). In A6 cells, PI3K activity and SGK phosphorylation are high in the absence of insulin and are further stimulated by it (Figs. 3 and 6; (34)). Consistent with this observation, although both basal and mineralocorticoid-induced Na\(^+\) transport are highly sensitive to PI3K inhibitors, exogenous activators of PI3K such as insulin are not absolutely required (Figs. 2 and 6 and Ref. 5). Although an explanation for this observation will require further investigation, it is plausible that an intracellular factor such as K-Ras activates PI3K directly (23, 37, 40). Other possible nonhormonal activators of PI3K include extracellular matrix components acting through integrins (7) or (less likely) autocrine factors acting through receptor tyrosine kinases (33).

**A mechanistic view of SGK-stimulated ENaC activity.** It seems likely that SGK stimulates ENaC-mediated Na\(^+\) transport by phosphorylating either ENaC subunits themselves or ENaC regulatory proteins (or both). Our data indicate that SGK physically interacts with the \(\alpha\)-ENaC and \(\beta\)-ENaC subunits (Fig. 8) in vitro. However, we have not been able to demonstrate direct phosphorylation of ENaC subunits by SGK (data not shown), consistent with recent reports showing that mutation of several possible kinase target residues in ENaC subunits did not affect their ability to be stimulated by SGK (1, 22). These observations are consistent with the view that SGK is recruited to its target sites by physical interaction with ENaC subunits (s) but that the ENaC subunits themselves are not targets of SGK phosphorylation. Perhaps the direct target of SGK phosphorylation is a component of the ENaC regulatory machinery. In this regard, striking parallels between the regulation of GLUT-4-mediated glucose transport and ENaC-mediated Na\(^+\) transport become apparent, perhaps reflecting common themes that underlie the hormonal control of protein trafficking events via intracellular signaling kinases (18, 24, 31). It should be noted that although the interaction data of Fig. 8 are consistent with some recent reports (22, 44), they are not consistent with others (1). Additional investigation will be required to determine whether SGK interacts with ENaC subunits in vivo and to further assess the physiological relevance of such an interaction. In particular, because both phosphorylated and nonphosphorylated forms of SGK interact with ENaC (Fig. 8 and data not shown), it is important to determine whether nonphosphorylatable mutants of SGK have dominant negative activity. Such an observation would have the interesting implication that the ratio of nonphosphorylated to phosphorylated SGK, not simply the absolute level, would be an important determinant of ENaC activity. This could explain the relatively large effects of LY-294002 and insulin in the absence of mineralocorticoids: LY-294002 decreases, whereas insulin increases, the ratio of nonphosphorylated to phosphorylated SGK.

According to the schematic view shown in Fig. 9, SGK is recruited to ENaC-containing vesicles by the ENaC subunits themselves and phosphorylates vesicle proteins that regulate plasma membrane vesicle fusion. Although the bulk of evidence now appears to favor changes in ENaC localization as paramount in increasing apical Na\(^+\) transport, it is possible that changes in ENaC open probability might also play a role (12). In either case, the dual regulation of SGK, its abundance through a transcriptional mechanism, and its activity through a PI3K-dependent pathway, provides an attractive mechanism for the context-dependent regulation of Na\(^+\) transport by mineralocorticoids and insulin.

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