The concentration in the blood of the potassium ion ($K^+$), an important determinant of cell membrane potential, is tightly regulated within a narrow range. The excretion of $K^+$ occurs mainly in the kidney through processes involving glomerular filtration, tubular reabsorption, and secretion. The transepithelial $K^+$ secretion in the kidney takes place predominantly in the aldosterone-sensitive distal nephron and involves $K^+$ uptake into cells by the basolateral sodium-potassium pump and exit into lumen through apical $K^+$ channels, which include the $Ca^{2+}$-activated maxi-K channel and the renal outer medullary potassium channel, ROMK (also known as Kir1.1).\(^1\)\(^2\)

ROMK channel undergoes constitutive clathrin-dependent endocytosis, which regulates the density of channel at the cell surface, thus controlling renal $K^+$ secretion.\(^3\) Recently, WNK (with-no-lysine [K]) kinases have been identified as important regulators of the cell surface abundance of ROMK. WNKs are serine-threonine protein kinases with an unusual position of the catalytic lysine in subdomain I instead of
subdomain II. Mammalian WNK family includes four members, WNK1-4, which share 85 to 90% sequence identity in their kinase domain.\textsuperscript{4-6} Mutations in WNK1 and WNK4 in humans cause an autosomal-dominant disease called pseudohypoaldosteronism type 2 (PHA2), featuring hypertension and hyperkalemia.\textsuperscript{2} Studies have shown that WNK1 and WNK4 regulate renal Na\textsuperscript{+} and K\textsuperscript{+} transporters, and dysregulation of these transporters contributes to hypertension and hyperkalemia in PHA2.

WNKs regulate renal Na\textsuperscript{+} transport through both catalytic and noncatalytic mechanisms. With respect to the catalytic mechanism of regulation, WNK1 and 4 phosphorylate and activate OSR1 (oxidative stress-responsive kinase-1) and its related kinase SPAK (Ste20-related proline-alanine-rich kinase), which in turn phosphorylate and activate the thiazide-sensitive sodium-chloride co-transporter NCC and the bumetanide-sensitive sodium-potassium-2 chloride cotransporter NKCC.\textsuperscript{7,8} WNK1 and 4 can also regulate ENaC and NCC via noncatalytic mechanisms that involve protein–protein interaction with serum- and glucocorticoid-induced kinase-1 (SGK1) for the regulation of ENaC and with transporter directly for the regulation of NCC.\textsuperscript{9,10} With respect to K\textsuperscript{+} transport, WNK1 and 4 stimulate endocytosis of ROMK via a kinase-independent mechanism that involves a direct interaction with an endocytic scaffold protein, intersectin.\textsuperscript{11}

Compared with the downstream effects of WNKs, the physiologic upstream regulators of WNKs are less understood. Vitari et al.\textsuperscript{12} showed that IGF1 induces phosphorylation of endogenous WNK1 in cultured human embryonic kidney (HEK) cells at threonine-60 (equivalent to threonine-58 of rat WNK1). The effect of IGF1 is through activation of phosphatidylinositol 3-kinase 3-kinase (PI3K), leading to activation of the 3-phosphoinositide–dependent protein kinase-1 (PDK1) and protein kinase B (PKB)/Akt1. Phosphorylation of WNK1 by Akt1 does not affect its kinase activity or subcellular distribution.\textsuperscript{12} Jiang et al.\textsuperscript{13} reported that insulin induces a similar phosphorylation of WNK1, which underscores the inhibition of cell proliferation of 3T3-L1 preadipocytes by insulin. Xu et al.\textsuperscript{14} reported that WNK1 activates SGK1 through direct protein–protein interactions independently of WNK1 kinase activity. Xu et al.\textsuperscript{15} further showed that phosphorylation of rat WNK1 at threonine-58 by the IGF1–Akt1 pathway enhances the ability of WNK1 to stimulate SGK1 kinase activity, leading to activation of ENaC. Others have also shown that insulin stimulates ENaC via Akt1, although the role of WNK1 was not studied.\textsuperscript{15} The mechanism for WNK1 regulation of ROMK and ENaC are fundamentally distinct, raising an interesting question as to whether phosphorylation of WNK1 by PI3K-activating hormones, such as insulin and IGF1, affects its regulation of ROMK. We investigate this question in this study.

RESULTS

Effects of Serum Deprivation, Insulin, and IGF1 on ROMK
Barium-sensitive K\textsuperscript{+} currents were measured by ruptured whole-cell recording from HEK cells transfected with ROMK. Under the condition of symmetrical 140 mM [K\textsuperscript{+}], ROMK-mediated K\textsuperscript{+} currents showed characteristic weak inward rectification (Figure 1A, left). No currents were observed in mock-transfected cells. To allow for testing the effect of insulin and IGF1 on ROMK, we first examined the effect of serum deprivation. After culturing in the serum-containing media for 48 hours to allow maximal expression of the channel, ROMK-transfected cells were deprived of serum for 3 to 25 hours before ruptured whole-cell recording. As shown, ROMK current density (normalized to capacitance, pA/pF) increased progressively with increasing duration of serum deprivation (Figure 1A, right). Six hours after serum deprivation, current density was significantly higher than that before deprivation. ROMK currents continued to increase and reached a plateau at 16 to 20 hours (Figure 1A). Addition of insulin thereafter caused a significant inhibition of ROMK currents in 30 minutes (Figure 1B). ROMK currents reached the nadir at approximately 2-hour incubation with insulin (Figure 1B). The dose–response relationship for inhibition of ROMK was examined by incubating insulin ranging from 1 to 100 nM for 2 hours. Insulin significantly inhibited ROMK at a concentration as low as 1 nM (Figure 1C). The concentration for half-maximal inhibition (IC\textsubscript{50}) of ROMK for insulin was estimated at 3.2 nM (Figure 1C). For reference, the plasma concentration of insulin in normal individuals is 10 to 150 pM at fasting but may reach 300 to 800 pM after carbohydrate meals.\textsuperscript{16} IGF1 and insulin act on similar membrane receptors and elicit overlapping cellular responses.\textsuperscript{17} Accordingly, we found a similar inhibition of ROMK by IGF1, with an IC\textsubscript{50} for IGF1 estimated at 18.5 ng/ml (= 2.4 nM; Figure 1D). The normal basal plasma concentration of IGF1 in humans is around 50 to 100 ng/ml.\textsuperscript{18}

Effect of Insulin and IGF1 Is Dependent on PI3K and WNK1-T58 Phosphorylation
To study whether the inhibition of ROMK by insulin and IGF1 requires PI3K, ROMK-transfected cells were incubated with insulin or IGF1 with or without a specific PI3K inhibitor, wortmannin. In these experiments, we also compared the effects of insulin and IGF1 on ROMK with or without serum. We found that 100 nM of insulin caused a significant inhibition of ROMK even in the presence of serum (Figure 2A), indicating that the receptors are not maximally occupied by insulin present in the serum. For comparison, IGF1 at 100 ng/ml did not cause further inhibition of ROMK in the presence of serum. As before, serum deprivation increased ROMK currents, and application of insulin or IGF1 thereafter inhibited the currents. Co-application of wortmannin (WM, 100 nM) completely abolished the effect of insulin and IGF1 on ROMK (Figure 2A), indicating that the effect of insulin and IGF1 depends on PI3K.

Stimulation of PI3K by hormones or growth factors produces 3-phosphoinositides in the plasma membrane and leads to activation of downstream AGC kinases, Akt1 and SGK1, via multiple concerted actions.\textsuperscript{19} First, it stimulates the mammalian target of rapamycin complex-2 (mTORC2) to phosphorylate Akt1 or SGK1 at the hydrophobic motif. Phosphorylation...
of Akt1 or SGK1 by mTORC2 enhances its binding with PDK1, which phosphorylates Akt1 or SGK1 at the T-loop to activate its catalytic activity. Finally, the recruitment and binding of PDK1 to Akt1 is also facilitated by the production of 3-phosphoinositides in the plasma membrane. To understand the role of PI3K in WNK1 regulation of ROMK, we examined the phosphorylation status of endogenous Akt1 and WNK1 in HEK cells using respective residue-specific phospho-antibodies. In the absence of serum, there was a basal level of phosphorylation of Akt1 at threonine-308 (T308) of the T-loop and serine-473 (S473) of the hydrophobic motif, respectively (Figure 2B, lane 4). Serum and/or insulin increased phosphorylation of Akt1 at both residues (lanes 1, 2, and 5). Wortmannin abrogated basal and serum or insulin-stimulated phosphorylation of Akt1 (lanes 3 and 6). Phosphorylation of WNK1 at threonine-60 (T60 = T58 of rat WNK1) was not detectable in the absence of serum (lane 4) but was enhanced by serum and/or insulin in a wortmannin-sensitive manner (lanes 1, 2, and 5). Serum and insulin also stimulated wortmannin-sensitive phosphorylation of overexpressed SGK1 at the T-loop (threonine-256) (Figure 2B, bottom two gels highlighted in box). Overexpressed SGK1 was used because endogenous SGK1 was below detection by our antibody.

Cell surface abundance of ROMK was examined using a biotinylation assay. Serum deprivation increased ROMK surface abundance (Figure 2C, lane 2 versus 3). Insulin and IGF1 decreased ROMK surface abundance in a wortmannin-sensitive manner (lanes 4 to 6). These results support the notion that insulin and IGF1 inhibit ROMK by enhancing its endocytosis, in which WNK1 may play a role.

To confirm the role of WNK1 in the PI3K-mediated regulation of ROMK, we knocked down endogenous WNK1 using small interference RNA (siRNA). Efficacy of WNK1 siRNA was validated by blotting endogenous WNK1 in HEK cells (Figure 2D). Cells transfected with WNK1 siRNA (“WNK1 siRNA”, white bar) or control oligonucleotides (“Control oligo”, black bar) had similar ROMK currents in the absence of serum (“serum-control oligo”, black bar) or control oligonucleotides (“Control oligo”, black bar) but not in cells expressing exogenous T58A mutant to examine the role of T58 phosphorylation on WNK1 (Figure 2E). Insulin inhibited ROMK in cells expressing endogenous (“vector”) or exogenous WNK1 ["WNK1(1-491)/WT"] but not in cells expressing exogenous T58A mutant of WNK1 ["WNK1(1-491)/T58A"]. Overexpression of WNK1-T58A exerted a dominant-negative effect on endogenous WNK1 (data not shown). These results strongly support the notion that phosphorylation on T58 of WNK1 is important for inhibition of ROMK by hormones or growth factors that activate PI3K.
Akt1 and SGK1 Phosphorylate WNK1 at Threonine-58

In Vitro and In Vivo

Although WNK1 has been reported as a substrate for Akt1 and SGK1, the efficacy of WNK1 phosphorylation by different status of Akt1 and SGK1 has not been clearly clarified yet. In this study, we decided to examine kinase activity of Akt1 and SGK1 systematically with the goal to guide our physiologic studies. We first examined the ability of exogenously expressed Akt1 and SGK1 to phosphorylate WNK1 in vitro, with or without coexpressed PDK1. Expressed epitope-tagged Myc-PDK1, HA-Akt1, or Flag-SGK1 was immunoprecipitated from HEK cell lysates by respective antibodies. Purified bacterial His-tagged fragment of WNK1 consisting of amino acids 1 to 119 was used as the substrate. T58A mutant WNK1 was used as the control for specific phosphorylation at T58. As shown, PDK1 by itself did not phosphorylate WNK1 (Figure 3A, lane 1). For Akt1, both wild-type Akt1 and myristoylated Akt1, but not their kinase dead mutant, phosphorylated WNK1 (lane 2 to 4). Although myristoylated Akt1 is reported catalytically more active than wild type toward certain substrates, we did not find this using WNK1 as a substrate. Co-expression of PDK1 did not further enhance the in vitro kinase activity of Akt1 (lane 8 to 10). For SGK1, only S422D (not wild-type or kinase-dead mutant) phosphorylated WNK1 in the absence of PDK1 (lanes 5 to 7). The SGK1-S422D mutant with serine-422 in the hydrophobic motif substituted by aspartate is constitutively active because it does not require phosphorylation by mTOR for activation by PDK1. Co-expression of PDK1 enhanced the kinase activity of WT and SGK1-S422D (lanes 11 to 13). The increase in the kinase activity on the S422D mutant was so much that phosphorylation on WNK1 also occurred at residue(s) other than T58 [see lane 11, on WNK1(1-119)/T58A mutant]. The averaged relative kinase activity of Akt1 and SGK1 (normalized to “Akt1-Myr” in lane 2, which is given as 1), representing specific phosphorylation on WNK1-T58 [i.e., after subtracting signal on wild type of WNK1(1-119)] from three independent experiments is shown in bar graph in the bottom.

We further examined the activity of these kinases acting in vivo (i.e., in intact cells). WNK1(1-220) (which lacks kinase domain thus avoiding autophosphorylation) was co-expressed with epitope-tagged Akt1, SGK1, and/or PDK1 as indicated. Phosphorylation at T58 was probed by anti-WNK1-T58 phospho-antibody. T58A mutant of WNK1(1-220) was used as a negative control (Figure 3B, lane 14, labeled as “M” for mutant). As shown, there was a low basal level of phosphorylation on WNK1(1-220) (lane 1), presumably from the endogenous Akt1. Myristoylated and wild-type Akt1, but not kinase-dead mutant, caused phosphorylation of WNK1 above the basal level (lanes 2 to 4). Coexpression with PDK1 slightly enhanced kinase activity of myristoylated Akt1 but not wild-type Akt1 (lanes 8 to 10). The increase in the kinase activity of myristoylated Akt1 by PDK1 in vivo, but not in vitro, may be caused by preferential targeting of the myristoylated Akt1 to the cell membrane, thus increasing co-localization with
PDK1. As in vitro studies, S422D and wild-type SGK1 phosphorylated WNK1 in vivo (lanes 5 and 6). PDK1 slightly enhanced SGK1 kinase activity but not as much as the effect found in the in vitro experiments (lanes 11 and 12). Differences in the ratio of substrate relative to kinase and/or the efficiency of kinase may account for the discrepancy. To our surprise, expression of kinase-dead SGK1 caused some phosphorylation of WNK1 above the basal level, although the activity was less compared with wild-type or S422D mutant (lanes 7 and 13). At the moment, we do not have a good explanation for the finding except to speculate that it may be caused by altered activity of endogenous Akt1. Overall, these results from in vitro and in vivo kinase assays support the idea that Akt1 and SGK1 can phosphorylate WNK1 at T58.

**Akt1 Inhibits ROMK through WNK1**

The finding that a supraphysiologic concentration of insulin can further inhibit ROMK in the presence of serum (Figure 2A) suggests that normally the endogenous Akt1 and WNK1 are not maximally activated. Consistent with the idea, exogenous WNK1 inhibits ROMK (Figure 2E; see also ref. 21). We thus asked whether overexpression of Akt1 may inhibit ROMK by enhancing WNK1-T58 phosphorylation. We used myristoylated Akt1 because it can be enhanced by PDK1 in intact cells (Figure 3B). Myristoylated Akt1 ("Akt1-Myr") inhibited ROMK but did not cause additional effect when WNK1(1-491) was co-transfected (Figure 4A). Kinase dead mutant of Akt1 (Akt1-KD) did not cause inhibition of ROMK but reversed WNK1(1-491)–mediated inhibition. Thus, Akt1 and WNK1 act on the same pathway.
To confirm the role of Akt1 is from phosphorylation on T58 of WNK1, we showed that WNK1(1-481)/T58A had no effect on ROMK but reversed myristoylated Akt1-mediated inhibition of ROMK (Figure 4B). Knocking down endogenous WNK1 by siRNA prevented the inhibition of ROMK by myristoylated Akt1 (Akt1-Myr). In each panel, ROMK current density (pA/pF at −100 mV) was represented as mean ± SEM (n = 6). *P < 0.05 between indicated groups by unpaired two-tailed t test. **P < 0.01. NS, not statistically significant. Equal protein expression was confirmed by Western blot. (D) Effect of insulin on membrane abundance of ROMK with or without endogenous Akt1. Cells were transfected with ROMK and control oligonucleotide or Akt1 siRNA (200 nM each) and deprived of serum for 16 hours.

SGK1 Inhibits ROMK through WNK1 and Works Together with Akt1

We next examined the potential role of Akt1, another member of the AGC kinase family that can also mediate downstream effect of PI3K, in regulating WNK1 inhibition of ROMK. Similar to Akt1, constitutively active SGK1 mutant, S422D, inhibited ROMK but did not cause an additional effect when WNK1(1-491) was co-expressed. Kinase-dead SGK1 did not inhibit ROMK but reversed the inhibition caused by WNK1 (Figure 5A). In the presence of the T58A mutant of WNK1(1-491), even the constitutively active form of SGK1 did not inhibit ROMK (Figure 5B). Knocking down endogenous WNK1 by siRNA also abrogated the effect of SGK1 on ROMK (Figure 5C). Thus, exogenous Akt1 and SGK1 showed a similar inhibitory effect on ROMK, and both effects depend on WNK1-T58 phosphorylation. Next, we examined potential synergistic effects of Akt1 and SGK1 on ROMK by silencing endogenous Akt1 and/or SGK1 using siRNA in the absence (“vector”) or presence of exogenous WNK1 [“WNK1(1-491)”). Knocking down Akt1 or SGK1 individually increased ROMK current significantly with or without exogenous WNK1 (Figure 5D). The effect of knocking down both Akt1 and SGK1 is greater than knocking down each individually in the “WNK1(1-491)–transfected but not in the “vector”–transfected group. Differences in the abundance of WNK1 substrate likely account for the different results. In summary, both Akt1 and SGK1 phosphorylate WNK1 and contribute to its regulation of ROMK. The importance of Akt1 versus SGK1 in vivo will depend on the relative abundance of each respective kinase and WNK1 in the setting.
Inhibition of ROMK by SGK1 via Enhanced Endocytosis and Not by Phosphorylation of ROMK

WNK1 inhibits ROMK by enhancing endocytosis through a dynamin-dependent, clathrin-mediated pathway.\(^3\)\(^2\)\(^1\)\(^2\)\(^2\) This effect of WNK1 requires an interaction with intersectin.\(^1\)\(^1\) It has also been reported that SGK1 can directly phosphorylate on ROMK1 at serine-44, although this effect is believed to result in an increase of the cell surface abundance of ROMK.\(^2\)\(^3\)\(^2\) We found that co-expression of a dominant-negative (“DN”) intersectin (“ITSN”) or dynamin prevented inhibition of ROMK by SGK1-S422D (Figure 6A). These results, together with our previous reports that these experimental maneuvers abolish inhibition of ROMK by WNK1, support that phosphorylation of WNK1-T58 by SGK1 leads to inhibition of ROMK by increasing endocytosis of ROMK. In further support of this idea, we found that SGK1-S422D inhibited ROMK bearing a mutation of serine-44 (S44D) and wild-type ROMK (Figure 6B).

Kidney-Specific WNK1 Blocks SGK1 Effect on ROMK without Interfering with Phosphorylation on WNK1

WNK1 has multiple alternatively spliced isoforms, including the full-length WNK1 (also known as long WNK1) and a kidney-specific WNK1 (KS-WNK1) that lacks most of the kinase domain and preceding amino acids in the N terminus.\(^2\) Long WNK1 contains T58, the target of Akt1/SGK1. In contrast, KS-WNK1 lacks T58. We showed that KS-WNK1 binds and antagonizes long WNK1–induced inhibition of ROMK.\(^2\)\(^1\) Here, we asked whether KS-WNK1 antagonizes the effect of long WNK1 in the presence of Akt1/SGK1. As reported previously, KS-WNK1 reversed WNK1(1-491)–induced inhibition of ROMK (Figure 7A, left three bars). The ability of KS-WNK1 to antagonize WNK1(1-491)–induced inhibition of ROMK was unaltered in the presence of exogenous constitutively active SGK1 (SGK1-S422D) (Figure 7A, last bar on the right). KS-WNK1 may antagonize long WNK1 inhibition of ROMK by interfering with its phosphorylation by Akt1/SGK1 (Figure 7B, mechanism “1”) or interfering with its interaction with downstream effectors of endocytosis, such as intersectin (mechanism “2”). To distinguish between these two possibilities, we examined the effect of KS-WNK1 on serum-induced phosphorylation of WNK1(1-491) in HEK cells coexpressed with SGK1-S422D. Serum deprivation decreased T58 phosphorylation on WNK1, which was enhanced by SGK1-S422D (lanes 1 to 3; Figure 7C). Coexpression of KS-WNK1 did not affect phosphorylation of WNK1 at T58 (lanes 4 to 6). KS-WNK1 alone had no
effect on WNK1 phosphorylation (lane 7). Thus, KS-WNK1 likely affects WNK1 interaction with downstream effectors.

**DISCUSSION**

ROMK undergoes constitutive clathrin-mediated endocytosis. WNK kinases including WNK1 and WNK4 inhibit ROMK by increasing endocytosis. We previously reported that WNK1 and 4 interact with intersectin, an endocytic scaffold protein that binds dynamin and other endocytic accessory proteins. The interaction with intersectin leads to stimulation of endocytosis of ROMK, probably by enhancing the recruitment and assembly of endocytic machinery. In this study, we showed that activation of PI3K by insulin, IGF1, and likely by other serum growth factors enhances endocytosis of ROMK through phosphorylation of T58 on WNK1. This effect on ROMK via WNK1 depends on two members of AGC kinases: Akt1 and SGK1. As summarized in Figure 8, activation of PI3K stimulates mTORC2 to phosphorylate Akt1 and SGK1 at S473 and S422 in the hydrophobic motif, respectively. Phosphorylation by mTORC2 allows binding of PDK1, which phosphorylates Akt1 and SGK1 at T308 and T256 in the T-loop, respectively, to activate their catalytic activity. Activated Akt1 and SGK1 phosphorylate WNK1 at T58, leading to enhanced endocytosis of ROMK via an intersectin-dependent mechanism.

This mechanism of regulation of ROMK by Akt1 and/or SGK1 via WNK1 has several potential physiologic or pathophysiologic relevancies. One of these is the maintenance of K⁺ homeostasis during chronic (approximately 1 week) K⁺ deficiency. IGF1 is produced in the kidney, and production is upregulated by chronic dietary K⁺ restriction. Upregulation of IGF1 is believed to play a role in the K⁺ deficiency–induced renal hypertrophy. An increase in the level of IGF1, nonetheless, can decrease renal K⁺ secretion via ROMK, contributing to K⁺ conservation during K⁺ deficiency. In support of the idea that IGF1 inhibits renal K⁺ secretion in vivo, intravenous administration of IGF1 in humans decreases renal K⁺ excretion without significant changes in the filtered load of K⁺. Conversely, mice with liver-specific deletion of IGF1 have approximately 80% reduction in the circulating IGF1 and increased renal K⁺ excretion despite a normal filtered load of K⁺. Finally, inhibition of PI3K increases the density of native ROMK channels in mouse CCD, providing further support for the physiologic role of PI3K in the inhibition of ROMK and renal K⁺ secretion.

Insulin also activates PI3K and thus may be another upstream signal that uses the Akt1/SGK1-WNK1 pathway to inhibit renal K⁺ excretion. A physiologic role of insulin in decreasing renal K⁺ excretion in vivo, however, is not universally accepted. Although it is known that insulin decreases urinary K⁺ excretion, some suggested that the effect is entirely from the decrease in the plasma K⁺ caused by the intracellular shift. In contrast, others showed that the decrease in urinary K⁺ excretion by insulin was not only due to the decrease in plasma K⁺ but also due to the decrease in renal K⁺ secretion.
K$^+$ secretion caused by insulin infusion in humans is more than the decrease in the filtered load, supporting that insulin inhibits renal K$^+$ excretion in vivo.$^{30,31}$ In support of this idea, application of insulin to the basolateral bath of isolated perfused rabbit CCD inhibits the net transepithelial K$^+$ secretion.$^{32}$ The $IC_{50}$ for inhibition of K$^+$ secretion in the CCD is 500 nM, which is higher than the normal fasting level of insulin (10 to 150 pM) but within the normal postprandial level (300 to 800 pM).$^{16}$ The minimal concentration of insulin required for inhibition of ROMK in our studies is 1 nM. This value is not far from the effective concentration of insulin in the CCD and the postprandial levels in vivo, considering that different experimental systems are compared. The inhibition of ROMK-mediated renal K$^+$ secretion by insulin, if it occurs in vivo, will help to maintain the plasma K$^+$ level in the postprandial state during which a very active intracellular K$^+$ shift occurs.

Our finding that SGK1 inhibits ROMK is different from other reports that SGK1 stimulates ROMK.$^{23,33}$ The in vivo importance of our finding is supported by several animal stud-

ies. The abundance of ROMK in the apical membrane of distal nephron in mice homozygous for Sgk1 deletion is higher than that in the wild-type littermates.$^{34}$ The increase in the ROMK abundance in Sgk1 knockout mice was suggested to be caused by a compensatory response to hyperkalemia caused by reduced Na$^+$ reabsorption and thus reduced electrical driving force for K$^+$ secretion. However, in mice with double knockout of Sgk1 and Sgk3 (in which Na$^+$ wasting is evident in normal Na$^+$ diets), the fractional urinary K$^+$ excretion is higher than that in wild-type littermates, despite a normal blood K$^+$ level and an impairment in Na$^+$ reabsorption in the double knockout mice.$^{35}$ These results support that SGK1 (perhaps together with SGK3 or isoforms) inhibits ROMK in vivo. It is possible that SGK1 can also exert a stimulatory effect on ROMK under different physiologic contexts or presence of certain co-regulators. Our present cell-based study, nonetheless, provides strong evidence to support the hypothesis that Akt1/SGK1-mediating phosphorylation of WNK1 can mediate inhibition of ROMK and renal K$^+$ secretion by PI3K-activating hormones. Future experiments will examine the effect of insulin and IGF1 on K$^+$ homeostasis in mice with WNK1, SGK1, and/or Akt1 deletions.

Aldosterone is a positive regulator of SGK1.$^{36}$ Our finding that SGK1 inhibits ROMK may seem to be counterintuitive to the fact that aldosterone stimulates renal K$^+$ secretion. However, besides SGK1, aldosterone also stimulates KS-WNK1,$^{37}$ which antagonizes the effect of SGK1 phosphorylation of (long) WNK1 on ROMK (see Figures 7 and 8). Thus, whereas insulin and IGF1 activate the Akt1/SGK1-WNK1 signaling cascade to inhibit ROMK, aldosterone activates two opposing effects on the signaling cascade (the negative and positive effect via SGK1 and KS-WNK1, respectively) and therefore may not have a net effect on ROMK. The principal mechanism for aldosterone stimulation of K$^+$ secretion is likely the increase in the electrical driving force for K$^+$ secretion secondary to enhanced Na$^+$ reabsorption, rather than via the SGK1-WNK1 cascade.

### CONCISE METHODS

**DNA Constructs and Reagents**

pEGFP-ROMK1, pCMV5-Myc-WNK1, pIRES-Flag-KS-WNK1, and dominant-negative intersectin and dynamin have been described previously.$^{21}$ The plasmids encoding N-terminal 60 amino acids truncated SGK1 or isoforms) inhibits ROMK.
siRNA were 5'-H11032 nucleotides (Dharmacon RNA Technology) for human WNK1 (Stratagene) and confirmed by sequencing. Sense and anti-sense oligonucleotides were generated by site-directed mutagenesis (QuickChange kit; Stratagene) and confirmed by sequencing. Sense and anti-sense oligonucleotides for human WNK1 were 5'-GACC GGCAUCGUAGACdTdT-3' and 5'-AAGGGGCCAUCGUAGACdTdT-3'. Sense and anti-sense oligonucleotides for human AKT1 were 5'-GACC GGCAUCGUAGACdTdT-3' and 5'-GACAAAAAGCAGGCGG-GUCdTdT-3'. Sense and anti-sense oligonucleotides for human Akt1 were 5'-GACC GGCAUCGUAGACdTdT-3' and 5'-UGACAAAGCAGGCGG-GUCdTdT-3'.

**Whole-Cell Patch-Clamp Recording of ROMK Channels**

After 48-hour transfection, cells were trypsinized and plated on poly-l-lysine–coated coverslips. Whole-cell ROMK currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) as described previously.11 The pipette resistance was around 1.5 to 3 MΩ. Green fluorescence of GFP-ROMK in transfected cells was identified by epifluorescent microscopy. The pipette solution contained 140 mM KCl and 10 mM HEPES (pH 7.2); the bath solution contained 140 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES (pH 7.4). The cell membrane capacitance and series resistance were monitored and compensated (>75%) electronically. The voltage protocol consisted of 0-mV holding potential and 500-ms steps from −100 to 100 mV in 25-mV increments. ClampX 9.2 software (Axon Instruments) was used for data acquisition. Current density was calculated by dividing current at −100 mV (pA; measured at 25°C) by capacitance (pF). Results were shown as mean ± SEM (n = 6–10). Each experiment (i.e., set of results shown in each panel of a figure) was repeated two to four times.

**Surface Biotinylation Assay**

For biotinylation of cell surface ROMK, cells were washed with ice-cold PBS and incubated with 0.75 ml PBS containing 1.5 mg/ml EZ-link-NHS-SS-biotin (Thermo Scientific) for 1 hour at 4°C. After quenching with glycine (100 mM), cell were lysed in a RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1% Triton X-100, 0.5% DOC, and 0.1% SDS) containing protease inhibitor cocktail. Biotinylated proteins were precipitated by streptavidin-agarose beads (Thermo Scientific). Beads were subsequently washed four times with PBS containing 1% Triton X-100. Biotin-labeled proteins were eluted in sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes for Western blotting. ROMK proteins on the membrane were detected using anti-GFP horseradish peroxidase conjugate antibody. The biotinylation experiment was performed three times with similar results.
Statistical Analysis
Data analysis and curve fitting were performed with Prism (v5.03) software (GraphPad Software, San Diego, CA). Data are presented as mean ± SEM. Statistical comparisons between two groups of data were made using the two-tailed unpaired t test. Multiple comparisons were determined using one-way ANOVA. Time course and dose–response curves were fitted by nonlinear regression analysis. Statistical significance was defined as P < 0.05 for single comparison and P < 0.01 for multiple comparisons.

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DISCLOSURES

These experiments were performed by C.-J.C. in partial fulfillment of the requirements of the PhD degree at the University of Texas Southwestern Medical Center at Dallas.

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